Regulation of Specific Cell Clusters in TCR-T Cells Responding to Differential Expression of Tumor PD-L1

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Abstract

PD-L1 signaling is essential in regulating T cell function and keeping the balance of tumor microenvironment, but its role in modifying TCR-T cell cytotoxicity remains unknown. MART-1-specific TCR-T cells (TCR-TMART-1) were stimulated by MEL-526 tumor cells expressing different proportions of PD-L1 and used to perform cytotoxicity assays and single-cell RNA sequencing. Percentage changes of different specific cell clusters were analyzed. The percentage of cluster HLA-DR+CD38+CD8+ was upregulated after antigen stimulation, and tumor PD-L1 modified TCR-T cell function through downregulating the percentages of HLA-DR+CD28+CD8+ and HLA-DR+CD38+CD8+ subsets which were higher in TCR-TMART-1 than in Tnull.

Index terms— TCR-T, PD-L1, scRNA-seq, cell clusters, gene expression.

1 Regulation of Specific Cell Clusters in TCR-T Cells Responding to Differential Expression of Tumor PD-L1

Introduction D-L1 (programmed death-ligand 1) takes participation in regulating T cell-mediated immune responses for tumor evasion from the immune system, thus promotes cancer development and progression [1]. PD-L1 is also known as CD274 or B7-H1 and is one ligand for PD-1 (programmed death-1). PD-L1 is wildly expressed on tumor cells of various types of malignancies, including melanoma, while PD-1 is highly expressed in tumor-infiltrating lymphocytes **??**2]. PD-L1 interacts with PD-1 resulting in T cell dysfunction and exhaustion, but the effect of tumor PD-L1 expression on TCR-T (T-cell receptor-engineered T cells) cell function has not been comprehensively studied. TCR-T cell therapy has great potential in mitigating tumor development, especially for solid tumors. The number of clinical trials with TCR-T cell therapy is increasing each year, and among them, the most targeted cancer type is melanoma [3]. Therefore, it's important to investigate how tumor PD-L1 expression affects TCR-T cell functionality.

In our study, single-cell mRNA sequencing (scRNA-seq) was performed to investigate MART-1specific TCR-T cells responding to different proportions of PD-L1 + melanoma cells. Distribution of specific cell clusters such as HLA-DR + CD28 + CD8 + and HLADR + CD38 + CD8 + was modified with increasing ratio of tumor PD-L1.

2 II.

3 Results

4 a) TCR-T MART-1 killed MEL-526 tumor cells efficiently at E:T ratio of 1:1

HLA-A*0201/MART-1-specific TCR sequence was obtained from T cells after stimulation with MART-1 (aa27-35, LAGIGILTV) peptide (data unpublished) and designed as TCR MART-1 (Fig. 1). To avoid mispairing with

endogenous TCR [4], TCR MART-1 ? and ? chains were fused with the constant region of murine TCR and synthesized before cloned into the lentiviral vector (Fig. 1). After transfection of TCRMART-1 lentivirus, CD8 + T cells expressed TCR MART-1 or not were designed as TCR-T MART-1 and T null , respectively. TCR-T MART-1 and T null cells were stimulated with peptide-loaded MEL-526 melanoma cells at different E:T ratios (1:1, 1:2, and 1:4. Fig. **??**) to assess the killing capacity. Compared to T null , TCR-T MART-1 killed tumor cells more efficiently, especially at E:T ratio of 1:1 (Fig. **??**).

5 b) Regulation of specific T cell clusters responding to different proportions of PD-L1+ tumor cells

To verify the effect of tumor PD-L1 expression on TCR-T cell function, MEL-526 cells expressing low, intermediate, and high levels of PD-L1 (data unpublished, designed as PD-L1 low , PD-L1 int , and PD-L1 high , respectively and PD-L1 expression ratio was about 3%, 50%, 100%) were incubated with TCR-T MART-1 (50% TCR MART-1 +). The percentage of specific CD8 + T cell clusters, including CX3CR + , HLA-DR + , HLA-DR + CD28 + , and HLA-DR + CD38 + , were analyzed in T cells (Fig. **??**). The percentages of CX3CR + , HLA-DR + , and HLADR + CD28 + clusters were decreased, while the proportion of HLA-DR + CD38 + subsetwas increased after antigen stimulation (Fig. **??**). Furthermore, the ratios of HLA-DR + CD28 + and HLA-DR + CD38 + subpopulations were reduced by the increasing proportion of tumor PD-L1 (Fig. **??**).

