Title:

The coordinated actions of TIM-3 on cancer and myeloid cells in the regulation of tumorigenicity and clinical prognosis in clear cell renal cell carcinomas

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Running title: Tumor and myeloid cell TIM-3 in RCC

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Conflict of interest

All authors declare that there is no conflict of interests.
Abstract

Clear cell renal cell carcinoma (ccRCC) is one of most common cancers in urogenital organs. Although recent experimental and clinical studies showed the immunogenic properties of ccRCC as illustrated by the clinical sensitivities to various immunotherapies, the detailed immunoregulatory machineries governing the tumorigenicity of human ccRCC remain largely obscure. In this study we demonstrated the clinical significance and functional relevance of T-cell immunoglobulin and mucin domain-containing molecule-3 (TIM-3) expressed on tumor cells and myeloid cells in patients with ccRCC. TIM-3 expression was detected on cancer cells and CD204+ tumor-associated macrophages (TAM), and higher expression level of TIM-3 was positively correlated with shorter progression-free survival (PFS) in patients with ccRCC. We found that TIM-3 expression was detected on a large number of tumors, and there was significant correlation between an increased number of TAMs and high expression level of TIM-3 in patients with ccRCC. Furthermore, TIM-3 rendered RCC cells with the ability to induce resistance to sunitinib and mTOR inhibitors, the standard regimen for patients with ccRCC, as well as stem cell activities. TIM-3 expression was induced on CD14+ monocytes upon long-term stimulation with RCC cells, and TIM-3-expressing myeloid cells play a critical role in augmenting tumorigenic activities of
TIM-3-negative RCC cells. More importantly, treatment with anti-TIM-3 mAb suppressed its tumorigenic effects in \textit{in vitro} and \textit{in vivo} settings. These findings indicate the coordinated action of TIM-3 in cancer cells and in myeloid cells regulates the tumorigenicity of human RCC.

\textbf{Introduction:}

Kidney cancer is the fifteenth most common cancer in the world and the global incidence rate is 4 cases per 100,000 persons. The incidence rate is significantly higher in the North American, Australian and European regions (1). Clear cell renal cell carcinoma (ccRCC) is the most common histologic type in kidney cancers. Overall median progression free survival (PFS) is 12 month in ccRCC and 17 months in non-clear cell RCCs (2). Although nearly 80\% of ccRCC cases are considered to be cured by resection, the median overall survival (OS) for patients with metastatic RCC is less than 3 years (3). Therefore, suitable markers for predicting outcomes are necessary to guide clinical therapeutic management.

T-cell immunoglobulin (Ig) and mucin domain-containing molecule-3 (TIM-3), also known as hepatitis A virus cellular receptor 2, is widely expressed on immune cells such as monocytes/macrophages, dendritic cells, natural killer cells and T cells (4,5).
Signaling via TIM-3 is generally involved in the regulation of immune responses via negatively regulating T-helper type 1-cell viability and interferon secretion (3, 6-8). Recently TIM-3 expression has also been found on melanoma, liver cancer and lung cancer cells. In these cancers, higher TIM-3 expression correlated with poor clinical prognosis (9-11).

In the present study, we demonstrated that, in patients with ccRCC, TIM-3 is frequently expressed on tumor tissues and higher TIM-3 expression levels are significantly associated with shorter PFS. Furthermore, TIM-3 on tumor cells and myeloid cells coordinately contributed to the tumorigenic activities of RCCs. These findings suggest that TIM-3 may serve as a useful biomarker for predicting prognosis and a potential therapeutic target for improving therapeutic responses in patients with ccRCC.

**Materials and Methods**

*Tissue samples*

In total, 91 paraffin-embedded tissue samples (not tissue array), derived from patients diagnosed with ccRCC, who had undergone curative surgery between 1998 and 2008 at University Hospital of Occupational and Environmental Health, and whose samples
were pathologically confirmed as ccRCC, were selected for this study (12). Cases with massive necrosis were not selected. Data for PFS and cancer-specific OS were obtained from the medical records of these patients. All samples were obtained with written informed consent from patients in accordance with protocols approved by each university review board. Tissue samples were fixed in 10% neutral buffered formalin and were embedded in paraffin per routine methods.

