CX3CR1 delineates temporally and functionally distinct subsets of myeloid-derived suppressor cells in a mouse model of ovarian cancer

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Expression of the chemokine receptor CX3CR1 has been used to identify distinct populations within the monocyte, macrophage and dendritic cell lineages. Recent evidence indicates that CX3CR1-positive subsets of myeloid cells play distinct and important roles in a wide range of immunological maladies, and thus the use of CX3CR1 expression has leveraged our understanding of the myeloid contribution to a multitude of diseases. Here we use CX3CR1 expression as a means to identify a novel nongranulocytic CX3CR1-negative myeloid population that is functionally distinct from the previously described CX3CR1-positive cellular subsets within the CD11b-positive cellular compartment of ascites from ovarian tumor-bearing mice. We functionally identify CX3CR1-negative cells as myeloid suppressor cells and as a cellular subset with pathological specificity. Importantly, the CX3CR1-negative cells exhibit early IL-10 production in the ovarian tumor microenvironment, which we have shown to be critically tied to suppression and additional myeloid-derived suppressor cell accumulation, and we now show that this cellular population actively contributes to tumor progression. Furthermore, we demonstrate that the CX3CR1-negative population is derived from the recently described CX3CR1-positive macrophage/dendritic cell precursor cell. These studies provide a greater understanding of the generation and maintenance of regulatory myeloid subsets and have broad implications for the elucidation of myeloid function and contributions within the tumor microenvironment.

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Expression of the chemokine receptor CX3CR1 is a hallmark of committed macrophage/dendritic cell precursors (MDPs) and variable expression of CX3CR1 has recently been used to identify distinct populations within the monocyte, macrophage and dendritic cell lineages. In mice, blood monocytes have been divided into two populations based on high and low expression of CX3CR1 relative to one another. These two subsets can also be identified in the blood by the differential expressions of the Ly6C antigen of GR-1, CD62L and CCR2.1,2 Accumulating evidence indicates that CX3CR1-positive myeloid cells play an important role in a wide range of immunological maladies including colitis,3,4 burn healing,5 pulmonary vaccinia infection,6 central nervous system injury,7 liver fibrosis,8 ovarian cancer,9 diabetes10 and obesity.10,11 Moreover, in different contexts, the CX3CR1-positive cells can be immunostimulatory (protection in a vaccinia lung infection model8 and drive bactericidal defenses during septic peritonitis12) but can also be immunoregulatory including inhibition of T cell-mediated colitis,3 accumulation of transforming growth factor-β-producing macrophages in wounds13 and limiting airway hyperresponsiveness.14

We recently employed CX3CR1-green fluorescent protein (CX3CR1-GFP) reporter mice to characterize the tumor-infiltrating myeloid cells in the ID8vβ murine model of ovarian cancer. From these studies we observed a progressive accumulation of CX3CR1-positive myeloid cells during tumor progression in the tumor microenvironment.9 Among the CX3CR1+ cell subsets, we identified that early tumor development is marked by the presence of infiltrating CX3CR1+ GR-1-positive cells, but is progressively dominated by a population of suppressive CX3CR1+ GR-1high cells that express CD11b, CD115 and variable levels of F4/80 and CD11c; the loss of GR-1 expression on the CX3CR1-positive cells seen later in tumor progression seems to be a consequence of entering the peritoneum rather than preferential recruitment of a specific monocyte subset.9 Importantly, both subsets of CX3CR1+ cells were identified to function as myeloid-derived suppressor cells (MDSCs) within the ovarian tumor microenvironment. Accumulated evidence from our lab and others has clearly demonstrated that MDSCs within the ovarian tumor microenvironment are critical to ovarian cancer progression9,13–18 and, specifically, that MDSC-derived interleukin-10 (IL-10) plays a crucial role in sculpting the microenvironment. IL-10 is both produced by, and acts upon, the bulk nongranulocytic CD11b-positive myeloid compartment in the ascites19–21 and is intricately tied to clinical tumor progression.22–24 Notably, the IL-10-expressing MDSCs enforce the suppressive tumor microenvironment, are potently inhibitory to T-cell activity, and accumulation of the MDSCs, characterized to date as CX3CR1 positive, over the course of tumor progression favors...
tumor growth. Thus, use of CX3CR1 expression to distinguish subpopulations of tumor-infiltrating myeloid cells provides a powerful mechanism to dissect the phenotypic and functional changes in the myeloid compartment that occur during tumor progression.

