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Original Article

Silencing the expression of Cbl-b enhances the immune activation of T lymphocytes against RM-1 prostate cancer cells *in vitro*

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Abstract

Background: The ubiquitin ligase Cbl-b potently modulates T lymphocyte immune responses and is critical in modulating tumor-induced immunosuppression. The influence of Cbl-b in modulating T lymphocyte activity against prostate cancer remains ill defined. We have determined the effects of silencing Cbl-b expression in T lymphocytes and their subsequent cytotoxic activity against prostate cancer cells.
Methods: T lymphocytes were isolated from the spleens of C57BL/6 mice. Lipofectamine-directed transfection of T lymphocytes with specific small interfering RNA (siRNA) silenced Cbl-b expression, which was confirmed by Western immunoblotting. The siRNA species were chosen that promoted the greatest transfection efficiency and dampened Cbl-b expression in T lymphocytes. The expression of CD69, CD25, and CD71 by the transfected T lymphocytes was determined by flow cytometry. T lymphocyte proliferation was assessed by CCK-8 assay. Enzyme-linked immunosorbent assay (ELISA) was used to measure the secretion of interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-β. The objective was to compare the cytotoxic activity of transfected T lymphocytes and nontransfected (i.e., negative control) T lymphocytes against the murine prostate cancer cell line target RM-1 *in vitro*.
Results: We selected a specific siRNA that decreased T lymphocyte Cbl-b expression to 15%. The siRNA-transfected T lymphocytes showed higher proliferation; higher CD69, CD25, and CD71 expression ($p < 0.001$); and higher IL-2, IFN-γ, and TNF-β secretion ($p < 0.05$), compared to the nontransfected cells. Transfected T lymphocytes were also more potent at killing RM-1 prostate cancer cells, compared to the negative control *in vitro*.
Conclusion: Silencing Cbl-b significantly enhanced T lymphocyte function and T lymphocyte cytotoxicity activity against a model prostate cancer cell line *in vitro*. This study suggests a potentially novel immunotherapeutic strategy against prostate cancer.
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Keywords: adoptive immunotherapy; Cbl E3 gene silencing; prostate cancer; T lymphocytes; ubiquitin protein ligase

1. Introduction

Prostate cancer is the second most prevalent cancer in men and accounts for approximately 14% of all male cancers worldwide.[1] The treatment of advanced prostate cancer is predominantly achieved with hormone therapy. However, most patients over time develop metastatic disease, despite androgen ablation. This state is referred to as castration-resistant prostate cancer.[2] Patients presenting with castration-resistant prostate cancer have limited treatment options available to them, and a poor prognosis.

Evidence has shown that a defective immune response may be present in the setting of prostate cancer. Despite the presence of immunological effector T cells, which recognize tumor-associated antigens in prostate tissue, T cells are actively tolerant to the tumor, and become incapable of

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mediating a tumoricidal response.[3] In addition, the tumor microenvironment is highly immunosuppressive, which is closely associated with the augmented expression of transforming growth factor (TGF)-β and the presence of CD4+ regulatory T cells.[4,5] A key question is how to effectively activate tumor-specific and cell-mediated immunity in adopting immunotherapeutic targeting of prostate cancer. Several Phase I/II clinical trials have demonstrated that treating individuals with dendritic cell-based immunotherapeutic vaccines can effectively activate T cell-mediated immunity, which results in greatly improved median overall survival rates and a relative reduction in the risk of death, compared to treating individuals with a placebo.[6–8] Thus, strengthening the antitumor immune response, especially by enhancing T cell activation, provides a promising immunotherapeutic approach for targeting prostate cancer.

The first E3 ligase to be identified as a major participant in cell-mediated immunity was Cb1-b, whose key function is to ensure a delicate balance between T cell activation, immunological tolerance, and autoimmunity *in vivo*.[9] When total Cbl-b is ablated, T cells exhibit a hyperactivated state, a predilection to spontaneous autoimmunity, and markedly increased secretion of interleukin (IL)-2, IL-17, and interferon gamma (IFN-γ). The total ablation of Cb1-b moreover promotes spontaneous tumor rejection in gene knockout mouse models *in vivo*. This has been demonstrated in cancers such as leukemia,[10] skin cancer,[11] TC1 tumor cells,[12] and lymphoma.[13] However, to the best of our knowledge, we have not seen any reports in the context of Cb1-b and prostate cancer. Furthermore, small interfering RNAs (siRNAs) hold great promise as a reversible therapeutic tool, compared to gene knockout approaches. Bearing that in mind, we used sequence-specific siRNA to silence the expression of Cbl-b and studied changes in the immunoreactivity of splenic T cells in a mouse model. We also assessed the immunological and tumoricidal activity of the transfected T cells against the prostate cancer cell line RM-1 *in vitro*.

