論 文 の 内 容 の 要 旨

論文題目　Investigation of immune checkpoint CD200-CD200R axis within non-small cell lung cancer

（非小細胞肺癌における免疫チェックポイントCD200-CD200R axisの検討）

氏　名　蘇　英晗

## Thesis Summary

Laboratory of Cancer Biology ID: 47-187304　Yinghan Su

**Introduction**

Tumor is a chaotic complex, which is including not only cancer cells but also stromal cells, tumor-infiltrating immune cells and extracellular matrix. As one of the most abundant stromal cell types, cancer-associated fibroblasts (CAFs) were widely reported about their important roles in tumor progression and therapeutic process. Beyond indirect cell-cell interaction in shaping a tumor-favor immune microenvironment, studies have shown that CAFs may also present immune checkpoints.

Based on a further understanding in both immunology and TME, 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, and this mechanism is hijacked by cancer cells. Immune checkpoint inhibitors (ICIs) have already been administered as first-line treatments for advanced-stage non-small cell lung cancer (NSCLC) patients.

CD200R and its ligand, CD200, have also been reported to be functional immune checkpoint molecules. The potential of CD200-CD200R has been widely discussed in the context of diseases or therapies related to the immune system. Many previous studies have claimed that CD200 expressed by cells of melanoma, ovarian cancer, breast cancer and hepatocellular carcinoma, which is a prognosis factor or even a biomarker for cancer stem cells. Meanwhile, CD200R was reported as a prognosis factor in lung cancer, and high stromal CD200R expression was strongly connected with advanced disease stage.

Although ICIs have already achieved a great achievement in treatments of several types of cancer, there are still certain ratios of patients who show no response to ICI therapy. Investigations in reliable biomarker and novel checkpoints are intensely required. In this study, we attempted to investigate CD200-CD200R axis within NSCLC and reported that the varies expression of CD200R in NSCLC tumor tissue and blood taken from the dissected pulmonary arteries (PA) and confirmed its co-expression with three other immune checkpoint molecules.

**Results**

1. *Investigation of CD200 RNA and protein expression within CAF library*

In order to confirm the genericity of CD200-expressing CAFs among NSCLC patients, we investigated CD200 RNA and surface protein expression level with Real-time polymerase chain reaction (RT-PCR) and Fluorescence-activated cell sorting (FACS) analysis among our pre-established CAF library. Four cases among 19 CAF samples were constantly express CD200 at both mRNA and membrane protein level (shown in Fig. 1)

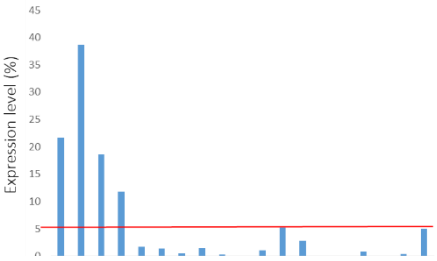


**Fig. 1. CD200 protein expression (FACS) within CAF library**

2. *Investigation of CD200R expression within tumor-infiltrating leukocytes and peripheral blood mononuclear cells (PBMCs) extracted from pulmonary artery (PA) blood in NSCLC*

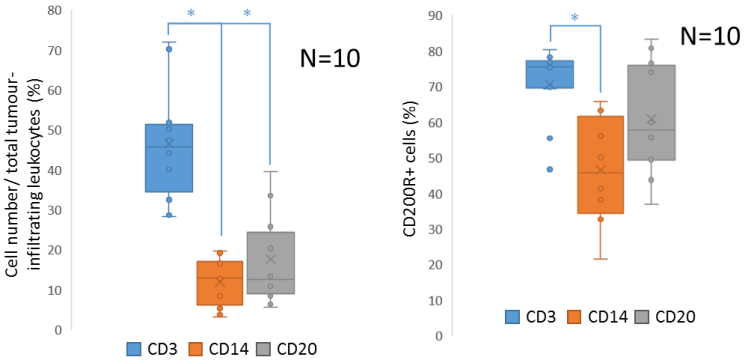


**Fig. 2A & 2B. CD200R expressing cells within TILs**

To confirm whether the expression of CD200R is mainly limited to cell populations of the myeloid lineage, tumor-infiltrating leukocytes were collected through the enzymatic digestion of dissected tumor specimens for flow cytometry analysis (shown in Fig. 2A, 2B). Among the infiltrating immune cells, CD3+ T cells accounted for an average of 46.55% of all the extracted cells, 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, and this expression was significantly higher than that of CD14+ cells (46.64%) and CD20+ cells (61.00%).