We now report a population of CX3CR1-negative MDSCs within the ovarian tumor microenvironment that are temporally and functionally distinct from the CX3CR1-positive cells. With the use of a novel combination of CX3CR1 and IL-10 reporter mice, we have identified a population of CX3CR1-negative MDSCs that express F4/80 and CD11b and are functionally immunosuppressive. Furthermore, we demonstrate that this CX3CR1-negative population is derived from the recently described CX3CR1-positive MDP. Temporal IL-10 production over the course of tumor progression, and this suggests that the CX3CR1-negative cells are critical for the initiation and generation of the tumor microenvironment and may influence the phenotype and function of subsequent infiltrating myeloid cells. Importantly, a novel cellular depletion method based on IL-10 expression reveals that the early IL-10-producing cells, dominated by CX3CR1-negative MDSCs, functionally contribute to tumor progression. These studies provide a greater understanding of the generation and maintenance of regulatory myeloid subsets and have broad implications for myeloid function.

**RESULTS**

The nongranulocytic CD11b compartment in the tumor microenvironment comprises CX3CR1-positive and CX3CR1-negative myeloid populations

Our previous work and other reports indicate that resident peritoneal macrophages are CX3CR1 negative, whereas monocytes and MDSCs recruited in inflammation and ovarian cancer are CX3CR1 positive.2,9 We recently identified a population of tumor-infiltrating CD11b+, IL-10-producing myeloid cells in the ascites to be critically tied to ovarian tumor progression through enforcement of a suppressive tumor microenvironment that included both the CX3CR1-positive and -negative subsets.13 Therefore, we undertook phenotypic and functional analyses of the CX3CR1-positive and -negative populations in the peritonea of naive and tumor-bearing mice to further clarify the lineage and contributions of the two populations, particularly with regard to the poorly described CX3CR1-negative cells.

When we compared naive and tumor-bearing CX3CR1-GFP reporter mice with wild-type tumor-bearing mice, we observed that in the tumor microenvironment CX3CR1 expression divides the bulk CD11b-positive cells into two populations of CD11b-positive, CSF-1 receptor-positive and Ly6C-low cells (Figure 1a). Within a naive peritoneal lavage, the population of CX3CR1-negative resident macrophages represents approximately two-thirds of the cells, whereas in the ascites it accounts for only a tenth of the total cells. In addition,

**Figure 1** CX3CR1 expression delineates two distinct tumor-infiltrating myeloid populations that potently suppress T-cell reactivity. (a) Representative flow plots from naive peritoneal lavages and ascites of tumor-bearing CX3CR1-GFP mice compared with ascites from wild-type tumor-bearing mice. Stained for CD11b, CD11c, Ly6C, and CX3CR1-GFP. Gates shown identify the CX3CR1-negative macrophage population. (b) CX3CR1-negative and -positive cell subsets were sorted by flow cytometry and titrated into a mixed splenocyte reaction stimulated with anti CD3 to test for suppressive capacity, with averages and s.d. shown. The results represent three independent experiments. (c) Total numbers of CX3CR1-positive and -negative cells within the peritonea of naive mice, and tumor-bearing mice over the course of tumor progression (n=3 mice). **P<0.01 in comparison with stimulated (open bar) cells. The s.d. values are shown.
the CX3CR1-negative population expressed slightly higher levels of F4/80 compared with the CX3CR1-positive cells in the naive peritoneum, although less dramatically in the tumor peritoneum, and was notably negative for Ly6G (Supplementary Figure S1). Parallel analyses of wild-type mice lacking the CX3CR1-GFP transgene confirmed that the CX3CR1-low cells are indeed CX3CR1 negative (Figure 1a). Thus, CX3CR1-GFP expression denotes two distinct populations within the tumor that share similar expression of other common myeloid markers.

