Original Paper

Enhancement of Anti-Leukemia Immunity by Leukemia-Derived Exosomes Via Downregulation of TGF- β 1 Expression

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Key WordsTgf- β 1 • Exosomes • Leukemia immunotherapy**Abstract**

Background/Aims: Minimal residual leukemia cells (MRLs) are difficult to eradicate through traditional treatment and therefore remain to be a major threat to the long-term survival of leukemia patients. Tumor-derived exosomes (TEXs), which carry tumor associated antigens (TAA), may be a potential cell-free tumor vaccine for the specific eradication of MRLs. However, TEXs are intended to be less immunogenic due to exosomal TGF- β 1. To further optimize the efficacy of TEX-based vaccines, we investigated whether exosomes from TGF- β 1 silenced leukemia cells (LEX_{TGF- β 1si}) had an increased potential to induce a specific antitumor effect compared with non-modified exosomes. **Methods:** Exosomal TGF- β 1 was downregulated via lentiviral shRNA silencing of *TGF- β 1* in leukemia cells. The characteristics of LEX_{TGF- β 1si} were determined via electron microscopy, western blot analysis, and flow cytometry. The antitumor effect of LEX_{TGF- β 1si} was evaluated by detecting the properties of LEX_{TGF- β 1si}-pulsed dendritic cells (DCs), CD4⁺ T-cell proliferation, Th1 cytokine secretion, specific CTL activity, and NK cell function. Moreover, to verify the superiority of LEX_{TGF- β 1si} immunization, LEX_{TGF- β 1si} was subcutaneously injected into DBA/2 mice: either followed by tumor challenge or tumor bearing. **Results:** The lentiviral shRNA silencing of *TGF- β 1* in parental leukemia cells successfully downregulated the TGF- β 1 level in leukemia cell derived exosomes (LEX). LEX_{TGF- β 1si} was uptaken by DCs and was more potent in promoting DC function by upregulating the surface expression of costimulatory factors and MHC class II molecules, while inducing the secretion of IL-12p70 and TNF- α . Furthermore, immunization with LEX_{TGF- β 1si} facilitated CD4⁺ T-cell proliferation and Th1 cytokine secretion, and stimulated stronger specific cytotoxic lymphocyte (CTL) response and nature killer (NK) cell cytotoxicity more efficiently compared to non-modified LEX. In mice models, immunization with LEX_{TGF- β 1si} resulted in a more potent capability to inhibit tumor growth and to prolong survival, suggesting that LEX_{TGF- β 1si} was more effective in both protective and therapeutic antitumor tests than non-modified LEX.

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Conclusions: These data suggested that down-regulation of exosomal TGF- β 1 effectively induced potent anti-tumor immunity. Our strategy of optimizing exosome vaccine may have promising potential for leukemia immunotherapy.

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Introduction

The majority of patients with leukemia can be brought into clinical remission; however, relapses following chemotherapy are frequent since minimal residual leukemia cells (MRL) are difficult to completely eradicate through traditional treatments such as high-dose chemotherapy and allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1]. Therefore, there is a pressing need to develop a novel potential strategy to effectively eradicate MRL with the aim of preventing relapse, while improving disease-free survival (DFS) and overall survival (OS).

Recently, exosome-based immunotherapy has generated considerable attention [2, 3], because it offers potential as a novel tumor vaccine to effectively eliminate minimal residual disease (MRD), thus prolonging DFS. It has been reported that most tumor cells constitutively secrete exosomes and furthermore, the exosomes derived from tumor cells (TEXs) are highly enriched in tumor associated antigens (TAA) and in a series of immune-related proteins such as histocompatibility complex (MHC) molecules and heat shock proteins (HSPs) [4, 5]. Accumulated evidence has demonstrated that TEXs could initiate a very potent and specific antitumor immunity with preventive or therapeutic effects [5-7]. In a previous study, we have demonstrated that leukemia cells can release exosomes, which harbor native antigens from their parental cells, thus providing a rationale for the development of leukemia cell-derived exosomes (LEX) as an attractive anti-leukemia vaccine for the targeted eradication of MRLs [6, 8]. However, an antitumor immunity induced by TEXs without manipulation is less effective and even prone to facilitate immune tolerance [9, 10]. Accordingly, a TEX-based anti-tumor vaccine remains to be optimized in achieving improved immunogenic efficiency.

