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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Abstract

Clear cell renal cell carcinoma (ccRCC) is one of the most common cancers in urogenital organs. Although recent experimental and clinical studies have shown 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 TIM-3 on tumor cells and myeloid 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 vitro and 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.

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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 months 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 this 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. Recombinant M-CSF proteins (500 ng/mL) were also administered i.p. to support the survival of transferred human monocytes in vivo, as shown by a previous report (15). Additional

CTCATGTTTTCACATCTTCCCTTTGACTG-3

Cell cultures
were used. Counting by these two investigators, and the averages of the results expressing cells were selected with G418 (400 μg/mL) for 48 hours. 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 used 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 20 ng/mL epithelial growth factor and 10 ng/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 were counted under the microscope.

In vivo tumor initiation assay
NSG mice were purchased from The Jackson Laboratory. 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 s.c. into NSG mice (ranged from 1 × 10^4 to 1 × 10^5 cells/mouse), and tumor growth was measured once a week during the entire experimental period.

Flow cytometry
For in vitro analysis, untreated ACHN cells or those stimulated with PKH26-labeled TIM-3+ or TIM-3− macrophages were stained with anti-BrdUrd mAbs or anti-CD14 mAbs (BioLegend). BrdUrd labeling was performed using the FITC BrdUrd Flow Kit according to the manufacturer’s instructions (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 s.c. into NSG mice (1 × 10^7/mouse) in conjunction with i.v. administration of CD14+ macrophages obtained from cancer patients (1 × 10^5/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 (500 μg/mL) were also administered i.p. to support the survival of transferred human monocytes in vivo, as shown by a previous report (15). Additional

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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, and Pou5F) were quantified by real-time PCR using SYBR Green Gene Expression Assays (Applied Biosystems).

Immunohistochemistry
A goat-polyclonal antibody against TIM-3 was purchased from LifeSpan BioSciences. Mouse monoclonal anti-CD204 antibody (SRA-E5; Transgenic) was used for detecting tumor-associated macrophages (TAM; ref. 13). For TIM-3 immunostaining, Get Signal (Toyobo) was used as an antibody diluent. Secondary antibodies were purchased from Nichirei, 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 in accordance with protocols approved by each university review board. Prior to coculture experiments, monocytes were labeled with PKH26 fluorescence (Sigma). Three human RCC cell lines (ACHN, 786-O, and Caki-1) were obtained from the American Type Culture Collection (ATCC). 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. Myeloid cell–macrophage interactions (Fw, 5'-TCAAGATCTCGACGCTCATGTTCATCACTCCCCCTTTGACGCTG-3'; Rv, 5'-CGGTGGA-TCCCGTGCCATGCGAAGGCGACAACCC-3'), and inserted into pPRIME-dsRed plasmid. The plasmids were transfected into human ccRCC cells with Lipofectamine 3000 for 48 hours according to the manufacturer’s instructions (Life Technologies). TIM-3–expressing cells were selected with G418 (400 μg/mL) for 14 to 21 days. After selection, the mRNA levels of human TIM-3 were approximately 250 times higher in the transfected cells (Fig. 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 (50 ng/mL) or rapamycin (50 ng/mL) for 48 hours. 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 used as described previously (14).

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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'-TCAGATCTCGACGCTCATGTTCATCACTCCCCCTTTGACGCTG-3'; Rv, 5'-CGGTGGA-TCCCGTGCCATGCGAAGGCGACAACCC-3'), and inserted into pPRIME-dsRed plasmid. The plasmids were transfected into human ccRCC cells with Lipofectamine 3000 for 48 hours according to the manufacturer’s instructions (Life Technologies). TIM-3–expressing cells were selected with G418 (400 μg/mL) for 14 to 21 days. After selection, the mRNA levels of human TIM-3 were approximately 250 times higher in the transfected cells (Fig. 3C). The empty pPRIME-dsRed plasmid was used as a control.
mice were treated with i.p. 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.

Statistical analysis
Statistical analyses were carried out using JMP10 (SAS Institute) and StatMate III (ATOMS). The Kruskal–Wallis test, the Kaplan–Meier method, and the Cox hazard test were used to analyze the clinical course associations. The Student t test was used for two-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 (Fig. 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 noncancer tissues, TIM-3 was detected in a subset of immune cells (Fig. 1A) and renal tubules (Fig. 1B). As shown in Fig. 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 (Fig. 1C and D). The patients with strong (score 2) TIM-3 expression in cancer cells showed the shortest PFS (Fig. 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 (Fig. 1E).