As the CX3CR1-negative population identified here is part of the larger CD11b compartment that we previously studied with T cell-suppressive activity,7 we next interrogated the specific suppressive capability of the CX3CR1-negative cells. We sorted CX3CR1-negative and -positive populations from tumor-bearing mice and titrated them into a mixed splenocyte reaction. Notably, the CX3CR1-negative population was functionally as suppressive as the CX3CR1-positive population that we had previously demonstrated to be robustly suppressive in this assay (Figure 1b). To assess the relative functional contribution of the two populations to the overall tumor microenvironment, we calculated total numbers of each within the peritoneum over the course of tumor progression. The total numbers of CX3CR1-negative cells increased during tumor progression, although in fewer total numbers of cells than the CX3CR1-positive cells (Figure 1c). These studies demonstrate that the CX3CR1-negative fraction in the CD11b compartment is a critical component of the suppressive population of myeloid cells in the tumor. Importantly, by functional activity, this now identifies a CX3CR1-negative MDSC population in addition to the previously described CX3CR1-positive MDSC population.

IL-10 is produced within the ovarian tumor microenvironment by two distinct myeloid populations as assessed with the use of CX3CR1-GFP/IL-10BiT reporter mice

Our previous description of CX3CR1 expression as a means to identify and track tumor-infiltrating MDSCs in syngeneic ID8-VEGF-A/BDNF29 (ID8vb) murine ovarian tumors allows us to specifically measure the molecular and cellular contribution of this subset of CD11b-positive cells to tumor progression.9 To determine the extent that cellular subsets contribute to tumor-supportive IL-10 production, we generated CX3CR1-GFP/IL-10BiT dual reporter mice by crossing CX3CR1-GFP mice25 with IL-10BiT reporter mice.26 These mice permit us to assess the CX3CR1-delineated cellular subsets within the ID8vb ascites for IL-10 production within the tumor microenvironment. In our previous analyses9 we discerned the previously-described CX3CR1-hi and -lo subsets of myeloid cells2 in the ascites by relative GFP expression, but because of a lack of detectable differences in function and phenotype distinguishing these cells in the ascites, we will refer to them collectively as CX3CR1-positive cells in these studies.

Analyses of advanced ascites from the CX3CR1-GFP/IL-10BiT tumor-bearing mice revealed IL-10 production from both the CX3CR1-positive and -negative populations of cells (Figures 2a and b). Approximately one-quarter of the CX3CR1-positive cells and 10% of the CX3CR1-negative population were IL-10 reporter positive when compared with the negative control of cells derived from a CX3CR1-GFP mouse lacking the IL-10BiT reporter (Figure 2b). The IL-10-producing cells from the CX3CR1-positive population represented ~7.5% of the cells in the ascites, whereas the IL-10-producing cells from the CX3CR1-negative population represented a smaller 2.5% (Figure 2c). In order to confirm the reporter staining, we sorted CX3CR1-negative or -positive cells from the CD11b-positive compartment in the ascites of tumor-bearing mice. The sorted cells were cultured with or without lipopolysaccharide stimulation for 72 h and IL-10 production in the supernatants was assessed by ELISA. We detected equivalent production of IL-10 per 10^5 cells from both populations, demonstrating that both populations are producing IL-10 directly ex vivo (Figure 2d). Thus, positive and negative CX3CR1 expression delineates two distinct populations of IL-10-producing cells within the CD11b compartment in the tumor microenvironment. Importantly, this reveals that the CX3CR1-negative population contributes to IL-10 expression within the tumor microenvironment.
Differential temporal contributions of IL-10 production in the tumor microenvironment by distinct MDSC subsets

In light of our previous findings in murine models of ovarian cancer that IL-10 signaling drives myeloid suppression, the contribution of the CX3CR1-negative population to IL-10 in the tumor microenvironment suggests a role in the development and maintenance of suppression within the tumor. Thus, we next endeavored to define the relationship of this population to CX3CR1-positive monocytes and MDSC that have previously been described to be critical to the IL-10 network.19

We used the CX3CR1-GFP/IL-10BiT mice to analyze the temporal IL-10 production by the MDSC subsets in tumor-bearing mice over the course of tumor progression. At 2 weeks post tumor injection, we observed no IL-10 reporter staining in the blood monocytes (Figure 3a), and very little reporter in the infiltrating peritoneal CX3CR1-negative population (Figure 3), anti-Thy1.1 antibody was administered to tumor-bearing mice at days 7 and 10 following tumor inoculation. This effectively reduced the IL-10-expressing cells 24 h after depletion (Figure 4a) as well as a week later (Figure 4b); as an internal control, it is worth noting that the CX3CR1-positive (and Thy1.1-low) cells were not depleted. Depletion of IL-10-expressing cells modestly reduced CD45-negative cells recovered from the peritoneum of tumor-bearing mice.

