Different immunological effects of the molecular targeted agents sunitinib, everolimus and temsirolimus in patients with renal cell carcinoma

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Abstract. Treatment with molecular targeted agents together with immune checkpoint inhibitors will most likely improve the efficacy of current cancer immunotherapy. Because molecular targeted agents not only directly affect cancer cells, but also influence immune cells and modulate the tumor microenvironment, a better understanding of the overall immunological effects of these drugs will contribute to the rational design of combination therapies. Therefore, this study performed extensive immune monitoring of patients' peripheral blood mononuclear cells (PBMCs) to investigate the immunological effects of the molecular targeted agents sunitinib, everolimus and temsirolimus, which have been widely used for the treatment of renal cell carcinoma (RCC). Immunophenotyping and functional analysis of PBMCs revealed that these molecular targeted agents exerted different immunological effects on patients with RCC. Sunitinib decreased the percentage of early-stage myeloid-derived suppressor cells (eMDSCs) and increased natural killer cells, but did not affect the phenotypes and effector functions of $CD4^+$ or $CD8^+$ T cells. Everolimus decreased effector regulatory T cells, but also decreased IL-2-producing $CD4^+$ T cells and increased dysfunctional $CD8^+$ T cells. Conversely, temsirolimus decreased programmed cell death protein 1^+CD8^+ T cells and eMDSCs, but increased interferon-γ and tumor necrosis factor-α double producers at the same time as decreasing dysfunctional $CD8^+$ T cells, albeit not significantly. In conclusion, although everolimus and temsirolimus are mTOR inhibitors, their effects on overall T-cell functions are very different. Therefore, although it may increase the risk of immune-related toxicity, temsirolimus is expected to offer the best outcome when combined with other immunomodulators for the development of cancer immunotherapy.

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Key words: renal cell carcinoma, sunitinib, everolimus, temsirolimus, mTOR inhibitor, immune response

Introduction

Renal cell carcinoma (RCC) is a genitourinary cancer with a high mortality rate (1,2). Major subtypes of RCC include clear cell RCC (ccRCC), papillary RCC and chromophobe RCC, of which ccRCC is the most common and accounts for most cancer-related deaths (3).

The standard of care for localized RCC is surgical excision; however, it has been reported that overall distant recurrence rates at 5 years after surgical resection are 27.6 and 64% for localized and locally advanced (nodal) disease, respectively (4). Approximately one-third of patients already have metastatic disease at diagnosis (5). For the treatment of metastatic or recurrent RCC, cytokines such as interferon (IFN)-α and high-dose IL-2 are the standard of care before the introduction of sunitinib (6). Tyrosine kinase inhibitors, including sunitinib (7), sorafenib (8), pazopanib (9) and axitinib (10), which inhibit hypoxia-inducible factor and vascular endothelial growth factor (VEGF) signaling, are the mainstay of treatment for advanced RCC in the front-line setting in addition to other targeting therapies, such as the anti-VEGF monoclonal antibody bevacizumab (11), and the mTOR inhibitors everolimus (12) and temsirolimus (13). Recently, multi-kinase inhibitors, including cabozantinib (14) and lenvatinib (15), have also been approved for the treatment of metastatic RCC (mRCC).

At present, immune checkpoint inhibitors are the standard first- and second-line treatments for RCC. The anti-programmed cell death protein 1 (PD-1) antibody nivolumab has been approved for the treatment of patients whose previous therapy has failed (16); treatment with nivolumab together with the anti-cytotoxic T-lymphocyte-associated protein 4 antibody ipilimumab has been approved as first-line treatment (17). Furthermore, pembrolizumab (anti-PD-1) plus axitinib has been shown to be superior to sunitinib for the first-line treatment of mRCC regardless of risk groups with an acceptable safety profile (18). These results resulted in a change to the National Comprehensive Cancer Network and European Urological Association guidelines (19,20). However, not all patients benefit, and response rates of 25% with nivolumab alone (16) and 42% with the combination therapy (17) have been documented. In addition, many immune-related adverse events have been reported, and no predictive biomarker is available to select which patients will benefit from which treatment. Therefore, although these checkpoint inhibitors are promising, there is still an urgent need to improve the treatment of RCC.

Combinations of molecular targeted agents with immune checkpoint inhibitors are now beginning to be extensively studied (21). Because molecular targeted agents not only impact directly on cancer cells but also affect immune cells and modulate the tumor microenvironment (22,23), a better understanding of the immunological properties of these drugs will contribute to the rational design of combination therapies. This study performed extensive immune monitoring of patients' peripheral blood mononuclear cells (PBMCs) to investigate the immunological effects of the molecular targeted agents sunitinib, everolimus and temsirolimus.

Materials and methods

Patients. This clinical study analyzed the immunological impact of molecular targeted agents in patients with RCC and was conducted at The University of Tokyo Hospital. The research protocol was approved by the Ethical Committee of The University of Tokyo (approval no. 3652) and written informed consent was obtained from each patient before they entered the study. All procedures in the present study were performed according to the ethical standards of the institutions and were in conformity with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Blood was collected before treatment started and after 4 weeks of treatment. Between June 2012 and November 2015, 31 patients (seven favorable, 20 intermediate and four poor risk patients, according to the Memorial Sloan Kettering Cancer Center risk criteria) were enrolled to the present study (24); 11, 12 and 8 patients received sunitinib, everolimus and temsirolimus, respectively, according to the then current guidelines for RCC treatment (Table I).

PBMC isolation and flow cytometry. Peripheral blood samples were collected twice, just before treatment and after 4 weeks of treatment. PBMCs were isolated by density gradient centrifugation at 1,100 x g for 20 min at room temperature using Lymphoprep™ (cat. no. 1114547; Alere Technologies AS), and cryopreserved in Bambanker™ freezing medium (cat. no. CS-02-001; Nippon Genetics Co., Ltd.). Cryopreserved PBMCs were thawed in RPMI-1640 (cat. no. 189-02025; Wako Pure Chemical Industries, Ltd.) supplemented with 50 IU/ml Benzonase® Nuclease (cat. no. E1014; Sigma-Aldrich; Merck KGaA). Cells ($2x10^5$) were blocked with 10 μl Clear Back (cat. no. MTG-001; MBL International Co.) for 5 min at room temperature and then stained with 100 μl phosphate-buffered saline containing 1% FBS (cat. no. 17012; Sigma-Aldrich; Merck KGaA) and 0.1% sodium azide (cat. no. 195-11092; Wako Pure Chemical Industries, Ltd.) with antibodies (1:100 dilution) against human leukocyte antigen (HLA)-DR (cat. no. 347367; BD Biosciences), tumor necrosis factor (TNF)-α (cat. no. 557996; BD Biosciences), CD8 (cat. no. 6603861; Beckman Coulter, Inc.), CD28 (cat. no. 6607111; Beckman Coulter, Inc.), 7-AAD Viability Dye (cat. no. A07704; Beckman Coulter, Inc.), CD3 (cat. no. A07746; Beckman Coulter, Inc.), CD19 (cat. no. A07769; Beckman Coulter, Inc.), CD56 (cat. no. A07788; Beckman Coulter, Inc.), NKG2D (cat. no. A08934; Beckman Coulter, Inc.), CD8 (cat. no. B08467; Beckman Coulter, Inc.), CD45RA (cat. no. IM2711U; Beckman Coulter, Inc.), CD3 (cat. no. 300328; BioLegend, Inc.), CD4 (cat. no. 300512; BioLegend, Inc.), CD4 (cat. no. 300514; BioLegend, Inc.), CD4 (cat. no. 300521; BioLegend, Inc.), CD4 (cat. no. 300538; BioLegend, Inc.), CD8 (cat. no. 300926; BioLegend, Inc.), CD11b (cat. no. 301325; BioLegend, Inc.), CD14 (cat. no. 301828; BioLegend, Inc.), CD16 (cat. no. 302006; BioLegend, Inc.), CD20 (cat. no. 302304; BioLegend, Inc.), CD25 (cat. no. 302606; BioLegend, Inc.), CD28 (cat. no. 302906; BioLegend, Inc.), CD33 (cat. no. 303408; BioLegend, Inc.), CD45 (cat. no. 304012; BioLegend, Inc.), CD45RA (cat. no. 304112; BioLegend, Inc.), CD95 (cat. no. 305624; BioLegend, Inc.), CD56 (cat. no. 318304; BioLegend, Inc.), forkhead box P3 (FOXP3; cat. no. 320212; BioLegend, Inc.), CD57 (cat. no. 322312; BioLegend, Inc.), CD15 (cat. no. 323020; BioLegend, Inc.), PD-1 (cat. no. 329919; BioLegend, Inc.), DNAX accessory molecule 1 (DNAM1; cat. no. 338306; BioLegend, Inc.), Ki67 (cat. no. 350514; BioLegend, Inc.), killer cell lectin-like receptor subfamily G member 1 (KLRG1; cat. no. 368605; BioLegend, Inc.), IL-2 (cat. no. 500306; BioLegend, Inc.), IFN-γ (cat. no. 502522; BioLegend, Inc.), CD19 (cat. no. 11-0199-42; eBioscience; Thermo Fisher Scientific, Inc.), CCR7 (cat. no. FAB197P; R&D Systems, Inc.), lymphocyte activation gene 3 protein (LAG-3; cat. no. FAB2319P; R&D Systems, Inc.) and T cell immunoglobulin and mucin protein 3 (TIM-3; cat. no. FAB2365G; R&D Systems, Inc.). Mouse IgG1, κ (cat. no. 400114; BioLegend, Inc.), Mouse IgG1, κ (cat. no. 400122; BioLegend, Inc.), Mouse IgG1 (cat. no. 400134; BioLegend, Inc.), Mouse IgG1, κ (cat. no. 400134; BioLegend, Inc.), Mouse IgG1, κ (cat. no. 400158; BioLegend, Inc.), Mouse IgG2a, κ (cat. no. 400212; BioLegend, Inc.), Mouse IgG2a, κ (cat. no. 400222; BioLegend, Inc.), Mouse IgM, κ (cat. no. 401609; BioLegend, Inc.), Rat IgG2A (cat. no. IC006G; R&D Systems, Inc.), Mouse IgG2a (cat. no. A12689; Beckman Coulter, Inc.), and Goat IgG (cat. no. IC108P; R&D Systems, Inc.) were used as isotype controls. Dead cells were excluded by staining with Zombie Yellow™ Fixable Viability kit (cat. no. 423104; BioLegend, Inc.) or Fixable Viability Dye eFluor™ 780 (cat. no. 65-0865-18; eBioscience; Thermo Fisher Scientific, Inc.).

