A role for CCL2 in both tumor progression and immunosurveillance

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Abbreviations: IM, inflammatory monocyte; MCA, 3-methylcholanthrene; MCP1, monocyte chemoattractant protein 1; MDSC, myeloid-derived suppressor cell; TAM, tumor-associated macrophage; WT, wild-type

The chemokine CCL2, which is best known for its chemotactic functions, is expressed not only by immune cells, but also by several types of malignant and stromal cells. CCL2 has been shown to exert both pro- and anti-tumor effects. However, recent results demonstrate a main role for CCL2 in tumor progression and metastasis, suggesting that this chemokine may constitute a therapeutic target for anticancer drugs. Mammary carcinoma models, including models of implantable, transgenic, and chemically-induced tumors, were employed in the setting of Ccl2 or Ccr2 knockout mice or CCL2 neutralization with a monoclonal antibody to further investigate the role of the CCL2/CCR2 signaling axis in tumor progression and metastatic spread. In our implantable tumor models, an anti-CCL2 monoclonal antibody inhibited the growth of primary malignant lesions in a biphasic manner and reduced the number of metastases. However, in Ccl2−/− or Ccr2−/− mice developing implanted or transgenic tumors, the number of pulmonary metastases was increased despite a reduction in the growth rate of primary neoplasms. Transgenic Mtag.Ccl2−/− or Mtag.Ccr2−/− mice also exhibited significantly earlier disease onset. In a chemical carcinogenesis model, anti-CCL2 monoclonal antibody inhibited the growth of established lesions but was ineffective in the tumor induction phase. In contrast to previous studies indicating a role for CCL2 in the establishment of metastases, we have demonstrated that the absence of CCL2/CCR2-signaling results in increased metastatic disease. Thus, the CCL2/CCR2 signaling axis appears to play a dual role in mediating early tumor immunosurveillance and sustaining the growth and progression of established neoplasms. Our findings support the use of anti-CCL2 therapies for the treatment of established breast carcinoma, although the complete abrogation of the CCL2 signaling cascade may also limit immunosurveillance and support metastatic spread.

Introduction

Chemokines are chemotactic cytokines that facilitate cell migration by directing their movement along concentration gradients.1 Chemokines have pleiotropic and context-dependent effects on the pathobiology of cancer, including tumor-promoting and/or tumor-suppressing activities. CCL2 (also known as monocyte chemoattractant protein 1, MCP1) is a chemokine that exerts potent chemotactic, stimulatory, and mitogenic effects on mononuclear cells.2 Elevated CCL2 expression levels in the tumor microenvironment3 as well as high circulating concentrations of this chemokine have been associated with poor prognosis in breast carcinoma patients.4 CCL2 has also been shown to stimulate the migration of mammary carcinoma cell lines5 and to mediate the recruitment of specific monocyte populations that support the establishment of metastatic disease.6 The secretion of CCL2 by tumor-infiltrating immune cells, malignant cells, and other stromal cells suggests that CCL2 mainly supports tumor progression.7 Together with the capacity of CCL2 to attract tumor-promoting and immunosuppressive cell types8 or their precursors, provides a strong rationale for attempting to therapeutically reduce CCL2 levels in the setting of established neoplasms.9 However, CCL2 may also act to attract antitumor immune cells10–12 and is required for efficient immunosurveillance, implying that the inhibition of CCL2 may promote neocarcinogenesis as well as the development of metastases. Thus, although there is evidence that CCL2 may mediate both pro- and antitumor effects, accumulating data suggest that the tumor-promoting effects of CCL2 outweigh its potential antitumor activity, at least in experimental model systems based on implantable cancers. These models do not necessarily recapitulate the slow and chronic nature of natural oncogenesis.13 Studies addressing...
the long-term effects of CCL2-targeting interventions and their influence on the immunosurveillance of primary cancers and metastases are indeed lacking. Thus, further investigation is required before the CCL2/CCR2 axis becomes an established target for the treatment of breast carcinoma and/or other cancers.

