



# Targeting the CCL2/CCR2 Axis in Cancer Immunotherapy: One Stone, Three Birds?

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CCR2 is predominantly expressed by monocytes/macrophages with strong proinflammatory functions, prompting the development of CCR2 antagonists to dampen unwanted immune responses in inflammatory and autoimmune diseases. Paradoxically, CCR2-expressing monocytes/macrophages, particularly in tumor microenvironments, can be strongly immunosuppressive. Thus, targeting the recruitment of immunosuppressive monocytes/macrophages to tumors by CCR2 antagonism has recently been investigated as a strategy to modify the tumor microenvironment and enhance anti-tumor immunity. We present here that beneficial effects of CCR2 antagonism in the tumor setting extend beyond blocking chemotaxis of suppressive myeloid cells. Signaling within the CCL2/CCR2 axis shows underappreciated effects on myeloid cell survival and function polarization. Apart from myeloid cells, T cells are also known to express CCR2. Nevertheless, tissue homing of Treg cells among T cell populations is preferentially affected by CCR2 deficiency. Further, CCR2 signaling also directly enhances Treg functional potency. Thus, although Tregs are not the sole type of T cells expressing CCR2, the net outcome of CCR2 antagonism in T cells favors the anti-tumor arm of immune responses. Finally, the CCL2/CCR2 axis directly contributes to survival/growth and invasion/metastasis of many types of tumors bearing CCR2. Together, CCR2 links to two main types of suppressive immune cells by multiple mechanisms. Such a CCR2-associated immunosuppressive network is further entangled with paracrine and autocrine CCR2 signaling of tumor cells. Strategies to target CCL2/CCR2 axis as cancer therapy in the view of three types of CCR2-expressing cells in tumor microenvironment are discussed.

**Keywords:** CCL2, CCR2, cancer immunotherapy, macrophages, T regulatory cells

## INTRODUCTION

A broad array of chemokines and chemokine receptors regulate physiological and pathological processes, including tumorigenesis (1). CC-chemokine receptor 2 (CCR2) is highly expressed by a subset of Ly6Chi monocytes with strong proinflammatory functions (2). CCR2 deficiency markedly reduces Ly6Chi monocytes trafficking out of bone marrow and to sites of inflammation (3–5). CCR2 deficiency also reduces Th1 response and the severity of experimental autoimmune diseases (6, 7).

Similarly, deletion of CCR2+ monocytes has a profound impact on immunity to infection and autoimmunity (8, 9). Despite this, CCR2 antagonism as a treatment for autoimmune diseases has been met with the disappointing results (10). Further, CCR2 antagonism has been found to exacerbate autoimmune diseases, suggesting an opposite immune regulatory role for CCR2, although the basis of anti-inflammatory role of CCR2 remains undefined (11). As the role of CCR2 in regulation of autoimmunity remains contradictory, overwhelming evidence supports that the CCL2/CCR2 axis activity largely favors progression and metastasis of tumor by attracting suppressive monocytes and Tregs (12, 13), though any chemokine/chemokine receptor can have both pro-tumor and anti-tumor action (1). Beyond chemotaxis, CCR2 can also directly impact the function of myeloid cells and T cells in a less defined fashion. This review will separately discuss the chemotactic and non-chemotactic effects of the CCL2/CCR2 axis on monocytes/macrophages, T cells and tumor cells. We surmise that two main types of suppressive immune cells (e.g. monocytic myeloid suppressors and Treg cells) in suppressive tumor microenvironments, are more dependent on CCR2 signaling and thus the net outcome of CCR2 signaling favors tumor progression and metastasis. Together with the paracrine and autocrine CCR2 signaling of tumor cells, CCR2 has a significant role in tumor growth and metastasis. Thus, targeting the CCL2/CCR2 axis may be a plausible avenue in cancer therapy, particularly for many solid tumors belonging to the “cold tumor” family.

## CCR2 LIGANDS AND CCR2 SIGNALING

Since being reported in 1994 (14), CCR2 is the second most studied chemokine receptor after CCR5 (based on PubMed) and continues to be actively investigated as a potential drug target for many diseases, ranging from autoimmune diseases, diabetes and chronic pain syndromes, to atherosclerosis, HIV and cancer. Over 2 decades, structure, expression, expression regulation of CCR2 and its ligands, and CCR2 signaling has been detailed (15). Here, we provide brief discussion on this aspect of CCL2/CCR2 biology, as more detailed dissection of cell type specific expression of and functions of CCL2/CCR2 is covered in corresponding sections of this review.

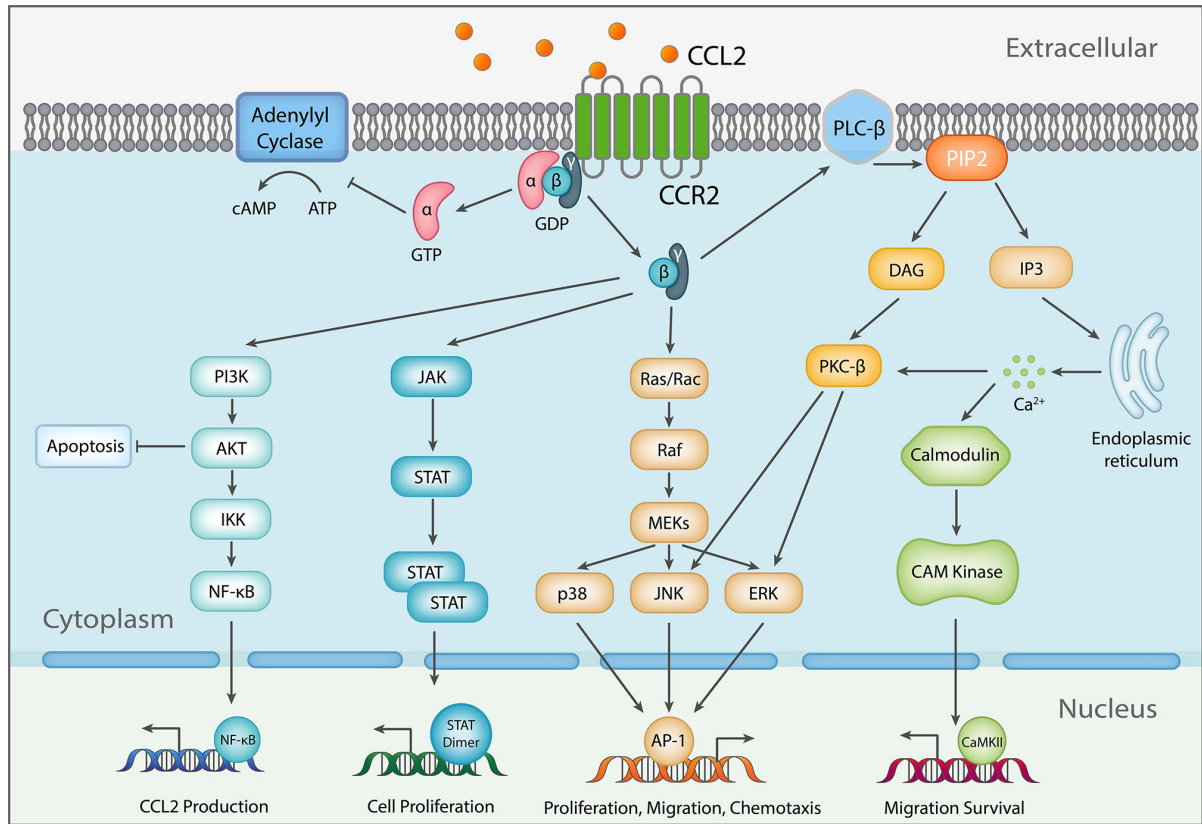
CCR2 belongs to the chemokine receptor subfamily of human Class A G protein-coupled receptors (GPCRs). In humans, two isoforms CCR2A and CCR2B differ in their C-terminal which can result in different signaling properties. CCR2 is known to be expressed by monocytes/macrophages. Consequently, deficiency grossly affects traffic of monocytes/macrophages (3, 6). Nevertheless, CCR2 is also expressed by various cell types including Tregs (16), CD4<sup>+</sup> T cells (17), CD8<sup>+</sup> T cells (18); NKTs (19),  $\gamma\delta$ T cells (20), B cells (21), plasmacytoid dendritic cells (22), basophils (23), stem cells (24), endothelial cells (25), microglia (26), muscle cells (27) and tumor cells (28). Expression of CCR2 is subject to regulation by many various factors. CCR2 expression by monocytes can be upregulated by plasma cholesterol, peroxisome proliferator-activated receptor gamma

ligands and salt (29, 30). On the other hand, hypoxia-induced HMGB1 downregulates CCR2 expression by monocytes (31). CCR2 on human NK cells can also be induced by IL-2 (32).