Table I. Demographic and clinical characteristics of the patients.

Variables	Sunitinib (n=11)	Everolimus (n=12)	Temsirolimus (n=8)
Age (years)	65 (20-83)	69 (53-84)	70 (62-85)
Sex			
Male	10 (91%)	5 (42%)	5 (63%)
Female	1 (9%)	7 (58%)	3 (38%)
Histology			
Clear cell renal cell carcinoma	8 (73%)	10 (83%)	3 (38%)
Papillary cell renal cell carcinoma	2 (18%)	1 (8%)	2 (25%)
Chromophobe renal cell carcinoma	0 (0%)	0 (0%)	1 (13%)
Other types	1 (9%)	1 (8%)	2 (25%)
Karnofsky performance status			
≥80	8 (73%)	10 (83%)	5 (63%)
<80	3 (27%)	2 (17%)	3 (38%)
MSKCC risk criteria			
Favorable	2 (18%)	4 (33%)	1 (13%)
Intermediate	9 (82%)	7 (58%)	4 (50%)
Poor	0 (0%)	1 (8%)	3 (38%)
Prior systemic treatments			
0	9 (82%)	0 (0%)	5 (63%)
1	2 (18%)	1 (8%)	1 (13%)
≥2	0 (0%)	11 (92%)	2 (25%)
Median duration of treatment (days)	91	161	43
Best overall response			
CR	0 (0%)	0 (0%)	1 (13%)
PR	4 (36%)	1 (8%)	1 (13%)
SD	4 (36%)	6 (50%)	3 (38%)
PD	3 (27%)	5 (42%)	3 (38%)

CR, complete response; MSKCC, Memorial Sloan Kettering Cancer Center; PD, progressive disease; PR, partial response; SD, stable disease.

For intracellular cytokine staining, cells were stimulated with 10 ng/ml PMA (cat. no. P1585; Sigma-Aldrich; Merck KGaA) and 1 μg/ml ionomycin (cat. no. I0634; Sigma-Aldrich; Merck KGaA) in the presence of 10 μg/ml brefeldin A (cat. no. B7651; Sigma-Aldrich; Merck KGaA) at 37˚C for 4 h. Cytokine-producing cells were then evaluated by intracellular cytokine staining, which was conducted according to the manufacturer's instructions using IntraPrep Permeabilizaton Reagent (cat. no. A07803; Beckman Coulter, Inc.). Cells were cultured at 37˚C for 30 min with 80 μM 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG; cat. no. 23002-v; Peptide Institute, Inc.) in glucose-free RPMI-1640 containing 10% FBS in order to analyze the uptake of the glucose analog. To analyze mitochondria, cells were cultured with MitoTracker Green (MTG; cat. no. M7514; Invitrogen; Thermo Fisher Scientific, Inc.) or tetramethylrhodamine, ethyl ester (TMRE; cat. no. 87917; Abcam) for 30 min at 37˚C. Stained cells were analyzed on a Gallios flow cytometer (Beckman Coulter, Inc.) and data processed using Kaluza software (version 2.1; Beckman Coulter, Inc.) and FlowJo (version 7.6.5; FlowJo, LLC).

Statistical analysis. Comparison of results was performed with the Wilcoxon signed-rank test using GraphPad Prism 5 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of molecular targeted agents on the composition of the PBMC population. The effects of sunitinib, everolimus or temsirolimus on the composition of PBMCs were examined by flow cytometry using samples from patients with RCC before and 4 weeks after treatment initiation. The frequencies of $CD3^+$ T cells, $CD19^+$ B cells, $CD14^+$ monocytes and $CD56^+CD3^-$ natural killer (NK) cells were assessed in each patient (Figs. 1A-D and S1). The frequencies of T cells, B cell and monocytes were either increased or decreased by sunitinib; the individual differences and variations were such that no significant differences emerged when all patients were grouped together. Conversely, $CD56^+CD3^-$ NK cells were consistently increased from 27.8±11.9 to 36.0±14.5% ($P=0.001$) following treatment with sunitinib (Figs. 1D and S2). In patients who received everolimus or temsirolimus, no significant

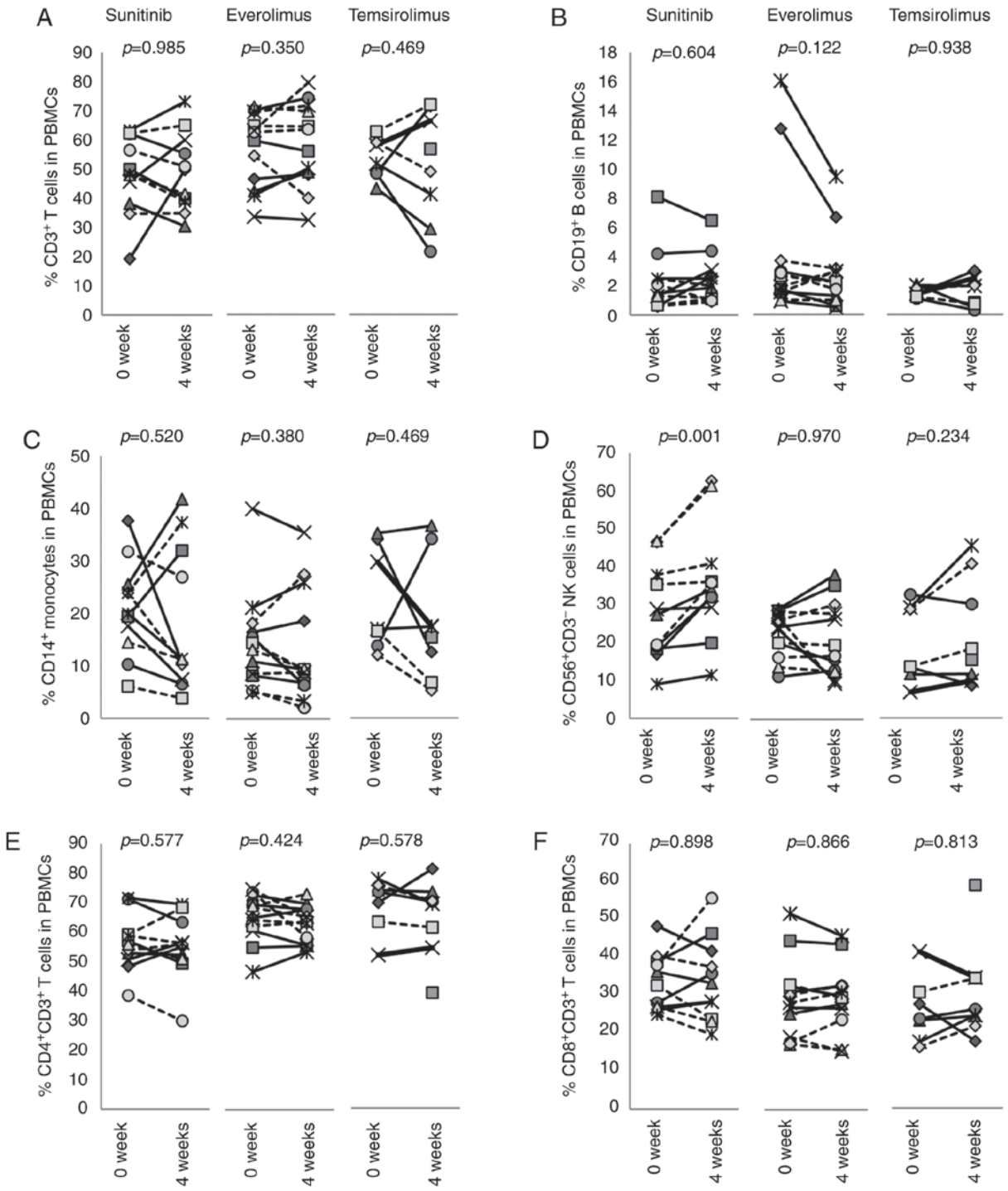

Figure 1. Effects of treatment of patients with RCC with molecular targeted agents on the distribution of immune cells in PBMCs. The effects of treatment with sunitinib, everolimus or temsirolimus on PBMCs were examined by flow cytometry. Percentages of (A) $CD3^+$ T cells, (B) $CD19^+$ B cells, (C) $CD14^+$ monocytes, (D) $CD56^+CD3^-$ NK cells, (E) $CD4^+CD3^+$ T cells and (F) $CD8^+CD3^+$ T cells in PBMCs before treatment (0 week) and after 4 weeks of treatment in each individual case are shown. NK, natural killer; PBMCs, peripheral blood mononuclear cells.

