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Protective Role of the Inflammatory CCR2/CCL2 Chemokine Pathway through Recruitment of Type 1 Cytotoxic γδ T Lymphocytes to Tumor Beds

Telma Lancã¡,* Maria Fernanda Costa,† Natacha Goûnuelles-Sousa,* Margarida Rei,* Ana Rita Grosso,* Carmen Penido,†,‡ and Bruno Silva-Santos*§

Tumor-infiltrating lymphocytes (TILs) are important prognostic factors in cancer progression and key players in cancer immunotherapy. Although γδ T lymphocytes can target a diversity of tumor cell types, their clinical manipulation is hampered by our limited knowledge of the molecular cues that determine γδ T cell migration toward tumors in vivo. In this study we set out to identify the chemotactic signals that orchestrate tumor infiltration by γδ T cells. We have used the preclinical transplantable B16 melanoma model to profile chemokines in tumor lesions and assess their impact on γδ T cell recruitment in vivo. We show that the inflammatory chemokine CCL2 and its receptor CCR2 are necessary for the accumulation of γδ TILs in B16 lesions, where they produce IFN-γ and display potent cytotoxic functions. Moreover, CCL2 directed γδ T cell migration in vitro toward tumor extracts, which was abrogated by anti-CCL2 neutralizing Abs. Strikingly, the lack of γδ TILs in TCRδ-deficient but also in CCR2-deficient mice enhanced tumor growth in vivo, thus revealing an unanticipated protective role for CCR2/CCL2 through the recruitment of γδ T cells. Importantly, we demonstrate that human Vδ1 T cells, but not their Vδ2 counterparts, express CCR2 and migrate to CCL2, whose expression is strongly deregulated in multiple human tumors of diverse origin, such as lung, prostate, liver, or breast cancer. This work identifies a novel protective role for CCL2/CCR2 in the tumor microenvironment, while opening new perspectives for modulation of human Vδ1 T cells in cancer immunotherapy. The Journal of Immunology, 2013, 190: 000–000.

T he recent success of the adoptive transfer of T lymphocytes to cancer patients has provided a great boost to immunotherapy (1). Consequently, there is increasing interest in manipulating lymphocyte populations capable of eliminating tumor cells. γδ T cells, which constitute 1–10% of human PBLs, are endowed with potent cytotoxic properties and have been shown to be important players in cancer immune surveillance (2). The antitumor role of γδ T cells has also been attributed to their rapid and abundant provision of cytokines like IFN-γ and TNF-α (3, 4). Importantly, tumor cell recognition by γδ T cells does not depend on MHC-mediated Ag presentation (2, 5), which may represent a key advantage to target advanced stages of cancer progression. Moreover, γδ T cells express high levels of a variety of activating NK receptors, thus conferring on them hybrid T cell/NK tumor recognition machinery (6).

Seemial work with animal models has shown that the genetic absence of γδ T cells rendered mice significantly more susceptible to chemically induced (7), transplantable (3), spontaneous (8), and transgenic (9) tumors in vivo. Furthermore, extensive in vitro studies with human γδ T cells have demonstrated they can efficiently kill tumor cells of multiple tissue origins (10, 11).

The antitumor properties of γδ T cells have been exploited in a series of cancer clinical trials focused on Vγ9Vδ2 cells, the major subset in human peripheral blood. As these cells are specifically reactive to nonpeptidic prenyl pyrophosphates ("phosphoantigens") (12), which are available in clinical grade, they can be selectively activated in vivo or expanded in vitro for adoptive cell transfer (ACT). Although a number of trials obtained some promising results (10–33% objective responses in cancer patients) with the endogenous activation of Vγ9Vδ2 cells, the overall clinical performance of γδ T cells, particularly in ACT protocols, has been modest (11, 13).