Figure 3 IL-10 production is initiated in CX3CR1-negative MDSCs and is subsequently detected in CX3CR1-positive MDSCs in tumor-bearing mice. Blood and peritoneal samples from 2-week (a) and 4-week (b) ID8¥/tumor-bearing mice were assessed for IL-10 reporter staining. Plots of CX3CR1-GFP/IL-10BiT reporter staining and histograms indicate reporter staining in gated populations in CX3CR1-GFP/IL-10BiT mice (black) in comparison with CX3CR1-GFP control mice (gray, filled) representative of results from ≥5 mice. (c) Staining from the peritoneum of naïve CX3CR1-GFP/IL-10BiT reporter.
mice a week later, suggesting impaired tumor growth (Figure 4c). Importantly, the depletion of the Thy1.1+/IL-10+ cells had durable and measurable effects, as this acute depletion of the Thy1.1+ cells prolonged median survival increased by nearly 3 weeks (Figure 4d).

These data demonstrate a critical functional role for the CX3CR1-negative population of MDSCs during early tumor progression and led us to ask whether migration of myeloid cells into the tumor microenvironment prompts the IL-10 expression.

Exposure to the tumor microenvironment is sufficient to induce IL-10 production in CX3CR1 myeloid subsets

The ability of IL-10 to enhance its own production suggested to us that the establishment of a tumor-supportive microenvironment early in tumor progression may be important in programming subsequent infiltrating monocytic cells to an IL-10-producing MDSC phenotype. To better understand the kinetics of IL-10 reporter induction on infiltrating CX3CR1-positive cells upon arrival in the tumor microenvironment, we made use of the GR-1 duality of CX3CR1-positive cells. As we previously reported, although both the GR-1-positive and -negative monocytic populations are capable of infiltrating the tumor microenvironment, expression of GR-1 is lost within 48 h. Thus, CX3CR1-positive cells that express GR-1 have recently migrated into the tumor microenvironment. Analyses of the CX3CR1-positive cells in the ascites revealed that the GR-1-positive subset, which represents a minitum of cells in the ascites (consistent with our previous findings), produced very little IL-10 by reporter

Figure 4 Depletion of early IL-10-expressing MDSCs inhibits tumor progression. ID8βtumor-bearing IL-10BiT reporter mice were injected with 500μg of anti-Thy1.1 antibody on days 7 and 10 following tumor inoculation to deplete IL-10-expressing Thy1.1+, cells. This lowered the CX3CR1-negative IL-10 reporter-positive staining 48 h after antibody depletion (gate set using non-BiT CX3CR1 control mouse) (a). (b) Thy1.1 IL-10 reporter staining 1 week after depletion. (c) CD45-negative peritoneal cells recovered 1 week after depletion. (d) Thy1.1-mediated depletion of the IL-10-expressing MDSCs on days 7 and 10 in ID8β tumor-bearing IL-10BiT reporter mice results in prolonged survival (Kaplan–Meier analysis; n = 5 mice, two independent experiments; *P<0.05). N/S, not significant (P>0.05).
staining (Figures 5a and b). However, the GR-1<sup>+</sup> subset was drastically increased in IL-10 reporter staining (Figure 5b). A direct comparison of the two subsets revealed that the GR-1-negative population had significantly higher IL-10 reporter staining that was not present in the GR-1-negative cells from naive mice (Figure 5b). This suggests that tumor-infiltrating GR-1-positive monocyteic cells increase production of IL-10 after migration into the tumor. Consistent with in <i>vitro</i> studies by Sinha <i>et al.</i>,<sup>27</sup> these data suggest that IL-10 production was induced in MDSCs after infiltration into the tumor microenvironment and exposure to the tumor milieu may enforce IL-10 production and suppressive functions in myeloid cells. From mice with advanced tumors, we did detect low levels of IL-10 reporter staining in blood monocyteic cells (Figure 3b). Further analyses indicated that this reporter staining was predominantly on the GR-1-negative CX3CR1 monocyteic subset, but it appeared lower than the reporter expression of this subset in the ascites. This suggests that blood monocytes also increase IL-10 production; however, these methods do not allow us to directly determine whether the GR-1-positive subset increases IL-10 expression upon arrival in the tumor.