differences in the frequencies of $CD3^+$ T cells, $CD19^+$ B cells, $CD14^+$ monocytes or $CD56^+CD3^-$ NK cells were seen after 4 weeks of treatment in all patients as a group. The frequencies of $CD4^+$ and $CD8^+$ T cells were not changed by any of these molecular targeted agents (Fig. 1E and F).

Effects of molecular targeted agents on T cell phenotypes. To determine the phenotypes of T cells, PBMCs were stained for CD3, CD4, CD8, CCR7 and CD45RA (Figs. 2 and S3). Naïve, effector memory, central memory and terminal effector memory T cells were defined as $CCR7^+CD45RA^+$, $CCR7^+CD45RA^-$, $CCR7^-CD45RA^-$ and $CCR7^-CD45RA^+$, respectively. The results revealed that none of the three drugs affected these memory markers.

This study also examined the expression of inhibitory receptors and activation markers on $CD4^+$ and $CD8^+$ T cells (Figs. 3, 4 and S4). Sunitinib reduced the percentage of $LAG\text{-}3^+CD4^+$ T cells (Fig. 3E, P=0.044), whereas the

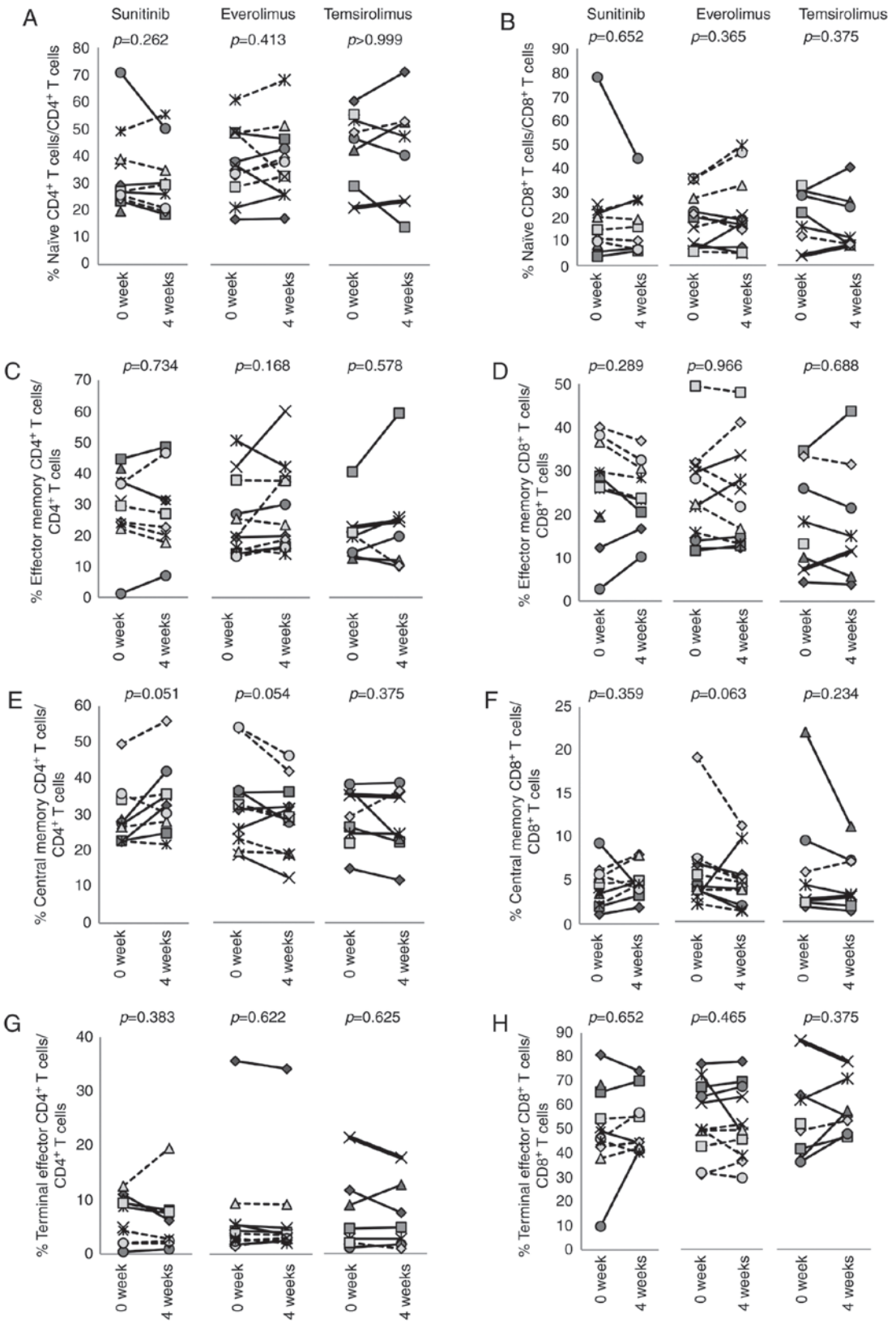

Figure 2. Effects of treatment with molecular targeted agents on T-cell phenotypes. The percentages of (A) naïve $CD4^+$, (B) naïve $CD8^+$, (C) effector memory $CD4^+$, (D) effector memory $CD8^+$, (E) central memory $CD4^+$, (F) central memory $CD8^+$, (G) terminal effector memory $CD4^+$ and (H) terminal effector memory $CD8^+$ T cells were determined in patients' peripheral blood mononuclear cells. Percentages of T cells with each phenotype in patients treated with sunitinib, everolimus or temsirolimus before treatment (0 week) and after 4 weeks of treatment in each individual case are shown.

expression of other molecules, including PD-1 and TIM-3 was not changed (Fig. 3A and C). Conversely, 4 weeks of treatment with everolimus increased the expression of TIM-3 on $CD4^+$ (P=0.031) and $CD8^+$ T cells (P=0.033; Fig. 3C and D). In addition, everolimus increased $NKG2D^+CD4^+$ T cells (P=0.035) and decreased $DNAM1^+CD8^+$ (P=0.021)