Tumor-induced immunosuppressive cascades have been widely reported in both patients and murine tumor models and are likely to constitute a significant impediment against natural immunosurveillance as well as antitumor immunotherapeutic regimens.14 Myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) have previously been shown to be major drivers of impaired immune system functions in tumor-bearing hosts, to significantly interfere with antitumor immunity,13,15 and to promote tumor growth through a number of mechanisms.16 MDSCs are characterized as a heterogeneous mixture of myeloid cells that express both CD11b and Gr1 on their surface (in mice) and mediate immunosuppressive effects in tumor-bearing hosts, both systemically and in the tumor microenvironment. Recent findings have demonstrated that tumor-induced MDSCs secrete elevated levels of CCL2.17 Since CCL2 is a major chemotactic factor for monocytes, inhibition of the CCL2/CCR2 axis should decrease the recruitment of both TAMs and MDSCs to the tumor site, hence reducing primary tumor growth.18 However, CCL2 also plays a central role in immunosurveillance. Thus, its inhibition may de facto favor neocarcinogenesis as well as the establishment of metastases.

We took advantage of a variety of mammary carcinoma murine models, including implantable, transgene-driven and 3-methylcholanthrene (MCA)-induced cancers, to investigate how inhibiting the CCL2/CCR2 signaling axis affected primary tumor growth and metastatic spread. Our findings support a model whereby CCL2 plays a dual function in promoting both immunosurveillance and tumor progression. The balance between these two activities of CCL2 appears to be skewed toward the tumor-augmentation, at least in established disease settings. In addition, if CCL2 or CCR2 are lacking in the tumor induction phase, metastatic disease is favored. These data expand upon recent results supporting the CCL2-mediated promotion of metastatic tumor growth. Our findings support the potential of CCL2-targeted therapies to treat patients bearing established breast carcinomas but also underscore that the timing and/or intensity of CCL2-inhibitory strategies is critical in determining the influence of such interventions on metastatic disease.

Results

Reduced growth of primary tumors but increased metastatic spread in Ccl2 or Ccr2 knockout mice. To investigate the role of host-derived CCL2 on tumor progression, we orthotopically implanted syngeneic AT-3 mammary carcinoma cells into wild-type (WT), Ccl2−/− or Ccr2−/− C57BL/6 mice. The growth of primary AT-3 tumors was significantly retarded in Ccl2−/− and Ccr2−/− mice as compared with their WT counterparts (Fig. 1A). However, the enumeration of spontaneous pulmonary metastases revealed a significant increase in the metastatic burden among both Ccl2−/− (P = 0.0109) and Ccr2−/− (P = 0.0002) mice, as compared with WT animals (Fig. 1B), with the strongest phenotype being observed for the Ccr2−/− genotype. Thus, following the orthotopic implantation of syngeneic malignant cells in hosts lacking CCL2 or CCR2 expression, the numbers of lung metastases was increased despite a reduction in primary tumor growth.

Reduction in tumor-induced systemic MDSC accumulation. During tumor progression, MDSCs accumulate both systemically and at the tumor site. MDSCs play a significant role in tumor progression and host immunosuppression and have previously been shown to produce high levels of CCL2.17 We therefore investigated whether defects in the host CCL2/CCR2 signaling axis would influence the systemic accumulation of this immunosuppressive cell type. The tumor-induced splenomegaly ensuing the orthotrophic implantation of AT-3 cells is primarily due to MDSCs.18 We observed a decreased splenomegaly in Ccl2−/− and Ccr2−/− mice implanted with AT-3 cells as compared with WT animals (Fig. 1C). This turned out to be due for the most part to a reduced accumulation of CD11b+Gr1+ MDSCs (Fig. 1D). The lack of CCL2 or CCR2 expression in the host resulted therefore in a reduced accumulation of systemic (spleen) tumor-promoting and immunosuppressive MDSCs.

