SUMMARY

Monocytes and macrophages are major components of the tumor microenvironment, but their contributions to human cancer are poorly understood. We used molecular profiling combined with functional assays to investigate the role of these cells in human renal cell carcinoma (RCC). Blood monocytes from RCC patients displayed a tumor-promoting transcriptional profile that supported functions like angiogenesis and invasion. Induction of this protumor phenotype required an interleukin-1 receptor (IL-1R)-dependent mechanism. Indeed, targeting of IL-1-IL-1R axis in a human RCC xenograft model abrogated the protumor phenotype of tumor-associated macrophages (TAMs) and reduced tumor growth in vivo. Supporting this, meta-analysis of gene expression from human RCC tumors showed IL1B expression to correlate with myelomonocytic markers, protumor genes, and tumor staging. Analyzing RCC patient tumors confirmed the protumor phenotype of TAMs. These data provide direct evidence for a tumor-promoting role of monocytes and macrophages in human cancer and indicate IL-1-IL-1R as a possible therapeutic target.

INTRODUCTION

The causal link between inflammation and cancer is now well established (Coussens et al., 2013; Grivennikov et al., 2010; Hanahan and Weinberg, 2011; Mantovani et al., 2008). Monocytes and macrophages represent the major inflammatory infiltrate associated within most solid tumors (Mantovani et al., 2008; Noy and Pollard, 2014), and their recruitment and activation at these sites is largely regulated by tumor-derived signals including chemokines, cytokines, and endogenous signals (Mantovani et al., 2008). The role of these cells in promoting tumor progression has been revealed primarily by studies involving their depletion or accumulation in spontaneous and transplanted murine tumor models (Biswas and Mantovani, 2010; Biswas et al., 2008; Coussens et al., 2000; Lin et al., 2006; Noy and Pollard, 2014). In contrast, our knowledge of the role played by monocytes and macrophages in human cancers remains limited. Most of the available human data come from epidemiological studies that demonstrate a correlation between increased macrophage density and poor prognosis in various cancers (including thyroid, breast, cervix, lung, and liver) (Bingle et al., 2002; Zhang et al., 2012) and with poor responses to chemotherapy (DeNardo et al., 2011). High circulating monocyte counts have also been associated with poor survival in patients with other cancers such as melanoma, head and neck cancer, and malignant pleural mesothelioma (Burt et al., 2011). However, an in-depth characterization of monocytes and macrophages in human cancer together with the mechanism(s) responsible for “educating” these cells to a tumor-promoting phenotype in vivo is still lacking.

We investigated the role played by monocytes and macrophages in human renal cell carcinoma (RCC), which is the most common type of kidney cancer in humans and the third most common urological cancer after prostate and bladder cancer. The incidence of RCC has steadily increased over the last 20 years, leading to estimates that approximately 58,240 patients will have been diagnosed by the start of this decade, with 13,040 cases presenting in the United States alone (Chow et al., 2010; Howlader et al., 2000). RCC is characterized by a lack of early warning symptoms, a variety of clinical manifestations, resistance to chemotherapy and radiation therapy, and most importantly, a high rate of metastasis (Koul et al., 2011). Indeed, around 50% of RCC patients with localized disease subsequently develop metastatic disease, and the 5-year survival for metastatic disease is only 9%. Although targeted antiangiogenic agents in the form of mammalian target of rapamycin (mTOR) inhibitors, tyrosine kinase inhibitors, and monoclonal antibodies...
are increasingly being used (Motzer et al., 2007a, 2007b) and have delivered improvements in progression-free survival, RCC still remains a challenging disease to treat. A better understanding of the cellular and molecular interactions that contribute to RCC progression is therefore necessary to support the future development of more effective therapeutic strategies.

The present study reports a tumor-promoting role for monocytes and macrophages in human RCC and identifies a molecular mechanism responsible for polarizing these cells toward a protumor phenotype. We further demonstrate that in vitro and in vivo targeting of this mechanism not only prevents monocytes and macrophages from adopting a protumor phenotype but also favors the acquisition of an antitumor phenotype, resulting in decreased tumor growth in a xenograft model of human RCC. These findings define a critical component of tumor progression in human RCC and identify a potential target pathway for future therapeutic interventions.

**RESULTS**

**Blood Monocytes from RCC Patients Exhibit a Distinct Transcriptomal Profile**

In order to clarify the role played by monocytes in human RCC progression, we first performed transcriptomal profiling of these cells in RCC patients. Blood monocytes from RCC patients (RCC monocytes) and healthy donors (monocytes) were isolated and compared by microarray analysis, using the 48K genome-wide human Illumina HT-12v4 microarray (as described in the Supplemental Experimental Procedures section and shown in Figure S1A available online). Principal component analysis (PCA) and hierarchical clustering clearly segregated the monocyte transcriptome profile of healthy controls from that of RCC patients, suggesting the populations to be transcriptomally distinct (Figures 1A and 1B). Limma differentially expressed gene (DEG) analysis of the transcriptome revealed differential modulation of 2,384 genes (1,054 upregulated; 1,330 downregulated; FDR < 0.05) in RCC monocytes compared with control monocytes (Figure S1B). These DEGs were then grouped using gene ontology (GO) biological processes, which identified immune-related genes as the most significant upregulated gene function group in RCC monocytes (Figure 1C).
We initially focused on the expression of immune-related genes that encode cytokines, chemokines, and growth factors because these are known to shape the tumor microenvironement (Biswas et al., 2008; Lewis and Pollard, 2006; Mantovani et al., 2008). As indicated in Table 1, RCC monocytes consistently displayed upregulation of a large number of proinflammatory cytokine and chemokine genes (e.g., TNF, IL1A, IL1B, IL24, CCL3, CCL3L1, CCL5, CCL7, CCL20) relative to monocytes obtained from healthy controls. Importantly, RCC monocytes also exhibited upregulation of several “protumor” genes including PTGS2 (encoding COX2), IL8, VEGFA, MMP19, MMP10, CXCR4, and HIF1A, which are known to mediate key processes in tumor development (Table 1; Mantovani et al., 2008; Murdoch et al., 2008). Figure 1D shows a heatmap representation of selected proinflammatory and protumor genes that were differentially expressed in RCC monocytes compared with healthy control monocytes.

Because the tumor microenvironement can polarize myelomonocytic cells (Biswas and Mantovani, 2010), we next aimed to determine whether the gene-expression profile of RCC monocytes was indicative of their polarization status. RCC monocytes were screened for the differential expression of a panel of M1- or M2-polarization related genes (Martinez et al., 2006, 2013; Murray et al., 2014). The profiling data for these genes indicated that RCC monocytes express a mixture of both M1 and M2 genes rather than exhibiting a distinct M1 or M2 phenotype (Figure 1E).

Taken together, the transcriptome data indicated that RCC monocytes possess a distinct gene expression profile suggestive of altered function in human cancer. We therefore sought to further validate these data and to functionally characterize RCC monocytes in subsequent experiments. A complete list of the DEGs can be found in Table S1 available online.