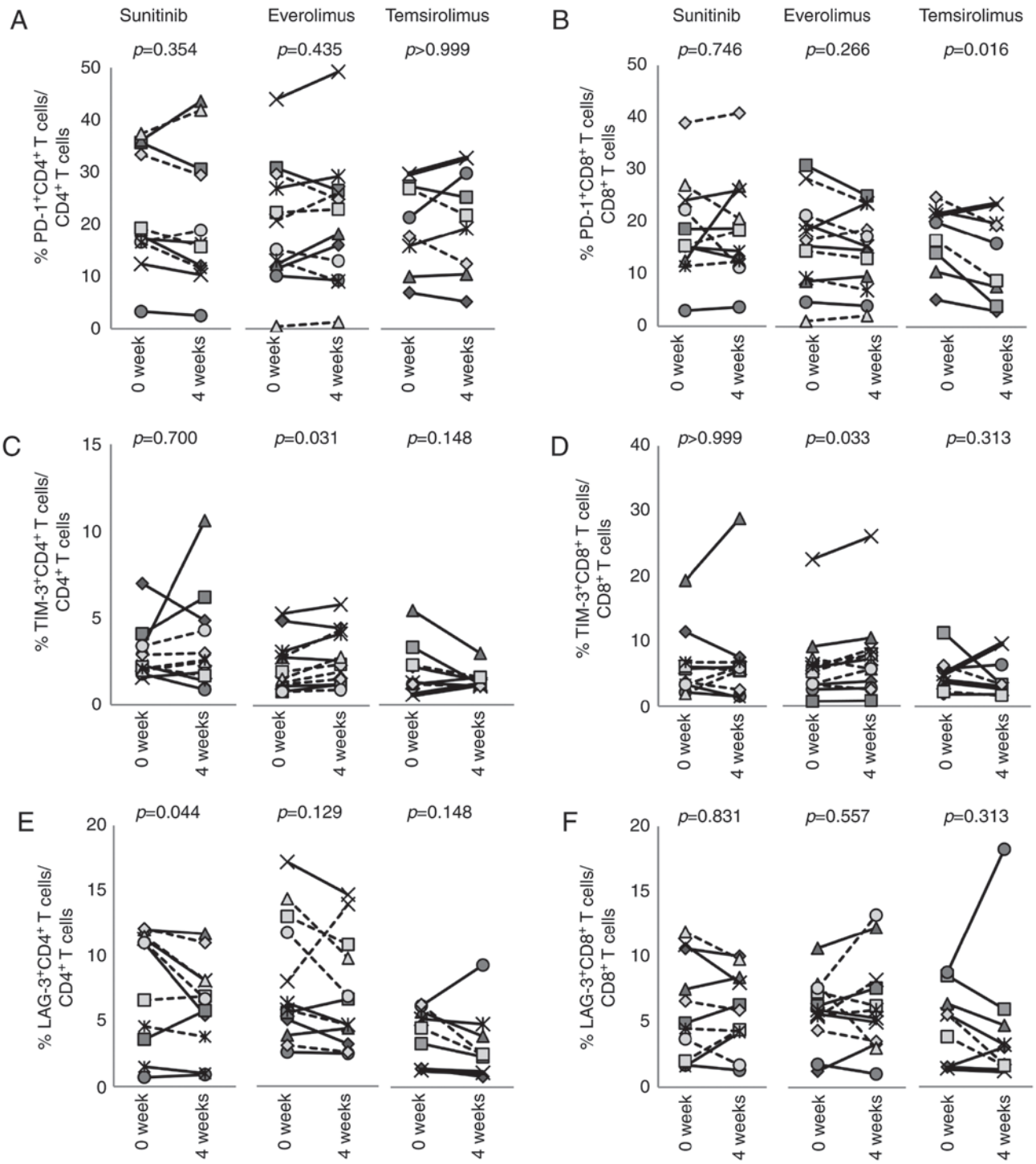

Figure 3. Effects of treatment with molecular targeted agents on the expression of checkpoint molecules by $CD4^+$ and $CD8^+$ T cells. The effects of sunitinib, everolimus or temsirolimus on the expression of (A and B) PD-1, (C and D) TIM-3 and (E and F) LAG-3 on (A, C and E) $CD4^+$ and (B, D and F) $CD8^+$ T cells are shown. Percentages of $CD4^+$ or $CD8^+$ T cells expressing checkpoint molecules before treatment (0 week) and after 4 weeks of treatment in each individual case are shown. LAG-3, lymphocyte activation gene 3 protein; PD-1, programmed cell death protein 1; TIM-3, T cell immunoglobulin and mucin protein 3.

and $CD95^+CD8^+$ T cells (P=0.016; Fig. 4A, D and F). Temsirolimus treatment had no effect on the expression of these molecules on $CD8^+$ T cells, with the exception that $PD\text{-}1^+CD8^+$ T cells decreased from 16.8±6.2% to 12.7±7.4% (P=0.016; Fig. 3B).

Effects of molecular targeted agents on inhibitory cells. The frequencies of regulatory T (T_{Reg}) cells and myeloid-derived suppressor cells (MDSCs) in PBMCs were enumerated (Fig. 5). T_{Reg} cells were subdivided into two populations: $CD45RA^+FOXP3^{low}$ naïve T_{Reg} cells (Fig. 5A) and $CD45RA^-FOXP^{high}$ effector T_{Reg} cells (Figs. 5B and S5) (25). There were more effector T_{Reg} cells than naïve T_{Reg} cells in these patients' PBMCs, and their frequencies were not affected by sunitinib. However, effector T_{Reg} cells were significantly decreased from 4.36±2.1 to 3.1±1.7% by everolimus (P=0.014)

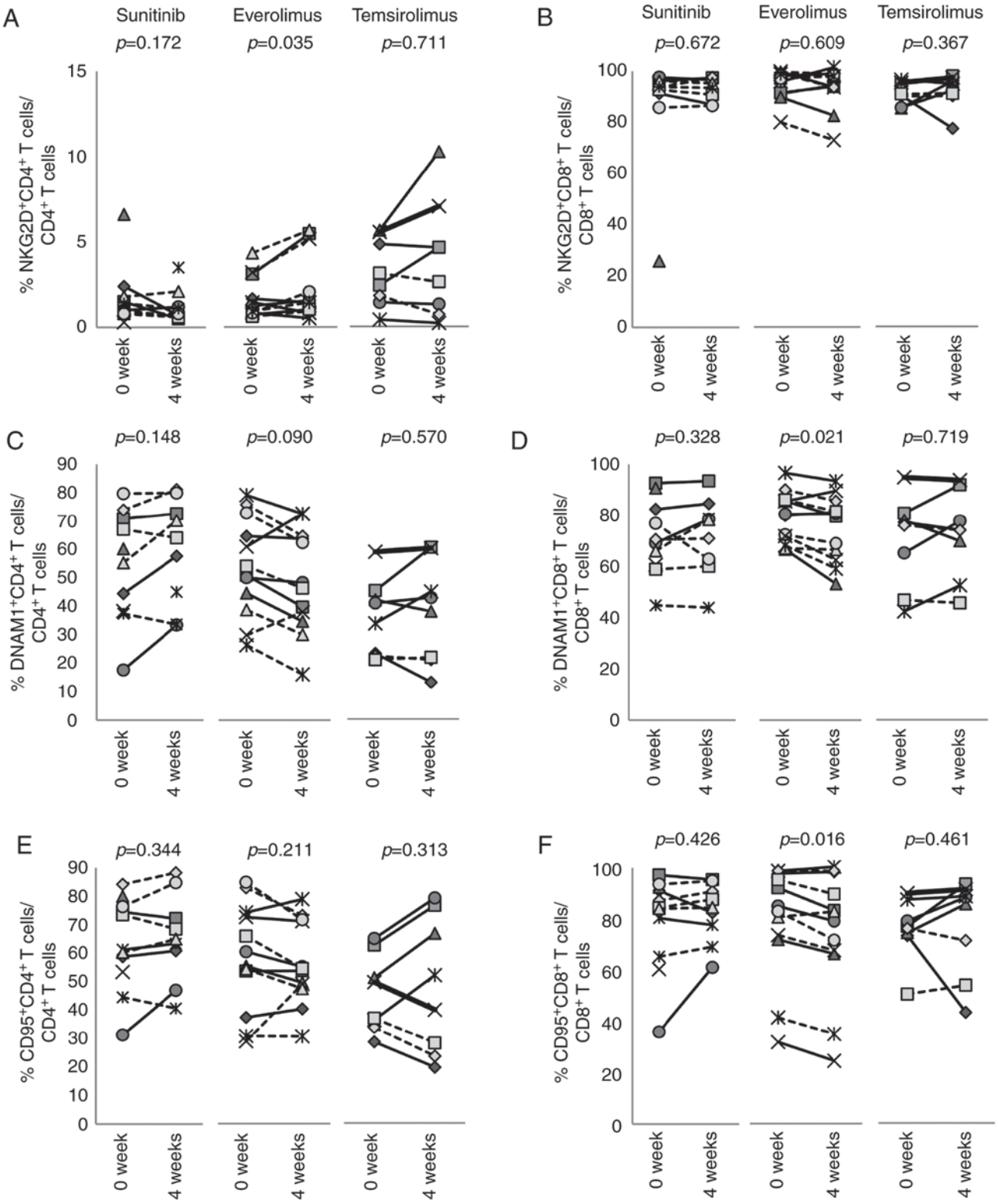

Figure 4. Effects of treatment with molecular targeted agents on the expression of activation markers by $CD4^+$ and $CD8^+$ T cells. The effects of sunitinib, everolimus or temsirolimus on the expression of (A and B) NKG2D, (C and D) DNAM1 and (E and F) CD95 on (A, C and E) $CD4^+$ and (B, D and F) $CD8^+$ T cells are shown. Percentages of $CD4^+$ or $CD8^+$ T cells expressing activation markers before treatment (0 week) and after 4 weeks of treatment in each individual case are shown. DNAM1, DNAX accessory molecule 1.

and although not statistically significant, temsirolimus also decreased the percentage of effector T_{Reg} cells from 3.2±1.6 to 2.1±1.3% (P=0.078).

