A role for CCL2 in both tumor progression and immunosurveillance

Ming Li[1,2], Deborah A Knight[1,2], Linda A Snyder[3], Mark J Smyth[1,2], and Trina J Stewart[4,*]

[1]Cancer Immunology Program; Peter MacCallum Cancer Centre; East Melbourne, VIC Australia;
[2]Sir Peter MacCallum Department of Oncology; The University of Melbourne; East Melbourne, VIC Australia;
[3]Janssen R&D LLC; Radnor, PA USA; [4]Griffith Health Institute; Griffith University; Gold Coast, QLD Australia

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 *chemo*tactic cyto*kines* 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 microenvironment[3] 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 lines[5] 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 types[8] 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 cells[10–12] and is required for efficient immunosurveillance, implying that the inhibition of CCL2 may promote neo-carcinogenesis 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 anticancer 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 monocytic cells, 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 expands 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 orthotopic implantation of AT-3 cells is primarily due to MDSCs.[19] 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 (splenic) 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**).
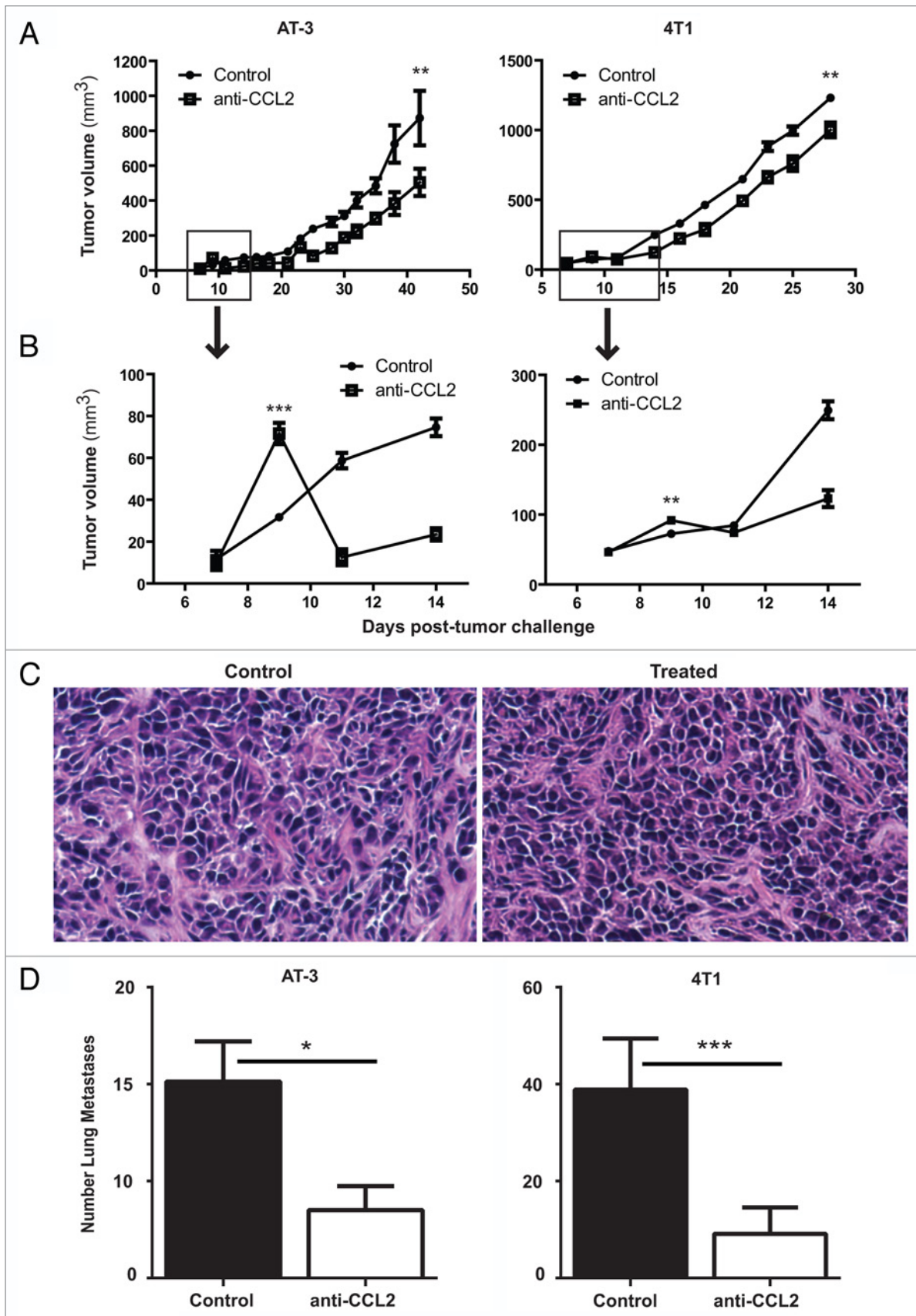
However, we noted that mice treated with anti-CCL2 monoclonal antibody displayed a significant, very early increase in tumor volume upon the initiation of therapy as compared with
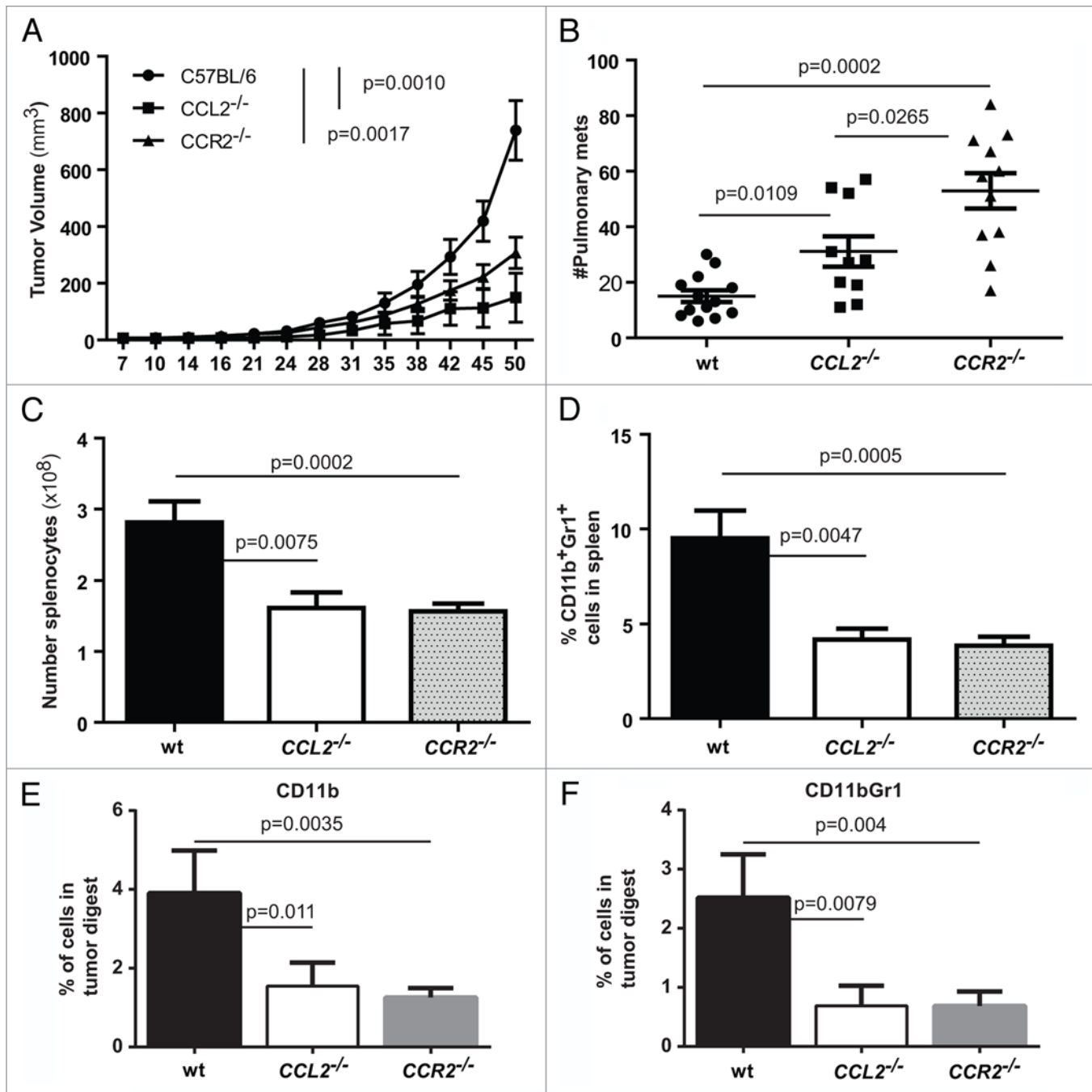
Figure 1. Effect of host-derived CCL2 or CCR2 on tumor growth. Groups (n = 10–12) of wild-type (WT), *Ccl2⁻/⁻*, and *Ccr2⁻/⁻* C57BL/6 mice were challenged orthotopically with 5×10^5 AT-3 tumor cells. (**A**) Tumor growth was monitored twice weekly. Statistical analyses were performed on data obtained at day 50 post-implantation, before mice started to be euthanized due to tumor size. (**B**) Metastases were enumerated in the lungs of mice once primary tumors had reached a comparable terminal size. (**C–F**) Spleens and primary tumor tissues were taken at the same time as the lungs and dissociated into single-cell suspensions, followed by cytofluorometric studies. Results are representative of at least 2 independent experiments. Graphs represent means ± SEM and p values were calculated using the Mann–Whitney U-test.

