

# Cbl-b gene silencing in splenic T lymphocytes as a therapeutic strategy to target the prostate cancer RM-1 cell tumors in immune competent mice

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**Abstract. – OBJECTIVE:** In recent years, the field of cancer immunotherapy has become a research hotspot and is currently faced with numerous challenges. The objective of this study was to assess the success of cbl-b gene silencing in splenic T lymphocytes as an immune strategy to target the murine prostate cancer RM-1 cells *in vitro* and solid tumors *in vivo*.

**MATERIALS AND METHODS:** For this purpose, cbl-b gene-specific siRNA was designed, synthesized, and was transfected into mouse splenic T lymphocytes, followed by assessment of T cell activation, TH1 cytokine production, and *in vitro* cytotoxicity against RM-1 cell targets. For *in vivo* cytotoxicity studies, first the RM-1 tumor model was established in immune competent mice that were later tumor-injected with splenic T lymphocytes transfected with specific shRNA for cbl-b gene silencing.

**RESULTS:** The data show that the cbl-b gene silencing in T lymphocytes resulted in an enhanced surface expression of CD69 activation marker, elevated production of interleukin (IL)-2 and interferon (IFN)- $\gamma$ , and their increased cytotoxicity as effectors against RM-1 prostate cancer cells. The tumor injection with cbl-b shRNA-transfected T lymphocytes also resulted in significant reduction of the tumor size as compared with controls.

**CONCLUSIONS:** cbl-b gene silencing strategy enhanced the immune function of T lymphocytes, increased their cytotoxic potential against RM-1 prostate cancer cells, as well as caused significant suppression of the tumor growth in immune competent mice.

## Key Words

cbl-b, Prostate cancer, siRNA, shRNA, T lymphocytes, Tumor immunity.

## Introduction

Prostate cancer is the most common malignancy in elderly males. In China, the incidence of this disease has been less than that in the Western countries. However, there is an increasing trend observed in the incidence of the disease in this country. In China, 80.6% patients with definitive diagnosis of prostate cancer were identified to be at the advanced and late stages of this disease, most of whom were radically unresectable<sup>1</sup>. Endocrine therapy and adjuvant radiotherapy remain to be the mainstream of current interventions. However, many patients may suffer from androgen-independent prostate cancer which remains poorly responsive to endocrine therapy and adjuvant radiotherapy, resulting in poor overall survival (OS) rates. As the 4<sup>th</sup> interventional option, tumor immunotherapy based on tumor biological treatment has received increasing attention in recent years. Cumulative evidence has pointed to the development of immune tolerance regarding tumor-specific lymphocytes in local microenvironment, resulting in failure of tumor immunity<sup>2-6</sup>. Therefore, mono-immunotherapy

tended at increasing the tumor-specific antigen presenting cells, such as administering *in vitro* expanded tumor-infiltrating lymphocytes (TILs), may not be successful. The development of tumor-induced immune tolerance is a multi-factorial process that induces lymphocyte suppression, resulting in failure of T cells to produce an effective immune attack against tumor cells.

Importantly, correlations between prostate cancer progression and paracrine TGF- $\beta$  signaling as well as between tumor invasion/metastasis and expression profile of inhibitory CD28 ligand B7-H3/B7x have been reported<sup>3-7</sup>. The key to the success of antitumor immunity and immunotherapy is how to effectively activate this population of tumor-specific immune cells or reverse immune tolerance or immune incompetent state. In this regard, E3 ubiquitin ligase Casitas B-lineage Lymphoma proto-oncogene B (cbl-b) gene silencing strategy may help overcome the afore-mentioned challenges. Cbl-b is a negative regulatory molecule of T cells and it plays a vital role in T cell activation<sup>7-9</sup>; cbl-b gene defect could not only directly activate cytotoxic T cells (CTLs) in the absence of costimulatory ligands<sup>9-13</sup>, but also reduced the sensitivity of T cells to suppressive cytokines such as TGF- $\beta$  and suppressive T regulatory cells (Tregs)<sup>14</sup>. Cbl-b gene deletion in mouse model studies was associated with decrease of tumor incidence and regression of existing tumors<sup>14,15</sup>. Cbl-b, a member of cbl family, was first isolated from human breast cancer cells<sup>16</sup>. The cbl family contains negatively regulating signal molecules that are widely distributed inside the body. Cbl-b gene is located on chromosome 3 and contains 19 exons; cbl-b protein has molecular weight of ~108kDa and acts as a signal molecule of various membrane receptors. It has distinct structural domains, including a highly conservative N-terminal, which mediates the binding process of cbl-b protein with diverse intracellular molecules. Cbl-b exerts its role as a conjunction or scaffold molecule during the process of cell activation. In these domains, RING domains are dominant and contain E3 ubiquitin chains which are associated with ubiquitin ligase activity of cbl-b for target protein degradation and negative regulation of intracellular signal transduction<sup>17</sup>. This negative regulation is vital to maintain an appropriate immune response and cbl-b suppression may induce autoimmune disease. Cbl-b deletion was associated with thymic hyperplasia, upregulation of

CD3 marker on T cells, increased Zap70 activities, and enhanced T cell proliferation in response to CD3 antibody stimulation<sup>18</sup>.

Herein, based on an immunocompetent tumor-bearing mouse model of prostate cancer, cbl-b-specific siRNA was transfected into T lymphocytes to silence cbl-b expression in order to: (i) reverse the immune tolerance of tumor-specific lymphocytes; (ii) improve the immune vitality of tumor-specific lymphocytes; and (iii) evaluate suppressive effects on prostate cancer and its clinical application potential. Moreover, the mechanisms of tumor immune escape and antitumor immunity were also investigated.

## Materials and Methods

### Mice

Thirty adult male (6 weeks old), healthy C57BL/6 mice were procured from the Chinese Academy of Science. The animals were housed in individual cages under 12h/12h dark/light cycle and were offered feed and water *ad libitum*. The study protocol was approved by the institutional Committee on Animal Research and Ethics (CARE) and all animal experiments performed conformed to the national and international guidelines for animal use in research.

