CXCL10 and CCL2 mRNA expression in monocytes is inversely correlated with the HLA-DR lower fraction of monocytes in patients with renal cell carcinoma

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Abstract. Circulating cluster of differentiation (CD)14$^+$ human leukocyte antigen (HLA)-DR$^{low/-}$ monocytes, those with a lower HLA-DR expression or are negative for HLA-DR, are considered to be involved in systemic immunosuppression in patients with several malignant tumors. However, few studies have investigated in detail the gene expression profile of CD14$^+$HLA-DR$^{low/-}$ monocytes. In the present study, the mRNA expression levels of immune-associated molecules in CD14$^+$ monocytes isolated from healthy donors and patients with renal cell carcinoma (RCC) were analyzed. Consistent with previous studies, the percentage of HLA-DR$^{low/-}$ cells in CD14$^+$ monocytes was significantly increased in patients with RCC compared with healthy donors. In 3 of the 4 patients who underwent surgical resection of the primary tumor, the percentage of CD14$^+$HLA-DR$^{low/-}$ cells was significantly decreased following surgery. The mRNA expression levels of cyclooxygenase 2, transforming growth factor β, interleukin 6R, chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 10 (CXCL10), oncostatin M, and vascular endothelial growth factor-A in CD14$^+$ monocytes were quantified using reverse transcription-quantitative polymerase chain reaction. The results of the present study revealed that increased expression levels of CCL2 and CXCL10 were inversely correlated with the percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes. This suggested that monocytes in RCC patients were immunologically suppressed, and that immunosuppression in RCC patients may be due, in part, to the dysfunction of circulating monocytes.

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Introduction

Systemic immunosuppression in cancer patients is considered to affect the progression of cancer, and therefore the treatment outcome (1,2). Renal cell carcinoma (RCC) is known to be resistant to conventional chemotherapeutic agents and is known to induce an immunosuppressive environment. Although tyrosine kinase inhibitors (TKIs), including sorafenib and sunitinib, are widely utilized for the treatment of patients with metastatic RCC and TKIs are expected to act as adjuvants for immunotherapeutic effects (3,4), the anticancer effects of TKIs may be unable to overcome the immunosuppressive microenvironment of RCC hosts (5).

Previous studies have indicated that myeloid lineage cells, including tumor-associated macrophages, inflammatory monocytes and myeloid-derived suppressor cells (MDSCs), have a significant role in cancer-induced immunosuppression (6,7). MDSCs were initially described in murine cancer models. However, it remains to be elucidated which cell populations in humans are comparable to murine MDSCs (6). Several studies have revealed that cancer patients exhibit an increase in the number of cluster of differentiation (CD)14$^+$ human leukocyte antigen (HLA)-DR$^{low/-}$ cells, those with a lower HLA-DR expression or are negative for HLA-DR, circulating in the blood (8-10). CD14$^+$HLA-DR$^{low/-}$ cells that were isolated from cancer patients were identified to suppress T-cell activation in culture, and thus these CD14$^+$HLA-DR$^{low/-}$ monocyte populations were considered to act as MDSCs (6,8-10).

In the present study, in order to investigate the immunological characteristics of human monocyte populations, HLA-DR and the gene expression of immune-associated molecules in circulating CD14$^+$ myeloid cells from RCC patients were evaluated.

Materials and methods

Blood samples. The protocol of the present study was approved by the Kumamoto University Review Board (ethical permit no. 509; Kumamoto University Hospital, Kumamoto, Japan). All healthy donors and patients reviewed the study objectives

Table I. Data from healthy donors and renal cell carcinoma patients.