Increased TAM infiltration in cases with higher TIM-3 expression
Because we had previously shown that CD204+ TAMs 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 (Fig. 2A), and the number of TAMs was significantly correlated with the staining intensity of TIM-3 (Fig. 2B). The number of TAMs was associated with a worse OS; however, the correlation with PFS was not statistically significant upon analysis (Fig. 2C and Table 2). Although a quantitative evaluation of TIM-3+ TAMs was too difficult to...
TIM-3 on RCC cells contributes to anticancer 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, because 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 (Fig. 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 with control ACHN cells, as shown by increased sphere-forming activities (Fig. 3B). We next examined whether TIM-3 confers cancer-stem cell characteristics to RCC cells. The mRNA levels of genes that 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 TIM-3− ACHN cells (Fig. 3D). However, tumor formation was observed when TIM-3+ ACHN cells were s.c. inoculated into NSG mice at the smaller numbers (10^5 cells per mouse), whereas TIM-3− ACHN tumor cells needed larger numbers (10^6 cells per mouse) to initiate tumor formation (Fig. 3E). These results suggest that TIM-3 promotes tumorigenicity of RCC cells by inducing cancer-stem cell properties.

Table 1. TIM-3 expression in cancer cells and clinicopathologic parameters

<table>
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<tr>
<th>Age, y</th>
<th>Score 0</th>
<th>Score 1</th>
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<td>9</td>
<td>24</td>
<td>5</td>
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<td>392</td>
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<td>28</td>
<td>7</td>
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</tr>
<tr>
<td>M</td>
<td>17</td>
<td>37</td>
<td>5</td>
<td>&gt;0.05</td>
<td>399</td>
<td>&gt;0.05</td>
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<tr>
<td>F</td>
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<td>15</td>
<td>7</td>
<td></td>
<td>364</td>
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<td></td>
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<tr>
<td>T1</td>
<td>20</td>
<td>19</td>
<td>3</td>
<td>&lt;0.001</td>
<td>389</td>
<td>&gt;0.05</td>
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<tr>
<td>T2−T4</td>
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<td>33</td>
<td>9</td>
<td></td>
<td>387</td>
<td></td>
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<tr>
<td>Nuclear grade</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G1, G2</td>
<td>42</td>
<td>42</td>
<td>6</td>
<td>&lt;0.001</td>
<td>373</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>G3, G4</td>
<td>17</td>
<td>10</td>
<td>6</td>
<td></td>
<td>454</td>
<td></td>
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</table>

NOTE: Correlation with TIM-3 was tested using a cumulative χ² test. Correlation with the number of CD204+ cells was tested using the Mann-Whitney U test. Underline, statistically significant.

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, the Kaplan-Meier analyses of PFS and OS were performed. D, TIM-3 expression was observed on TAMs. E, the Kaplan-Meier analysis of PFS was performed to investigate the correlations between TIM-3+ TAMs and clinical prognosis.
TIM-3+ myeloid cells contribute to anticancer 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). Because we show that TIM-3 was also expressed on TAMs in ccRCC in this study (Fig. 2C), we next investigated the contribution of TIM-3+ TAMs in coculture experiments and in a mouse model. ACHN cells and PKH26-labeled monocytes were directly cocultured for 2 weeks, and TIM-3 expression was examined by FACS (Fig. 4A). After coculture, TIM-3 expression was observed on CD14+ monocytes, although TIM-3 was not detected on the

Table 2. Univariate Cox regression analysis of PFS and OS

<table>
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<th>PFS</th>
<th>OS</th>
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<td></td>
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<tr>
<td>Age, &lt;60 vs. 60</td>
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<td>1.3 (0.6–2.9) 0.48 0.9 (0.4–2.7) 0.99</td>
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<tr>
<td>Gender, M vs. F</td>
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<td>1.3 (0.6–3.0) 0.49 2.3 (0.2–0.7) 0.28</td>
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<tr>
<td>Stage, T1 vs. T2+4</td>
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<td>3.5 (1.6–7.8) 0.001 2 (0.8–5.4) 0.16</td>
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<tr>
<td>Nuclear grade, G1+2 vs. G3+4</td>
<td>74/77</td>
<td>5.5 (2.5–11.8) 0.001 3.9 (1.5–9.9) 0.007</td>
</tr>
<tr>
<td>TIM-3, score 0 vs. score 1+2</td>
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<td>6.1 (1.8–37.7) 0.002 3.7 (0.7–68) 0.12</td>
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<td>CD204, &lt;300 vs. 300</td>
<td>45/46</td>
<td>1.6 (0.8–3.6) 0.18 3.1 (1.1–11.0) 0.029</td>
</tr>
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</table>

Abbreviations: CI, confidence interval; HR, hazard ratio. Underline, statistically significant.