As a complimentary method to demonstrate IL-10 induction in CX3CR1-positive monocytes upon infiltration into the tumor microenvironment, we adoptively transferred CX3CR1-GFP/IL-10BiT dual reporter peripheral blood mononuclear cells by intraperitoneal injection into tumor-bearing mice. Compared with IL-10 reporter staining before transfer, at 48 h after being transferred into the tumor microenvironment we detected increased IL-10 reporter staining, with an average of 44% of the transferred CX3CR1-positive cells inducing IL-10 reporter staining (Figure 5c). Together, these data indicate that exposure to the tumor microenvironment is sufficient to induce production of IL-10, a marker of suppressive function and phenotype. Thus, the establishment of the tumor microenvironment may represent a critical tipping point in regulating the innate response, which modulates the adaptive response, against an ovarian tumor.

The tumor microenvironment alters maturation/differentiation of the CX3CR1 cellular populations

The identification of both phenotypic and functional similarities between the CX3CR1-positive and -negative myeloid populations during tumor growth led us to interrogate the origin of the CX3CR1-negative population. Likely origins included from the pretumor resident peritoneal macrophage population, infiltration and conversion of CX3CR1-positive monocytes, or from some other myeloid, etc.

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**Figure 5** IL-10 reporter is induced in infiltrating CX3CR1-positive monocyteic MDSCs. (a) GR-1-positive and -negative CX3CR1-positive cells in the naive peritoneum, or ascites of tumor-bearing CX3CR1-GFP/IL-10BiT mice, were analyzed for IL-10 reporter staining. Plot shows GR-1-positive and -negative gates. (b) Histogram indicates reporter staining on gated populations from CX3CR1-GFP/IL-10BiT mice showing GR-1 negative (black) compared with GR-1 positive (gray, filled) with quantification of IL-10 reporter staining from gated populations (a) by mean fluorescence intensity (MFI). (c) Plots demonstrating reporter staining on CX3CR1-GFP-positive cells before (left dot plot) and after transfer (right dot plot) of peripheral blood mononuclear cells (PBMCs) from CX3CR1-GFP/IL-10BiT mice intraperitoneally (i.p.) into a wild-type tumor-bearing recipient, with comparative histogram of IL-10 reporter staining before (gray filled) and after transfer (black) showing percentage positive for reporter staining with s.d.; <i>n</i> = 3 independent experiments, three mice. Statistical significance (*<i>P</i>&lt;0.05) and s.d. is shown.
blood-derived intermediary between the bone marrow and peritoneum. The observation that the CX3CR1-negative population is maintained at a relatively stable frequency within the peritoneum during tumor progression (Figure 1) despite the dramatic increases in total peritoneal cellularity indicates that this population must be increasing in number either through local proliferation or new recruitment.

A number of studies have investigated the role of CX3CR1-positive blood-borne monocytes and their precursor, the MDP, in populating the host myeloid compartment during resting and inflammatory conditions.28,29 These studies indicate that monocytes and their precursor can give rise to multiple tissue macrophages and myeloid cell subsets; however, these studies do not report whether these cells can give rise to CX3CR1-negative MDSCs. In studies by Fogg et al.,28 the MDP and monocytes, although to a dramatically lesser extent, were demonstrated in vivo to seed resident splenic macrophage and dendritic cell populations negative for CX3CR1. The authors also report that culture of the MDP with GM-CSF yields CD11bCD11c double-positive cells that are negative for CX3CR1. We asked whether this loss of CX3CR1 expression could occur in vivo in a peritoneal tumor setting in order to determine whether CX3CR1-positive precursor cells give rise to the subsequent CX3CR1-negative MDSCs. To address this question, we generated MDP chimeric mice by fluorescence-activated cell sorted GFP-positive MDPs that were negative for lineage markers for CD11b, CD11c, CD3, GR-1, NK1.1 and B220 from the bone marrow of CX3CR1-GFP mice. These congenically marked cells were mixed with wild-type bone marrow and used to reconstitute lethally irradiated wild-type mice. This experimental setup allowed us to identify MDP-derived (CD45.1 congenic) cells in these mice and to assess their expression of CX3CR1. We injected MDP chimeric mice with ID8vβ tumors and assessed the ability of these CX3CR1-positive precursors to give rise to congenically marked CX3CR1-negative cells in the ascites. We found that after reconstitution and tumor progression, the MDPs do indeed give rise to CX3CR1-negative cells in the ascites (Figure 6a), with wild-type tumor-bearing mice stained for CD45.1 as a negative control (Figure 6a). Interestingly, very few GFP-positive progeny, which we were able to detect immediately after reconstitution (data not shown), were observed in the ascites. However, the time course of this experiment extends beyond the replicative lifespan of MDP, and the

