Tumor-associated macrophages mediate immune suppression in the renal cancer microenvironment by activating the 15-lipoxygenase-2 pathway

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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 TAM isolated from human RCC produce substantial amounts of the pro-inflammatory chemokine CCL2 and immunosuppressive cytokine IL-10, in addition to enhanced eicosanoid production via an activated 15-lipoxygenase-2 (15-LOX2) pathway. TAM 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 TAM. In addition, TAM isolated from RCC were capable of inducing in T lymphocytes the pivotal T regulatory cell transcription factor FOXP3 and the inhibitory CTLA-4 co-receptor. 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 TAM often present in RCCs display enhanced 15-LOX2 activity which contributes to RCC-related inflammation, immune suppression and malignant progression. Further, we show that TAM mediate the development of immune tolerance through both 15-LOX2-dependent and 15-LOX2-independent pathways. We propose that manipulating LOX2-dependent arachidonic acid metabolism in the tumor microenvironment could offer new strategies to block cancer-related inflammation and immune escape in renal cell carcinoma patients.

PRECIS: Findings define a specific lipoxygenase that is essential for tumor-associated macrophages to nurture the tumor microenvironment by driving immune escape.
INTRODUCTION

Renal cell carcinoma (RCC) is the most common type of kidney cancer (1). Although chemotherapeutic and radiation therapies are the standard treatment for most metastatic malignancies, their effect in disseminated RCC has shown little impact on progression-free or overall survival (2). On the other hand, since RCC is considered an immunogenic tumor, it responds to immunotherapeutic intervention thereby fostering continued interest in exploiting the immune system’s ability 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 anti-tumor 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). TAM 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 (AA) in the tumor microenvironment (12). Augmented AA metabolism results in increased production of eicosanoids such as prostaglandin, thromboxane, leukotriene and hydroxyeicosatetraenoic acid (HETE), all of which are biologically active lipids. Free AA can be metabolized by two key enzymes: cyclooxygenase (COX) and lipoxygenase (LOX).

Numerous clinical and pharmacological studies have demonstrated that COX-2 expression and enhanced PGE$_2$ 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 cells,
including myeloid APCs and their precursors (13). The role of LOX isoforms in the progression of human cancers remains largely unexplored.

Here, we demonstrate that macrophages infiltrating human kidney cancer tissues (RCC TAM) secrete immunosuppressive cytokine IL-10 and pro-inflammatory chemokine CCL2. Furthermore, RCC TAM contribute to immune evasion via induction of tolerogenic FOXP3+ and IL-10-secreting T cells as well as via up-regulation of inhibitory receptor CTLA-4 expression in autologous T cells. RCC TAM displayed high expression of 15-lipoxygenase2 (15-LOX2) and produced substantial amounts of 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE). Inhibition of lipoxygenase activity led to down-regulation of production of IL-10, CCL2 by RCC TAM 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 immune suppression and tumor evasion.

MATERIALS AND METHODS

Patients. 51 cancer patients with diagnosed 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: (a) a new diagnosis of histologically confirmed cc RCC; (b) no prior treatment including surgery, chemotherapy, or radiation for kidney cancer; and (c) no other known malignancy. Detailed characteristics of the patients can be found in Supplemental Materials section (Suppl. Table S1).
**Isolation of TAM.** To obtain single cell suspension from RCC tumors, solid fresh cancer and normal kidney tissues were disaggregated with collagenase cocktail. For isolation of TAM, we first lysed red blood cells with ACK buffer and then depleted tumor cell suspension from CD15⁺ and CD33⁺ MDSC 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-AAD using flow cytometry and trypan blue exclusion assays.

**Morphological characterization of TAM.** Freshly isolated CD11b⁺CD15⁻CD33⁻ cells were spun on slides and stained with a Hema3 Stat Pack kit (Fisher Scientific). Non-specific esterase activity was determined using naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase kit (Sigma-Aldrich).