### Collection of splenic lymphocytes and culture

A healthy mouse was sacrificed by cervical dislocation and was immersed in 75% alcohol for 2-3 min for disinfection. After transferring to an ultra clean bench, the abdominal cavity of mouse was cut open using small pair of scissors and spleen was removed. After excising the outer membrane and connective tissues, the residual mass was placed in a sterile Petri dish containing small amount of physiological saline. The spleen was scratched with the frosted face of two slides, and washed with saline. Harvested fluid was filtered and 4 ml of filtrate was gently overlaid on 8ml of lymphocyte separation medium and the tube was centrifuged in swinging bucket rotor at a speed of 1200  $\times$ g for 20 min. Splenic lymphocytes visible as a buffy coat at the interface were gently collected, mixed with normal saline for washing, centrifuged at 800  $\times$ g for 5 min and the supernatant was discarded. After a total of 3 washings, lymphocyte pellet was dislodged by finger tapping, mixed with lymphocyte medium and cells were counted.

Mouse splenic lymphocytes were cultured ( $2 \times 10^6$  cells/ml) using lymphocyte medium containing ConA (0.05  $\mu\text{g/ml}$ ) in a 6-well plate (2 ml per well) and incubated at  $37^\circ\text{C}$  in a humidified  $\text{CO}_2$  (5%) incubator for 3 days. Fresh culture medium (1 ml) was replenished after 2 days by replacing the same volume from each well. In the event of prolonged incubation, culture medium was replaced as before after every 2-3 days as required.

### **siRNA transfection**

Four cbl-b specific siRNAs were designed and synthesized by Shanghai Jikai Gene Co. Ltd. (China) and the sequences were as follows: siRNA-1: 5'-UCC CAA GCU UCA GUU GAA Att-3'; siRNA-2: 5'-CCC UGA UUU AAC CGG AUU Att-3'; siRNA-3: 5'-CCA UCU CAU UGC CAU AAU Gtt-3'; siRNA-4: 5'-UGA GAU GCC CUG AUA UUA Att-3'. Mouse splenic lymphocytes cultured in a 6-well plate for 3 days were washed twice, resuspended to have a single cell suspension in Opti-MEM by repeated pipetting, and cells were seeded (100  $\mu\text{l/well}$ ) in a 96-well plate at a concentration of  $1 \times 10^5$  cells/ml. Lipofectamine<sup>TM</sup>2000 liposomes (0.5  $\mu\text{l}$ ) and cbl-b siRNA (1.5  $\mu\text{l}$ ) were added to 5  $\mu\text{l}$  of Opti-MEM, gently mixed together and incubated at room temperature for 20 min. The transfection mixture was added drop wise to designated well, dispersed by gentle tapping before adding anti-CD3 (20  $\mu\text{l}$ ), and under ambient conditions for 6h, after which, 20  $\mu\text{l}$  of heat-inactivated fetal bovine serum (FBS) was added and incubated at  $37^\circ\text{C}$  in a humidified  $\text{CO}_2$  (5%) incubator for another 48h. The transfection efficiency was 89.42% (data not shown).

### **Flow cytometry**

After 48h incubation, cbl-b-shRNA transfected cells were harvested by centrifugation at  $1200 \times g$  for 5 min and suspended in 1ml of lymphocyte culture medium. Cells were counted, washed thrice in phosphate buffered saline (PBS) and suspended ( $1 \times 10^6$ ) in 100  $\mu\text{l}$  saline and incubated with 1  $\mu\text{l}$  FITC-conjugated anti-CD69 mouse antibody at room temperature for 30 min in the dark. Cells were washed thrice as before and resuspended in 500  $\mu\text{l}$  of PBS. Cells were analyzed by flow cytometry (Becton and Dickinson, Franklin Lakes, NJ, USA) and data analyzed by CellQuest software. A total of  $1 \times 10^5$  cells were analyzed for each sample and results were expressed as percentages of fluorescent positive cells.

### **Western Blot**

Samples (each containing 100  $\mu\text{g}$  protein) were mixed with  $2 \times$  sample buffer, boiled in  $100^\circ\text{C}$  water bath and loaded in gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was run first at 100 V for 10 min and then at 80 V for 2-3h. Proteins were electro-transferred to PVDF membrane at 80V for 1.75h. The membranes were blocked with 1%BSA in tris buffered saline tween (TBST) at room temperature with shaking for 1h. The samples were incubated with primary antibody (1:1000 diluted) at  $4^\circ\text{C}$  overnight with shaking. Samples were washed twice with TBST (5 min each time), followed by TBS wash twice (5min each time), then incubated with secondary antibody (1:1500 diluted) at room temperature for 45 min with shaking. Samples were washed with TBS for three times. The images were captured and band intensity of target proteins was analyzed using GelAnalyse imaging analysis software. The results were expressed as band intensity of target protein divided by that of internal reference.

### **RM-1 prostate cancer cell culture**

RM-1 tumor cells, originally derived from C57BL/6 mice, were rapidly defrosted at  $37^\circ\text{C}$  in a water bath, washed twice with physiological saline solution and cultured ( $10^3$  cells/ml) using RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin in a cell culture flask and incubated at  $37^\circ\text{C}$  in a humidified  $\text{CO}_2$  (5%) incubator for 2 days. Tumor cells were regularly passaged by trypsinization (0.05% v/v trypsin, 0.53 mM EDTA) during the logarithmic growth phase as required.

### **T lymphocyte proliferation assay**

After 48h incubation, cbl-b siRNA-transfected lymphocytes were incubated with cell counting kit (CCK)-8 solution (10  $\mu\text{l/well}$ , at a volume ratio of 1:10) at  $37^\circ\text{C}$  in a humidified (5%  $\text{CO}_2$ ) incubator for 1-4h. The absorbance values were determined using microplate reader at 450 nm and 630 nm. Triplicate readings were taken and the results were expressed as the average of three readings.