MDSCs can also be subdivided into polymorphonuclear (PMN)-MDSCs, monocytic MDSCs (M-MDSCs) or early-stage MDSCs (eMDSCs) (26) defined as $CD14^-CD11b^+CD15^+$ (or $CD66b^+$), $Lin(CD3/19/20/56)^-CD11b^+CD14^+HLA\text{-}DR^{low/-}CD15^-$, and $Lin^-CD14^-CD15^-HLA\text{-}DR^-CD33^+$, respectively (Fig. S6). Since the present study utilized cryopreserved PBMCs that were isolated by density-gradient

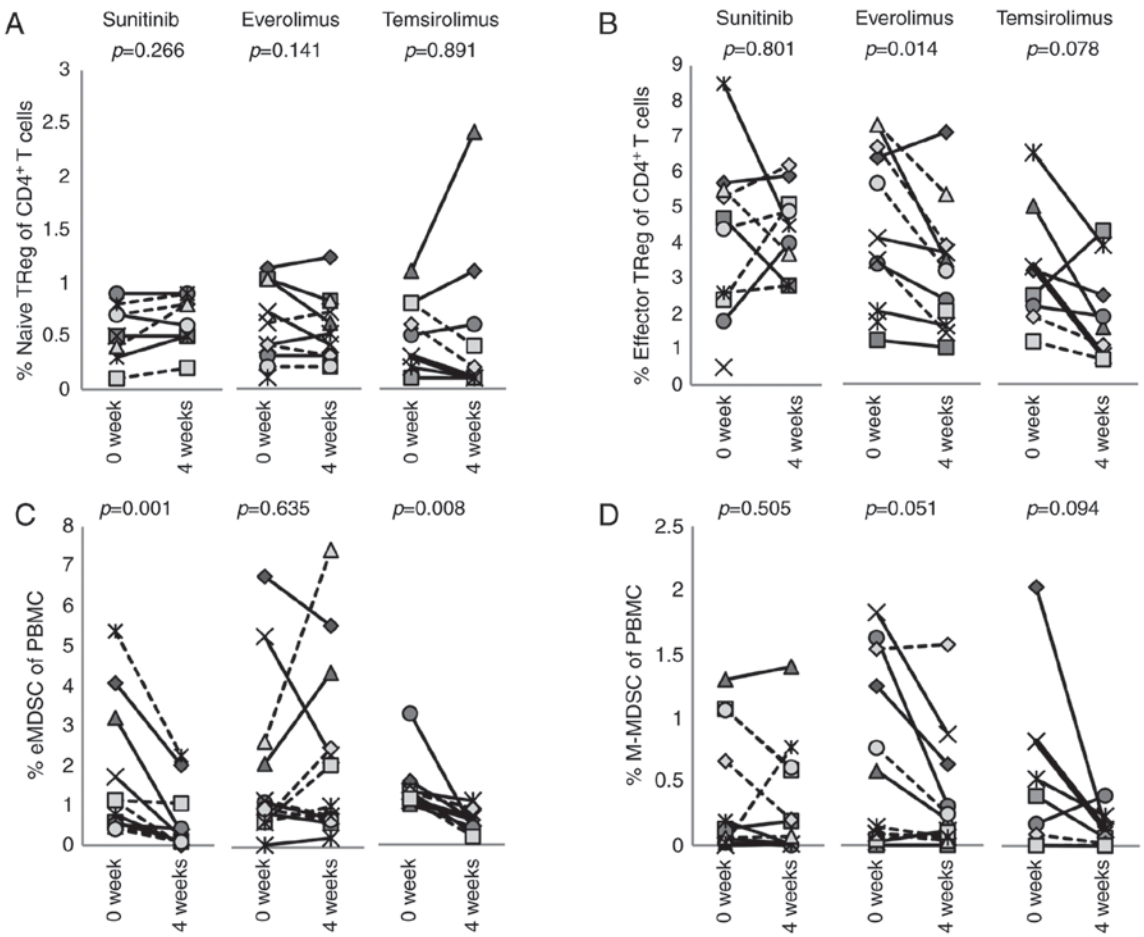

Figure 5. Effects of treatment with molecular targeted agents on T_{Reg} cells and MDSCs. The effects of sunitinib, everolimus or temsirolimus on (A) naïve T_{Reg} cells, (B) effector T_{Reg} cells, (C) eMDSCs and (D) M-MDSCs are shown. Data before treatment (0 week) and after 4 weeks of treatment in each individual case are shown. eMDSCs, early-stage MDSCs; M-MDSCs, monocytic MDSCs; MDSCs, myeloid-derived suppressor cells; T_{Reg}, regulatory T.

centrifugation with Lymphoprep™, most PMN-MDSCs were lost to the study. Therefore, this study primarily evaluated eMDSCs (Fig. 5C) and M-MDSCs (Fig. 5D). Sunitinib reduced the percentage of eMDSCs (P=0.001), but not M-MDSCs (Figs. 5C and D, and S7). Similarly, temsirolimus reduced the percentage of eMDSCs from 1.5±0.69 to 0.58±0.28% (P=0.008). In contrast, everolimus did not affect either eMDSCs or M-MDSCs.

Effects of molecular targeted agents on senescent T cells. Aging is associated with a decline of the immune system known as immunosenescence, which could influence the efficacy and safety profile of immunotherapy. It is known that immunosenescence is accompanied by an increase in $CD28^-$, $KLRG1^+$, $CD152^+$, $CD45RO^+$ and $CD57^+$ cells; $CD28^-CD57^+KLRG1^+CD3^+$ T cells are defined as immunosenescent T cells (Fig. S8). This study revealed that 4 weeks of treatment with any of the three agents analyzed had no impact on the percentage of senescent T cells (Fig. 6).

Effects of molecular targeted agents on T-cell metabolism. T-cell phenotypes and functions are closely associated with cellular metabolism. Therefore, this study examined the effect of molecular targeted agents on this variable (Fig. 7). Glucose uptake was evaluated by 2-NBDG incorporation, and mitochondrial mass and membrane potential were determined by MTG and TMRE, respectively (Figs. S9 and S10). Sunitinib and temsirolimus did not affect 2-NBDG uptake by $CD4^+$ or $CD8^+$ T cells (Fig. 7A and B), whereas the effect of everolimus differed substantially between individuals. Thus, the mean fluorescent intensity (MFI) of 2-NBDG in $CD4^+$ or $CD8^+$ T cells was either increased or decreased by the treatment. Sunitinib and temsirolimus did not affect the TMRE or MTG of T cells in any patient; however, everolimus decreased the MFI of MTG from 117,194±10,626 to 106,488±11,724 (P=0.016) in $CD4^+$ T cells (Fig. 7C) and from 82,767±16,244 to 64,020±11,349 (P=0.016) in $CD8^+$ T cells (Fig. 7D), with no effect on TMRE (Fig. 7E and F).

Effects of molecular targeted agents on T-cell functionality. T-cell functionality was evaluated by intracellular cytokine staining for the production of IFN-γ, TNF-α and IL-2 following stimulation with PMA and ionomycin (Figs. S11-S13). The results revealed that the percentages of $CD4^+$ T cells producing these cytokines were not changed following treatment of patients with sunitinib or temsirolimus (Fig. 8A, C and E). In everolimus-treated patients, the percentage of IL-2-producing $CD4^+$ T cells was slightly decreased from 43.6±12.6 to 39.2±12.7 after 4 weeks (P=0.026), whereas the frequencies of IFN-γ- or TNF-α-producing $CD4^+$ T cells were not changed (Fig. 8C and E).

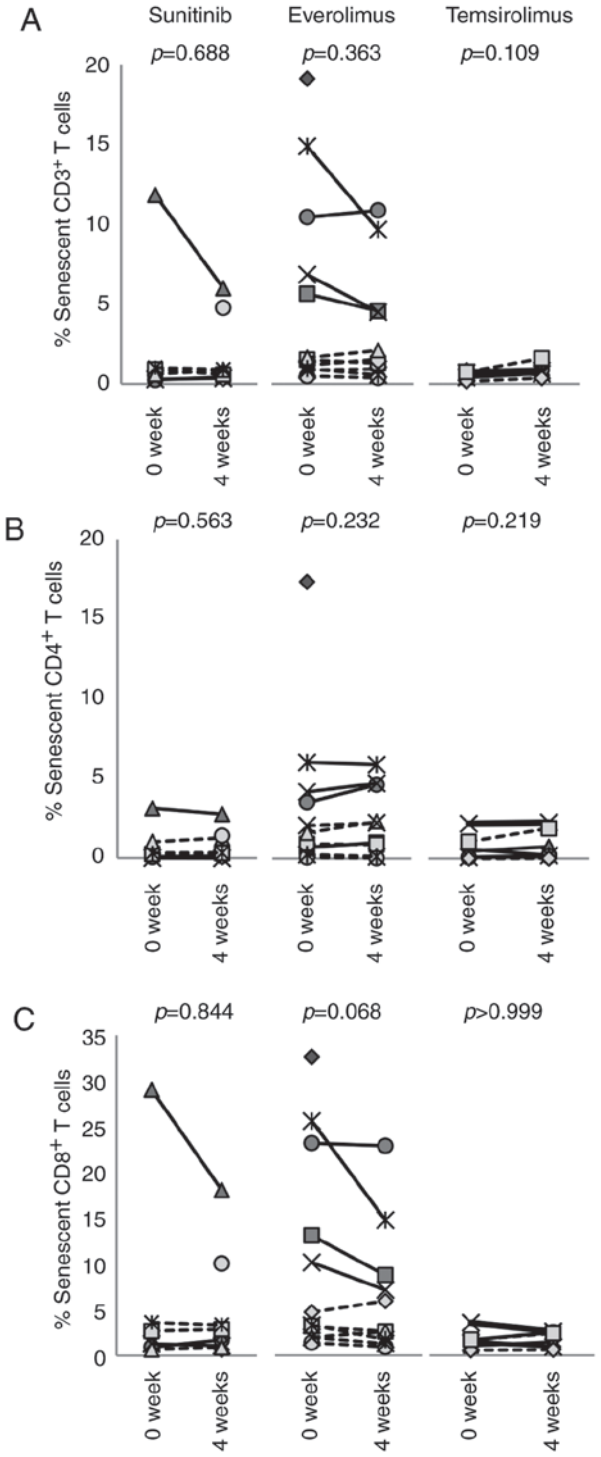

Figure 6. Effects of treatment with molecular targeted agents on senescent T cells. $CD28^{-}CD57^{+}KLRG1^{+}CD3^{+}$ T cells are defined as immunosenescent. The effects of sunitinib, everolimus or temsirolimus on these immunosenescent (A) $CD3^{+}$ T cells, (B) $CD4^{+}$ T cells and (C) $CD8^{+}$ T cells in peripheral blood mononuclear cells were examined by flow cytometry. Percentages of T cells bearing senescence markers before treatment (0 week) and after 4 weeks of treatment in each individual case are shown. KLRG1, killer cell lectin-like receptor subfamily G member 1.

