Deletion of Cbl-b inhibits CD8\(^+\) T-cell exhaustion and promotes CAR T-cell function

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ABSTRACT

Background Chimeric antigen receptor (CAR) T-cell therapy is an emerging option for cancer treatment, but its efficacy is limited, especially in solid tumors. This is partly because the CAR T cells become dysfunctional and exhausted in the tumor microenvironment. However, the key pathways responsible for impaired function of exhausted cells remain unclear, which is essential to overcome CAR T-cell exhaustion.

Methods Analysis of RNA-sequencing data from CD8\(^+\) tumor-infiltrating lymphocytes (TILs) led to identification of Cbl-b as a potential target. The sequencing data were validated using a syngeneic MC38 colon cancer model. To analyze the in vivo role of Cbl-b in T-cell exhaustion, tumor growth, % PD1\(^+\)Tim3\(^+\) cells, and expression of effector cytokines were analyzed in cbl-b\(^{-/-}\) and cbl-b\(^{+/-}\) mice. To evaluate the therapeutic potential of Cbl-b depletion, we generated a new CAR construct, hCEAscFv-CAR, that recognizes human carcinoembryonic antigen (CEA). cbl-b\(^{-/-}\) and cbl-b\(^{+/-}\) CEA-CAR T cells were generated by retroviral transduction. Rag\(^{-/-}\) mice bearing MC38-CEA cells were injected with cbl-b\(^{-/-}\) and cbl-b\(^{+/-}\) CEA-CAR T cells, tumor growth, % PD1\(^+\)Tim3\(^+\) cells and expression of effector cytokines were analyzed.

Results Our results show that the E3 ubiquitin ligase Cbl-b is upregulated in exhausted (PD1\(^+\)Tim3\(^+\)) CD8\(^+\) TILs. CRISPR-Cas9-mediated inhibition of Cbl-b restores the effector function of exhausted CD8\(^+\) TILs. Importantly, the reduced growth of syngeneic MC38 tumors in cbl-b\(^{-/-}\) mice was associated with a marked reduction of PD1\(^+\)Tim3\(^+\) CD8\(^+\) TILs. Depletion of Cbl-b inhibited CAR T-cell exhaustion, resulting in reduced MC38-CEA tumor growth, reduced PD1\(^+\)Tim3\(^+\) cells and increased expression of interferon gamma (IFN-\(\gamma\)), tumor necrosis factor alpha (TNF-\(\alpha\)) and tumor cell killing. Our results demonstrate that deficiency of Cbl-b overcomes endogenous CD8\(^+\) T-cell exhaustion, and deletion of Cbl-b in CAR T cells renders them resistant to exhaustion. Our results could facilitate the development of efficient CAR T-cell therapy for solid tumors by targeting Cbl-b.2

INTRODUCTION

Adoptive transfer T-cell therapy is an emerging option for cancer treatment,1 but its efficacy is limited, especially in solid tumors, because the effector CD8\(^+\) T-cells that promote antitumor immunity become dysfunctional and exhausted in the tumor microenvironment (TME). Exhausted T cells exhibit progressive loss of effector function (expression of interferon gamma (IFN-\(\gamma\)), tumor necrosis factor alpha (TNF-\(\alpha\)) and tumor cell killing) and express inhibitory receptors (PD1, Tim3, and Lag3).2 However, the key pathways responsible for impaired function of exhausted cells remain unclear.

Post-translational modification mediated by ubiquitin conjugation plays an indispensable role in immune cells. Ubiquitination involves a cascade of biochemical reactions through ubiquitin activating (E1) enzymes, ubiquitin-conjugating (E2) enzymes, and ubiquitin ligase (E3) enzymes. The E3 ubiquitin ligases are critical components of this system because they recognize and target specific target proteins for ubiquitination.3

Cbl-b is an E3 ubiquitin ligase that belongs to the Really Interesting New Gene (RING) family. Cbl-b contains an N-terminal tyrosine kinase-binding domain, a RING finger, and a C-terminal proline-rich sequence, and can thus function as both an E3 ligase and a molecular adaptor.3 Here, we demonstrate that Cbl-b is upregulated in exhausted CD8\(^+\) Tumor-infiltrating lymphocytes (TILs) and plays a crucial role in dysfunction of tumor-reactive TILs.

MATERIALS AND METHODS

Mice

C57BL/6, Rag\(^{-/-}\) and cbl-b\(^{-/-}\) mice were housed in microisolator cages, and the experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of UT Southwestern Medical Center.
Figure 1  Cbl-b is upregulated in exhausted CD8+PD1+Tim3+ TILs. (A) Geo dataset (GSE85947) was analyzed and normalized read counts (TPM) for Cbl-b are shown. (B) Volcano plot of differentially expressed genes (−log10 p value vs log2 fold change) in PD1+Tim3+ versus PD1–Tim3– TILs. (C) MC38 tumors were implanted subcutaneously into C57BL6 mice; PD1+Tim3+ and PD1–Tim3– TILs were sorted; and Cbl-b messenger RNA levels were analyzed by real-time PCR. (D) Immunoblot of Cbl-b protein using Fluorescence-activated cell sorted (FACS) PD1+Tim3+ versus PD1–Tim3– TILs. The data are representative of three independent experiments. Statistics are mean±SD, calculated by Student’s t-test (two-tailed). TIL, tumor-infiltrating lymphocyte. TPM, transcript per million; IB, Immunoblotting, FACS, Fluorescence-activated cell sorting.