### Table 1. Upregulation of Selected Inflammatory and Protumor Genes in RCC Monocytes

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Full Name</th>
<th>Fold change (Log 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL3</td>
<td>Homo sapiens chemokine (C-C motif) ligand 3 (CCL3), mRNA.</td>
<td>2.43</td>
</tr>
<tr>
<td>CCL3L1</td>
<td>Homo sapiens chemokine (C-C motif) ligand 3-like 1 (CCL3L1), mRNA.</td>
<td>2.39</td>
</tr>
<tr>
<td>CCL4L2</td>
<td>Homo sapiens chemokine (C-C motif) ligand 4-like 2 (CCL4L2), mRNA.</td>
<td>3.16</td>
</tr>
<tr>
<td>CCL5</td>
<td>Homo sapiens chemokine (C-C motif) ligand 5 (CCL5), mRNA.</td>
<td>2.14</td>
</tr>
<tr>
<td>CCL7</td>
<td>Homo sapiens chemokine (C-C motif) ligand 7 (CCL7), mRNA.</td>
<td>3.16</td>
</tr>
<tr>
<td>CCL20</td>
<td>Homo sapiens chemokine (C-C motif) ligand 20 (CCL20), mRNA.</td>
<td>4.05</td>
</tr>
<tr>
<td>CXCL2</td>
<td>Homo sapiens chemokine (C-X-C motif) ligand 2 (CXCL2), mRNA.</td>
<td>3.11</td>
</tr>
<tr>
<td>TNF</td>
<td>Homo sapiens tumor necrosis factor (TNF superfamily, member 2) (TNF), mRNA.</td>
<td>2.83</td>
</tr>
<tr>
<td>IL1A</td>
<td>Homo sapiens interleukin 1, alpha (IL-1A), mRNA.</td>
<td>2.65</td>
</tr>
<tr>
<td>IL1B</td>
<td>Homo sapiens interleukin 1, beta (IL-1B), mRNA.</td>
<td>3.48</td>
</tr>
<tr>
<td>IL24</td>
<td>Homo sapiens interleukin 24 (IL-24), transcript variant 2, mRNA.</td>
<td>1.55</td>
</tr>
</tbody>
</table>

### RCC Patient Monocytes Display a Proinflammatory and Tumor-Promoting Phenotype

To further validate the gene-expression profile of RCC monocytes, a panel of differentially modulated genes was chosen from Table 1 and assessed by quantitative PCR (qPCR). qPCR analysis confirmed significant increase in the expression of proinflammatory cytokine and chemokine genes TNF, IL1A, IL1B, CCL3, CCL3L1, CCL5, CCL7, CCL20 in RCC monocytes compared with monocytes from healthy controls (Figure 2A). Accordingly, we were able to confirm the upregulated expression of TNF-α, IL-1β, IL-6, and CCL3 proteins in RCC monocyte culture supernatants (Figure 2B). Further confirmation of the microarray data was achieved by qPCR validation of the upregulated expression of protumor genes IL8, VEGFA, PTGS2, CXCR4, and MMP10 in RCC monocytes compared with control monocytes (Figure 2C). Consistent with these data, elevated protein expression of proangiogenic factors VEGFA and IL-8 were also detected in RCC monocyte culture supernatants as compared with control monocytes (Figure 2D).

These data indicated that RCC monocytes upregulate key protumor genes and proteins that have been reported to support angiogenesis and metastasis (Murdoch et al., 2008). To test whether RCC monocytes could actually promote such...
Figure 2. RCC Monocytes Exhibit a Proinflammatory and Tumor-Promoting Phenotype

(A) qPCR showing expression of proinflammatory cytokine and chemokine genes in RCC monocytes. Gene names indicated on the x axis. Data are mean ± SEM (healthy: n = 4–6 and patient: n = 7–9).

(B) Amount of indicated cytokines and chemokines detected in the cell-free culture supernatant of RCC monocytes versus monocytes. Data are mean ± SEM (n = 3–4).

(C) qPCR analysis of protumor gene expression in RCC monocytes. Data are mean ± SEM (healthy: n = 4–6 and patient: n = 9).

(D) Amount of proangiogenic factors detected in the culture supernatant of RCC monocytes versus monocytes. Data are mean ± SEM (n = 3).

(E) Angiogenesis assay showing endothelial tube formation on matrix gel induced by RCC monocytes. Left panel shows quantitative analysis of tube formation (n = 3). Right panel shows inhibition of tube formation by α-VEGFR. Quantitation and bright field image of tube formation from one experiment is shown. Data are mean ± SEM.

(F) RCC monocytes enhance the invasion of tumor cells in an in vitro invasion assay. Left panel shows quantitative analysis of tumor cell invasion (n = 2). Right panel shows inhibition of tumor cell invasion by MMP inhibitor. Quantitation and bright field image of tumor cell invasion from one experiment is shown. Data are mean ± SEM. Optical density is shown as a measure of amount of invaded cells. (E and F) #p < 0.05, versus RCC-Mo+α-control/vehicle. In all panels, *p < 0.05, versus Mo.
Role of Monocytes and Macrophages in Human Cancer

Immunity

Protopumor Phenotype of RCC Monocytes Is Regulated by a Nuclear Factor-κB-MyD88 Pathway

We next sought to determine the molecular mechanism(s) that support monocytes acquisition of a protumor phenotype. Because NF-κB is a master regulator of many genes known to modulate tumor development, e.g., TNFA, IL6, VEGFA, PTGS2, and MMP (hereafter termed “protumor genes”) (Biswas and Lewis, 2010; Karin and Greten, 2005), we began by investigating whether NF-κB activation was responsible for inducing a protumor phenotype in RCC monocytes. In order to achieve this, we first generated “tumor-conditioned” monocytes by coculturing monocytes obtained from healthy donors with a RCC tumor cell line (RCC4) in a transwell plate. After 48 hr coculture, RCC-conditioned monocytes exhibited marked increase in the expression of protumor genes, angiogenic activity, and invasive function compared with unconditioned monocytes (Figures S2A–S2C). Similar results were obtained for monocytes cocultured with another RCC cell line (Caki-2) (Figure S2D). These data indicate that RCC cells can “condition” normal monocytes to adopt a protumor phenotype similar to that observed in RCC patients and could therefore be used to probe the mechanisms that support monocyte acquisition of tumor-promoting functions.

RCC-conditioned monocytes displayed evidence of NF-κB activation as indicated by enhanced IκBα phosphorylation and nuclear translocation of p65 NF-κB (Figures 3A and 3B). The role of NF-κB activation in RCC-conditioned monocytes was further investigated using a specific inhibitory peptide for the NF-κB regulator, IKKγ. RCC-conditioned monocytes treated with the inhibitory peptide displayed a marked decrease in protumor genes TNF, IL6, VEGFA, and PTGS2 expression compared with conditioned monocytes that received a control peptide (Figure 3C). Functionally, these inhibitor-treated cells also showed marked downregulation of angiogenic activity and invasive behavior (Figures 3D and 3E). Together, these results suggest that the protumor phenotype of RCC-conditioned monocytes is regulated by NF-κB activation.

We further analyzed whether the protumor phenotype of RCC-conditioned monocytes relied on the MyD88 signaling pathway, upstream of NF-κB. RCC-conditioned monocytes exposed to a MyD88 inhibitory peptide displayed a marked decrease in the expression of TNF, PTGS2, IL6, and VEGFA compared with monocytes exposed to the control peptide (Figure 3F). Functionally, RCC-conditioned monocytes were impaired in their ability to stimulate angiogenesis and tumor cell invasion in vitro after treatment with the MyD88 inhibitory peptide (Figures 3G and 3H). Collectively, these observations support the involvement of the MyD88 signaling pathway in driving the protumor phenotype of RCC-conditioned monocytes.

IL-1-IL-1R Signaling Shapes the Protopumor Phenotype of RCC Monocytes

Elevated IL-1β expression was detected in the plasma of RCC patients (Figure S3A). Because IL-1 signals through the MyD88-NF-κB pathway (Biswas and Lewis, 2010) and could induce protumor genes (Figure S3B), we focused subsequent studies on investigating the involvement of IL-1-IL-1R in mediating the protumor phenotype of monocytes.

In order to assess the role of IL-1-IL-1R in inducing protumor gene expression by RCC-conditioned monocytes, we supplemented Monocyte-RCC cocultures with human recombinant IL-1 receptor antagonist (IL-1RA), to inhibit IL-1-IL-1R signaling. IL-1RA treatment significantly inhibited the expression of protumor genes in RCC-conditioned monocytes (Figure 4A). Because TNF is another primary cytokine that is implicated in RCC (Chuang et al., 2008), we checked in parallel the effect of adding TNF neutralizing antibody in addition to IL-1RA in the above experiments. However, addition of anti-TNF antibody (α-TNF+IL-1RA) did not have any further inhibitory effect (Figure 4A, compare IL-1RA versus α-TNF+IL-1RA). Similarly, adding anti-TNF antibody alone failed to show a significant inhibition in the expression of most protumor genes (Figure S3C).

