Global Journal of Medical Research: F
Diseases
Volume 20 Issue 8 Version 1.0 Year 2020
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

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GJMR-F Classification: NLMC Code: QU 300

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I. Introduction

PD-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.

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

II. Results

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TCR sequence was obtained from T cells after stimulation with MART-1 (aa27-35, LAGIGILTV) peptide (data unpublished) and designed as TCR_{MART-1} (Fig. 1).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} α and β 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. 2) 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. 2).

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. 3). 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. 3). Furthermore, the ratios of HLA-DR^+CD28^+ and HLA-DR^+CD38^+subpopulations were reduced by the increasing proportion of tumor PD-L1 (Fig. 3).

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 $CX3CR^-$ cells (Fig. 5A). After gene oncology (GO) analysis, T cell activation and cell-cell adhesion related signaling were enriched in the CX3CR+ cluster (Fig. 5B).

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. 6A). Endocytic vesicle membrane signaling was enriched in the HLA-DR^+CD8^+subset (Fig. 6B).

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. 7A). 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. 7B), which was much similar to that of the $CX3CR^+CD8^+$population (Fig. 5B).

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.

III. 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. 3, 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. 3). 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. 3), 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. 2).

IV. 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. Materials and Methods

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 μg/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.

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.

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 α and β, respectively. TCR_{MART-1}-encoded DNA was then synthesized by GeneScript (Nanjing, China) and ligated into a lentiviral vector, pRRLSIN.cPPT.PGK (Addgene, 12252).

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.

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

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the blood of HLA-A*0201-restricted 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 12-well plate. To promote infection efficiency, polybrene was added into the medium at the final concentration of 2μg/mL, and the well plate was centrifuged at 800g for 30 minutes. T cells were then expanded and maintained in T cell medium.

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.

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.

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.

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.

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.

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.

Acknowledgements

We sincerely thank the support provided by China National GeneBank. This research was supported by National Natural Science Foundation of China (No. 81903159), Guangdong Provincial Key Laboratory of Genome Read and Write (No. 2017B030301011), the Shenzhen Municipal Government of China Peacock Plan (No. KQTD2015033017150531), and Science, Technology and Innovation Commission of Shenzhen Municipality (No. JCYJ20170817150015170).

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.

Declaration of interests

The authors declare no competing financial interest.

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Figure legend

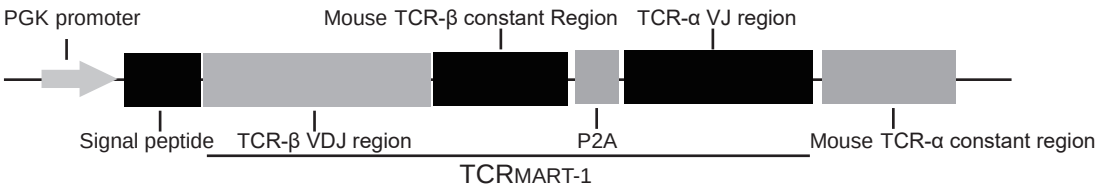

Figure 1: Schematic design of MART-1$_{26-35}$ peptide-specific TCR sequence. The transcription of TCR_{MART-1} was driven by PGK promoter. Mouse constant regions were used to reduce the mispairing with endogenous TCR. TCRβ and TCRα were linked by P2A.

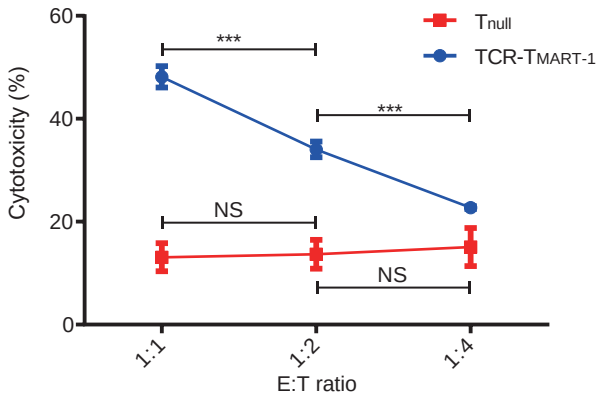

Figure 2: Cytotoxicity of T_{null} and TCR-T_{MART-1} against peptide-loaded MEL-526 cells. The in vitro killing assays were performed at different E: T ratios (1:1, 1:2, and 1:4). Data were generated from three individual replicates and shown as mean ± SD. 2-tailed unpaired t-tests were used to calculate p-values, *: p＜0.05; **: p＜0.01; ***: p＜0.001; NS: Not significant.