CCL2 is the prototype chemokine binding to CCR2, and the CCL2-CCR2 pairing is the most relevant for CCR2 function. Nevertheless, CCR2 can be activated by other chemokines including CCL7 (MCP-3) (33, 34), CCL8 (MCP-2) (35), CCL12 (MCP-5), CCL13 (MCP-4) (36), CCL16 (37). In general, chemokines other than CCL2 have been less explored for their contribution to CCR2-mediated function.

Ligation of the chemokine receptor CCR2 leads to activation of multiple downstream signaling pathways (**Figure 1**). The CCR2-mediated signal transduction starts with CCL2/CCR2 binding and activation of GPCR (39) and then activates PI3K/Akt pathway (40–42), RAC GTPase pathway (43), PKC-dependent pathway (44), and JAK/STAT pathway. Conceivably, activation of different pathways affects different biological processes, ranging from cell survival, proliferation, migration, and differentiation. The downstream activation of PI3K/Akt pathway protects tumor cells from death and promotes proliferation (45, 46). Activated PI3K induces activation of protein kinase B (Akt) *via* phosphorylation at Thr308 and Ser473 by PDK1 and PDK2, respectively (40, 41, 47), which in turn mediates up-regulation of survivin and down-regulation of autophagosome formation *via* promoting mammalian target of rapamycin (mTOR) activation (42, 48). Thereafter, the crucial survival protein, survivin, inhibits two major programmed cell death pathways – apoptosis (49–51) and autophagic death (52). Consequently, it allows tumor cells, such as PC3 and VCaP prostate cancer cells, to survive from cell death stimuli, like nutrition starvation (42). In addition, it has been shown that PI3K/Akt plays a central role in chemotaxis by inducing IKK $\alpha$   $\beta$  phosphorylation, which in turn increases NF- $\kappa$ B transactivation and consequently promotes MMP-9 expression (53–55). MMP-9 aids in cell migration through degrading the extracellular matrix (ECM) (56), while CCL2 induces migration of other CCR2-expressing cells. Activation of downstream MEK/ERK pathway (57) can result in up-regulation of gene expression, such as MMP-9 (58), which promotes migration. Additionally, PKC is activated as a downstream signal of G-protein dissociation, and prompts activation of JNK and ERK to promote cancer cell migration (59, 60). CCL2/CCR2 signaling also triggers the JAK/STAT pathway by activating Janus kinase 2 (JAK2) (61), and thereby triggers downstream pathways, including STAT1, STAT3, and STAT5 (62, 63), which further inhibit apoptosis and induce extravasation and expansion of tumor cells like colon carcinoma (64).

As mentioned above human CCR2 has two forms: CCR2A and CCR2B. They differ only in their terminal carboxyl tails and cellular location (14, 65). We discuss here some differences in expression and function which may have implications in inflammation and cancer immunity. CCR2B was the predominant isoform a mRNA level in human monocytes (65). In some pathological conditions, the CCR2 show cell specific expression (66). For example, mononuclear cells in disease lesions show strong expression of CCR2A but not CCR2B (66).



**FIGURE 1** | Schematic diagram of CCL2 signaling. As a response to CCL2 binding at the N-terminus, extracellular loops and transmembrane bundle of CCR2, the intracellular G-protein  $\alpha$  subunit dissociates from the CCR2 and the  $\beta\gamma$  subunit. The  $\alpha$  subunit then inhibits adenylyl cyclase (AC) function resulting in decreased cyclic adenosine monophosphate levels. In contrast, the  $\beta\gamma$  subunit signaling induces gene expression via several pathways, further inducing changes to cellular function [Adapted from (38)].

Further, CCR2 isoforms shows preferential induction by inflammatory stimulation (67). Exposure of fibroblast like synoviocytes of patients with RA to sCD40L caused strong upregulation of CCR2A but not of CCR2B protein expression. CCR2A is also notably frequently overexpressed in glioblastoma (68). In stable transfected Jurkat cells, CCR2A and CCR2B show some subtle difference in CCL2-eminated signaling (69). CCL2 induced a transient Ca(2+) flux in the CCR2B transfected cells but not in the CCR2A transfected cells. Together, CCR2A and CCR2B may have differences in their function and distribution.

### CHEMOTACTIC AND NON-CHEMOTACTIC EFFECTS OF CCL2/CCR2 AXIS ON MONOCYTES/MACROPHAGES

#### Functional Conundrum of CCR2-Expressing Monocytes/Macrophages: Inflammatory or Suppressive?

Monocytes consist of different subsets and are the prime source of tissue macrophages. The dominant subset of mouse

CD11b<sup>+</sup>Ly6C<sup>+</sup> monocytes and human CD14<sup>+</sup> monocytes express CCR2. Early work has established that CCR2 expressing mouse Ly6C<sup>hi</sup> monocytes are potent producers of proinflammatory cytokines (2). Consequently, deletion of CCR2<sup>+</sup> monocytes/macrophages profoundly reduces immunity to infection and autoimmunity (8). However, MØs do not always function as proinflammatory and immuno-stimulatory. Rather, they have a broad spectrum of function that depends on their surrounding environmental cues. Particularly, many soluble factors modulate MØ function and their corresponding molecular programming. Interferon (IFN)- $\gamma$ /LPS has been found to induce proinflammatory M1 MØs with a potent ability to produce cytokines priming Th1, Th17 and CTL response while IL-4 induces alternatively activated M2 MØs with a more immunosuppressive role (70). Many other cytokines also influence MØ functional polarization. For example, GM-CSF and M-CSF have been shown to generate distinct MØ populations with certain M1 and M2 characteristics from mouse bone marrow (BM) precursors (71). Despite GM-CSF being considered a M1 MØ inducer *in vitro* and *in vivo* for both mouse and human MØs (9, 71–75), high levels of GM-CSF have also been associated with development of suppressive M2-like

MØs (76, 77). Of note, pre-treatment of MØs with CCL2 selectively inhibited IL-12 production in response to IFN- $\gamma$  and *Staphylococcus aureus* (78).

In the context of cancer, MØs also function in a spectrum from tumoricidal, immuno-stimulating to immunosuppressive. MØs within the local tumor microenvironment (TME) generally polarize to be immunosuppressive. There are two terms “Tumor associated macrophages (TAM)” and monocytic myeloid derived suppressor cells (M-MDSCs) to describe closely connected monocytic myeloid cells in TMEs (79, 80). M-MDSCs are present in blood and lymphoid organs and defined as CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>-</sup>. They develop towards TAM that express lower levels of Ly6C and higher levels of MHCII, F4/80 and CX3CR1 (79). Abundant evidence shows that TAMs are derived from Ly6C<sup>+</sup>CCR2<sup>+</sup> monocytes in various experimental tumor models (81–85). Due to the heterogenous nature of TMEs, factors that polarize TAMs into suppressive M2 like MØs are also likely heterogenous. Consequently, functional programs of TAMs can also be versatile, ranging from cancer initiation, angiogenesis, immune suppression to tumor metastasis (86). Given their CCR2<sup>+</sup> cell origin, the CCL2/CCR2 axis is critically for TAM development, *via* chemotactic as well as non-chemotactic mechanisms.

## The Role of CCL2/CCR2 Axis in Monocyte Recruitment

Early work with CCR2 deficient mice firmly establishes a role for CCR2 in monocyte traffic and recruitment (6, 87). Firstly, CCR2 contributes to monocyte egress from bone marrow, leading to monocytopenia in the absence of CCR2 (87). Secondly, CCR2 is critically required for influx of inflammatory monocytes/MØs in respond to innate inflammatory stimulation (6). Results from adoptive transfer of WT and CCR2<sup>-/-</sup> monocytes also support that CCR2 is required for migration of monocytes from the blood to inflamed tissues (87). Impairments in monocyte migration have also been reported in CCL2<sup>-/-</sup> mice (88). Interestingly, deficiency of another CCR2 ligand MCP-3 but not MCP-2 and MCP-5 also profoundly reduces monocyte egress from bone marrow (87).