**Cell isolation from blood.** Peripheral blood mononuclear cells (PBMC) from whole blood of RCC patients 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 NDGA, COX-2 inhibitor NS 398 and arachidonic acid were obtained from Cayman Chemical. Antibodies against 15-LOX2 were purchased from Oxford Biomedical Research, anti-15-LOX1 antibody were from Cayman Chemical. All antibodies used for flow cytometry were purchased from (BD Bioscience). 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, Tie-2 as indicated in the text. Expression of FOXP3 and IL-10 was assessed using intracellular flow cytometry staining kit from BD Biosciences. Cytometry data were acquired using 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 using 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), performed on an ABI PRISM 7900 (Applied Biosystems) using the following specific TaqMan®Gene expression assays: 15-lipoxygenase-1 (*Alox15*); 15-lipoxygenase-2 (*Alox15B*), cyclooxygenase 2 (*Ptgs2*), forkhead box protein P(*Foxp3*), cytotoxic T-lymphocyte antigen (*Ctla4*). All samples were run in triplicate, and amplification data were analyzed using AB Prism Sequence Detection Software, version 2.2.1. Relative quantification was calculated according to ∆∆Ct 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 μg/lane) were subjected to 10% SDS-PAGE, and blotted onto PVDF membranes. Membranes were blocked for 1 hour at room temperature with 5% dry skimmed milk in TBS (20 mM Tris-HCl, pH 7.6, 137 mM 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 Ab conjugated with HRP. Results were visualized by chemiluminescence detection using a SuperSignal West Pico substrate (Thermo Scientific). To confirm equal loading membranes were stripped using Restore™ Western blot stripping buffer (Thermo Scientific) and reprobed with antibody against β-Actin (Santa Cruz Biotechnology, Inc).

**ELISPOT assay.** The ELISPOT assays were performed using the human IL-10 ELISPOT Kit (BD Bioscience). Briefly, 96-well MultiScreen™ plates (Millipore) were coated with anti-human IL-10 mAb in PBS. After overnight incubation at 4°C, the wells were repeatedly washed with PBS and immobilized with anti-CD3 mAb for 2h at 37°C. After incubation cells were washed again and 100µl of X-VIVO culture media were added to each well for 1h at room temperature. 100,000 T cells were incubated with 50,000 TAM from the same patient and anti-CD28 mAb (10µg/ml) for 24h, 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 was evaluated using the ELISPOT plate reader and software from Cellular Technology Ltd.

**Secretion of 15(S)-HETE and PGE2.** Analysis of 15(S)-HETE and PGE2 production were performed using an EIA kits and protocols developed by Cayman Chemical.

**Measurement of CCL2 and IL-10 production by ELISA.** Both production of CCL2 and IL-10 were measured using commercial ELISA kits purchased from BD Bioscience.

**Statistical analysis.** Values are expressed as mean ± SD. Unpaired Student’s *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 utilized a flow cytometric approach. Fresh, surgically removed cancerous and normal kidney tissues from patients with RCC (detailed information on patients is shown in Suppl. 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 RCC patient in Fig. 1A (left panel), 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 co-expressed HLA-DR. Conversely, only rare CD11b-negative cells were observed in normal kidney tissue (Fig. 1A, right panel). Analysis of CD45⁻CD11b⁻ fraction of tumor-infiltrating cells revealed that majority of those cells was presented by tumor-infiltrating T lymphocytes and NK (data not shown), which is consistent with earlier observations (14, 15).

Characterization of tumor-associated macrophages isolated from RCC tissue

The majority of RCC-infiltrating myeloid cells co-express MHC class II molecule (Fig. 1A) suggesting that these cells belong to monocyte/macrophage cell lineage and most likely are represented by tumor-associated macrophages (TAM)(16). In contrast, the minor HLA-DR-negative CD11b cell population is likely comprised of MHC class II-negative myeloid-derived
suppressor cells (MDSC) or granulocytic myeloid cells. We developed a protocol to isolate myeloid cell population from RCC tissue that is enriched for TAM (see Materials and Methods). Under these protocol conditions viability of isolated cells routinely exceeded 90% as determined by expression of 7-AAD using flow cytometry and trypan blue exclusion assays (Suppl. Fig S2). Microphotographs of freshly isolated populations of CD11b⁺CD15⁻CD33⁻ myeloid cells show that these mononuclear cells displayed macrophage morphology (Fig. 1B, left panel) and were positive for esterase activity, an enzyme specific for monocytes/macrophages (Fig. 1B, central panel). In culture they acquired a typical macrophage shape (Fig. 1B, right panel). 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 Suppl. Fig. S1). The majority of cells co-expressed 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, CD40 (Suppl. Fig. S2 and Suppl. Table S2). In addition, isolated cells co-expressed variable levels of CD36 and PD-L1 (Suppl. Fig. S1, S2 and Suppl. Table S2). Based on these results, we conclude that CD11b⁺HLA-DR⁺ cells isolated from human RCC tissues represent tumor-associated macrophages.