We also examined whether IL-1RA treatment altered the protumor functions of RCC-conditioned monocytes. Figures 4B and 4C show that supernatants from RCC-conditioned monocytes that had received IL-1RA treatment displayed a significant reduction of angiogenesis and tumor cell invasion. Similar results were reproduced in monocytes cocultured with another metastatic RCC cell line, A498 (Figures 4D–4F). Together, these results clearly indicate a role for the IL-1-IL-1R pathway in promoting a protumor phenotype in RCC monocytes.

Disruption of IL-1-IL-1R Signaling Alters Macrophage Phenotype and Restricts Tumor Growth In Vivo

In order to validate our in vitro data in an in vivo system, we established a human RCC4 xenograft model in SCID mice (detailed in Supplemental Experimental Procedures). The xenograft tumors showed substantial infiltration of F4/80+ TAMs (constituting >10% of the total live tumor cell population) (Figures S4A and S4B, left panel). To trace the origin of these TAMs, we used a previously reported fate-mapping approach for labeling blood monocytes in vivo with fluorescent latex beads that could then be tracked in the tumors (Movahedi et al., 2010). Figure S4C shows the infiltration of labeled inflammatory monocytes
Figure 3. Protumor Phenotype of RCC Monocytes Is Regulated by NF-κB-MyD88 Pathway

(A) Immunoblot for phospho-IκBα (p-IκBα) expression in monocytes cocultured with RCC cells (Mo+RCC) versus monocytes cultured alone (Mo).

(B) Nuclear translocation of p65 NF-κB in Mo+RCC (see insets in merge panel).

(C) Downregulated expression of the indicated genes in Mo+RCC treated with IKKγ inhibitor peptide. Data are mean ± SD representative of two independent experiments; *p < 0.05, versus control peptide-treated Mo+RCC.
(CD11b+Ly6C^hi/F4/80^lo) and TAMs (CD11b+Ly6C^lv/Ly6C^hi/F4/80^lo) in the RCC tumors with monocyte percentage decreasing, while TAM percentage increases from day 8 to 14 of tumor growth. These results indicate inflammatory blood monocytes to infiltrate the RCC tumors and differentiate into TAMs. Moreover, TAMs showed protumor gene expression, as revealed by upregulation of TNF, IL6, IL1B, PTGS2, VEGFA, and MMP10, compared to peritoneal macrophages (PECs) from tumor-free animals (Figure S4D). Further evidence for their protumor role came from a monocyte-macrophage depletion experiment, using liposom cladronate injection, which significantly reduced tumor growth in our RCC xenograft model (Figure S4E).

We next investigated the involvement of IL-1-IL-1R signaling in driving the protumor phenotype of TAMs in vivo using the above xenograft tumor model. Initial immunohistochemistry revealed the presence of IL-1β in the RCC4 tumor tissues (Figure S4B, right). To directly assess the role of IL-1R signaling in driving TAMs to adopt a protumor phenotype and support disease progression in vivo, we injected tumor-bearing animals with recombinant IL-1RA or PBS intratumorally on days 7, 9, and 11 after tumor implantation (as described in Supplemental Experimental Procedures). Tumor take was monitored throughout and TAMs were analyzed after sacrifice on day 20-21. IL-1RA treatment resulted in a decrease in tumor growth (Figure 5A). TAMs from IL-1RA-treated mice showed a marked downregulation of protumor genes as compared to TAMs from PBS-treated mice (Figure 5B). In line with this, culture supernatants of TAMs from IL-1RA-treated tumor showed lesser angiogenic activity and tumor cell invasion, indicating diminished protumor functions (Figures 5C and 5D). The ability of α-VEGFR2 antibody and MMP inhibitor to block angiogenesis and tumor cell invasion induced by TAM supernatants mechanistically links these functions to VEGFA and MMP10 expression by TAMs. Additionally, in vivo imaging confirmed reduced angiogenesis and MMP activity (= invasion) in situ in the tumors of IL-1RA-treated mice (Figure 5E). Finally, our observations were also reproduced in another RCC xenograft model using the aggressive A498 line (Figure S5A). Decreased tumor growth (Figure S5B) and abrogation of TAM protumor gene expression and function were noted in the IL-1RA-treated animals (Figures S5C–S5E). However, TAM infiltration did not show any significant change between PBS- and IL-1RA-treated tumors in both our xenograft models (Figure S5F).

As TAMs have been considered as polarized macrophages, we wondered whether IL-1RA could change the polarization status of these cells. TAMs from PBS-treated, tumor-bearing mice displayed a IL12B^lo/IL10^lo/NOS2^lo/ARG1^hi gene-expression profile, which is characteristic to murine M2 macrophages. In contrast, TAMs from IL-1RA-treated tumor-bearing mice showed increased IL12B and NOS2 expression accompanied by decreased IL10 and ARG1 expression, suggestive of a skewing toward the so-called M1-like phenotype with respect to these markers (Figure 5F).

Taken together, the multiple evidences present above demonstrate that in vivo targeting of the IL-1-IL-1R pathway by IL-1RA could indeed abrogate the protumor gene expression and functions of TAMs resulting in decreased tumor growth.

**Genetic Blockage of IL-1-IL-1R Pathway Inhibits Tumor Growth and Protumor Phenotype of TAMs**

Consistent with the above data, we also demonstrate that RCC4 xenografts display reduced growth in Il1r1^−/− SCID mice (Figure 6A) and that TAMs from these animals exhibit reduced expression of protumor genes compared with their WT counterparts (Figure 6B). To specifically prove that the IL-1R pathway in macrophages was essential for their tumor-promoting role, we adoptively transferred WT or Il1r1^−/− macrophages into our RCC xenograft model and assessed its impact on tumor growth (treatment regime is described in Supplemental Experimental Procedures). Indeed, adoptive transfer of Il1r1^−/− macrophages resulted in reduced tumor growth, in vivo angiogenesis, and MMP activity (Figures 6C and 6D), indicating that the IL-1R pathway in macrophages was instrumental in regulating the tumor-promoting role. These observations provide additional support for the integral in vivo role of IL-1-IL-1R signaling in shaping the protumor phenotype of host macrophages and in driving RCC tumor progression.

**IL-1B Expression Correlates with Cancer Stage, Myelomonocytic, and Protumor Gene Expression in RCC**

Our mechanistic studies demonstrated a central role for the IL-1-IL-1R pathway in polarizing monocytes-macrophages toward a protumor phenotype and in supporting RCC progression. We therefore investigated whether there was an association between IL-1 expression, monocyte-macrophage profile, and expression of protumor genes in tumor tissues obtained from RCC patients. To this end, we performed a meta-analysis of tumor gene-expression data from a cohort of 34 RCC patients with a tumor node staging, available on Oncomine (Rhodes et al., 2004; Yang et al., 2005). We observed that higher IL1B expression in tumors significantly correlated with advanced tumor stages (Figure 7A). Moreover, increased IL1B expression correlated with expression of protumor genes including PTGS2 and IL6 and that of monocyte-macrophage-specific markers like CD14, CD11b, and CD163 (Figure 7B). Accordingly, tumor sections from RCC patients displayed substantial infiltration of CD163^+ TAMs (Figure 7C). In line with this, CD163 gene expression in the Yang et al. (2005) data set significantly correlated with the expression of other macrophage-specific markers (CD14, CD11b) and protumor genes (PTGS2, IL8, IL6), implicating the expression of these protumor genes by TAMs (Figure 7D).

