Expansion of CCR8+ Inflammatory Myeloid Cells in Cancer Patients with Urothelial and Renal Carcinomas

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Abstract

Purpose: Chemokines are involved in cancer-related inflammation and malignant progression. In this study, we evaluated expression of CCR8 and its natural cognate ligand CCL1 in patients with urothelial carcinomas of bladder and renal cell carcinomas.

Experimental Design: We examined CCR8 expression in peripheral blood and tumor tissues from patients with bladder and renal carcinomas. CCR8-positive myeloid cells were isolated from cancer tissues with magnetic beads and tested in vitro for cytokine production and ability to modulate T-cell function.

Results: We show that monocytic and granulocytic myeloid cell subsets in peripheral blood of patients with cancer with urothelial and renal carcinomas display increased expression of chemokine receptor CCR8. Upregulated expression of CCR8 is also detected within human cancer tissues and primarily limited to tumor-associated macrophages. When isolated, CD11b+CCR8+ cell subset produces the highest levels of proinflammatory and proangiogenic factors among intratumoral CD11b myeloid cells. Tumor-infiltrating CD11b+CCR8+ cells selectively display activated Stat3 and are capable of inducing FoxP3 expression in autologous T lymphocytes. Primary human tumors produce substantial amounts of the natural CCR8 ligand CCL1.

Conclusions: This study provides the first evidence that CCR8+ myeloid cell subset is expanded in patients with cancer. Elevated secretion of CCL1 by tumors and increased presence of CCR8+ myeloid cells in peripheral blood and cancer tissues indicate that CCL1/CCR8 axis is a component of cancer-related inflammation and may contribute to immune evasion. Obtained results also implicate that blockade of CCR8 signals may provide an attractive strategy for therapeutic intervention in human urothelial and renal cancers. Clin Cancer Res; 19(7); 1670–80. ©2013 AACR.

Introduction

Emerging evidence indicates importance of inflammation in tumor initiation and progression. However, information on specific mechanisms or mediators of cancer-related inflammation in human cancers is still limited (1, 2). Recent studies show that a substantial portion of inflammatory cells in human tumor tissues is represented by CD11b+ myeloid cells that include large populations of tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC; ref. 3). TAMs represent an abundant and heterogeneous cell population in the tumor microenvironment and they play a key role in tumor development (4, 5). For example, although M1-oriented TAMs constitute a critical component of the antitumor immune response, they are frequently subverted in the tumor microenvironment into alternatively activated M2 type that promotes tumor progression.

Chemokines and their receptors are involved in malignant progression (2, 6). Some chemokines, such as CCL1, CCL2, CCL17, and CCL22, have been shown to promote M2 and T-helper (Th2)2 polarization in tumors that subvert the immune system by establishing a microenvironment of immune cells and cytokines that suppress specific antitumor responses. Hence, it is critical to study the mechanisms by which specific chemokines and their receptors mediate inflammatory cells traffic into tumor tissue and their functions. Despite the fact that chemokines are abundantly expressed in tumors, there is little information concerning chemokine receptor expression in circulating or tumor-infiltrating leukocytes in human patients with cancer.

CCR8 is a chemokine receptor that was initially described as a Th2 cell–restricted receptor (7, 8). CCR8 is believed to...
CCR8+ Inflammatory Myeloid Cells in Cancer Patients

Translational Relevance

In the current study, we show that progression of human urothelial and renal carcinomas is associated with increased expression of chemokine receptor CCR8. Upregulated CCR8 expression was detected in peripheral and tumor-infiltrating myeloid cell subsets. At the functional level, we show that CD11b+CCR8+ cell subset may contribute to immune evasion in human cancers through elevated secretion of proinflammatory [interleukin (IL)-6, CCL3, CCL4] and proangiogenic (VEGF, IL-8) factors as well as through induction of FoxP3 expression in autologous T lymphocytes. We conclude that increased numbers of CCR8+ myeloid cells as well as enhanced production of CCL1 by primary tumors represent a common feature of bladder and renal carcinomas, thus implying relevance of CCR8/CCL1 axis to the cancer-related inflammation and immune evasion.

Statistical analysis

Values are expressed as mean ± SD. Unpaired Student t test was used to determine statistical significance between groups (GraphPad Prism; GraphPad Software, Inc.). The criterion for significance was set at P < 0.05. The flow cytometric data shown are representative of at least 3 separate determinations.

