Available online at www.sciencedirect.com

ScienceDirect

Journal of the Chinese Medical Association 77 (2014) 630–636

www.jcma-online.com

Original Article

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

Shu-Kui Zhou, Wei-Hua Chen, Zheng-Duo Shi, Shun-Ping Wang, Liang Li, Xiao-Fei Wen, Yue-Min Wang*

Department of Urology, East Hospital, Tongji University, Shanghai, China

Received October 16, 2013; accepted March 18, 2014

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.

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

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Yue-Min Wang, Department of Urology, East Hospital, Tongji University, 150, Jimo Road, Shanghai 200120, China.
E-mail address: wangyuemin@hotmail.com (Y.-M. Wang).

http://dx.doi.org/10.1016/j.jcma.2014.03.008

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

CD3 functional antibody was afterwards added to each well wherein the final concentration of the mouse CD3 functional antibody was 1 μg/mL. After an incubation period of 6 hours at 37°C, 80 μL of FCS was added to each well. T cells that had been transfected with scrambled siRNA served as the negative control. The transfection efficiency was measured by flow cytometry using fluorescent-labeled siRNA (NC-FAM) using the exact same methods as described previously.

2.5. Western immunoblot detection of Cbl-b protein expression

At 48 hours after transfecting the T cells, Cbl-b proteins were extracted using the total protein extraction kit in accordance with the manufacturer's protocol. Protein concentrations were determined by the Lowry method. Total proteins (30–50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 1% bovine serum albumin in Tris-buffered saline Tween-20 (TBST) at room temperature for 1 hour, and then incubated overnight with the indicated primary antibodies (at 1:1000 dilution) at 4°C. After washing with TBST, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (at 1:1500 dilution) for 45 minutes at room temperature. Visualization of the antibody-bound protein bands was achieved by using an enhanced chemiluminesence reagent. The protein bands were quantified by the computerized imaging program Quantity One (Bio-Rad, CA, USA). Data were normalized to the reference bands corresponding to the housekeeping protein β-actin.

2.6. Flow cytometric assay of CD69, CD25, and CD71 expression

The cell surface expressions of CD69, CD25, and CD71 were assessed after culturing for 12 hours, 24 hours, and 48 hours. All cells were washed twice in phosphate-buffered saline buffer, after which the cells were stained with a combination of anti-PE-CD69, anti-Percp-cy5.5-CD25, or anti-CD71-FITC for 30 minutes at room temperature in the dark. The cells were washed twice in phosphate-buffered saline buffer and samples were evaluated by flow cytometry. Data were analyzed using the CellQuest software program (Becton Dickinson and Company, San Jose, CA, USA).

2.7. ELISA assay of cytokine secretion

Mouse T cells at a density of 2×10^5 cells/mL were seeded into 24-well microplates and cultured in the presence of mouse CD3 functional antibody (1 μg/mL). After 48 hours, the supernatants were collected and centrifuged to remove the transfected cells. In accordance with the manufacturer's instructions, transfected T cell culture supernatants were measured by ELISA for the secretion of IL-2, INF-γ, and TNF-β.

2.8. Assay of T lymphocyte proliferation

Cell proliferation was measured by the CCK-8 assay. After 48 hours, the transfected T cell suspensions were transferred to a 96-well orifice plate as 100-μL cell suspensions Ten microliters of the CCK-8 reagent were then added to each well at a volume ratio of 10:1. The plated cells were incubated for another 4 hours at 37°C in a 5% CO_2 and fully humidified cell culture incubator. The absorbance was measured for each well at a wavelength of 450 nm using a microplate reader (Bio-Rad Model 3550; Bio-Rad).

2.9. Assay of the killing activity of transfected T lymphocytes against RM-1 cells

The killing activity of transfected T cells against the RM-1 prostate cancer target cells was measured as previously described.[14] The RM-1 cells were cultured in 96-well microtiter plates at a density of 1×10^4 cells/well and in a total volume of 100 μL. Transfected T cells prepared as described previously, and T cells that were transfected with scrambled siRNA (i.e., negative control) were used as the effector (E) cells. Effector T cells were added at a density of 80×10^5 cells/well, 40×10^5 cells/well, 20×10^5 cells/well, and 10×10^5 cells/well to give a corresponding E/T ratio of 80:1, 40:1, 20:1, and 10:1, respectively. The plates were then incubated for 48 hours at 37°C in a 5% CO_2 and fully humidified incubator and subjected to the CCK-8 assay. The killing activity was calculated according to the following equation:

$$\text{Killing activity}(\%) = [(\text{ODE} + \text{ODT}) - \text{ODE} + \text{T}]/\text{ODT} \times 100\%$$

in which ODT is the optical density value of the target cell controls, ODE + T is the optical density value of the test samples, and ODE is the optical density value of the effectors cell controls.

