

Tumor-Associated Macrophages Mediate Immunosuppression in the Renal Cancer Microenvironment by Activating the 15-Lipoxygenase-2 Pathway

Irina Daurkin¹, Evgeniy Eruslanov¹, Taryn Stoffs¹, George Q. Perrin¹, Chester Algood¹, Scott M. Gilbert¹, Charles J. Rosser³, Li-Ming Su¹, Johannes Vieweg¹, and Sergei Kusmartsev^{1,2}

Abstract

Renal cell carcinoma (RCC), the most common human kidney cancer, is frequently infiltrated with tumor-associated macrophages (TAM) that can promote malignant progression. Here, we show that TAMs isolated from human RCC produce substantial amounts of the proinflammatory chemokine CCL2 and immunosuppressive cytokine IL-10, in addition to enhanced eicosanoid production via an activated 15-lipoxygenase-2 (15-LOX2) pathway. TAMs isolated from RCC tumors had a high 15-LOX2 expression and secreted substantial amounts of 15(*S*)-hydroxyeicosatetraenoic acid, its major bioactive lipid product. Inhibition of lipoxygenase activity significantly reduced production of CCL2 and IL-10 by RCC TAMs. In addition, TAMs isolated from RCC were capable of inducing in T lymphocytes, the pivotal T regulatory cell transcription factor forkhead box P3 (FOXP3), and the inhibitory cytotoxic T-lymphocyte antigen 4 (CTLA-4) coreceptor. However, this TAM-mediated induction of FOXP3 and CTLA-4 in T cells was independent of lipoxygenase and could not be reversed by inhibiting lipoxygenase activity. Collectively, our results show that TAMs, often present in RCCs, display enhanced 15-LOX2 activity that contributes to RCC-related inflammation, immunosuppression, and malignant progression. Furthermore, we show that TAMs mediate the development of immune tolerance through both 15-LOX2-dependent and 15-LOX2-independent pathways. We propose that manipulating LOX-dependent arachidonic acid metabolism in the tumor microenvironment could offer new strategies to block cancer-related inflammation and immune escape in patients with RCC. *Cancer Res*; 71(20); 6400–9. ©2011 AACR.

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer (1). Although chemo- and radiation therapies are the standard treatment modality for most metastatic malignancies, their effect in disseminated RCC has shown little impact on progression-free or overall survival (2). On the other hand, as RCC is considered an immunogenic tumor, it responds to immunotherapeutic intervention, thereby fostering continued interest in exploiting the ability of immune system to recognize and eradicate renal malignancies (3). However, despite its high immunogenicity and the marked

presence of immune cells in the RCC tumor tissue, the majority of affected patients fail to develop effective antitumor immune responses, implying that the ability of the local immune response to control tumor growth is severely impaired (4).

Tumor-associated macrophages (TAM) are prominent components of solid tumors and exhibit distinct phenotypes in different microenvironments (5–7). TAMs originate from recruited myeloid cells, such as blood monocytes or myeloid-derived suppressor cells (MDSC), which contribute to tumor evasion from immune system (8, 9). Tumor-induced immune dysfunction is associated with enhanced cancer-related inflammation (10, 11), frequently caused by aberrant metabolism of arachidonic acid in the tumor microenvironment (12). Augmented arachidonic acid metabolism results in increased production of eicosanoids, such as prostaglandin, thromboxane, leukotriene, and hydroxyeicosatetraenoic acid (HETE), all of which are biologically active lipids. Free arachidonic acid can be metabolized by 2 key enzymes: cyclooxygenase (COX) and lipoxygenase (LOX). Numerous clinical and pharmacologic studies have shown that COX-2 expression and enhanced prostaglandin E2 (PGE2) production are frequently attributed to inflammation-associated cancers such as lung, colon, bladder, and prostate cancer (12). Moreover, the expression of inducible COX-2 enzyme has been detected in both tumor cells and inflammatory tumor-infiltrating

Authors' Affiliations: Departments of ¹Urology and ²Anatomy and Cell Biology, University of Florida, Gainesville; and ³MD Anderson Cancer Center Orlando, Orlando, Florida

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I. Daurkin and E. Eruslanov contributed equally to this work.

Corresponding Author: Sergei Kusmartsev, Cancer & Genetics Research Center, University of Florida, 2033 Mowry Rd, Rm. 459, Gainesville, FL 32610. Phone: 352-273-8235; Fax: 352-273-8335; E-mail: s.kusmartsev@urology.ufl.edu

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cells, including myeloid adenomatous polyposis coli and its precursors (13). The role of LOX isoforms in the progression of human cancers remains largely unexplored.

Here, we show that macrophages infiltrating human kidney cancer tissues (RCC TAM) secrete immunosuppressive cytokine interleukin-10 (IL-10) and proinflammatory chemokine CCL2. Furthermore, RCC TAMs contribute to immune evasion via induction of tolerogenic forkhead box P3 (FOXP3⁺) and IL-10-secreting T cells as well as via upregulation of inhibitory receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression in autologous T cells. RCC TAMs displayed high expression of 15-lipoxygenase-2 (15-LOX2) and produced substantial amounts of 15(*S*)-hydroxyeicosatetraenoic acid [15(*S*)-HETE]. Inhibition of LOX activity led to downregulation of production of IL-10 and CCL2 by RCC TAMs and also to reduction of TAM-mediated induction of IL-10 in T cells. Our data indicate that enhanced metabolism of arachidonic acid in the RCC tumor microenvironment, mediated by an activated 15-LOX2/15(*S*)-HETE pathway, affects the immune function of macrophages and T lymphocytes, thus promoting local immunosuppression and tumor evasion.

Materials and Methods

Patients

A total of 51 patients with a diagnosis of clear cell renal cell carcinoma (cc RCC) that underwent full or partial nephrectomy at Department of Urology, University of Florida, Gainesville, FL, were enrolled in the study. Clinical specimens were obtained following informed consent, as approved by the Institutional Review Board. All patients selected for entry into the study met the following criteria: (i) a new diagnosis of histologically confirmed cc RCC, (ii) no prior treatment including surgery, chemotherapy, or radiation for kidney cancer, and (iii) no other known malignancy. Detailed characteristics of the patients can be found in Supplementary Table S1.

