Tumor-Infiltrating T Cells Concurrently Overexpress CD200R with Immune Checkpoints PD-1, CTLA-4, and TIM-3 in Non-Small-Cell Lung Cancer

Yinghan Su^{a, b} Shota Yamazaki^{a, b} Ryo Morisue^{b, c} Jun Suzuki^{b, d}
Toshiaki Yoshikawa^e Tetsuya Nakatsura^e Masahiro Tsuboi^d Atsushi Ochiai^{a, f}
Genichiro Ishii^{a, b, g}

^aLaboratory of Cancer Biology, Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Kashiwa, Japan; ^bDivision of Pathology, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa, Japan; ^cDepartment of Hepatobiliary and Pancreatic Surgery, National Cancer Center Hospital East, Kashiwa, Japan; ^dDepartment of Thoracic Surgery, National Cancer Center Hospital East, Kashiwa, Japan; ^eDivision of Cancer Immunotherapy, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa, Japan; ^fExploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa, Japan; ^gDepartment of Pathology and Clinical Laboratories, National Cancer Center Hospital East, Kashiwa, Japan

Keywords

CD200R · Cytotoxic T-lymphocyte-associated protein 4 · Immune checkpoints · Programmed cell death protein 1 · Tumor-infiltrating leukocytes

Abstract

Introduction: CD200R has been reported to be the receptor for the immune checkpoint molecule CD200 and can transduce immune-suppressive signals. In this study, we mainly focused on the expression level of CD200R in T cells in pulmonary artery (PA) blood and non-small-cell lung cancer (NSCLC) tumor tissue. **Methods:** Immune cells were isolated from dissected tumor samples and PA blood of NSCLC patients and analyzed with multiparameter flow cytometry. The co-expression of CD200R with other immune check-

points, including programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), was also investigated. **Results:** CD200R expression was observed on the surface of approximately 75% of T cells among tumor-infiltrating leukocytes (TILs). Compared to T cells extracted from TILs, only 55% of T cells extracted from PA blood exhibited CD200R expression. Moreover, with higher expression of CD200R, the expression of other immune checkpoints, including PD-1, CTLA-4, and TIM-3, was also increased in tumor-infiltrating T cells compared to T cells in PA blood. **Conclusions:** Our results showed that those tumors were dominated by T cells expressing CD200R together with other checkpoints, which suggests a phenotypic change after T cell infiltration into the tumor, such as T cell exhaustion.

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Introduction

The discovery of immune checkpoints has proven to be one of the most important advancements for cancer therapy development. Immune checkpoints are the key molecules that act as brakes to restore immune homeostasis after diseases, and those molecules are hijacked by cancer cells to inhibit immune activities. The currently best-studied checkpoints are programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Immune checkpoint inhibitors (ICIs) have already been administered as first-line treatments for advanced-stage non-small-cell lung cancer (NSCLC) patients. Compared with standard chemotherapy, ICIs have led to promising, durable responses and exhibited possible benefits for long-term survival [1, 2].

CD200R and its ligand, CD200, have also been reported to be functional immune checkpoint molecules. To date, it is well documented that the interaction between CD200 and CD200R triggers an immune suppression signal that inhibits the RAS/MAPK signal cascades, resulting in a decrease in the production of inflammatory cytokines and an increase in anti-inflammatory cytokine secretion [3–7].

The potential of CD200-CD200R has been widely discussed in the context of diseases or therapies related to the immune system, such as arthritis, infection, and transplantation [8–10]. Many previous studies have claimed that CD200 expressed by melanoma, ovarian cancer, breast cancer, and hepatocellular carcinoma can interact with CD200R, which is a prognostic factor or even a biomarker for cancer stem cells [11–14]. Meanwhile, CD200R was reported as a prognostic factor in lung cancer, and high stromal CD200R expression was strongly connected with non-adenocarcinoma histological types (e.g., squamous cell carcinoma and other subtypes) and also with advanced disease stage [15].

The expression of CD200R is restricted to the surfaces of immune cells, such as monocytes, macrophages, neutrophils, and dendritic cells from the myeloid lineage as well as T and B cells from the lymphoid lineage [3–5, 16]. On the other hand, CD200 is reported to be widely distributed among cells of both hematopoietic and nonhematopoietic origins, such as neurons, endothelial cells, and fibroblasts, as well as among multiple types of cancer [17–20].