### **T lymphocyte in vitro cytotoxicity assay**

RM-1 prostate cancer cells (designated as target cells) were centrifuged at  $800 \times g$  for 5 min after trypsin digestion, followed by addition of lymphocyte preparation medium (1 ml). Cell suspension (0.1 ml) was mixed with 0.4% Trypan blue for

counting, cell concentration was adjusted to  $1 \times 10^6/\text{ml}$ , and  $10 \mu\text{l}$  of cell suspension was added to each well of a 96-well plate. T lymphocytes with or without *cbl-b* gene transfection (designated as effector cells) were inoculated into triplicate wells at the effect: target (E:T) ratios of 40:1, 20:1, and 10:1 and the volume was adjusted to  $100 \mu\text{l}$  in each well. Effector-target coculture (triplicate) plates were then incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  humidified incubator for 24h, 48h, or 72h, followed by CCK-8 assays (Shanghai Bogu Biological Co., Shanghai, China). The CCK-8 assay allows for sensitive colorimetric determination of the cell viability in cytotoxic or cell proliferation assays. In this assays, Dojindo's highly water soluble tetrazolium (WST)-8 salt is reduced by dehydrogenase activities in live cells to generate a yellow color formazan dye which is soluble in tissue culture media. Thus, the amount of formazan dye generated by cell dehydrogenases is directly proportional to the number of live cells present. The assay is based on 3 simple steps: (1) addition of CCK-8 solution; (2) incubation; and (3) optical density (OD) measurement at 450nm, which were performed following the manufacturer's instructions. The rate of killing by T lymphocytes was determined as follows:

$$\text{T lymphocyte killing rate (\%)} = \frac{\text{ODE} + \text{T} - \text{ODE}/\text{ODT}}{1 - (\text{ODE} + \text{T} - \text{ODE}/\text{ODT})} \times 100$$

Where, ODE+T: OD values of 'effector plus target' cells; ODE: OD values of effector cells at corresponding concentrations; and ODT: OD values of target cells at corresponding concentrations.

#### ***Inoculation of RM-1 cells into C57BL/6 mice***

After trypsinization of RM-1 prostate cancer cells in logarithmic growth phase, the cells were washed twice with physiological saline solution and resuspended in culture medium. C57BL/6 mice were transferred to ultra-clean bench, fur near the right armpit was trimmed off using curved scissors, and skin was sterilized with 75% alcohol. RM-1 tumor cell concentration was adjusted to  $2 \times 10^7/\text{ml}$  and 0.1 ml of cell suspension was mixed with biological glue on ice at a ratio of 1:1 and the preparation was injected (0.2 ml) into the right armpit and the tumor growth profile was examined periodically. The diameters of tumors were measured using a Vernier caliper and documented once per three days after initiation of tumor formation. The tumor growth curve was also plotted.

#### ***T lymphocyte inoculation into C57BL/6 mice***

T lymphocytes transfected with plasmids containing *cbl-b* shRNA were homogenized with culture medium by pipetting to render a cell concentration of  $1 \times 10^7/\text{ml}$ . Then, lymphocyte suspension (0.5 ml) was administrated subcutaneously into the tumor site using a 1 ml syringe as the tumor size attained 0.2 cm in diameter.

#### ***Statistical Analysis***

The data obtained were analyzed by multi-factor analysis of variance (ANOVA) using SPSS14.0 statistical software (SPSS Inc., Chicago, IL, USA). A *p*-value of  $< 0.05$  was considered to be statistically significant.

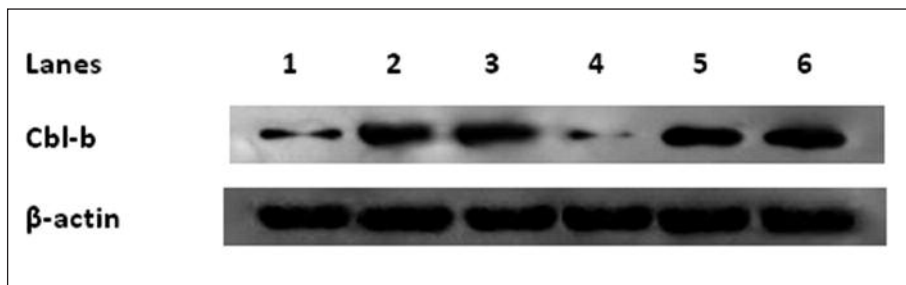
## **Results**

#### ***Cbl-b protein expression after siRNA transfection***

Total proteins were extracted following the manufacturer's instructions using total protein extraction kit (Shanghai Bogu Biological Co. Shanghai, China) from four batches of *cbl-b* siRNA-transfected T lymphocytes as well as from control group and blank group (untransfected) lymphocytes. Protein concentrations were determined by using Folin-phenol kit, expression of *cbl-b* protein was determined by Western blot and results were analyzed using ShineTech Gel-Analyse image processing software. *Cbl-b* protein suppression rates in 4 siRNA-transfected T lymphocytes batches (Lanes 1 through 4; Figure 1) were found to 62%, 13%, 8%, and 85%, respectively, while negative control (mock-transfected) and blank control (untransfected) lymphocytes had comparable *cbl-b* protein expression.

#### ***Surface expression of T cell activation marker CD69 after cbl-b gene silencing***

Surface expression of CD69 which is considered a useful marker for the early T lymphocytes activation in mixed populations was also determined by flow cytometry. The expression of CD69 was significantly upregulated ( $6.37 \pm 0.40\%$ ) following siRNA-mediated suppression of *cbl-b* in T lymphocytes as compared with its expression in control ( $1.94 \pm 0.81\%$ ) and blank ( $1.85 \pm 0.20\%$ ) groups. The representative data from three independent experiments are shown (Table I; Figure 2).