Immunohistochemistry

A goat-polyclonal antibody against TIM-3 was purchased from LifeSpan BioSciences (Seattle, WA). Mouse monoclonal anti-CD204 antibody (SRA-E5; Transgenic, Kumamoto, Japan) was used for detecting TAMs (13). For TIM-3 immunostaining, Can Get Signal (Toyobo, Tokyo, Japan) was used as an antibody diluent. Secondary antibodies were purchased from Nichirei (Tokyo, Japan) and reactions were visualized using a diaminobenzidine substrate system (Nichirei). Two investigators, who were blinded to any information about the samples, evaluated the infiltration of CD204⁺ cells and TIM-3⁺ cancer cells. CD204⁺ cells in six randomly selected areas were counted by these two investigators, and the averages of the results were used.

Cell cultures

CD14⁺ monocytes were isolated from peripheral blood mononuclear cells (PBMC)
obtained from healthy volunteers using CD14-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The blood mononuclear cells from patients or healthy donors were obtained with written informed consent from healthy volunteers in accordance with protocols approved by each university review board. Prior to co-culture experiments, monocytes were labeled with PKH26 fluorescence (Sigma). Three human RCC cell lines (ACHN, 786-O, Caki-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). CD14⁺ monocytes and human RCC cells were cocultured in serum-free media supplemented with human EGF and β-FGF on the ultra-low attachment plates. In this condition, the tumor cell growth kinetics was low while the cell viability was preserved, and the ratio of tumor cells and monocytes remained constant during the experimental periods. Mycoplasma tests were performed using a polymerase chain reaction (PCR) detection kit (Takara Bio Inc., Otsu, Japan).

**Construction of human TIM-3 gene plasmid**

The human TIM-3 mRNA (Accession number: NM_032782.4) was isolated from TIM-3⁺ tumor-infiltrating monocytes using primers for full-length amplification (FW 5'-TCAGATCTCGAGCTCATGTTTTCACATCTTCCCTTTGACTG -3'
RV 5'- CGGTGGATCCCCGTGGCATTGCAAAGCGACAACC -3’), and inserted into pPRIME-dsRed plasmid. The plasmids were transfected into human ccRCC cells with
Lipofectamine 3000 for 48 hrs according to the manufacturer’s instruction (Life Technologies). TIM-3-expressing cells were selected with G418 (400 μg/mL) for 14-21 days. After selection, the mRNA levels of human TIM-3 was approximately 250 times higher in the transfected cells (Figure 3C). The empty pPRIME-dsRed plasmid was used as a control.

Apoptosis assay

ACHN cells were transfected with human TIM-3 or control gene-introduced plasmids, and cells were then treated with sunitinib (50nM) or rapamycin (50nM) for 48 hrs. Caspase-3 activity in the tumor cell lysates was quantified with a colorimetric assay kit according to the manufacturer’s instructions (Invitrogen). In all indicated experiments, monoclonal antibodies (mAb) recognizing human TIM-3 (Clone F38-2E2: 10 μg/mL) were employed as described previously (14).

Sphere formation assay

ACHN cells were transfected with human TIM-3 or control genes, or stimulated with TIM-3⁺ or TIM-3⁻ macrophages for 7 days. The cells were then cultured in ultra-low attachment culture dishes (Corning) in DMEM/F-12 serum-free medium supplemented with 20ng/mL epithelial growth factor and 10ng/mL basic-fibroblast growth factor-2 (PeproTech). Cell digestions with trypsin and cell passages were performed every 3
days, and the size and number of spheres was counted under the microscope.

Quantitative RT-PCR

mRNA was isolated from TIM-3⁺ or TIM-3⁻ ACHN cells, and the genes associated with cancer stem cell properties (Twist1, Snail, Pou5F) were quantified by real-time PCR using SYBR Green Gene Expression Assays (Applied Biosystems).

In vivo tumor initiation assay

NSG mice were purchased from Jackson Laboratories. All experiments were conducted under a protocol approved by the animal care committees of Hokkaido University (Sapporo, Japan). For analysis of ACHN tumor-initiating activities, ACHN cells were injected subcutaneously into NSG mice (ranged from $1 \times 10^4$ and $1 \times 10^5$ cells/mouse), and tumor growth was measured once a week through the entire experimental period.