Figure 6 Tumor-infiltrating myeloid cells generated from the MDPs lose CX3CR1 expression, but tumor-transferred monocytes maintain CX3CR1 expression. (a) Ascites from ID8vβ tumor-bearing wild-type mouse (left) and MDP-reconstituted (right) mouse were assessed for CX3CR1-negative (gate shown), congenically marked cells. Results are representative of two independent experiments, 6 mice. (b) Sorted CX3CR1-GFP-positive cells from naive blood (left plot) were adoptively transferred into the peritonea of tumor-bearing wild-type mice and assessed for GFP expression 4 days post transfer (right plot) with quantification of CX3CR1-positive and -negative subsets in the congenic gate with s.d. (n=4, 4 mice). (c) Percentages of CX3CR1-positive cells pregated for CD11b-Ly6G in bone marrow cultured overnight, and with or without addition of tumor ascites plasma for an additional 24 h (n=9, 3 independent experiments). Statistical significance (*P<0.05 and ***P<0.001) and s.d. is shown.
initial GFP+ precursors likely all died or lost expression of CX3CR1 upon maturation into the CX3CR1-negative subset that we were able to detect. These data indicate that CX3CR1-positive precursor cells give rise to CX3CR1-negative myeloid populations in vivo.

The tumor microenvironment promotes accumulation of CX3CR1-positive MDSC during tumor progression

Although the MDP transfers indicate that CX3CR1-positive cells in the bone marrow can give rise to the CX3CR1-negative cells in the ascites, whether this occurs before tumor growth through monocyte repopulation of resident cells or at some specific time during tumor progression could not be determined in the chimeric mice. To clarify whether this can occur within the tumor microenvironment, we performed intratumoral transfers of congenically marked CX3CR1-GFP cells sorted from naive blood. We discovered a substantial CX3CR1-positive population in the ascites as late as 5 days post transfer (Figure 6b) that suggests that these cells persist in the tumor microenvironment for extended periods of time as GFP-positive cells. This persistence indicated that during advanced tumor progression, infiltrating CX3CR1-positive cells maintain CX3CR1 expression for long periods of time within the ascites, at a time when the MDP-derived cells in the chimera experiments were already in place and had already undergone CX3CR1 downregulation.

To test this model, we cultured CX3CR1-GFP bone marrow in vitro to monitor the stability of the CX3CR1-positive cells stimulated with tumor plasma. Normally the CX3CR1-positive cells decrease over time in culture with or without stimulating stimuli. When cultured in the presence of tumor plasma, we observed a marked increase in the persistence of the CX3CR1-positive fraction within the CD11b+Ly6G− cells in the cultured bone marrow (Figure 6c), and observed a converse in the percentage of CX3CR1-negative cells. This supports the hypothesis that the tumor microenvironment and, specifically, soluble mediators in the ascites enforce the accumulation of the CX3CR1-positive myeloid population in the tumor microenvironment.

DISCUSSION

Among recent advancements in the characterization of the mononuclear phagocyte system, CX3CR1 has emerged as a valuable marker with both phenotypic and functional importance in normal and pathological myeloid biology. However, despite work from several groups using CX3CR1 as a defining marker of mononuclear cells and cell subsets,2,8,9,25 how CX3CR1 expression delineates cellular function remains poorly understood. Here, we have utilized CX3CR1 expression to subset distinct CD11b-positive myeloid cells in the ID8β mouse model of ovarian cancer that were previously inseparable by other common myeloid markers. Importantly, our previous focus on CX3CR1-positive cells excluded this newly appreciated suppressive CX3CR1-negative fraction within the CD11b+CD115+Ly6G− compartment. Identification of the CX3CR1-negative population, and distinguishing it from the CX3CR1-positive population (the collective CX3CR1-hi and -low populations), within the myeloid compartment in the tumor has allowed us to shed new light on myeloid dynamics and function with regard to IL-10 control of tumor progression.