Patient	Age, years	Gender	Tumor classification	Surgery	Histology	Size, cm	White blood cells, cells/μl	Lymphocytes, %	Monocytes, %	CD14+HLADR$^{low/-}$, %
HD1	37	M	-	-	-	-	-	-	-	14
HD2	58	F	-	-	-	-	-	-	-	16
HD3	49	F	-	-	-	-	-	-	-	13
HD4	58	M	-	-	-	-	-	-	-	10
HD5	57	M	-	-	-	-	-	-	-	10
P1	64	M	T3a(rt),cT1a(lt)	Yes	Clear cell	6.5 (rt), 2.5 (lt)	3700	34	5.9	38
P2	62	F	T1a	Yes	Clear cell	3.1	3200	45	6.0	29
P3	61	M	T2a	Yes	Clear cell	7.1	4600	54	4.5	24
P4	64	F	T1a	Yes	Papillary	2.5	6100	53	6.3	24
P5	51	M	T1a	Yes	Clear cell	3.0	4100	70	5.4	18
P6	75	F	T1b	Yes	Clear cell	6.5	6300	68	8.6	35
P7	65	M	-	Yes	-	-	7000	65	5.1	31
P8	71	M	T1a(rt),T1b(lt)	Yes	Papillary	2.0 (rt), 4.8 (lt)	6500	51	4.8	12
P9	73	F	T1a	Yes	Clear cell	4.0	6800	47	3.9	34
P10	81	F	T1a	Yes	Clear cell	3.3	2900	44	5.5	10
P11	66	M	T1a	Yes	Papillary	3.0	6600	63	4.1	17
P12	47	M	multiple(NA)	No	Papillary	-	2500	66	5.7	12
P13	42	M	T1a	Yes	Clear cell	1.8	4000	49	4.5	14
P14	82	F	T1b	Yes	Clear cell	4.7	7400	56	4.3	20
P15	77	F	T1b(rt),T1a(lt)	Yes	Clear cell	4.7 (rt), 1.8 (lt)	3700	44	7.5	28
P16	57	F	T1b	Yes	Clear cell	5.0	4200	44	5.5	31
P17	79	M	T3a	Yes	Clear cell	7.0	5600	67	6.4	8
P18	69	M	T3a	Yes	Clear cell	-	5900	65	6.8	23
P19	51	M	T1b	Yes	Clear cell	5.0	7100	66	3.7	10
P20	66	F	T3a	Yes	Clear cell	7.0	7200	81	5.2	45
P21	75	M	T4	No	Clear cell	-	5100	66	8.9	40
P22	37	M	T4	Yes	Clear cell	10.0	3900	61	10.6	23

HD, healthy donor; P, patient; rt, right; lt, left; -, not applicable; CD, cluster of differentiation; HLA-DR^{low}, human leukocyte antigen-DR lower fraction.

and agreed to provide a blood sample based on consent in accordance with the Declaration of Helsinki. The clinical data of all patients and healthy donors is summarized in Table I. Additional blood samples were collected from 4 of the patients 2-3 months after surgery. All patients had not received treatment with TKIs or immunotherapy prior to sample collection. Patients with chronic renal failure and diabetes mellitus were excluded from the present study.

Isolation of peripheral blood mononuclear cells and CD14$^+$ monocytes. Peripheral blood mononuclear cells (PBMCs) were obtained from 30-ml blood samples using Lymphoprep™ (Axis-Shield Density Gradient Media; Alere Technologies GmbH, Jena, Germany) according to the manufacturer's protocols. Half of the PBMCs were suspended in CELLBANKER® medium (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) and were stored in liquid nitrogen. The remaining half of the PBMCs were used for isolation of CD14$^+$ monocytes using CD14 MicroBeads (Miltenyi Biotec, Inc., Auburn, CA, USA) according to the manufacturer's protocols.

Flow cytometry. PBMCs ($5x10^5$/tube) were treated with Fc Receptor Blocking Solution and subsequently stained with mouse monoclonal fluorescein isothiocyanate-labeled anti-human CD14 (catalog no., 325604; clone, HCD14; 1:20) and phycoerythrin-labeled anti-human HLA-DR (catalog no., 307606; clone, L243; 1:20) antibodies. Fc receptor Blocking Solution and all antibodies, including mouse monoclonal isotype-matched control antibodies (clones, MOPC-173 and MOPC-21; catalog no's., 400212 and 400110, respectively; 1:20), were obtained from BioLegend, Inc. (San Diego, CA, USA). The stained cell samples were analyzed using a FACSverse™ and FACSuite software (BD Biosciences, San Jose, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted with RNAiso Plus (catalog no., 6110A; Takara Bio Inc., Otsu, Japan). RNA was reverse-transcribed using the PrimeScript RT Reagent kit and DNase (catalog no., 2270A) from Takara Bio, Inc. The complementary DNA product (25 μl) was amplified using qPCR at 94˚C for 5 min, then 40 cycles of 94˚C for 30 sec and 60˚C for 30 sec. qPCR was performed using *Taq*Man polymerase with SYBR Green fluorescence (Takara Bio, Inc.) with an ABI PRISM® 7300 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA). The relative expression level was determined using the $2^{-\Delta\Delta Cq}$ normalization method (11). The sequences of the primers were designed using the Primer3 website (version 0.4.0; avaliable from http://bioinfo.ut.ee/primer3-0.4.0/) and were synthesised by Hokkaido System Science Co., Ltd. (Tokyo, Japan). The primer seqeunces are shown in Table II. The internal control gene used was β-actin, and 3 parallel wells were set up for each DNA sample (25 μl/well). The data was representative of ≥2 independent experiments.