Flow cytometry

For in vitro analysis, untreated ACHN cells or those stimulated with PKH26-labeled TIM-3⁺ or TIM-3⁻ monocytes were stained with anti-BrdU mAbs or anti-TIM-3 mAbs (Biolegend). BrdU labelling was performed using the FITC BrdU Flow Kit according to the manufacturer’s instruction (BD-Biosciences). For in vivo assays, EpCAM⁺ tumor
cells or CD68+ tumor-infiltrating macrophages were analyzed by flow cytometry using mAbs specifically recognizing human EpCAM, CD68, CD163 and MHC class II (Biolegend). Cell acquisition and analysis were performed with a FACS-Canto (Becton-Dickinson).

**Myeloid cell-mediated in vivo tumorigenesis**

For *in vivo* tumor-myeloid cell interaction analysis, ACHN cells were injected subcutaneously into NSG mice (1 x 10^5/mouse) in conjunction with intravenous administration of CD14+ macrophages obtained from cancer patients (1 x 10^6/mouse). The blood mononuclear cells from patients were obtained with written informed consent in accordance with protocols approved by each university review board. Recombinant M-CSF proteins (500μg/mL) were also administered intraperitoneally to support the survival of transferred human monocytes *in vivo*, as shown by previous report (15). Additional mice were treated with intraperitoneal injections of control Ig or the anti-TIM-3 mAb. Tumor growth was measured on the indicated days, and the number of human macrophages in the tumors was evaluated for each mouse.

**Statistics**

Statistical analyses were carried out using JMP10 (SAS Institute, Chicago, IL) and StatMate III (ATOMS, Tokyo). The Kruskal-Wallis test, Kaplan-Meier method, and
COX hazard test were used to analyze the clinical course associations. The Student’s t test was used for 2-group comparisons in in vitro and in vivo studies, and data are expressed as means ± SD. A value of $P < 0.05$ was considered statistically significant.

**Results:**

**Higher expression of TIM-3 in cancer cells is associated with shorter PFS**

Immunostaining of ccRCC tissues revealed TIM-3 expression on both immune and cancer cells (Figure 1A). TIM-3 was mainly expressed on cell surface membranes in cancer cells, and TIM-3-positive cancer cells were detected in 63 of 91 cases. In non-cancer tissues, TIM-3 was detected in a subset of immune cells (Figure 1A) and renal tubules (Figure 1B). As shown in figure 1A, the staining intensity of TIM-3 varied from case to case and therefore the staining intensities for cancer cells were classified into three groups (score 0, negative or weak; score 1, intermediate; score 2, strong). The correlations between TIM-3 expression and clinicopathologic factors were then analyzed. As a result of this analysis, we found that the presence of TIM-3-positive cancer cells was preferentially detected in cases with higher clinical T stage and nuclear grade (Table 1), and was significantly associated with shorter PFS, but not with OS (Figure 1C, 1D). The patients with strong (score 2) TIM-3 expression in cancer cells
showed the shortest PFS (Figure 1C). Statistical analysis was also performed in patients with lower clinical stage (T1) and lower nuclear grade (grade 1 and 2), and the presence of TIM-3-positive cancer cells was significantly associated with shorter PFS in both groups (Figure 1E).

**Increased TAM infiltration in cases with higher TIM-3 expression**

Because we had previously shown that CD204$^+$ tumor-associated macrophages (TAM) are intimately involved in cancer cell activation (16), we hypothesized that TAM-derived factors induce TIM-3 expression in cancer cells. To test this hypothesis, serial sections were stained using anti-CD204 antibody, and the correlation of CD204 staining with TIM-3 expression was analyzed. The number of CD204$^+$ TAMs was increased in TIM-3-expressing cases (Figure 2A), and the number of TAMs was significantly correlated with the staining intensity of TIM-3 (Figure 2B). The number of TAMs was associated with a worse OS; however, the correlation with PFS was not statistically significant upon analysis (Figure 2C, Table 2). Although a quantitative evaluation of TIM-3$^+$ TAMs was too difficult to perform in tissue sections of patients with high expression level (score 3) of TIM-3, we observed some TAMs also expressed TIM-3 in tissue sections of no or low expression level (score 0 and 1) of TIM-3 (Figure 2D).
TIM-3⁺ TAMs were counted and the average percentage of TIM-3⁺ TAMs among CD204⁺ TAMs was found to be 15.7%. Then patients were divided into two groups and statistical analysis demonstrated that patients with higher number of CD204⁺ TAMs and higher percentage of TIM-3⁺ TAMs had shorter PFS (Figure 2E).