TAMs can be derived from both resident MØs and monocyte-derived MØs (84, 85). CCR2 clearly contributes to the influx of monocytes/MØs to tumors, particularly metastatic sites to generate TAMs (12, 80, 84, 89). In a MMTV-PyMT (PyMT) mammary tumor model (84), depletion of tumor-associated monocytes significantly reduced TAMs. Of note, compared to resident mammary tissue MØs, TAMs had a higher proliferative capacity (84). Further, TAM populations in this model expressed Vcam1 but did not express typical M2 markers such as Ym1, Fizz1, and Mrc1 (84). In a gliomas model, CCR2 deficiency also reduced CD11b<sup>+</sup>/Ly6C<sup>hi</sup>/PD-L1<sup>+</sup> MDSCs and inhibition of CCR2 increased median survival (90). Fittingly, a combination of CCR2 inhibition and anti-PD-1 increased survival of glioma-bearing mice (90).

CCR2 mediated recruitment of blood monocytes or M-MDSCs to tumor tissues results in a high abundance of TAMs that is often associated with poor clinical outcomes in patients.

In human breast cancer, the level of CCL2, produced by monocytic cells and tumor cells, was associated significantly with TAM accumulation and was a significant indicator of early relapse (91) and vessel invasion of tumor cells (92). However, in another study with breast carcinoma, CCL2 expression was not found to be closely correlated with MØ infiltrations, although carcinomas showed higher levels of CCL2 mRNA than normal breast tissue (93). In esophageal carcinogenesis, high expression of CCL2 also correlated with TAM accumulation and was associated with poor prognosis (94). Overall, there is support that recruitment of TAM by CCL2 has clinical significance.

## The Role of CCL2/CCR2 Axis in Survival/Proliferation and Functional Polarization of Monocytes/Macrophages

As the chemotactic role of CCL2/CCR2 axis is well-established, the non-chemotactic role of CCL2/CCR2 axis is less understood but has been receiving recent attention (95). CCL2/CCR2 signaling can directly impact on survival/proliferation, functional polarization, effector molecule secretion, autophagy, killing, and adhesion of monocytes/MØs (95). We briefly discuss here the non-chemotactic roles of the CCL2/CCR2 axis: survival/proliferation and functional polarization of monocytes/MØs, particularly in the context of TAMs.

Human monocytes can be separated into CD14<sup>+</sup>CD16<sup>-</sup> classical monocytes, CD14<sup>+</sup>CD16<sup>+</sup> intermediate monocytes, and CD14<sup>lo</sup>CD16<sup>+</sup> nonclassical monocytes. Classical monocytes have a very short circulating lifespan (about 1 d). Most classical monocytes leave the circulation or die, and the remaining cells become intermediate monocytes. Intermediate monocytes have a longer lifespan (about 4 days) before transitioning to nonclassical monocytes. Nonclassical monocytes have the longest lifespan in the blood, of around 7 days, before either leaving the circulation or dying (96). Similarly, mouse monocyte subsets follow a similar pattern (97).

Homeostasis of immune cells is critically regulated by the BCL-2 regulated apoptosis pathway. Several pro-survival members of the BCL-2 family including BCL-2, BCL-xL, A1, MCL-1, and BCL-w promote survival of immune cells (98). However, survival of monocytes/macrophages at steady state is not essentially affected by loss/inhibition of BCL2 (99), BCL-xL (100, 101), MCL-1 (102, 103) or A1 (104). CCL2 induces upregulation of Bcl-2, Bcl-XL and cFLIP and protects monocytes from apoptosis under serum deprivation stress (105). During inflammation, CCL2 also linked to MØ survival (106). Nevertheless, current knowledge is still very limited regarding survival control of TAMs and the contribution of axis of CCL2/CCR2 to survival of monocytes/TAMs. As TAMs replenishment requires the continuous recruitment of M-MDSC and monocytes, TAMs also expressed high levels of Ki67 staining and EdU incorporation, indicating cell proliferation and self-renewal (84). CCL2, as well as CCL3 and CCL14 can induce MØ proliferation (107).

CCL2 also seems to affect functional polarization of MØs. Related to the suppressive nature of TAMs and M-MDSCs, CCL2 has been found to induce human CD206<sup>+</sup> MØs (105). Inclusion of CCL2 in monocytes cultured with M-CSF or GM-



CSF also increased M2 markers *in vitro* (108). Pre-treatment of MØs with CCL2 selectively inhibited IL-12 production in response to IFN- $\gamma$  and *Staphylococcus aureus* (78). Therefore, CCL2/CCR2 signaling axis can promote the polarization of M2 like MØs. However, it is somewhat unclear how CCL2 induces M2 polarization in the above tumor context. Notably in certain conditions, deficiency of CCL2 or CCR2 biases for M2 polarization (109, 110), highlighting the complexity of functional polarization of MØs. Overall, tumor microenvironment contains many factors favoring the development of M2 like TAMs, CCL2/CCR2 signaling axis not only play a major role in recruitment of monocytes/MØs to contribute to functional polarization of MØs but also can directly polarize MØs. The significance of the direct signaling to functional polarization of MØs remains to be further explored.

### The Role of CCL2/CCR2 Axis in Monocytes/MØs-Mediated Tumor Metastasis

Contribution of CCR2-mediated recruitment of monocytes in cancer metastasis is nicely demonstrated in a study with monocyte transfer (12). Adoptive transfer of Ly6C<sup>hi</sup>CD11b<sup>+</sup> cells from wt but not CCR2<sup>-/-</sup> mice resulted in preferential recruitment of monocytes to a tumor cell challenged lung (12). Transfer of human CCR2-expressing CD14<sup>+</sup> monocytes into CSF-1 supplemented nude mice with inoculation of human MDA-MB-231-derived metastatic breast cancer cells also led to selective tumor recruitment of CD14<sup>+</sup> monocytes to lung (12). Neutralization of CCL2 or CCR2 deficiency blocks monocyte recruitment, reduces MØs interacting with tumor cells and more importantly reduces lung metastasis (12). Notably, monocyte-derived VEGF has a role in promoting tumor extravasation (12). Fittingly, CCL2 induced chemotaxis of human endothelial cells and induced the formation of blood vessels *in vivo* (12). Neutralization of CCL2 also inhibits chemotaxis of human endothelial cells, increases survival and inhibits lung metastases in mice bearing human breast carcinoma cells (111). Tumor metastasis involves epithelial-mesenchymal transition (EMT). At molecular level, a group of matrix metalloproteinases (MMPs) plays a critical role in the process. Cells of the monocyte/MØ lineage secrete diverse MMPs in large quantities, stimulated by many cellular and soluble factors (112). Conceivably, CCL2/CCR2 signaling recruits abundant TAMs to produce MMPs in response to intra-tumoral environmental cues. Nevertheless, direct CCR2 signaling on macrophages also modulates MMP production (113).

### CCR2 ON T CELLS MEDIATES CHEMOTACTIC AND NON-CHEMOTACTIC FUNCTIONS

#### CCR2 Is Expressed by T Cell Subsets and Contributes to Tissue Homing of T Cells

It has been known for a long time that the chemotactic axis of the CCL2/CCR2 also directly contributes to T cell chemotaxis (114).

Phenotyping of chemoattracted T lymphocytes shows that CCR2-expressing T cells have an activated/memory phenotype (114). Further, Th1 cells have been shown to have higher expression of CCR2 and are more responsive to CCL2 induced migration (17). Activated but not naïve CD8<sup>+</sup> T cells during viral infection also express CCR2 (18). Of note, CCR2 expression by T cells also shows location and disease preference (115). CCR2<sup>+</sup> lymphocytes, predominantly CD4<sup>+</sup> T cells but not CD8<sup>+</sup> T cells, are increased in ileal Crohn's disease (CD), but not colonic CD or in ulcerative colitis. Such an increase in CCR2<sup>+</sup> lymphocytes in ileal CD is limited to lesions, without any increase in circulating CCR2<sup>+</sup> T lymphocytes (115). Functionally, experiments with CCR2<sup>-/-</sup> mice provided the evidence that CCR2 deficiency greatly impacts on Th1 and Th2 responses (6, 116, 117). However, in most cases, the contributions of CCR2 to myeloid cells and T cells in induction of a T cell response has not been delineated.