Statistical analysis. The Mann-Whitney U and Spearman's rank correlation tests were performed for statistical analysis using StatMate (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table II. List of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')
TGF-β	F: TTGCTTCAGCTCCACGGAGAA
	R: ACGTAGTACACGATGGGCAGC
CXCL10	F: CGCTGTACCTGCATCAGCATTAG
	R: CTGGATTCAGACATCTCTTCTCACC
PTGS2 (COX2)	F: ACTATGGCTACAAAAGCTGGGAAG
	R: ATCATCAGGCACAGGAGGAAG
VEGFA	F: CAGGAGTACCCTGATGAGATCG
	R: TCTGCATGGTGATGTTGGAC
IL-6R	F: CACGACTCTGGAAACTATTCATGC
	R: AGGACCCCACTCACAAACAAC
CCL2	F: GTGTCCCAAAGAAGCTGTGATCT
	R: TGTCCAGGTGGTCCATGGA
OSM	F: GCCCAGGATTTGGAGAGGTCTGG
	R: GCGATGGTAGCCATGCAGGAACCT
β-actin	F: ATTCCTATGTGGGCGACGAG
	R: AAGGTGTGGTGCCAGATTTTC

F, forward; R, reverse; TGF, tumor growth factor; CXCL10, chemokine (C-X-C motif) ligand 10; PTGS, prostaglandin-endoperoxide synthase; COX; cyclooxygenase; VEGF, vascular endothelial growth factor; IL; interleukin; CCL2, chemokine (C-C motif) ligand 2; OSM, oncostatin M.

Results

CD14$^+$HLA-DR$^{low/-}$ monocyte percentage is increased in patients with RCC. In total, 22 patients with RCC and 5 age-matched healthy donors were enrolled in the present study (Table I). The mean percentage of CD14$^+$HLA-DR$^{low/-}$ cells in CD14$^+$ monocytes isolated from PBMCs was 23.9% in the RCC patients and 12.6% in the healthy donors. This difference was statistically significant ($P=0.035$; Fig. 1A and B). For 4 patients, the percentage of CD14$^+$HLA-DR$^{low/-}$ cells was compared pre- and post-surgery (2-3 months later). A significant reduction in the percentage of CD14$^+$HLA-DR$^{low/-}$ cells was observed following surgical resection in 3 of the 4 patients (Fig. 1C). By contrast, the percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes demonstrated no correlation with age, gender, tumor classification or other laboratory data (Table I).

mRNA expression of chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 10 (CXCL10) is negatively associated with the percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes. The present study analyzed the mRNA expression of immune-associated molecules in CD14$^+$ monocytes, and subsequently evaluated the association between mRNA expression levels and frequency of CD14$^+$HLA-DR$^{low/-}$ monocytes. This method of analysis was used due to the fact that the mRNA expression of CD14$^+$HLA-DR$^{low/-}$ monocytes could not be directly measured, as the total number of CD14$^+$HLA-DR$^{low/-}$ monocytes was too low to undergo mRNA extraction. mRNA was extracted from 16 patient and 4 healthy donor samples. A total of 6 patient samples and 1 healthy donor