Using another independent approach involving a gene set enrichment analysis (GSEA), we showed a significant enrichment (p = 0.012) of our earlier identified protumor gene panel and
monocyte-macrophage marker genes (viz: TNF, IL6, VEGFA, IL8, MMP10, CD11b, MMP10, IL1B, PTGS2, CD163, CSF1R, CD14, and CD68) in stage IV (advanced) versus stage I (early) tumor samples of the Yang et al. (2005) data, inferring the presence of protumor TAMs. Confirming this protumor phenotype of TAMs, we demonstrated that TAMs isolated from our RCC patient tumors upregulated expression of protumor genes (TNF, IL6, IL1B, PTGS2, VEGFA, IL8, and MMP10) (Figure 7E) and protumor functions like angiogenesis and invasion, as compared to macrophages from healthy donors (Figures 7F and 7G). This was consistent with the protumor phenotype of RCC monocytes, in vitro RCC “conditioned” monocytes, and TAMs in the RCC xenograft model, described earlier (Figures 2, S2, and 5).

Together, the above analysis further supports a significant relationship between IL1B expression, TAM recruitment and expression of protumor genes, and malignant progression of human RCC. Additionally, it also confirmed the protumor phenotype of TAMs in our RCC patient tumors.

DISCUSSION

The present study reports a tumor-promoting role for monocytes and macrophages in human RCC and identifies a crucial contribution of IL-1-IL-1R pathway in shaping the tumor-promoting phenotype of these cells. Monocytes from RCC patients displayed a distinct transcriptomal profile that was characterized by a proinflammatory and tumor-promoting gene signature. Proinflammatory cytokines and chemokines support cancer-related inflammation (Hanahan and Weinberg, 2011; Mantovani et al., 2008), while expression of molecules such as VEGFA,
IL-8, COX2, and MMP10 supports key processes in tumor development. Accordingly, enhanced gene expression of these molecules in monocytes from RCC patients (and in RCC-conditioned monocytes generated in vitro) was linked to enhanced tumor-promoting functions, including significant increases in angiogenic and tumor cell invasive activity. The same tumor-promoting function and gene profile was mirrored in the TAMs isolated from RCC patients and human (RCC) xenograft tumors. Previous studies in mouse models of spontaneous breast and skin cancer have identified a role for myelomonocytic cells in mediating the "angiogenic switch," malignant progression, and metastasis (Andreu et al., 2010; Lin and Pollard, 2007; Qian et al., 2011). However, such a role for these cells in human cancer needs to be demonstrated. Our studies on monocytes and macrophages from RCC patients provide direct evidence of a protumor role for these cells in a human cancer. In support, epidemiological studies have previously correlated macrophage infiltration with poor prognosis in several human cancers like breast, cervix, prostate, gastric, and Hodgkin’s lymphoma (Bin-}

**Figure 5. IL-1-IL-1R Pathway Disruption Modulates Tumor Progression In Vivo**

(A) Kinetics of tumor growth. Data are mean ± SEM from a representative experiment with four mice per group. Experiment was repeated three times independently. *p < 0.05 versus PBS-treated group.

(B) Gene expression analysis of TAMs from PBS- or IL-1RA-treated mice assessed by qPCR. Data are mean ± SEM from three independent experiments (total of 13–15 mice per group). *p < 0.05 versus PBS-treated group.

(C) Angiogenesis and (D) tumor invasion assay performed with supernatants of TAMs from either treatment groups. Data are mean ± SEM from one experiment with four mice per group. *p < 0.05 versus PBS-treated group.

(E) In vivo imaging of angiogenic and MMP activity in the tumors using the Angiosense and MMPlense dyes as described in Supplemental Experimental Procedures. White arrow indicates tumor site.

(F) Polarization of TAMs from PBS- or IL-1RA-treated mice. Data are mean ± SEM from three independent experiments (total of 13–15 mice per group) *p < 0.05, #p < 0.05 versus expression of the corresponding gene in PBS-treated group. See also Figures S4 and S5.

IL-8, COX2, and MMP10 supports key processes in tumor development. Accordingly, enhanced gene expression of these molecules in monocytes from RCC patients (and in RCC-conditioned monocytes generated in vitro) was linked to enhanced tumor-promoting functions, including significant increases in angiogenic and tumor cell invasive activity. The same tumor-promoting function and gene profile was mirrored in the TAMs isolated from RCC patients and human (RCC) xenograft tumors. Previous studies in mouse models of spontaneous breast and skin cancer have identified a role for myelomonocytic cells in mediating the “angiogenic switch,” malignant progression, and metastasis (Andreu et al., 2010; Lin and Pollard, 2007; Qian et al., 2011). However, such a role for these cells in human cancer needs to be demonstrated. Our studies on monocytes and macrophages from RCC patients provide direct evidence of a protumor role for these cells in a human cancer. In support, epidemiological studies have previously correlated macrophage infiltration with poor prognosis in several human cancers like breast, cervix, prostate, gastric, and Hodgkin’s lymphoma (Bin-}

Besides exerting protumor activity by modulating cancer-related inflammation, angiogenesis, and invasion, as observed in RCC monocytes, they can also promote tumor progression by skewing and/or suppressing antitumor T cell responses via factors like iNOS, Arginase1, B7-H1, and B7-H4. In human HCCs, B7-H1+ peritumoral monocyte-macrophages suppressed cytotoxic activity of B7-H1 receptor-expressing CD8+ T cells (Kuang et al., 2009). Similarly, myeloid-derived suppressor cells (MDSCs), by expressing Arginase 1 and iNOS, mediate suppression of T cell responses in several human cancers (Gabrilovich and Nagaraj, 2009). However, B7-H1, B7-H4, iNOS, and Arg1 failed to show up among the significantly modulated genes in the human RCC monocytes profiling. While our data favor direct protumor activity of monocytes and macrophages in RCC, their relative role in modulating tumor-induced immunosuppression needs further study.

Although protumor activity has been generally linked to M2 polarization of macrophages, depending on the plasticity of these cells, their spatiotemporal location, and the type of tumor
involved, diverse polarized states of macrophages (viz M1-like, M2-like, or a mixture of both) have been implicated in tumor promotion (Biswas and Mantovani, 2010; Biswas et al., 2008; Cav- 
nar et al., 2013; Franklin et al., 2014; Movahedi et al., 2010). This also supports the growing evidence that the phenotype of these cells in vivo is more complicated than clear-cut M1 or M2 polarized states (Murray et al., 2014). Indeed, microarray of RCC monocytes failed to reveal a specific polarization profile but rather showed a mixed expression of M1 and M2 genes. Considering the in vivo plasticity of monocyte-macrophase, their lack of clear-cut polarization phenotype in vivo, the paucity of conserved M1 versus M2 markers (between mice and human), and the fact that some genes can promote both M1 or M2 states depending on their context, assessing these cells in terms of a few M1 and M2 markers may not provide a complete picture of their in vivo phenotype (Murray et al., 2014). Instead, defining these cells by their function would be more relevant in such in vivo settings. Following such a functional definition, our data from RCC monocytes, tumor “conditioned” monocytes and TAMs demonstrate a consistent protumor phenotype, charac-

terized by the upregulation of the same protumor genes, func-
tions, and mechanistic pathway, irrespective of the M1-M2 markers.