2.10. Statistical analysis

The data were expressed as the mean ± standard deviation (SD). The figures show the mean ± standard error of the mean (SEM). The differences between the groups were determined by the Student t test. An alpha value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS analysis software (IBM, NY, USA).

3. Results

3.1. The determination of T lymphocyte transfection efficiency and Cbl-b protein expression

After 6 hours of transfection, the efficiency of T cell transfection was determined by flow cytometry. The transfection efficiency of the T cells remained relatively stable, and the highest transfection efficiency was 87.13% (Fig. 1A). Green fluorescence was observed by fluorescence microscopy of T cells transfected with fluorescein amidite (FAM)-siRNA (Fig. 1B).

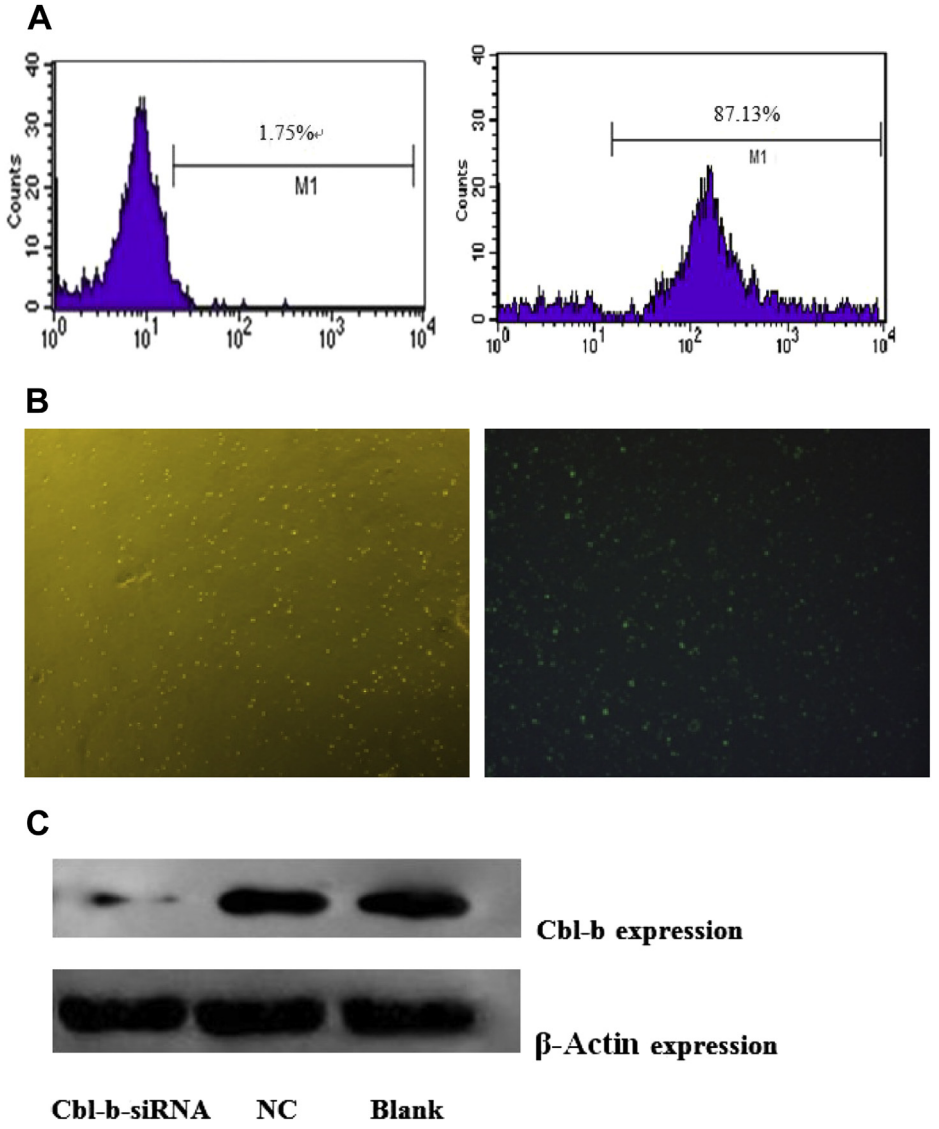

Fig. 1. (A) Graph of the efficiency of Cbl-b siRNA-transfected T cells showing that the transfection rate of FAM-siRNA-transfected T cells is 87.13%. Other data are not shown. However, the control cells not transfected with FAM-siRNA is only 1.75%. (B) T cells analyzed under fluorescence microscope 6 hours after transfection (×100 objective lens). Most T cells emit fluorescence. (C) The expression of Cbl-b protein in the Cbl-b siRNA group, the negative control group, and the blank group, as determined by Western immunoblot analysis. All aforementioned experiments were repeated three times and the results are very similar. FAM = fluorescein amidite; NC = negative control; siRNA = small interfering RNA.

FAM is related to the green fluorescent group with blue excitation at a wavelength of 480 nm. With a fluorescence pattern scattered in the cytoplasm, FAM green fluorescence was only observed in successfully transfected T cells. After 48 hours of transfection, Western immunoblot analysis of Cbl-b protein expression was determined (Fig. 1C) and compared to the negative control and the blank groups. The expression of Cbl-b protein in the transfected T cell group was significantly lower.

3.2. Silencing the Cbl-b gene promotes cell surface expression of CD69, CD25, and CD71