When T cells were further divided into T null and TCR-T MART-1 , the percentages of HLA-DR + CD28 + and HLA-DR + CD38 + clusters were higher in TCR-T MART-1 than in T null (Fig. 4). Consistently, the percentages of HLA-DR + CD28 + and HLA-DR + CD38 + clusters in both T null and TCR-T MART-1 was downregulated with the increasing proportion of tumor PD-L1 (Fig. 4). c) CX3CR + CD8 + cluster was characterized by GZMA expression Differentially expressed genes (DEGs) were analyzed in these specific clusters. Except for CX3CR1, the expression of cytotoxic genes GZMA and NKG7 and chemokine CCL5 was upregulated in the CX3CR+ cluster, while the expression of IL2RA, XCL1, and GZMB were downregulated compared to CX3CRcells (Fig. 5A). After gene oncology (GO) analysis, T cell activation and cell-cell adhesion related signaling were enriched in the CX3CR+ cluster (Fig. 5B).

6 d) HLA-DR + CD8 + cluster was characterized by IL32 and GZMA expression

In addition to GZMA and CCL5 expression, which was upregulated in the CX3CR + cluster as well, the expression of cytokine IL32 was increased in the HLA-DR + CD8 + cluster (Fig. **??**A). Endocytic vesicle membrane signaling was enriched in the HLA-DR + CD8 + subset (Fig. **??**B).

7 e) HLA-DR + CD28 + CD8 + cluster was characterized by CD52 expression

The expression of CD52 was increased in addition to CD28 in the HLA-DR + CD28 + CD8 + cluster (Fig. **??**A). Though not so dramatic as that of CD52, the expression of CCL5 and JAK1, which are essential for cytokine signaling, was upregulated as well. Leukocyte activation related pathways were enriched in this cluster (Fig. **??**B), which was much similar to that of the CX3CR + CD8 + population (Fig. 5B).

8 f) HLA-DR + CD38 + CD8 + cluster was characterized by GZMB expression

One characteristic of the HLA-DR + CD38 + CD8 + cluster was the upregulated expression of GZMB (Fig. 8A), which plays a critical role in T cell cytotoxicity. Metabolic process-related signaling pathways were enriched (Fig. 8B), indicating the active status of this cluster.

9 III.

10 Discussion

CX3CR1 expression on CD8 + T cells is associated with cytotoxic capability [5,6]. Consistently, DEG analysis of CX3CR + CD8 + cluster was characterized by upregulated expression of cytotoxic genes GZMA and NKG7 (Fig. 5A) and T cell activation signaling was top enriched in this cluster (Fig. 5B). But the percentage of CX3CR + CD8 + cluster was quite low in T cell populations (Fig. **??**, Fig. 4), indicating a weak role of this cluster under the circumstances. HLA-DR + CD8 + T cells are considered activated cytotoxic T lymphocytes [7], and HLA-DR + CD28 + CD8 + T cells showed telomerase activity with proliferative potential **??**8]. The percentages of clusters CX3CR + CD8 + , HLA-DR + CD8 + , and HLA-DR + CD28 + CD8 + were downregulated, in contrast, the proportion of HLA-DR + CD38 + CD8 + cluster which was defined as activated T cells during the acute phase of viral infections [9], was upregulated after antigen stimulation compared to that in unstimulated Ctrl group (Fig. **??**). The results indicated various changes inproportions of different cell subsets, though they

might have similar functions. On another aspect, the percentages of clusters HLA-DR + CD28 + CD8 + and HLA-DR + CD38 + CD8 + were decreased with the increased proportion of PD-L1 + tumor cells (Fig. **??**), implying the inhibition of tumor PD-L1 on the percentages of clusters HLA-DR + CD28 + CD8 + and HLA-DR + CD38 + CD8 + might result in the damage on TCR-T cell cytotoxicity (unpublished data).

The percentages of clusters HLA-DR + CD28 + CD8 + and HLA-DR + CD38 + CD8 + were higher in TCR-T MART-1 than in T null , while there was no significant change in the distribution of subsets CX3CR + CD8 + and HLA-DR + CD8 + between T null and T MART-1 (Fig. 4). It might be the reason why TCR-T MART-1 were more cytotoxic than T null (Fig. **??**).

11 IV.

12 Conclusions

In conclusion, the landscape of different functional cell clusters in T null and TCR-T MART-1 responding to different proportions of PD-L1 + MEL-526 cells loaded with MART-1 27-35 peptide was provided in this study.

V.