Previous reports on the role of myelomonocytic cells in tumor progression have focused mainly on macrophages, while the contribution of blood monocytes in cancer development has re-

mained unclear. Three recent studies in mouse mammary carcin-

oma model have indicated that inflammatory monocytes are precursors of TAMs and can contribute to tumor metastasis and immunosuppression (Cas- 

o et al., 2010; Movahedi et al., 2010; Qian et al., 2011). Our present findings provide transcrip-
tomic and functional demonstration of the protumor role of blood monocytes in human cancer. Our biochemical studies in RCC-conditioned monocytes indicated that the expression of protumor genes, as well as tumor-promoting func-
tions like angiogenesis and invasion, are regulated through a MyD88-dependent NF-κB pathway. These data are in accordance with a report that used coculture of syn-
genic mouse ovarian cancer cells with bone-marrow-derived macrophages deficient in MyD88 or IKKβ to demonstrate a key role for these signaling molecules in regulating tumor inva-
siveness (Hagemann et al., 2008). MyD88-dependent signaling was also implicated in tumorigenesis in a mouse model of sponta-

taneous ApcMin/+ intestinal cancer (Rakoff-Nahoum and Medzhitov, 2007). In the current report, we used both in vitro and in vivo approaches to uncover a key role for IL-1-IL-1R, up-
stream of MyD88, in driving the “protumor” phenotype of monocyte and macrophage in RCC. This is supported by several lines of evidence. First, gene-expression data indicated upregulation of IL1A and IL1B and its receptor IL1R1 (but not its antagonist, IL1RN) in RCC monocytes. Second, in vitro treatment of RCC-conditioned monocytes with IL-1RA (an antagonist of IL-1-IL-1R1 signaling) impaired their expression of protumor genes and protumor activity (angiogenesis and invasion). Third, in vivo studies in a xenograft model of human RCC demonstrated that IL-1RA injection impaired the expres-
sion of protumor genes and functions by TAMs and restricted tumor growth, angiogenesis, and invasion (MMP activity). Fourth, TAMs from Il1r1−/− mice displayed a marked reduction in protumor gene expression that correlated with reduced tumor growth. Finally, adoptive transfer experiments with WT or Il1r1−/− macrophages in our RCC xenograft model proved the specific contribution of macrophage IL-1R pathway in tumor promotion. Together, these data support a role for IL-1-IL-1R pathway in driving the tumor-promoting phenotype of monocytes and macrophages in human RCC, thereby regulating disease progression.
Role of Monocytes and Macrophages in Human Cancer

In support of these findings, an IL-1R-MyD88 signaling pathway was recently reported to mediate keratinocyte transformation and tumorigenesis (Cataisson et al., 2012). Oncogenic keratinocytes were shown to employ an IL-1α-IL-1R-MyD88 autocrine loop to induce inflammation and promote oncogenesis. In a separate transgenic mouse model, stomach-specific overexpression of human IL-1β was reported to induce spontaneous gastric inflammation-associated cancer accompanied by the recruitment and activation of myeloid-derived suppressor cells (MDSCs) (Tu et al., 2008). Other studies in a murine chemical carcinogenesis model have previously demonstrated a role for IL-1β in the recruitment of myeloid and endothelial cells, and in angiogenesis and invasive potential of tumor cells (Carmi et al., 2009; Krelin et al., 2007). Together, these studies indicate that the tumorigenic effect of the IL-1-IL-1R pathway might either be intrinsic to transformed cells or could be mediated by indirect crosstalk between transformed cells and stromal cells (e.g., immune cells, endothelial cells, cancer-associated fibroblasts [Erez et al., 2010]). In human RCC, we show an IL-1-IL-1R-dependent mechanism to be crucial in shaping the tumor-promoting phenotype of monocytes and macrophages. Accordingly, increased IL-1β serum concentrations, its positive correlation with tumor size, and elevated IL-1β expression in tumor supernatants have been mentioned in some RCC patient studies (van Rossum et al., 2009; Yoshida et al., 2002). We also noted an increased IL-1β (but not IL-1α) in the plasma of RCC patients, as compared to healthy donors. A separate study further demonstrated that higher IL-1β expression is associated with more malignant human RCC cell lines and correlates with increased invasive ability (Chuang et al., 2008). Alternatively, proinflammatory cytokines such as IL-6 and TNF-α have also been implicated in the pathogenesis of RCC (Balkwill, 2009; Dosquet et al., 1994; Koo et al., 1992; Yoshida et al., 2002). However, it is worth noting that IL-1 being a primary cytokine is known to induce and amplify the production of both IL-6 and TNF (Granowitz et al., 1992). Although our data suggest the IL-1-IL-1R pathway as a crucial mediator of the protumor effects of monocytes and macrophages in human RCC (and although blocking TNF-α did not show any significant effect over IL-1RA on these cells in vitro), we cannot exclude the simultaneous involvement of other cytokine/receptor crosstalks.

By conducting a meta-analysis on tumor gene-expression data in a cohort of 34 RCC patients (Yang et al., 2005), we were able to demonstrate a positive correlation between tumor stage and intratumoral expression of IL1B. Expression of IL1B significantly correlated with the expression of protumor genes (including IL6 and PTGS2) and myelomonocytic markers (including CD14, CD11b, and CD163). Intriguingly, infiltration of CD163+ macrophages into human RCC tissues has recently been suggested to predict poor prognosis (Komohara et al., 2011). In our meta-analysis, we observed that CD163 gene expression in human RCC tumors is significantly correlated with the expression of IL1B, IL6, and IL8, and with macrophage markers including CD11b and CD14. GSEA analysis on this data set also showed significant enrichment of monocyte-macrophage specific markers and their protumor genes preferentially in the advanced (stage IV) tumor tissues (which correlates with increased IL1B expression). Taken together, these observations provide further evidence of a significant role for IL1B expression in regulating TAM recruitment/transcriptional profile and in disease progression in human RCC. However, it may be pointed out that although blood monocytes and TAMs in our study showed a consistent protumor phenotype, divergence arising out of their different tissue localization is expected. Studies in mice have illustrated how different tissue microenvironments (Gautier et al., 2012) and spatial localization within tumors can influence monocyte and macrophage transcriptional profiles (Movahedi et al., 2010). However, such issues are at its infancy in the human settings. Future studies involving an in-depth, side-by-side comparison of blood monocyte and TAM transcriptome from the same patient (and if possible at different disease stages) should bring to light the conserved and divergent features of these cell types and their plasticity during human cancer progression, thereby providing further insight for specific targeting options.

In conclusion, the present study reports a transcriptomic and molecular profiling of blood monocytes in human RCC, revealing their direct role in tumor promotion. We have further demonstrated that IL-1-IL-1R signaling critically regulates the tumor-promoting phenotype of monocytes and macrophages in this cancer. Based on these data, it is possible that therapeutic manipulation of IL-1-IL-1R signaling in human cancers could be used to block the protumor role of monocytes and macrophages and reprogram them to restrict cancer progression in vivo. IL-1R antagonists such as Anakinra (used for treating autoinflammatory diseases and arthritis) are already available in the clinics. Hence, the potential efficacy of anti-IL-1 therapy for selected human cancers merits further investigation.

**EXPERIMENTAL PROCEDURES**

**Quantitative PCR**

Cells were lysed with Trizol (Life Technologies, Invitrogen) and total RNA was prepared using the RNAeasy kit (QIAGEN) according to the manufacturer’s instructions. Typically, 0.5 μg of total RNA was reverse transcribed and the cDNA used for quantitative PCR analysis on an iCycler iQ5 Real-Time PCR detection system (BioRad) as per manufacturer’s instructions. In all cases, target gene expression was normalized to the expression of the housekeeping gene, B-actin. Relative gene expression was calculated using the standard 2-ΔΔCt method.

**Isolation and Culture of Human Blood Monocytes**

Mononuclear cells were isolated from blood obtained from healthy volunteers through the blood bank of Health Sciences Authority, Singapore, using Ficoll-Hypaque Plus (Amersham Biosciences) density gradient centrifugation. Monocyte isolation was performed using the CD14+ monocyte isolation kit from Miltenyi Biotec (Bergisch Gladbach) according to the manufacturer’s instruction. Isolated monocytes were cultured in IMDM medium (IMDM-modified HyClone medium containing HEPES and 4 mM L-glutamine; Thermo Scientific) supplemented with 5% (v/v) human serum and 100 U/ml penicillin-streptomycin, and used as indicated in the Results.