Next, peripheral blood mononuclear cells (PBMCs) were collected from the pulmonary artery (PA) blood of resected lung specimens. In contrast to tumor-infiltrating T cells, only 55.06% of CD3+ T cells extracted from PA blood were found to have CD200R expression. To further characterize CD200R+ T cells, magnetic-activated cell sorting was conducted to purify CD3+ T cells from all PBMCs, to evaluate the expression levels of CD200R on CD4+ and CD8+ T cells (shown in Fig. 2C).

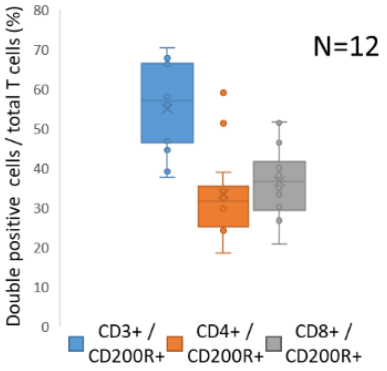


**Fig. 2C. CD200R+ cells within PBMCs extracted from PA blood**

3. *Construction of the co-culture system and CD200-CD200R axis interaction between CAFs and immune cells*

To investigate the CD200-CD200R axis, a co-culture system has been constructed, which the expanded T cells (extracted from PA) were seeded with either CD200-negative or CD200-positive CAFs (shown in Fig. 3).

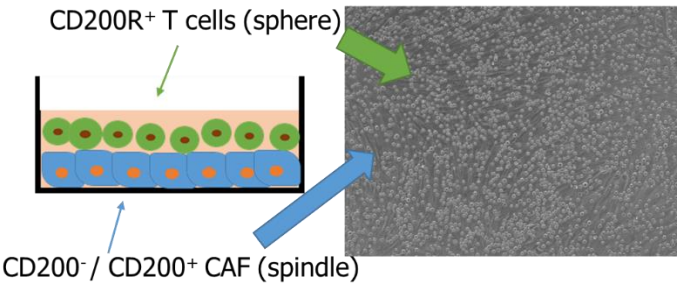


**Fig. 3. Co-culture system for CD200-CD200R axis interaction between CAFs and T cells**

For the functional assay, T cell apoptosis and necrosis levels were checked with Annexin V and propidium iodide (PI) by FACS analysis, which no significant differences of apoptosis or necrosis levels were found among 3 samples. The cytokine expression pattern was first checked with RT-PCR. However, neither pro-inflammatory cytokines (IFN-γ; TNF-α) nor anti-inflammatory cytokines (TGF-β, IL-4, IL-10 and IL-13) expressed by T cells has shown a significant difference between co-culture with CD200-negative or CD200 overexpressed CAFs (shown in Fig. 4).

To further confirm the interaction between CD200 and CD200R, the recombinant CD200 protein was added into the culture medium of T cells in different concentration. No dose-dependent pattern of cytokine expression was observed. In addition, a blocking antibody of CD200R was added into medium of the co-culture system to interfere with the CD200 and CD200R interaction between cells. As showed in Figure 5, the up-regulation of IL-4 cannot be cancelled by introducing CD200-blocking antibody.