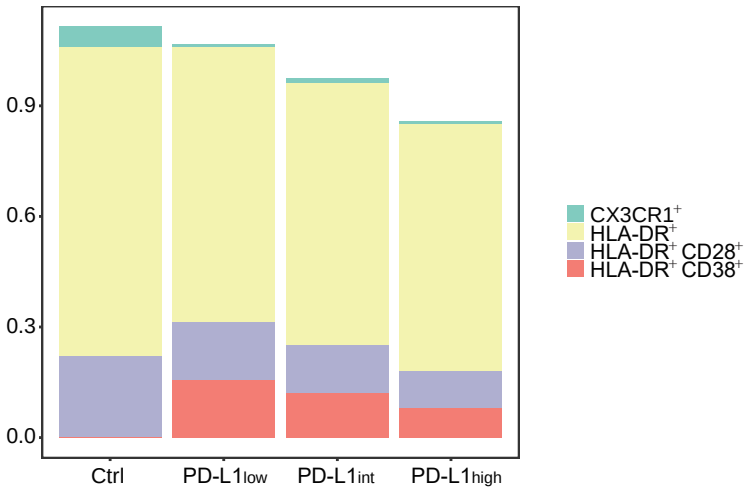

Figure 3: Compositions of specific cell clusters in T cells responding to tumor PD-L1. The proportions of clusters $CX3CR1^+$, $HLA\text{-}DR^+$, $HLA\text{-}DR^+CD28^+$, and $HLA\text{-}DR^+CD38^+$ in T cells stimulated with antigen or not.

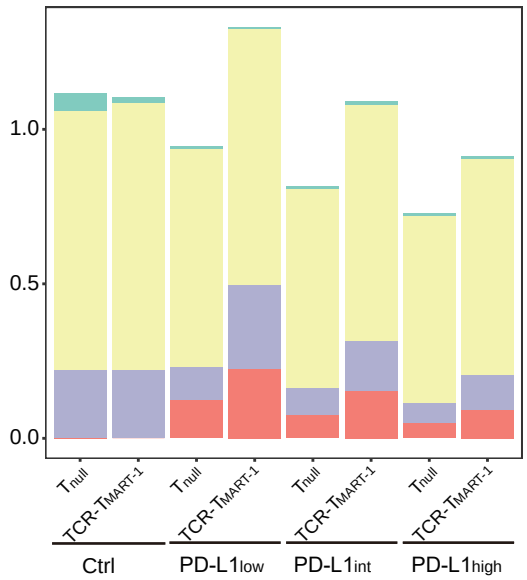

Figure 4: Compositions of specific cell clusters in T_{null} and TCR-T_{MART-1} responding to tumor PD-L1. The proportions of clusters CX3CR1^{+}, HLA-DR^{+}, HLA-DR^{+}CD28^{+}, and HLA-DR^{+}CD38^{+} in T_{null} and TCR-T_{MART-1} stimulated with antigen or not.