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

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.

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.
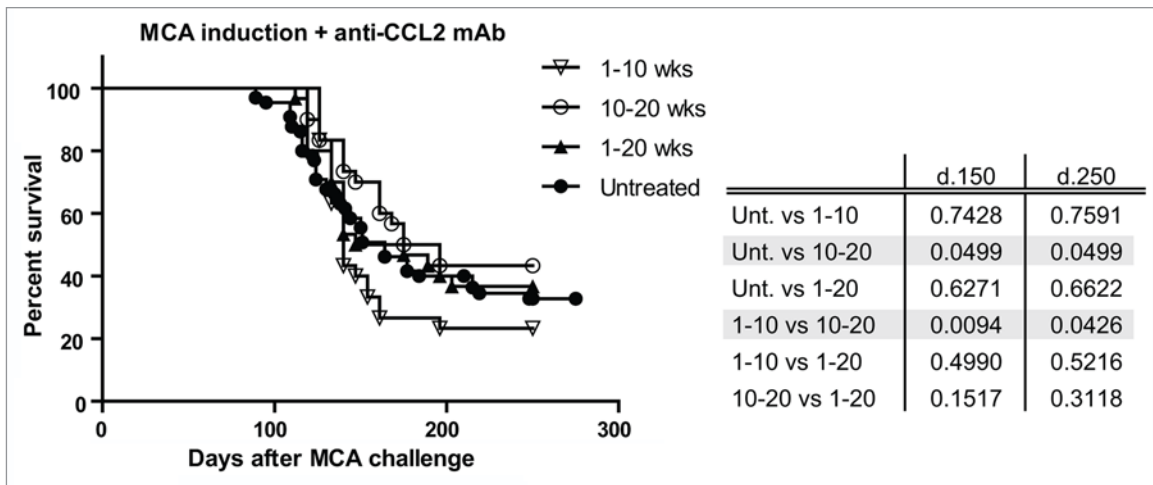
Anti-CCL2 monoclonal antibody reduces the amount of spontaneous pulmonary metastases. In view of previous reports demonstrating a reduction in the pulmonary metastatic burden in response to anti-CCL2 monoclonal antibody,[6,20] the results obtained in *Ccl2⁻/⁻* or *Ccr2⁻/⁻* mice were somewhat surprising. 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 *Ccl2⁻/⁻* or *Ccr2⁻/⁻* mice, and oncogenesis and tumor progression were monitored over time (**Fig. 4A**). The absence of *Ccl2* or *Ccr2* significantly accelerated the development of palpable tumors (**Fig. 4B**). However, the earlier appearance of palpable lesions in *Ccl2⁻/⁻* 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 *Ccl2⁻/⁻* or *Ccr2⁻/⁻* transgenic hosts. The effects of the *Ccl2⁻/⁻* 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 *Ccl2* (*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.

Figure 3. Anti-CCL2 monoclonal antibody therapy is only effective against established tumors. (**A**) Groups of 30 C57BL/6 mice were injected with 100 µg 3-methylcholanthrene (MCA) s.c. and monitored for tumor growth over 250 d. Mice were administered i.p. with 200 µg anti-CCL2 monoclonal antibody per week either early in tumor induction phase (1–10 wk), once neoplastic lesions were established (10–20 wk), or continuously (1–20 wk). Mice were euthanized once tumors had reached a predetermined size limit complying with ethical guidelines and the same day was recorded for the determination of survival curves. *P* values were calculated at 2 time points using the Gehan–Breslow–Wilcoxon test: 150 d after the MCA challenge, when anti-CCL2 therapy had ceased (week 21), and at the end of the experiment (day 250).