The cell surface expression of CD69, CD25, and CD71 were assessed by flow cytometric analysis 12 hours, 24 hours, and 48 hours after the cells had been transfected. The mean percent expression for CD69 was significantly higher in the transfected group (68.44 ± 5.89%) than in the negative control group (24.29 ± 2.06%) or the blank group (26.91 ± 2.77%; $p < 0.001$). The mean percentage value of CD25 was significantly higher in the transfection group (19.27 ± 1.84%) than in the negative control group (8.18 ± 0.71%) or the blank group (9.25 ± 0.65%; $p < 0.001$). The mean percentage value of CD71 was significantly higher in the transfection group (43.26 ± 3.89%) than in the negative control group (23.99 ± 1.97%) or the blank group (28.09 ± 2.15%; $p < 0.001$). Fig. 2 shows the results.

3.3. Silencing the Cbl-b gene augments the secretion of IL-2, IFN-γ, and TNF-β

The secretion of IL-2, IFN-γ, and TNF-β in the T cell supernatant was measured 48 hours after the cells were transfected (Fig. 3). The secretion levels of IL-2 and TNF-β

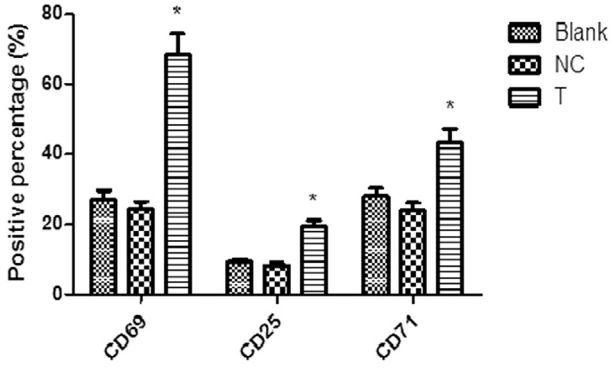

Fig. 2. Cell surface expression of CD25, CD69, and CD71 on transfected T cells, as determined by flow cytometry. The graph shows the comparative expression of CD69, CD25, and CD71 by the transfected T cells, which is markedly greater than the expression levels of the negative control or blank groups (*$p < 0.001$). The expression values (%) are expressed as the mean ± standard error of the mean ($n = 3$). NC = negative control; T = T cell.

were significantly higher in the transfected T cell group in comparison to either the negative control group or the blank group ($p < 0.001$). The secretion of IFN-γ in the transfection group was only marginally enhanced in the transfected T cell group, compared to the negative control or blank control groups ($p < 0.05$).

3.4. Silencing the Cbl-b gene augments T cell proliferation

After 48 hours of transfection, T cell proliferation was evaluated by the CCK-8 assay (Fig. 4). The absorbance values (A450) of the transfected T cell group, negative control group, and blank group were 0.763 ± 0.063, 0.356 ± 0.039, and 0.383 ± 0.015, respectively. The proliferation was markedly higher in the transfected T cell group than in the blank or control groups ($p < 0.001$).