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

It has been shown that TAM arise from myelomonocytic cells of bone marrow origin (MDSC, monocytes) which are recruited by cancer cells to the tumor site. Increased and constant presence of TAM in RCC tumors suggests existing 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 for chemokine receptor CCR2 which 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 TAM isolated from RCC. Although CCL2 production varied widely among the patients, the cancer tissues consistently expressed higher levels than normal kidney tissue (Fig 2A). TAM isolated from RCC tumors also secreted substantial amounts of CCL2.

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

TAM 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 appears that these immune cells in RCC are dysfunctional as they fail to destroy the malignant cells. Recent studies demonstrate 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 TAM could contribute to the enhanced IL-10 production by T cells. To this end, we mixed TAM isolated from RCC tumor tissue with autologous T cells purified from peripheral blood of RCC patients. These T cells were then stimulated with anti-CD3/CD28 antibodies and IL-10 production was measured using an ELISPot assay. When RCC-derived TAM or CD3/CD28-stimulated T cells were cultured separately, both TAM 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 Suppl. Fig S3, B). Since both TAM and T cells are capable of secreting IL-10, it was not clear what cell type could enhance production of cytokine upon mixing. To clarify this issue, we mixed fresh isolated TAM with activated autologous T cells, co-cultured for forty eight hours and then measured intracellular IL-10 expression in TAM and T cells using flow cytometry. Obtained results indicate (Suppl. Fig. S4) that both TAM and T cells increase IL-10 expression in co-culture conditions.

Another mechanism that cancers employ in order to escape the immune system is induction of FOXP3+ regulatory T cells (T regs) or up-regulation of CTLA-4 expression in T cells upon activation. A high frequency of T regs and increased expression of CTLA-4 have been linked to poor prognosis in patients with cancer. We tested the ability of TAM to induce FOXP3 and CTLA-4 expression in T cells. TAM isolated from RCC tumor tissue were mixed with purified autologous PBMC-derived CD3+ T cells from RCC patients in a cell ratio of 1:2 and T cells were stimulated with CD3/CD28 mAbs. Levels of FOXP3, TGF-β1 and CTLA-4 gene expression were measured by qRT-PCR. As shown in Fig. 2D, macrophages 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-β1 (data not shown). TAM-mediated induction of CTLA-4 and FOXP3
protein expression in T cells has also been confirmed using Western blotting analysis (Suppl. Fig. S5, A) and flow cytometry (Fig. 5E), respectively.

**RCC-infiltrating macrophages display enhanced eicosanoid production mediated by up-regulated 15-LOX2 expression**

TAM 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 prostaglandin E2 (PGE2) and 15 (S)-hydroxyeicosatetraenoic acid (15(S)-HETE), by primary RCC tumor tissue, normal kidney tissue and RCC TAM. We found that levels of PGE2 secreted by primary RCC tumor tissue and RCC TAM were relatively low and about similar to those found in supernatants from normal kidney tissue (Fig. 3A). At the same time, both whole RCC tumors tissues and isolated TAM produce substantially increased amounts of 15(S)-HETE as compared to normal kidney tissue (Fig. 3B).

15(S)-HETE is a metabolite of arachidonic acid that is formed by 15-lipoxygenase. In humans, two distinct subtypes of 15-LOX exist: 15-lipoxygenase-1(15-LOX1) and 15-lipoxygenase-2 (15-LOX2). We measured the expression of 15-LOX1 (alox15), 15-LOX2 (alox15b) and COX2 (ptgs2) genes in macrophages isolated from RCC. Our results indicate that the expression of 15-LOX2 and COX2 genes in RCC-infiltrating macrophages was substantially up-regulated in comparison to whole RCC tumor tissue (Fig. 3C). Expression of the 15-LOX1 gene was undetectable in both RCC TAM and whole RCC tumor tissues. Increased expression of 15-
LOX2 in RCC TAM was confirmed by Western blot analysis (Fig. 3D and Suppl. Fig. S5, C). In addition, we found that expression of 15-LOX2 protein could be induced in monocytes from peripheral blood of RCC patients 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 TAM, whole RCC tumor or normal kidney tissue (Fig. 3F). Despite relatively high levels of COX-2 mRNA expression in TAM, its protein expression is fairly low and sometimes could not be detected by Western blot (Suppl. Fig. S5, B). This is consistent with low levels of PGE2 secreted by RCC TAM (Fig. 3A).