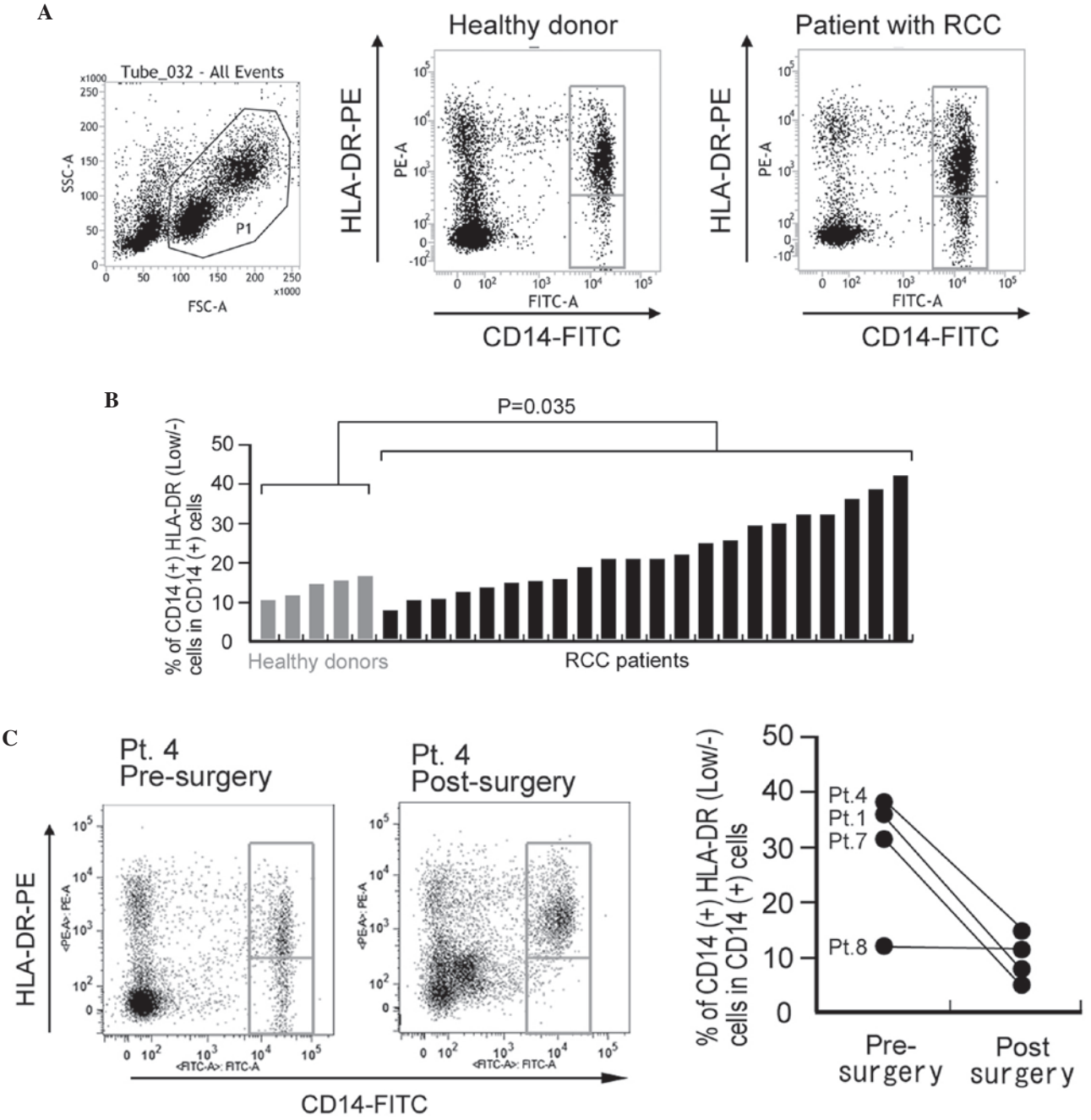

Figure 1. Percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes in circulating blood is increased in patients with RCC. (A) Representative FACS analysis of CD14$^+$HLA-DR$^{low/-}$ monocytes in the peripheral blood mononuclear cells of a healthy control donor and a patient with RCC. (B) The percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes in circulating CD14$^+$ monocytes of healthy donors and patients was analyzed. The difference between the groups was statistically analyzed using the Mann-Whitney U test. (C) The percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes in blood samples of 4 RCC patients pre-surgery and post-surgery. Left panel: Representative FACS analysis of pt.4. Right panel: Quantification of all patients. CD, cluster of differentiation; HLA, human leukocyte antigen; RCC, renal cell carcinoma; HLA-DR$^{low/-}$, cells with a lower HLA-DR expression or are negative for HLA-DR; FSC, forward scatter; SSC, side scatter; PE, phycoerythrin; FITC, fluorescein isothiocyanate; Pt, patient; FACS, fluorescence-activated cell sorting.

sample were excluded due to the low quality of the mRNA. Extremely low levels of gene expression of interleukin (IL)-6, IL-10, programmed death-1 (PD-1), PD-1 ligand (PD-L1), PD-L2, nitric oxide synthase 2, indoleamine 2,3-dioxygenase 1 and arginase 1 were observed in the preliminary analysis of microarray data (data not shown). The gene expression levels of cyclooxygenase 2 (COX2), transforming growth factor β (TGF-β), IL-6R, CCL2, CXCL10, oncostatin M (OSM) and vascular endothelial growth factor A (VEGF-A) were quantified using RT-qPCR (Fig. 2). Increased expression levels of CCL2 and CXCL10 were significantly correlated with a reduced percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes ($P<0.010$; $R=-0.58$; and $P=0.013$; $R=-0.55$; respectively).