Reduced proportion of CD11b+ cells in malignant lesions developing in Ccl2−/− or Ccr2−/− mice. Since CCL2 is a major pro-inflammatory mediator and the tumor-infiltrating leukocyte (TIL) profile has a significant influence over tumor progression, we investigated whether changes in the TIL profile would account for the inhibition of primary tumor growth observed in CCL2- and CCR2-deficient mice. The lack of CCL2 in the host (Ccl2−/− mice) as well as the inability to respond to it (Ccr2−/− mice) resulted in decreased proportions of CD11b+ and CD11b+Gr1+ (MDSC) cells within primary neoplastic lesions (Fig. 1E and F). Conversely, there were no significant differences in the frequency of CD4+, CD8+, or γδ T cells, B cells, CD11c+ cells, natural killer (NK), or NKT cells observed within the digests of tumors developing in Ccl2−/−, Ccr2−/−, or WT mice (data not shown). Thus, Ccl2−/− or Ccr2−/− tumor-bearing hosts manifested a preferential inhibition in the recruitment of monocytes, macrophages, or MDSCs into the tumor environment.

Anti-CCL2 monoclonal antibody inhibits primary tumor growth in a biphasic manner. We employed an anti-CCL2 monoclonal antibody to further investigate the role of CCL2 in tumor progression and to determine whether this intervention may have therapeutic applications. Two different implantable tumor models were used in this aim, namely the implantation of AT-3 or 4T1 mammary carcinoma cells to the mammary fat-pad of C57BL/6 and BALB/c mice, respectively. Once tumors became palpable (established), mice were treated weekly with anti-CCL2 monoclonal antibody. At the end of the experiments, we observed a significant inhibition in the growth of both AT-3 (P = 0.0115, at day 42 post-implantation) and 4T1 (P = 0.0068, at day 29 post-implantation) primary lesions in anti-CCL2 monoclonal antibody-treated mice as compared with animals receiving isotype control antibody (Fig. 2A).

However, we noted that mice treated with anti-CCL2 monoclonal antibody displayed a significant, very early increase in tumor volume upon the initiation of therapy as compared with
observed in numerous experiments (n > 12), altogether including more than 100 mice per group. Of note, the early increase in tumor volume in response to anti-CCL2 monoclonal antibody rapidly reverted over time, ultimately resulting in the inhibition of primary tumor growth. We hypothesize that this finding reflects the inhibition of CCL2-mediated immunosurveillance.

controls animals (Fig. 2B). Such an increase was slight due to the small size of malignant lesions but was highly significant in most experiments (e.g., in the AT-3 model at day 9 post-implantation, \( P < 0.0001 \); in the 4T1 model at day 9 post-implantation, \( P = 0.0029 \)). Despite the limitations associated with the assessment of small tumor sizes by means of a caliper, this phenomenon was
**Figure 2.** For figure legend, see page e25474-5.
Thus, although overall anti-CCL2 monoclonal antibody inhibited the growth of primary mammary carcinoma lesions, this therapeutic approach initially enhanced tumor growth and was therefore considered to act in a biphasic manner.

To discard the possibility that the early increase in tumor volume observed in response to anti-CCL2 monoclonal antibody would be due to an increase in the number of TILs, a histological investigation of tumors obtained 2 and 4 d after the initiation of anti-CCL2 therapy was conducted (Fig. 2C). As determined by a trained veterinary pathologist, at these early time points, tumors were predominantly infiltrated by lymphocytes, with low numbers of neutrophils, plasma cells, and macrophages. Although the precise composition of TILs was difficult to quantify by this approach, we observed no obvious increase in the abundance of TILs that could account for the increased tumor size resulting from anti-CCL2 therapy. Moreover, there were no significant differences in the morphology of tumors isolated 2 and 4 d after the administration of anti-CCL2 monoclonal antibody (Fig. 2C; data not shown). Due to the small size of malignant lesions, a cytofluorometric analysis of TILs could not be conducted at this stage of tumor progression. We concluded that the early increase in tumor size following anti-CCL2 therapy was not a result of increased inflammatory cell infiltrate or edema.