Although ICIs have already had great achievement in the treatment of several types of cancer, there is still a proportion of patients who show no response to ICI therapy. Investigations in reliable biomarkers and novel check-

points are intensely required in order to enhance the efficacy of ICIs. In this study, we investigated the expression of CD200R in NSCLC tumor tissue and blood taken from dissected lung pulmonary artery (PA) and confirmed its co-expression with three other immune checkpoint molecules.

Materials and Methods

Tumor-Infiltrating Leukocyte (TIL) Extraction

Human lung specimens were obtained from the surgically resected lung tissues of NSCLC patients at National Cancer Center Hospital East. The tissue specimens were cut into small pieces with sterilized scissors and tweezers. The minced specimens were then transferred to a 50-mL centrifuge tube with pre-prepared BD Horizon™ Dri Tumor & Tissue Dissociation Reagent (BD Biosciences, Inc., Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The mixture was placed in a 37 °C water bath for 30 min with frequent agitation until the tissue was enzymatically dissociated into a single-cell suspension. The enzymatic reaction was stopped by washing with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) with 1% bovine serum albumin (Life Technologies, Carlsbad, CA, USA) and ethylenediaminetetraacetic acid (final concentration 2 mM; Sigma-Aldrich). The cell suspension was pooled with the stored supernatant and filtered through a 70-µm filter (Corning Inc., Corning, NY, USA). After centrifugation, the pelleted samples were treated with BD Pharm Lyse Buffer (BD Biosciences, Inc.) to remove the erythrocytes. After centrifugation, the TIL layers in the tubes were collected and counted with a hemocytometer for downstream assays.

Peripheral Blood Mononuclear Cell (PBMC) Isolation and Collection

Human peripheral blood was isolated with a heparin (Mochida Pharmaceutical Co., Ltd, Tokyo, Japan)-coated 10-mL syringe from the PA of surgically resected primary lung cancer tissues as previously reported [21]. PBMCs were isolated through centrifugation with BD Vacutainer Evacuated Blood Collection Tubes (BD Biosciences, Inc.) according to the manufacturer's instructions. After centrifugation, the blood mononuclear cell layers in the tubes were collected and counted with a hemocytometer for downstream assays.

T Cell Purification

The cells obtained from the tumor tissue or PA blood were washed with PBS, quantified, and positively selected by passing through a magnetic-activated cell sorting (MACS) medium column with anti-CD3 antibody-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of CD3+ T cells was further confirmed with flow cytometry analysis.

Fluorescence-Activated Cell Sorting (FACS) Analysis

The expression of cell surface antigens was determined by using multicolor immunofluorescence staining and a minimum of 10,000 events were counted with the FACS Diva software (BD Biosciences, Inc.). The conjugated antibodies used were CD3-APC (clone: UCHT1), CD14-APC (clone: 63D3), CD20-APC (clone: 2H7), CD4-

APC, CD4-Alexa Fluor 700 (clone: RPA-T4), CD8a-APC, CD8a-Alexa Fluor 700 (clone: HIT8a), CD200R-PE, CD200R-APC (clone: OX-108), CD279-PE (CD279: PD-1; clone: EH12.2H7), CD152-PE (CD152: CTLA-4; clone: L3D10), and CD366-PE (CD366: T cell immunoglobulin and mucin domain-containing protein 3 [TIM-3]; clone: F38-2E2; BioLegend, San Diego, CA, USA). Fluorescence-conjugated mouse IgG1 (PE, APC & Alexa Fluor 700; clone: MOPC-21) and mouse IgG2b (APC; clone: MPC-11) κ isotype Ctrl antibodies (BioLegend) were used as negative controls. Detailed information on all antibodies is provided in online supplementary Table 1 (see www.karger.com/doi/10.1159/000511557 for all online suppl. material). In brief, 100 μ L of cell suspension (containing 5×10^5 cells) were incubated with 5 μ L of conjugated antibodies for 1 h at 4 °C in the dark. Excess antibodies were removed by washing the cells with PBS (containing 3% FBS). Then, FACS analysis was performed by using a FACS Aria or FACS Accuri C6 plus (BD Biosciences, Inc.). Single-spectrum staining of the aforementioned reagents was used for compensation. The final data analysis was conducted with Flowing Software version 2.51, developed by Perttu Terho (accessed from www.flowingsoftware.com).