Results

CCR8-expressing myeloid cell subset in peripheral blood of cancer patients

Tumors are known to secrete large amounts and a wide range of chemokines that affect function of host’s immune and inflammatory cell subsets, including myeloid cells of bone marrow origin (14). Given enhanced production of chemokines by tumors, we hypothesized that to respond to those tumor-derived chemokines, some myeloid cell subsets in patients with cancer might display upregulated expression of certain chemokine receptor(s). To examine this possibility, we measured expression of several chemokine receptors, including CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR2, and CXCXR4, in myeloid cells obtained from peripheral blood of patients that have been diagnosed with bladder cancer or RCC (Supplementary Table S2A). As exemplified by paired healthy donor and patients with cancer in Fig. 1A and Supplementary Fig. S1, among screened receptors, CCR8 expression was markedly upregulated in patients with bladder and kidney cancers. As can be seen, CD33+ myeloid cells in patients with cancer constitute of 2 cell subpopulations (CD33\textsuperscript{high} and CD33\textsuperscript{low}). This reflects the presence of monocytic and granulocytic MDSC populations in peripheral blood of patients with cancer (15). Significantly, both monocytic CD33\textsuperscript{high}CD11b+ and granulocytic CD33\textsuperscript{low}CD11b+ MDSCs in peripheral blood of patients with cancer displayed increased expression of CCR8. Together, in 12 patients with cancer, we consistently observed elevated expression of CCR8 in CD11b+ myeloid cells in comparison to healthy donors (Supplementary Table S2B).

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Translated relevance

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Characterization of CCR8-expressing myeloid cells obtained from peripheral blood

We examined whether CCR8 is functional by treating peripheral blood mononuclear cells (PBMC) obtained from patients with bladder cancer with CCR8 ligand CCL1 and measuring CCL1-elicited intracellular Ca\(^{2+}\) influx using Fluo-4–based assay. Results presented in Fig. 1B show that in vitro stimulation of PBMCs from a patient with bladder cancer (bottom) but not from a healthy donor (top) with recombinant human CCL1 induced Ca\(^{2+}\) influx in gated CD11b cells. Because CD11b cells also express variable levels of another chemokine receptor CXCR4, we compared the Ca\(^{2+}\) influx induced by CCL1 and the CXCR4 ligand SDF-1. As shown in Supplementary Fig. S2, both chemokines stimulated the Ca\(^{2+}\) mobilizations, albeit SDF1 elicited a stronger response. Collectively, these results showed that CCR8-expressing cells readily respond to the stimulation with CCL1 to induce Ca\(^{2+}\) influx, suggesting that myeloid cells in patients with cancer express functional CCR8. In addition to Ca\(^{2+}\) mobilization, we also examined whether CCL1-mediated signaling could stimulate production of reactive oxygen species (ROS) in myeloid cells. Obtained results indicate that treatment of CD11b cells isolated from peripheral blood of patients with cancer with CCL1 had no effect on ROS production (data not shown).

In separate experiments, we found that expression of CCR8 also can be induced in myeloid cells by culturing them in the presence of primary bladder tumor-conditioned medium (TCM; Fig. 1C and Supplementary Table S3A) and the addition of CCL1 to cells pretreated with TCM induced Ca\(^{2+}\) influx (Supplementary Fig. S2C). To test whether TCM-mediated induction of CCR8 expression in myeloid cells associates with activation of specific transcription factors, we compared phosphorylation status of
extracellular signal–regulated kinase (ERK), Stat1, and Stat3 in TCM-treated CD11b cells that were isolated from peripheral blood of healthy donors. Results presented in Fig. 2A and Supplementary Table S3B indicate that exposure of normal myeloid cells to the TCM promoted a strong increase in Stat3 phosphorylation, a relatively weak ERK phosphorylation, and no effect on Stat1 phosphorylation. Addition of Stat3 inhibitor S3I-201 completely prevented the TCM-mediated upregulation of CCR8 in the myeloid cells (Fig. 2B). Distinctly, the inhibition of active ERK with U0126 or PD098059 showed no impact on the TCM-regulated expression levels of CCR8. These data indicate that TCM-induced CCR8 expression in myeloid cells depends on Stat3 activity.