3.5. Silencing Cbl-b gene expression augments T cell killing of RM-1 cells in vitro

At 48 hours after transfection, T cells were cocultured with RM-1 cells in 100 μL of 1:1 mixture of RPMI 1640 culture medium. The killing activity was analyzed by the CCK-8 assay. The killing activity of the transfected T cells (i.e., the effectors) against RM-1 prostate cancer cells (i.e., the targets) was higher than the killing activity in the negative control group or blank group at each effect to target ratio (Fig. 5, $p < 0.05$). The killing activity was incrementally enhanced as the effector to target ratio increased, and reached a maximum (62.24 ± 3.82%) at the effector to target ratio of 80:1.

4. Discussion

T cells can be specifically activated to eradicate cancer, and thus have a critical role in antitumor immunity. The stimulation of a tumor-specific T cell response has more theoretical advantages than other forms of cancer treatment. On one hand, T cells can continue to proliferate and be activated in response to tumor-associated immunogenic proteins expressed by a particular cancer until all tumor cells are eradicated. By contrast, immunological memory can be generated to kill antigen-bearing tumor cells should they recur.[15] T cell activation is a series of complex signal transduction pathways that are initiated by molecular interactions. Cbl-b actively regulates several key signaling molecules such as ZAP70, P85, VAV, PLC-γ, and PKC-θ. Each of these signaling molecules are involved in T cell activation and collectively establish the aneroid state.[16] Cbl-b knockout T cells hyperproliferate and, interestingly, bypass the requirement for CD28 costimulation.[17] Furthermore, Cbl-b participates in peripheral T cell tolerance by regulating resistance to CD4+ T regulatory cells and TGF-β-induced immunosuppression.[18] It may be that prostate cancer is more immunogenic than previously understood and is able to induce spontaneous autoantibodies in patients.[19] In addition, prostate cancer progresses relatively slowly, which provides a sufficient period of time for the host to generate an optimal antitumor immune response.[20] Therefore, Cbl-b may provide novel opportunities for the development of an effective immunotherapeutic strategy to target prostate cancer.

In the current study, we investigated whether silencing the Cbl-b gene could effectively promote the activation and proliferation of T cells using siRNA technology. We cultured cells *in vitro* with an antimouse CD3 functional antibody (1 μg/mL) that can specifically react with the mouse CD3 complex and initiate intracellular biochemical pathways that drive cellular activation and proliferation. However, the complete activation of peripheral T cells is regulated by the T cell receptor complex and costimulatory signals that are provided by antigen-presenting cells such as dendritic cells.[21] For example, CD28 has an essential role in discriminating activation from tolerance signaling.[17] Only stimulation through the T cell receptor alone leads to an anergic state of naïve T cells. However, Cbl-b knockout T cells produce high levels of IL-2 and proliferate vigorously after anti-CD3 stimulation alone.[22]Activated proliferating T cells express several surface molecules, including CD25, CD69, and CD71, which are usually poorly expressed or absent altogether on resting cells and are thus referred to as “activation antigens”.[23] A combination of flow cytometric analysis of the expression of these markers with cell proliferation assays may improve our understanding of events leading to efficient cell-mediated immune responses.[24] The cell surface presence of CD69, CD25, and CD71 are sequentially expressed after mitogenic stimulation (e.g., Con A). We found that the efficiency gradually decreased with time under the conditions of Cbl-b gene silencing. Thus, we designed experiments to observe the expression of CD69, CD25, and CD71 at the time points of 12 hours, 24 hours, and 48 hours. In addition, cytokines affect proliferation, differentiation, and functional activation of T cells that regulate cellular and humoral immune responses. By binding to specific cell surface receptors, IL-2 drives the proliferation of T cells, augments the cytotoxic activity of T cells, and generates lymphokine-activated killer cells *in vivo*.[25]