MC38-carcinogenic embryonic antigen (CEA) tumor and CD8+ TIL isolation
cbl-b−/− and cbl-b+/+ mice were injected subcutaneously with 1×106 MC38-CEA cells and tumor growth was analyzed. Kaplan-Meier estimate was used to analyze the survival of mice. Mice were counted as dead when the tumor diameter reached the length limit of 15 mm. CD8+ TILs were isolated using CD8 TIL Microbeads (Miltenyi).

Construction of retroviral vector (hCEA scFv-GFP) containing chimeric antigen receptor (CAR)
DNA sequences encoding scFv anti-CEA chimeric receptors were synthesized by Genescript (New Jersey, USA and cloned into the m1928z-GFP retroviral construct by replacing the CD19scFv region between CD8 leader (CD8L) sequence and CD8 hinge region (CD8TM) at the Nool restriction site. The chimeric gene constructs were composed of the mouse CD8 signal peptide (CD8L), scFv-anti-CEA mAb, a membrane proximal hinge region along with the transmembrane domain, and mouse stimulatory domain, which included mouse CD28 and mouse CD3ζ.

CRISPR-Cas9 targeting
Three top-ranking guide sequences against Cbl-b exons were designed and cloned into lent-CRISPR v2 plasmid (Addgene 52961). HEK293FT cells (Thermo Fisher) were transfected with lent-CRISPR v2 plasmid, along with packaging plasmid pMD2.G (Addgene 12259) and pSAX2 (Addgene 12260). Lentivirus was harvested after 48–72 hours of incubation, passed through 0.45 μm filter and stored at −80°C. The details of the guide RNAs targeting Cbl-b gene are as follows:
Cbl-b guide RNA1: GATGGGATGTGCAATCTCGTG. Cbl-b guide RNA2: GCACCTGTCGCTTTACCGCG. Cbl-b guide RNA3: GGCAGAGTTGGAAATGCAGA.

Retroviral transduction, CAR T-cell production and adoptive transfer
CD8+ T-cells were stimulated for 24 hours with anti-CD3 clone (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies and were cultured with mIL-2 (100 U/mL) and transduced with the retrovirus by spinoculation method. On the day of adoptive transfer, GFP+ cells were sorted by flow cytometry and (GFP+/CD8+ CAR T cells) adoptively transferred via tail-vein injections.

Statistical analysis
Statistical analysis was performed with the Prism V.8.0 software, and all results were summarized as mean±SD. Differences between groups were evaluated by two-tailed Student’s t-test. Log-rank test was used for survival analysis. A p value of <0.05 was considered as statistically significant.

RESULTS
Cbl-b is upregulated in PD1+Tim3+ exhausted TILs
Cbl-b has been shown to be an important negative regulator of T cells, and deletion of Cbl-b in T cells results in rejection of established tumors. Analysis of published RNA-sequencing data (GSE85947) showed that messenger RNA (mRNA) encoding the Cbl-b gene was preferentially upregulated in PD1+Tim3+ exhausted CD8+ TILs in the CT26 colon carcinoma model (figure 1A,B). Further, analysis of the published sequencing results showed that Cbl-b expression was upregulated in exhausted CD8+ TILs in multiple tumor models (GSE123738 and GSE85947), as well as in a lymphocytic choriomeningitis virus infection model (GSE88987). To confirm these sequencing results, we adopted the syngeneic MC38 colon cancer model. As shown in figure 1C, real-time PCR analysis of RNA isolated from PD1+Tim3+ TILs showed increased expression of Cbl-b compared with PD1–Tim3– cells. Increased Cbl-b expression in PD1+Tim3+ TILs was further confirmed by immunoblotting (figure 1D). These data collectively suggest that Cbl-b expression is preferentially upregulated in exhausted CD8+ TILs in the TME.
Inhibition of Cbl-b restores the effector function of exhausted CD8+ TILs

To test the hypothesis that upregulated Cbl-b expression in exhausted CAR T cells is a significant factor leading to decreased effector function, we examined the role of Cbl-b in CD8+ TILs using CRISPR-Cas9-mediated deletion of Cbl-b. We knocked out Cbl-b in PD1+Tim3+ TILs from MC38 tumor-bearing C57BL/6 mice. Cbl-b deficiency in PD1+Tim3+ TILs significantly reduced the number of PD1+Tim3+ cells in tumors in Cbl-b–/– mice compared with Cbl-b+/+ mice (figure 2A–D).