To evaluate the immune function of CCR8-expressing cells, we examined effects of CCL1 on cytokine production by myeloid cells obtained from peripheral blood of cancer patients. To this end, CD11b+ cells were isolated from PBMCs of healthy donors and patients with cancer and were cultured in the presence of CCL1 for 24 hours. Cell supernatants were collected and analyzed for the presence of 10 cytokines and chemokines, including IL-1β, TNF-α, IL-6, IL-8, VEGF, basic fibroblast growth factor (FGF), platelet-derived growth factor (PDGF)-BB, CCL2, CCL3, and CCL4 using Multiplex cytokine assay. Obtained results indicate that treatment of peripheral myeloid cells from patients with both bladder (Fig. 3A) and kidney (Fig. 3B) cancer exhibited enhanced IL-6 production. Furthermore, we found that CCL1 stimulates IL-6 production by peripheral CD11b+ cells from patients with cancer in a dose-dependent manner (Fig. 3C and Supplementary Fig. S3). Remarkably, CCL1-induced IL-6 production appears to be chemokine-specific and it was not observed following in vitro treatment with, for example, SDF-1 (Fig. 3D).

**CCR8+ cell subset can be found among CD11b myeloid cells infiltrating human cancer tissues**

In addition to the peripheral blood, we also measured the levels of CCR8 expression in human tumor infiltrates (Fig. 4 and Supplementary Table S4). Analysis of freshly obtained, surgically removed high-grade invasive urothelial carcinoma of bladder revealed that a large portion of tumor-infiltrating CD11b+ cells co-expressed CCR8 (Fig. 4A, left). Specifically, in bladder cancer tissues, expression of CCR8 was associated with CD11b+CD206+ TAMs but not with tumor-infiltrating CD3+ T lymphocytes (TIL; Fig. 4B, left). Similarly, expression of CCR8 in human RCC tissue was predominantly associated with CD11b+HLA-DR+CD68+ TAMs (Fig. 4A, right) and, again, not with CD3+ TILs (Fig. 4B, right). These results indicate that in human cancer tissues, CCR8 expression is limited to tumor-infiltrating CD11b myeloid cell subsets including TAMs.
has to be noted that TAMs arise from recruited blood monocytes or MDSCs (16), suggesting that circulating CCR8⁺ myelomonocytic cells in blood (Fig. 1A) might be precursors of CCR8⁺ TAMs.

Characterization of tumor-infiltrating CD11b⁺ CCR8⁺ myeloid subset

We next investigated the functional characteristics of CCR8-expressing myeloid cells infiltrating human cancer tissue. CD11b⁺CCR8⁺ and CD11b⁺CCR8⁻ myeloid cell subsets were isolated from RCC tissues using magnetic beads. Freshly isolated and hematoxylin and eosin (H&E)-stained CCR8⁺ myeloid cells strongly resembled tissue macrophages, whereas similarly treated CCR8⁻ fraction of tumor-infiltrating myeloid cells exhibited heterogeneous composition that was comprised of various types of myeloid cells, including polymorphonuclear neutrophils (Fig. 4C). Admittedly, we could not isolate the tumor-infiltrating CCR8⁺ cells from human bladder cancer due to the small size of available tumor tissues. CCR8⁻ and CCR8⁺ cell subsets isolated from RCC tissues were cultured for 24 hours and collected cell supernatants were assayed for presence of cytokines and chemokines using 10-plex cytokine assay. Analysis of cytokine production by CCR8⁻ and CCR8⁺ myeloid cell subsets is shown in Fig. 5A. It appears that tumor-infiltrating CCR8⁺ cells secreted significantly more IL-6, VEGF, CCL3, and CCL4, but twice less PDGF-BB than their CCR8⁻ counterparts. No significant differences were observed between those cell subsets in the production of TNF-α, IL-1β, IL-8, CCL2, and basic FGF (data not shown).