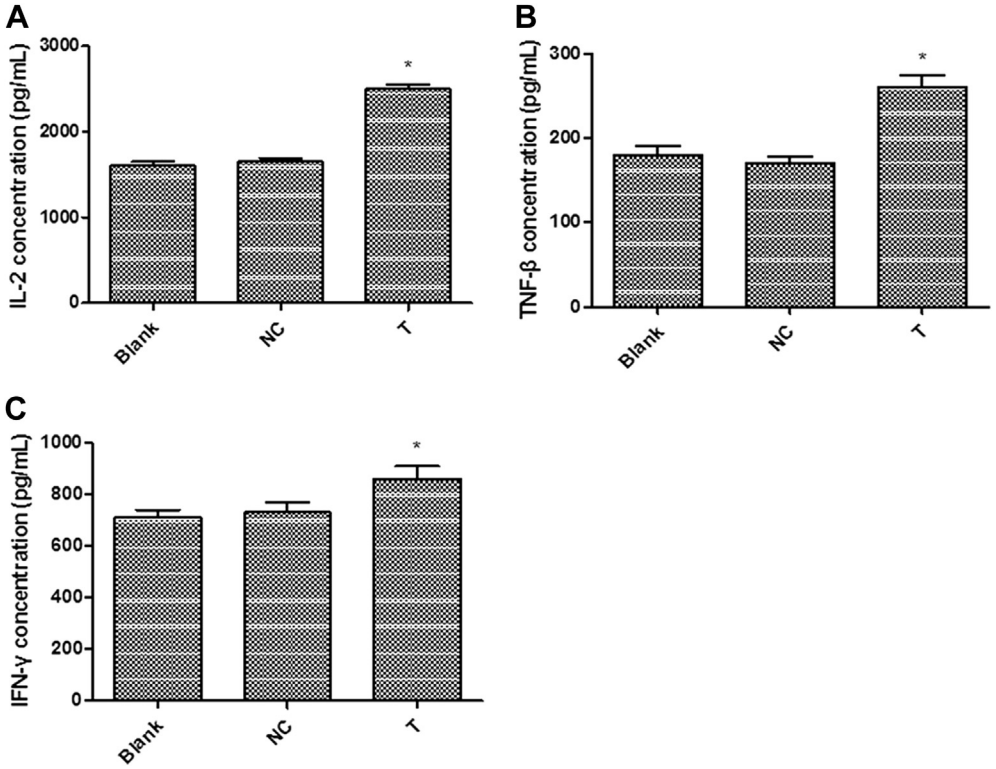

Fig. 3. Changes in cytokine secretion by transfected T cells at 48 hours after transfection. The secretion of IL-2, TNF-β, and IFN-γ in the T cell supernatant was measured by ELISA. (A) The secretion of IL-2 by the transfected T cells is significantly greater than its secretion by the negative control group or blank group (*$p < 0.001$). (B) The secretion of TNF-β by the transfected T cells is significantly greater than its secretion by the negative control group or blank group (*$p < 0.001$). (C) The secretion of IFN-γ by the transfected T cells is significantly greater than its secretion by the negative control group or blank group (*$p < 0.05$). Cytokine secretion values are presented as the mean ± the standard error of the mean ($n = 18$). ELISA = enzyme-linked immunosorbent assay; FAM = fluorescein amidite; IFN = interferon; IL = interleukin; NC = negative control; siRNA = small interfering ribonucleic acid; T = T cell; TNF = tumor necrosis factor.

The secretion of IFN-γ by activated T cells (and other immune cells) serves many important immunopotentiating effects such as regulating several aspects of the immune response; stimulating antigen presentation through Class I and Class II major histocompatibility complex receptors; and through its effects on T cell proliferation and apoptosis.[26] By contrast, TNF-β is primarily secreted by T cells and promotes thymocyte proliferation, activation, and differentiation, and it is widely involved in inflammation and in the immune response.[27] Our study showed that silencing the expression of the Cbl-b gene can induce T cell proliferation (Fig. 4) and the secretion of IL-2, IFN-γ, and TNF-β (Fig. 3). Silencing the Cb1-b gene also

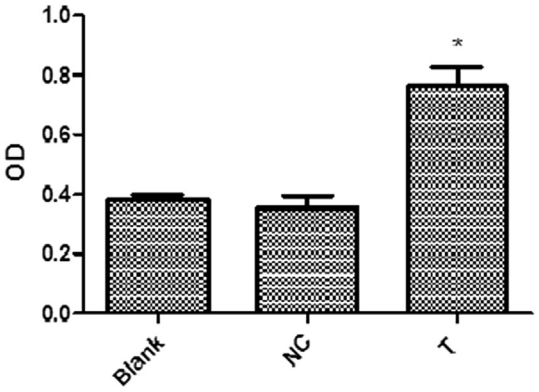

Fig. 4. The change in proliferation of transfected T cells after 48 hours. The proliferation rate of the transfected T cells is markedly greater than that of the negative control group or blank group (*$p < 0.001$). The data are expressed as the mean ± the standard error of the mean ($n = 18$). NC = negative control; OD = optical density; T = T cell.