Discussion

The present study evaluated the mRNA expression of immune-associated molecules in circulating CD14$^+$ monocytes. The number of CD14$^+$HLA-DR$^{low/-}$ monocytes is considered to be a prognostic factor; however, an evaluation

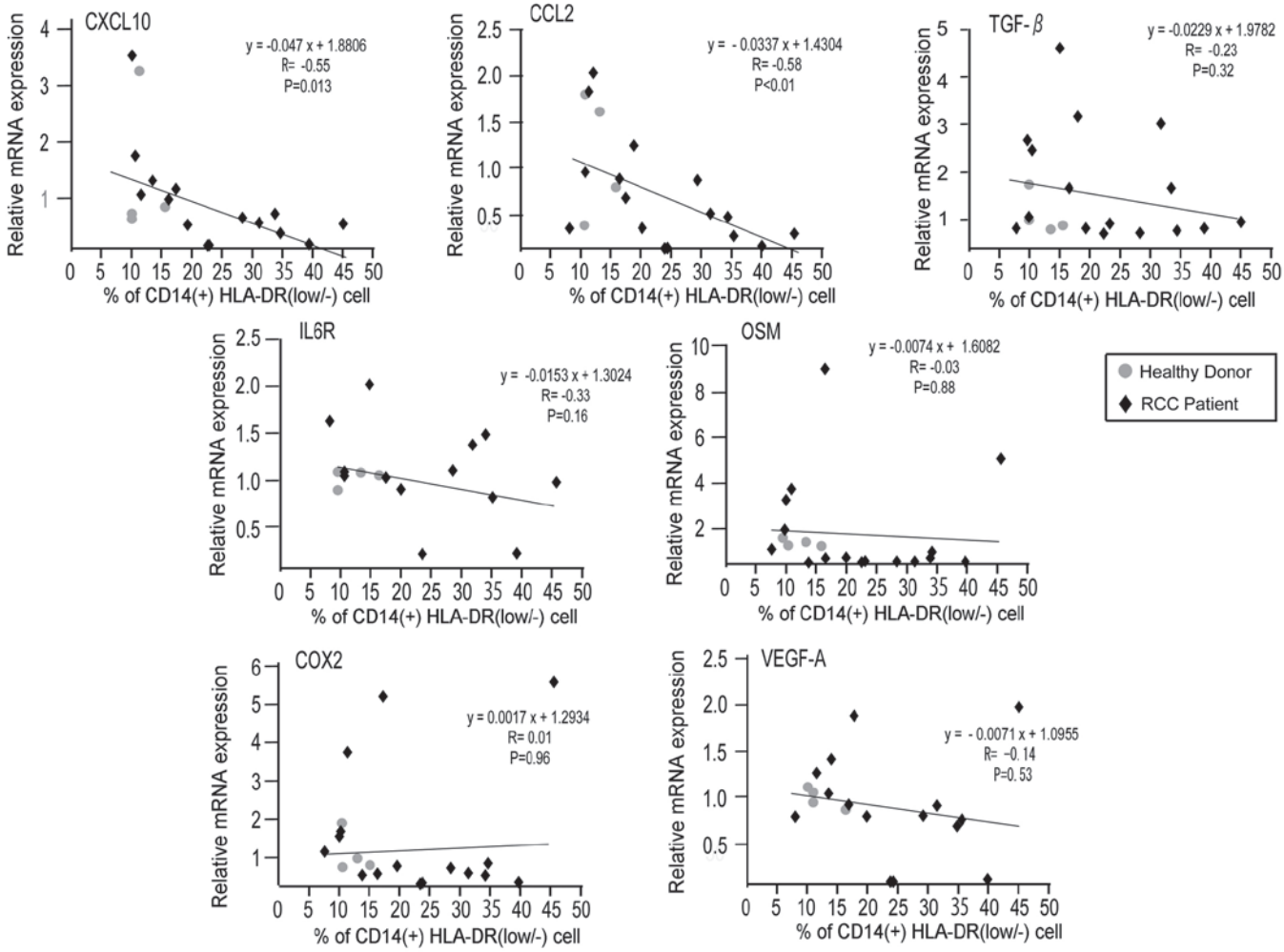

Figure 2. mRNA expression of immune-associated genes is negatively associated with a low percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes. The mRNA expression of COX2, TGF-β, IL-6R, CCL2, CXCL10, OSM and VEGF-A in CD14$^+$ monocytes of healthy donors and RCC patients was examined by reverse transcription-quantitative polymerase chain reaction. The correlation between indicated gene expression and the percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes in CD14$^+$ monocytes was evaluated using Spearman's rank correlation test. CD, cluster of differentiation; HLA, human leukocyte antigen; HLA-DR$^{low/-}$, cells with a lower HLA-DR expression or are negative for HLA-DR; COX2, cyclooxygenase 2; TGF-β, transforming growth factor β; IL, interleukin; CCL2, chemokine (C-C motif) ligand 2; CXCL10, chemokine (C-X-C motif) ligand 10; OSM, oncostatin M; VEGF, vascular endothelial growth factor; RCC, renal cell carcinoma.

of the association with the clinical prognosis of patients was not performed by the present study. A high number (>40%) of CD14$^+$HLA-DR$^{low/-}$ monocytes was significantly associated with a poorer clinical prognosis in patients with chronic lymphocytic leukemia (12). In addition, an increased number of CD14$^+$HLA-DR$^{low/-}$ monocytes was demonstrated to be an independent prognostic factor in patients with non-small lung cancer (13). The present study revealed that the number of CD14$^+$HLA-DR$^{low/-}$ monocytes was associated with CXCL10 and CCL2 expression, and in a previous study a high CXCL10 expression in cancer tissues was demonstrated to be associated with a favorable survival rate in patients with esophageal cancer (14). Therefore, it may be of interest to investigate whether the serum concentration of CXCL10 or CCL2 is a useful prognostic marker for patients with cancer. A higher frequency of CD14$^+$HLA-DR$^{low/-}$ monocytes in RCC patients was observed compared with healthy donors, confirming observations made in previous studies (8-10). Notably, he present study showed that the frequency of CD14$^+$HLA-DR$^{low/-}$ monocytes reverted to normal following surgical resection