Together, these data provide strong evidence that RCC-infiltrating macrophages display enhanced eicosanoid production due to activated 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-unfiltrating 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 tissue, RCC tumor tissue and RCC TAM. Our data suggest that it is unlikely that those cytokines could be involved in up-regulated 15-LOX2 expression in macrophages associated with RCC (Fig. 4 A, 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 AA-related enzymes 15-LOX2 or COX2, whose expression was up-regulated in
TAM, were involved in the regulation of CCL2 production, we cultured RCC TAM in the presence of pharmacological 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 COX2 inhibitor NS-398, significantly reduced production of CCL2 by TAM 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 TAM also was markedly inhibited in presence of lipoxygenase inhibitor NDGA (Fig. 5B). Importantly, no cell toxicity was observed for NDGA. We then asked whether this lipoxygenase inhibitor could also prevent enhanced IL-10 production observed in co-cultured TAM and T cells (Fig. 2C). Results presented in Fig. 5C demonstrate that pretreatment of TAM isolated from RCC with NDGA led to substantial reduction in the amount of immunosuppressive IL-10 produced in mixed culture of TAM and T lymphocytes, whereas constant presence of LOX inhibitor in the co-culture resulted in complete prevention of IL-10 production in co-cultured 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 immune suppression mediated by TAM thus enhancing anti-tumor immune response in patients with advanced RCC.

In addition, we also examined whether inhibition of LOX activity in RCC TAM could reduce their ability to induce CTLA-4 or FOXP3 expression in the T lymphocytes. Results shown in Fig. 5D and 5E indicate that pre-treatment of TAM with LOX inhibitor NDGA did not affect this immune regulatory 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 tumor-associated macrophages in the human cancer progression (25, 26), including RCC. TAM play a complex role in cancer progression via production of pro-angiogenic cytokines that stimulates tumor angiogenesis, secretion of multiple biologically active factors with immunomodulatory activity, inhibiting T-cell function and exerting pro-tumorigenic effects (6, 7, 16). Production of lipid mediators including eicosanoids, which are arachidonic acid metabolites, is important component of activated macrophages including tumor-associated macrophages. Cancer progression is frequently associated with deregulated production of eicosanoids (12). Here we demonstrate that progression of human RCC is associated with enhanced eicosanoid metabolism mediated by lipoxygenase pathway in RCC TAM.

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 up-regulated levels of 15-LOX2 expression and secrete substantial amounts of the arachidonic acid metabolite 15(S)-HETE. Moreover, exposure of blood monocytes from renal cell carcinoma patients to RCC-conditioned medium also promoted up-regulation of 15-LOX2 but not 15-LOX1 in differentiating myelomonocytic cells. Second, RCC TAM produce substantial amounts of the pro-inflammatory chemokine CCL2 and the immunosuppressive cytokine IL-10 in a lipoxygenase-dependent manner. Third, RCC-infiltrating macrophages are capable of inducing immune tolerance via induction of CTLA-4 and FOXP3 expression. These
data underscores the importance of RCC-infiltrating macrophages in the development of immune suppression and tolerance observed in renal cell carcinoma patients.

In addition to up-regulated 15-LOX2, RCC-infiltrating TAM display increased gene expression of another eicosanoid-forming enzyme COX2 that utilizes arachidonic acid to form prostanoids. However, both COX-2 protein expression and PGE2 production by primary RCC tissues or isolated TAM were fairly low suggesting that arachidonic metabolism in RCC tumor microenvironment is shifted to lipoxygenase 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 selective COX-2 inhibitor celecoxib could not improve therapeutic effects of IFN-alpha therapy of RCC patients.

Lipoxygenases (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 hepoxylin 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 anti-tumor 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 anti-proliferative effects and might be associated with generation of anti-tumor 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 tumor-associated macrophages, was associated with elevated production of immunosuppressive and pro-inflammatory factors. Emerging evidence indicates that expression of 15-LOX2 in
human macrophages could be up-regulated by hypoxia (34), while 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 immune suppression.

