Targeting CXCR4-dependent immunosuppressive Ly6C<sup>low</sup> monocytes improves antiangiogenic therapy in colorectal cancer

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Antiangiogenic therapy with antibodies against VEGF (bevacizumab) or VEGFR2 (ramucirumab) has been proven efficacious in colorectal cancer (CRC) patients. However, the improvement in overall survival is modest and only in combination with chemotherapy. Thus, there is an urgent need to identify potential underlying mechanisms of resistance specific to antiangiogenic therapy and develop strategies to overcome them. Here we found that anti-VEGFR2 therapy up-regulates both C-X-C chemokine ligand 12 (CXCL12) and C-X-C chemokine receptor 4 (CXCR4) in orthotopic murine CRC models, including SL4 and CT26. Blockade of CXCR4 signaling significantly enhanced treatment efficacy of anti-VEGFR2 treatment in both CRC models. CXCR4 was predominantly expressed in immunosuppressive innate immune cells, which are recruited to CRCs upon anti-VEGFR2 treatment. Blockade of CXCR4 abrogated the recruitment of these innate immune cells. Importantly, these myeloid cells were mostly Ly6<sup>+</sup>C<sup>−</sup> monocytes and not Ly6<sup>+</sup>C<sup>+</sup> monocytes. To selectively deplete individual innate immune cell populations, we targeted key pathways in Ly6<sup>+</sup>C<sup>−</sup> monocytes (Cx3cr1<sup>−/−</sup> mice), Ly6<sup>+</sup>C<sup>−</sup> monocytes (CCR2<sup>−/−</sup> mice), and neutrophils (anti-Ly6G antibody) in combination with CXCR4 blockade in SL4 CRCs. Depletion of Ly6<sup>+</sup>C<sup>−</sup> monocytes or neutrophils improved anti-VEGFR2-induced SL4 tumor growth delay similar to the CXCR4 blockade. In CT26 CRCs, highly resistant to anti-VEGFR2 therapy, CXCR4 blockade enhanced anti-VEGFR2-induced tumor growth delay but specific depletion of Ly6G<sup>+</sup> neutrophils did not. The discovery of CXCR4-dependent recruitment of Ly6<sup>+</sup>C<sup>−</sup> monocytes in tumors unveiled a heretofore unknown mechanism of resistance to anti-VEGF therapies. Our findings also provide a rapidly translatable strategy to enhance the outcome of anti-VEGF cancer therapies.

Significance

The survival benefit of antiangiogenic therapies for cancer patients has been limited, potentially due to intrinsic/acquired resistance. Deciphering and targeting resistance mechanisms are critical to improving treatment outcome, especially in cancers where antiangiogenic therapies are standard of care, such as colorectal cancer (CRC). Consistent with our clinical findings, we found up-regulation of CXCL12/CXCR4 in orthotopic CRC models and conditional Apc mutant spontaneous rectal tumors after anti-VEGFR2 treatment. CXCR4 signaling recruited immunosuppressive innate immune cells such as Ly6<sup>+</sup>C<sup>−</sup> monocytes and Ly6G<sup>+</sup> neutrophils to the CRCs, conferring resistance to VEGFR2 blockade. Furthermore, we successfully targeted these pathways genetically and pharmacologically, including with an FDA-approved agent Plerixafor (AMD3100), which significantly enhanced treatment response. These strategies have the potential for rapid clinical translation.


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axis in regulation of immune cells has been investigated, including a subset of monocytes (i.e., Ly6C<sub>high</sub> monocytes) (12–17), whether CXCR4 is involved in regulation of another subset of monocytes (i.e., Ly6C<sub>low</sub>) monocytes is not known.