All three drugs had a greater impact on $CD8^{+}$ T cells than $CD4^{+}$ T cells. The frequency of IL-2-producing $CD8^{+}$ T cells was decreased by sunitinib from a pretreatment level of 20.0±8.9 to 15.1±7.8% after 4 weeks (P=0.002) (Fig. 8B). However, IFN-γ or TNF-α single-producer $CD8^{+}$ T cells (Fig. 8D and F), and IFN-γ and TNF-α double-producers (Fig. 9D and F) were not affected. In everolimus-treated patients, the percentages of IFN-γ-producing $CD8^{+}$ T cells were significantly reduced from 63.6±18.5 to 59.4±19.6% (P=0.021), and the percentages of IL-2- and TNF-α-producing $CD8^{+}$ T cells were decreased from 23.1±10.9 to 20.9±9.6% (P=0.129) and from 67.8±18.1 to 63.2±19.8% (P=0.052), respectively (Fig. 8B, D and F). While frequencies of IFN-γ and TNF-α double-producers were not changed (Fig. 9D and F), the percentage of dysfunctional $CD8^{+}$ T cells that could not produce any of these three cytokines was increased from 22.0±15.2 to 26.4±16.6% (P=0.012) (Fig. 9J), suggesting that everolimus treatment has some immunosuppressive activity on $CD8^{+}$ T cells. Conversely, temsirolimus treatment increased the percentage of cytokine-producing $CD8^{+}$ T cells. Although not statistically significant, differences between pretreatment and 4 week samples for single cytokine producers were altered, and the percentage of IFN-γ and TNF-α double-producers was increased from 45.7±19.6 to 59.2±16.1% by temsirolimus treatment (P=0.023) (Fig. 9D and F). Reciprocally, albeit not significantly, the percentage of dysfunctional $CD8^{+}$ T cells that could not produce any of these three cytokines was decreased from 20.7±10.9 to 12.6±0.11% (P=0.109) (Fig. 9J). These results suggested that temsirolimus may have a positive impact on cytokine production of $CD8^{+}$ T cells.

Discussion

This study performed flow cytometric immunophenotyping and assessed the functionality of PBMCs from patients with RCC receiving the molecular targeted agents sunitinib, everolimus or temsirolimus, in order to assess the immunological impact of these drugs. It was revealed that these molecular targeted agents had different effects on the distribution and functionality of numerous immune cells in the peripheral blood.

The therapeutic landscape of advanced RCC has totally changed since nivolumab was approved by the United States Food and Drug Administration in 2015 as second-line therapy for mRCC (16), and the combination of nivolumab plus ipilimumab was approved as first-line therapy for intermediate and poor-risk patients (17). In addition, pembrolizumab plus axitinib has been revealed to be superior to sunitinib in first-line management of mRCC, regardless of the risk groups, with an acceptable safety profile (18). The Javelin Renal-101 trial revealed the PD-L1 blocker avelumab plus axitinib to be more efficacious than sunitinib (27). Furthermore, the IMmotion151 trial demonstrated that atezolizumab plus bevacizumab prolonged progression-free survival compared with sunitinib with a favourable safety profile (28). Ongoing phase III trials aim to investigate the combination of nivolumab plus cabozantinib compared to sunitinib (Checkmate-9ER, NCT01984242), and pembrolizumab plus lenvatinib compared to lenvatinib plus everolimus or sunitinib (Clear, NCT02811861). Thus, a large number of trials are testing the combination of immune checkpoint inhibitors and other molecular targeted agents.

For establishing effective combination immunotherapy for mRCC, it is important to understand the immunological properties of molecular targeted therapy. The mode of action

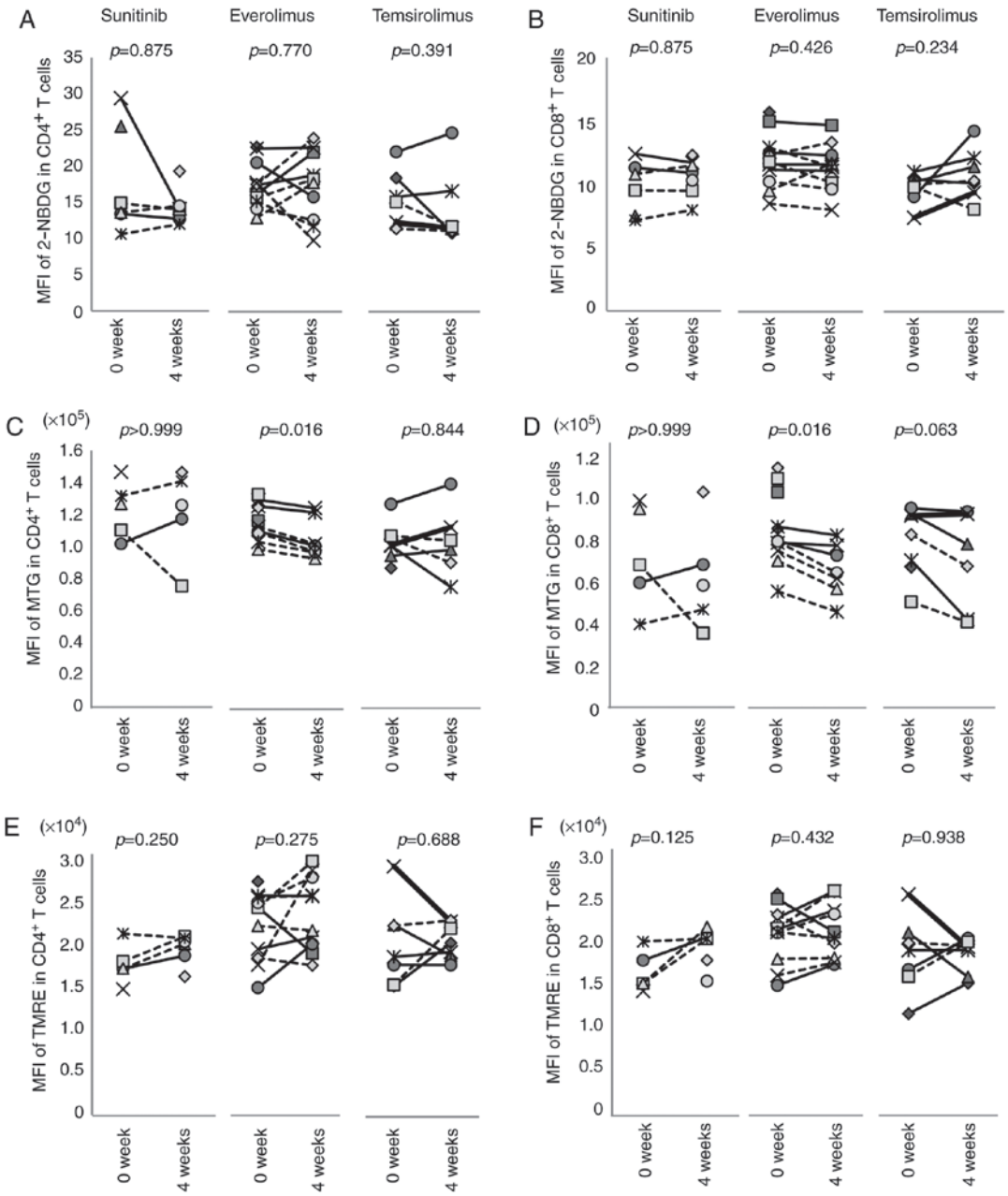

Figure 7. Effects of treatment with molecular targeted agents on T-cell metabolism. The effects of sunitinib, everolimus or temsirolimus on the uptake of (A and B) 2-NBDG, (C and D) MTG staining and (E and F) TMRE staining of (A, C and E) $CD4^+$ and (B, D and F) $CD8^+$ T cells are shown. MFI before treatment (0 week) and after 4 weeks of treatment in each individual case is indicated. 2-NBDG, 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose; MFI, mean fluorescence intensity; MTG, MitoTracker Green; TMRE, tetramethylrhodamine, ethyl ester.

of molecular targeted agents is to inhibit oncogenic activation of several processes required for the growth, survival and proliferation of cancer cells. They are used in the clinical setting in cancer therapy, and in the case of mTOR inhibitors also for immunosuppression. However, the effects of molecular targeted agents on the immune system may be more complex than previously thought. A variety of immune cell populations infiltrate into the tumor and contribute to the complexity of the tumor microenvironment, which can either promote or limit tumor progression and result in anti- or pro-tumor immunity (29,30). Therefore, understanding the effects of molecular targeted agents on overall immune responsiveness *in vivo* is important. Because current checkpoint blockade therapies depend on the invigoration of pre-existing anti-tumor T cells (31), this study focused particularly on the distribution of T-cell subsets and their functions in patients with RCC, particularly during early treatment, to determine the effects of several molecular targeted agents.