Public Database Analysis

To explore whether the results could be generalized to a wider population, an analysis was conducted of a TCGA mRNA expression dataset that included 584 primary lung adenocarcinoma patients by using the cBioPortal for Cancer Genomics web site [22, 23]. Based on the median CD200R1 gene expression, all samples were divided into two groups (the CD200R-high and CD200R-low groups). The correlations between the gene expression of CD200R1 and the gene expression of six different immune checkpoint molecules and another nine immune-related genes were evaluated.

Statistical Analysis

The significance of differences between the two groups was examined by using Student's *t* test (paired or unpaired *t* tests as indicated), and *p* < 0.05 was considered statistically significant.

Results

CD200R Expression Was Observed Mainly in CD3+ TILs in NSCLC

To confirm whether the expression of CD200R is mainly limited to cell populations of the myeloid lineage, TILs were collected through the enzymatic digestion of dissected tumor specimens for flow cytometry analysis (Fig. 1a; online suppl. Fig. 1A, 1B). Among the infiltrating immune cells, CD3+ T cells accounted for an average of 46.55% of all extracted cells (Fig. 1b), which was significantly higher than the proportions of monocytes (CD14+; 11.94%) and B cells (CD20+; 17.61%). In addition, CD200R was expressed on the surface of 70.52% of CD3+ T cells (Fig. 1c), and this expression was significantly higher than that of CD14+ cells (46.64%) and CD20+ cells (61.00%).

Next, PBMCs were collected from the PA blood of resected lungs, and a similar proportion of CD200R+ cells

were observed; of these CD200R+ cells, T cells were most abundant among the extracted immune cells (online suppl. Fig. 1C). In contrast to tumor-infiltrating T cells, only 55.06% of the CD3+ T cells extracted from PA blood were found to have CD200R expression (Fig. 1d). To further characterize CD200R+ T cells, MACS was conducted to purify CD3+ T cells from all PBMCs to evaluate the expression levels of CD200R on CD4+ and CD8+ T cells (online suppl. Fig. 1D). As Figures 1d and e show, both CD4 (73.71%) and CD8 (66.05%) T cells expressed CD200R.

A Higher Proportion of CD200R Expression Was Observed in Tumor-Infiltrating T Cells than in Peripheral Blood T Cells

To further confirm the differences in CD200R expression between tumor-infiltrating T cells and PA blood-derived T cells, MACS-sorted CD3+ T cells from PA and TILs (online suppl. Fig. 2A, B) were analyzed. Twelve samples of T cells showed that tumor-infiltrating T cells were dominated by CD200R+ T cells, compared to peripheral blood T cells (Fig. 2a). Moreover, similar results were obtained in three paired samples of PA blood and tumor tissue (Fig. 2b). On the other hand, CD8+ cells tend to be the main reason for this increase in the CD200R+ population (Fig. 2c), while those numbers in CD4+ cell might differ from sample to sample (Fig. 2d).

Co-Expression of CD200R and Other Immune Checkpoint Molecules

Given that CD200R is an immune checkpoint molecule which regulates immune cell activity, we investigated cells collected from the paired PA blood and tumor specimens by FACS analysis to determine whether other immune checkpoint molecules may be upregulated and co-expressed with CD200R. An average of 52.02% of tumor-infiltrating T cells expressed both PD-1 and CD200R (Fig. 3a, left). In comparison, only an average of 23.21% of T cells extracted from PA blood expressed both PD-1 and CD200R. Similarly, co-expression of PD-1 and CD200R was also significantly increased among CD4+ (1.61-fold) and CD8+ (1.79-fold) T cells when comparing T cells derived from PA blood and those derived from tumor tissue (Fig. 3a, right; online suppl. Fig. 3A and 3B, left, and 3C, 3D). For CTLA-4 and TIM-3, both of these checkpoints showed a limited level of expression on the surface of CD3+ T cells derived from PA blood (CTLA-4: 1.19%; TIM-3: 0.89%; Fig. 3b and c, left). Among tumor-infiltrating T cells, CTLA-4 was co-expressed with CD200R on 21.88% of CD4+ T cells and 20.75% of CD8