**Figure 1.** Suppression of the cbl-b protein expression. Suppression of cbl-b protein expression in the mouse splenic T lymphocytes following transfection with 4 different siRNAs was determined by Western blot, using β-actin housekeeping gene as internal control. Lanes 1 through 6 represent siRNA-1, siRNA-2, siRNA-3, siRNA-4, blank (untransfected) and control (mock-transfected) lymphocytes, respectively. The cbl-b protein suppression induced by siRNA-1, siRNA-2, siRNA-3, and siRNA-4 was 62%, 13%, 8%, and 85%, respectively. The representative blot from three independent experiments is shown.

**IL-2 and IFN-γ production by T lymphocytes after cbl-b gene silencing**

The expression of IL-2, a Th1 cytokine that is centrally involved in the growth and proliferation of T lymphocytes as well as their differentiation into ‘effector’ T cells, was determined in T cell culture supernatants using commercial ELISA kit (Shanghai Bogu Biological Co., Shanghai, China) and following the manufacturer’s instructions. IL-2 secretion by T lymphocytes was significantly upregulated ( $p < 0.01$ ) after siRNA-mediated cbl-b gene suppression in experimental group ( $852.78 \pm 38.17$  pg/ml) as compared with its expression in control ( $629.46 \pm 37.00$  pg/ml) and blank ( $593.83 \pm 15.98$  pg/ml) groups (Table II).

Also, the expression of interferon (IFN)-γ, a cytokine that is critical for innate and adaptive immunity against viral/intracellular pathogens or tumors and is secreted by both T lymphocytes and natural killer (NK) cells, was determined in T cell culture supernatants using commercial ELISA kit (Shanghai Bogu Biological Co., Shanghai, China) and following the manufacturer’s instructions. IFN-γ secretion by T lymphocytes was signifi-

cantly upregulated ( $p = 0.002$ ) after siRNA-mediated cbl-b gene suppression in experimental group ( $208.19 \pm 7.92$  pg/ml) as compared with its expression in control ( $167.08 \pm 10.97$  pg/ml) and blank ( $163.76 \pm 7.35$  pg/ml) groups. The representative data from three independent experiments are shown in Table II.

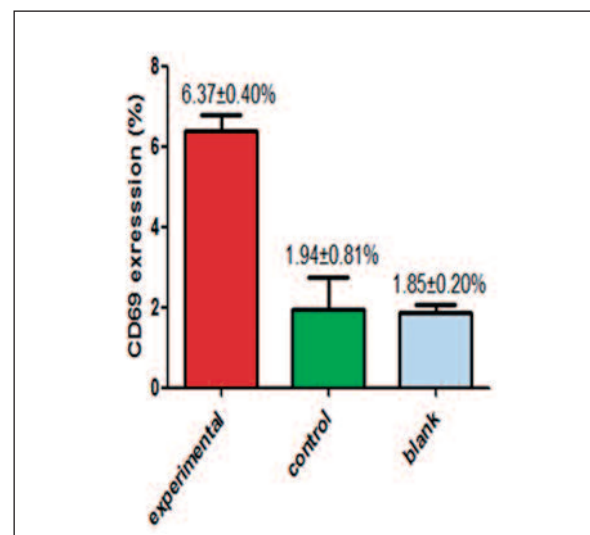
**T lymphocyte proliferative responses following cbl-b gene silencing**

T lymphocyte proliferative responses were also assessed following siRNA-mediated suppression of cbl-b gene by using CCK-8 kit as described earlier in materials and methods. Consistent with increased IL-2 expression, T lymphocyte prolifer-

**Table I.** Surface expression (%) of activation marker CD69 on blood T lymphocytes after cbl-b gene silencing

Groups	CD69 expression (%)
Experimental group (cbl-b siRNA-transfected)	6.37±0.40
Control group (mock-transfected)	1.94±0.81
Blank group (untransfected)	1.85±0.20

Experimental vs. Control:  $q = 13.594$  ( $p < 0.001$ ); Experimental vs. Blank:  $q = 14.29$  ( $p < 0.001$ ); Control vs. Blank:  $q = 0.697$  ( $p = 0.640$ ).



**Figure 2.** Surface expression of CD69 T cell activation marker. Surface expression (%) of CD69 activation marker was determined by flow cytometry on blood T lymphocytes following transfection with cbl-b siRNA-4. The representative histograms from three independent experiments are shown.

**Table II.** IL-2 and IFN- $\gamma$  concentrations in T cell supernatants after cbl-b gene silencing

Groups	IL-2 (pg/ml)	IFN- $\gamma$ (pg/ml)
Experimental group (cbl-b siRNA-transfected)	852.78 $\pm$ 38.17	208.19 $\pm$ 7.92
Control group (mock-transfected)	629.46 $\pm$ 37.00	167.08 $\pm$ 10.97
Blank group (untransfected)	593.83 $\pm$ 15.98	163.76 $\pm$ 7.35

Experimental vs. Control:  $q = 12.169$  ( $p < 0.001$ ); Experimental vs. Blank:  $q = 14.08$  ( $p < 0.001$ ); Control vs. Blank:  $q = 1.890$  ( $p = 0.230$ ).

ation was also enhanced significantly ( $p < 0.05$ ) in the experimental group (OD = 1.43 $\pm$ 0.02) as compared with control group (OD = 0.542 $\pm$ 0.03). The representative data from three independent experiments are shown in Table III.

### T lymphocytes-mediated killing of RM-1 prostate tumor cells

T lymphocytes-mediated killing of RM-1 prostate tumor cells was determined using CCK-8 commercial kit as describe in materials and methods. Higher cytotoxic activity by T lymphocyte effectors was observed in the experimental group as compared with control in effector-target co-culture assays. The highest cytotoxicity (82.02%) was observed at an effector-to-target ratio of 40:1 at 24h (Figure 3; Table IV). Consistent with this finding, more extensive lymphocytic infiltration together with plaques of floating dead tumor cells were observed in cocultures at 24h (supplementary Figure S-1).