Discussion

CCL2 has been implicated in a number of tumor-promoting activities including the recruitment of TAMs and MDSCs, as well as the stimulation of tumor cell invasiveness.[5,9,21] Given these important roles in tumor progression, CCL2 appears a promising target for the development of novel anticancer therapeutics. However, contrasting experimental findings obtained with the manipulation of the CCL2/CCR2 signaling, axis as well as the potentially important role that CCL2 plays in host immune responses and immunosurveillance, indicate that the CCL2/CCR2 pathway must be investigated further before being validated as a target for anticancer therapy. The present study highlights the important role that CCL2 plays in tumor-associated inflammation and supports the notion that this chemokine can mediate opposing effects on tumor progression, as it appears to enhance 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.[17] 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 cells[11] or γδ T cells[10] 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 small and/or developing tumors. However, once neoplastic lesions reach a "critical mass," CCL2 appears to predominantly play a tumor-promoting function.

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
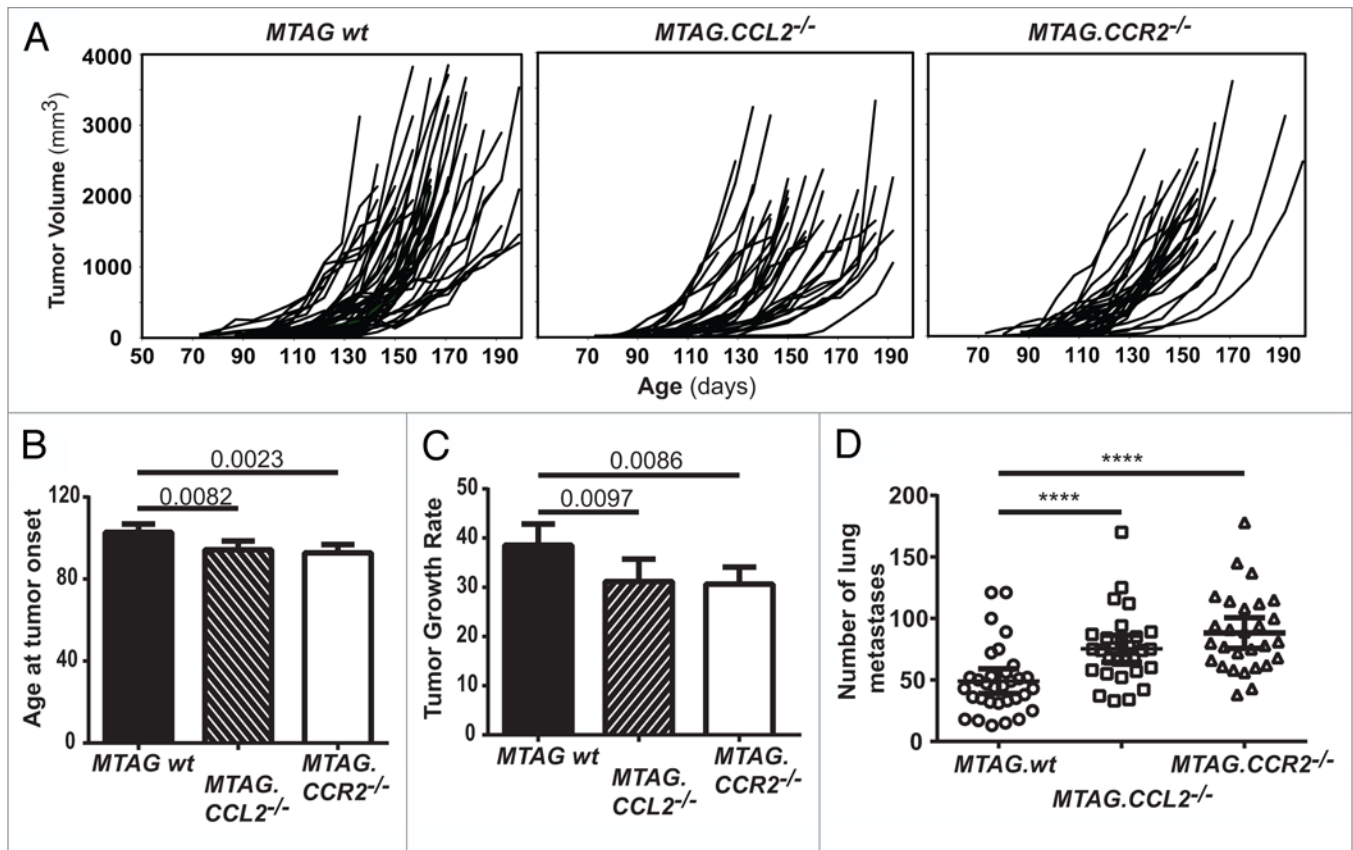
Figure 4. Accelerated oncogenesis but reduced growth rates of established neoplastic lesions in mice lacking CCL2 or CCR2 expression. The growth of developing primary tumors was measured weekly, starting from the development of palpable tumors through to euthanasia (performed when tumors reached a predetermined size limit complying with ethical guidelines). The volumes of individual lesions were summed to obtained total tumor volume in each mouse. (**A**) Progressive tumor growth of each individual mouse in groups of *Mtag* (n = 48), *Mtag.Ccl2*$^{-/-}$ (n = 33) and *Mtag.Ccr2*$^{-/-}$ (n = 32) mice. (**B**) The day at which tumors became palpable (onset) is reported as mean age ± 95% CI. (**C**) Tumor growth rate is reported as mean ± 95% CI. Tumor growth rate was determined as the ratio between final tumor volume (mm^3) and the number of days of tumor progression (age of mouse at euthanasia − age of mouse when tumor became palpable; in days). (**D**) Lungs were taken from *Mtag* (n = 32), *Mtag.Ccl2*$^{-/-}$ (n = 29) and *Mtag.Ccr2*$^{-/-}$ (n = 29) and compared in terms of metastatic burden. Metastases were counted in a blinded fashion on H&E-stained sections from paraffin-embedded blocked lungs. Statistical significance was determined by Mann–Whitney U-tests. ****$P < 0.0001$.