One of the explanations for its relatively modest efficacy may be the high enrichment of TGF- β 1 in TEXs [11]. It has been shown that exosomal TGF- β 1 could facilitate TEX-mediated immunosuppression via multiple mechanisms such as impairing NK cell cytotoxicity [12], inhibiting dendritic cell maturation [13], blocking cytokine secretion [14], inducing regulatory T-cells [15], and inhibiting an effective CTL response [16]. Therefore, the pleiotropic effects of TEX-contained TGF- β 1 should be counteracted to ultimately improve the efficacy of TEX-based vaccines. Previous studies have shown that downregulating TGF- β expression or blocking the TGF- β -related pathway in vaccine formulations could effectively enhance antitumor immunity [17-20]. Therefore, it may be assumed that downregulating TGF- β 1 expression in LEX may improve the immunogenicity of LEX-based tumor vaccines.

It has been proved feasible to artificially modify protein content of exosomes through genetically engineering parental cells. For example, modified TEXs can be obtained from tumor cells that were genetically engineered with IL-2, TNF, and CD40L [21-23]. Therefore, in this study, we used a lentiviral vector containing the TGF- β 1 small hairpin RNA (shRNA) to downregulate or silence TGF- β 1 expression in L1210 leukemia cells. We then investigated the anti-leukemia efficacy of exosomes from TGF- β 1 silenced leukemia cells (LEX_{TGF- β 1si}). Our data demonstrated that LEX_{TGF- β 1si} could promote both maturation and function of DCs, augment CD4⁺ T-cell proliferation, and increase Th1 cytokine secretion. Moreover, anti-leukemia CTL response and NK cell-mediated cytotoxicity could also be more potently induced by LEX_{TGF- β 1si} than by exosomes derived from non-manipulated L1210 cells and L1210 cells, which were transduced with a scrambled shRNA. Furthermore, vaccination with LEX_{TGF- β 1si} exhibited more significant inhibitory effects on tumor growth and prolonged survival in mice models as both preventive and therapeutic cancer vaccines. Our findings suggest that downregulation of the TGF- β 1 level in TEX-based vaccines via silencing the TGF- β 1 expression on parental cancer cells may be a promising approach to enhance anti-tumor immunity.

Materials and Methods

Reagents

RPMI-1640 medium, fetal bovine serum (FBS), and serum-free medium AIM-V were purchased from Invitrogen (Shanghai, China). Rabbit anti-mouse shock protein 70 (HSP70), CD9, and CD63 antibodies were purchased from Cell Signaling Technology (Shanghai, China). Recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF), recombinant mouse interleukin (rhIL)-4, rhIL-2, and lipopolysaccharide (LPS) were purchased from PeproTech (Shanghai, China). Rabbit anti-mouse TGF- β 1 antibodies were purchased from Abcam (Shanghai, China). PE-labeled anti-CD11c, PE-cyanine7 conjugated anti-CD80, PE-conjugated anti-MHC II (I-A/I-E), and APC-labeled anti-CD86 were purchased from eBioscienc (Shanghai, China). EasySep™ Mouse CD4⁺ and CD8⁺, T-cell isolation kits, and NK Cell enrichment kits were purchased from Stem cell Technologies (Vancouver, Canada).

Cell lines and animals

The murine acute lymphoblastic leukemia cell lines, L1210 cell and p388 cell were purchased from the Shanghai Institute for Biological Science (Shanghai, China) and were cultured in RPMI 1640 medium, containing 10% FBS, 100 u/ml penicillin, and 100 mg/ml streptomycin. Pathogen-free DBA/2 female mice at six to eight weeks of age were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and were housed in standard environmental conditions. All animal protocols were approved by the Ethics Committee of the Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