Regulatory T cells (Treg) also express CCR2 (16, 118). Notably, FoxP3<sup>+</sup> cells, particularly CD62L<sup>-</sup> ones, expressed higher levels of CCR2 than FoxP3<sup>-</sup> T cells (16). Fittingly, Ag-primed FoxP3<sup>+</sup> T cells migrate more efficiently to nonlymphoid tissues than FoxP3<sup>-</sup> T cells, although it is not clear from the study whether CCR2 mediates the migration (16). In mixed chimera mice containing both WT and CCR2<sup>-/-</sup> cells (controlling for differences in myeloid cell counts), there was a profound and selective defective accumulation of CCR2<sup>-/-</sup> FoxP3<sup>+</sup> cells in liver, lung and adipose tissue (119, 120). In an allograft model with Treg transfer, CCR2<sup>-/-</sup> Tregs migrate poorly to the inflamed grafts. Consequently, transfer of CCR2<sup>-/-</sup> Tregs did not improve allograft survival (118). High levels of CCR2 ligand also results in preferential local accumulation of Tregs (121). Overall, Tregs, compared to conventional T cells, seem to more rely on CCR2 for migration to tissue.

### CCR2 Directly Regulates Function of Conventional T Cells and Tregs

There is also *in vitro* and *in vivo* evidence supporting non-chemotactic roles for CCR2 in regulation of T cell differentiation. When CD4<sup>+</sup> T cells were activated either by antigen-pulsed APCs or polyclonal stimuli in the presence of CCL2 *in vitro*, T cells showed an increase in production of IL-4, but not IFN- $\gamma$  (122), implying a role independent of chemotaxis. In order to investigate the intrinsic activity of CCR2 on T cells independent of the innate cellular microenvironment, naïve CD45RB<sup>hi</sup> CD4<sup>+</sup>, CD45RB<sup>lo</sup> effector/memory CD4<sup>+</sup> or CD25<sup>+</sup> Tregs from WT and CCR2<sup>-/-</sup> mice were transferred into immunodeficient host RAG1<sup>-/-</sup> mice (123). Mice transferred with naïve or effector CD4<sup>+</sup> cells from WT but not CCR2<sup>-/-</sup> donors caused significant weight loss in recipient mice. However, the numbers of donor T cells accumulated in the colon were not significantly different between WT and CCR2<sup>-/-</sup> cells (123). Rather, CCR2<sup>-/-</sup> T cells showed defective production of IFN- $\gamma$  and IL-17 and were biased towards Treg differentiation. In support of an early *in vitro* study (122), CCR2<sup>-/-</sup> CD4<sup>+</sup> T cells cross-linked using anti-CD3/CD28 produced less cytokines IL-17F, IL-22, IFN- $\gamma$ , and IL-10 without impacting on cell proliferation and CCL2 seems to mediate the effect (123). This also occurs under Th17 and Th1 polarizing

conditions. In contrast, CCR2<sup>-/-</sup> CD4<sup>+</sup> T cells in the presence of TGFβ differentiated more efficiently into Foxp3<sup>+</sup> Tregs (123). At a signaling level, the levels of p-Akt were significantly reduced in CCR2<sup>-/-</sup> T cells following T cell activation. Although GPCR chemokine receptors induce activation of the PI3K/Akt network (**Figure 1**), it is not clear from the study what the up-stream signals leading to CCR2-mediated PI3K/Akt activation are.

Similar to conventional T cells, CCR2 may also directly impact on Treg function. *In vivo*, CCR2<sup>-/-</sup> Tregs are less capable of suppressing alloimmunity compared to WT Tregs when they are directly transferred into graft tissue (118). Although CCR2 deficiency does not grossly affect accumulation of Tregs in lymphoid organs (118, 119), we found that Foxp3<sup>+</sup> CD4<sup>+</sup> T cells in lymphoid organs and peripheral tissues of CCR2<sup>-/-</sup> mice express lower levels of CD25 (119), bearing in mind that CD25 expressed by Tregs can serve as an IL-2 sink to execute immune suppression (124). Fittingly, we found that CCR2<sup>-/-</sup> Tregs *in vitro* were less effective at suppressing T cell proliferation. The signaling events mediated by CCR2 leading to CD25 upregulation are currently unclear. *In vitro* innate activation by CpG can lead to CD25 upregulation on FoxP3<sup>+</sup> cells in a CCR2 dependent manner but CpG did not stimulate the overt upregulation of CD25 when FoxP3<sup>+</sup> T cells were isolated and cultured alone. Further CCL2 alone could not phenocopy CpG stimulation (119). Of note, in CCR2-DTR mice expressing diphtheria toxin receptor (DTR) under control of the *Ccr2* locus, DT (diphtheria toxin) treatment deleted CD11b<sup>+</sup>Ly6C<sup>+</sup> monocytes/MØs but did not significantly deplete Tregs and conventional T cells, likely due to lower expression level of CCR2 on lymphocytes (119).

Overall, there is evidence supporting the idea that CCR2 directly regulates the function of conventional T cells and Tregs. However, the findings are rather patchy and contradictory. For example, CCR2 deficiency has a negative impact on Treg function in some studies (118, 119), but not others (123). Thus, the significance of this aspect of CCR2 function in the context of chemotactic roles of CCR2 in migration of myeloid cells and T cells remains to be elucidated.

### CCR2 Preferentially Mediates the Recruitment of Tregs to Tumors

CCR2 can conceivably affect homing of different T cell subsets to tumoral tissues. Loyher et al. had comprehensively investigated the role of CCR2 in recruitment of Tregs and conventional T cells to tumor in different tumor models, as well as human oral squamous cell carcinoma (OSCC) (13). In mice injected subcutaneously with a tumor cell line, tumoral accumulation of Tregs but not conventional CD4<sup>+</sup> CD25<sup>-</sup>Foxp3<sup>-</sup> T cells was significantly reduced by CCR2 deficiency. The preferential tumor recruitment of Tregs was intrinsic to Tregs since parabiosis experiments between WT and Foxp3-EGFP CCR2<sup>-/-</sup> mice show that EGFP<sup>+</sup> CCR2<sup>-/-</sup> Tregs which migrated to the tumor tissue represented only 20% of total Treg in both WT hosts and CCR2<sup>-/-</sup> hosts. Live imaging also revealed that 36% WT were highly motile while only 12% CCR2<sup>-/-</sup> Tregs belonged to the highly motile group. Using

CCL2 binding as a way detecting CCR2-expressing cells, tumor Tregs contained >50% CCR2<sup>+</sup> while proportions of CCR2<sup>+</sup> Tregs in circulation and draining LNs were much lower (13). Together, the data supports that CCR2 represents a major Treg homing receptor in tumor contexts. Apart from cell homing, CCR2<sup>+</sup> Tregs had a heightened ability to produce IL-10 and self-renew than CCR2<sup>-/-</sup> counterparts, and also expressed higher levels of CD39 but not CD73 when tumor Tregs were separated into two cohorts according to their CCR2 expression (13). Interestingly to note, low dose cyclophosphamide has been shown to selectively deplete CCR2<sup>+</sup> Tregs, probably due to their higher activating and proliferating state (13).

## CCR2 SIGNALING IN CANCEROUS CELLS PROMOTE SURVIVAL/GROWTH AND METASTASIS

CCL2 can be produced by multiple cell types in the tumor environment, while CCR2 can also be expressed by multiple cell types including tumor cells. As illustrated above, the signaling axis of CCL2/CCR2 in monocytes/macrophages and T cells has a great impact on cancer immunity, and accumulating evidence also supports the concept that cancerous cells can directly employ CCR2 to promote survival/growth and metastasis. Here we provide an expanded discussion on the topic.