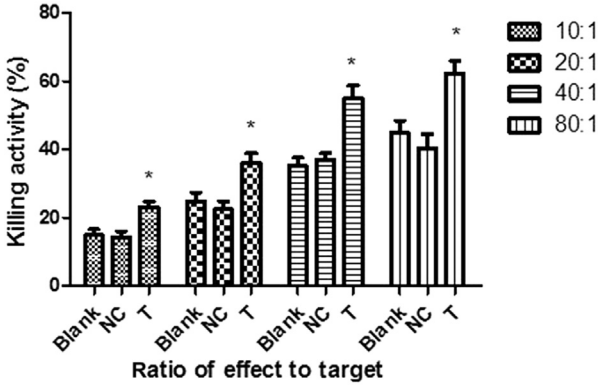

Fig. 5. The percent killing activity between the effector T cells and prostate cancer cell line targets after 48 hours of coculture. T cells were cocultured with RM-1 cells, and the killing activity determined by Cell Counting Kit-8 (KeyGen Biotech, Nanjing, China). The values are presented as the mean ± the standard error of the mean ($n = 18$). The killing activity of transfected T cells against targeted RM-1 prostate cancer cells at each ratio was higher than the activity in the negative control group or blank group (*$p < 0.05$). However, there is no difference between the killing activity at the effector:target (E:T) ratio of 40:1, compared to the ratio of 80:1 ($p = 0.078$). NC = negative control; T = target cell.

augmented the expression of CD69, CD25, and CD71 (Fig. 2). Thus, siRNA-mediated silencing of the Cbl-b gene promotes the activation and proliferation of T cells *in vitro*.

We furthermore studied whether silencing the Cbl-b gene could improve the cytotoxic activity of T cells against RM-1 prostate cancer cells *in vitro*. The RM-1 prostate cancer cell line originated from C57BL/6 mice.[28] Our data indicated that T cells transfected with Cbl-b-specific siRNA displayed a greater killing ability against RM-1, compared to control T cells that were transfected with scrambled siRNA at different E:T ratios (Fig. 5). Our observations suggested that silencing Cbl-b by siRNA enhanced the cytotoxic activity of T cells against RM-1 prostate cancer cells and thus provided a proof-of-concept that this approach is indeed possible. This approach could be a promising therapeutic option in the treatment of prostate cancer and, interestingly, could indicate a similar role for targeting Cbl-b in leukemia,[10] skin cancer,[11] TC1 tumor cells,[12] and lymphoma.[13] The effect of inhibiting tumors by an approach such as we have described in the current report certainly requires further verification in animal models. In this way, the mechanisms involved could be explored. Previous studies in animal models showed that the Cbl-b knockout mutation enhanced CD8+ T cell-mediated antitumor immunity. This observation could be explained by the fact that these cells may respond to antigenic stimulation, independent of CD28 costimulation, and thus bypass the suppressive effect otherwise mediated by TGF-β secreted by the tumor environment. Whether a similar mechanism operates *in vitro* also remains to be determined. We speculate that T cells transfected with siRNA demonstrate enhanced cytotoxic activity against RM-1 prostate cancer cells, partly because of CD28-independent activation and the pattern of the secreted cytokines. The killing ability of the transfected T cells interestingly was not significantly different when the effector T cell to target cell ratio was 40:1 or 80:1. We thus speculate that the cytotoxicity activity of T cells had achieved a saturation point when the ratio was 40:1. In conclusion, our results indicate that silencing Cbl-b gene expression by specific siRNA can enhance the cytotoxicity activity of T cells against RM-1 prostate cancer cells *in vitro* and it does so by promoting the immune activation of T lymphocytes.