**Table III.** T lymphocyte proliferative response following cbl-b gene suppression

Groups	Optical Density (OD)
Experimental group (cbl-b siRNA-transfected)	1.43 $\pm$ 0.02
Control group (mock-transfected)	0.542 $\pm$ 0.03
Blank group (untransfected)	0.528 $\pm$ 0.07

Experimental vs. Control:  $q = 119.571$  ( $p < 0.001$ ); Experimental vs. Blank:  $q = 121.553$  ( $p < 0.001$ ); Control vs. Blank:  $q = 1.982$  ( $p = 0.211$ ).

### T lymphocytes-mediated cytotoxicity in prostate cancer mouse model

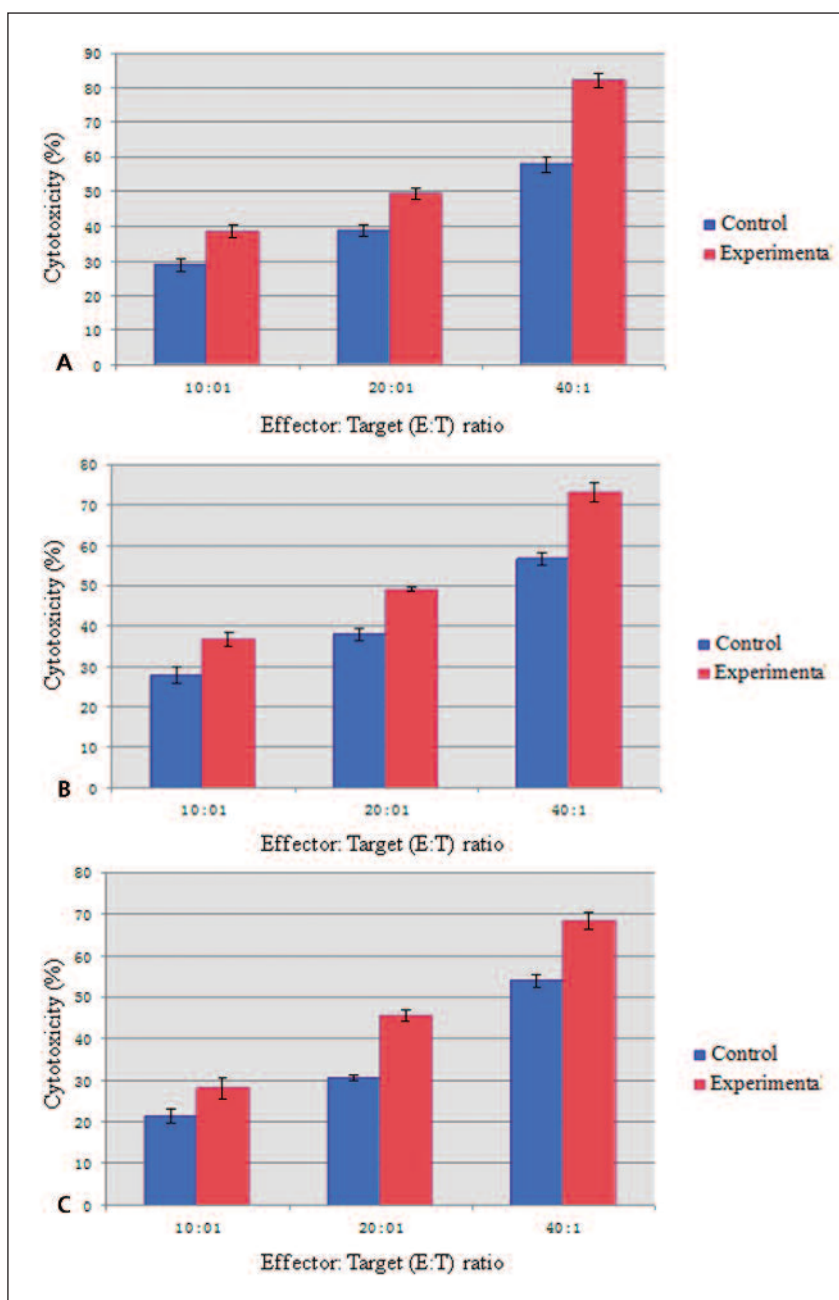
C57BL/6 mice were inoculated subcutaneously in the armpit with RM-1 prostate cancer cells to establish a mouse tumor model. The tumor nodule started to appear at about 7 days post-inoculation and, as shown in Figure 4, tumor of nearly 2cm size was observed at 20 days. The tumor biopsy shows successful establishment of solid tumor with typical histopathological features of abundant cytoplasm, abnormal cellular morphology, mitotic fat and skeletal muscle infiltration, and angiogenic blood vessel growth (supplementary Figure S-2).

Following intra-tumor injection with cbl-b shRNA-transfected T lymphocytes, the tumor size (diameter in cm) was measured every 3 days, starting at the 9<sup>th</sup> day onward. The rate of tumor growth in experimental group (inoculated with shRNA-transfected T lymphocytes) was significantly lower than control (untransfected T lymphocytes) and blank (un-inoculated) groups (Figure 5). As expected, the tumor regression was induced following intra-tumor injection with splenic T lymphocytes transfected with cbl-b shRNA. The tumor at 4 weeks (Figure 6) was remarkably smaller in experimental mice (inoculated with shRNA-transfected T lymphocytes) as compared with control (inoculated with non-transfected T lymphocytes) and blank mice (un-inoculated); and at necropsy performed at 6 weeks, the tumor diameter (cm) in experimental mouse was significantly smaller than in control mouse (Figure 7).