### Tumor Cells Express CCR2 and Produce CCL2

Expression of CCR2 by cancer cells is rather widespread, although the levels of expression may be extremely heterogenous. A study on osteosarcoma revealed CCR2 mRNA expression in all samples (125). In renal cell carcinoma, >50% metastatic tumors were CCR2<sup>+</sup> (126). Not surprisingly, AML patients (65%) also had CCR2 expressing cancer cells (127). Interesting to note, the study did not observe a direct correlation between CCR2 expression by qPCR vs protein expression by western blot or flow cytometry, implying that post-translational events such as protein degradation could contribute to CCR2 regulation. We surveyed the literature for CCR2 expression by various human tumor cell lines as well as patient samples and found that all types of solid and blood cancers expressed either CCR2 (**Table 1**). The human protein atlas database ([www.proteinatlas.org](http://www.proteinatlas.org)) also showed expression of CCR2 mRNA and protein by multiple types of cancers. Of note, CCR2 expression by tumor cells is also subject to regulation by its own ligands, as CCL2 has been shown to downregulate CCR2 in tumor cells (159). CCR2 expression by tumor cells can respond in a paracrine fashion to CCL2 produced by various non-tumor cells, tumor cells can also produce CCL2 which acts on CCR2 on tumor cells *via* an autocrine fashion. Notably, many tumor cell lines also produce CCL2 (**Table 1**). Comparison of cancer and non-cancer tissues indicated that a higher proportion of prostate cancer tissue samples produced CCL2 (160). In most cases, expression of CCR2 or CCL2 predicts metastasis and poor

**TABLE 1** | Expression of CCR2 and CCL2 by human tumor lines and patient samples.

Type of tumor cell	Cell line	CCR2	CCL2	Reference
<b>Breast carcinoma</b>	MDA-MB-231	+	+/-	(128–130)
	MCF10A	+	-	(131)
	MCF7	+	+	(129)
	4T1	+	+	(128, 129)
	<b>Patient samples</b>	+	+	(132)
<b>Osteosarcoma</b>	MG63	+	+	(133)
	U2OS	+	+	(133)
	HOS	+	+	(133)
	<b>Patient samples</b>	+	+	(125)
	<b>Non-small cell lung carcinoma</b>	A549	+	+
NCI-H460		-	+	(134)
NCI-H1299		+	+	(128)
LC99A		-	-	(134)
<b>Patient samples</b>		+	+	(135)
<b>Hepatocellular carcinoma</b>	Huh-7	+	+	(136, 137)
	HepG2	+	+	(136, 138)
	Hep3B,		+	(136, 138)
	MHCC-97L,		+	(136)
	MHCC-97H	+	+	(136, 138)
	LM3		+	(136)
	SMMC-7721		-	(136, 138)
	<b>Patient samples</b>	+	+	(136, 138)
<b>Oophoroma</b>	OVCAR-3	+	+	(139)
	SK-OV-3	+	+	(139, 140)
	<b>Patient samples</b>	+	+	(139)
	<b>Gastric adenocarcinoma</b>	SGC7901		+
BGC823			+	(141)
GES-1		<b>Inducible</b>		(141)
GC1401, GC1415 and GC1436		-		(142)
<b>Patient samples</b>		+	+	(141, 143)
<b>Prostatic cancer</b>		PC-3	+	+
	DU145	+		(128)
	LNCap	+		(128)
	C42B	+		(128)
	<b>Patient samples</b>	+		(128)
	<b>Pancreatic cancer</b>	Panc-1		+
PC13			+	(144)
PT45P1			+	(144)
Capan-1			+	(144)
MiaPaca-2			+	(144)
<b>Patient samples</b>			+	(145)
<b>Renal carcinoma</b>	786-O	+	+	(146)
	CaKi-1	+	+	(146)
	<b>Patient samples</b>	+	+, CCL7+	(126, 147)
<b>Melanoma/epidermoid carcinoma</b>	LM16-R cells		+	(148, 149)
	A431		+	(150)
	<b>Patient samples</b>		+	(148, 149)
<b>Glioblastoma/meningioma</b>	IOMM Lee		+	(151)
	SF-3061		+	(151)
	<b>Patient samples</b>	+	+	(152, 153)
<b>Colorectal cancer</b>	HCT116	+		(154)
	SW480	+		(154)
	SW1116p21 cells		+	(155)
	<b>Patient samples</b>	+	+	(154–156)
<b>Head and neck cancer</b>	<b>Patient samples</b>	+		(157)
	Kas	+/-		(127, 128)
<b>Blood cancer</b>	Raw264.7	+	+	(43)
	THP-1	+	+	(127)
	NB4	+	+	(158)
	U937	+/-	+	(127, 159)
	<b>Patient samples</b>	+		(127)

survival. For example, CCR2 mRNA expression correlates with prostate cancer progression and pathological stages (128, 161).

Another important player in tumor microenvironment is cancer-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs) (162). CAFs can promote cancer progression by multiple mechanisms. One mechanism is to produce CCL2 (163). Interestingly, CAFs from breast cancer patients but not normal human mammary fibroblasts expressed high levels of CCL2 mRNA in response to activation by various breast cancer cells (163). CAF-derived CCL2 could induce cancer stem cells by activating NOTCH signaling. Consequently, knockdown of CCL2 in CAFs inhibits *in vivo* tumorigenesis cells (163). In support, murine fibroblast activation protein (FAP)<sup>+</sup> CAFs were a major source of CCL2 (164). In the system, FAP activates STAT3–CCL2 signaling cascade to recruit TAMs and promoted tumor growth in a murine liver cancer model. Fittingly, in human intrahepatic cholangiocarcinoma, high expressions of stromal FAP and CCL2 were negatively associated with ICC patient survival (164). In a murine skin cancer model, CAFs also produced CCL2 to retain TAMs and promote skin carcinogenesis (165). In a study with human liver CAFs and cancer lines, CAFs could up-regulate CCL2 expression in cancer cells (166). Thus, CAFs can participate tumor progression and metastasis *via* CCL2/CCR2 axis.

### CCR2 Signaling Promotes Tumor Metastasis/Invasion

In this section, we mainly consider tumor metastasis/invasion from the angle of direct CCR2 signaling of tumor cells. A large body of evidence comes from *in vitro* investigations with tumor cell lines of different types. THP-1 cells are known for high expression of CCR2 and undergo enhanced migration in the presence of CCL2 (127). Such migration was significantly reduced in the presence of a neutralizing CCL2 monoclonal antibody or blocking CCR2 monoclonal antibody. On the other hand, the CCR2<sup>lo</sup> U-937 cell line showed no CCL2-induced migration (127). Similarly, migration studies with patient AML samples could also be blocked by an Ab to CCR2 or CCR2 inhibitor (127). For solid tumors, CCL-2 enhanced the migration and invasion of MCF-7 cells (128, 167). Migration of RCC cells in the presence of a CCR2 antagonist or neutralizing CCL2 antibody was also markedly decreased compared with control cells and enhanced by supplemented CCL2 (128, 146).

As discussed, MMPs are a family of zinc-dependent endoproteinases and facilitate tumor cell invasion/metastasis. Within the MMPs, MMP-9 has been shown to degrade the extracellular matrix (ECM) to remove physical barriers for metastasis, increase cell motility, and promote angiogenesis (56, 168). *In vitro* studies have shown that CCL2 can increase MMP-9 expression in human chondrosarcoma cells (55), in NSCL A549 cell migration (167) and in human breast cancer MCF-7 cells (169). Upstream signaling leading to MMP-9 upregulation involves activation of protein kinases MEK/ERK and NF- $\kappa$ B (55). Thus, the CCL2/CCR2 axis not only regulates

MPP production and therefore tumor invasion/metastasis *via* its action on TAMs, but also *via* its action on tumor cells directly.

### CCR2 Signaling Promotes Tumor Growth/Survival

The direct action of CCR2 on tumor cells can also lead to survival and proliferation of tumor cells. CCL2 has been found to promote survival and proliferation of THP-1 (127), prostate cancer line PC3 cells (43), renal cell carcinoma lines 786-O and CaKi-1 cells and lung carcinoma line A549 cells (134). Accordingly, cells which were grown in serum-free medium and treated with a CCL2 neutralizing antibody or a CCR2 antagonist (RS504393) had significantly decreased proliferation of tumor cells compared with untreated control cells (134, 146). Of note, endogenous CCL2 expression levels appear to be sufficient for the maximum proliferation rate of 786-O and CaKi-1 cells but not for the maximum migratory ability of RCC cells (146). Related to cancer treatment, CCL2 significantly reduced apoptosis of mouse and human breast cancer cells induced by gentamicin or 5-FU treatment as determined by cleaved caspase-3 expression (129). CCR2<sup>-/-</sup> cancer cells were also more sensitive to CTL-mediated killing, compared to WT cancer cells (170). However, it has also been reported that CCL2 and GM-CSF-CCL2 fusion protein induced BAX expression and apoptosis in CCR2<sup>+</sup> EG7 but not CCR2<sup>-</sup> B16 cells (171). At a signaling level, activation of the PI3 kinase/Akt pathway was found to be vital for the CCL2-mediated proliferative effects of prostate cancer epithelial cells (43). Moreover, CCL2 can promote the viability, proliferation and invasiveness of endometrial stromal cells through the Akt and MAPK/Erk1/2 signal pathways (172). However, understanding of cell death pathways influenced by the CCL2/CCR2 is still very limited.