Lentivirus vector Construction and cell infection

The siRNA sequence was designed and constructed to specifically target the mouse TGF- β 1 sequence by the Shanghai Hanbio, Ltd., Shanghai, China. A scrambled shRNA sequence was used as negative control. The shRNA-expressing vectors were synthesized through inserting annealed siRNA encoding sequences into digested pHBV-U6-ZsGreen vectors (Shanghai Hanbio, Ltd., Shanghai, China). The recombinant plasmid DNAs were then transfected into *Escherichia coli* cells. After identifying successful ligation via PCR and DNA sequencing, these vectors were packaged with 293T cells through transient co-transfection. The harvested lentiviruses were concentrated and tittered. For L1210 cell infection, cells were pre-dispensed into 24-well plates at 1×10^5 cells per well and then infected with shRNA-containing lentivirus at a multiplicity of infection (MOI) of 100. The Green Fluorescent Protein (GFP)-positive cells were sorted via flow cytometry and interference efficiency was evaluated via RT-PCR and western blot analysis. The utilized shRNA sequence was as follows: shRNA targeting mouse TGF- β 1: Top strand: GAT CCG CGG AGA GCC CTG GAT ACC AAC TAT TTT CAA GAG AAA TAG TTG GTA TCC AGG GCT CTC CGT TTT TTC; Bottom strand: AAT TGA AAA AAC GGA GAG CCC TGG ATA CCA ACT ATT TCT CTT GAA AAT AGT TGG TAT CCA GGG CTC TCC GCG. Scramble shRNA as negative control: Top strand: GAT CCC CTT CTC CGA ACG TGT CAC GTT TCA AGA GAA CGT GAC ACG TTC GGA GAA TTT TTG GAA A-3; Bottom strand: AGC TTT TCC AAA AAT TCT CCG AAC GTG TCA CGT TCT CTT GAA ACG T GAC ACG TTC GGA GAA GGG.

Isolation of exosomes via continuous centrifugations

L1210 cells were pre-cultured in FBS-free AIMV medium for 24 hours to avoid contaminations from the serum. After that, the culture supernatant was collected as a source of exosome isolation. Exosomes were accumulated via continuous centrifugations as previously described [6] (300 g for 10 min to remove cells, 1, 200 g for 30 min, and 10, 000 g for 1 h to remove cellular debris, 100, 000 $\times$ g for 1 h to pellet exosomes). The harvested exosome pellets were re-suspended in ice-cold PBS and re-concentrated via an ultra-centrifugation step (100, 000 g for 1 h). The concentration of exosomal proteins was measured with the Bradford assay. The exosomes derived from non-modified L1210 cells were termed LEX, while exosomes from L1210 cells that were transfected with the lentiviral vector containing the scrambled shRNA sequence and TGF- β 1 shRNA were termed LEX_{nullvec} and LEX_{TGF- β 1si}, respectively.

Electron microscopic and western blot analyses of the exosome

Exosome samples were stained according to the negative staining procedures; the morphology of exosome preparations was then visualized under a Philips CM12 transmission electron microscope operating at 80 kV. Western blot analysis for the exosome associated proteins, CD9, CD63, and Hsp70

were conducted as described previously [22]. Primary antibodies were all purchased from Cell signaling technology (Shanghai, China).

Exosome uptake analysis

To investigate whether $\text{LEX}_{\text{TGF-}\beta 1\text{si}}$ could be uptake by DCs or not, DCs were co-cultured with CFSE stained $\text{LEX}_{\text{TGF-}\beta 1\text{si}}$ for 1 hour ($10\mu\text{g}/1 \times 10^6$ DC). These DCs were then collected, plated on glass-slides, and fixed in methanol for 15 min at -20°C . Slides were mounted in Elvanol and observed under a Leica TCS SP2 laser confocal microscope.

To further investigate the uptake rate of $\text{LEX}_{\text{TGF-}\beta 1\text{si}}$, the DCs which co-cultured with CFSE stained $\text{LEX}_{\text{TGF-}\beta 1\text{si}}$ for 1-12 hours were labeled with PE-labeled anti-CD11c antibody, and then analyzed by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA)

The secretion levels of IL-12p70 and tumor necrosis factor (TNF)- α from DCs which were stimulated for 24 h, and the IFN- γ and IL-2 released from CD4^+ T-cells were detected by ELISA kits following the manufacturer's instructions. The concentration of each sample was determined based on a standard curve.

Flow cytometric analysis

To quantify TGF- β 1 content in exosomes, $30\mu\text{g}$ exosome preparations were incubated with aldehyde/sulfate latex beads (Invitrogen, Shanghai, China) at 4°C overnight. After that, the reaction was blocked by adding 100 mmol/L glycine to the system. The exosome-loaded beads were washed twice in PBS buffer containing 1% FBS and then stained with specific antibodies against mouse TGF- β 1 or against isotype control. Following that, beads were analyzed via a BD FACScan TM flow cytometer and the fluorescence intensity was analyzed with Flowjo.