In this study, building on our previous clinical finding that CXCR4 is up-regulated in rectal cancer post-bevacizumab therapy, we targeted CXCR4 using AMD3100, an FDA approved agent, which resulted in significant improvement of anti-VEGFR2 therapy in clinically relevant orthotopic murine CRC models. Mechanistically, we found that CXCR4 is critical for tumor infiltration of Ly6C<sub>low</sub> monocytes and that blockade of Ly6C<sub>low</sub> monocyte recruitment improves the efficacy of anti-VEGFR2 therapy in CRCs. Thus, targeting CXCR4-mediated Ly6C<sub>low</sub> monocyte infiltration is a potential strategy to enhance the efficacy of anti-VEGF treatments for CRC patients.

**Results**

**Antiangiogenic Therapy Increases Expression of CXCL12 and CXCR4 in CRCs.** We treated two different orthotopic colon cancer models—SL4 and CT26—as well as a spontaneous rectal tumor model in Apc conditional knockout mice with a monoclonal antibody against VEGFR2 (DC101) targeting VEGF receptor 2 (VEGFR2) (18). We have previously demonstrated that CXCL12 and CXCR4 expression increases in primary human rectal tumors of patients treated with bevacizumab monotherapy (10, 11). Consistent with these clinical data (11), we found a significant increase in CXCR4 and CXCL12 expression after DC101 treatment in both spontaneous rectal tumors and established CRCs (Fig. 1 A–D and Figs. S1–S14). These findings indicate that our CRC models recapitulate the response of primary human CRCs to bevacizumab.

**Blockade of CXCR4 Enhances the Antitumor Effect of Anti-VEGFR2 Therapy.** We next examined the role of CXCL12/CXCR4 signaling in the resistance to anti-VEGFR2 therapy in CRCs using an FDA-approved CXCR4 inhibitor (AMD3100) (19). In both SL4 and CT26 models, we found that treating with AMD3100 significantly enhanced the antitumor effect of anti-VEGFR2 (DC101) therapy, resulting in delayed tumor growth and increased survival of animals (Fig. 1 E–H and Figs. S2–S4). Interestingly, SL4 tumors were more sensitive to DC101 therapy than CT26 tumors, representing the heterogeneity in patient response. As we have previously shown (9), SL4 and CT26 tumor size was ∼45% and ∼70% of that of the control after 12 d of DC101 monotherapy (Fig. 1 G and H). In addition, the extent of tumor growth inhibition by the combined therapy was similar in both SL4 and CT26 models. These data indicate that the mechanism making CT26 less sensitive to anti-VEGFR2 treatment was abolished by the blockade of CXCR4. These data show that targeting CXCR4 enhances the efficacy of antiangiogenic therapy in both CRC models, regardless of their sensitivity to anti-VEGFR2 monotherapy.

**Blockade of CXCR4 Alleviates Anti-VEGFR2 Therapy-Induced Lymphocyte Reduction but Does Not Directly Affect Tumor Cell Proliferation.** Next, we sought the mechanisms of how the blockade of CXCR4 enhances the antitumor effect of anti-VEGFR2 therapy. First, we tested whether the blockade of CXCR4 directly inhibited proliferation of cancer cells themselves. MITT (methyl thiazolyl tetrazolium) assay results showed that CXCR4 is not involved in the regulation of colon cancer cell proliferation (Fig. S5). Given the important role of CXCR4 in chemotaxis of leukocytes (20, 21), we next examined the effect of AMD3100 and DC101 on the immune microenvironment. We analyzed lymphoid populations in SL4 and CT26 tumors and found fewer CD4<sup>+</sup> (SL4, 3.7 ± 0.8% in control vs. 1.7 ± 0.3% in DC101 and CT26, 4.4 ± 0.9% in control vs. 2.5 ± 0.4% in DC101) and CD8<sup>+</sup> T cells (SL4, 4.1 ± 0.8% in control vs. 2.3 ± 0.7% in DC101 and CT26, 8.9 ± 1.7% in control vs. 4.1 ± 1.2% in DC101) in the DC101-treated group (Fig. S6). We also found that AMD3100 treatment restored the number of T lymphocytes to the level of control rat IgG-treated tumors (Fig. S6). Given that effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells are known to express CXCR4 in some contexts (22, 23), we measured the expression of CXCR4 on these lymphocytes. However, to our surprise, we observed only a negligible level of CXCR4 expression on T lymphocytes in the CRC microenvironment (Fig. S7). Based on these findings, we explored whether the effect of AMD3100 treatment on the inhibition of T-cell infiltration is indirect and mediated by other immune cell types expressing CXCR4.