A key parameter in these immunotherapy strategies is the migration and homing of effector T cells to the tumor site, which is mostly controlled by chemokines and their receptors (14). However, the mechanisms that direct γδ T cells toward tumors remain largely unknown. This is of utmost relevance, not only for Vγ9Vδ2 cells but also for their Vδ1 counterparts (typically 10–35% of γδ PBLs), which are known to expand significantly in cancer patients (15) and to infiltrate multiple human tumor types (16–20) while also producing IFN-γ and being strongly cytotoxic against tumor cells. In fact, our recent work has characterized a population of NKp30+ Vδ1 cells with enhanced tumor-killing capacity, compared with that of Vγ9Vδ2 cells (21), which may have an important application in cancer immunotherapy. Moreover, tumor-infiltrating Vδ1 cells were numerically enriched and displayed enhanced cytotoxicity when compared with Vδ2 cells in a collection of 74 primary cutaneous melanomas (19). In addition, in hematological malignancies, Vδ1 cells have been shown to be
the main responding γδ T cell subset against acute leukemic blasts, and the importance of this antitumor reactivity is reflected in the strong correlation between donor-derived γδ T cells and long-term survival of patients who underwent allogeneic bone marrow transplantation (22, 23).

Various chemokines have been implicated in γδ T cell migration (24, 25). Human γδ T cells have been shown to migrate in vitro toward CCL2, CCL3, CCL4, CCL5, CXL10, CXCL11, and CXCL12 (25–28). In vivo studies with mice have further allowed dissection of the role of homeostatic or inflammatory chemokines, depending on whether they directly γδ T cell migration in the absence or presence of inflammatory stimuli. Thus, particular chemokines are responsible for the migration of thymic-derived γδ T cells to particular tissues at late embryonic or perinatal stages, leading to the establishment of local intraepithelial compartments (29). Namely, skin homing is controlled by CCL27 (27), whereas gut homing is directed by CCL25 (30). In contrast, CCL2 (31, 32), CCL25 (33), and CXCL10 (34) have been shown to control murine γδ T cell migration upon allergic (31, 33) or microbial challenge (32, 34). Strikingly, it is yet unknown which chemokine(s) among this large family of cytokines mediate γδ T cell recruitment to tumor beds. In this study, we have used a widely validated (3, 35–40) preclinical tumor model, based on the transplantable B16 melanoma cell line, to identify the chemokines and chemokine receptors involved in γδ T cell infiltration in vivo. Importantly, these data were validated in activation and migration assays using both murine and human γδ T cells, which allowed us to establish a critical role for the CCR2/CCL2 pathway in tumor infiltration by γδ T cells and its potential impact on Vδ1 T cell–based immunotherapy.

Materials and Methods

Mice
Female mice were used at 6–10 wk of age. C57BL/6 (B6) mice were obtained from Charles River or The Jackson Laboratory; B6.Tcrd−/− and B6.Ccr2−/− mice were obtained from The Jackson Laboratory; B6.Ccr2−/− mice were provided by the Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil).

In vivo B16 tumor model
A total of $5 \times 10^4$ B16-F0 melanoma cells (American Type Culture Collection) were injected (in 50 µl PBS) s.c. into the backs of anesthetized mice. Tumor size was measured with a caliper in two perpendicular dimensions every 2–3 d.

Analysis of tumor-infiltrating leukocytes
Tumors were harvested and treated for 30 min (in shaker at 37˚C) with 5 mM HMB-PP (Echelon Biosciences) in the presence of 100 U/ml IL-2. The top wells were loaded with 5 × 10^5 γδ T cells. After 3 h incubation at 37˚C, filters were removed and cells that migrated to the lower chamber were counted. Chemotaxis index was calculated as the ratio of cells migrating toward rCCL2 (or the tumor extracts) and cells randomly migrating across the membrane.

Real-time quantitative PCR
Total RNA was extracted and reverse transcribed, and quantitative PCR was performed as described (21). Primer sequences and methodology details are available upon request.

Analysis of human γδ T cells
PBLs were isolated as described (21), and Vδ1 and Vδ2 T cell subsets were either analyzed ex vivo or sorted for in vitro cultures. Cells were activated for 7 d (in RPMI 1640 medium) with 4 µg/ml PHA (Sigma-Aldrich) or 10 nM HMB-PP (Echelon Biosciences), in the presence of 100 U/ml IL-2. CCR2 surface expression was assessed by flow cytometry using the CCR2-PerCP/Cy5.5 mAb (BioLegend).