Fig. 1. CD200R-expressing cells within TILs and peripheral blood mononuclear cells extracted from PA blood. **a** FACS analysis of CD200R+ cells in CD3 (left), CD14 (middle), and CD20 (right) cells. **b** Proportion of CD3+ T cells, CD14+ monocytes, and CD20+ B cells within extracted TILs ($n = 10$). * $p < 0.05$ according to Student's t test. **c** Proportion of CD200R+ cells within each cell popu-

lations ($n = 10$). * $p < 0.05$ according to Student's t test. **d** Proportion of T cells with CD3+/CD200R+, CD4+/CD200R+, or CD8+/CD200R+ among all CD3+ T cells extracted from PA blood ($n = 12$). **e** Proportion of CD200R+ cells within the CD4 and CD8 T cell populations ($n = 12$). FACS, fluorescence-activated cell sorting; PA, pulmonary artery; TILs, tumor-infiltrating leukocytes.

cells, which was significantly upregulated compared to paired PA samples (Fig. 3b, c; online suppl. Fig. 3A, B, middle and right). Moreover, as Figure 3c shows, the co-expression of TIM-3 and CD200R was limited to CD8 T cells and showed differences between the PA and TIL samples.

Public Database Analysis Confirmed a High Level of Immune Checkpoint Expression in the CD200R-High Group

To confirm that the co-expression of CD200R and other immune checkpoints can be generalized to a wider population, an analysis of a TCGA lung adenocarcinoma

Fig. 2. Comparison of the proportions of CD200R+ T cells in PA blood and TILs. **a** Proportion of CD200R+ T cells in CD3+ cells from the PA and TIL (unpaired) samples ($n = 12$). * $p < 0.05$ according to unpaired Student's t test. **b** Proportion of CD200R+ T cells in CD3+ cells from the PA and TIL (paired) samples ($n = 3$). Analyzed with paired Student's t test. **c** Proportion of CD200R

among CD4+ T cells between paired PA and TIL samples ($n = 3$). Analyzed with Student's t test. **d** Proportion of CD200R in CD8+ T cells in the paired PA and TIL samples ($n = 3$). Analyzed with Student's t test. PA, pulmonary artery; TIL, tumor-infiltrating leukocyte.

database was introduced into this study. Based on the median CD200R mRNA (CD200R1) expression level, 517 samples were separated into the CD200R-high and CD200R-low groups. The mRNA expression levels of six immune checkpoint molecules, including PD-1 (PDCD1), CTLA-4, TIM-3 (HAVCR2), lymphocyte-activation gene 3 (LAG-3), T cell immunoreceptor with Ig and ITIM do-

main (TIGIT), and indoleamine-pyrrole 2,3-dioxygenase, and the correlations of these molecules with CD200R1 were investigated. All six immune checkpoint molecules showed significantly higher expression levels in the CD200R-high group (Fig. 4). At the same time, the other immune-related genes also showed a similar tendency (online suppl. Fig. 4).

Fig. 3. Co-expression of other immune checkpoints in T cells. **a** Proportion of T cells co-expressing CD200R and the immune checkpoint PD-1 within CD3+ (left), CD4+, and CD8+ T cells (right) from the paired PA and TIL samples ($n = 3$). Analyzed with Student's t test. **b** Proportion of T cells co-expressing CD200R and the immune checkpoint CTLA-4 within CD3+ (left), CD4+, and CD8+ T cells (right) from the paired PA and TIL samples ($n = 3$). Analyzed with Student's t test. **c** Proportion of T cells co-express-

ing CD200R and the immune checkpoint TIM-3 within CD3+ (left), CD4+, and CD8+ T cells (right) from the paired PA and TIL samples ($n = 3$). Analyzed with Student's t test. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PA, pulmonary artery; PD-1, programmed cell death protein 1; TIL, tumor-infiltrating leukocyte; TIM-3, T cell immunoglobulin and mucin domain-containing protein 3.

Fig. 4. Expression of CD200R in lung adenocarcinoma and its correlation with immune checkpoints according to public database analysis. Comparing the co-expression of mRNA between samples, separated according to medium expression value (CD200R-low and CD200R-high group). Cohort: lung adenocarcinoma

(TCGA, Firehose Legacy), 517 samples derived from 516 patients. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IDO, indoleamine-pyrrole 2,3-dioxygenase; LAG-3, lymphocyte-activation gene 3; PD-1, programmed cell death protein 1; TIGIT, T cell immunoreceptor with Ig and ITIM domains.

Fig. 5. Compared with the inflow from the PA, T cells within the cancer tissue show a significantly higher proportion of CD200R expression together with some other immune checkpoints, which suggests that a phenotypic change has occurred within the tumor microenvironment. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PA, pulmonary artery; PD-1, programmed cell death protein 1; TILs, tumor-infiltrating leukocytes.