**TIM-3 on RCC cells contributes to anti-cancer drug resistance and tumorigenic activities**

To determine the effect of tumor-derived TIM-3 in this model, we transfected human TIM-3 gene into cells of the human RCC line, ACHN, since no TIM-3 expression was detected on any of the human RCC cell lines used in our study (data not shown). Although TIM-3 did not significantly influence the growth of ACHN cells *in vitro* (data not shown), TIM-3-expressing ACHN cells manifested increased resistance to sunitinib and the mTOR inhibitor, rapamycin (Figure 3A). Sunitinib and mTOR inhibitors have been approved as standard anticancer regimens against human RCC (17, 18). Indeed, TIM-3⁺ ACHN cells have superior self-renewal activities compared to control ACHN cells, as shown by increased sphere-forming activities (Figure 3B). We next examined whether TIM-3 confers cancer-stem cell characteristics to RCC cells. The mRNA levels of genes which have been reported to be associated with renal cancer-stem cell
properties, such as Twist1, Snail and Pau5F1 (19), were compatible between TIM-3\(^+\)
and TM-3\(^-\) ACHN cells (Figure 3D). However, tumor formation was observed when
TIM-3\(^+\) ACHN cells were subcutaneously inoculated into NSG mice at the smaller
numbers (10\(^4\) cells per mouse), whereas TIM-3\(^-\) ACHN tumor cells needed larger
numbers (10\(^5\) cells per mouse) to initiate tumor formation (Figure 3E). These results
suggest that TIM-3 promotes tumorigenicity of RCC cells by inducing cancer-stem cell
properties.

\textit{TIM-3}\(^+\) myeloid cells contribute to anti-cancer drug resistance and tumorigenic
activities of RCC cells

Recent studies have revealed that TIM-3 on myeloid cells mediates
immunoregulatory functions, leading to impaired antitumor immunosurveillance (20).
Since we show that TIM-3 was also expressed on TAMs in ccRCC in the present study
(Figure 2C), we next investigated the contribution of TIM-3\(^+\) TAMs in co-culture
experiments and in a mouse model. ACHN cells and PKH26-labelled monocytes were
directly co-cultured for 2 weeks and TIM-3 expression was examined by FACS (Figure
4A). After co-culture TIM-3 expression was observed on CD14\(^+\) monocytes, although
TIM-3 was not detected on the human RCC cell lines examined in this study (ACHN,
Caki-1, MAMIYA, 786-0) (Figure 4B, and data not shown). Next we isolated TIM-3+ and TIM-3- monocytes from tumor-monocyte mixtures to evaluate the significance of TIM-3 in cell-cell interactions. We investigated whether TIM-3 on monocytes influences the self-renewal properties of RCC cells, and found that the sphere-forming capacity of ACHN cells was augmented by co-culture with TIM-3+ monocytes (Figure 3B). Next, TIM-3+ or TIM-3- monocytes were co-cultured with ACHN cells for 72 hrs. TIM-3+ monocytes rendered ACHN cells with the ability to promote proliferation and resist apoptotic cell death mediated by sunitinib and rapamycin compared to ACHN cells alone or those co-cultured cells with TIM-3- monocytes (Figure 4C and 4D). Moreover, the treatment with anti-TIM-3 mAb resulted in theduced proliferative and chemoresistant phenotypes of ACHN cells specifically mediated by TIM-3+ monocytes (Figure 4C and 4D). Together, these findings highlight the significant involvement of TIM-3 expression on myeloid cells in human RCC tumorigenicity.