We recently showed that macrophage-infiltrating human RCCs exhibit potent regulatory activity (17). Specifically, isolated TAMs were able to induce tolerogenic transcription factor FoxP3⁺ in T lymphocytes as well as to enhance IL-10 production. To evaluate the effects of CCR8⁺ myeloid cells on T lymphocytes, we isolated CD11b⁺CCR8⁺, CD11b⁺ CCR8⁻ myeloid subsets as well as total CD11b cells from RCC tissue and co-cultured them with autologous T cells for 48 hours. Intracellular expression of FoxP3 in CD4 cells was measured using flow cytometry. In addition, we collected cell supernatants and measured the concentration of IL-10 using ELISA. Data presented in Fig. 5B and Supplementary Fig. S4 clearly indicate that enriched CCR8⁺ myeloid cell subset exhibits superior ability to induce FoxP3 in comparison to CCR8⁻ myeloid cells or total tumor-infiltrating CD11b cells. However, we were not able to obtain consistent data to show that CCR8⁺ myeloid cells have a superior
ability to induce IL-10 (data not shown). Overall, these data indicate that tumor-infiltrating CD11b+CCR8+ cell subset may contribute to immune evasion and progression of human cancers through enhanced secretion of proinflammatory (IL-6, CCL3, CCL4) and proangiogenic (VEGF) factors as well as through induction of FoxP3 expression in autologous T lymphocytes.

**Primary human cancers secrete large amounts of CCL1**

Having showed that expression of CCR8 in myeloid cells during tumor progression is increased, we next evaluated the levels of natural CCR8 ligand CCL1 in patients with cancer. Established human bladder and RCC cancer cell lines, freshly obtained primary bladder and kidney cancer tissues and CD11b cells isolated from RCC tissues were cultured for 24 hours. Cell-free supernatants were collected and analyzed for CCL1 presence using commercial ELISA. As shown in Fig. 6A, both bladder and RCC human cancer cell lines (SW780 and A498, respectively) did not secrete detectable levels of CCL1, whereas primary human tumors secreted substantial amounts of the chemokine (bladder cancer: 118 ± 44 pg/mL and renal carcinoma: 30.5 ± 7 pg/mL). Moreover, tumor-infiltrating myeloid CD11b+ cells were the primary source of CCL1 among the tumor cell suspension as CD11b+ myeloid cells isolated from renal carcinoma tissues produced 98 ± 36 pg/mL of CCL1. In addition, we measured levels of CCL1 in peripheral blood (plasma) obtained from patients with cancer. Results indicate that levels of CCL1 in plasma of patients with cancer with urothelial or renal carcinomas are very low (<5 pg/mL) and in most cases undetectable (data not shown). These results indicate that primary human cancers

![Figure 4. CCR8+ cell subset detected among tumor-infiltrating CD11b myeloid cells. A, tumor tissues were obtained from patients with cancer with invasive urothelial carcinoma of bladder or RCCs. Single-cell suspensions were prepared from tumor tissues as specified in Materials and Methods and stained with fluorochrome-labeled antibodies against surface markers CD11b, CCR8, CD206, HLA-DR, as well as intracellular CD68. 7-Aminoactinomycin D (7-AAD)—positive cells in tumor cell suspension were excluded from analysis. Expression of indicated markers was assessed using flow cytometry. B, tumor single-cell suspensions were labeled with fluorochrome-conjugated antibodies against surface markers CD3 and CCR8. Expression of indicated markers was assessed using flow cytometry. C, cytomorphologic characterization of tumor-infiltrating CCR8+ myeloid cell subset. CCR8+ myeloid cells were enriched from human RCC tumor tissues. Purity of isolated cell population exceeded 85%. Top left, expression of CCR8 in CCR8-enriched cell fraction. Top right, isolated CCR8+CD11b+ cells were cultured on chamber vessel tissue culture glass slide (BD Falcon) and then stained with the Hema3 Stat Pack Kit (Fisher Scientific). Bottom, microphotographs of freshly isolated and H&E-stained tumor-infiltrating CCR8+ (left) and CCR8− (right) myeloid cell populations. Results are shown for 1 patient. Similar results were obtained from 3 patients.](a)
secrete substantial amounts of CCL1 and tumor-infiltrating myeloid cells represent a major source of the chemokine.