**CXCR4 Is Predominantly Expressed by Monocytes and Neutrophils in the Immune Microenvironment in CRCs.** While we found that CD4<sup>+</sup>/CD8<sup>+</sup> T lymphocytes and natural killer cells do not appreciably express CXCR4, we found abundant expression of CXCR4 in myeloid cell...
populations in CRC tissues upon DC101 treatment (Fig. S7). Consistent with these findings and our previous studies in other types of tumors (24), we observed an increase in CD11b\(^+\)Gr1\(^+\) cells in DC101-treated tumors shown by flow cytometry (Fig. S8). In fact, the CD11b\(^+\)Gr1\(^+\) cells represent a heterogeneous mixture of myeloid cells that include monocytes and neutrophils. Thus, we subdivided the CD11b\(^+\)Gr1\(^+\) myeloid cells and analyzed these subpopulations separately (i.e., Ly6C\(^{low}\) monocytes and Ly6G\(^{+}\) neutrophils) based on their immunophenotype using appropriate surface markers. These CD11b\(^+\)Gr1\(^+\) cells were previously implicated in resistance to anti-VEGF therapy (2, 25). We also found abundant expression of CXC4 in another monocyte subset—Ly6C\(^{low}\) monocytes—which do not belong to the CD11b\(^+\)Gr1\(^+\) myeloid cells (Fig. S7). Of note, these myeloid cells are devoid of F4/80 and CD11c expression. Hence, they do not appear to be tumor associated macrophages (TAMs) or dendritic cells (DCs) (Fig. S9).

**Blockade of CXCR4 Inhibits Anti-VEGFR2 Therapy-Induced Tumor Infiltration of Ly6C\(^{low}\) Monocytes and Ly6G\(^{+}\) Neutrophils.** We have previously analyzed these three different myeloid cell subpopulations expressing CXCR4 (i.e., Ly6C\(^{low}\) monocytes, Ly6C\(^{high}\) monocytes, and Ly6G\(^{+}\) neutrophils) during the course of anti-VEGFR2 therapy (9). Consistent with our prior work, we found DC101 treatment significantly increased the number of Ly6C\(^{low}\) monocytes on day 5 and day 12 (Fig. 2) in SL4 and CT26 tumors. Also, there was a significant increase in the number of neutrophils on day 12 after DC101 treatment in both tumor models (Fig. 2 B and D). The number of Ly6C\(^{high}\) monocytes was much lower than the other two subpopulations and did not change upon anti-VEGFR2 therapy (Fig. 2). In addition, the fractions of Ly6C\(^{low}\) monocytes and Ly6G\(^{+}\) neutrophils to CD45\(^{+}\) cells were higher in CT26 than in SL4 tumors after DC101 treatment, which seems consistent with the differences in growth behavior of CT26 ("DC101 resistant" as shown in Fig. 1 F and H) and SL4 tumors ("DC101 sensitive") (Fig. 1 E and G).

In both SL4 and CT26 tumors, consistent with our previous findings (9), we observed that Ly6C\(^{low}\) monocytes infiltrate into tumors before neutrophils (Fig. 2). Furthermore, AMD3100 treatment inhibited DC101-induced Ly6C\(^{low}\) monocyte and neutrophil accumulation (Fig. 2 B and D), which is consistent with our hypothesis and leads to the improved antitumor efficacy that we observed (Fig. 1 E and F).