## Discussion

Cbl-b, a member of Cbl protein family, is a key gene responsible for the immune function regulation. As a negative regulatory gene, it plays a vital role in the activation of adjacent T cells<sup>19,20</sup>. The strategy of cbl-b gene silencing could not only directly stimulate CTLs in the absence of co-stimulatory ligands to produce massive IL-2 cytokine, but also reduce the sensitivity of T-cells to suppressive cytokines such as TGF- $\beta$ , and subsequently kill tumor cells through active immunization which suggests a promising potential for their use in the field of tumor immune intervention. Accordingly, four cbl-b specific siRNAs were designed and synthesized, and subsequently transfected into mice splenic lymphocytes using liposomes as vector. The transfec-

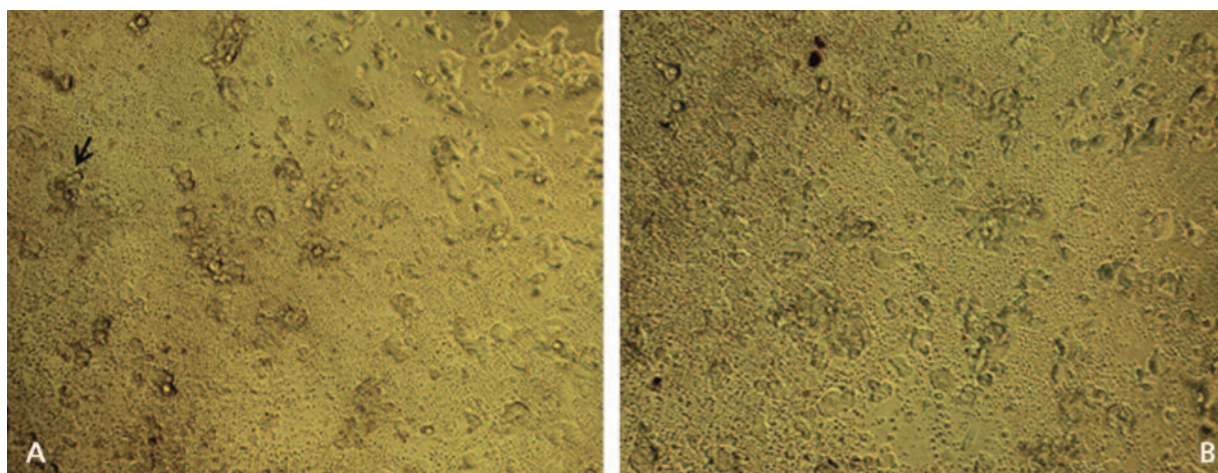
**Figure 3.** In vitro cytotoxicity of T lymphocytes against RM-1 prostate cancer cells. T lymphocytes-mediated killing (%) of RM-1 tumor cells, as determined by using CCK-8 commercial kit, is shown at (A) 24h; (B) 48h; and (C) 72h. Significantly higher cytotoxicity was observed by T lymphocyte after siRNA-mediated cbl-b gene silencing as compared with control. The highest cytotoxicity of 82.02% was observed at an effector to target (E:T) ratio of 40:1 at 24h. The data obtained from four independent experiments are shown.



**Table IV.** Cytotoxicity (%) of cbl-b siRNA-transfected and untransfected T lymphocytes against RM-1 prostate cancer cells.

Duration	Cbl-b siRNA-transfected lymphocytes			Untransfected lymphocytes		
	10:1	20:1	40:1	10:1	20:1	40:1
24h	38.36±1.82	49.27±1.66	82.03±1.82	28.76±1.83	38.67±1.75	57.94±2.31
48h	36.68±1.76	49.03±0.57	73.17±2.31	27.74±2.11	37.89±1.47	56.60±1.46
72h	28.08±2.53	45.51±1.24	68.31±1.97	21.33±1.78	30.47±0.69	53.78±1.58

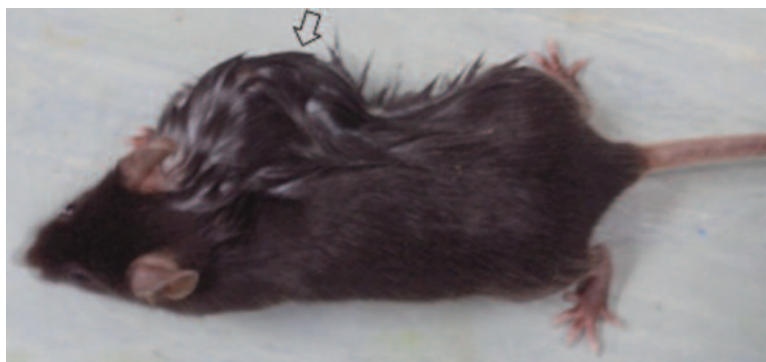
Significant differences were observed between Cbl-b siRNA-transfected and untransfected cells with regard to time (24h, 48h, 72h) and effector: target ratios (10:1, 20:1, 40:1) (All *p*-values <0.05).



**Figure S-1.** Lymphocytic infiltration of RM-1 prostate cancer cell cultures at 24h (magnification: 100×). Cbl-b gene silencing via siRNA transfection induced T lymphocyte activation and extensive lymphocytic infiltration of RM-1 prostate cancer cells was observed at 24h in experimental group (A) as compared with control group (B). Plaques of floating dead cells (arrow) were frequently visible in experimental cocultures.

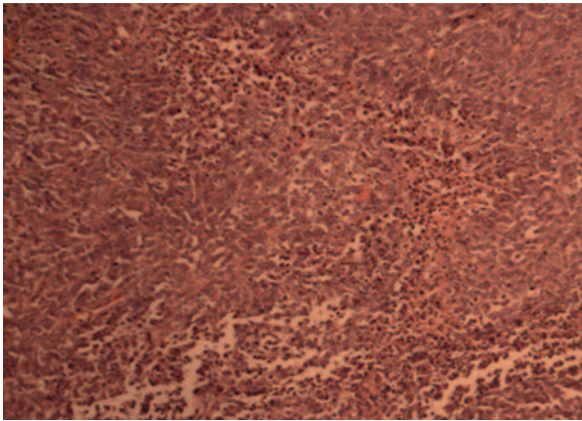
tion efficiency was found to be 89.42% which was higher than that obtained through other transfection methods used elsewhere. In order to further assess the siRNA-mediated suppressive effect on cbl-b gene expression, cbl-b protein expression was determined by Western blot at 48h which indicated the variable degree of cbl-b gene expression by 4 siRNAs used and siRNA-4 induced the highest gene expression at protein level (85%) in mouse splenic T lymphocytes. Different silencing effects are generated by siRNAs targeting different nucleotides of the same mRNA transcribing into variable expression of cbl-b protein. In this study, we found that siRNA-4 induced the maximum suppression in cbl-b gene expression. Consistent with this finding, it was reported earlier that the effective suppression of gene expression could be observed by only few siRNAs<sup>21</sup>.