To elucidate what cell subset among tumor-infiltrating CD11b cells produce CCL1, we measured CCL1 levels in CCR8+ and CCR8− myeloid cells isolated from human RCC tissues. As can be seen in Fig. 6B, CCR8− myeloid cells secreted significantly more CCL1 than CCR8+ subsets in 3 of 4 examined patients with cancer. Therefore, it is likely that certain myeloid cell type among intratumoral CCR8+ of 4 examined patients with cancer. Therefore, it is likely that certain myeloid cell type among intratumoral CCR8 of 4 examined patients with cancer. Therefore, it is likely that certain myeloid cell type among intratumoral CCR8+ myeloid cells is dependent upon Stat3 activation. Therefore, we next evaluated Stat3 phosphorylation status in tumor-infiltrating CD11b+CCR8+ cell subset isolated from human RCCs. Data presented in Fig. 6C convincingly show that phosphorylated Stat3 is predominantly and selectively present in tumor-infiltrating CCR8+CD11b+ cell subset. In addition, we found that levels of ERK phosphorylation are higher in the CCR8+ than in their CCR8− counterparts (Fig. 6C). Together, these data show the CCR8-expressing myeloid cells have activated Stat3 and ERK pathways.

Discussion

Tumor growth is associated with abnormal myelopoiesis and enhanced cancer inflammation as a result of secretion by tumor cells of various bioactive substances including growth factors, cytokines, chemokines, and lipids. These factors stimulate mobilization of heterogeneous myeloid cells into blood circulation, promote the accumulation of MDSCs in tumor hosts, and provide constant recruitment of myeloid cells to the tumor site. Increased presence of myeloid cells within tumor tissues associates with poor prognosis through increased tumor angiogenesis, tissue
remodeling, and suppression of antitumor immune responses (20–25). We and others have shown earlier that MDSCs mediate immune evasion in tumor host by inducing antigen-specific CD8 T-cell tolerance (26–28). Mechanistically, these cells are capable of suppressing T-cell responses through peroxynitrite production in an arginase and/or iNOS-dependent manner (27, 29–31). Furthermore, MDSCs directly promote angiogenesis and tissue remodeling via enhanced production of proangiogenic mediators such as VEGF and MMP9 (21, 32). Upon recruitment to tumor tissue, MDSCs frequently differentiate into more mature myeloid cell subsets such as TAMs. The TAMs represent an abundant and heterogeneous cell population in the tumor microenvironment and they play a key role in tumor development (4, 33). Several lines of evidence suggest that TAMs promote tumor invasion, angiogenesis, tissue remodeling and metastasis, and associate with poor prognosis. TAMs also can have an inhibitory role in the development of antitumor immunity through the production of immunosuppressive cytokines. Despite recent advances in our understanding of the multiple roles of bone marrow–derived myeloid cells in tumor progression, heterogeneity of myeloid cells in tumor tissues as well as lack of specific markers for discrete cell subsets still represent significant obstacles for further progress. Establishing such biomarkers has the potential to enhance clinical prognosis and provide insights into relevant pathways of immune suppression used by the specific cancer.

In this study, we measured expression of multiple chemokine receptors (CCR1, CCR2, CCR3 CCR4, CCR5, CCR6, CCR7, CCR8, CXCR2, and CXCR4) in myeloid cells obtained from peripheral blood of patients with bladder cancer and RCCs. We found that among screened chemokine receptors only CCR8 expression was consistently and strongly upregulated in CD11b+ myeloid cells from patients with cancer as compared with healthy donors. Importantly, both monocytic CD33highCD11b and granulocytic CD33lowCD11b MDSCs in blood obtained from the
patients with cancer showed increased expression of CCR8. Upregulated expression of CCR8 was also detected in tumor tissues obtained from patients with bladder cancer and RCCs. Furthermore, CCR8 expression in cancer tissues appears to be limited to the tumor-infiltrating CD11b myeloid cells and primarily to TAMs. In addition, it should be noted that CD3+ T lymphocytes infiltrating human renal and bladder tumors did not express CCR8.