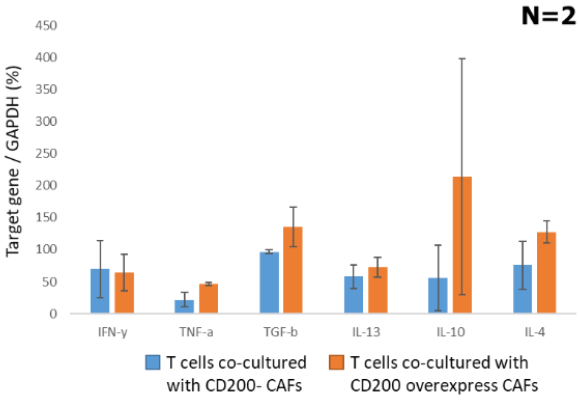


**Fig. 4. Cytokine expression were checked with RT-PCR among two T cell samples**

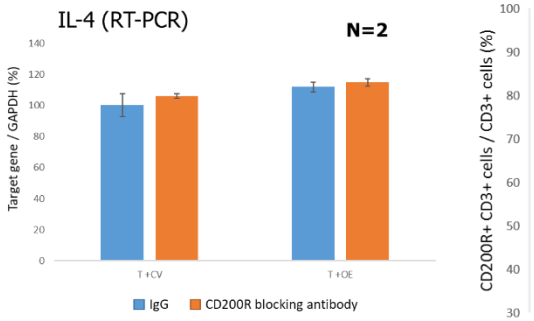


**Fig. 5. Investigate CD200-CD200R interaction between CAFs and T cells**

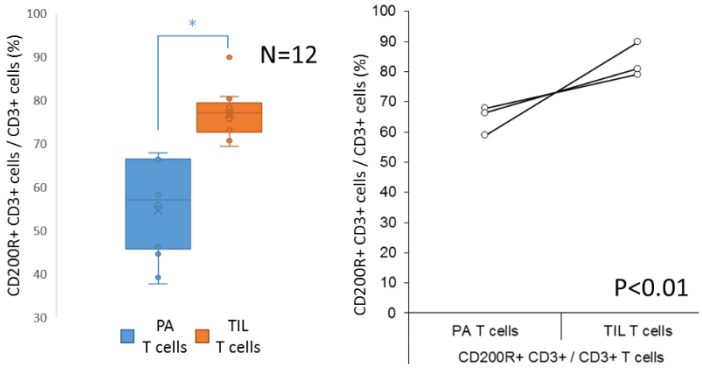


**Fig. 6. Comparison of the proportions of CD200R expressing T cells in PA blood and TILs.**

4. *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 paired PA blood and TIL samples 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 (shown in Fig. 6A). Moreover, similar results were obtained in 3 paired samples of PA blood and tumor tissue (shown in Fig. 5B).

*5. Co-expression of CD200R and other immune checkpoints*

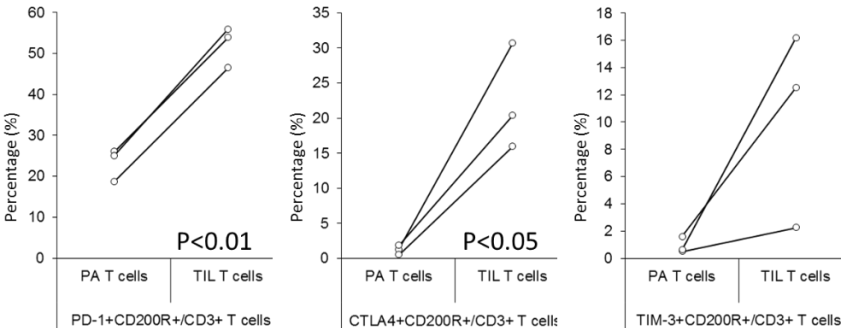


**Fig. 7. Co–expression of other immune checkpoint molecules in CD200R expressing T cells**

Paired specimens were analysed to determine whether other immune checkpoint molecules may be co-expressed with CD200R (shown in Fig. 7). An average of 52.02% of tumor-infiltrating T cells expressed both PD-1 and CD200R. In comparison, only an average of 23.21% of T cells extracted from PA blood expressed both PD-1 and CD200R. 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%; TIM3: 0.89%). 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 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. All six immune checkpoints showed significantly higher expression levels in the CD200R-high group.

**Discussion & Conclusion**

In collusion, our research investigated the expression level of a pair of immune checkpoint molecules, CD200 in CAFs, and CD200R in PA blood and TILs within NSCLC. Through checking CAF library which was pre-established within our lab, CD200+ CAFs were found about roughly 20-25% in NSCLC samples. In addition, our result indicated that TILs were dominated by CD200R-expressing T cells. This suggested that CD200R may potentially not only participate in the innate immune response but also have a role in the adaptive immune response. Moreover, the expression of CD200R largely overlapped with that of other immune checkpoint molecules. Unfortunately, the established co-culture system was not suitable to confirm or ensure the interaction among CD200-expressing CAFs and CD200R-expressing immune cells would be one of the main limitations of this study.

Many studies have confirmed that the exhausted or dysfunctional T cells simultaneously express multiple immune checkpoints. It is widely accepted that before T cells entered the terminal exhausted stage, dysfunction of T cells might be refined through the introduction of ICIs, such as anti-PD1. 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. 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.

**Publication as the first author**

1. **Su Y**, Yamazaki S, Morisue R, Suzuki J, Yoshikawa T, Nakatsura T, Tsuboi M, Ochiai A, Ishii G. Tumor-infiltrating T cells concurrently overexpress CD200R with immune checkpoints PD-1, CTLA-4 and TIM-3 in non-small cell lung cancer. *Pathobiology.* [Epub ahead of print]. doi: 10.1159/000511557