As summarized in Table II, sunitinib treatment decreased the percentage of eMDSCs and increased NK cells; sunitinib did not affect the phenotypes and effector function of $CD4^+$ and $CD8^+$ T cells. Everolimus decreased effector T_{Reg} cells. However, it also decreased IL-2-producing $CD4^+$ T cells and increased the percentage of dysfunctional $CD8^+$ T cells. In contrast, temsirolimus decreased the percentage of PD-1^+CD8^+ T cells and eMDSCs; the percentage of IFN-γ and TNF-α double-producers was increased and

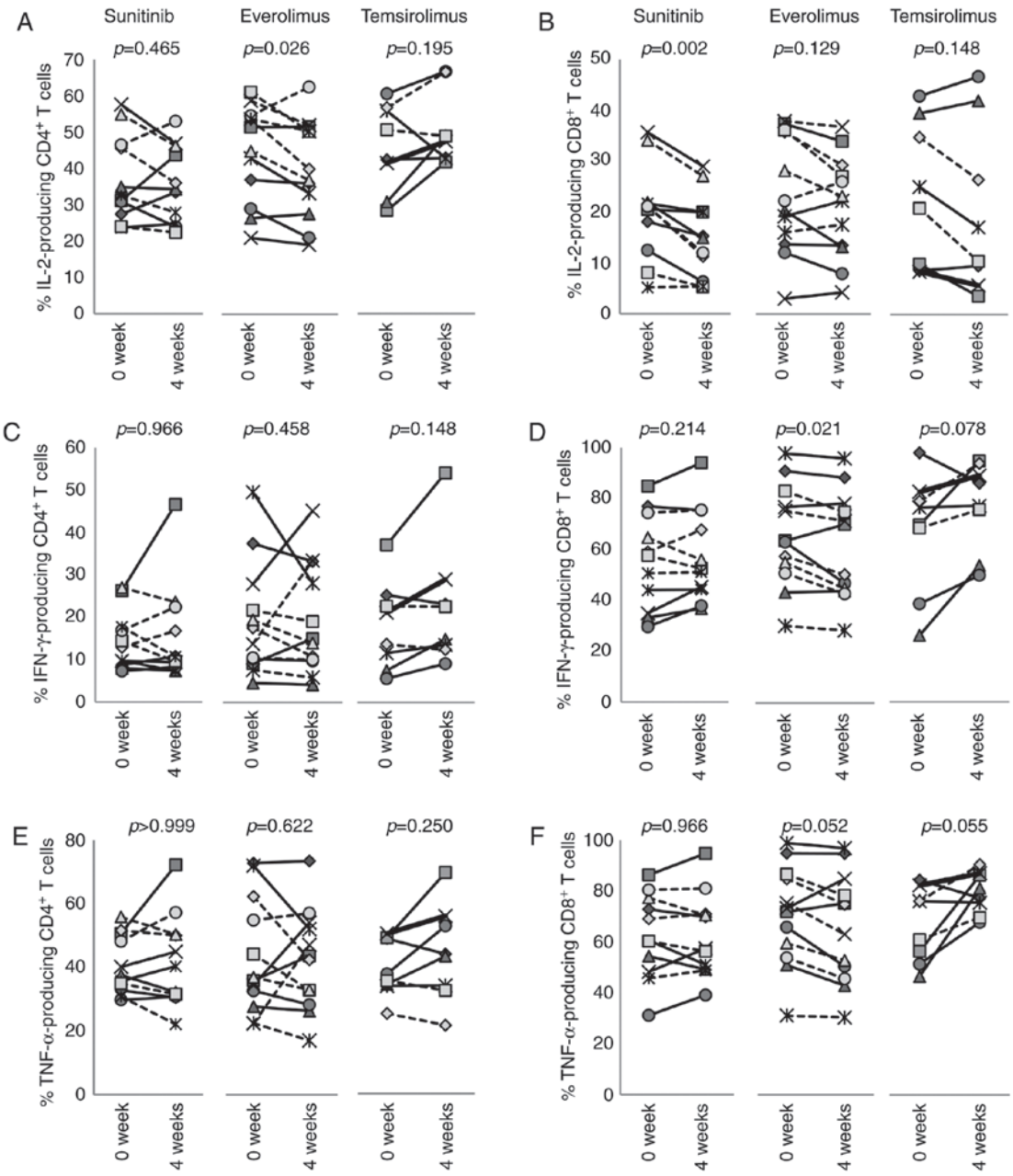

Figure 8. Effects of treatment with molecular targeted agents on cytokine-producing T cells. The effects of sunitinib, everolimus, and temsirolimus on (A and B) IL-2-, (C and D) IFN-γ- and (E and F) TNF-α producing (A, C and E) $CD4^+$ and (B, D and F) $CD8^+$ T cells were established by intracellular cytokine staining. Percentages of cytokine producing $CD4^+$ or $CD8^+$ T cells before treatment (0 week) and after 4 weeks of treatment in each individual case are indicated. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

the percentage of dysfunctional $CD8^+$ T cells was decreased. Although everolimus and temsirolimus are both mTOR inhibitors, they behaved as a potential immunosuppressor and immunostimulator, respectively. The underlying molecular mechanisms responsible for these different immunological effects are not clear, and several other differences between these two drugs were observed. In addition, everolimus increased the expression of TIM-3 on $CD4^+$ and $CD8^+$ T cells, and also affected 2-NBDG uptake and the MFI of MTG. These results suggested that everolimus has more direct effects on T cells than temsirolimus and that it may predominantly suppress effector functions.

It has been reported that sunitinib and sorafenib may inhibit T-cell activation, proliferation and cytokine production (32,33). On the other hand, it has been suggested that sunitinib reduces T_{Reg} cells (34) and MDSCs (35); and may therefore potentiate antitumor immune responses (36). This study observed decreasing percentages of eMDSCs in response to sunitinib treatment; however, the overall T-cell response was not affected by 4 weeks of sunitinib treatment.

The expansion of T_{Reg} cells has been reported in patients with mRCC treated with everolimus (37,38), which induced an overall increased level of immunosuppression via an increase in T_{Reg} cells and MDSCs (38,39). In the present study, no increase was observed in T_{Reg} cells, but an increase in dysfunctional $CD8^+$ T cells was detected in everolimus-treated patients. This may be at least partly because the mTOR pathway is directly involved in the control of T-cell proliferation and functions,