**In Anti–VEGFR2-Sensitive SL4 Tumors, Blockade of CXCR4-Dependent Infiltration of Ly6C\(^{low}\) Monocytes or Ly6G\(^{+}\) Neutrophils Improves Efficacy of Anti-VEGFR2 Therapy.** Next, we performed a series of experiments depleting individual subsets of myeloid cells. Ly6C\(^{low}\) monocytes express a high level of CX3CR1, and their infiltration is dependent on this chemokine receptor (26–28). We specifically inhibited the infiltration of Ly6C\(^{low}\) monocytes by using C3sr1\(^{−/−}\) mice as described previously (9, 29, 30). Using C3sr1\(^{−/−}\) mice, we also confirmed our earlier observation of impaired recruitment of Ly6G\(^{+}\) neutrophils, which arrive after Ly6C\(^{low}\) monocytes (9) (Fig. S10). DC101 monotherapy in wild-type mice exhibited modest tumor growth delay and survival benefit (Fig. 3 A and S4). AMD3100 treatment in combination with DC101 in wild-type mice showed a further improvement in antitumor efficacy (Fig. 3 A and S4). The antitumor efficacy of combinatorial treatment of AMD3100 and DC101 in wild-type mice was comparable to that of DC101 monotherapy in C3sr1\(^{−/−}\) mice (Fig. 3A). However, there was no further improvement in antitumor efficacy by AMD3100 treatment in C3sr1\(^{−/−}\) mice (Fig. 3A). Using Ccr2\(^{−/−}\) mice to specifically inhibit infiltration of Ly6C\(^{high}\) monocytes, there was no difference in antitumor efficacy from any of the tested treatments between wild-type and Ccr2\(^{−/−}\) mice (Fig. 3B). Lastly, using an anti-Ly6G neutralizing antibody to deplete neutrophils, antitumor efficacy was improved when combined with DC101 (Fig. 3C), similar to the effect of combined AMD3100 and DC101.

**In Anti–VEGFR2-Resistant CT26 Tumors, Blockade of both Ly6C\(^{low}\) Monocyte and Ly6G\(^{+}\) Neutrophil Infiltration Is Needed to Improve the Efficacy of Anti-VEGFR2 Therapy.** As shown, CT26 tumors are more refractory to anti-VEGFR2 (Fig. 1 E and F) and have a higher number of Ly6C\(^{low}\) monocytes than SL4 tumors (Fig. 2). Thus, we asked whether the inhibition of neutrophil infiltration in CT26 tumors is sufficient to improve the efficacy of anti-VEGFR2 therapy as observed in SL4 tumors (Fig. 3C). We used anti-Ly6G antibody to specifically deplete neutrophils in the CT26 model. Interestingly, even with the successful depletion of neutrophils after anti-Ly6G antibody treatment, we did not observe any significant improvement in antitumor efficacy from the combination treatment of DC101 and anti-Ly6G antibody compared with DC101 monotherapy (Fig. 4). Meanwhile, when we treated the AMD3100 and anti-Ly6G antibody group with a CX3CR1 antibody, there was a further intrinsic enhancement in antitumor efficacy compared with DC101 monotherapy in CT26 tumors (Fig. 4 and Fig. S4B). Thus, we...
conclude that blockade of CXCR4—which efficiently inhibits infiltration of both Ly6C<sup>low</sup> monocytes and neutrophils—is more effective than targeting neutrophils alone. Therefore, it is critical to target Ly6C<sup>low</sup> monocytes to improve anti-VEGFR2 therapy efficacy especially in CRCs highly refractory to the treatment.

Discussion

In this study, we first verified that the findings from our preclinical CRC models recapitulate the key findings of our previous clinical studies on CRC patients with anti-VEGF therapy—namely up-regulation of CXCL12/CXCR4 pathway after a single dose of bevacizumab (31). Our preclinical models allowed a causal study of this signaling pathway using a clinically available CXCR4 inhibitor (AMD3100). Furthermore, we found that targeting Ly6C<sup>low</sup> monocytes is essential to improve antitumor efficacy of anti-VEGFR2 therapy in tumors highly resistant to the therapy, such as CT26. The elevated numbers of Ly6C<sup>low</sup> monocytes in CT26 tumors may explain, at least in part, why CT26 tumors are more resistant than SL4 tumors to anti-VEGF therapy and why blocking neutrophils using anti-Ly6G antibody was not sufficient to improve the antitumor efficacy of DC101 treatment (Fig. 4). Importantly, as AMD3100 targets both Ly6C<sup>low</sup> monocytes and neutrophils, these findings suggest a possible route for clinical translation for improving antiangiogenic treatment of colorectal cancer.