Before analyzing the phenotype of DCs, DCs were pre-stimulated by PBS, LEX, $\text{LEX}_{\text{nullvec}}$, $\text{LEX}_{\text{TGF-}\beta 1\text{si}}$ or LPS respectively for 24 h. Following that, DCs were harvested and stained with PE-cyanine 7-labeled anti-CD80, PE-labeled anti-MHC Ia/Ib, and APC-labeled anti-CD86 and then analyzed with FACScan.

CD4+ T-cell proliferation assay

Splenic CD4^+ T-cells from immunized mice were isolated for seven days after the last immunization with the EasySep™ mouse CD4^+ T-cell isolation kit. The purified CD4^+ T-cells ($1 \times 10^5/\text{well}$) were then co-cultured with irradiated L1210 ($1 \times 10^4/\text{well}$) or p388 cells ($1 \times 10^4/\text{well}$), which were used as the control target for 72 h in presence of PHA ($20\mu\text{g}/\text{ml}$) at 37°C in an incubator containing 5% CO_2 . Thereafter, [^3H] thymidine ($0.5\mu\text{Ci}$ per well) was added for an additional 16 h. Subsequently, the cells were harvested and [^3H] thymidine uptake was detected with a MicroBeta counter (Beckman Coulter, Krefeld, Germany).

Mixed lymphocyte reaction (MLR)

DCs from DBA/2 mice were stimulated with $10\mu\text{g}/\text{ml}$ of exosomes, $100\mu\text{g}/\text{ml}$ PBS, or $1\mu\text{g}/\text{ml}$ LPS for 48 hours, respectively. Then, the DCs were irradiated with 4000 rad and used as stimulators. Allogeneic T-cells, which were isolated with the EasySep™ T cell separation kit, were used as responders (0.1×10^6 cells/well). DC/T-cell co-culture was maintained for 72 h at 37°C in RPMI 1640 complete medium. Following that, [^3H] thymidine ($0.5\mu\text{Ci}/\text{well}$) was added per well. After a further 24 h of co-culture, T-cell proliferation was determined via [^3H] thymidine incorporation.

Cytotoxicity assay

Single-cell suspensions of splenocytes were obtained from immunized mice seven days after final stimulation. To evaluate NK cell cytotoxicity against YAC-1 cells, NK cells were isolated via negative selection with the EasySep™ mouse NK Cell Enrichment Kit. For the CTL assay, splenic CD8^+ T-cells were isolated from immunized mice seven days after the final stimulation, using the EasySep™ mouse CD8^+ T-cell Isolation Kit, re-stimulated with irradiated L1210 cells with mouse IL-2 ($20\text{IU}/\text{ml}$) IL-2 for seven days, and then harvested as effector cells. L1210 cells were used as specific target cells and p388 cells served as the control target cells; both were seeded in a 96-well plate at 1×10^4 cells/well. The magnitude of the cytotoxic response at different effector/target (E/T) ratios was evaluated via LDH assay and specific lyses (%) were calculated as previously described [6].

Animal study

To evaluate the protective effects of exosome vaccines, we subcutaneously (s.c.) injected 100 μ L PBS, 10 μ g LEX, 10 μ g LEX_{null vec} and 10 μ g LEX_{TGF- β 1si} into the mouse's inner side of the right hind limbs on Day 0. The immunization was boosted twice on Day 7 and Day 14. On Day 21, the immunized mice were s.c. challenged with L1210 cells (0.5×10^6 cells/mouse) into the lateral thigh. The mortality of the mice and the tumor size was monitored and recorded every two days. Tumor size was calculated as length $\times$ width² $\times \pi / 6$. Percentage of increase life-span (%ILS) was calculated as follows: $100 \times (\text{the median day of death of the exosome treated tumor bearing mice} - \text{median day of death of mice in PBS group}) / \text{median day of death of mice in PBS group}$. Percentage of tumor growth delay (%TGD) on Day 22 was calculated as follows: $100 - 100 \times (\text{the mean tumor volume for the mice treated by exosomes} / \text{the mean tumor volume for the mice in PBS group on})$.

To examine the therapeutic effects of exosome, the tumor-bearing mice were pre-established via inoculation of L1210 cells (0.5×10^6 cells/mouse) on the lateral portion of right thigh s.c. on Day 0. For tumor treatment, 100 μ L PBS, 10 μ g LEX, 10 μ g LEX_{null vec} and 10 μ g LEX_{TGF- β 1si} were s.c. injected in the inner side of the right thighs on the established tumor-bearing mice, respectively. An identical treatment was repeated on Day 10 and Day 15. Mice were monitored every two days for the evaluation therapeutic efficacy. The survival rate, tumor size, %ILS and %TGD were recorded as mentioned above.