## DEVELOPMENT OF CCR2 ANTAGONISM IN CANCER THERAPY: HOW DO WE GET THERE?

### Clinical and Preclinical Studies Targeting CCL2/CCR2 Axis: An Update

Perhaps owing to the critical role of CCR2 in the traffic of inflammatory monocytes, CCR2 antagonists have been developed by many pharmaceutical companies for potential use in the treatment of rheumatoid arthritis, asthma, diabetes, inflammatory bowel disease and multiple sclerosis, cardiovascular diseases (173) (Tables 2, 3). However, CCR2 antagonists had not generated favorable outcomes in several Phase II clinical studies (173). Redundancy of the target, poor drug-like properties, insufficient drug level and many other factors could all contribute to the failed clinical trials with small molecules targeting chemokine receptors (173).

Despite the setbacks in development of CCL2/CCR2 in clinical studies, particularly for inflammatory conditions, the detrimental roles of the CCL2/CCR2 axis in cancer setting have increasingly



**TABLE 2** | Antagonists targeting CCR2-CCL2 axis.

Product	Developer	Stage	Target	Indications	Status	References/Trial ID
AZD2423	AstraZeneca	II	CCR2	Chronic obstructive pulmonary disease (COPD)	Inactive	NCT01215279
BMS-687681	Bristol-Myers Squibb	Pre	CCR2, CCR5	Cancer	Active	(174)
BMS-741672	Bristol-Myers Squibb	II	CCR2	Type II diabetes	Inactive	NCT00699790
BMS-813160	Bristol-Myers Squibb	I/II	CCR2, CCR5	Pancreatic cancer, Colorectal cancer, Liver cancer, NSCL	Active	NCT03184870; NCT04123379
BMS-813160	Bristol-Myers Squibb	II	CCR2, CCR5	Type II diabetes	Active, Inactive	NCT01752985
CCX140	ChemoCentryx, Vifor Pharma	II	CCR2	Type II diabetes, Fibrosis	Active	(175), NCT01447147.
CCX872	ChemoCentryx	I/II	CCR2	Pancreatic cancer	Active, Inactive	NCT02345408, NCT03778879
Genicriviroc	Takeda Pharmaceuticals, Dong-A Pharma, Tobira Therapeutics (AbbVie)	II	CCR2, CCR5	HIV	Inactive	(176)
Genicriviroc	Takeda Pharmaceuticals, Dong-A Pharma, Tobira Therapeutics (AbbVie)	III	CCR2, CCR5	NASH, HIV, COVID19	Active, Inactive	NCT03028740
CNTX-6970	Centrexion Therapeutics	I	CCR2	Pain	Active	NCT03787004
INCB8696	Incyte	I	CCR2	MS	Inactive	(177)
INCB3344	Incyte	Pre	CCR2	MS, RA	Inactive	(178)
INCB3284	Incyte	Pre	CCR2	Undefined inflammation	Inactive	(179)
INCB10820	Incyte, Pfizer	Pre	CCR2, CCR5	Autoimmune diseases	Inactive	(180)
JNJ-17166864	Johnson & Johnson	II	CCR2	Allergic rhinitis	Inactive	NCT00604123
JNJ-27141491	Johnson & Johnson	Pre	CCR2	MS	Inactive	(181)
JNJ-41443532	Johnson & Johnson	II	CCR2	Type II diabetes	Inactive	NCT01230749
MK-0812	Merck & Co.	II	CCR2	RA, MS	Inactive	NCT00239655
PF-04136309	Incyte, Pfizer	II	CCR2	Pancreatic cancer, Arthritic pain, Chronic hepatitis	Inactive	NCT02732938; NCT00689273; NCT01226797
PF-04634817	Pfizer	II	CCR2, CCR5	Type II diabetes	Inactive	NCT01712061
RAP-103	Creative Bio-Peptides	Pre	CCR2, CCR5, CCR8	Neuropathic Pain	Active	(182)
RO5234444	Roche	Pre	CCR2	Type II diabetes	Inactive	(183)
SSR150106	Sanofi	II	CCR2	RA	Inactive	NCT00545454
Tropifexor+	Allergan (AbbVie), Novartis	II	CCR2, CCR5, FXR	NASH	Active	CLJC242A2201J; NCT03517540
WXSH0213	WuXi AppTec, Zhongsheng Pharmaceuticals	Pre	CCR2, CCR5	NASH	Active	(184)
Bindarit	Angelini	II	CCL2	Type II diabetes, Atherosclerosis	Inactive	NCT01109212
NOX-E36	NOXXON Pharma AG	II	CCL2	Type II diabetes	Inactive	NCT01547897
NOX-E36	NOXXON Pharma AG	Pre	CCL2	Liver fibrosis	Inactive	(185)

been appreciated so that the CCL2/CCR2 axis remains to be actively targeted as candidates for immunotherapy (28) (Tables 2, 3). We surmise above that CCR2 has enhancing effects on two main types of immunosuppressive immune cells to promote tumor progression and metastasis. Thus, together with direct effects on tumor cells, targeting the CCL2/CCR2 axis likely generates multiple attacks on multiple unfavorable cell types (including cancer cells) within the TME (Figure 2). In view of the rather disappointing developmental history of CCR2 antagonism, and the now increased understanding of mode of action of the CCL2/CCR2 axis, we attempt to discuss the strategies that could facilitate tailored application of CCR2 antagonism in cancer therapy.

## Can Elimination of CCR2-Expressing Cells Offer Advantage Over Functional Blocking of CCR2?

In a study dissecting the origin of TAM (84), the TAM percentage in CCR2<sup>-/-</sup> mice in the MMTV-PyMT (PyMT) mammary tumor model was found not to be significantly different compared to WT controls. However, in CCR2-DTR PyMT mice expressing diphtheria toxin receptor (DTR) under control of the *Ccr2* locus, DT (diphtheria toxin) treatment resulted in a large depletion of tumor-associated monocytes and significant reduction of TAM numbers (84). CCR2 expression by TAMs could also render them sensitive to DT

**TABLE 3** | Antibodies targeting CCR2-CCL2 axis.

Product	Developer	Stage	Target	Indications	Status	Reference/Clinical Trail No.
Anti-CCR2	Pfizer, Amgen,	Pre	CCR2	Solid tumor, Inflammation	Active	US9238691B2
Anti-CCR2	MRC, U.Regensburg	Pre	CCR2	MS, RA	Active	US9068002B2
Anti-CCR2	Sorrento	Pre	CCR2	MS	Inactive	(186)
Anti-CCR2, CSF-1R	Elstar	Pre	CCR2, CSF-1R	Inflammation	Not Clear	WO1997031949A1
CCL2-LPM	Osprey Pharmaceuticals	I	CCR2	IgA nephropathy	Inactive	NCT00856674
Plozalizumab (MLN1202)	Takeda Pharmaceuticals	II	CCR2	Solid tumors	Inactive	NCT02723006
Plozalizumab (MLN1202)	Takeda Pharmaceuticals	II	CCR2	Atherosclerosis	Inactive	NCT00715169
VET2-L2 (oncolytic virus)	Astellas Pharma, Kalivir Immunotherapeutics	Pre	CCR2, leptin, IL-2	Solid tumors	Active	(187)
ABN912	Novartis	I	CCL2	RA	Inactive	(188)
Carlumab (CNTO 888)	Johnson & Johnson	II	CCL2	Solid tumor, Prostate cancer	Inactive	NCT01204996, NCT00992186 (189),
Carlumab (CNTO 888)	Johnson & Johnson	II	CCL2	Idiopathic pulmonary fibrosis, Tumor	Inactive	NCT00786201
ABN912	Novartis	Pre	CCL2	Tumor	Not Clear	(190)
Anti-CCL2	Shire Human Genetic Therapies	Pre	CCL2	Scleroderma	Not Clear	WO2013177264A1

treatment (191). Thus, there is a difference between functional deficiency of CCR2 and elimination of CCR2<sup>+</sup> myeloid cells (either TAM precursors or TAM themselves). Similarly, deletion of TAMs but not CCR2 deficiency slows the growth of mouse lung adenocarcinomas (192). Further, the same contrast of deletion vs inhibition could also apply to CCR2 expressing Tregs and tumor cells. We need to consider whether elimination of CCR2-expressing cells offers advantage over functional blocking when developing a CCR2 antagonist.