of the primary tumor. This observation indicated that unknown molecules derived from RCC tissues may affect the frequency of CD14$^+$HLA-DR$^{low/-}$ monocytes. The combined observations of the present study suggested that modification of circulating monocytes by cancer-derived factors may be involved in immunosuppression in patients with RCC. However, to the best of our knowledge, there has been no evidence (such as mRNA expression levels of immune-associated molecules) that has been able to directly support the possibility that immunosuppressive molecules are expressed by CD14$^+$HLA-DR$^{low/-}$ monocytes. The present study speculated that increased expression levels of immunosuppressive molecules would be observed in circulating monocytes along with the high percentage of CD14$^+$HLA-DR$^{low/-}$ monocytes. However, the results of the present study revealed that the expression of major immunosuppressive molecules, including COX2, OSM, TGF-β and VEGF-A, was not correlated with the frequency of CD14$^+$HLA-DR$^{low/-}$ monocytes. By contrast, increased expression levels of CXCL10, which is associated with immune activation, and CCL2, which is known

to be secreted from activated monocytes and macrophages, were significantly associated with a reduced percentage of $CD14^{+}HLA\text{-}DR^{low/-}$ monocytes (15). The results of the present study suggested that the expression of inflammatory cytokines is typically decreased in $CD14^{+}HLA\text{-}DR^{low/-}$ monocytes.

In conclusion, the present study did not observe an increase in the expression of any immune-associated molecules investigated in $CD14^{+}$ monocytes isolated from patients with RCC. However, increased mRNA expression of CCL2 and CXCL10 was inversely correlated with the frequency of $CD14^{+}HLA\text{-}DR^{low/-}$ monocytes, suggesting that cytokine expression may be suppressed in these cells. As the dysfunction of monocytes appears to be attributable to immunosuppression in RCC patients, we are currently in the process of identifying the molecules that are derived from cancer tissue and are able to suppress monocytes. Additional investigation regarding the strategy required to overcome the dysfunction of $CD14^{+}HLA\text{-}DR^{low/-}$ monocytes and to generate immune stimulatory myeloid lineages is required in order to achieve an improved outcome for cancer patients.

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