13 Materials and Methods

14 a) Cell culture

HEK293T (ATCC, CRL-11268) cell line was purchased from ATCC, and MEL-526 (BNCC340404) cell line was purchased from BNCC, and they were cultured in DMEM (Gibco, 21063029) added with 10% fetal bovine serum (Hyclone, SH30084.03HI), penicillin (100 IU/mL), and streptomycin (50 ?g/mL) at 37? and 5% CO2. CD8 + T cells were cultured in HIPP-T009 (Bioengine, RG0101302) containing 2% fetal bovine serum (Hyclone, SH30084.03HI), IL-2 (20 ng/ml), IL-7 (10 ng/ml) and IL-15 (10 ng/ml) at 37? and 5% CO 2 .

15 b) Peptide

HLA-A*0201-restricted MART-1 peptide ELAGIGILTV) was synthesized by GenScript (Nanjing, China). The peptide was stored at 10 mg/ml in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) at -20°C.

16 c) Plasmid construction

The constant regions of TCR MART-1 sequence, which was identified from our previous work (data unpublished), were replaced by mouse TCR constant region ? and ?, respectively. TCR MART-1 -encoded DNA was then synthesized by GeneScript (Nanjing, China) and ligated into a lentiviral vector, pRRLSIN.cPPT.PGK (Addgene, 12252).

17 d) Lentivirus production

To produce lentivirus, 293T cells were transfected with a lentiviral vector containing the gene of interest and the packaging constructs (PsPAX2 and PMD2G). The culture medium was collected 72 h after transfection and filtered with 0.45 uM filters (Sartorius). Subsequently, the virus was concentrated by ultracentrifugation at 35,000 rpm for 90 min.

18 e) Generation of MART-1 peptide-specific TCR-T cells

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the blood of HLA-A*0201restricted healthy donors with informed consent. CD8 + T cells were purified from PBMC via human CD8 MicroBeads (MiltenyiBiotec) and activated with T Cell TransAct (MiltenyiBiotec); 36-48 h after activation, CD8 + T cells were transduced with lentivirus in a 6-well or 12well plate. To promote infection efficiency, polybrene was added into the medium at the final concentration of 2?g/mL, and the well plate was centrifuged at 800g for 30 minutes. T cells were then expanded and maintained in T cell medium.

19 f) In vitro killing assays

TCR-T cells were co-cultured with target cells labeled with Carboxyfluoresceinsuccinimidyl ester (CFSE; Invitrogen) at different E: T ratios for 24 h. Cells were then collected and stained with PI for FACS analysis. The cytotoxicity was calculated with the proportion of PI + CFSE + cells divided by the proportion of CFSE + cells.

20 g) Statistical analysis

PRISM 6 (GraphPad Software) and RStudio were used for data analysis. *P<0.05, **P<0.005, ***P?0.001. Error bars represented the Mean±SD.

21 h) Differential gene expression analysis

Seurat FindMarkers were used for DEG analysis. DEGs of each subset were generated relative to all the remained cells. Then DEGs were identified as the criteria: FDR adjusted p value of F test < 0.01.

22 i) Gene set enrichment analysis

The "enrichGO" function in the "clusterProfiler" package was used to perform GO analysis with the corresponding default parameters. Pathways with the q value <0.05 corrected by FDR were used for further analysis.

23 j) Data availability

The data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA: https://db.cngb.org/cnsa/) of CNGBdb with accession numberCNP0001109.

24 k) Ethics approval and consent to participate

The study was approved by the Institutional Review Board on Bioethics and Biosafety of BGI. Written informed consent forms were regularly obtained from all donors.

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Figure legend

PD-L1high PD-L1int PD-L1low T n u ll T C R -T M A R T -1 T n u ll T C R -T M A R T -1 T n u ll T C R -T M A R T -1 T n u ll T C R -T M A R T -1

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Figure 1: Figure 1 :