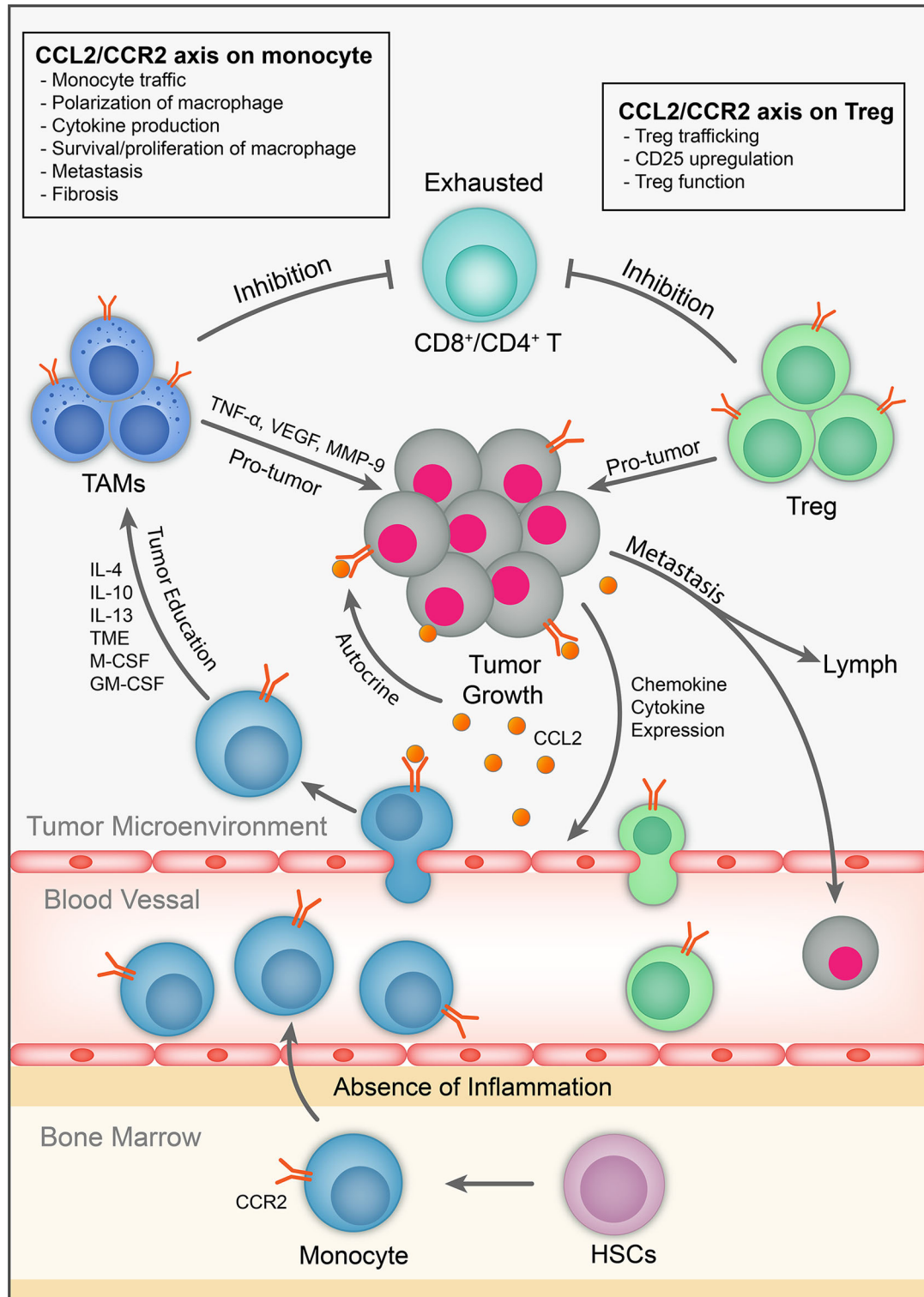
For clinical studies, how can we achieve the above different outcomes? Arguably, classical CCR2 antagonists could only block CCR2 function, but not directly delete the CCR2-expressing cells. Similarly, neutralization Abs to CCL2 are also unlikely deplete CCR2-expressing cells. On the other hand, administration of an anti-CCR2 antibody could drastically decrease the total number of monocytes/MØs in the peritoneal fluid but not the number of infiltrating granulocytes and lymphocytes, although it was not conclusive whether that reduction was caused by direct deletion (193). The same Ab was used subsequently to delete Ly6C<sup>+</sup> monocytes during infection (194). Most depleting Abs, likely containing a human IgG1 Fc region could trigger Fc-dependent effector mechanisms including complement-dependent cytotoxicity (CDC), antibody-dependent cell cytotoxicity (ADCC), and phagocytosis (195). Thus, design of proper anti-CCR2 Abs needs taking mode of action into consideration. As several anti-CCR2 Abs are under development (Table 3), it would be of interest whether anti-tumor efficacy links to their cell depleting property, and whether the depleting effect of anti-CCR2 Abs could be enhanced by antibody drug conjugation *via* antibody-guided delivery of cytotoxic drugs. Of note, anti-human CD64 antibody (clone H22) conjugated to a cytotoxic agent dimeric pyrrolbenzodiazepine (dPBD) depleted CD163<sup>+</sup> TAMs in humanized mice inoculated with human oral squamous cell carcinoma HSC-4 cells (196). Thus, the depleting effect of anti-CCR2 Abs could be enhanced by

antibody drug conjugation *via* antibody-guided delivery of cytotoxic drugs.

Depletion of suppressive immune cells by targeting CCL2/CCR2 axis could also consider Treg cells. Within the TME, Treg cells consist of a large proportion of human tumor CD4<sup>+</sup> infiltrates (197). Importantly, deletion of Tregs abrogates immunological unresponsiveness and promotes anti-tumor response (198). As discussed, Tregs express CCR2 (16, 118) and tumor Tregs contained an even higher proportion of CCR2<sup>+</sup> Tregs (13). Importantly, CCR2 preferentially mediates tissue homing of Tregs including tumor tissue (13, 119, 120). Radiotherapy in cancer induces the recruitment of CCR2<sup>+</sup> Tregs that contribute to treatment resistance (199). Notably, a phase 1 trial with a CCR2 antagonist in non-metastatic pancreatic cancer patients demonstrated that CCR2 inhibition decreases the number of tumor-infiltrating Tregs (200). Apart from Treg trafficking, CCR2 also regulates CD25 expression on Tregs and Treg function (119). Together, it may be important to consider the role of CCR2 in regulating traffic and function of Tregs when targeting CCR2 in cancer therapy.

### Does the Promiscuity of CCL2 and CCR2 Partnership Affect the Targeting Efficacy?

As discussed above, although CCL2 is a prototype CCR2 ligand, CCR2 can also partner with several other CCR2 agonists including CCL7 (MCP-3), CCL8 (MCP-2), CCL13 (MCP-4) and CCL12 (MCP-5) (201). Interestingly, deficiency of CCR2 ligand CCL7 but not CCL8 and CCL12 could reduce monocyte egress from bone marrow (87). Conversely, CCL2 has been reported to bind CCR4 on activated Tregs (202) and CD8<sup>+</sup> T cells (203). Blocking the CCL2/CCR4 axis in Tregs evokes an anti-tumor immune response in a model of head and neck squamous cell carcinoma (202) while blocking of CCL2/CCR4 axis on CTLs could be detrimental as argued in the study (203).



**FIGURE 2** | The role of CCL2/CCR2 axis in tumor immunology. CCL2 is expressed by immune cells, cancer and stromal cells in the TME. It exhibits chemotactic and non-chemotactic effects on CCR2-expressing monocytes/macrophages. It also has chemotactic and non-chemotactic effects on CCR2-expressing T cells, particularly Tregs. It also induces tumor cell proliferation/survival and metastasis in autocrine and paracrine fashion.

As a further complication, human CCR2 has two isoforms. As aforementioned, induction of CCR2A and CCR2B and signaling outcomes of CCR2A and CCR2B could be different. It remains to be known whether these underappreciated aspects of the mode of action of CCR2 and CCL2 could impact on the efficacy of different targeting strategies.