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 demonstrate that TAM 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 immune suppression observed in patients with advanced cancer (4). Elevated IL-10 production in tumor microenvironment also may 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 appears that macrophages infiltrating RCC also possess tolerogenic function since they are able to up-regulate 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 co-stimulatory molecules, is a negative regulator of T cell activation, also
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 TAM to induce FOXP3 or CTLA-4 in T cells is not associated with enhanced 15-LOX2 pathway in macrophages, since LOX inhibitor could not prevent TAM-mediated expression of FOXP3 and CTLA-4 (Fig. 5D and 5E).

Taken together, our data demonstrate that tumor-associated macrophages play an important role in the development of immune suppression 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 pro-inflammatory 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 CCL2-mediated recruitment of CCR2-expressing monocytic myeloid cells (Suppl. Fig S6), which upon arriving in tumor site, might differentiate in immunosuppressive, IL-10-secreting and 15-LOX2-expressing TAM. In addition, tumor-infiltrating macrophages could also exert tolerogenic effect on T cells by inducing FOXP3 and CTLA-4 in lipoxygenase-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 immune suppression 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 immune suppression/tolerance in patients with advanced RCC.
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FIGURE LEGENDS

Figure 1. Characterization of RCC-infiltrating macrophages. A: Fresh, surgically removed cancerous (left panel) and normal kidney (right panel) tissues from patients with renal cell carcinoma 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 four color flow cytometry.

B: Morphology of tumor-associated macrophages isolated from human RCC tissue. Tumor-infiltrating CD11b⁺CD15⁻CD33⁻ myeloid cells were purified with magnetic beads and stained with hematoxylin/eosin (left panel) and nonspecific esterase (central panel), or cultured in complete medium for 24 hours before microphotographs were taken (right panel).

C: Phenotype of tumor-associated macrophages isolated from RCC. RCC TAM were isolated using magnetic beads as described in section 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.

Figure 2. Regulatory function of RCC-infiltrating macrophages. A: Comparative levels of CCL2 produced by RCC TAM, whole RCC and normal kidney tissues. Single cell suspensions (10⁶/ml) of fresh RCC tissues, normal kidney tissues and isolated TAM from three RCC patients were cultured in serum-free medium for 24 h. Levels of CCL2 in free-cell supernatants were measured in triplicates using ELISA kit. Average concentrations ± SD are shown. *, p<0.05.
**B:** Tumor-associated macrophages from large tumors produce more IL-10. TAM were isolated from four RCC patients with different size of tumor and cultured in serum-free medium for 24 hours (5 x10^4 cells/per well). Cell-free culture supernatants were assayed for IL-10 using commercial ELISA kit. Assays were run in triplicates. Average IL-10 concentrations are shown.

**C:** Co-incubation of TAM and activated T cells enhances IL-10 production. IL-10 production was measured using ELISPOT assay. Fresh RCC TAM (5 x10^5) and purified autologous T cells (1 x10^5) from the peripheral blood of RCC patients were mixed or cultured alone in ELISPOT plate. T cells were stimulated with anti-CD3/CD28 Abs (1 and 5µg/ml, respectively). Assays were run in quadruplicates. IL-10 ELISPOT results obtained from three RCC patients are shown (Average ± SD) (C).* p<0.05.

**D:** RCC TAM promote up-regulation of CTLA-4 and FOXP3 gene expression in T cells. Purified T cells (control) or mixture of 5x10^5 RCC TAM and 1x10^6 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 using qRT-PCR assay. Samples were run in triplicates. Average fold changes (± SD) for three RCC patients are shown *, p<0.05.

**Figure 3.** RCC TAM have up-regulated expression of 15-lipoxygenase2 (15-LOX2) and produce elevated amounts of arachidonic acid metabolite 15 (S)-HETE. **A:** PGE2 production. Single cell suspensions (10^6/ml) of normal kidney tissues, whole RCC tumor tissues, and RCC TAM from the RCC patients were cultured in serum-free medium for 24 h. Levels of PGE2 in free-cell supernatants were measured in triplicates using ELISA kit. Data for six RCC patients are shown.
**B:** 15(S)-HETE production. Single cell suspensions (10^6/ml) of normal kidney tissues, whole RCC tumor tissues, and RCC TAM from RCC patients were cultured in serum-free medium for 24 h. Levels of 15(S)-HETE in free-cell supernatants were measured in triplicates using ELISA kit. Data for six RCC patients are shown.