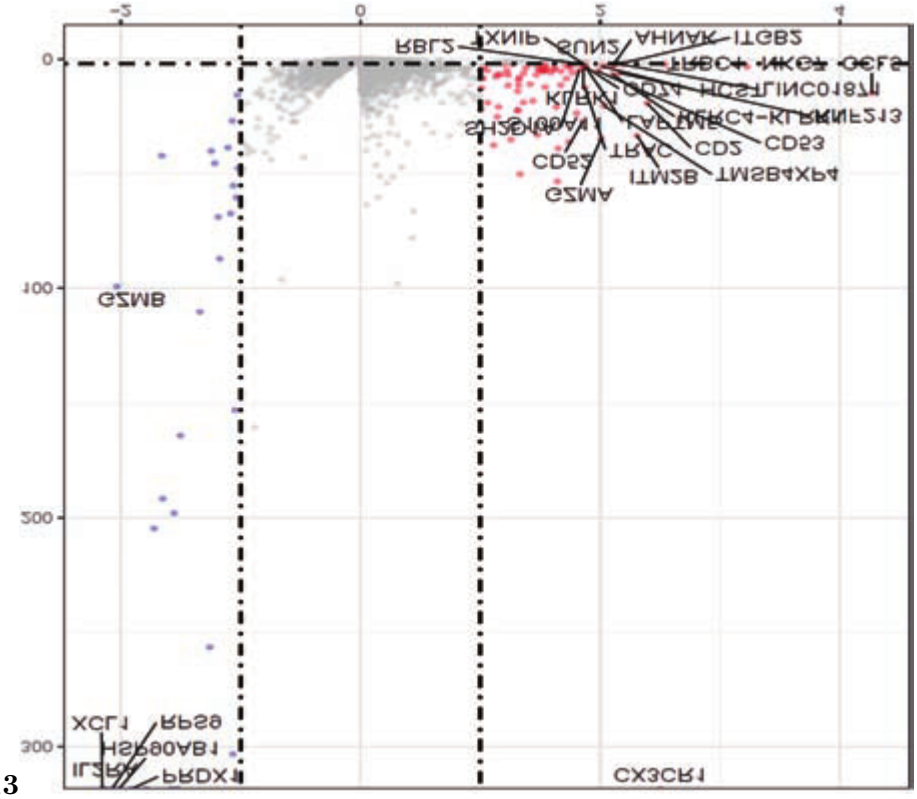

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Figure 2: Figure 2 : 1 EFigure 3 :

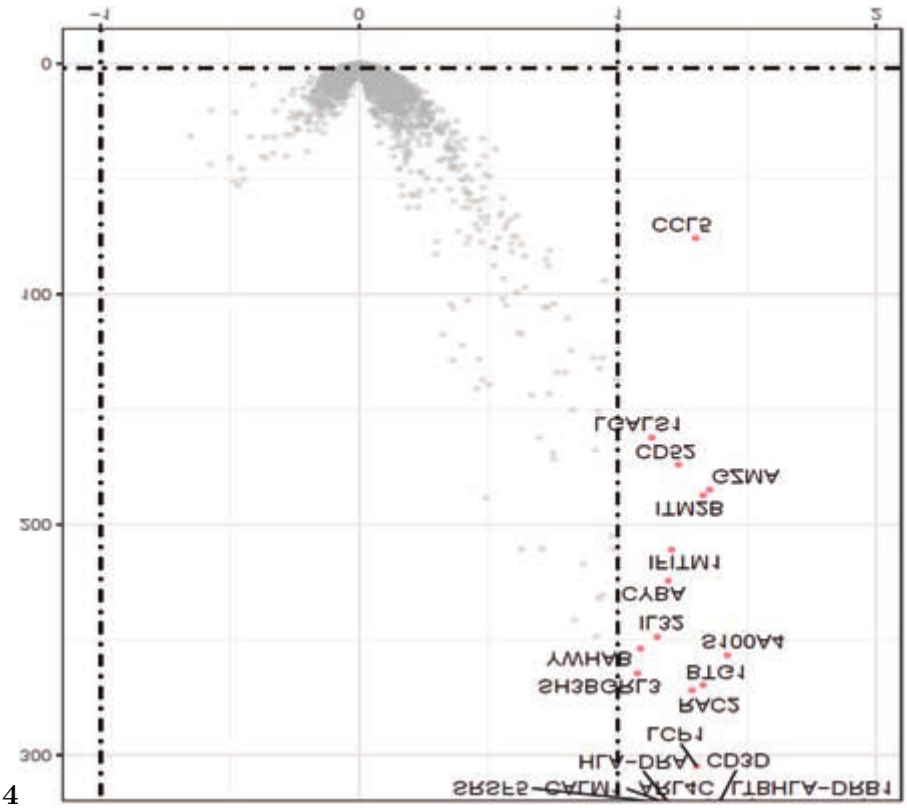

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Figure 3: Figure 4 :