Statistical analysis

Experiments were performed in triplicate. Data are present as the mean $\pm$ SD or SEM. The Log-rank test was used to analyze survival data and differences between the two groups which were analyzed via Students' t-test. The statistical significance was determined at $p < 0.05$.

Results

Silencing TGF- β 1 of parental cells decreases the TGF- β 1 level on exosomes

When L1210 cells were transduced with a mock or TGF- β 1 shRNA-incorporated lentivirus, neither cell growth, nor viability was affected (data not shown). As shown in Fig. 1a under electron microscopy, LEX_{TGF- β 1si} was a population of micro-vesicles with 40-100 nm in diameter and was consistent with the typical morphology of exosomes [24]. To determine whether knockdown of TGF- β 1 expression in L1210 cells could effectively affect the protein pattern on an exosomal level or not, Western blot analysis was performed. It has been shown that all types of leukemia cell-derived exosome preparations exhibited the typical exosome associated proteins, CD9, CD63, and Hsp70. Interestingly, we found that there was no TGF- β 1 detectable in LEX_{TGF- β 1si}. However, a certain amount of the TGF- β 1 could be detected in LEX and LEX_{null vec} preparations (Fig. 1b). Moreover, the amount of TGF- β 1 in LEX_{TGF- β 1si} was further quantified by flow cytometry. As shown in Fig. 1c, TGF- β 1 was abundantly enriched on the membrane of non-modified LEX and LEX_{null vec}. However, exosomes derived from L1210 cells infected with TGF- β 1 shRNA-incorporated lentiviral vectors loaded significantly less TGF- β 1 than other LEXs. Besides, the median fluorescence intensity (MFI) value of TGF- β 1 in LEX_{TGF- β 1si} is significantly lower than that in LEX and LEX_{null vec} ($p < 0.01$). This indicates that silencing TGF- β 1 expression on parental leukemia cells successfully down-regulated TGF- β 1 expression in LEXs.

LEX_{TGF- β 1si} could facilitate the function of dendritic cells

TEXs could be predominantly taken up by DCs and exert a different effect on DC due to its sophisticated components. TGF- β 1 is known to inhibit the maturation of DC as well as to impair their capacity to present antigen, while initiating T-cell responses. However, DC plays a critical role in initiating adaptive immunity. Therefore, we evaluated the effect of LEX_{TGF- β 1si} on DCs *in vitro*. DCs were incubated with LEX_{TGF- β 1si} (10 μ g), which was pre-labeled with CFSE. Confocal fluorescence microscopy was used to detect CFSE expression on DCs. It has been shown that CFSE-positive DCs can be detected after 1 hour of incubation (Fig. 2a). To further investigate the uptake rate of LEX_{TGF- β 1si}, the percentage of the CFSE positive

Fig. 1. Characterization of LEX_{TGF- β 1si}. (a) Exosomes derived from TGF- β 1 silenced L1210 cells were visualized via electron microscopy. They are dimpled micro-vesicles, ranging between 40 and 100 nm. The scale bar is 100 nm. (b) Western blot analysis of CD9, CD63, HSP70 and TGF- β 1 in exosomes preparations. (c) Exosome preparations were absorbed onto latex beads and the membrane bound TGF- β 1 level on exosome was measured via flow cytometry. The median fluorescence intensity (MFI) of TGF- β 1 in each groups were analyzed and the data are collected from three independent experiments and presented as mean values $\pm$ SD. * p < 0.05 and ** p < 0.01.

DCs were detected by flow cytometry after incubating for 1-12 hours. As shown in Fig. 2b, CFSE-positive DCs ($16.8 \pm 2.1\%$) were detectable as early as 1 h after incubation, and then the percentage reached a plateau 6 hours after incubation. These suggested that LEX_{TGF- β 1si} could be taken up and internalized by DCs efficiently and stably (Fig. 2b). Furthermore, we examined whether LEX_{TGF- β 1si} could influence the maturation and function of DCs or not. As expected, after exosome stimulation for 24 hours, the expression of MHC II, CD80, and CD86 on the DC surface as well as the IL-12p70 and TNF- α secretion from DCs was significantly upregulated compared to immature DCs. Among these, LEX_{TGF- β 1si} exerted the most significant effect, which was comparable to the effect of LPS (Fig. 2c, d, e, p < 0.05), suggesting that DC can be more efficiently activated and matured by LEX_{TGF- β 1si} than by non-modified LEX. Moreover, the MLR assay also revealed that LEX_{TGF- β 1si} stimulated DCs were more potent initiators in inducing T-cell proliferative response than LEX or LEX_{null vec} treated-DCs at 1:5 and 1:20 of stimulator/responder ratios (Fig. 2f, p < 0.05). In summary, these data suggest that LEX_{TGF- β 1si} can promote phenotypic and functional maturation of dendritic cells more efficiently.