**C:** Expression of 15-LOX2 and COX2 genes in RCC TAM is up-regulated. Total RNA was isolated from RCC TAM and whole RCC tumor tissue from 6 cancer patient using RNeasy kit (Qiagen). qRT-PCR analysis was performed using an Applied Biosystems Prism 7900HT Fast Real-Time PCR System. Samples were run in triplicates. Data for six RCC patient ± SD are shown.

**D:** Enhanced expression of 15-lipoxygenase-2 (15-LOX2) protein in RCC TAM. Cell lysates were prepared from RCC tumor tissue (line 1), RCC TAM (2) and normal kidney tissue (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 RCC patient (line 1); CD14^+ cells from the same patient cultured in complete culture medium for 3 days (2); CD14^+ cells from the same patient cultured in the presence of RCC TCM (30%) for 3 days (3).

**F:** Expression of 15-LOX1 protein in: RCC tumor tissue (line 1), RCC TAM (2), or normal kidney tissue (3), positive Western Ready Control (4).

**Figure 4. Levels IL-4 and IL-13 secreted by primary RCC tumor tissues.** Single cell suspensions of RCC tumor tissues, normal kidney tissues and RCC TAM were prepared as described in Materials and Methods. Each cell suspensions (10^6/ml) were cultured for 24 hours in serum free media. Collected cell-free supernatants were assayed for presence of IL-13 and IL-
4 using commercial ELISA kits. Samples from six RCC patients were used for measurement of IL-13 and IL-4.

**Figure 5. The lipoxygenase pathway controls regulatory function of RCC TAM.**

A: LOX inhibitor NDGA, but not COX2 inhibitor NS398, reduced production of CCL2 by RCC TAM. 1x10^6/ml of RCC TAM were cultured in serum-free medium in the presence of vehicle control, NDGA (20 µM) and NS398 (50 µM) for 24 h. Collected cell-free supernatants were assayed for CCL2 using ELISA kit. Data ± SD for one RCC patient are shown. *, p<0.05. Similar results were obtained from four RCC patients.

B: Lipoxygenase inhibitor NDGA inhibits secretion of IL-10 by RCC TAM. Isolated TAM were cultured in serum-free medium in the presence of vehicle control, NDGA (20 µM) and NS398 (50 µM) for 24 h. The concentration of IL-10 in cell free supernatants was measured using ELISA kit. Data ± SD for one patient are shown. *, p<0.05. Similar results were obtained from four RCC patients.

C: The LOX inhibitor NDGA reduces IL-10 production in co-culture of RCC TAM and autologous T cells. Fresh RCC TAM (5 x10^4) and purified autologous T cells (1 x10^5) from the peripheral blood of RCC patients were mixed or cultured alone in 96-well plates. T cells were stimulated with anti-CD3/CD28 Abs (1 and 5µg/ml, respectively). IL-10 concentration was measured in cell supernatants (collected after 48 hours of culture) using ELISA. Data presented as: T cells alone (1); TAM alone (2); mixture of TAM and T cells (3); TAM pretreated with NDGA (25 µM) for two hours before co-culturing with T cells (4); NDGA (25 µM) was added to the co-culture of TAM and T cells at the initial point and was present in co-culture for 48
hours (5). IL-10 concentration ± SD for one RCC patient are shown*, p<0.05. Similar results were obtained from two patients.

**D, E:** Lipoxygenase inhibitor NDGA could not prevent RCC TAM-mediated expression of CTLA-4 and FOXP3 in T lymphocytes. 1x10^6 purified T cells (control) or mixture of 5 x10^5 TAM and 1x10^6 autologous T cells were cultured together. TAM were pretreated with vehicle control or NDGA (25 µM) for two hours before co-culturing with T cells. T cells were stimulated with CD3/CD28 Abs. Forty eight hours later, cells were collected. Expression of CTLA-4 was measured using qRT-PCR assay. Samples were run in triplicates. Fold changes (± 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 three RCC patients.
Figure 1
Figure 3
Figure 5
Tumor-associated macrophages mediate immune suppression in the renal cancer microenvironment by activating the 15-lipoxygenase-2 pathway

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