Bioinformatic analysis
Microarray data were obtained from previously published studies using the Human Exon 1.0 ST platform and available in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) for breast cancer (GSE3692), colorectal cancer (GSE24551), gastric cancer (GSE27342), liver cancer (GSE12941), lung adenocarcinoma (GSE12236), oral squamous cell carcinoma (GSE25099), ovarian cancer (GSE29156), and primary and metastatic prostate cancer (GSE21034). Determination of significant deregulation was determined by AltAnalyze application (41), using a moderated t test with Benjamin–Hochberg p value correction.

Statistics
The statistical significance of differences between populations was assessed using the Student t test and is indicated when significant as *p < 0.05, **p < 0.01, and ***p < 0.001.

Study approval
All experiments were performed in compliance with relevant laws and institutional guidelines and have been approved by local ethics committees.

Results

Cytotoxic γδ T cells infiltrate B16 lesions and delay tumor growth in vivo
We selected the murine B16 melanoma cell line for transplantation, given its extensive validation as a relevant preclinical tumor model (3, 35–40) and the solid evidence for γδ T cell infiltration in human melanoma (19). Palpable tumors were detected 7–9 d after s.c. injection of B16-F0 cells, and they were measured until day
At 14, at which time tumor infiltrates were analyzed by flow cytometry. Approximately 1 in 40 infiltrating lymphocytes was found to be a γδ T cell mostly expressing either Vγ4 or Vγ1 TCR chains (Fig. 1A), like lymph node (circulating) but not skin-resident γδ T cells (42). γδ TILs also expressed CD27 (Fig. 1B) and ifng (Fig. 1C), characteristic of the most abundant peripheral γδ T cell subset (43). IFN-γ (but not IL-17 or IL-4) production was also detected in tumor-draining lymph nodes (Supplemental Fig. 1A and data not shown). Importantly, γδ-TILs expressed the cytotoxic effector molecules pfnb and gzmb (Fig. 1C) and were able to kill B16 tumor cells in vitro (Fig. 1D). Furthermore, mice lacking γδ T cells developed significantly larger tumors than did WT controls at each time point analyzed (Fig. 1E), thus indicating accelerated tumor cell growth in vivo (Supplemental Fig. 1B). These results, which are consistent with and further expand on a previous study by Gao et al. (3), demonstrate a nonredundant protective role for cytotoxic and IFN-γ–producing (type 1) γδ-TILs in the B16 tumor model, making it a particularly suitable system to analyze the in vivo requirements of γδ T cell recruitment.

**CCR2 ligands accumulate in B16 lesions lacking γδ T cells**

We next used this tumor model to identify the chemokines involved in γδ T cell recruitment in vivo. We hypothesized that B16 lesions in TCRδ-deficient mice would accumulate chemokines normally consumed by γδ T cells during tumor infiltration (in WT mice). We therefore used a chemokine array to analyze protein extracts derived from B16 tumors isolated (at day 14 post transplantation) from WT or TCRδ-deficient mice. Among the 25 chemokines profiled (Fig. 2, Supplemental Fig. 2), the CCR2 ligands CCL2 and CCL12 were significantly overexpressed in TCRδ-deficient mice (Fig. 2). This pattern was highly selective: in particular, it did not extend to CXCL9 and CXCL11 (Fig. 2) or CXCL16 (Supplemental Fig. 2), all implicated in CD8+ TIL recruitment; or to CXCL12 (Fig. 2) and CCL19 (Supplemental Fig. 2), typically associated with lymph node homing. Furthermore, CCL25 and CCL27, previously shown to control γδ T cell migration under homeostatic or inflammatory conditions (24, 33), were also equally expressed in WT and TCRδ-deficient mice (Supplemental Fig. 2). These data suggested a potential selective role for CCR2 ligands in governing γδ T cell recruitment to B16 tumors.

**Murine γδ T cells express CCR2 and migrate toward CCL2 in vitro**

Building on previous results, we focused on the components of the CCR2/CCL2 pathway and assessed their relevance for γδ T cell migration. Surface CCR2 expression was detected on 20–30% of...
γδ T cells isolated from spleen or peripheral lymph nodes (Fig. 3A). Both Vγ1+ and Vγ4+ cells constitutively expressed CCR2 (Fig. 3B and data not shown) and markedly upregulated it upon activation (Fig. 3B, 3C). Importantly, when purified γδ T cells were incubated with increasing doses of recombinant CCL2, they responded robustly by fluxing calcium (Fig. 3D) and migrating in a Transwell system (Fig. 3E). To establish a direct link to the tumor model, we prepared protein extracts from excised B16 tumors (grown in vivo) and used them as “bait” for purified γδ T cells in vitro. We observed a striking migration (chemotaxis index ∼30) of γδ T cells toward the B16 protein extracts and, critically, this was significantly inhibited by neutralizing anti-CCL2 mAbs (Fig. 3F). These data clearly demonstrate that murine γδ T cells use the CCR2/CCL2 chemokine pathway to migrate toward CCL2-rich B16 tumors in vitro.