At the functional level, we showed that CD11b+ CCR8+ cell subset may contribute to immune evasion and progression of human cancers through elevated secretion of proinflammatory (IL-6, CCL3, CCL4) and proangiogenic (VEGF, IL-8) factors as well as through induction of FoxP3 expression in autologous T lymphocytes. Mechanistically, we found that tumor-derived factors promote CCR8 expression in myeloid cells in a Stat3 activation–dependent manner, and CCR8+ myeloid cells isolated from human cancer tissue display phosphorylated Stat3. Signaling by Stat3 is a major intrinsic pathway for cancer inflammation because it is frequently activated in malignant cells and is capable of regulating the expression of a large number of genes that are crucial for inflammation (1). Activation of Stat3, which is a leading pathway in regulation of both cancer-related inflammation and tumor immunity (1, 34), can be achieved with several proinflammatory agents including IL-6, which is produced primarily by bone marrow–derived myeloid cells (1). Here, we show that human renal carcinoma tumor-infiltrating CCR8+ myeloid cells represent a major source of IL-6 and, moreover, IL-6 production by CCR8+ cells can be stimulated by treatment with CCL1. Importantly, elevated levels of these cytokines are frequently observed in cancer and are associated with cancer-related inflammation (35). Taken together, our observations provide evidence for enhanced CCL1/CCR8 signal pathway in cancer and suggest that CCL1/CCR8 axis represents an important regulatory component in inflammation that associates with human cancer progression.

In addition to the infiltration with CCR8-expressing myeloid cells, we also observed that primary human tumors secreted substantial amounts of CCL1. We found that RCC-infiltrating myeloid cells produced significantly higher amounts of CCL1 than whole RCC tissue or epithelial cancer cell lines. Previous studies showed that CCL1 exhibits a unique pattern of regulation associated with a distinct form of M2-type monocyte activation that participates in macrophage-dependent regulatory circuits of innate and adaptive immunity (36). CCL1 also induces regulatory T-cell recruitment (37) and promotes T112 immune response (38, 39). Interestingly, in mice subjected to chronic hepatic injury, CCR8-expressing liver macrophages were necessary for CCL1-directed migration of inflammatory but not classically activated monocytes (11). CCR8 deficiency also protected the liver against injury, ameliorating inflammatory responses, and hepatic fibrogenesis. Furthermore, absence of CCR8 enhances innate immunity during septic peritonitis (40) and a recent report indicated that mouse macrophages could upregulate CCR8 in an autocrine manner upon CCL1 binding (41).

However, information available on the role of CCL1 and CCR8 in the progression of tumors particularly of human cancers is very limited. A recent study, on the basis of measurement of gene expression and single-nucleotide polymorphism analyses, evidenced a link between CCL1 expression and development of breast cancer in women (42). Antibody-mediated neutralization of CCL1 in mice harboring mammary carcinomas significantly reduced the rate of CD4 T-cell conversion into regulatory T cells (43), suggesting a role for CCL1 (presumably through cognate receptor CCR8) in tumor-associated immunosuppression. Other studies showed that CCL1 exhibits proangiogenic activity through stimulation of chemotaxis of endothelial and vascular smooth muscle cells (12, 13, 44), reinforcing the possible involvement in cancer progression.

On the basis of our results, we propose a working model for the contribution of CCR8-expressing myeloid cells to immune evasion in cancer (Fig. 6D). CCL1 is produced primarily by CCR8+ tumor-infiltrating myeloid cells. Upon binding to the CCR8 on CCR8+ myeloid cells, CCL1 promotes production of IL-6 that, in turn, activates Stat3 in target cells. Activation of Stat3 promotes expression of a whole range of proinflammatory, proangiogenic, and immunosuppressive genes. In addition, CCR8+ myeloid cells are highly effective in induction of FoxP3 expression in T lymphocytes. The emerging picture suggests that CD11b+ CCR8+ myeloid cell subset could exert a marked tumor-promoting effect.

Overall, we conclude that enhanced production of CCL1 by primary human cancers and increased numbers of CCR8+ myeloid cells represent a common feature of human bladder and renal carcinomas, thereby suggesting the contribution of CCR8/CCL1 axis to cancer inflammation, immune evasion, and tumor angiogenesis via stimulation of pro-inflammatory and pro-angiogenic cytokines/chemokines and induction of tolerogenic transcription factor Foxp3 in CD4 T lymphocytes. Further studies are needed to evaluate possible correlation of CCL1 expression with clinical parameters and overall contribution of CCR8-expressing cells and its ligand CCL1 to the development and progression of cancers. Finally, our data also suggest that blockade of CCR8 signaling may provide a novel strategy for therapeutic intervention in human cancers.

Disclosure of Potential Conflicts of Interest

J. Vieweg is a consultant/advisory board member of American Urological Association. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: Y. Daaka, S. Kusmartsev
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Stoffs, W.-J. Kim, I. Daurkin, L.-M. Seo
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Stoffs, S. Kusmartsev
Study supervision: S. Kusmartsev
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