**TIM-3 serves as a therapeutic target for suppressing RCC growth in a myeloid cell-dependent manner**

Finally, we evaluated the impact of TIM-3 on *in vivo* tumorigenicity. For this purpose, ACHN cells were injected subcutaneously into NSG mice in the presence of
control Ig or anti-human TIM-3 mAb (F38-2E2), and in vivo tumor formation was evaluated at the indicated times. To examine the involvement of human myeloid cells in the regulation of TIM-3-mediated antitumor responses, NSG mice were treated with human M-CSF (500μg/kg per day) twice per week to improve human myeloid cell reconstitution. During this procedure, adoptive transfer of cancer patients-derived monocytes (1 x 10⁶/mouse) was performed via intravenous injection (Figure 5A). Although co-transfer of monocytes had little impact on the tumor growth compared to non-transfer controls, tumor growth was significantly suppressed by treatment with anti-TIM-3 mAb in the monocytes-transferred groups (Figure 5B). We detected the expression of human TIM-3 in tumor-infiltrating CD68⁺ myeloid cells, but not in RCC cells (Figure 5C, D). TIM-3⁺ cells are observed in tumor tissues in the monocytes-transferred groups, but not in the control groups (Figure 5D). The numbers of TIM-3⁺ myeloid cells were not changed by treatment with anti-TIM-3 mAb (Figure 5D). Interestingly, treatment with anti-TIM-3 mAb decreased the infiltration of CD163-expressing M2 macrophages in the tumor tissues of the monocyte-transferred group, suggesting that TIM-3 may support the differentiation of pro-tumor myeloid cells in the tumor microenvironments (Figure 5E). Overall, these findings provide clear evidence that TIM-3-mediated regulation of cancer cell-myeloid cell interactions serves as a
critical pathway supporting RCC tumorigenicity.

**Discussion:**

Accumulating evidence has revealed that TIM-3 expressed on tumor cells and tumor-associated immune cells has diverse tumorigenic activities mediated through recognition of multiple ligands such as galectin-9, high-mobility group box 1 (HMGB1), and phosphatidylserine (7, 21, 22). Galectin-9 recognizes TIM-3 expressed on exhausted CD8⁺ T lymphocytes and triggers apoptosis by antagonizing human leukocyte antigen B (HLA-B)-associated transcript 3 (Bat3)-mediated survival signals. HMGB1 binds TIM-3 on dendritic cells and suppresses innate immune signals mediated by nucleic acid-pattern-recognition receptors (PRR) (7, 23). Furthermore, TIM-3 is detected on various tumor cells and tumor-initiating cells, and contributes to tumor-initiating and tumor-promoting activities (24, 25). Thus, TIM-3 has pleiotropic functions that influence multiple immunologic and biologic properties of various types of cells.

Importantly, TIM-3 expressed on tumor cells and tumor-infiltrating immune cells may serve as a key sentinel linking impaired tumor immunosurveillance with amplified tumorigenicity in the tumor microenvironment. Although the ligand recognized
specifically by tumor cells including RCC cells remains to be identified, recent studies
suggest that TIM-3 activates the NF-κB pathway via phosphorylation of cytoplasmic
tyrosine kinase motifs in murine B16 melanoma cells (26). Moreover, NF-κB functions
as a critical hub governing multiple modes of oncogenic processes, including anti-
apoptotic pathways, inflammatory carcinogenesis and the acquisition of cancer-stem
cell properties (27, 28). Thus, it is of interest to evaluate whether the TIM-3-NF-κB axis
may be a key pathway that coordinately stimulates intrinsic oncogenic signals and
immune-mediated carcinogenic pathways, such as shown in our current study in which
TIM-3 mediates tumor- and myeloid cell-mediated regulation of RCC tumorigenesis. It
is noteworthy that the same antibody exerts antitumor effects by coordinately targeting
TIM-3 on cancer cells and myeloid cells, raising the possibility that inhibition of TIM-3
may create a cancer microenvironment that antagonizes tumorigenicity and stimulates
endogenous cancer immunosurveillance.