Discussion

In this study, we demonstrated that CD200R was highly expressed in tumor-infiltrating T cells in NSCLC, and CD200R was highly co-expressed with multiple immune checkpoint molecules (Fig. 5). Most studies only focused on CD200R expression and its function among myeloid lineage cells [5, 7, 18]. However, our results demonstrated that both B and T cells express CD200R. Based on the current model of cancer-immune cycle [24], those T cells collected from cancer patients' PA were recruited from nearby lymph nodes and were activated by antigen-presenting cells. The PA blood used in the experiments was considered to be the inflow tract into the lung tissue, delivering blood circulating immune cells into the tumor. The significant differences of the immune checkpoint profile between the inflow tract of PA

blood and accumulated tumor-infiltrating immune cells suggested that significant changes to their phenotype may have happened, and these changes might be due to the changes in the surrounding microenvironments, which is also reinforced by the results of public database analysis.

It is well reported that most tumor-infiltrating T cells have entered the stage of exhaustion or dysfunction [25]. Many studies have confirmed that the exhausted or dysfunctional T cells simultaneously express multiple immune checkpoint molecules, including PD-1, CTLA-4, TIM-3, LAG-3, and TIGIT [26–29]. Different levels of checkpoint co-expression represent highly heterogeneous T cell populations with different stages of exhaustion, e.g., a subpopulation of T cells expressing both PD-1 and TIM-3 was well reported as a terminal exhausted group with no polyfunctionality retained [30–32]. It is widely accepted that before T cells differentiate into the terminal exhausted stage, dysfunction of T cells might be revised through the introduction of ICIs, such as anti-PD-1 [33], which can tip the balance of immune microenvironments from cold (inhibitory) to hot (activating). One previous study claimed that CD4 T cells increased CD200R expression level and loss of multifunctional potential during chronic infection [34], which might have happened similarly inside tumor tissue. The fact that CD200R is also largely co-expressed with other checkpoints inside tumors suggests that tumor-infiltrating T cells have entered the exhaustion stage. A combination of CD200R and other checkpoints might be able to act as a biomarker which can specify a subgroup of T cells with a certain level of exhaustion. Furthermore, a combinational blockage strategy of PD-1 and CTLA-4 has been designed, aiming to broaden the horizon for ICIs [35, 36]; targeting CD200R together with other checkpoints could be a promising candidate to restore T cell activities. Although an inhibitor of CD200 has currently entered into a clinical trial for chronic lymphocytic leukemia and multiple myeloma [37], the results of a lung cancer study claimed that CD200 might be a favorable prognostic factor [15]. Moreover, our previous study indicated that CD200 might potentially augment cancer cell sensitivity to chemotherapy [17, 38], which suggests that CD200 may potentially have functions other than immune modulation.

For revealing different stages of T cell infiltration and their connections to the CD200-CD200R axis, the better approach might be to construct a reliable model, such as a mouse or organoid model. Introducing a stabilized model will be helpful for downstream metabolic

analysis and epigenetic studies that may further elucidate the subpopulations of CD200R+ T cells and exhausted cells. It also provides an option of inhibitor administration which can further confirm the mechanism of the CD200-CD200R axis and the potential of targeting CD200R.

In conclusion, our research investigated the expression level of an immune checkpoint, CD200R, in PA blood and TILs in NSCLC. The tumor-infiltrating T cells were more strongly dominated by CD200R-expressing T cells compared to the T cells from PA blood. Moreover, the expression of CD200R largely overlapped with that of other immune checkpoint molecules, including PD-1, CTLA-4, and TIM-3. Considering this finding, co-expression of CD200R and other immune checkpoints might be a good biomarker of T cell phenotypic changes or even a potential target for immune therapy.

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Statement of Ethics

This retrospective study was approved by the National Cancer Ethical Review Board (IRB approval number 2005-043).

Conflict of Interest Statement

No conflicts of interest are involved in this study.

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Author Contributions

Y. Su: design, arrangement, conduction of the work, collection and analysis of all data, writing and editing of the manuscript including all pictures. S. Yamazaki and J. Suzuki: help with design. R. Morisue: helping with statistical analysis. T. Yoshikawa and T. Nakatsura: help with MACS techniques. M. Tsuboi: provision of all surgery specimens. A. Ochiai: help with design and statistical analysis. G. Ishii: coordination of the research, editing and revision of the manuscript.

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