There have been several reports showing that TAMs mediate resistance to anti-VEGF therapy in different types of cancers (2, 32). It is also known that VEGF inhibition promotes maturation and activation of DCs, which leads to an increase in intratumoral effector T-cell numbers (5). In this study, we found Ly6C<sup>low</sup> monocytes are the key players that drive resistance to anti-VEGFR2 therapy in colorectal cancer.

We have previously found reduced blood vessels after anti-VEGFR2 therapy (9), which indicates that alternative angiogenesis mechanisms are not the chief cause of CRC resistance to anti-VEGFR2 therapy. We have also found progressively increased hypoxia in parallel with the decrease in the intratumoral vessel density in these CRCs over the course of DC101 treatment compared with the control (9). It has been shown that antiangiogenic treatment up-regulates the expression of CXCL12 in tumors via hypoxia (1). Indeed, the protein level of CXCL12 increased on days 5 and 12 in proportion to the degree of hypoxia (Fig. S1C). CXCL12 is also produced by tumor-infiltrating Ly6C<sup>low</sup> monocytes and Ly6G<sup>+</sup> neutrophils (9). We also found a predominant expression of CXCR4 in CD11b<sup>+</sup> monocytes and neutrophils compared with the lymphoid populations—as measured by flow cytometry (Fig. S7). Thus, DC101 treatment appears to trigger a positive feedback loop between the expression of CXCL12 and the infiltration of CXCR4-expressing Ly6C<sup>low</sup> monocytes and Ly6G<sup>+</sup> neutrophils in CRCs.

Here, we showed that the blockade of CXCR4 significantly improves antitumor efficacy when combined with antiangiogenic therapy. Blockade of CXCR4 with AMD3100 treatment significantly inhibited DC101-induced tumor infiltration of Ly6C<sup>low</sup> monocytes and neutrophils in both SL4 and CT26 tumors. However, AMD3100 treatment did not completely abolish infiltration of Ly6C<sup>low</sup> monocytes and neutrophils. This could possibly be due to the fact that some Ly6C<sup>low</sup> monocytes and neutrophils are recruited to tumors in a CXCR4-independent manner or that the dose we used to block CXCR4 signaling is not sufficiently high. Nevertheless, the majority of Ly6C<sup>low</sup> monocytes and neutrophils infiltrate into the tumor in a CXCR4-dependent manner, and their infiltration can be efficiently blocked by targeting the CXCR4 axis. It would be of interest to further characterize the CXCR4-independent Ly6C<sup>low</sup> monocytes and neutrophils and/or to examine the effect of a higher dose of AMD3100 on the infiltration of these cells in future studies.