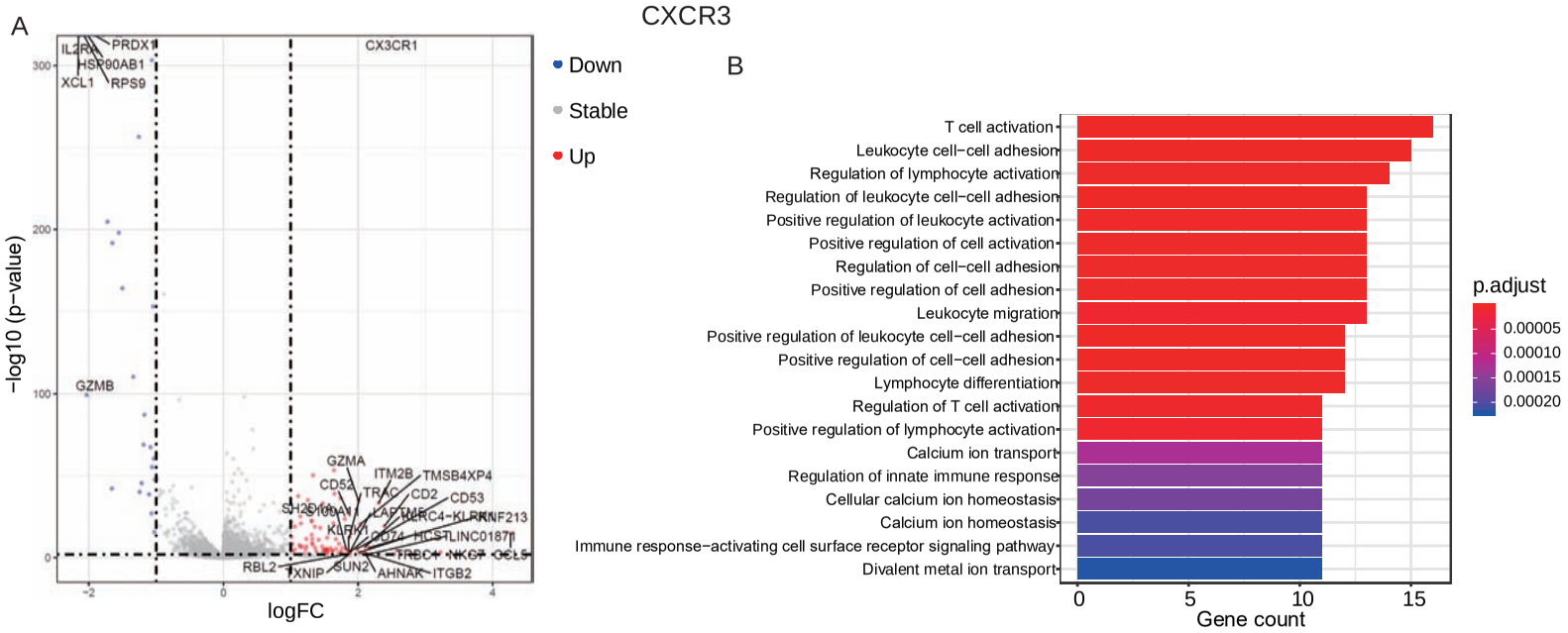

Figure 5: Characteristics of cluster CX3CR1⁺CD8⁺. A, Volcano plot showing differentially expressed genes between CX3CR1+ and CX3CR1- T cells. Each red or blue dot denotes an individual upregulated or downregulated gene (|logFC| >= 1 and p.value< 0.01). B, Bar plot showing the top 20 pathways of CX3CR1⁺CD8⁺ T cells. The color represents p.value and the x-axis represents gene count.