Acknowledgments

We are grateful to Huiming Mao and Ying Lu for their expert technical assistance and for flow cytometry (FACS) analysis.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin D. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;**127**:2893–917.
2. Drake CG. Prostate cancer as a model for tumour immunotherapy. *Nat Rev Immunol* 2010;**10**:580–93.
3. Anderson MJ, Shafer-Weaver K, Greenberg NM, Hurwitz AA. Tolerization of tumor-specific T cells despite efficient initial priming in a primary murine model of prostate cancer. *J Immunol* 2007;**178**:1268–76.
4. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 2005;**5**:263–74.
5. Barrack ER. TGF beta in prostate cancer: a growth inhibitor that can enhance tumorigenicity. *Prostate* 1997;**31**:61–70.
6. Higano CS, Small EJ, Schellhammer P, Yasothan U, Gubernick S, Kirkpatrick P, et al. Sipuleucel-T. *Nat Rev Drug Discov* 2010;**9**:513–4.
7. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010;**363**:411–22.
8. Kantoff PW, Schuetz TJ, Blumenstein BA, Glode LM, Bilhartz DL, Wyand M, et al. Overall survival analysis of a phase II randomized controlled trial of a poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J Clin Oncol* 2010;**28**:1099–105.
9. Agdalena P, Josef MP. Cbl-b in T-cell activation. *Semin Immunopathol* 2010;**32**:137–48.
10. Stromnes IM, Blattman JN, Tan X, Jeevanjee S, Gu H, Greenberg PD. Abrogating Cbl-b in effector CD8+ T cells improves the efficacy of adoptive therapy of leukemia in mice. *J Clin Invest* 2010;**120**:3722–34.
11. Loeser S, Loser K, Bijker MS, Rangachari M, van der Burg SH, Wada T, et al. Spontaneous tumor rejection by Cbl-b-deficient CD8+ T cells. *J Exp Med* 2007;**204**:879–91.
12. Paolino M, Thien CB, Gruber T, Hinterleitner R, Baier G, Langdon WY, et al. Essential role of E3 ubiquitin ligase activity in Cbl-b–regulated T cell functions. *J Immunol* 2011;**186**:2138–47.
13. Chiang JY, Jang IK, Hodes R, Gu H. Ablation of Cbl-b provides protection against transplanted and spontaneous tumors. *J Clin Invest* 2007;**117**:1029–36.
14. Xu HS, Wu YW, Xu SF, Sun HX, Chen FY, Yao L. Antitumor and immunomodulatory activity of polysaccharides from the roots of *Actinidia eriantha*. *J Ethnopharmacol* 2009;**125**:310–7.
15. Disis ML, Bernhard H, Jaffee EM. Use of tumour-responsive T cells as cancer treatment. *Lancet* 2009;**373**:673–83.
16. Duan L, Reddi AL, Ghosh A, Dimri M, Band H. The Cbl family and other ubiquitin ligases: destructive forces in control of antigen receptor signaling. *Immunity* 2004;**21**:7–17.
17. Huang F, Gu H. Negative regulation of lymphocyte development and function by the Cbl family of proteins. *Immunol Rev* 2008;**224**:229–38.
18. Wohlfert EA, Callahan MK, Clark RB. Resistance to CD4+CD25+ regulatory T cells and TGF-beta in Cbl-b-/- mice. *J Immunol* 2004;**173**:1059–65.
19. Wang X, Yu J, Sreekumar A, Varambally S, Shen R, Giacherio D, et al. Autoantibody signatures in prostate cancer. *N Engl J Med* 2005;**353**:1224–35.
20. Harzstark AL, Small EJ. Immunotherapeutics in development for prostate cancer. *Oncologist* 2009;**14**:391–8.
21. Acuto O, Michel F. CD28-mediated costimulation: a quantitative support for TCR signalling. *Nat Rev Immunol* 2003;**3**:939–51.
22. Chiang YJ, Kole HK, Brown K, Naramura M, Fukuhara S, Hu RJ, et al. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* 2000;**403**:216–20.
23. Fraser JD, Strauss D, Weiss A. Signal transduction events leading to T-cell lymphokine gene expression. *Immunol Today* 1993;**14**:357–62.
24. Caruso A, Licenziati S, Corulli M, Canaris AD, De Francesco MA, Fiorentini S, et al. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry* 1997;**27**:71–6.
25. Asano Y, Kaneda K, Hiragushi J, Tsuchida T, Higashino K. The tumor bearing state induces augmented responses of organ-associated lymphocytes to high-dose interleukin-2 therapy in mice. *Cancer Immunol Immunother* 1997;**45**:63–70.
26. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. *Annu Rev Immunol* 1997;**15**:749–95.
27. Vassalli P. The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 1992;**10**:411–52.
28. Zhang AL, Russell PJ. Paclitaxel suppresses the growth of primary prostate tumours (RM-1) and metastases in the lung in C57BL/6 mice. *Cancer Lett* 2006;**233**:185–91.