Fig. 2. $LEX_{TGF-\beta 1si}$ more effectively induced phenotypic and functional maturation of dendritic cells. Immature dendritic cells were stimulated with each type of leukemia-derived exosomes for 24 h. Immature DCs stimulated with PBS were used as negative control, while LPS-matured DCs were used as positive control. (a) 20 μ g CFSE-labeled $LEX_{TGF-\beta 1si}$ were incubated with 10^5 /ml dendritic cells (DCs) for 1 h and CFSE-positive DCs were detected under confocal fluorescence microscopy. (b) DCs were incubated with CFSE-labeled $LEX_{TGF-\beta 1si}$ for 1–12 hours, the percentage of CFSE positive DCs were detected by flow cytometry at different time points. (c) The costimulatory factors and major histocompatibility complex (MHC) class II molecules on the DC surface were analyzed via flow cytometry. $LEX_{TGF-\beta 1si}$ significantly promoted the phenotypic maturation of DCs. $LEX_{TGF-\beta 1si}$ promoted IL-12p70 (d) and TNF- α secretion (e) secretions from DCs. (f) $LEX_{TGF-\beta 1si}$ improved the stimulatory capacity of DCs. DCs pulsed with $LEX_{TGF-\beta 1si}$ stimulated the allogeneic CD4⁺ T-cells proliferation more effectively. * $p < 0.05$ and ** $p < 0.01$ denote statistically significant differences. Data are collected from three independent experiments and expressed as mean values $\pm$ SD.

$LEX_{TGF-\beta 1si}$ can promote CD4⁺ T-cell proliferation and Th1 cytokine production efficiently

CD4⁺ T-cells play a pivotal role in modulating the anti-tumor immune response. To investigate the antigen-specific CD4⁺ T-cell response induced by $LEX_{TGF-\beta 1si}$, splenic CD4⁺ T-cells from mice immunized with LEX, $LEX_{null\ vec}$ and $LEX_{TGF-\beta 1si}$ were isolated and evaluated

Fig. 3. Immunization with LEX_{TGF-β1si} more potently induced CD4⁺ T-cell proliferation and Type I cytokine secretion. DBA/2 mice were immunized with PBS or each type of exosomes for three times each with a 7-day interval. Seven days after the last immunization, the splenic CD4⁺ T-cells were separated from immunized mice and incubated with irradiated L1210 cells or p388 cells for 72 h. CD4⁺ T-cell proliferation was evaluated via ³H thymidine incorporation. Cytokine secretion was determined via ELISA. (a) T-cell proliferation. (b) and (c) show IFN-γ and IL-2 secretion from CD4⁺ T cell, respectively. Data are collected from three independent experiments and expressed as mean values ± SD.

for their response to L1210 cells or p388 cells *in vitro*. As shown in Fig. 3a, immunization with LEX, LEX_{multivec}, or LEX_{TGF-β1si} can enhance CD4⁺ T-cell proliferation response to L1210 cells, which is used as specific target. However, LEX_{TGF-β1si} immunization exerted the most significant effect on boosting CD4⁺ T-cell proliferation ($p < 0.05$). Furthermore, we measured the level of IFN-γ and IL-2, which were recognized as the indicator of Th1 response in the CD4⁺ T-cell culture medium. Under the stimulation of inactivated L1210 cells, it had been observed that the splenic CD4⁺ T-cells from mice immunized with LEX_{TGF-β1si} produced the highest level of IFN-γ and IL-2 compared with the mice of other groups (Fig. 3b and 3c). However, the control target p388 cells could neither stimulate the proliferation response nor the Th1 cytokine secretion of CD4⁺ T-cells from mice immunized with LEX_{TGF-β1si}, indicating that the CD4⁺ T-cell immune response induced by LEX_{TGF-β1si} was antigen-specific. In summary, these data suggested that LEX_{TGF-β1si} could more effectively induce CD4⁺ T-cell proliferative responses and Th1 cytokine production in an antigen-specific manner.