Anti-CCL2 monoclonal antibody reduces the amount of spontaneous pulmonary metastases. In view of previous reports demonstrating a reduction in the pulmonary metastatic burden in response to anti-CCL2 monoclonal antibody, the results obtained in Ccl2−/− or Ccr2−/− mice were somewhat surprising. We therefore went on to investigate whether the administration of anti-CCL2 monoclonal antibody to our murine models of mammary carcinoma could recapitulate what had been reported in other model systems. In agreement with previous reports, we demonstrated a significant decrease in the number of spontaneous pulmonary metastases in response to the administration of anti-CCL2 mAb to mice bearing established 4T1 (P = 0.0006) or AT-3 (P = 0.0311) mammary tumors (Fig. 2D). Thus, the discrepancy in the effects on metastatic burden of the genetic abrogation of Ccl2 or Ccr2 and the monoclonal antibody-mediated blockade of CCL2/CCR2-conveyed signals may depend on the timing of the interventions and/or on the degree of CCL2/CCR2 inhibition attained.

Anti-CCL2 therapy is efficient against established, carcinogen-driven tumors but not if administered during tumor initiation. To investigate the possible influence of anti-CCL2 monoclonal antibody on the immunosurveillance of early tumor growth or tumor initiation, a long-term experiment based on a model of chemical carcinogenesis was conducted. To this aim, groups of MCA-challenged mice were treated with anti-CCL2 monoclonal antibody over the tumor induction period (1–10 wk); once tumors were established (10–20 wk); or continuously during tumor progression (1–20 wk) (Fig. 3). In this context, anti-CCL2 therapy resulted in significant therapeutic effects only when administered to mice bearing established tumors (10–20 wk, P = 0.049 as compared with untreated mice). When this group was compared with animals that were treated only over the induction phase (1–10 wk), an even greater therapeutic effect was observed (P = 0.009), indicating that the administration of anti-CCL2 monoclonal antibody during the induction phase may actually have been detrimental. Although there was no significant difference in overall tumor progression between untreated mice and animals receiving anti-CCL2 therapy throughout tumor induction, an increased number of mice developed tumors in the latter group. There was also a 20% increase in the number of mice that developed tumors in the 1- to 10-wk group (23/30 = 77%) compared with the 10- to 20-wk treatment group (17/30 = 57%). Overall, these findings suggest that anti-CCL2 therapy is effective in the setting of established malignancies or beginning at a time after the tumor induction phase.

Deficiency in host CCL2 or CCR2 leads to earlier tumor initiation but a reduced growth rate of transgene-driven mammary carcinomas. A transgenic murine model of mammary carcinoma was used to study the effects of CCL2/CCR2 defects on tumor initiation and progression. In this study, MTag transgenic mice (which express the polyomavirus middle T antigen under the control of the MMTV-LTR promoter) were crossed with either Cdl2−/− or Ccr2−/− mice, and oncogenesis and tumor progression were monitored over time (Fig. 4A). The absence of Cdl2 or Ccr2 significantly accelerated the development of palpable tumors (Fig. 4B). However, the earlier appearance of palpable lesions in Cdl2−/− and Ccr2−/− mice was not followed by an aggressive tumor progression but rather resulted in a significantly reduced tumor growth rate (Fig. 4C). These data indicate that the CCL2/CCR2 signaling axis inhibits early oncogenesis but enhances the growth and progression of established lesions.