**Human Vδ1 T cells express CCR2 and migrate toward CCL2 in vitro**

Following these results in mice, we next investigated the potential role of CCR2/CCL2 in the migration of human γδ T cells. Total γδ PBLs clearly responded to increasing doses of recombinant CCL2 in vitro (Fig. 4A, Supplemental Fig. 3). Because human γδ-PBLs contain two major subsets that express either Vδ1 (5–30%) or Vδ2 (60–95%) chains, we determined CCR2 expression in both populations. Of interest, only Vδ1 PBLs expressed CCR2 either constitutively (Fig. 4B) or after activation with the mitogen PHA (in the presence of IL-2) (Fig. 4C). By contrast, Vδ2 PBLs lacked CCR2 expression under both conditions, as well as upon activation with the specific Vγ9Vδ2-TCR agonist HMB-PP (Fig. 4C, lower panel). Consistent with these data, Vδ1 (but not Vδ2) PBLs migrated (chemotaxis index >1) toward CCL2 in vitro (Fig. 4D). These data validate our findings in mice and suggest that CCR2/CCL2 may play a key role in the recruitment of human Vδ1 PBLs.

**CCL2 expression is deregulated in multiple human tumor types**

We next investigated how Ccl2 levels were regulated in human malignancies. We took a bioinformatics approach to inquire into public genome-wide Human Exon 1.0 ST microarray data. Comparison of tumors and corresponding healthy tissues revealed a significant deregulation of Ccl2 levels in most (6 of 9) tumor types analyzed (see Materials and Methods for details). On one hand, oral squamous cell carcinoma, breast cancer, and primary prostate cancer showed significant upregulation of Ccl2 expression (Fig. 4C).

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**FIGURE 2.** CCL2 and CCL12 accumulate in B16 tumor lesions lacking γδ T cells. Chemokine concentrations in protein extracts from day 14 tumors isolated from WT or Tcrd−/− mice, as measured by Quantibody Chemokine Mouse Array (Raybiotech). Bars indicate SD. n = 3; *p < 0.05, **p < 0.01.

**FIGURE 3.** Murine γδ T cells express CCR2 and migrate toward CCL2 in vitro. (A) Ex vivo CCR2 expression in splenic and lymph node γδ T cells (gated on CD3+ TCRγδ+ lymphocytes) from WT C57BL/6 mice was assessed by flow cytometry. Each dot represents one mouse. (B) CCR2 expression in resting (gray filled) and activated (black line) Vγ1+ T cells from lymph nodes. (C) CCR2 expression in activated Vγ1+ (gray filled) or Vγ4+ (black line) splenocytes. CD3+ TCRγδ+ cells were FACS sorted from spleen or lymph nodes; activated on plate-bound anti-CD3ε (3 μg/ml) and soluble anti-CD28 (3 μg/ml) mAbs for 48 h; and stained for Vγ1+ TCR, Vγ4+ TCR, and CCR2 for FACS analysis. Indicated percentages of CCR2+ cells were defined relative to negative control (without anti-CCR2 mAb). (D) Calcium influx assay on MACS-sorted γδ T cells from spleen and lymph nodes exposed to indicated concentrations of recombinant CCL2 in HBSS buffer. RFU, relative fluorescence units. (E and F) In vitro chemotaxis assays of MACS-sorted γδ T cells from spleen and lymph nodes toward recombinant CCL2 (E) or protein extracts derived from day 14 tumors of WT mice (F) in Transwell plates. Where indicated, anti-CCL2 neutralizing Ab was added. Bars indicate SD. **p < 0.01, ***p < 0.001.
5A). On the other hand, metastatic prostate cancer, liver cancer, and lung cancer displayed reduced Ccl2 levels (Fig. 5B). These data reveal a striking heterogeneity of Ccl2 levels in human tumors that may have critical implications for Vδ1 T cell recruitment or infiltration in immune surveillance and immunotherapy.