Immunization with LEX_{TGF-β1si} induced specific CTL response and NK cell cytotoxicity more efficiently

To further investigate the possibility and efficacy of applying LEX_{TGF-β1si} as tumor vaccine, CTL immune response and NK cell cytotoxicity were detected in immunized mice. The results of target cell lysis mediated via CTL showed that immunization with exosome vaccines

Fig. 4. Immunization with LEX_{TGF- β 1si} more efficiently induced specific CTL activity and NK cell cytotoxicity. The splenic cells were derived from immunized mice seven days after the last immunization. (a) CD8⁺ T-cells were isolated and then re-stimulated with irradiated L1210 (4000 rad) cells at the presence of mIL-2 in vitro for seven days. Following that, the separated viable CD8⁺ T-cells were referred to as effector cells. L1210 or p388 cells were served as target cells and mixed with effector cells at different ratios. (b) Isolated NK cells from splenic cells were served as responder cells against YAC-1 cells. *p<0.05 and **p<0.01 denoted statistically significant differences between E:T, effect cells and target cells; Data were collected from three independent experiments and expressed as mean values $\pm$ SD.

Fig. 5. LEX_{TGF- β 1si} immunization induced anti-leukemia preventive immunity. 6-8 week old DBA/2 mice were immunized with 10 μ g of each type of exosome formulations or injected with 100 μ l PBS on day 0 (prime), day 7 (boost I), and day 14 (boost II). On day 21, mice were subcutaneously challenged with 5×10^5 L1210 cells. Each group contained 10 mice. (a) Tumor volume was measured after tumor challenge with calipers. (b) Survival rate of immunized mice until 50 days after tumor challenge.

could induce a certain level of CTL response against L1210 cells, compared to no detectable CTL response induction in mice via PBS injection. Moreover, the strongest CTL response was induced by LEX_{TGF- β 1si} immunization compared to those induced by LEX or LEX_{null vec} (Fig. 4a, p < 0.05). However, the CTL induced by LEX_{TGF- β 1si} did not show significant eradicating activities against p388 cells, indicating the CTL response induced by LEX_{TGF- β 1si} to be tumor-specific. Furthermore, it has been observed that LEX_{TGF- β 1si} immunization significantly enhanced the cytotoxic activity of NK cells against target cells compared to other groups (Fig. 4b, p < 0.05), whereas NK cells from mice immunized with other vaccine formulations responded poorly to YAC-1 cell. This suggests that NK cell-mediated anti-tumor immune response

could be more efficiently evoked via LEX_{TGF- β 1si}.

LEX_{TGF- β 1si} exerted a more powerful protective and therapeutic effect against tumor attack

To investigate protective antitumor immunity induced by LEX_{TGF- β 1si} *in vivo*, DBA/2 mice were pre-vaccinated with either PBS, LEX, LEX_{null vec}, or LEX_{TGF- β 1si} thrice with a 7-day interval, respectively. Animals were then subcutaneously challenged with 5×10^5 L1210 cells seven days after the last vaccination. Tumor growth and overall survival were monitored and recorded. As shown in Fig. 5a, immunization with LEX, LEX_{null vec}, or LEX_{TGF- β 1si} significantly inhibited tumor growth compared to uninhibited tumor growth in PBS-treated mice. However, the most significant inhibitory effect was exhibited in mice immunized with LEX_{TGF- β 1si}. As depicted in Table 1, LEX_{TGF- β 1si} immunization promoted *in vivo* tumor growth delay (%TGD 66.5 $\pm$ 3.3%) more efficiently than LEX(%TGD 35.1 $\pm$ 7.1%, $p < 0.01$) or LEX_{null vec}(%TGD 39.6 $\pm$ 8.0%, $p < 0.01$). Moreover, the survival of tumor-challenged mice was also most significantly prolonged by LEX_{TGF- β 1si} vaccination (Fig. 5b). The mean survival time (MST) of mice in the PBS group was 20 days (Table 1). Immunization with LEX or LEX_{null vec} could prolong MST to 24-25 days (% ILS: 20-25%). Moreover, MST was prolonged to 33 days after tumor challenge via LEX_{TGF- β 1si} vaccine (% ILS: 64%, Table 1). In summary, these results indicate that LEX_{TGF- β 1si} could induce a more powerful protective immunity against leukemia cells than LEX or LEX_{GFP}.