In addition, we found higher TIM-3 expression on RCC tumors closely correlated
with increased TAMs infiltration, raising the possibility that TIM-3 expression on tumor
cells might be regulated by cell-cell interactions with TAMs. We previously
demonstrated that macrophages induce signal transducer and activator of transcription 3
(Stat3) signal activation in RCC cells by direct cell-cell contact. Furthermore, the
engagement of membrane-type M-CSF expressed on cancer cells with CD115 on macrophages contributes to this cell-cell interaction (16). Moreover, TAMs support tumorigenic activities and trigger resistance to anti-cancer drugs by inducing cancer-stem cell properties in tumor cells (29-31). However, we did not observe myeloid cell-mediated induction of TIM-3 on RCC cell lines in vitro, although RCC tumors express TIM-3 in the vicinity of macrophages in clinical samples. A previous report suggested that various mediators preferentially produced from the tumor microenvironment, such as IL10, VEGF-A and arginase-I, were responsible for inducing TIM-3 expression on myeloid cells (7) though the downstream signals critical for transcriptional and translational regulation of TIM-3 remain obscure. Thus, it is likely that various mediators derived from complex networks of the tumor microenvironment are required; those from in vitro cultured myeloid cells may be insufficient for inducing TIM-3 expression on RCC cells. It will therefore be important to elaborate the detailed mechanisms whereby tumor-myeloid cell interactions impact TIM-3 expression on a variety of tumor cells including RCC cells.

The adaptive transfer of human CD14+ monocytes had little impact on in vivo tumor growth, whereas CD14+ monocytes increased tumorigenic activities of in vitro-cultured RCC cell lines. Since complexity of the tumor microenvironment serves as a critical
factor to determine the direction of myeloid cell-mediated regulation of tumorigenicity, it is likely that the tumor microenvironment influences the differentiation of transferred human monocytes towards subsets with pro-tumor or antitumor properties. We demonstrated that treatment with anti-TIM-3 mAb suppressed tumor growth when human monocytes were adoptively transferred, and this observation might indicate that blocking of TIM-3 would induce antitumor properties in transferred human monocytes. Thus, TIM-3 may be a critical regulator of myeloid cell plasticity in the tumor microenvironment.

In conclusion, we demonstrate herein the significant involvement of TIM-3 on cancer cells and myeloid cells in the regulation of RCC tumorigenesis. High expression of TIM-3 on cancer tissues with increased infiltration of TAMs serves as useful biomarker to predict poor clinical prognosis for patients with ccRCC. TIM-3 on cancer cells is involved in the resistance to sunitinib and small molecule inhibitors of mTOR. TIM-3 on myeloid cells interacts with cancer cells to increase tumorigenicity and trigger resistance to anticancer drugs. The essential function of TIM-3 on cancer cells and myeloid cells underscores the inhibition of TIM-3 as a suitable strategy with which to rewire tumorigenic microenvironments and enhance the clinical efficacy of standard anticancer regimens in patients with RCC.
Acknowledgments

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Conflict of interest

The authors have no financial competing interests to declare.

References:


expressed in melanoma cells and is upregulated in TGF-beta stimulated mast cells.


14. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-


Figure legends:

**Figure 1** Immunohistochemical determination of TIM-3 expression in ccRCC. (A) The intensity of TIM-3 immunohistochemical staining was classified into three groups (Score 0, 1, and 2). TIM-3⁺ immune cells are indicated as arrowhead. (B) TIM-3 expression was weakly detected in renal tubules in non-cancer kidney tissue. (C) The Kaplan-Meier analysis of PFS and (D) OS were performed to investigate the correlations between TIM-3 expression and clinical prognosis. (E) Kaplan-Meier analysis was performed in patients with lower stage (T1) or lower nuclear grade (grade 1,2).

**Figure 2** Immunohistochemical determination of CD204 expression. (A) Immunostaining of CD204 was performed to detect TAMs. (B) The correlation between the number of TAMs and TIM-3 expression was analyzed using the Kruskal-Wallis test. (C) Kaplan-Meier analyses of PFS and OS were performed. (D) TIM-3 expression was observed on TAMs. (E) Kaplan-Meier analysis of PFS was performed to investigate the correlations between TIM-3⁺ TAMs and clinical prognosis.