Isolation of TAMs

To obtain single-cell suspension from RCC tumors, solid fresh cancer and normal kidney tissues were disaggregated with collagenase cocktail. For isolation of TAMs, we first lysed red blood cells with ACK buffer and then depleted tumor cell suspension from CD15⁺ and CD33⁺ MDSCs using a cocktail of anti-CD15 and anti-CD33 antibody-conjugated magnetic beads and MACS columns (Miltenyi Biotec). Finally, the remaining myeloid cells were isolated by positive selection of CD11b cells from CD15/CD33-negative fraction using anti-CD11b antibody-conjugated magnetic beads and MACS columns (Miltenyi Biotec). The viability of isolated cells routinely exceeded 90%, as determined by the expression of 7-aminoactinomycin D (7-AAD), by flow cytometry and trypan blue exclusion assays.

Morphologic characterization of TAMs

Freshly isolated CD11b⁺CD15⁻CD33⁻ cells were spun on slides and stained with the Hema3 Stat Pack Kit (Fisher Scientific). Nonspecific esterase activity was determined with

naphthol AS-D chloroacetate (specific esterase) kit and α -naphthyl acetate (nonspecific esterase) kit (Sigma-Aldrich).

Cell isolation from blood

Peripheral blood mononuclear cells (PBMC) from whole blood of patients with RCC were separated by gradient density centrifugation. CD14-positive monocytes were isolated from the PBMC fraction using an anti-CD14 antibody conjugated with magnetic beads and MACS columns (Miltenyi Biotec). T cells were isolated from the PBMC fraction using human T-cell enrichment columns (R&D Systems, Inc.) according to the manufacturer's protocol.

Reagents and medium

The LOX inhibitor nordihydroguaiaretic acid (NDGA), COX-2 inhibitor NS 398, and arachidonic acid were obtained from Cayman Chemical. Antibodies against 15-LOX2 were purchased from Oxford Biomedical Research, and anti-15-LOX1 antibodies were from Cayman Chemical. All antibodies used for flow cytometry were purchased from BD Biosciences. Serum-free X-VIVO15 and AIM-V media were obtained from Invitrogen.

Flow cytometry

Cells were labeled with antibodies against CD11b, CD45, HLA-DR, CD15, CD33, CD68, CD163, CD206, IL4Ra, CD83, CD80, PDL-1, PDL-2, CD1a, CD80, C56, CD40, and Tie-2 as indicated in the text. Expression of FOXP3 and IL-10 was assessed by intracellular flow cytometry staining kit from BD Biosciences. Cytometry data were acquired with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with CXP software (Beckman Coulter).

Total RNA purification and real-time PCR analysis

Total RNA was extracted with an RNeasy Plus kit (QIAGEN). The quality and quantity of RNA samples were determined by Agilent RNA 6000 Nano Chip (Agilent Technologies). cDNA from purified total RNA was produced with MultiScribe reverse transcriptase (Applied Biosystems) according to the manufacturer's protocol. Fifty nanograms of template cDNA was used in TaqMan real-time PCR (2 minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C, 1 minute at 60°C, for 45 cycles), carried out on an ABI PRISM 7900 system (Applied Biosystems), by the following specific TaqMan Gene expression assays: 15-lipoxygenase-1 (*Alox15*); 15-lipoxygenase-2 (*Alox15B*), COX-2 (*Ptgs2*), *Foxp3*, and *Ctla4*. All samples were run in triplicate, and amplification data were analyzed with AB Prism Sequence Detection Software, version 2.2.1. Relative quantification was calculated according to $\Delta\Delta C_t$ method (Applied Biosystems), using a statistical confidence of 99.9%.

Western blotting

Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific) containing protease and phosphatase inhibitors. Whole-cell lysates (30 μ g per lane) were subjected to 10% SDS-PAGE gel and blotted onto polyvinylidene difluoride membranes. Membranes were blocked for 1 hour at room temperature with 5% dry skimmed milk in

TBS [20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl plus 0.1% (v/v), Tween 20] and probed with appropriate primary antibodies overnight at 4°C. Membranes were washed and incubated for 1 hour at room temperature with secondary antibody conjugated with horseradish peroxidase. Results were visualized by chemiluminescence detection with a SuperSignal West Pico substrate (Thermo Scientific). To confirm, equal loading membranes were stripped with Restore Western blot stripping buffer (Thermo Scientific) and reprobated with antibody against β -actin (Santa Cruz Biotechnology, Inc.).

ELISPOT assay

The ELISPOT assays were conducted by the Human IL-10 ELISPOT Kit (BD Biosciences). Briefly, 96-well MultiScreen plates (Millipore) were coated with anti-human IL-10 monoclonal antibody (mAb) in PBS. After overnight incubation at 4°C, the wells were repeatedly washed with PBS and immobilized with anti-CD3 mAb for 2 hours at 37°C. After incubation, cells were washed again and 100 μ L of X-VIVO culture media were added to each well for 1 hour at room temperature. A total of 100,000 T cells were incubated with 50,000 TAMs, from the same patient, and anti-CD28 mAb (10 μ g/mL) for 24 hours at 37°C. At the end of incubation, plates were extensively washed and developed according to the manufacturer's specification. Samples were run in quadruplicates. The numbers of spots were evaluated by the ELISPOT plate reader and software from Cellular Technology Limited.

Secretion of 15(S)-HETE and PGE2

Analysis of 15(S)-HETE and PGE2 production were conducted by EIA kits and protocols developed by Cayman Chemical.

Measurement of CCL2 and IL-10 production by ELISA

Production of both CCL2 and IL-10 was measured with commercial ELISA kits purchased from BD Bioscience.

Statistical analysis

Values are expressed as mean \pm 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.

Results

RCC is infiltrated with inflammatory cells of hematopoietic origin

To explore the cellular composition of RCC infiltrates, we used a flow cytometric approach. Fresh, surgically removed cancerous and normal kidney tissues from patients with RCC (detailed information on patients is shown in Supplementary Table S1) were used for analysis of tumor-infiltrating cells. First, we examined the numbers and phenotypes of RCC tumor-infiltrating myeloid cells using the myeloid lineage cell marker CD11b. CD11b-positive myeloid cells were observed in all analyzed RCC tissues. As expected, the numbers of tumor-infiltrating CD11b-positive cells varied significantly among analyzed samples (ranging from 8% to 19% of all

nucleated cells in tumor tissues). As illustrated for one patient with RCC in Fig. 1A (left), renal carcinoma tissue contained a substantial number of CD45-positive cells of hematopoietic origin including CD45⁺CD11b⁺ (9% in this sample) and CD45⁺CD11b⁻ tumor-infiltrating cells (31.9%). Most RCC-infiltrating CD11b-positive cells coexpressed HLA-DR. Conversely, only rare CD11b-positive cells were observed in normal kidney tissue (Fig. 1A, right). Analysis of CD45⁺CD11b⁻ fraction of tumor-infiltrating cells revealed that majority of those cells were presented by tumor-infiltrating T lymphocytes and natural killer cells (data not shown), which is consistent with earlier observations (14, 15).