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 nmol/L 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 patients’ 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, Twist, Snail, and Pou5F1 in TIM-3+ or TIM-3− ACHN cells were quantified by RT-PCR. D, TIM-3+ or TIM-3− ACHN cells (10^4−10^5 per mouse) were inoculated s.c. into NSG mice, and tumor growth was measured. Similar results were obtained in three independent experiments. *, P < 0.05. ns, not statistically significant.
human RCC cell lines examined in this study (ACHN, Caki-1, MAMiYA, and 786-0; Fig. 4B; and data not shown). Next, we isolated TIM-3⁺ and TIM-3⁻/C0 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 coculture with TIM-3⁺ monocytes (Fig. 3B). Next, TIM-3⁺ or TIM-3⁻/C0 monocytes were cocultured with ACHN cells for 72 hours. TIM-3⁺ monocytes rendered ACHN cells with the ability to promote proliferation and resist apoptotic cell death mediated by sunitinib and rapamycin compared with ACHN cells alone or those cocultured with TIM-3⁻/C0 monocytes (Fig. 4C and D). Moreover, the treatment with anti–TIM-3 mAb resulted in the educated proliferative and chemoresistant phenotypes of ACHN cells specifically mediated by TIM-3⁺ monocytes (Fig. 4C and D). Together, these findings highlight the significant involvement of TIM-3 expression on myeloid cells in human RCC tumorigenicity.

**Figure 4.**
Increased tumorigenic activities of RCC cells stimulated with TIM-3-expressing myeloid cells. A, schema of the experimental design. B, PKH26-labeled CD14⁺ monocytes isolated from PBMCs of patients (5 × 10⁵) were cocultured with unlabeled ACHN cells (1 × 10⁵) 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 (10 μg/mL) for 7 days. Cell proliferation was then analyzed by quantifying BrdUrd uptake with flow cytometry. D, ACHN cells cocultured with CD14⁺ monocytes as described above were treated with 50 nmol/L 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. ns, not statistically significant.

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 s.c. 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 (500 μg/kg/d) twice per week to improve human myeloid cell reconstitution. During this procedure, adoptive transfer of human-derived monocytes (1 × 10⁵/mouse) was performed via i.v. injection (Fig. 5A). Although cotransfer of monocytes had little impact on the tumor growth compared with nontransfer controls, tumor growth was significantly suppressed by treatment with anti–TIM-3 mAb in the monocyte-transferred groups (Fig. 5B). We detected the expression of human TIM-3 in tumor-infiltrating CD68⁺ myeloid cells, but not in RCC cells (Fig. 5C and D). TIM-3⁺ cells are observed in tumor tissues in the monocyte-transferred groups, but not in the...
control groups (Fig. 5D). The numbers of TIM-3+ myeloid cells were not changed by treatment with anti–TIM-3 mAb (Fig. 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 protumor myeloid cells in the tumor microenvironments (Fig. 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 (6, 7, 21). 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; refs. 7, 22). Furthermore, TIM-3 is detected on various tumor cells and tumor-initiating cells and contributes to tumor-initiating and tumor-promoting activities (23, 24). 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 (25). Moreover, NF-κB functions as a critical hub governing multiple modes of oncogenic processes, including antiapoptotic pathways, inflammatory...
carcinogenesis, and the acquisition of cancer-stem cell properties (26, 27). 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 this 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 have 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 anticancer drugs by inducing cancer-stem cell properties in tumor cells (28–30). 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 affect TIM-3 expression on a variety of tumor cells, including RCC cells.

The adaptive transfer of human CD14+ monocytes had little impact on in vitro tumor growth, whereas CD14+ monocytes increased tumorigenic activities of in vitro–cultured RCC cell lines. Because 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 toward subsets with protumor 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 a 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tumor and Myeloid Cell TIM-3 in RCC


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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