**Figure 3** Increased tumorigenic activities of TIM-3-expressing RCC cells. (A) ACHN
cells transfected with human TIM-3 or control gene plasmid were treated with 50 nM of sunitinib or rapamycin for 6 hours and then assayed for cell death by measuring active caspase-3. Similar results were observed in three experiments. (B) Bulk ACHN cells, TIM-3⁺ or TIM-3⁻ ACHN cells, or those stimulated with TIM-3⁺ or TIM-3⁻ CD14⁺ monocytes isolated from patient’s PBMCs were then cultured with three passages in ultra-low attachment plates. The size (left) and numbers (right) of formed spheres generated per 100,000 cells were determined. (C) mRNA expression of TIM-3, Twist1, Snail, and Pou5F in TIM-3⁺ or TIM-3⁻ ACHN cells were quantified by RT-PCR. (D) TIM-3⁺ or TIM-3⁻ ACHN cells (10⁴-10⁵ per mouse) were inoculated subcutaneously into NSG mice and tumor growth was measured. Similar results were obtained in three independent experiments. *p<0.05

**Figure 4** Increase tumorigenic activities of RCC cells stimulated with TIM-3-expressing myeloid cells. (A) Schema of the experimental design. (B) PKH26-labelled CD14⁺ monocytes isolated from PBMCs of patients (5 x10⁵) were co-cultured with unlabeled ACHN cells (1 x10⁵) for 14 days. TIM-3 expression on monocytes (PKH26⁺) or ACHN cells (PKH26⁻) was evaluated by flow cytometry. (C) ACHN cells were cultured with TIM-3⁺ or TIM-3⁻ monocytes, and treated with anti-TIM-3 mAb or
control Ig (10μg/mL) for 7 days. Cell proliferation was then analyzed by quantifying BrdU uptake with flow cytometry. (D) ACHN cells cocultured with CD14+ monocytes as described above were treated with 50 nM of sunitinib or rapamycin for 6 hours and then assayed for cell death by measuring active caspase-3. Similar results were obtained in three independent experiments. *p<0.05

**Figure 5** The significance of TIM-3-expressing myeloid cells in an animal model. (A) Schema of the *in vivo* experiment. (B) NSG mice (n=5 per group) were intraperitoneally treated with M-CSF at 500ug/kg twice per week. CD14+ monocytes isolated from patients’ PBMCs were transferred intravenously into NSG mice along with subcutaneous injections of ACHN tumor cells. The tumor volume in each mouse treated as described above was measured 4 weeks after tumor challenge. *p<0.05. (C) The frequencies (%) of CD68+ populations within tumor. (D) The frequencies (%) of TIM-3+ populations within CD68+ macrophages. (E) TIM-3+ cells were observed by immunohistochemistry. (F) The frequencies (%) of CD68+ total and CD68−CD163+ M2 macrophages infiltrating tumor tissues were evaluated by flow cytometry. Similar results were obtained in four independent experiments. *p<0.05
Table 1: TIM-3 expression in cancer cells, and clinicopathologic parameters

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<td>&gt;0.05</td>
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<tr>
<td>G3,G4</td>
<td>17</td>
<td>1</td>
<td>10</td>
<td>6</td>
<td></td>
<td>454</td>
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</table>

Correlation with TIM-3 was tested using a cumulative Chi-square test.
Correlation with the number of CD204-positive cells was tested using Mann-Whitney U-test.
Table 2: Univariate cox regression analysis of PFS and OS

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th>PFS</th>
<th></th>
<th></th>
<th></th>
<th>OS</th>
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<tbody>
<tr>
<td></td>
<td>No. of patients</td>
<td>HR</td>
<td>95% CI</td>
<td>P value</td>
<td>HR</td>
<td>95% CI</td>
<td>P value</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age, &lt;60 v ≧60</td>
<td>38/53</td>
<td>1.3</td>
<td>0.6 to 2.9</td>
<td>0.48</td>
<td>0.9</td>
<td>0.4 to 2.7</td>
<td>0.99</td>
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</tr>
<tr>
<td>Gender, M v F</td>
<td>59/32</td>
<td>1.3</td>
<td>0.6 to 3.0</td>
<td>0.49</td>
<td>2.3</td>
<td>0.2 to 0.7</td>
<td>0.28</td>
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<tr>
<td>Stage, T1 v T2+3+4</td>
<td>42/49</td>
<td>3.5</td>
<td>1.6 to 7.8</td>
<td><strong>0.001</strong></td>
<td>2</td>
<td>0.8 to 5.4</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear grade, G1+2 v G3+4</td>
<td>74/17</td>
<td>5.5</td>
<td>2.5 to 11.8</td>
<td><strong>&lt;0.001</strong></td>
<td>3.9</td>
<td>1.5 to 9.9</td>
<td><strong>0.007</strong></td>
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<tr>
<td>TIM-3, score 0 v score 1+2</td>
<td>27/64</td>
<td>6.1</td>
<td>1.8 to 37.7</td>
<td><strong>0.002</strong></td>
<td>3.7</td>
<td>0.7 to 68</td>
<td>0.12</td>
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<tr>
<td>CD204, &lt;300 v ≧300</td>
<td>45/46</td>
<td>1.6</td>
<td>0.8 to 3.6</td>
<td>0.18</td>
<td>3.1</td>
<td>1.1 to 11.0</td>
<td><strong>0.029</strong></td>
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</tbody>
</table>