2. Methods

2.1. Reagents and antibodies

Anti-CD3-FITC (clone 145-2C11), anti-CD69-PE (clone H1.2F3), anti-CD25- Percp-cy5.5 (clone PC61), anti-CD71-FITC (clone C2F2), and mouse T lymphocyte enrichment set-DM were all obtained from BD Biosciences Pharmingen (San Diego, CA, USA). Antimouse CD3 functional antibody (clone 17A2) was purchased from eBioscience (San Diego, CA, USA). The cell counting kit-8 and the total protein extraction kit were both obtained from KeyGen Biotech (Nanjing, China). The Lowry protein assay kit was obtained from Sangon Biotech (Shanghai, China). The antibody targeted against Cbl-b was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Concanavalin A (Con A) and peroxidase-conjugated rabbit antimouse immunoglobulin G were obtained from Sigma (Saint Louis, MS, USA). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). Roswell Park Memorial Institute Medium (RPMI) 1640 culture medium, Opti-MEM medium, and fetal calf serum (FCS) were all obtained from Gibco (Grand Island, NY, USA). Cytokine-detecting enzyme-linked immunosorbent assay (ELISA) kits were obtained from Wuhan Boster Biological Technology (Hubei, China). The cytokines used were IL-2, IFN-γ, and tumor necrosis factor (TNF)-β.

2.2. Mice and prostate cancer cell line

Male C57BL/6 mice were obtained from the Chinese Academy of Sciences (Shanghai, China) and were maintained under specific pathogen-free conditions. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (Tongji University, Shanghai, China). Mice were sacrificed at age 6–8-weeks-old. The mouse prostate cancer cell line (RM-1) was obtained from Shanghai Cellular Institute of The China Scientific Academy (Shanghai, China) and maintained in RPMI 1640 culture medium supplemented with heat-inactivated FCS (10%), penicillin (100 U/mL), and streptomycin sulfate (100 μg/mL). The RM-1 cell line was cultured at 37°C in 5% carbon dioxide (CO_2) and in a fully humidified incubator. The Cbl-b siRNA and control siRNA were obtained from GeneChem (Shanghai, China). The sequence of the Cbl-b siRNA was 5'-UGAGAUGCCCUG AUAUUAAtt- 3' (sense strand) and 5'-UUAAUAUCAGGGCAU CUCAtt-3" (antisense strand).

2.3. T lymphocytes enrichment and identification

BD IMag Mouse T Lymphocyte Enrichment microbeads (BD Biosciences) were used for the negative selection of T lymphocytes from male C57BL/6 mice spleen. In accordance with the manufacturer's protocol, we isolated $1–2 \times 10^6$ T lymphocytes for each mouse spleen. The T lymphocytes were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, and 5 μg/mL Con A. The T cells were stained with fluorescein isothiocyanate (FITC)-conjugated antimouse CD3e mAb and detected by flow cytometry. The T cell purity was >95.9%. The T lymphocytes were cultured at 37°C in 5% CO_2 within a fully humidified incubator.

2.4. Transfection of T lymphocytes

The T cells were cultured to 80–90% confluence at the time of transfection. Single cell suspensions were prepared at a density of 2.0×10^5/mL and seeded to 24-well plates in a volume of 400 μL/well. The transfections were performed in accordance with the manufacturer's instructions. In brief, 2 μL of Lipofectamine 2000 (Invitrogen) and 6 μL of sequence-specific siRNA were added into 20 μL of opti-MEM (Gibco) reduced serum medium, mixed gently, and incubated for 5 minutes at room temperature. The diluted siRNA was combined with the diluted Lipofectamine 2000 (Invitrogen) and incubated for 20 minutes at room temperature. The siRNA-Lipofectamine 2000 complexes were added to each well containing the cells and the culture medium; 60 μL of mouse

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