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 *Ccl2*$^{-/-}$ 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). *Ccl2*^{-/-} (*B6.129S4-Ccl2*[tm1Rol]*/J*) mice were a kind gift from Dr Michael Hickey (Monash University), while *Ccr2*^{-/-} (*B6.129S4-Ccr2*[tm1Ifc]*/J*) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred on site. The C57BL/6J *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.Ccl2*^{-/-} or *Mtag.Ccr2*^{-/-} male mice were crossed with *Ccl2*^{-/-} 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 GlutaMax™, under standard conditions (37°C, 10% CO_2 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^2 × 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, *Ccl2*^{-/-} 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 matting 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 mm^3. 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 FACS 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 γδTCR (UC7-13D5). Appropriate isotype-matched antibodies were invariably employed as staining controls, cytofluorometric analyses were performed on a LSRII cytometer (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.

References

1. Stewart TJ, Smyth MJ. Chemokine-chemokine receptors in cancer immunotherapy. Immunotherapy 2009; 1:109-27; PMID:20635978; http://dx.doi.org/10.2217/1750743X.1.1.109

2. Xia Y, Frangogiannis NG. MCP-1/CCL2 as a therapeutic target in myocardial infarction and ischemic cardiomyopathy. Inflamm Allergy Drug Targets 2007; 6:101-7; PMID:17692033; http://dx.doi.org/10.2174/187152807780832265

3. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res 2000; 6:3282-9; PMID:10955814

4. Lebrecht A, Grimm C, Lantzsch T, Ludwig E, Hefler L, Ulbrich E, et al. Monocyte chemoattractant protein-1 serum levels in patients with breast cancer. Tumour Biol 2004; 25:14-7; PMID:15192307; http://dx.doi.org/10.1159/000077718

5. Youngs SJ, Ali SA, Taub DD, Rees RC. Chemokines induce migrational responses in human breast carcinoma cell lines. Int J Cancer 1997; 71:257-66; PMID:9139852; http://dx.doi.org/10.1002/(SICI)1097-0215(19970410)71:2<257::AID-IJC22>3.0.CO;2-D

6. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 2011; 475:222-5; PMID:21654748; http://dx.doi.org/10.1038/nature10138

7. Steiner JL, Murphy EA. Importance of chemokine (CC-motif) ligand 2 in breast cancer. Int J Biol Markers 2012; 27:e179-85; PMID:22865298; http://dx.doi.org/10.5301/JBM.2012.9345

8. Huang B, Lei Z, Zhao J, Gong W, Liu J, Chen Z, et al. CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. Cancer Lett 2007; 252:86-92; PMID:17257744; http://dx.doi.org/10.1016/j.canlet.2006.12.012