Increased metastatic disease in Cdl2−/− or Ccr2−/− transgenic hosts. The effects of the Cdl2−/− or Ccr2−/− genotype on the development of metastatic disease in MTag transgenic mice was also investigated. The enumeration of spontaneous pulmonary metastases revealed a significant increase of metastatic burden in hosts lacking either Cdl2 (P < 0.0001) or Ccr2 (P < 0.0001) as compared with control animals (Fig. 4D). Therefore, in a long-term model of tumor progression devoid of CCL2 or CCR2, a high level of metastatic disease and an accelerated oncogenesis was observed in spite of the overall slower rate of primary tumor growth.
that inflammation and the recruitment of MDSCs/TAMs play in tumor progression. The reduction in primary tumor burden and the decrease in tumor-promoting and immunosuppressive cell types that we observed support the use of anti-CCL2 strategies as a means to augment the therapeutic impact of other approaches that would benefit from a reduction in tumor-mediated immunosuppression. Also, conventional anticancer regimens may benefit from CCL2-targeting strategies, as TAMs have been shown to limit the efficacy of various forms of chemotherapy.25 Conversely, some types of immunotherapy that require the recruitment of antitumor immune effectors including T cells11 or γδ T cells10 may be inhibited by blocking the CCL2/CCR2 signaling axis.

The treatment of implantable tumors with anti-CCL2 monoclonal antibody results in a biphasic effect, with the early growth of primary lesions being enhanced despite an overall retardation in tumor progression (Fig. 2). These results were backed up by data obtained in the MCA carcinogenesis model, demonstrating that anti-CCL2 monoclonal antibody reduces tumor growth only when administered in the setting of established lesions. Conversely, the administration of anti-CCL2 monoclonal antibody during the tumor induction phase actually increased the numbers of mice that developed neoplasms (Fig. 3). Moreover, primary tumors progressed at a significantly slower rate in MTag.Ccl2−/− or MTag.Ccr2−/− transgenic mice than in control animals, in spite of an accelerated initiation phase (Fig. 4). These data support the hypothesis that CCL2 is required for the immunosurveillance of developing tumors and possibly metastatic cells while boosting the progression of established primary lesions.

Accumulating evidence identifies tumor- and metastases-associated macrophages as targets for cancer therapy.6,22–24 The elevated growth rate observed in WT and tumor-bearing hosts and mice receiving isotype-matched antibodies is probably due, for the most part, to the CCL2-mediated recruitment of tumor-supporting and/or immunosuppressive cell populations such as TAMs or MDSCs. Indeed, the reduction in primary tumor growth observed in the absence of CCL2 or CCR2 correlated with a decrease in systemic MDSC levels as well as in the proportion of TAMs and MDSCs within neoplastic lesions. It has previously been reported that the depletion of MDSCs using anti-Gr1 monoclonal antibodies can inhibit the growth of 4T1 mammary carcinomas in vivo, and that the inoculation of MDSCs together with malignant cells favors tumor growth.27 These results highlight the significant role that inflammation and the recruitment of MDSCs/TAMs play in tumor progression. The reduction in primary tumor burden and the decrease in tumor-promoting and immunosuppressive cell types that we observed support the use of anti-CCL2 strategies as a means to augment the therapeutic impact of other approaches that would benefit from a reduction in tumor-mediated immunosuppression. Also, conventional anticancer regimens may benefit from CCL2-targeting strategies, as TAMs have been shown to limit the efficacy of various forms of chemotherapy.25 Conversely, some types of immunotherapy that require the recruitment of antitumor immune effectors including T cells11 or γδ T cells10 may be inhibited by blocking the CCL2/CCR2 signaling axis.

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Qian et al. have recently reported that CCL2 mediates the preferential recruitment of inflammatory monocytes (IMs) to breast cancer pulmonary metastases, thus facilitating their growth.6 On the surface, these data are at odds with our findings, revealing...
that the lack of CCL2 or CCR2 in the host actually increases the metastatic burden in both implantable and transgenic models of spontaneous metastasis. This discrepancy may be due to the fact that Ccl2−/− or Ccr2−/− mice (as those used in our study) are characterized by continuous systemic abrogation of CCL2/CCR2 signaling, which may continually inhibit anticancer immunosurveillance. Such a long-term inhibition may indeed allow metastatic tumor cells in the periphery to escape immunosurveillance and traffic in greater numbers to the lungs. Conversely, in the recent study by Qian et al., the inhibition of CCL2 activity was only temporary (achieved with anti-CCL2 monoclonal antibodies). The stronger phenotype observed in Ccr2−/− mice was probably due to rather pleiotropic effects involving multiple chemokine ligands that can signal via this receptor (e.g., CCL7, CCL8, CCL12).1