Characterization of TAMs isolated from RCC tissue

The majority of RCC-infiltrating myeloid cells coexpress MHC class II molecule (Fig. 1A), suggesting that these cells belong to monocyte/macrophage cell lineage and most likely are represented by TAMs (16). In contrast, the minor HLA-DR-negative CD11b cell population is likely composed of MHC class II-negative MDSCs or granulocytic myeloid cells. We developed a protocol to isolate myeloid cell population from RCC tissue that is enriched for TAMs (see Materials and Methods). Under these protocol conditions, viability of isolated cells routinely exceeded 90%, as determined by expression of 7-AAD by flow cytometry and trypan blue exclusion assays (Supplementary Fig. S2). Microphotographs of freshly isolated populations of CD11b⁺CD15⁻CD33⁻ myeloid cells show that these mononuclear cells displayed macrophage morphology (Fig. 1B, left) and were positive for esterase activity, an enzyme specific for monocytes/macrophages (Fig. 1B, central). In culture, they acquired a typical macrophage shape (Fig. 1B, right). Flow cytometric analysis of isolated cells revealed that most of these cells express MHC class II molecule HLA-DR and macrophage markers CD163 and CD68 but not mannose receptor CD206 (Fig. 1C and Supplementary Fig. S1). The majority of cells coexpressed the pan-hematopoietic marker CD45. In contrast, they lacked expression of MDSC markers CD15 and CD33 (data not shown). These cells were also characterized by low or weak expression of dendritic cell markers CD1a, CD83, and CD40 (Supplementary Fig. S2 and Supplementary Table S2). In addition, isolated cells coexpressed variable levels of CD36 and PD-L1 (Supplementary Fig. S1 and S2 and Supplementary Table S2). On the basis of these results, we conclude that CD11b⁺HLA-DR⁺ cells isolated from human RCC tissues represent TAMs.

RCC-infiltrating TAMs secrete increased amounts of CCL2 and IL-10

It has been shown that TAMs arise from myelomonocytic cells of bone marrow origin (MDSCs and monocytes), which are recruited by cancer cells to the tumor site. Increased and constant presence of TAMs in RCC tumors suggests existence of specific mechanism that provides enhanced recruitment of blood myelomonocytic cells. One candidate for tumor-mediated recruitment of macrophage precursors in RCC is monocyte chemoattractant protein (CCL2) whose production is increased in many cancer types. CCL2 is a cognate ligand

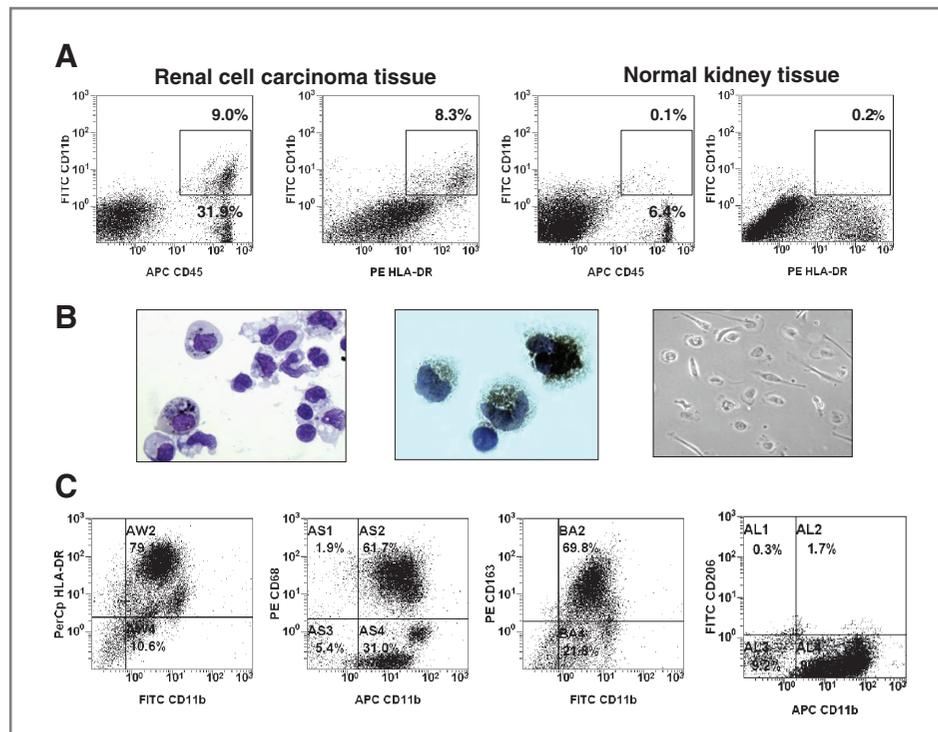


Figure 1. Characterization of RCC-infiltrating macrophages. **A**, fresh, surgically removed cancerous (left) and normal kidney (right) tissues from patients with RCC were used for analysis of tumor-infiltrated CD11b myeloid cells. Disaggregated tissues were labeled with fluorochrome-conjugated antibodies against CD11b, CD45, and HLA-DR. Expression of cell surface markers was assessed by 4-color flow cytometry. **B**, morphology of TAM isolated from human RCC tissue. Tumor-infiltrating CD11b⁺CD15⁻CD33⁻ myeloid cells were purified with magnetic beads and stained with hematoxylin/eosin (left) and nonspecific esterase (central) or cultured in complete medium for 24 hours before microphotographs were taken (right). **C**, phenotype of TAMs isolated from RCC. RCC TAMs were isolated using magnetic beads as described in Materials and Methods. Isolated cells were labeled with fluorochrome-conjugated antibodies against surface markers CD11b, HLA-DR, CD163, CD206, as well as intracellular CD68. Expression of the indicated markers was examined using flow cytometry. Results of one representative experiment are shown. FITC, fluorescein isothiocyanate.

for chemokine receptor CCR2 that is expressed by monocytes in peripheral blood and plays multiple roles in cancer including chemoattraction of circulating CCR2-positive monocytes/macrophages to the tumor vicinity (17). To evaluate levels of the CCL2 secreted by RCC, we measured the chemokine production by RCC cancer tissue, normal kidney tissue, and TAMs isolated from RCC. Although CCL2 production varied widely among the patients, the cancer tissues consistently expressed higher levels than normal kidney tissues (Fig. 2A). TAMs isolated from RCC tumors also secreted substantial amounts of CCL2.