**Discussion**

This study demonstrates that γδ T cells use the CCR2/CCL2 pathway to migrate toward tumors, where they exert a key non-redundant antitumor function. It also reveals a protective role for inflammatory CCR2/CCL2 chemokine signals, which have been mostly associated with promotion of tumor growth through recruitment of myeloid cells, particularly monocytes/macrophages (44, 45). Our analysis of tumor infiltrates in Ccr2−/− mice data confirmed these findings, while also showing a striking reduction in myeloid-derived suppressor cells, which can actively suppress antitumor CD8+ (46) and γδ T cells (T. Lanka and B. Silva-Santos, unpublished observations). However, and strikingly, the overall phenotype of Ccr2−/− mice (compared with that in WT controls) was enhanced tumor growth, which suggests a dominance of CCR2/CCL2 antitumor functions over their protumor effects in this preclinical model. This finding is consistent with a recent report showing increased tumor growth and metastasis in Ccl2−/− mice, associated with the infiltration of CD8+ and CD4+ T cells, but γδ T cells were not analyzed (47).

The balance between antitumor and protumor roles of CCL2 may depend on the composition of the tumor leukocyte infiltrates (relative abundance of each subset), as well on intrinsic CCL2 levels, because different leukocyte populations may have distinct thresholds for CCL2-mediated accumulation. This idea should be further investigated in various preclinical models and, most importantly, associated with clinical data on tumor infiltrates upon evaluation of CCL2 levels in situ.

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**FIGURE 4.** Human Vδ1 T cells express CCR2 and migrate toward CCL2 in vitro. (A) Relative fluorescence units (RFU) from calcium influx assays on in vitro expanded γδ T cells from human peripheral blood exposed to increasing concentrations of recombinant CCL2. (B and C) CCR2 expression on freshly isolated (B) or activated (C) Vδ1 and Vδ2 T cells from five healthy donors. Activation was performed for 7 d with either PHA or HMB-PP, always in the presence of IL-2. (D) Vδ1 or Vδ2 T cells were FACS sorted from peripheral blood of healthy donors and activated in vitro for 1 wk. Chemotaxis (Transwell) assays toward recombinant human CCL2 were performed for 3 h. Each dot represents one blood donor. *p < 0.05.
Although CCL2 has been previously implicated in mediating the recruitment of CD8+ T cells (47, 48), in our study CD8+ T cell numbers were unaffected in B16 lesions of Ccr2−/− or Ccl2−/− mice. It is possible that posttranslational inactivating modifications of CCL2 may prevent effective CD8+ T cell recruitment in WT mice, thus permitting rapid tumor growth. This suggestion follows from an elegant study showing that intratumoral reactive nitrogen species induce CCL2 nitration that prevents CD8+ T cell infiltration (49).

Notwithstanding, our study provides, to our knowledge, the first evidence for an antitumor role of CCL2 linked to γδ T cell recruitment, representing an important addition to the pleiotropic functions of CCL2 in the tumor microenvironment. It is notable that despite γδ T cells accounting for < 3% of leukocyte infiltrates in B16 lesions, their absence (in Tcrd−/− mice) results in a significant accumulation of CCL2 (Fig. 2).

Recently, members of our team have shown that CCL25 and its receptor CCR9 control the migration and lung recruitment of a subset of γδT cells committed to IL-17 production in a model of allergic pleurisy (33). In fact, the effect of CCL25 was selective to IL-17+ γδ cells—namely, not extending to IFN-γ-producing γδ T cells. In view of our data presented in this article, together with other studies by our team (31), we propose that, instead of CCR9/CCL25, the key determinants of murine IFN-γ+ γδ cell migration are CCR2/CCL2.