HR, hazard ratio; CI, confidential interval
Figure 1
Figure 2

A
Pt. 8 (TIM-3 score 0)
Pt. 4 (TIM-3 score 2)

CD204

CD204

50 μm

B
The number of CD204 (+) macrophages/mm²

P = 0.047

TIM-3 → Score 0 Score 1 Score 2

C

PFS

CD204 low (n=45)

CD204 high (n=46)

Logrank: P>0.05
Wilcoxon: P>0.05

OS

CD204 low (n=45)

CD204 high (n=46)

Logrank: P=0.024
Wilcoxon: P=0.028

D

TIM-3

CD204

50 μm

E

TIM-3 score 1.2

PFS

CD204 high (TIM-3+ TAM <15%) (n=59)

CD204 low (n=79)

CD204 high (TIM-3+ TAM ≥15%) (n=20)

Log-rank: P=0.025
Wilcoxon: P=0.006

Months

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Figure 3

A

Active caspase-3 (ng/ml)

DMSO
Sunitinib
Rapamycin

TIM3-
TIM3+

B

ACHN
TIM-3 (-) ACHN
ACHN TIM-3 (-) monocyte

TIM-3 (+) ACHN
ACHN TIM-3 (+) monocyte

C

TIM-3
Twist1
Snail
Pou5F1

Fold induction (β-actin)

control TIM3 control TIM3 control TIM3 control TIM3

0
1
2
3
4
5
6

10^4 cells

10^5 cells

Sphere forming cells (10^3)

D

10% FBS ACHN TIM3(-) ACHN TIM3(+) ACHN TIM3(-) monocyte + ACHN TIM3(+) monocyte + ACHN

TIM-3 (-) ACHN TIM-3 (+) ACHN

tumor area (mm^2)

Week)

0 1 2 3 4 5 6

0 10 20 30 40 50

10um
Figure 4

A) Monocytes → PKH26-labeled monocytes → ACHN cells (PKH26-) → Monocytes (PKH26+) → Evaluate TIM-3 expression by flow cytometry

B) 1st week 2nd week

Tumor

<table>
<thead>
<tr>
<th></th>
<th>0.0</th>
<th>0.1</th>
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<tbody>
<tr>
<td>0.0</td>
<td>99.8</td>
<td>0.1</td>
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</table>

Tumor + Monocytes

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<th>0.2</th>
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</thead>
<tbody>
<tr>
<td>0.1</td>
<td>82.1</td>
<td>17.6</td>
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</tbody>
</table>

C) TIM3

<table>
<thead>
<tr>
<th></th>
<th>1.1</th>
<th>2.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>80.2</td>
<td>16.3</td>
</tr>
</tbody>
</table>

D) BrdU+ ACHN cells (%)

- Control
- αTIM-3

* * *

Active caspase-3 (ng/ml)

- DMSO
- Rapamycin
- Sunitinib

* * *
Figure 5
Cancer Immunology Research

The coordinated actions of TIM-3 on cancer and myeloid cells in the regulation of tumorigenicity and clinical prognosis in clear cell renal cell carcinomas

Yoshihiro Komohara, Tomoko Morita, Dorcas A Annan, et al.

Cancer Immunol Res  Published OnlineFirst March 17, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/2326-6066.CIR-14-0156

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