One of the notable characteristics of TAMs is increased secretion of the immunosuppressive cytokine IL-10 (6, 18). As can be seen in Fig. 2B and Supplementary Fig. S3A, TAMs isolated from larger tumor produced higher amounts of IL-10. These data underscore relevance of IL-10-producing TAMs to immunosuppression observed in patients with late stages of cancer disease.

TAMs promote expression of FOXP3 and CTLA-4 in T lymphocytes

Besides myeloid cells, RCC frequently contain tumor-infiltrating lymphocytes (TIL), which are represented by various

proportions of CD4 and CD8 cells and are usually a major component of the RCC tumor microenvironment (19). Many TILs are specific for tumor-associated antigens. However, it seems that these immune cells in RCC are dysfunctional, as they fail to destroy the malignant cells. Recent studies show that CD4 and CD8 TIL frequently secrete increased amounts of the immunosuppressive factor IL-10 (20). It has been suggested that IL-10 plays a critical role in mediating an immunosuppressive tumor microenvironment and contributes to weakened T-cell effector functions in RCC. We examined whether RCC TAMs could contribute to the enhanced IL-10 production by T cells. To this end, we mixed TAMs isolated from RCC tumor tissue with autologous T cells purified from peripheral blood of patients with RCC. These T cells were then stimulated with anti-CD3/CD28 antibodies, and IL-10 production was measured by an ELISPOT assay. When RCC-derived TAMs or CD3/CD28-stimulated T cells were cultured separately, both TAMs and T cells secreted variable amounts of IL-10, as detected by ELISPOT assay. However, when cultured together, there was a strong and statistically significant increase of IL-10 production (Fig. 2C and Supplementary Fig. S3B). Because both TAMs and T cells are capable of secreting IL-10, it was not clear what cell type

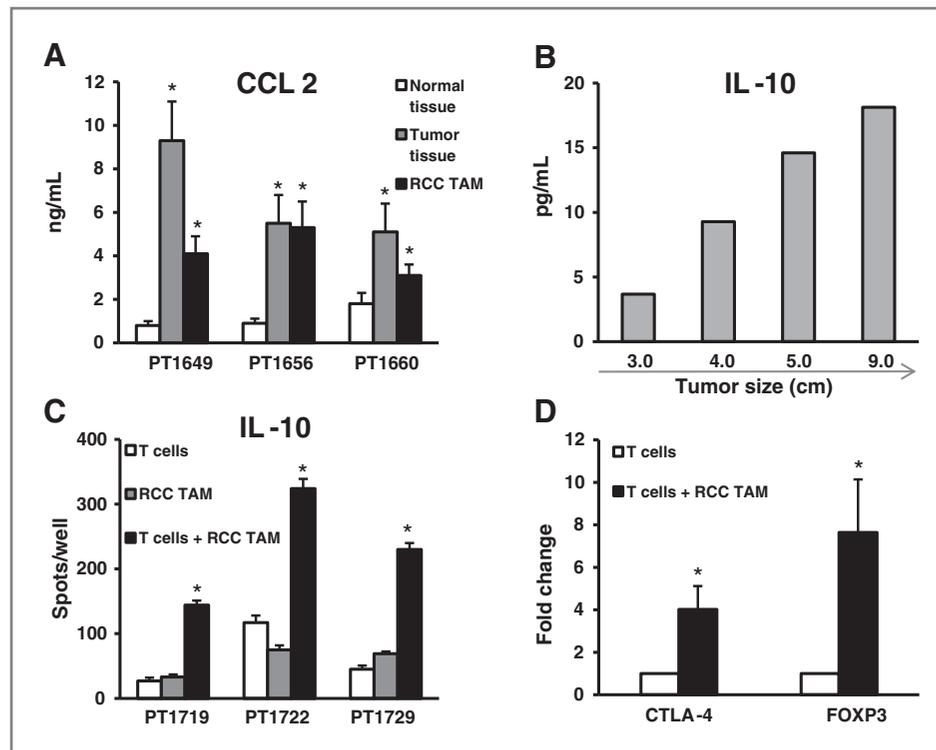


Figure 2. Regulatory function of RCC-infiltrating macrophages. **A**, comparative levels of CCL2 produced by RCC TAMs, whole RCC, and normal kidney tissues. Single-cell suspensions (10^6 per mL) of fresh RCC tissues, normal kidney tissues, and isolated TAMs from 3 patients with RCC were cultured in serum-free medium for 24 hours. Levels of CCL2 in free-cell supernatants were measured by ELISA kit. Average concentrations \pm SD are shown. *, $P < 0.05$. **B**, TAM from large tumors produces more IL-10. TAMs were isolated from 4 patients with RCC with different size of tumor and cultured in serum-free medium for 24 hours (5×10^4 cells per well). Cell-free culture supernatants were assayed for IL-10 by commercial ELISA kit. Assays were run in triplicates. Average IL-10 concentrations are shown. **C**, cocultivation of TAMs and activated T cells enhances IL-10 production. IL-10 production was measured by ELISPOT assay. Fresh RCC TAMs (5×10^4) and purified autologous T cells (1×10^5) from the peripheral blood of patients with RCC were mixed or cultured alone in ELISPOT plate. T cells were stimulated with anti-CD3/CD28 Antibodies (Abs; 1 and 5 μ g/mL, respectively). Assays were run in quadruplicates. IL-10 ELISPOT results obtained from 3 patients with RCC are shown (Average \pm SD; **C**), *, $P < 0.05$. **D**, RCC TAMs promote upregulation of *CTLA-4* and *FOXP3* gene expression in T cells. Purified T cells (control) or mixture of 5×10^5 RCC TAMs and 1×10^6 autologous T cells were cultured together. T cells were stimulated with CD3/CD28 Abs. Forty-eight hours later, cells were collected, and total RNA was isolated. Expression of *CTLA-4* or *FOXP3* was measured by qRT-PCR assay. Samples were run in triplicates. Average fold changes (\pm SD) for 3 patients with RCC are shown. *, $P < 0.05$.

could enhance production of cytokine upon mixing. To clarify this issue, we mixed fresh isolated TAMs with activated autologous T cells, cocultured for 48 hours, and then measured intracellular IL-10 expression in TAMs and T cells by flow cytometry. Obtained results indicate (Supplementary Fig. S4) that both TAMs and T cells increase IL-10 expression in coculture conditions.