Two important findings emerge from the temporal dynamics of the CX3CR1-negative MDSC population relative to our previous studies on IL-10 functions in the tumor microenvironment. First, these cells may represent an important initiator of a chain reaction of IL-10 signaling in the tumor microenvironment, on which we have shown the function and accumulation of infiltrating MDSCs depend.39 Therefore, the discovery that early IL-10 signaling from the CX3CR1-negative population, at a time when the CX3CR1-positive population that is present lacks this production, demonstrates a novel and important functional contribution of these cells during early tumor progression and enhances our understanding of myeloid cell dynamics and interactions in the peritoneum. This initiation likely represents a key switch between tumor-recruited myeloid cells capable of initiating and supporting adaptive antitumor immunity and tumor-suppressive myeloid cells that accumulate and inhibit adaptive antitumor immunity. This premise is further reinforced by our observation that the CX3CR1-negative population of MDSCs functionally contribute to tumor progression since depletion of the IL-10+CX3CR1-negative population significantly and substantially delays tumor progression.

Second, we wanted to determine whether CX3CR1-negative cells could arise from CX3CR1-positive precursor cells in the blood or bone marrow. By way of precedent, previous analyses of naive peritoneal macrophages indicate that the resident macrophage population (as documented to be CX3CR1 negative) is seeded almost exclusively by blood monocytes.30 However, more recent studies that assess the fate of CX3CR1-positive monocytes suggest that these monocytes do not give rise to CX3CR1-negative cells in the naive peritoneum within 18 h.2 However, both of these studies had an unrelated specific focus and remain incomplete. Here, we demonstrate that CX3CR1-positive MDPs are capable of giving rise to CX3CR1-negative peritoneal cells in vivo. Interestingly, when directly transferred into the tumor, CX3CR1-positive monocytes did not undergo this shift quite as strongly. This suggests that monocyte differentiation to CX3CR1-negative cells requires an additional in vivo stimulus before tumor infiltration, the process of transfer impaired this transition or that CX3CR1-negative cells recovered in tumor-bearing MDP mice arose before or early in tumor progression. The in vitro studies carried out here on bone marrow from CX3CR1-GFP mice support the foremost explanation. These experiments indicate that the tumor microenvironment drives the accumulation of CX3CR1-positive cells; however, whether this is through regulation of CX3CR1 gene expression or by increasing the survival of these cells in vitro and in vivo is unclear.

In summary, the regulation of antitumor immunity by myeloid populations within the tumor is emerging as an important aspect of tumor progression. Data indicate that these cells are involved in tumor escape from immune pressure, metastasis of tumor cells and, importantly, contribute significantly to blocking immunotherapeutic strategies. Thus, understanding the networks controlling the development, accumulation and function of myeloid cells is critical to advancing our understanding and potential interventions in disease. Here we have presented work focused on utilizing the CX3CR1 axis to improve our understanding of myeloid cell dynamics, the emergence of MDSCs within the tumor microenvironment, the initiation of the suppressive IL-10 network and control of the tumor microenvironment. These studies provide insight into progressive immune processes, as well as identify new targets and strategies to therapeutically intervene in tumors and other immune maladies.

METHODS

Mice

Female C57BL/6 mice (Mus musculus) were purchased from the National Cancer Institute (Fredricksburg, MD, USA). CX3CR1-GFP25 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). IL-10BiT reporter mice26 used in collaboration with Edward Usherwood (Dartmouth Medical School, Lebanon, NH, USA) were bred with CX3CR1-GFP mice to test this model, we cultured CX3CR1-GFP bone marrow in vitro to monitor the stability of the CX3CR1-positive cells stimulated with tumor plasma. Normally the CX3CR1-positive cells decrease over time in culture with or without stimulating stimuli. When cultured in the presence of tumor plasma, we observed a marked increase in the persistence of the CX3CR1-positive fraction within the CD11b+Ly6G− cells in the cultured bone marrow (Figure 6c), and observed the converse in the percentage of CX3CR1-negative cells. This supports the hypothesis that the tumor microenvironment and, specifically, soluble mediators in the ascites enforce the accumulation of the CX3CR1-positive myeloid population in the tumor microenvironment.
obtain CX3CR1/IL-10BiT double transgenic reporters. All animal experiments were approved by the Dartmouth Medical School Institutional Animal Care and Use Committee.