The different effects of CCL2 inhibition on the number of pulmonary metastases may also reflect differences in the degree of CCL2 inhibition attained, which in knockout mice is both complete and systemic, while upon the administration of anti-CCL2 monoclonal antibody, CCL2 may be inhibited to a partial extent (and perhaps preferentially in some anatomical compartments). The timing of CCL2 availability may also be important in this context, since the abrogation of CCL2 or CCR2 expression in knockout mice occurs throughout tumor initiation and progression, as opposed to the temporary reduction in CCL2 availability that results from anti-CCL2 therapy. Therefore, variations in the level and timing of CCL2 inhibition (in relation to the stage of disease) may be important issues for the therapeutic application of CCL2-targeting strategies. The CCL2-secreting capacity of tumors did not appear to compromise their ability to generate metastases since both Ccl2−/− transgenic tumors and Ccl2−/− AT-3 lesions generated a higher number of pulmonary metastases in hosts with systemic CCL2 or CCR2 defects compared with control mice. These data also suggest that the increase in metastatic burden resulting from the blockade of the CCL2/CCR2 signaling axis stems from the inhibition of immunosurveillance rather than an affect on cancer cell migration. Overall, our findings point to a compartmentalization of CCL2-mediated effects into a peripheral and a tumor-specific component.
Thus, in contrast to current understanding, CCL2 does not always mediate pro-metastatic effects and the CCL2-mediated recruitment of IMs is not an absolute requirement for the pulmonary seeding of malignant cells. We hypothesize that CCL2 plays a dual role in the context of oncogenesis and tumor progression, such that CCL2 facilitates immunosurveillance of small neoplastic lesions and the trafficking of metastatic cells, while promoting the growth of neoplastic lesions that have reached a "critical mass." This is the first study to demonstrate that CCL2 can have opposing effects on the development of metastatic disease and may provide guidance in the utilization and timing of CCL2-targeting strategies for anticancer therapy.

**Materials and Methods**

**Mice.** C57BL/6 mice at 8–10 wk of age were obtained from the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). Cdd2−/− (B6.129S4-Cd2tm1Shi/J) mice were a kind gift from Dr. Michael Hickey (Monash University), while Ccr2−/− (B6.129S4-Ccr2tm1Ifc/J) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred on site. The C57BL/6 J MMTV-PyMT transgenic mouse, herein termed Mtag, expresses the polyomavirus middle T antigen under the control of the MMTV-LTR promoter.26 Only female Mtag mice were used in these experiments and were obtained by breeding transgenic male mice with wild-type C57BL/6 female mice. Mtag, Cdd2−/− or Mtag Ccr2−/− male mice were crossed with Cdd2−/− or Ccr2−/− female mice to obtain transgenic mice lacking the expression of CCL2 or CCR2, respectively. All mice were maintained under specific pathogen-free conditions and used in accordance with institutional guidelines. All procedures were reviewed by the PMCC animal experimentation committee to confirm compliance to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

**Cell culture conditions.** AT-3 and 4T1 cells were cultured in DMEM or RPMI medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum (Moregate Biotech), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 1 mM L-glutamine GlutaMaxTM, under standard conditions (37°C, 10% CO2 or 5% CO2 for RPMI cultures).

**Tumor models.** Mtag transgenic mice develop autochthonous mammary carcinomas with 100% incidence, with eventual metastatic spread to the lungs.26 The long-term disease progression of this model closely mimics that of human breast carcinoma in morphological (disease stage), molecular,27 and immunological terms.13 This model allows us to investigate the chronic nature of long-term host-tumor interactions and permits the study of therapeutic strategies over an extended time frame.