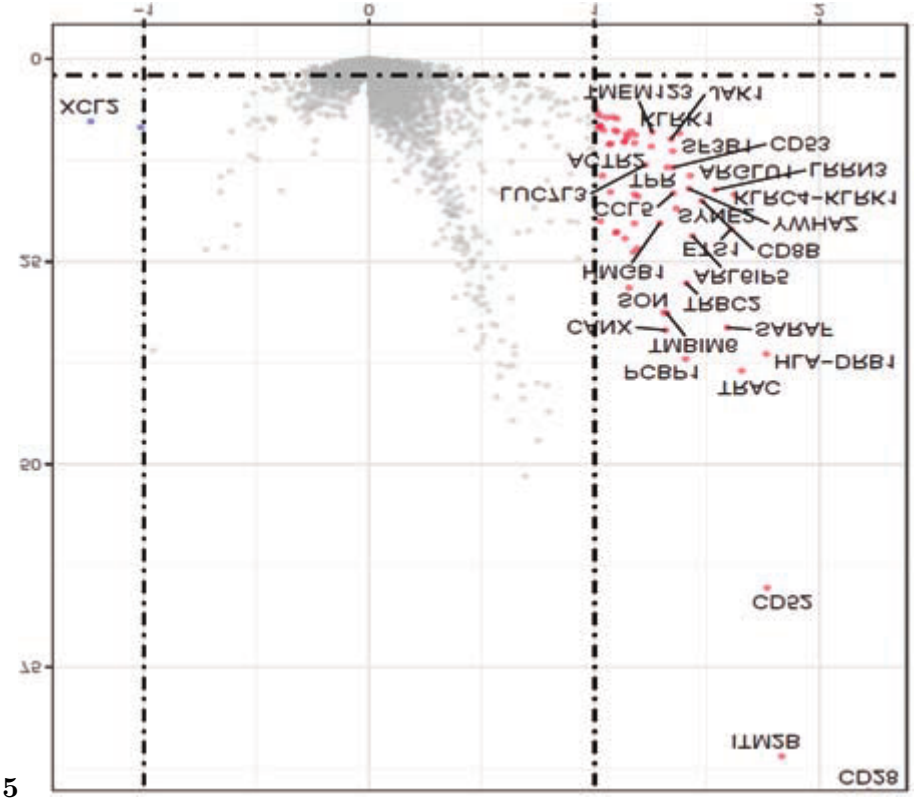

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Figure 4: Figure 5 :

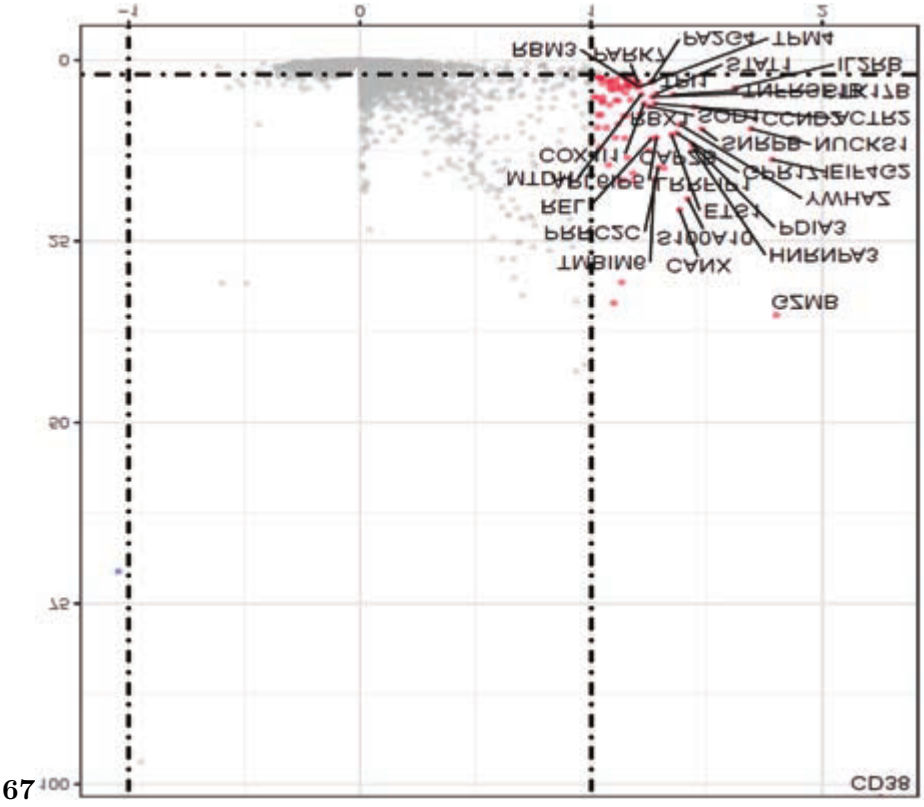

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Figure 5: Figure 6 :Figure 7 :

.1 Acknowledgements

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.2 Author contributions

Q.G. designed and supervised the project, wrote and revised the manuscript. S.L. performed the bioinformatic analysis. R.D., H.C. and Q.G. performed the experiments. B.K., Y.G. and X.D. helped with the manuscript revision.

.3 Declaration of interests

The authors declare no competing financial interest.

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