**RCC Patient Sample and Protocols**

Peripheral blood (10–15 ml) of 14 RCC patients with early or metastatic disease was obtained from the National University Hospital (NUH), National University Health System (NUHS), Singapore. Exclusion criteria at baseline included active infection, known immunocompromised states or other active immune conditions, and no chemotherapy or other immunomodulating treatments in the past 6 months. None of the patients had undergone any systemic anticancer therapy at the time of blood collection. Pathologically, the RCC cases
Figure 7. Increased IL1B Expression Correlates with Cancer Stage, Expression of Monocyte and Macrophage Markers and Protumor Genes in RCC Patients

(A) Bar graph shows the expression of IL1B gene across different tumor stages as revealed by meta-analysis of an ONCOMINE gene expression data set from RCC patients (n = 34), *p < 0.05 for comparisons indicated in the figure.

(B) Correlation analysis between IL1B gene expression and the expression of the indicated genes.

(C) Immunohistochemistry showing CD163+ macrophages (dark brown stained) in human RCC tumor tissue.

(legend continued on next page)
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consisted of 12 clear cell and 2 unclassified subtypes. Cancer staging for the 14 RCC cases were as follows: 11 stage IV, 2 stage III, and 1 stage II. Monocyte isolation from patient blood was done using the same method as described for monocytes from healthy volunteers. The culture conditions remained the same as described above. In addition, fresh RCC tumor tissues were also provided for 4 RCC cases (3 clear cell and 1 chromophobe subtype; cancer staging: 2 stage I, 1 stage II, 1 stage IV) kindly by the Tissue Repository, Department of Pathology, NUHS, Singapore. All study protocols were approved by the DSRB Ethics Committee (NHG, Singapore) and in accordance with the Helsinki Declaration of 2000. Informed consent was obtained from all human subjects included in this study.

Endothelial Tube Formation or Angiogenesis Assay
Angiogenesis assay on HUVEC cells was performed. In brief, HUVEC cells (2 × 10^5/well) were seeded on matrix gel in a 24-well plate and incubated with 500 μl of cell-free supernatant (20% v/v) of monocytes from healthy donors or RCC patients at 37°C for 6–8 hr. Endothelial tubule formation was accessed and photographed was taken at a magnification of 10× using an inverted microscope (Zeiss). Quantitative analysis of tube formation was done by counting the number of tubes in at least four different microscopic fields for each sample and represented as average number of tubes per field. Generation of cell-free monocyte supernatants used in the above assay is described in the Supplemental Experimental Procedures.

Tumor Cell Invasion Assay
Tumor cell invasion assay was performed using RCC cells on transwell inserts (8 μm) coated with matrix gel Geltrex, (Invitrogen Life Technologies). Inserts were then placed in a 24-well plate containing 500 μl cell-free supernatant (50% v/v) from monocytes or RCC monocytes and incubated at 37°C for 16 hr. After incubation, media was aspirated from the wells and inserts and noninvaded cells on the upper side of the membrane were removed with a cotton swab. Invaded cells attached to the bottom side of the membrane were fixed with cold methanol, air-dried, and stained with 0.1% (v/v) crystal violet solution. Inserts were washed and pictures were taken at a magnification of 10× using an inverted microscope (Zeiss). Stained cells were then lysed by 10% (v/v) acetic acid and the optical density was quantified in a plate reader at 595 nm (TECAN Infinite M200). Generation of cell-free monocyte supernatants used in the above assay is described in the Supplemental Experimental Procedures.

Statistical Analysis
Statistical significance was calculated by Student’s t test when comparing two groups or by one-way or two-way ANOVA when comparing three or more groups. A p value < 0.05 was considered as statistically significant.

Additional methods, including reagents, cell lines, mice, microarray and bioinformatics analysis, biochemical assays (immunoblotting, lumine assay, ELISA), and human RCC xenograft model are provided in the Supplemental Experimental Procedures.

ACCESSION NUMBERS
The transcriptome data reported in the paper have been deposited in the GEO database under accession number GSE38424.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.09.014.

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REFERENCES


(D) Correlation analysis between the gene expression of the macrophage marker CD163 and the indicated genes. For panels B&D: the number of patients, p values and r values are all indicated within the individual graphs.

(E) qPCR analysis of the indicated protumor genes in TAMs isolated from tumors of four RCC patients. Gene names are indicated on x axis. Monocytes (Mo) and monocyte-derived macrophages (MDMs) from healthy donors served as controls. Data are mean ± SEM (n = 4).

(F) Increased endothelial tube formation and (G) tumor cell invasion induced by the culture supernatants from TAMs as compared to those of MDMs (respective left panels). Abrogation of these events by α-VEGFR2 and MMP inhibitor is shown to demonstrate specificity (respective right panels). Left panels show TAMs from two patients, while one of these is shown in the right panel. Data are mean ± SEM; *p < 0.05 versus MDMs; # versus TAM-α-control/vehicle.

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Role of Monocytes and Macrophages in Human Cancer


Molecular Profiling Reveals a Tumor-Promoting Phenotype of Monocytes and Macrophages in Human Cancer Progression

Manesh Chittezhath, Manprit Kaur Dhillon, Jyue Yuan Lim, Damya Laoui, Irina N. Shalova, Yi Ling Teo, Jinmiao Chen, Revathy Kamaraj, Lata Raman, Josephine Lum, Thomas Paulraj Thamboo, Edmund Chiong, Francesca Zolezzi, Henry Yang, Jo A. Van Ginderachter, Michael Poidinger, Alvin S.C. Wong, and Subhra K. Biswas
SUPPLEMENTAL INFORMATION

Inventory of Supplementary Data

1. Supplemental Figures and Tables

**Figure S1.** Transcriptome analysis of blood monocytes from RCC patients. *Related to Figure 1.*

**Figure S2.** Monocytes co-cultured with RCC cell lines show an inflammatory and pro-tumor phenotype. *Related to Figure 3.*

**Figure S3.** Regulation of protumor genes in monocytes by IL-1. *Related to Figure 4.*

**Figure S4.** In vivo infiltration of TAMs and their expression of protumor genes in human RCC xenograft model. *Related to Figure 5.*

**Figure S5.** Blocking IL-1/IL-1RA pathway in vivo with IL-1RA modulates tumor progression in human A498 xenograft model. *Related to Figure 5.*

**Table S1.** List of differentially regulated genes in RCC-Mo as compared to Mo. *Related to Table 1.*

2. Supplemental Experimental Procedures

3. Supplemental References
Supplementary Figure S1: Transcriptome analysis of blood monocytes from RCC patients. Related to Figure 1

(A) A simplified flow diagram showing the layout of the experiment for the transcriptome analysis of blood monocytes from RCC patients (n=4) as compared to those from healthy donors (n=4). Further information on the experimental methodology and patients can be found in the Experimental Procedures section. (B) Pie-chart showing the quantitative distribution of unmodulated and modulated (DEGs) genes from the above transcriptome experiment. Heatmap represents total number of DEGs.
Supplementary Figure S2: Monocytes co-cultured with RCC cell lines show an inflammatory and protumor phenotype. Related to Figure 3

Normal monocytes (1.5x10^6/well) were plated in 12-well plates and co-cultured with RCC cell lines, RCC4 (A) or Caki2 (D) (2x10^5/well) seeded into the upper wells of the 0.4µm transwell inserts for 48h. After co-culture, inserts were removed, monocytes washed with PBS and RNA was extracted. Gene expression was detected by qPCR. Names on the x-axis indicate the genes assayed. (B) Enhanced endothelial tube formation and (C) tumor cell invasion induced by the cell-free supernatants of monocytes that had been co-cultured with RCC4 cells (Mo+RCC) as compared to supernatants from normal monocytes cultured alone (Mo). For these functional assays, monocytes were first co-cultured or not with RCC cells for 36h, then inserts were removed and monocytes incubated in fresh media for a further 12h. This culture supernatant was used for the assay. * p<0.02, versus Mo. Data shown in all panels are mean±SD from a representative experiment.
Supplementary Figure S3: Regulation of protumor genes in monocytes by IL-1. Related to Figure 4