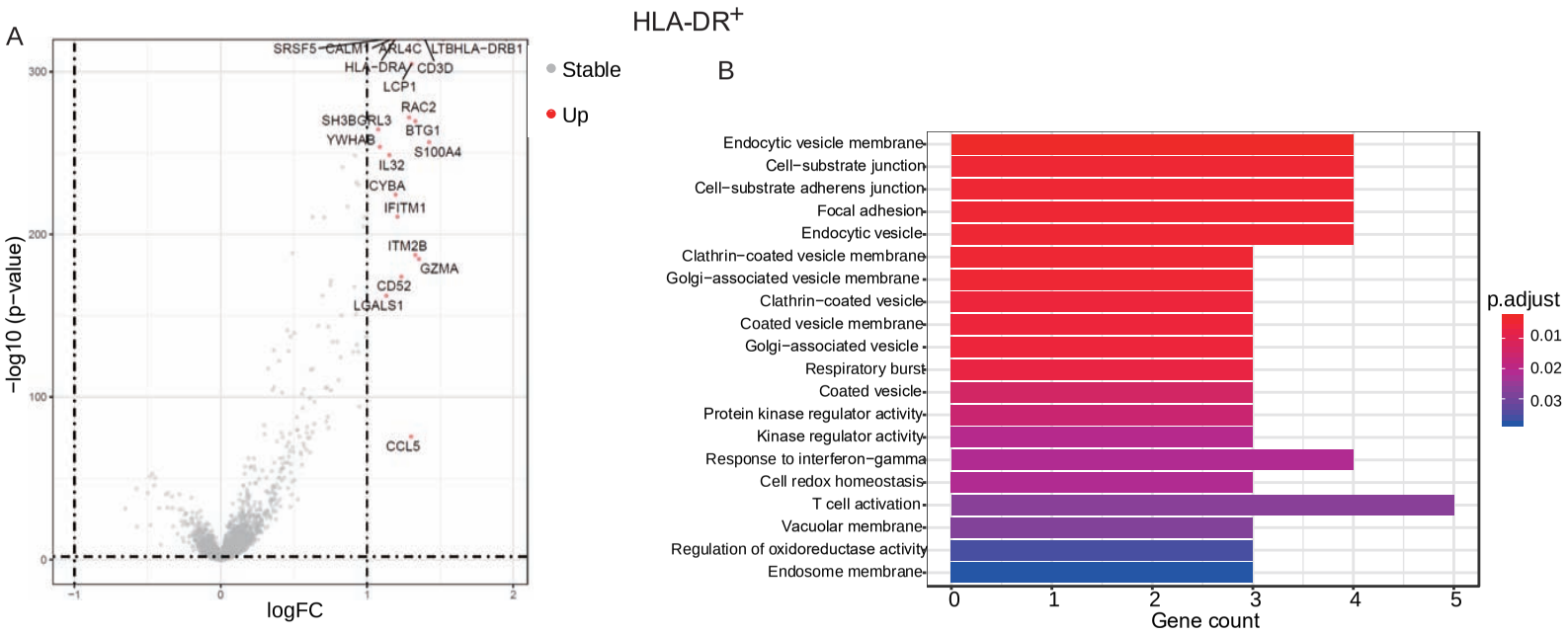

Figure 6: Characteristics of cluster HLA-DR$^+$CD8$^+$. A, Volcano plot showing differentially expressed genes between HLA-DR$^+$ and HLA-DR- T cells. Each red or blue dot denotes an individual upregulated or downregulated gene (|logFC| >= 1 and p.value< 0.01). B, Bar plot showing the top 20 pathways of HLA-DR$^+$CD8$^+$ T cells. The color represents p.value and the x-axis represents gene count.