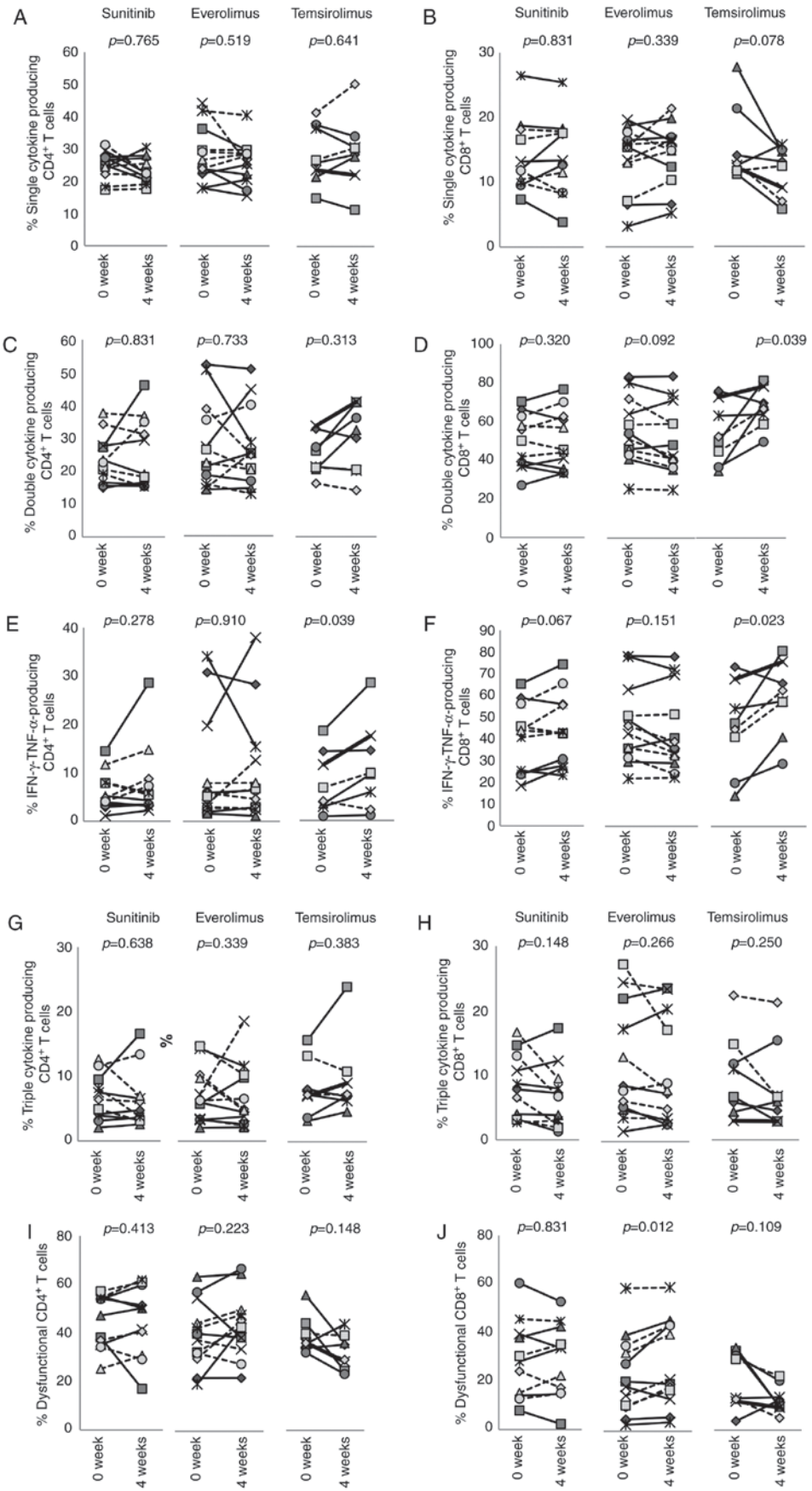

Figure 9. Effects of treatment with molecular targeted agents on polyfunctional T cells. The effects of sunitinib, everolimus or temsirolimus on polyfunctionality of (A, C, E, G and I) $CD4^+$ and (B, D, F, H and J) $CD8^+$ T cells were examined by intracellular cytokine staining. Production of IL-2, IFN-γ and TNF-α were simultaneously evaluated. T cells positive for one or more of these three cytokines were recognized as (A and B) single cytokine producers or (C and D) double cytokine producers. (E and F) T cells positive for IFN-γ and TNF-α but negative for IL-2 were present. (G and H) Triple cytokine producers and (I and J) dysfunctional T cells that could not produce any of these three cytokines were also detected. Percentages of $CD4^+$ and $CD8^+$ cytokine-producing T cells before treatment (0 week) and after 4 weeks of treatment in each individual case are indicated. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

and immune signals are integrated with cellular metabolism by mTOR signaling (40).

mTOR signaling is also associated with $CD8^+$ T cell differentiation programs (41). Activation of mTOR facilitates the differentiation of naïve T cells to activated and effector T cells, whereas its inhibition in effector T cells leads to memory T cell formation (42,43). Therefore, based on T-cell differentiation status, inhibition of mTOR should result in reduced $CD8^+$ effector T-cell numbers and functions, and an increase of memory T cells. Because the differentiation status of peripheral blood T cells varies in different individuals, this may explain the patient-to-patient variations observed in the effects of molecular targeted agents on T-cell immunity.

Immunosenescence, or age-associated changes to adaptive and innate immunity, has been associated with increased susceptibility to infection and cancer (44). Since the response to vaccination is reduced in the elderly, the efficiency of checkpoint blockade may also be associated with immunosenescence. None of the molecular targeted agents examined in the present study affected the frequency of $CD28^-CD57^+KLRG1^+$ immunosenescent T cells in PBMC. However, temsirolimus decreased $PD\text{-}1^+CD8^+$ T cells and enhanced cytokine production. This is consistent with a previous study. which reported that the mTOR inhibitor RAD001 decreased the percentage of $PD\text{-}1^+CD4^+$ and $CD8^+$ T cells, and improved the response to influenza vaccination (45). The low-grade inflammation observed in elderly people and continuous antigenic stimulation by chronic infection or cancer results in a slight activation of mTOR that can be ameliorated by mTOR inhibitors. Unlike everolimus, overall responses in temsirolimus-treated patients were more immunostimulatory, which may contribute to enhanced immune function and improve the quality of T-cell responses. Therefore, temsirolimus might be a good candidate for combination with other immunomodulators. Notably, a combination of temsirolimus with a CCR4 antagonist targeting this receptor that is highly expressed on T_{Reg} cells, together with cancer vaccination, has been reported to be more effective in amplifying functional tumor-specific $CD8^+$ T cells than monotherapy alone (46).

There are several limitations to the present study. Firstly, the sample size was small. Secondly, sunitinib is approved as a first-line option, whereas the mTOR inhibitors everolimus and temsirolimus are approved in the second-line setting and only in the first-line setting for patients with high-risk status (12,13). Therefore, baseline immunological conditions might have differed in the patients receiving these different agents. Nonetheless, the data obtained in the present study derive from real-world patients with RCC. They include comprehensive immunomonitoring that covers phenotype, function and metabolism, and this should be valuable for further comparisons. Thirdly, only the immune monitoring of patients' PBMCs was conducted; the changes in PBMC do not necessarily reflect changes in tumor tissue. The phenotypes and functions of tumor-infiltrating lymphocytes and PBMCs have been reported to differ in patients with RCC (47). However, tumor biopsies are generally costly, invasive, cause treatment delays and increase the risk of adverse events. The analysis of readily accessible peripheral blood is preferred for developing biomarkers with clinical utility. In fact, numerous studies provide compelling evidence that subtypes and status of PBMCs are associated with responses to immunotherapy (48).

Table II. Immunological effects of treatment of patients with renal cell carcinoma with molecular targeted drugs on peripheral blood mononuclear cells.

Treatment	NK	$TIM\text{-}3^+$ $CD4^+$	$LAG\text{-}3^+$ $CD4^+$	$PD\text{-}1^+$ $CD8^+$	$TIM\text{-}3^+$ $CD8^+$	eT_{Reg}	eMDSC	M-MDSC	2-$NBDG^+$ T	$TMRE^+$ T	MTG^+ T	$IL\text{-}2^+$ $CD4^+$ T	$IL\text{-}2^+$ $CD8^+$ T	$IFN\text{-}\gamma^+$ $CD8^+$ T	$TNF\text{-}\alpha^+$ $CD8^+$ T	$IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$ $CD8^+$ T	Dysfunctional $CD8^+$ T
Sunitinib	↑	→	↓	→	→	→	↓	↑	↑	↑	→	↑	↓	→	→	→	→	↑
Everolimus	→	↑	→	→	↑	↓	↑	↑	⇅	↑	↓	↓	↘	↓	↘	↑	↑	↑
Temsirolimus	→	→	→	↓	→	↗	↓	↑	↑	↑	↑	→	→	→	→	→	↑	↘

↑, increased; →, no change; ↓, decreased; ⇅, increased or decreased depending on the case. 2-NBDG, 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; eMDSC, early-stage MDSC; eT_{Reg}, effector regulatory T; IFN-γ, interferon-γ; LAG-3, lymphocyte activation gene 3 protein; M-MDSC, monocytic MDSC; MDSC, myeloid-derived suppressor cell; MTG, MitoTracker Green; NK, natural killer; PD-1, programmed cell death protein 1; TIM-3, T cell immunoglobulin and mucin protein 3; TMRE, tetramethylrhodamine, ethyl ester; TNF-α, tumor necrosis factor-α.

In conclusion, different immunological effects of the molecular targeted agents sunitinib, everolimus and temsirolimus were observed in patients with RCC. Everolimus tended towards suppression of T-cell functions, whereas temsirolimus increased T-cell functionality. Although it may increase the risk of immune-related toxicity, it may be proposed that temsirolimus combined with checkpoint blockade will result in enhanced activity of cancer immunotherapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YK, AH, KN, TKar and HM analyzed and interpreted the flow cytometry data. DY, TKaw, YS, TT, YY, MN, MS, AM, TN and HK recruited patients, collected samples and interpreted the data regarding RCC. KK conceived and designed the study, analyzed and interpreted the data, and wrote and revised the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This clinical study on the immunological impact of molecular targeted agents in patients with RCC was conducted at The University of Tokyo Hospital. All procedures in this study were performed following the ethical standards of the institutions, and in conformity with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The research protocol was approved by the Ethical Committee of The University of Tokyo (approval no. #3652). Written informed consent to participate in the study was obtained from each patient before they entered the study.

Patient consent for publication

Written informed consent was obtained from each patient before they entered the study.

Competing interests

The authors declare that have no competing interests.

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