(A) Elevated IL-1β in the plasma of RCC patients (n=11) as compared to those of healthy donors (n=6). Data are mean±SEM. (B) IL-1β induced the expression of protumor genes. Normal monocytes were treated with recombinant human IL-1β (20ng/ml) for 4h. Thereafter, monocytes were washed with PBS and RNA was extracted. Gene expression was detected by qPCR. Names on the x-axis indicate the names of the genes assayed. Data are mean±SEM. (C) Expression of the indicated protumor genes in RCC co-cultured monocytes (RCC+Mo) in the presence or absence of a neutralizing antibody against TNF (α-TNF) or its isotype control antibody (α-Con). Data are mean±SD (n=3-4). n.s., not significant.
Supplementary Figure S4: In vivo infiltration of TAMs and their expression of protumor genes in human RCC xenograft model. Related to Figure 5

Human RCC4 xenograft tumors were established in SCID mice as described in Supplemental Experimental Procedures and the tumors analyzed at day 20 following sacrifice. (A) Infiltration of TAMs is demonstrated by flow cytometric analysis of F4/80+CD11b+ cells and (B) immunohistochemistry staining for F4/80+ cells in tumor sections (left panel). Right panel shows immunohistochemistry staining for IL-1β in RCC4 tumor tissue. (C) Fate mapping of latex (lx) bead-labeled monocytes in the tumors at day 8 and 14. FACS plot showing frequency of monocytes and TAMs (out of Lx+CD11b+ cells) in the tumor. (D) Gene expression analysis of TAMs from RCC4 tumors as compared to peritoneal macrophages (PECs) from non-tumor animals. Data are mean±SD from a representative experiment. (E) Effect of clodronate-liposome treatment for monocyte-macrophage depletion on RCC4 tumor growth. Mice were treated with PBS-liposome (control) or clodronate-liposome as detailed in Supplemental Experimental Procedures. Data are mean±SEM and from one experiment with 4-5 mice per group. *p<0.05
Supplementary Figure S5. Blocking IL-1/IL-1RA pathway in vivo with IL-1RA modulates tumor progression in human A498 xenograft model. Related to Figure 5

(A) Schematic representation of the experimental plan for the in vivo IL-1RA treatment study in A498 xenograft model. Following injection of SCID mice with A498 cell line (Day 0), animals received 3 intratumoral injections (200µl) of IL-1RA (5µg/kg body weight) or PBS on 7th, 9th and 11th day. Tumor take was monitored every 2 days and analysis of TAMs was carried out on day 18-20. (B) Kinetics of tumor growth. *p<0.05 versus PBS-treated group. (C) Gene expression analysis of TAMs from PBS- or IL-1RA-treated tumor mice assessed by qPCR. (D) Angiogenesis and (E) tumor invasion assay performed with supernatants of TAMs from either treatment groups. The addition of α-VEGFR2 and MMP inhibitor (Inh) shows the involvement of these molecules in the respective assays (see Results). Data in all panels are mean±SEM and from a single experiment consisting of 5 mice per treatment group. (F) TAMs frequency in RCC xenografts in response to PBS versus IL-1RA treatments. Collective data from flow cytometric analysis of both RCC xenograft models, A498 and RCC4. Data are mean±SEM (n=5; RCC4: 3, A498: 2).
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines, reagents and cell culture

All murine cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen Life Technologies, NY) containing 4,500 mg/L D-glucose, L-glutamine without sodium pyruvate and sodium bicarbonate. Medium was supplemented with 10% FBS (HyClone, UT) and 100U/ml penicillin-streptomycin. HUVEC cells were cultured in EndoGRO medium containing low serum growth supplement (EndoGRO-LS, Millipore, MA). Human RCC cell lines and monocytes were cultured in complete IMDM medium (IMDM-modified HyClone medium containing HEPES and 4mM L-glutamine; Thermo Scientific, USA) supplemented with 5% (v/v) human serum and 100U/ml penicillin-streptomycin. Human umbilical vein endothelial cells (HUVEC), A498 and Caki-2 cells were purchased from American Type Culture Collection (ATCC), VA. Human RCC4 cells were a kind gift from Dr. M. Celeste Simon (Abramson Family Cancer Research Institute, University of Pennsylvania, US). The following reagents were purchased from the indicated sources: IKKγ NEMO binding domain (NBD) inhibitory peptide and MyD88 homodimerization inhibitory peptide set from Imgenex, CA; recombinant human interleukin-1 receptor antagonist (IL-1RA) and anti-human VEGFR2 antibody from R&D Systems, MN; MMP inhibitor GM6001, Merck Millipore, Germany; LEAF™ purified anti-human TNFα (clone MAB1) and anti-mouse CD11b PE-Cy7, Biolegends, CA; Novocastra™ lyophilized mouse monoclonal antibody CD163 (Leica Biosystems Newcastle Ltd, UK); Anti-mouse F4/80 (BM8) Alexa Flour 488, F4/80 APC and CD45 PE from eBioscience, CA; PBS-liposome and Clodronate-liposomes preparations were purchased from ClodLip B.V. (Amsterdam, Netherlands).

Mice

Severe combined immunodeficiency (SCID) mice were obtained from Biological Resource Center (BRC, A*STAR). NOD/SCID/Gamma (NSG) and Il1r1-/- mice (originally from The Jackson Laboratory, US) were obtained from mutant mouse collection core service of SglN. All animals were maintained in our animal facility. Mice were used between 6-8 weeks of age, in accordance with institutional guidelines proposed by the IACUC committee of the BRC, A*STAR, Singapore.
**Microarray and bioinformatics analysis**

Total RNA integrity was assessed by Agilent Bioanalyzer and the RNA Integrity Number (RIN) was calculated; all RNA samples had a RIN ≥ 7.3. Biotinylated cRNA was prepared starting from 50 ng of total RNA using the TargetAmp™ Nano-g™ Biotin-aRNA Labeling Kit for the Illumina® System (Epicentre). Labelled cRNAs were hybridized to the Illumina Human HT-12 v4 Beadchips for 17 hours at 58°C; the arrays were then washed and stained base on Illumina Wash Protocol and then scanned using BeadArray Scanner 500GX at BSF Microarray Facility. The images were analyzed using GenomeStudio Gene Expression v 1.8.0 according to the instructions provided by Illumina.

The intensity values of the array were extracted without background subtraction using BeadStudio. Intensities with detection p-value greater than 0.05 were replaced by the lowest positive intensity with detection p-value equal to or lower than 0.05. The resulting intensities were quantile normalized, and then subjected to an interquartile range filter of 0.40. Differentially expressed genes (DEGs) were determined from this subset with limma (Smyth, 2004), with a Benjamini-Hochberg false discovery rate of 0.05 and an absolute fold change of at least 2 (i.e. a log2 fold change of atleast 1). Multiscale bootstrap with default parameters was applied to the microarray data using the R package Pvclust (Suzuki and Shimodaira, 2006). Gene ontology and pathway analysis were carried out on the DEGs using DAVID (Huang da et al., 2009) and Ingenuity Pathway Analysis, respectively. Heatmaps were generated with MEV (Saeed et al., 2003). All bioinformatic analyses were enabled using Pipeline Pilot (www.accelrys.com). Transcriptome data has been deposited in the Gene Expression Omnibus (GEO) under the following reference number GSE38424.