Another mechanism that cancers use to escape the immune system is induction of *FOXP3*⁺ regulatory T cells (Tregs) or upregulation of *CTLA-4* expression in T cells upon activation. A high frequency of Tregs and increased expression of *CTLA-4* have been linked to poor prognosis in patients with cancer. We tested the ability of TAMs to induce *FOXP3* and *CTLA-4* expression in T cells. TAMs isolated from RCC tumor tissue were mixed with purified autologous PBMC-derived *CD3*⁺ T cells from patients with RCC in a cell ratio of 1:2, and T cells were stimulated with CD3/CD28 mAbs. Levels of *FOXP3*, *TGF- β 1*, and *CTLA-4* gene expression were measured by quantitative real-time PCR (qRT-PCR). As shown in Fig. 2D, macro-

phages infiltrating RCC tissue were able to stimulate mRNA expression of *CTLA-4* and *FOXP3* in activated T cells but did not affect expression of *TGF- β 1* (data not shown). TAM-mediated induction of *CTLA-4* and *FOXP3* protein expression in T cells has also been confirmed by Western blot analysis (Supplementary Fig. S5A) and flow cytometry (Fig. 5E), respectively.

RCC-infiltrating macrophages display enhanced eicosanoid production mediated by upregulated 15-LOX2 expression

TAMs are key orchestrators of chronic cancer-related inflammation and play a complex role in cancer progression. Important characteristic of cancer-related inflammation is deregulated metabolism of arachidonic acid and enhanced production of eicosanoids. It has also been shown that tumor-recruited myelomonocytic cells can further stimulate cancer-related inflammation via enhanced secretion of eicosanoids (13, 21). Here, we compared the production of major eicosanoids, such as PGE2 and 15(S)-HETE, by primary RCC tumor

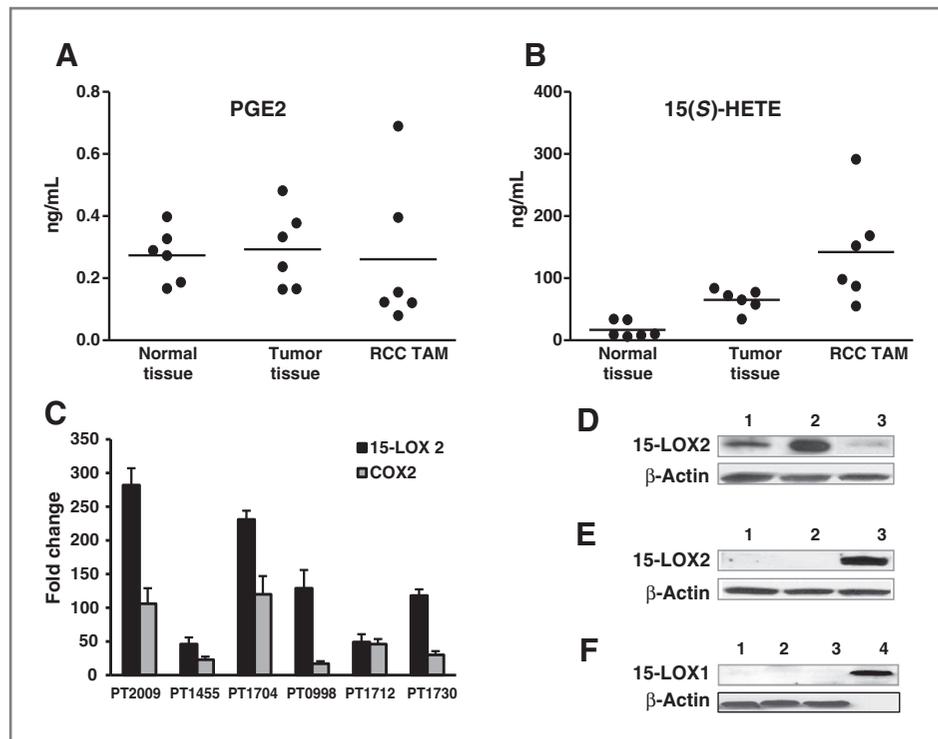


Figure 3. RCC TAMs have upregulated expression of 15-LOX2 and produce elevated amounts of arachidonic acid metabolite 15(S)-HETE. **A**, PGE2 production. Single-cell suspensions (10^6 per mL) of normal kidney tissues, whole RCC tumor tissues, and RCC TAMs from the patients with RCC were cultured in serum-free medium for 24 hours. Levels of PGE2 in free-cell supernatants were measured in triplicates by ELISA kit. Data for 6 patients with RCC are shown. **B**, 15(S)-HETE production. Single-cell suspensions (10^6 per mL) of normal kidney tissues, whole RCC tumor tissues, and RCC TAMs from patients with RCC were cultured in serum-free medium for 24 hours. Levels of 15(S)-HETE in free-cell supernatants were measured in triplicates by ELISA kit. Data for 6 patients with RCC are shown. **C**, expression of 15-LOX2 and COX-2 genes in RCC TAMs is upregulated. Total RNA was isolated from RCC TAMs and whole RCC tumor tissue from 6 patients with cancer by RNeasy kit (QIAGEN). qRT-PCR analysis was conducted using an Applied Biosystems Prism 7900HT Fast Real-Time PCR System. Samples were run in triplicates. Data for 6 patients with RCC \pm SD are shown. **D**, enhanced expression of 15-LOX2 protein in RCC TAMs. Cell lysates were prepared from RCC tumor tissue (line 1), RCC TAMs (line 2) and normal kidney tissue (line 3) from the same patient. **E**, 15-LOX2 expression can be induced in blood monocytes by culturing in the presence of RCC-conditioned medium. Freshly isolated CD14⁺ cells from the blood of patient with RCC (line 1); CD14⁺ cells from the same patient cultured in complete culture medium for 3 days (line 2), CD14⁺ cells from the same patient cultured in the presence of RCC TCM (30%) for 3 days (line 3). **F**, expression of 15-LOX1 protein in: RCC tumor tissue (line 1), RCC TAMs (line 2), or normal kidney tissue (line 3), positive Western Ready Control (line 4).

tissue, normal kidney tissue, and RCC TAMs. We found that levels of PGE2 secreted by primary RCC tumor tissue and RCC TAMs were relatively low and approximately similar to those found in supernatants from normal kidney tissue (Fig. 3A). At the same time, both whole RCC tumor tissues and isolated TAMs produce substantially increased amounts of 15(S)-HETE as compared with normal kidney tissue (Fig. 3B).