Cells and antibodies
ID8 cells transduced with Vpef-A and 8DE29 (referred to as ID8β) within this manuscript) were generated and maintained as previously described. Anti- mouse Fc Block (BD Biosciences, San Jose, CA, USA). Anti-mouse CD3 (145-2C11), Gr-1 (RB6-8C5), CD11b (M1/70), CD45.1 (A20) and Ly6G (HK.1.4) were purchased from eBioscience (San Diego, CA, USA). Anti-mouse Thy1.1 (OX-7), CD11c (N418), NK.1.1 (PK136), CD115 (AF598), F4/80 (BM8), Ly6G (1A8), CD45.2 (104) and BCL-2 (BCL/10C4) were purchased from Biolegend (San Diego, CA, USA).

Tumors and leukocyte isolation
Ovarian tumors were generated by intraperitoneal injection of 5 × 10^6 ID8β cells as previously described. At the indicated time points, ascites and blood were harvested from mice and the cellular fraction was collected. In addition, peritoneal cells from naive animals were collected by clear 3 ml lavages with ice-cold phosphate-buffered solution. Single-cell suspensions from bone marrow isolated from femurs were obtained by passing tissues through a M cell strainer (BD Biosciences, San Jose, CA, USA). Single-cell suspensions were treated with ACK lysis buffer (0.15 M NH4Cl, 1.0 mM KHCO3, 0.1 mM EDTA) to remove red blood cells. Cells were resuspended in 0.9% bovine serum albumin in phosphate-buffered solution or media for further analysis or sorting. For bone marrow cultures, whole bone marrow was seeded at a 1 × 10^6 cells per well in a 48-well plate, and cells were cultured overnight in complete medium, stimulated for 24 h the following day with 20% plasma isolated from tumor ascites and analyzed by fluorescence-activated cell sorting.

Fluorescence-activated cell sorting
Cells from mouse ID8β ascites, bone marrow from femurs and blood were incubated with Fc blocking antibody (clone 2.4G2) before staining with the indicated primary antibodies. Flow cytometry and cell sorting was done on the Accuri C6 and BD FACSaria (BD Biosciences). Flow data were analyzed using CFlow (BD Biosciences) and FlowJo 8.8.2 software (Treestar Inc., Ashland, OR, USA).

Chimeric mice
For MDP chimeric mouse studies, similar methods as described by Fogg et al. were employed. Briefly, bone marrow was collected from femurs, treated with red blood cell lysis buffer and washed and resuspended in phosphate buffered solution. ≥ 2 × 10^6 sorted Ly5.1 (CD45.1) DM3.5 (CX3CR1-GFP, lin negative (CD11b, CD1c, CD3, Gr-1, NK.1.1, B20.3)) of purity ≥ 90% were admixed with ≥ 1 × 10^6 whole C57BL/6 bone marrow. Mixed bone marrow was injected intravenously by paraorbital injection into irradiated (> 1000 rads) naive C57BL/6 recipients.

Immunosuppression assays and IL-10 enzyme-linked immunosorbent assay (ELISA)
Sorted CX3CR1 cell populations from naive and tumor-bearing mice (purity ≥ 90%) were cocultured at the indicated ratios with 10^6 naive splenocytes. Plated splenocytes were stimulated with 1 µg anti-CD3 (145-2C11; BioXCell, West Lebanon, NH, USA) and culture supernatants were collected after 72 h and analyzed for interferon-γ production using murine DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). In addition, sorted populations were plated at 1.5–3 × 10^6 cells per well with or without 100 ng/ml lipo polysaccharide stimulation (Sigma Aldrich, St Louis, MO, USA) and cultured for 72 h. Supernatants were assessed for IL-10 production per 10^6 cells using a murine DuoSet ELISA (R&D Systems).

Therapeutic depletion of IL-10-expressing cells
IL-10 reporter-positive cells were depleted in tumor-bearing IL-10BiT mice through intraperitoneal injection of anti-Thy1.1 antibody (500 µg) provided by Edward Usherwood (Dartmouth Medical School) on days 7 and 10 post tumor injection. Mice were killed on days 11 and 15 to assess peritoneal cell numbers and composition by flow cytometry. Additional mice were monitored and killed when they became moribund. Statistical analysis of survival was determined by Kaplan–Meier analysis.

Statistical analyses
All data were analyzed with Prism (GraphPad Software, La Jolla, CA, USA). Data were considered significant for P-values <0.05 when performing a two-tailed t-test or Kaplan–Meier analysis. Differences are noted as *P < 0.05, **P < 0.01 and ***P < 0.001. Sample sizes for each experiment are noted in the text. Values are shown as means ± s.d.

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