**Immunoblotting**

Monocyte protein extracts were prepared and processed for immunoblot as described earlier (Biswas et al., 2006). The following antibodies were used for the immunoblots: anti-phospho-IκBα, (Cell Signalling Technology Inc, MA) and anti-β-actin from Santa Cruz Biotechnologies Inc, CA.
Luminex assay for cytokines, chemokines and angiogenic factors

Monocytes from healthy donors or RCC patients (2x10^4 cells) were plated in 100µL IMDM media in 96-well flat-bottomed culture plate, overnight at 37°C. The conditioned media were collected, centrifuged and cell-free culture supernatants used for luminex and other functional assays like angiogenesis and tumor cell invasion. Cytokines, chemokines and angiogenic factors were assessed in the cell-free monocyte supernatants using Bioplex kits (Bio-Rad, CA), according to the manufacturer's protocol. Data was collected and analysed using the luminex-based LiquiChip system (Qiagen, CA). ELISA for human IL-1β was done using reagents from BioLegend, CA, as per manufacturer’s instructions.

Immunostaining for confocal microscopy

Briefly, monocytes were seeded onto glass coverslips and treated as indicated in the Results. Thereafter, cells were washed in PBS, fixed in 4% Paraformaldehyde for 10mins, washed three times with PBS, followed by permeabilization with 0.02% Triton-X for 5mins and again washed in PBS containing 0.5% BSA. Cells were then incubated with p65 NF-κB antibody (Cell Signalling Technology Inc, MA) for 1hr at room temperature, washed with PBS three times and incubated with Alexa-flour 488-conjugated secondary antibody (Molecular Probes, Life Technologies, NY) for 1 hr at room temperature. Cells were again washed three times with PBS and mounted with Vecta-Shield mounting media containing DAPI. Samples were visualized and images captured using an LSM Zeiss confocal microscope.

Animal studies in human RCC xenograft model

Human RCC xenograft model and the isolation of tumor associated macrophages (TAM): Age- and sex-matched SCID mice (6-8 weeks old) were injected on the right flank with a total of 1-2 million RCC4 or A498 cells per mouse. Tumor growth was monitored and the diameter of the growing tumor was measured in millimeters every 2 days by using a calliper. TAMs were isolated about 3 weeks after tumor implantation. Briefly, solid tumors were disaggregated by stirring in PBS containing 0.125% (wt/vol) Trypsin (BioWhitaker Inc, Lonza, MD) for 40 minutes at 37°C, followed by filtering the tumor suspension through a 100µm cell strainer (BD Falcon™, Singapore) and collected in a 50ml falcon tube. Cells were
then centrifuged and washed twice with wash buffer (PBS containing 5% v/v FBS and 0.5mM EDTA). TAMs were subsequently isolated by adherence to plastic as described previously (Sica et al., 2000). Typically, disaggregated tumor cells (70x10^6) were seeded in 150mm Petridish (Nuncleon, Demark) in a final volume of 20ml incomplete medium and, after 1 hour of incubation, non-adherent cells were vigorously washed-off. The majority of the adherent cells constituted the TAMs as confirmed by morphological and functional characteristics. PEC isolation was done according to the protocol described earlier (Sica et al., 2000). In all cases, PEC and TAM isolation was done on the same day, under same conditions and from the same age- and sex-matched mice batch. Isolation of TAMs from human RCC tumor tissue was also done using the same method except for using 1mg/ml Collagenase A and 0.1mg/ml DNase I (Roche Diagnostics, Germany) instead of Trypsin for tumor disaggregation, as described above.

**Fate mapping experiments:** Experiments involving the in vivo labelling of inflammatory monocytes with fluorescence latex beads and their subsequent fate mapping in the RCC4 xenograft model was done as described earlier (Movahedi et al., 2010; Tacke et al., 2006). Briefly, blood monocytes were first depleted by i.v. injection of clodronate-loaded liposomes. After 18 hours, fluorescent latex beads (Lx) were injected i.v. to label newly emigrated bone marrow monocytes (all Ly6C^{hi}, i.e. ‘inflammatory’ monocytes) in the bloodstream. After 24 hours, SCID mice were injected with 2x10^6 RCC4 cells to establish the RCC xenograft. Tumors were harvested after 8 and 14 days of tumor growth and checked whether the labelled ‘inflammatory’ monocytes (Ly6C^{hi} F4/80^{lo}) differentiated into TAMs (Ly6C^{hi/lo} F4/80^{hi}).

**Clodronate experiment:** Systemic injection of liposome-encapsulated clodronate is a widely reported method for monocyte-macrophage depletion, as described earlier (Gazzaniga et al., 2007; Sunderkotter et al., 2004; Van Rooijen and Sanders, 1994). Age and sex-matched SCID mice (6-8 weeks old) were injected on the right flank with a total of 1x10^6 RCC4 cells per mouse. On the following day, mice were injected intraperitoneally (i.p) with either liposomes in PBS (control) or clodronate-liposomes suspension in PBS (200µL/mice). The injection was continued every three days for a period of two weeks. Tumor growth was monitored every 2 days and mice sacrificed after day 15 for further analysis.
**IL-1RA in vivo treatment:** Following injection of SCID mice with RCC4 cells (i.e. tumor transplantation; day 0) as described above, the animals received three 200µl intratumoral injections of IL-1RA (5µg/kg body weight) or PBS on day 7, 9 and 11, respectively. Tumor take was monitored every 2 days and analysis of TAMs was carried out on day 20-21 following sacrifice.

**Macrophage adoptive transfer experiment:** WT and *Il1r1*⁻/⁻ murine bone marrow derived macrophages (BMDMs) were differentiated by culturing bone marrow cells in 20% (v/v) L929-conditioned media for 7 days. Age- and sex-matched NSG mice (6-8 weeks old) were injected on the right flank with a mixture of 1x10⁶ RCC4 cells and 2.5x10⁶ WT or *Il1r1*⁺⁺ BMDMs in 100µL PBS (day 0). Thereafter, the animals received two further intra-tumoral injections of 2.5x10⁶ WT or *Il1r1*⁻/⁻ BMDMs (in 100µL PBS) on day 3 and 7, post tumor implantation. Tumor growth was monitored and mice sacrificed after day 10 for further analysis.

**In vivo monitoring of angiogenesis and MMP activity**

In vivo monitoring of angiogenesis and MMP activity was done using fluorescent imaging agents AngioSense750EX and MMPSense680 (Perkin Elmer, Inc., MA) respectively. RCC tumor bearing mice were administered with either AngioSense750EX or MMPSense680 via tail vein injection (150µL/mice). 24 hours later, imaging using an IVIS Bio-imager was performed to visualize in vivo tumor angiogenesis and MMP activity.

**Meta-analysis, correlation and gene set enrichment analysis (GSEA)**

For meta-analysis, a gene expression dataset by Yang et al (Yang et al., 2005) for human RCC tumor showing cancer staging was chosen from Oncomine (Rhodes et al., 2007). The dataset contained gene expression from tumor tissues of 34 RCC patients (papillary subtype) consisting of the following cancer staging: Stage I-17; Stage II-2; Stage III 8; Stage IV: 7. Other clinic-pathological features are reported in the original paper (Yang et al., 2005). For each of the datasets, expression data files were normalized by Robust Microarray Averaging (RMA) and quantile normalization (Irizarry et al., 2003). The normalized expression values were log2 transformed before correlation calculation. Pearson’s method
was employed to calculate correlation coefficients and P values. Data manipulation and correlation analysis were performed using Pipeline Pilot (www.accelrys.com).

GSEA was performed on the Yang et al. Oncomine dataset using a panel of pro-tumor and monocyte-macrophage specific marker genes as defined in the Results section pertaining to meta-analysis, using the BROAD Institute software (Mootha et al., 2003; Subramanian et al., 2005).

SUPPLEMENTAL REFERENCES


Microenvironments Contain Functionally Distinct Subsets of Macrophages Derived from Ly6C(high) Monocytes. Cancer Res, 70, 5728-5739