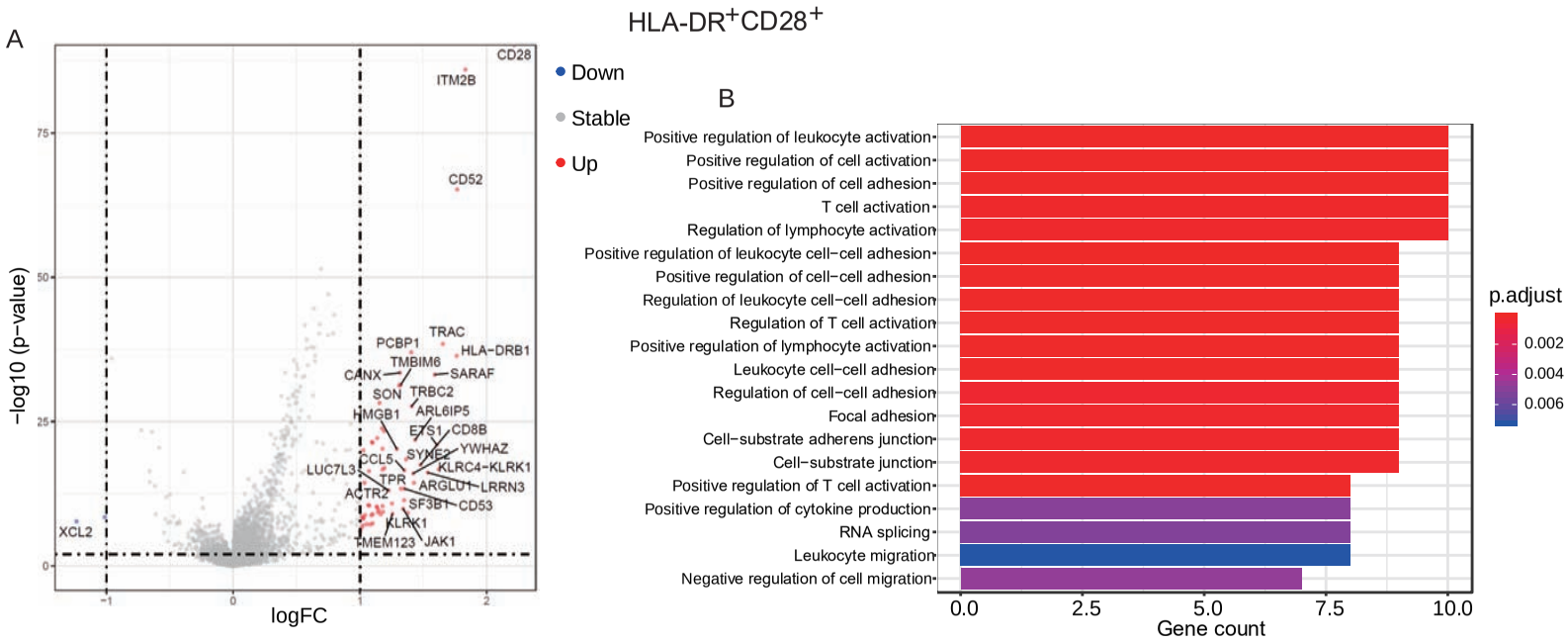

Figure 7: Characteristics of cluster HLA-DR$^+$CD28$^+$CD8$^+$. A, Volcano plot showing differentially expressed genes between HLA-DR$^+$CD28$^+$ and non-HLA-DR$^+$CD28$^+$ T cells. Each red or blue dot denotes an individual upregulated or downregulated gene (|logFC| >= 1 and p.value< 0.01). B, Bar plot showing the top 20 pathways of HLA-DR$^+$CD28$^+$CD8$^+$ T cells. The color represents p.value and the x-axis represents gene count.

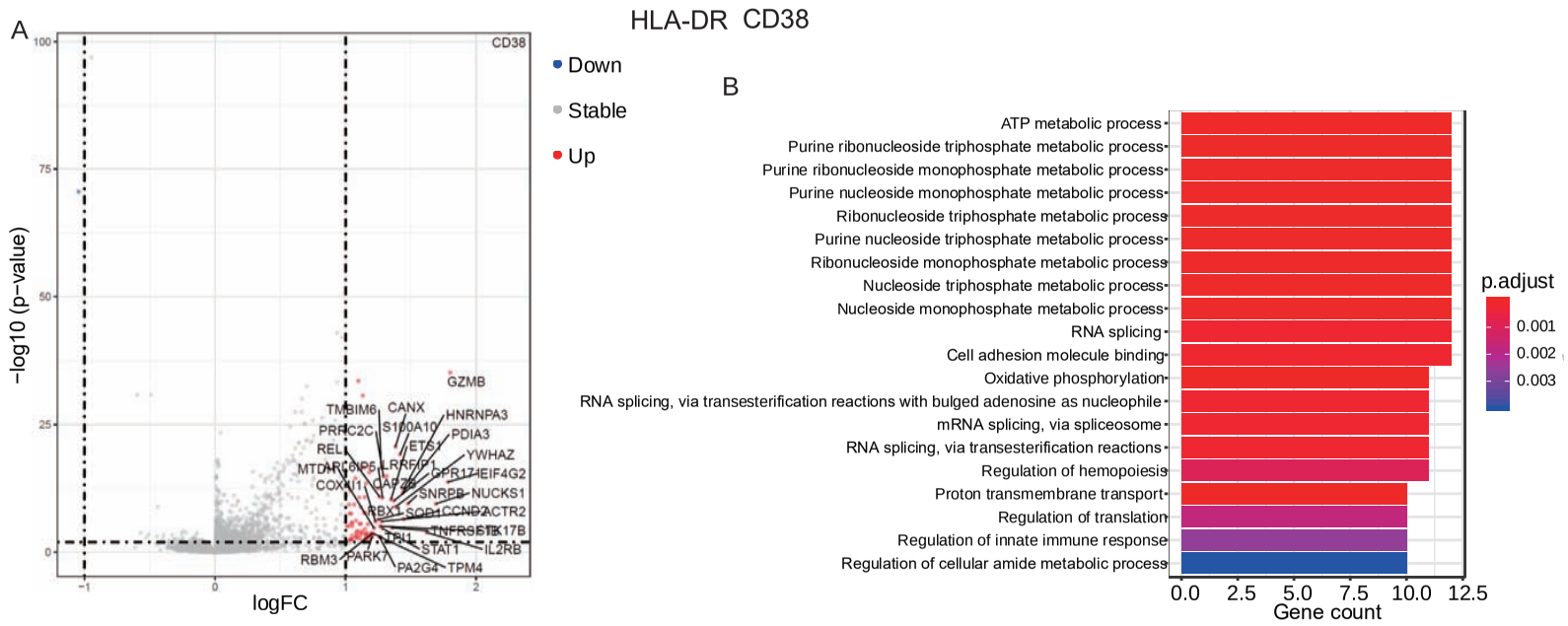

Figure 8: Characteristics of cluster HLA-DR$^+$CD38$^+$CD8$^+$. A, Volcano plot showing differentially expressed genes between HLA-DR$^+$CD38$^+$ and non-HLA-DR$^+$CD38$^+$ T cells. Each red or blue dot denotes an individual upregulated or downregulated gene (|logFC| >= 1 and p.value< 0.01). B, Bar plot showing the top 20 pathways of HLA-DR$^+$CD38$^+$CD8$^+$ T cells. The color represents p.value and the x-axis represents gene count.