9. Conti I, Rollins BJ. CCL2 (monocyte chemoattractant protein-1) and cancer. Semin Cancer Biol 2004; 14:149-54; PMID:15246049; http://dx.doi.org/10.1016/j.semcancer.2003.10.009

10. Lança T, Costa MF, Gonçalves-Sousa N, Rei M, Grosso AR, Penido C, et al. Protective Role of the Inflammatory CCR2/CCL2 Chemokine Pathway through Recruitment of Type 1 Cytotoxic γδ T Lymphocytes to Tumor Beds. J Immunol 2013; 190:6673-80; PMID:23686489; http://dx.doi.org/10.4049/jimmunol.1300434

11. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, et al. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. J Exp Med 2011; 208:1949-62; PMID:21930770; http://dx.doi.org/10.1084/jem.20101956

12. Rollins BJ, Sunday ME. Suppression of tumor formation in vivo by expression of the JE gene in malignant cells. Mol Cell Biol 1991; 11:3125-31; PMID:2038321

13. Stewart TJ, Lutsiak ME, Abrams SI. Immune consequences of protracted host-tumor interactions in a transgenic mouse model of mammary carcinoma. Cancer Invest 2008; 26:237-49; PMID:18317964; http://dx.doi.org/10.1080/07357900701708419

14. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol 2001; 166:678-89; PMID:11123353

15. Stewart TJ, Abrams SI. Altered immune function during long-term host-tumor interactions can be modulated to retard autochthonous neoplastic growth. J Immunol 2007; 179:2851-9; PMID:17709499

16. Bronte V, Serafini P, Apolloni E, Zanovello P. Tumor-induced immune dysfunctions caused by myeloid suppressor cells. J Immunother 2001; 24:431-46; PMID:11759067; http://dx.doi.org/10.1097/00002371-200111000-00001

17. Stewart TJ, Liewehr DJ, Steinberg SM, Greeneltch KM, Abrams SI. Modulating the expression of IFN regulatory factor 8 alters the protumorigenic behavior of CD11b+Gr-1+ myeloid cells. J Immunol 2009; 183:117-28; PMID:19542426; http://dx.doi.org/10.4049/jimmunol.0804132

18. Siveen KS, Kuttan G. Role of macrophages in tumour progression. Immunol Lett 2009; 123:97-102; PMID:19428556; http://dx.doi.org/10.1016/j.imlet.2009.02.011

19. Stewart TJ, Greeneltch KM, Reid JE, Liewehr DJ, Steinberg SM, Liu K, et al. Interferon regulatory factor-8 modulates the development of tumour-induced CD11b+Gr-1+ myeloid cells. J Cell Mol Med 2009; 13(9B):3939-50; PMID:20196788; http://dx.doi.org/10.1111/j.1582-4934.2009.00685.x

20. Loberg RD, Ying C, Craig M, Day LL, Sargent E, Neeley C, et al. Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. Cancer Res 2007; 67:9417-24; PMID:17909051; http://dx.doi.org/10.1158/0008-5472.CAN-07-1286

21. Izhak L, Wildbaum G, Jung S, Stein A, Shaked Y, Karin N. Dissecting the autocrine and paracrine roles of the CCR2-CCL2 axis in tumor survival and angiogenesis. PLoS One 2012; 7:e28305; PMID:22279523; http://dx.doi.org/10.1371/journal.pone.0028305

22. Chen Q, Zhang XH, Massagué J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. Cancer Cell 2011; 20:538-49; PMID:22014578; http://dx.doi.org/10.1016/j.ccr.2011.08.025

23. Hao NB, Lü MH, Fan YH, Cao YL, Zhang ZR, Yang SM. Macrophages in tumor microenvironments and the progression of tumors. Clin Dev Immunol 2012; 2012:948098; PMID:22778768; http://dx.doi.org/10.1155/2012/948098

24. Mukhtar RA, Nseyo O, Campbell MJ, Esserman LJ. Tumor-associated macrophages in breast cancer as potential biomarkers for new treatments and diagnostics. Expert Rev Mol Diagn 2011; 11:91-100; PMID:21171924; http://dx.doi.org/10.1586/erm.10.97

25. De Palma M, Lewis CE. Cancer: Macrophages limit chemotherapy. Nature 2011; 472:303-4; PMID:21512566; http://dx.doi.org/10.1038/472303a

26. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992; 12:954-61; PMID:1312220

27. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol 2003; 163:2113-26; PMID:14578209; http://dx.doi.org/10.1016/S0002-9440(10)63568-7