E3 ubiquitin ligase cbl-b is a key factor involved in immune regulation and cbl-b upregulation has been associated with development of peripheral tolerance and anergy of T cells<sup>22</sup>. Thus, cbl-b is critical for establishing the threshold for T cell activation. Since cbl-b functions as a negative regulator of T cell activation, we assessed the secretion of two immune regulatory T cell cytokines (IL-2 and IFN- $\gamma$ ) in the supernatants of mouse splenic T lymphocytes that were transfected with cbl-b-specific siRNA. IL-2 is the major factor for T cell activation and proliferation which is foundation of priming the feedback loop. IFN- $\gamma$  is a type II interferon that has antiviral, antitumor and immunoregulatory properties. As the data show, both IL-2 and IFN- $\gamma$  were found significantly upregulated in T cell supernatants of experimental group as compared with those of control or blank groups; thus, suggesting



**Figure 4.** Establishment of prostate cancer mouse model. C57BL/6 mice were inoculated subcutaneously in the right armpit with RM-1 prostate cancer cells, as described in Materials and Methods, to establish a mouse tumor model. The development of tumor nodule was visible at about 7 days post-inoculation and a solid tumor of ~2 cm size (arrow) was observed at 20 days.





**Figure S-2.** Tumor histopathology at 2 weeks. The tumor biopsy performed at about 2 weeks shows the successful establishment of solid tumor with typical histopathological features including tumor cell infiltration, abundant cytoplasm, abnormal cell morphology, clear nucleoli, mitotic fat and skeletal muscle infiltration, and angiogenic blood vessel growth.

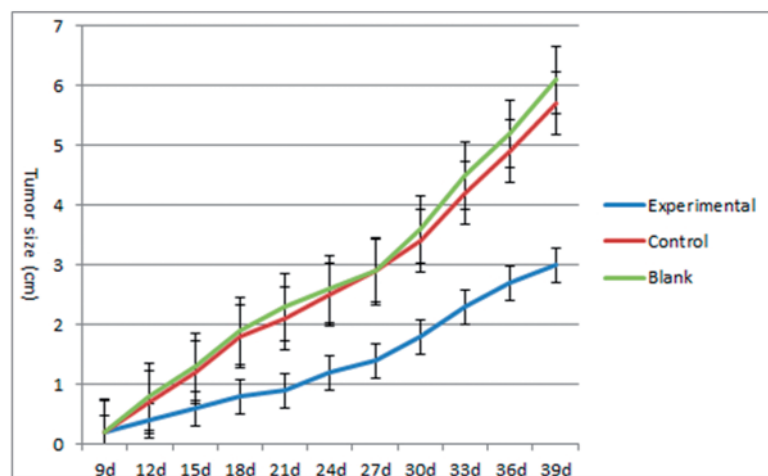
a significant amplification of the lymphocyte activity following *cbl-b* gene silencing. Consistent with our findings, Stromnes et al<sup>23</sup> also reported that siRNA knockdown of *Cbl-b* in CD8<sup>+</sup> effector T cell clones enhanced the IL-2/IFN- $\gamma$  production, restored T cell proliferative responses and increased target avidity.

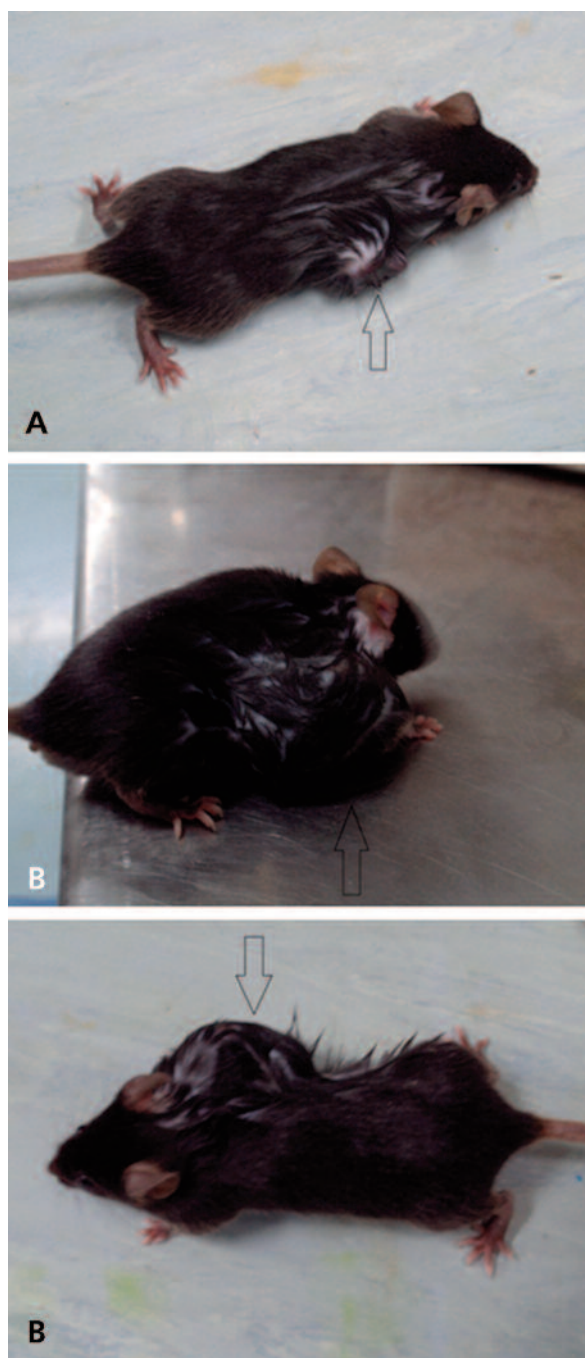
To further investigate the cytotoxicity of *cbl-b*-knockdown lymphocytes against tumor cells, T lymphocytes with or without *cbl-b* siRNA transfection were cocultured with RM-1 prostate cancer cells at E:T ratios of 40:1, 20:1 and 10:1. The *cbl-b* gene knockdown lymphocytes (i.e. siRNA transfected) showed signifi-