The 4T1 tumor cell line is a BALB/c-histocompatible breast carcinoma cell line that establishes as primary neoplastic lesions when injected subcutaneously or orthotopically into the mammary fat pad. The AT-3 mammary carcinoma cell line has been derived from a primary tumor developing in an Mtag mouse.15 AT-3 cells grow orthotopically or subcutaneously in syngeneic C57BL/6 mice and spontaneously metastasize to the lungs. Primary tumor growth was measured in 2 dimensions, using a digital caliper, and the tumor volume was calculated using the formula (width x length)/2, as previously described.13 In Mtag mice, total tumor volume was calculated as the sum of the volume of all neoplastic lesions developing in an individual mouse.

**Chemical carcinogenesis model.** Groups of WT, Cdd2−/− or Ccr2−/− C57BL/6 mice were injected subcutaneously on the hind flank with 100 μg of 3-methylcholanthrene (Sigma-Aldrich 213942) dissolved in 100 μL corn oil. MCA-induced tumors normally develop within 2–3 mo. Mice were monitored weekly for the development of fibrosarcomas over an experimental time course of 275 d. Once neoplastic lesions became palpable, their size was measured weekly and animals were euthanatized when at least one dimension reached 1.5 cm.

**Enumeration of pulmonary metastases.** In implantable and transgenic models, lung metastases was quantified in a blinded fashion by manual counting of hematoxylin and eosin (H&E)-stained sections from paraffin-embedded lungs. To this aim, lungs were excised following tracheal perfusion with 10% buffered formalin, which was used to inflate the lobes and fix the lung structure. Upon further fixation in formalin, lungs were dissected into individual lobes and strategically laid out on mating within a cassette for paraffin embedding. Lungs were then sectioned through to the largest surface of tissue and a couple of 4-μm thick sections were stained with H&E. The number of metastases and micrometastases were counted in a blinded fashion under a light microscope equipped with a 10× objective. In experiments based on 4T1 cells, macroscopic metastases were enumerated following lung perfusion with India ink and fixation in Fekete’s solution.

**Tumor-infiltrating leukocyte (TIL) studies.** Once tumors had reached the endpoint size (at least one dimension = 1.5 cm), they were excised and manually disrupted using fine dissection scissors to create a cellular slurry that contained no tumor pieces larger than 1 mm³. This cellular preparation was resuspended in a digestion solution containing 1 mg/mL collagenase, 0.1 mg/mL hyaluronidase, and 30 U/mL DNase in PBS and incubated at 37°C with shaking for approximately 30 min, or until a limited amount of particulate material remained. Single-cell suspensions were then obtained by means of a sieve to remove clumped material, washed twice and resuspended in PBS buffer (0.2% bovine serum albumin in PBS). Finally, cells were stained with antibodies specific for CD3 (17A2), CD4 (RM4-5), CD8 (53.6.7), CD11b (3A33), CD11c (N418), Gr1 (RB68C5), F4/80 (BM8), CD45R (RA3-6B2), CD19 (eBio1D3), NK1.1 (PK136), CD49B (DX5), TCRβ (H57-597), or 8εTCR (UC7-13D5). Appropriate isotype-matched antibodies were invariably employed as staining controls. Cytofluorometric analyses were performed on a LSRII cytomter (BD Biosciences), and data were analyzed using the Flowjo software (Tree Star Inc.). Gating of single cells using the FSC/W and SSC/W parameters and the exclusion of dead cells were routinely performed.

**Anti-CCL2 therapy.** Anti-mouse CCL2 monoclonal antibody (C1142) from Janssen R&D was administered intraperitoneally at a weekly dose of 200 μg/mouse in 200 μL PBS.

**Statistical analyses.** Two-sided Mann–Whitney U-tests were used to determine statistical significance when 2 data sets were
compared. When appropriate, the Gehan–Breslow–Wilcoxon test was employed. Statistical analyses were performed by means of the Prism v.6 (GraphPad Software). P values < 0.05 were considered as statistically significant. Graphed values represent either means with 95% confidence intervals (CIs) or means ± SEM.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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