15(S)-HETE is a metabolite of arachidonic acid that is formed by 15-LOX. In humans, 2 distinct subtypes of 15-LOX exist: 15-lipoxygenase-1(15-LOX1) and 15-LOX2. We measured the expression of 15-LOX1 (*Alox15*), 15-LOX2 (*Alox15B*), and COX-2 (*Ptgs2*) genes in macrophages isolated from RCC. Our results indicate that the expression of 15-LOX2 and COX-2 genes in RCC-infiltrating macrophages was substantially upregulated in comparison to whole RCC tumor tissue (Fig. 3C). Expression of the 15-LOX1 gene was undetectable in both RCC TAMs and whole RCC tumor tissues. Increased expression of 15-LOX2 in RCC TAMs was confirmed by Western blot analysis (Fig. 3D and Supplementary Fig. S5C). In addition,

we found that expression of 15-LOX2 protein could be induced in monocytes from peripheral blood of patients with RCC by culturing these cells in the presence of RCC tumor-conditioned medium (Fig. 3E). As expected from our qRT-PCR data, we could not detect expression of 15-LOX1 protein in freshly isolated RCC TAMs, whole RCC tumor, or normal kidney tissue (Fig. 3F). Despite relatively high levels of COX-2 mRNA expression in TAMs, its protein expression is fairly low and sometimes could not be detected by Western blot analysis (Supplementary Fig. S5B). This is consistent with low levels of PGE2 secreted by RCC TAMs (Fig. 3A). Together, these data provide strong evidence that RCC-infiltrating macrophages display enhanced eicosanoid production due to activation of 15-LOX2/15(S)-HETE pathway. It has been shown that expression of 15-LOX1 can be induced in monocytes/macrophages by IL-13 or IL-4 (22). To examine whether elevated expression of 15-LOX2 in RCC-infiltrating macrophages could also be associated with elevated production of IL-13 and IL-4 in RCC, we measured secretion of these cytokines by normal kidney

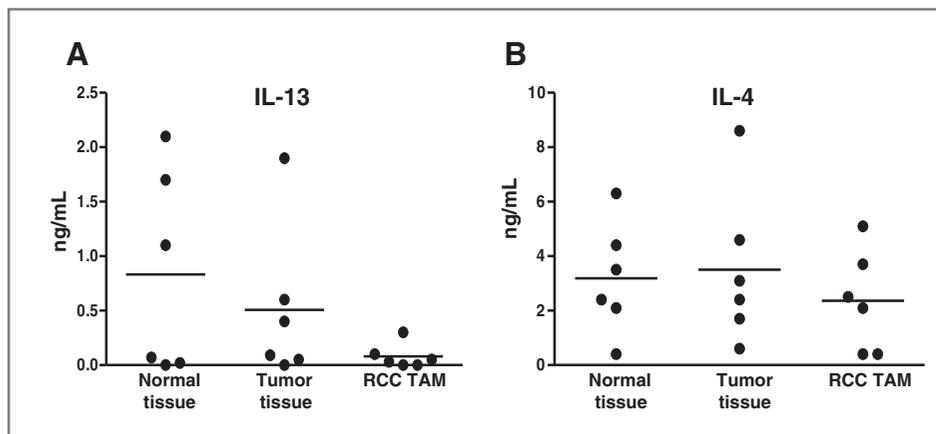


Figure 4. A and B, levels IL-4 and IL-13 secreted by primary RCC tumor tissues. Single-cell suspensions of RCC tumor tissues, normal kidney tissues, and RCC TAMs were prepared, as described in Materials and Methods. Each cell suspensions (10^6 per mL) were cultured for 24 hours in serum-free media. Collected cell-free supernatants were assayed for presence of IL-13 and IL-4 by commercial ELISA kits. Samples from 6 patients with RCC were used for measurement of IL-13 and IL-4.

tissue, RCC tumor tissue, and RCC TAMs. Our data suggest that it is unlikely that those cytokines could be involved in upregulated 15-LOX2 expression in macrophages associated with RCC (Fig. 4A and B).

15-LOX2 contributes to the cancer-related inflammation and immune evasion through regulation of CCL2 and IL-10 production

15-LOX pathway has been linked to the regulation of monocyte chemoattractant protein (CCL2) production (23, 24) that, in turn, has been shown to be involved in cancer-related inflammation via recruitment of CCR2-positive blood monocyte/macrophages to tumor site (7). To examine whether the arachidonic acid-related enzymes 15-LOX2 or COX-2, whose expression was upregulated in TAMs, were involved in the regulation of CCL2 production, we cultured RCC TAMs in the presence of pharmacologic LOX or COX-2 inhibitors and measured levels of CCL2 in cell-free supernatants. Results shown in Fig. 5A indicate that the LOX inhibitor NDGA, but not vehicle control or selective COX-2 inhibitor NS-398, significantly reduced production of CCL2 by TAMs isolated from RCC tumor. These data suggest an involvement of 15-LOX2 in the mechanism of the regulation of CCL2 production, thus implicating its role in the regulation of RCC-related inflammation.

Remarkably, production of IL-10 by RCC TAMs also was markedly inhibited in the presence of LOX inhibitor NDGA (Fig. 5B). Importantly, no cell toxicity was observed for NDGA. We then asked whether this LOX inhibitor could also prevent enhanced IL-10 production observed in cocultured TAMs and T cells (Fig. 2C). Results presented in Fig. 5C show that pretreatment of TAMs isolated from RCC with NDGA led to substantial reduction in the amount of immunosuppressive IL-10 produced in mixed culture of TAMs and T lymphocytes, whereas constant presence of LOX inhibitor in the coculture resulted in complete prevention of IL-10 production in cocultured T cells and macrophages. Collectively, these data suggest that targeting of 15-LOX2 could potentially represent a valuable measure to limit cancer-related inflammation mediated by CCL2 and attenuate immunosuppression mediated by

TAMs, thus enhancing antitumor immune response in patients with advanced RCC.

In addition, we also examined whether inhibition of LOX activity in RCC TAMs could reduce their ability to induce CTLA-4 or FOXP3 expression in the T lymphocytes. Results shown in Fig. 5D and E indicate that pretreatment of TAMs with LOX inhibitor NDGA did not affect this immunoregulatory function of RCC-infiltrating macrophages.