cantly higher ( $p < 0.05$ ) cytotoxicity against the tumor cells as compared with controls; and the highest killing (85%) was observed at E:T ratio of 40:1 at 24h. This observation suggests the possibility of amplifying cytotoxicity against RM-1 prostate cancer cells through the *cbl-b* gene knockdown/ablation strategy in *in vivo* model studies. Since target cell killing rates at 48h and 72h mutually differed non-significantly and were relatively less than killing observed at 24h, we propose that the time-dependent characteristic of siRNA interference may explain the reduced toxicity over time. Furthermore, since killing rates differed significantly ( $p < 0.05$ ) between E:T ratios used, the selection of an appropriate E:T ratio and assay time would be critical to the successful clinical application of this interventional strategy for prostate cancer immunotherapy. Since mice bear similarities to humans in many aspects of genetics, pathology, and biology, they have been widely used as animal model for cancer research<sup>24,25</sup>. Using state of the art technology, desirable gene mutations with regard to human disease can be designed and induced to generate the relevant murine models. Thus, mouse models can be employed as a powerful tool to integrate the fundamental and clinical cancer research in immune prevention and therapy.

In order to establish an immune competent mouse tumor model, RM-1 prostate cancer cells harvested from logarithmic culture phase were administered subcutaneously into the right armpit of 6-month old mice and the tumor development started at one week post-inoculation. Since the directly administered tumor cells in

**Figure 5.** Rate of tumor growth in C57BL/6 mice. Following intra-tumor injection with *cbl-b* shRNA-transfected T lymphocytes, the tumor size (diameter in cm) was measured every 3 days, starting at the 9<sup>th</sup> day onward. The rate of tumor growth in experimental group (inoculated with shRNA-transfected T lymphocytes) was significantly lower than control (untransfected T lymphocytes) and blank (un-inoculated) groups.





**Figure 6.** Tumors at 4 weeks in C57BL/6 mice. The tumor regression was induced following intra-tumor injection with splenic T lymphocytes transfected with *cbl-b* shRNA. The tumor was smaller in experimental (inoculated with shRNA-transfected T lymphocytes) mouse (A) as compared with control (inoculated with non-transfected T lymphocytes) mouse (B) and blank (un-inoculated) mouse (C).

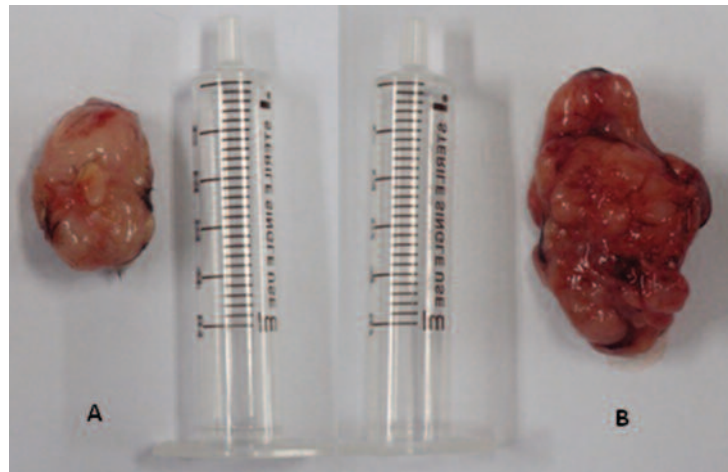
immune competent mice could undergo phagocytosis by effector cells, co-administration with biological glue allowed for tumor cell immobi-

lization at the site of inoculation, resulting in successful development of tumor. Thus, our immune-competent mouse model was different from the immune-deficient models that were previously used in related studies and has the potential benefits of economics, feasibility, and efficiency for further use in subsequent studies. Besides, siRNA-mediated *cbl-b* gene suppression validated in short-duration (3-day) *ex vivo* cytotoxicity assays was considered unsuitable for the *in vivo* cytotoxicity; we, therefore, used plasmids expressing more suitable and long-acting shRNA for *cbl-b* gene interference in mouse model studies. The data show that 3 out of 5 mice administered with *cbl-b* shRNA-transfected lymphocytes exhibited reduced tumor growth, higher activation status of the peripheral blood T lymphocytes, and enhanced IL-2/IFN- $\gamma$  production, whereas other two mice were comparable with controls. The difference might be due to variations in the individual immunobiological and genetic factors involved which needs to be further investigated. This study sets the stage for further investigations into prostate cancer immune intervention via the *cbl-b* gene silencing, however, one major caution relates to possible development of allergic auto-antigenic responses over time leading to autoimmune disease. Notwithstanding that such autoimmune diseases are often non-lethal, active preventive and therapeutic measures need to be considered in order to minimize any adverse reactions associated with gene immunotherapy. *Cbl-b* gene silencing may lead to autoimmune disease which, as compared to prostate cancer, is non-lethal; however, further studies will be required to investigate the possibilities in different animal models.

## Conclusions

Our *in vitro* study data show that siRNA-mediated *cbl-b* gene silencing induced T lymphocyte activation, increased cytotoxicity and enhanced production of immunoregulatory cytokines, such as IL-2 and IFN- $\gamma$ . Complement to these findings, mouse model study data show that the tumor-bed inoculation with *cbl-b* shRNA-transfected splenic lymphocytes leads to tumor regression as assessed by tumor size reduction. Taken together, *cbl-b* gene silencing strategy may be used as a useful tool for prostate cancer immunotherapy.

**Figure 7.** Tumor size at necropsy. The tumor size (diameter in cm) at necropsy, performed at 6 weeks post-inoculation, was significantly smaller in the experimental mouse (A) as compared with control mouse (B).



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### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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