Cbl-b deficiency leads to reduced PD1+Tim3+ TILs

To examine the in vivo role of Cbl-b in T-cell exhaustion and tumor growth, we inoculated cbb+/+ and cbb–/– mice with MC38-CEA cells. A markedly reduced growth of MC38 tumors was observed in cbb–/– mice compared with cbb+/+ mice (figure 3A,B). Our results are consistent with previous reports that cbb–/– mice mount a robust antitumor response against transplantated (TG-1, EL4, EG7, and B16 melanoma), chemically induced and spontaneous tumors. The increased antitumor activity in cbb–/– mice was attributed primarily to CD8+ T-cells. Similarly, we found an increased number of CD8+ T-cell infiltration into MC38 tumors in cbb–/– mice (online supplemental figure 1B).

To test if Cbl-b deficiency affected the CD8+ T-cell exhaustion, we isolated CD8+ TILs from Cbl-b+/+ and Cbl-b–/– mice, and real-time PCR analysis showed that cbb–/– TILs were sorted and expression of IFN-γ, TNF-α, and granzyme B was analyzed by real-time PCR. The data are representative of three independent experiments. Statistics are mean±SD, calculated by Student’s t-test (two-tailed).

Depletion of Cbl-b prevents CAR T-cell exhaustion

Exhaustion is a major barrier in the success of CAR T cells. Similar to endogenously exhausted CD8+ T-cells, Cbl-b was also upregulated in exhausted CAR T cells. Therefore, we investigated if depletion of Cbl-b would rescue T cells from exhaustion resulting in enhanced antitumor activity. We replaced CD19scFv with CEA-scFv (MFE23), which recognizes human carcinoembryonic antigen (hCEA) of a CAR construct against CD19, CD19scFv-CD28-CD3ζGFP, generating a new CAR construct, hCEAscFv-CD28-CD3ζGFP.
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We retrovirally transduced naïve CD8⁺ T-cells with either control (mock) (without hCEAscFv) or hCEAscFv-CD28-CD3ζ.GFP. The transduction efficiency varied between 60% and 65% (online supplemental figure 2A). We cocultured fluorescence-activated cell sorted GFP⁺ cells (online supplemental figure 2B) with MC38-CEA cells, a mouse colon cancer cell line expressing hCEA. Real-time PCR analysis showed that CEA-CAR T cells, but not mock transduced cells, expressed IFN-γ, TNFα, granzyme B, and IL-2 (online supplemental figure 2C).

Next, we injected MC38-CEA cells into the right flank of Rag⁻/⁻ mice, and on days 3 and 8, we inoculated with cbb⁻/⁻ or cbb⁻/⁻ CEA-CAR T cells. The use of cbb⁻/⁻ CAR T cells considerably enhanced the survival of Rag⁻/⁻ mice as shown in figure 4B. In addition, we observed significantly reduced tumor growth in Rag⁻/⁻ mice that received cbb⁻/⁻ CAR T cells compared with the mice that received cbb⁻/⁻ CAR T cells (figure 4C).
To investigate if Cbl-b deficiency affected CAR T-cell exhaustion, we employed adoptive transfer procedures. Mice were sacrificed 7 days post-transfer, and CD8+ TILs were sorted and analyzed. Consistent with our hypothesis, Cbl-b deficiency significantly reduced the exhaustion phenotype as measured by increased expression of exhaustion markers (PD1, Tim3).

**DISCUSSION**

Immunotherapy for cancer via checkpoint blockade and adoptive transfer of tumor antigen-specific CAR T cells have shown promising results. However, only a small subset of patients show complete remission with checkpoint blockade. Similarly, CAR T cells have been effective against hematopoietic malignancies but not against solid tumors. Several lines of evidence implicating exhaustion in limiting the potency of CAR T cells are available. Here, we demonstrate that deletion of Cbl-b potentiates the antitumor activity of CAR T cells. Since Cbl-b deficiency in T cells is linked to autoimmunity, we looked for signs of toxicity in mice that received Cbl-b−/- cells. No significant change in spleen size was observed between Rag−/− mice that received either Cbl-b−/- or Cbl-b+/+ cells. Similarly, histological analysis of HE-stained sections of colon, lung and liver tissues of mice that received Cbl-b−/- CAR T cells did not show any signs of toxicity (figure 4E). It was demonstrated that Cbl-b deficiency uncouples the requirement for costimulation for T cells and hence heightened antitumor activity.

**CONCLUSION**

Our studies demonstrate that deficiency of Cbl-b overcomes endogenous CD8+ T-cell exhaustion, and deletion of Cbl-b in CAR T cells render them resistant to exhaustion. Our results could facilitate the development of efficient CAR T-cell therapy for solid tumors by targeting Cbl-b.
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