Discussion

Despite the recent advances in mechanisms of tumor evasion from immune system, it is becoming increasingly apparent how little is known about the function of TAMs in the human cancer progression (25, 26) including RCC. TAMs play a complex role in cancer progression via production of proangiogenic cytokines that stimulates tumor angiogenesis, secretion of multiple biologically active factors with immunomodulatory activity, inhibiting T-cell function, and exerting protumorigenic effects (6, 7, 16). Production of lipid mediators including eicosanoids, which are arachidonic acid metabolites, is important component of activated macrophages including TAMs. Cancer progression is frequently associated with deregulated production of eicosanoids (12). Here, we show that progression of human RCC is associated with enhanced eicosanoid metabolism mediated by LOX pathway in RCC TAMs.

Furthermore, our results establish a link between enhanced metabolism of arachidonic acid in RCC-infiltrating macrophages and local immunosuppression in the RCC tumor microenvironment. This is supported by several lines of evidence. First, RCC-infiltrating macrophages have upregulated levels of 15-LOX2 expression and secrete substantial amounts of the arachidonic acid metabolite 15(S)-HETE. Moreover, exposure of blood monocytes from patients with RCC to RCC-conditioned medium also promoted upregulation of 15-LOX2 but not 15-LOX1 in differentiating myelomonocytic cells. Second, RCC TAMs produce substantial amounts of the proinflammatory chemokine CCL2 and the immunosuppressive cytokine IL-10 in a LOX-dependent manner. Third,

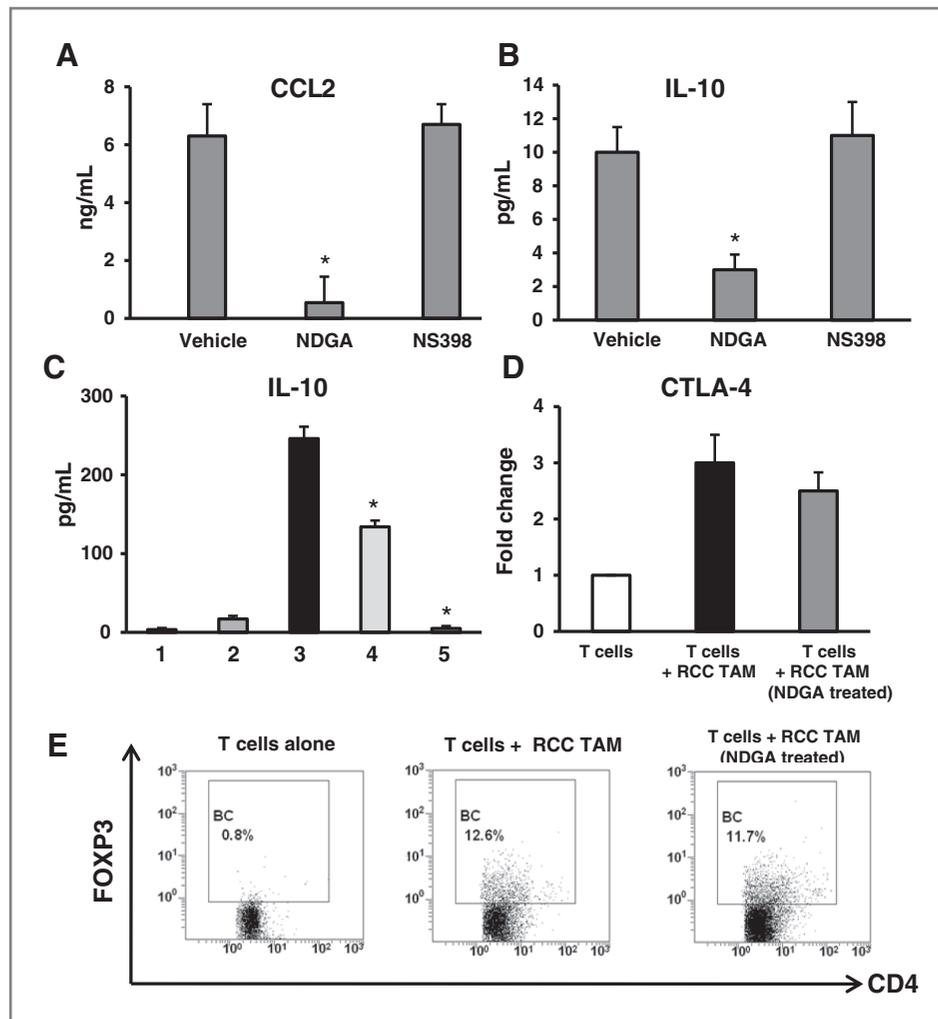


Figure 5. The LOX pathway controls regulatory function of RCC TAMs. **A**, LOX inhibitor NDGA, but not COX-2 inhibitor NS398, reduced production of CCL2 by RCC TAMs. A total of 1×10^6 per mL of RCC TAMs were cultured in serum-free medium in the presence of vehicle control, NDGA (20 $\mu\text{mol/L}$), and NS398 (50 $\mu\text{mol/L}$) for 24 hours. Collected cell-free supernatants were assayed for CCL2 by ELISA kit. Data \pm SD for one patient with RCC are shown. *, $P < 0.05$. Similar results were obtained from 4 patients with RCC. **B**, LOX inhibitor NDGA inhibits secretion of IL-10 by RCC TAMs. Isolated TAMs were cultured in serum-free medium in the presence of vehicle control, NDGA (20 $\mu\text{mol/L}$), and NS398 (50 $\mu\text{mol/L}$) for 24 hours. The concentration of IL-10 in cell-free supernatants was measured by ELISA kit. Data \pm SD for one patient are shown. *, $P < 0.05$. Similar results were obtained from 4 patients with RCC. **C**, the LOX inhibitor NDGA reduces IL-10 production in coculture of RCC TAMs and autologous T cells. Fresh RCC TAMs (5×10^4) and purified autologous T cells (1×10^5) from the peripheral blood of patients with RCC were mixed or cultured alone in 96-well plates. T cells were stimulated with anti-CD3/CD28 Abs (1 and 5 $\mu\text{g/mL}$, respectively). IL-10 concentration was measured in cell supernatants (collected after 48 hours of culture) by ELISA. Data presented are as follows: T cells alone (1); TAMs alone (2); mixture of TAMs and T cells (3); TAMs pretreated with NDGA (25 $\mu\text{mol/L}$) for 2 hours before coculturing with T cells (4); NDGA (25 $\mu\text{mol/L}$) was added to the coculture of TAMs and T cells at the initial point and was present in coculture for 48 hours (5). IL-10 concentration \pm SD for one patient with RCC are shown. *, $P < 0.05$. Similar results were obtained from 2 patients. **D** and **E**, LOX inhibitor NDGA could not prevent RCC TAM-mediated expression of CTLA-4 and FOXP3 in T lymphocytes. A total of 1×10^6 purified T cells (control) or mixture of 5×10^5 TAMs and 1×10^6 autologous T cells were cultured together. TAMs were pretreated with vehicle control or NDGA (25 $\mu\text{mol/L}$) for 2 hours before coculturing with T cells. T cells were stimulated with CD3/CD28 Abs. Forty-eight hours later, cells were collected. Expression of CTLA-4 was measured by qRT-PCR assay. Samples were run in triplicates. Fold changes (\pm SD) are shown for one patient. Expression of FOXP3 in CD4 cells was evaluated using flow cytometry. Results are shown for one patient. Similar results were obtained from 3 patients with RCC.