Recently, we found that Ly6C<sup>low</sup> monocytes and neutrophils infiltrate into tumors and confer resistance to anti-VEGFR2 therapy by secreting high amounts of immunosuppressive cytokines such as IL-10. These immunosuppressive cytokines inhibit adaptive immunity in the tumors, characterized by low numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as their dysfunctional phenotype (i.e., increased PD-1<sup>+</sup> and reduced Granzyme B expression) (9). Consistent with this, we observed the restoration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and tumor growth delay when we added AMD3100.
Before implantation, we cultured SL4 murine CRC cells in DMEM/F12 medium containing 10% FBS. We then intraperitoneally injected 100 mg/kg of ketamine and 10 mg/kg of xylazine to anesthetize 8- to 10-week-old C57BL/6 mice. For orthotopic CT26 CRC model, we intraperitoneally injected 100 mg/kg of ketamine and 10 mg/kg of xylazine to anesthetize 8- to 10-week-old BALB/c mice. We then removed abdominal hair using a hair clipper and made a 5-mm-long midline incision. Through the incision we exteriorized the cecum, and then injected 10 μL of cell/ Matrigel mixture containing 5 × 10⁶ cells from the serosal side into the cecum wall between the mucosa and serosa. We used a 27-gauge needle with an insulin syringe for the cell/Matrigel mixture injection (51, 52). We put back the cecum to the abdominal cavity, closed the abdominal wall with 5–0 polypropylene sutures (Covidien), and then closed the abdomen using surgical staples. We monitored tumor size by assessing blood Gaussia luciferase (Gluc) activity (53, 54) or by using a high-frequency ultrasound imaging system two times a week. We used Apc conditional knockout mice as we previously described (18) for spontaneous rectal tumor model. All animal procedures followed NIH Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Blood Gluc Assay for Monitoring SL4 Tumor Growth. We transduced SL4 cells with lentivirus encoding the Gluc gene to generate the SL4-Gluc cell line. We measured the activity of secreted Gluc to monitor SL4 tumor growth as described previously (53, 54). We collected blood from the tumor-bearing mice every 2 or 3 d. We used a GloMax 96 Microplate Luminometer (Promega) to measure the blood Gluc activity.

Ultrasound Imaging. We monitored tumor growth by using a high-frequency ultrasound imaging system two times a week (Vevo 2100 system, VisualSonics) using a probe with a 40-MHz frequency (M5500S). We then intraperitoneally injected 100 mg/kg of ketamine and 10 mg/kg of xylazine to anesthetize mice and kept them on a heated platform. We then removed abdominal hair using a hair clipper and applied ultrasound gel on the skin. After sterilizing the skin, we inserted the ultrasound probe into the cecum wall. We used M5500S probe. We measured the long diameter (LD) and short diameter (SD) from the images and calculated tumor volume as (LD × SD²)/2.
Flow Cytometry. We performed flow cytometry as described previously [55]. We harvested tumor tissues and chopped using a surgical scissors. We incubated chopped tumor tissues for 30 min in 1.5 ml medium of collagenase type 1A, 1.5 mg/ml of hyaluronidase, and 2 mg/ml of DNase for 1 h in a 37 °C incubator. We filtered the digested tissue using 70-μm cell strainers followed by filtering with 40-μm cell strainers. We incubated single cell suspensions for blocking with an anti-mouse CD16/CD32 antibody. We then stained the cell suspensions with the following antibodies: CD45 (clone 30-F11), B220 (clone RA3-6B2), CD49b (clone DX5), CD90 (clone 52-2.1), NK1.1 (clone PK136), Ter119 (clone TER-119), Ly-6E (clone M31/14.15.2), CD4 (clone RM4-5), CD8 (clone S3-6.7), CD25 (clone PG6), FoxP3 (clone FJK-16B), CD11b (clone M1/70), F4/80 (clone BMB), CD11c (clone HL3), Gr1 (clone RB6-8C5), Ly6C (clone HK1.4), and Ly6G (clone 1AB) (BD Biosciences). We added 7-amo-acid-Acidine Orange (7AAAD) reagent (eBioscience) to the stained tubes to rule out dead cells just before running flow cytometry. Monocytes were defined as CD45<sup>+</sup> Lin<sup>−</sup> F4/80<sup>−</sup> CD11c<sup>−</sup> Ly<sup>-</sup>6C<sup>−</sup> or Ly<sup>+</sup>6G<sup>−</sup> population. Neutrophils were defined as CD45<sup>+</sup> Lin<sup>−</sup> F4/80<sup>−</sup> CD11c<sup>−</sup> Ly<sup>+</sup>6G<sup>−</sup> population. We used LSRII flow cytometer (Becton Dickinson) and analyzed the data with Flowjo software (Tree Star).

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