Related to the above context, significant and broader redundancy exists for cross talk between various chemokine receptors and ligands (10). Many chemokine receptors such as CCR1, CCR2, CCR5 and CXCR3 have all been implicated in the pathophysiology of multiple sclerosis and rheumatoid arthritis. It leads to the proposal to target more than one chemokine receptor (10). Similarly, the redundancy of the chemokines is also implicated. For example, as CCL7 acts a ligand for CCR2 for monocyte traffic (87), CCL7 can also act as a ligand for CCR1, CCR3, CCR5 and CCR10 (204). In a model of lung metastasis of breast cancer, CCR2<sup>+</sup> MØs upon activation by CCL2 produce CCL3 to induce CCR1-mediated TAM retention in the lung (205), supporting involvement of multiple chemokines and receptors in tumor metastasis. Thus, combination therapies targeting multiple chemokines/chemokine receptors in the form of a single molecule had been proposed to counter such redundancy (10). Recent advances in engineering of bispecific, trispecific and even tetraspecific antibodies as a single molecule (206) may help to realize such a tenet.

### Does CCR2 Antagonism Preferentially Affect Primary Tumor and Metastasis

It has been shown that tumor-derived CCL2 supports tumor growth, depending on CCR2 expression by host cells (136) in mouse models of transferred liver cancer cells. Treatment with a CCR2 antagonist reduced the liver tumor volume, inhibited the abdominal dissemination and metastasis of cancer cells and reduced the recurrence of HCC after surgery (136). Interestingly, in the model, the CCR2 antagonist had no therapeutic effect on tumor cells with no CCL2 expression. Thus, the CCL2/CCR2 axis has effect on control of primary tumor. Nevertheless, an adoptive transfer study indicated that inflammatory monocytes preferentially migrate to the lung with metastatic tumors over the primary tumor (12). Further, CCL2 produced by MØs is required for metastatic seeding of cancer cells rather than tumor growth at the primary and metastatic sites (205). Similarly, in another study with breast cancer lines, CCL2 overexpression by tumor cells promoted cancer metastasis to lung and bone. Accordingly, CCL2 neutralization reduces metastasis (207). Thus, the CCL2/CCR2 axis has a critical role in the multiple stages of cancer metastasis (201).

Immunotherapy of cancer has also been explored with local administration of immunotherapies into the tumor (208). Local depletion of Treg cells inside the tumor led to the eradication of highly aggressive tumors and the development of persistent antitumor memory in a mouse model (209). Local depletion of MØs has been attempted to manipulate local immune responses in non-tumor models (210, 211). Conceivably, local depletion of CCR2<sup>+</sup> TAM, Treg and tumor cells as well as local neutralization of ligand CCL2 in TME will likely enhance anti-tumor arm of immunity.

Although CCR2 antagonism in cancer is predominantly discussed in the context of solid tumors, the CCL2/CCR2 axis is also implicated in blood cancers. Not surprisingly, human monocytoid AML samples expressed CCR2 and CCL2 (212). Like solid tumors, the CCL2/CCR2 axis in AML also affects MØ phenotype in leukemia-bearing mice (213). Consequently, blockade of the CCL2/CCR2 axis affects migration and signaling of AML cells and MØs (213). In addition, CCR2 is expressed on a small fraction of human B lymphocytes in peripheral blood (PB) and tonsils (21, 214) and the cell-surface expression of CCR2 in different types of B-cell lymphomas can be rather high in patients with chronic lymphocytic leukemia (CLL) (215). Recruitment of monocytes towards the CLL lesion is also mediated by CCR2 (216). In a mouse model of multiple myeloma, osteoclasts have been found to express CCR2-targeting chemokines, and an anti-CCR2 monoclonal antibody was able to block osteoclast chemoattractant activity for multiple myeloma cells (61). Thus, there is evidence for the role of the CCL2/CCR2 axis in blood cancers.

### Can Combinational Therapy Broaden Application of CCR2 Antagonism in Cancer Therapy?

Development of CCR2 antagonism as monotherapy in autoimmune and inflammatory diseases has not been very successful, owing to many factors (10). As we highlighted above some aspects of how to target CCL2/CCR2 in cancer, we also contend that combination therapy may broaden and improve application of CCR2 antagonism as cancer therapy.

CCR2 antagonism has previously been combined with immune checkpoint inhibitors. In a mouse model transferred with bladder cancer line, combination therapy of a CCR2 antagonist and an anti-PD-L1 antibody showed significant synergy (217). The same combination also reduced murine melanoma pulmonary metastases and mammary fat pad tumor growth of implanted breast cancer cells (217). Notably, for bladder cancer growth and pulmonary metastases in the study, treatment with anti-PD-L1 did not overtly offer benefit (217). Similarly, in another study with glioma, tumor growth was not significantly inhibited by anti-PD-L1 monotherapy. However, anti-PD-L1 antibodies had a strong anti-tumor effect when combined with CCR2 deficiency or CCR2 inhibition (90). Immunologically, only combination treatment significantly enhanced intratumour T-cell responses (90). Synergy between CCR2 inhibition and immune checkpoint inhibitors was also reported for cutaneous T-cell lymphoma in a murine model (218). Collectively, these recent studies advocate targeting the CCL2/CCR2 axis to improve current immunotherapy with immune checkpoint inhibitors.

As combination with immune checkpoint inhibitors is a prime consideration, can CCR2 antagonism be combined with other strategies of immune modulation? Pancreatic ductal adenocarcinoma (PDAC) is considered to be resistant to immunotherapy. A recent study has highlighted the severe deficiency of conventional dendritic cells (cDC) with anti-tumor properties, specifically cDC1s in the presence of high



numbers of suppressive TAMs in both murine model and human patient samples (219). Mobilizing cDCs into early pancreatic lesions with Flt3L administration can improve IFN- $\gamma$  producing T cell response and disease stabilization. Furthermore, combination of a STING agonist (RR-S2-CDA) or CD40-agonist with Flt3L further increased the influx of cDC1s and enhanced intratumoural CD8<sup>+</sup> CTL and CD4<sup>+</sup> Th cell infiltration without Treg induction (219). Considering the positive outcome with CCR2 inhibition in pancreatic cancer (200), we speculate that a combination of CCR2 inhibition with the above strategies to increase cDCs would further warm up the TME in favor of anti-tumor responses.

Notably, changes in the TME could alter responsiveness to conventional cancer therapy. In the above study, cDC-directed therapy increased responsiveness of pancreatic cancer to radiation therapy (219). In the context of the CCL2/CCR2 axis, CCR2<sup>-/-</sup> mice have been found to respond better to treatment with doxorubicin or cisplatin (220). On the other hand, doxorubicin treatment can lead to CCL2 production by stromal cells from tumor microenvironment and contributes to drug resistance by recruitment of suppressive myeloid cells (220). These studies provide a rationale to combine CCR2 antagonism and chemotherapy. In clinical trials in pancreatic patients with a CCR2 inhibitor, combination therapy with FOLFIRINOX showed better overall survival compared to FOLFIRINOX monotherapy (200). Notably, clinical trials with anti-CCL2 antibody Carlumab, with or without combination chemotherapy did not offer significant efficacy, probably due to lack of long-term neutralization of CCL2 (221) or redundancy issue discussed above. Similar to chemotherapy, Radiotherapy could induce the CCR2-dependent recruitment of monocytes and CCR2<sup>+</sup> Tregs into the tumor (199). Similarly, radiotherapy increased the release of chemokines CCL2 and CCL5 in experimental tumor models. Fittingly, blockade of CCR2 and CCR5 improved the efficacy of radiotherapy in radioresponsive

tumors (222). Together, evidence supports combining CCR2 antagonism with chemotherapy and radiotherapy.

## OVERVIEW/CONCLUSIONS

CCR2 antagonism has been investigated as a therapeutic for autoimmune diseases and more recently for cancer over many decades. Suffice to say, clinical trials so far have not been very successful (10, 189, 223, 224). There are many factors contributing to the results (10). In the context of cancer, we highlight here the complex and sometimes underappreciated roles of the CCL2/CCR2 axis in regulating abundance and functions of monocytes/M $\phi$ s, T cells (particularly Tregs) and tumor cells (Figure 2). With the increase in the understanding of modes of action, expression regulation of CCL2/CCR2 in various immune cells, other stromal cells, and cancer cells, and improved and versatile delivery platforms including multiple specific Ab as a single biologic, antibody drug conjugation and PROTAC, as well as identification of biomarkers with predictive value, we contend that there are still potential solutions to generating effective therapeutics targeting CCR2/CCL2 axis in cancer.

## AUTHOR CONTRIBUTIONS

LF, XR, HY, and YZ wrote the manuscript. XR made the figures. XR, HY, and YZ made the tables. YZ supervised the writing. All authors contributed to the article and approved the submitted version.

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