RCC-infiltrating macrophages are capable of inducing immune tolerance via induction of CTLA-4 and FOXP3 expression. These data underscore the importance of RCC-infiltrating macrophages in the development of immunosuppression and tolerance observed in patients with RCC.

In addition to upregulated 15-LOX2, RCC-infiltrating TAMs display increased gene expression of another eicosanoid-

forming enzyme COX-2 that uses arachidonic acid to form prostanoins.

However, both COX-2 protein expression and PGE2 production by primary RCC tissues or isolated TAMs were fairly low, suggesting that arachidonic metabolism in RCC tumor microenvironment is shifted to LOX pathway. The relatively weak involvement of COX-2 pathway in RCC progression has

been recently indirectly supported by clinical trial (27), in which administration of the selective COX-2 inhibitor celecoxib could not improve therapeutic effects of IFN- α therapy of patients with RCC.

LOX are iron-containing enzymes that catalyze the deoxygenation of polyunsaturated fatty acid in lipids (22, 28). The specific bioactivities of LOXs include hydroperoxidase, leukotriene synthase, lipoxin synthase, and hepoxilin synthase. Supportive evidence suggests that 15-LOX1 resolves inflammation through the secretion of lipoxines, resolvines, and protectins (29) and positively regulates production of IL-12 (30), which is important for generation of antitumor immune response. In addition, 15-LOX1 was also identified as a suppressor of myeloproliferative disease (31). These and other data (32, 33) suggest that 15-LOX1 could exert antiproliferative effects and might be associated with generation of antitumor immunity. Consistent with these observations, we could not detect 15-LOX1 expression in tested samples of primary human RCC. In contrast, expression of 15-LOX2 was markedly enhanced. Moreover, increased expression of 15-LOX2 in RCC, particularly in TAMs, was associated with elevated production of immunosuppressive and proinflammatory factors. Emerging evidence indicates that expression of 15-LOX2 in human macrophages could be upregulated by hypoxia (34), whereas transgenic overexpression of this enzyme in prostate tissue promotes hyperplasia (35). We speculate that in contrast to inflammation-resolving function of 15-LOX1, expression of 15-LOX2 in tumor microenvironment could be associated with cancer-related chronic inflammation and local immunosuppression.

A recently proposed classification of macrophages suggests that IL-10 production is one of the most important and reliable characteristic of "regulatory macrophages" (18). The authors suggest that the presence of regulatory macrophages could negatively correlate with vaccine protection. Our results show that TAMs in advanced human RCC produce elevated levels of IL-10, implying that the proportion of regulatory macrophages among macrophages infiltrating RCC is increasing with tumor progression. The increased presence of IL-10-secreting regulatory macrophages in tumor site may contribute to the enhanced immunosuppression observed in patients with advanced cancer (4). Elevated IL-10 production in tumor microenvironment may also have significant impact on maturation and function of dendritic cells. It has been shown that IL-10 prevents the generation of monocytic dendritic cells and promotes differentiation of macrophages (36, 37). It is possible that TAM- and T cell-derived IL-10 might inhibit differentiation of myeloid antigen-presenting cells in tumor microenvironment by inducing a bystander effect on recruited surrounding monocytes.

It seems that macrophages infiltrating RCC also possess tolerogenic function, as they are able to upregulate expression of FOXP3 and CTLA-4 in autologous T lymphocytes. These molecules were implicated in mechanisms of T-cell unresponsiveness. FOXP3 transcription factor is a key to dominant immune tolerance (38). CTLA-4, which is a second counter-receptor for the B7 family of costimulatory molecules, is a negative regulator of T-cell activation and greatly contributes to T-cell unresponsiveness in cancer (39–41). It is obvious that TAM-mediated induction of FOXP3 and CTLA-4 expression in T lymphocytes favors tolerogenic conditions that allow tumor to escape immune response. At the same time, it seems that ability of TAMs to induce FOXP3 or CTLA-4 in T cells is not associated with enhanced 15-LOX2 pathway in macrophages, as LOX inhibitor could not prevent TAM-mediated expression of FOXP3 and CTLA-4 (Fig. 5D and E).

Taken together, our data show that TAMs play an important role in the development of immunosuppression and T-cell tolerance in human RCC through secretion of immunosuppressive factors and induction of immune unresponsiveness in T cells. Ability of RCC-infiltrating macrophages to produce immunosuppressive cytokine IL-10 and proinflammatory chemokine CCL2 is regulated by 15-LOX2 activity (Fig. 5A and B). It is plausible that enhanced 15-LOX2 activity in RCC tumor microenvironment could be directly implicated in the maintenance of RCC-related cancer inflammation via stimulation of CCL2-mediated recruitment of CCR2-expressing monocytic myeloid cells (Supplementary Fig. S6), which upon arriving in tumor site might differentiate in immunosuppressive, IL-10-secreting, and 15-LOX2-expressing TAMs. In addition, tumor-infiltrating macrophages could exert tolerogenic effect on T cells by inducing FOXP3 and CTLA-4 in LOX-independent fashion.

Therefore, enhanced metabolism of arachidonic acid in the RCC tumor microenvironment, mediated by an activated 15-LOX2/15(S)-HETE pathway, directly affects the function of recruited immune cells, thus promoting local immunosuppression and tumor evasion. Therapeutic approaches directed toward the manipulation of the 15-LOX2-mediated arachidonic acid metabolism in the tumor microenvironment could represent a novel strategy to counteract cancer-related inflammation and attenuate immunosuppression/tolerance in patients with advanced RCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tumor-Associated Macrophages Mediate Immunosuppression in the Renal Cancer Microenvironment by Activating the 15-Lipoxygenase-2 Pathway

Irina Daurkin, Evgeniy Eruslanov, Taryn Stoffs, et al.

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