Importantly, our study is, to our knowledge, the first to identify a chemokine pathway responsible for in vivo γδ T cell recruitment in a tumor setting. Furthermore, our demonstration that human Vδ1 PBLs also migrate toward CCL2 stresses the importance of investigating the role of this chemokine in Vδ1 T cell recruitment to inflammatory sites in human disease. In this context, our bioinformatics analyses revealed a deregulation of Ccr2 expression in multiple human tumor types. Oral squamous cell carcinoma, breast cancer, and primary prostate cancer showed significant upregulation of Ccr2 expression (Fig. 5A), which may render

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**FIGURE 5.** Ccl2 expression is deregulated in various human tumor types. Ccl2 expression levels in human tumor types (gray) and corresponding normal tissue (black) according to Human Exon 1.0 ST microarray data (see Materials and Methods for details). Data were analyzed using AltAnalyze with a moderated t test by the Benjamini–Hochberg p value correction (*adjusted p value < 0.05).

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**FIGURE 6.** The CCR2/CCL2 pathway is required for γδ T cell recruitment to B16 tumors in vivo. WT, Ccl2−/−, or Ccr2−/− mice were injected s.c. with 5 × 10^6 B16-F0 cells and sacrificed after 14 d. Numbers of total γδ (gated on CD3+ TCRγδ+ lymphocytes) (A and B) or CD8+ and CD4+ (C) TILs. (D) Tumor size, in experiments carried out in two animal facilities in Lisbon, Portugal (Instituto de Medicina Molecular) and Rio de Janeiro, Brazil (Fundação Oswaldo Cruz). Each dot represents one tumor/mouse. *p < 0.05.
these tumors particularly susceptible to V61 T cell recruitment. This finding is fully consistent with a study that showed very abundant V61 T cell infiltration in breast and prostate tumors (18). In contrast, liver and lung cancer displayed reduced Ccl2 levels (Fig. 5B), which is likely to confer resistance to V61 T cell infiltration. In fact, it is interesting to speculate that downregulation of Ccl2 expression may constitute an evasion strategy against V61 T cell–mediated immune surveillance. Along these lines, the prostate cancer series showed a strong reduction of Ccl2 expression between primary and metastatic tumors (Fig. 5A, 5B). Future studies should test the association between V61 T cell infiltrates and Ccl2 expression in situ, because CCL2 expression in tumors may constitute an important prognostic factor for future V61 T cell–based cancer immunotherapies.

Of note, human γδ T cells express other chemokine receptors like CCR5 and CXCR3, and have been shown to also migrate in vitro toward CCL3, CCL4, CCL5, CXL10, CXCL11, and CXCL12 (25–28). Thus, it will be worthwhile to assess the relative contributions of these pathways, in addition to CCR2/CCL2, to tumor infiltration by γδ T cells, particularly V61 PBLs.

Regarding the potential of V61 PBLs for ACT-based cancer immunotherapy, we have recently shown that this subset is uniquely endowed, among γδ T cells, with the capacity to express natural cytotoxicity receptors that enhance their killing of hematological (21) and solid (D.V. Correia and B. Silva-Santos, unpublished observations) tumors. Moreover, V61 cells were the dominant tumor-infiltrating T cell population found in a collection of 74 primary cutaneous melanomas (19). However, other studies by Peng and colleagues (18) detected poor infiltration of V61 T cells, as well as total γδ T cells (50), in melanoma biopsy specimens. By contrast, γδ T cells, and V61 cells in particular, were abundant in breast and prostate tumors. In vitro functional assays demonstrated that V61 cells derived from breast cancer biopsies inhibited the maturation and function of dendritic cells, and suppressed proliferation and IL-2 production of CD4+ T cells derived from breast cancer patients (50). These findings raise interesting questions for future investigation: Do distinct suppressive versus cytotoxic V61 T cell subsets exist? Do these differentially infiltrate tumor types, such as melanoma or breast cancer? Can the (breast or prostate) tumor microenvironment manipulate V61 T cells toward immunosuppression? In any case, we believe V61 T cells will be a key population to consider in developing cancer immunotherapy strategies.

On the basis of results presented in this article, we propose CCL2 as a promising target for manipulation of V61 T cells. Importantly, as blocking anti-CCL2 Abs are being evaluated in prostate and ovarian cancer clinical trials (51), our data also indicate caution regarding the unexpected effects of this therapeutic strategy. We therefore suggest that future research should aim at integrating the effects of CCL2 on tumor-infiltrating leukocyte subsets with strikingly distinct functions (52) in both preclinical models and cancer immunotherapy trials.

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Disclosures
The authors have no financial conflicts of interests.