The therapeutic effect of LEX_{TGF- β 1si} was evaluated in tumor-bearing mice. Tumor mass was not measured until 22 days after tumor inoculation. Similar to the effect in protective models, all of the tested exosome formulations significantly inhibited tumor growth in pre-established tumors (Fig. 6a). It is worth of noting that immunization with LEX_{TGF- β 1si} resulted in a most significant inhibitory effect on tumor growth (Table 2, %TGD 63.6 $\pm$ 5.8, $p < 0.05$). Moreover, vaccination with LEX_{TGF- β 1si} improved the survival rate and prolonged MST of tumor-bearing mice significantly compared to vaccination with either LEX or LEX_{null vec} (Fig. 6b, Table 2). 10% of the LEX_{TGF- β 1si} immunized mice survived until the end of observation time at 50 days. These results suggest that LEX_{TGF- β 1si} exhibited a more potent effect on slowing tumor growth and more efficiently prolonged the survival of vaccinated tumor-bearing mice than LEX and LEX_{null vec}.

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Discussion

Tumor vaccine is a strategy of cancer immunotherapy which is designed to presenting TAA for boosting specific antitumor immunity against tumor cells.

Table 1. The protective effect of LEX_{TGF- β 1si} vaccines. MST: median survival time; %TGD: percentage of tumor growth delay on Day22; % ILS: percentage of increased life span. ^a: LEX_{TGF- β 1si} group vs LEX group or LEX_{null vec} group. Data are representative of three independent experiments. %TGD was expressed as mean values $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ denote statistically significant differences

parameter s	PBS group	LEX group	LEX _{null vec} gr	LEX _{TGF-β1si} group
MST(days)	20.0	24.0	25	33.0
%TGD	--	35.1 $\pm$ 7.1	39.6 $\pm$ 8.0	66.5 $\pm$ 3.3 ^{a**}
% ILS	--	20	25	64

Table 2. The therapeutic effect of LEX_{TGF- β 1si} vaccines. MST: median survival time; %TGD: percentage of tumor growth delay on Day22; % ILS: percentage of increased life span. ^a: LEX_{TGF- β 1si} group vs LEX group or LEX_{null vec} group. Data are representative of three independent experiments. %TGD was expressed as mean values $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ denote statistically significant differences

parameter s	PBS group	LEX group	LEX _{null vec} gr	LEX _{TGF-β1si} group
MST(days)	19.5	25.0	26.0	33.0
%TGD	--	35.4 $\pm$ 8.1	41.9 $\pm$ 6.3	63.6 $\pm$ 5.8*
% ILS	--	23.1	33.3	69.2

Fig. 6. LEX_{TGF- β 1si} immunization induced anti-leukemia therapeutic immunity. 6-8 week-old DBA/2 mice were subcutaneously inoculated with 5×10^5 L1210 cells on day 0 and then vaccinated with 10 μ g of each type of exosomes or injected with 100 μ l PBS on day 5 (prime), day 10 (boost I), and day 15 (boost II). Each group contained 10 mice. (a) Tumor volume was measured from day 6 to day 22 with calipers. (b) Survival rates of tumor-bearing mice were recorded from day 6 to day 50.

TEXs, which harbor a panel of tumor associated antigens from the parental cell, have emerged as promising candidates for tumor vaccines due to easy and non-traumatic acquisition. However, according to previous studies, TEXs can be immune-stimulatory or immunosuppressive depending on the complexity of the components [25-29]. A previous study has demonstrated that leukemia cell-derived exosomes can induce specific antitumor immunity; however, with relatively low efficacy *in vivo* [6]. Others have shown that TEX alone was less immunogenic as tumor vaccine [30]. Therefore, to optimize the efficacy of TEX-based vaccines, attention should be paid to elucidate the underlying mechanisms, which compromised the immunogenicity of TEXs. It had been reported that the critical immunosuppressive cytokine TGF- β 1 was enriched in TEXs, and that it played an important role in TEX-mediated immunosuppression via compromising DC maturation, specific CTL response, NK cell cytotoxicity, and inducing regulatory B and T cells [12-14]. Accordingly, TGF- β 1 may play an important role in compromising TEX-induced specific anti-tumor immunity.

Evidence had shown that exosomes isolated from the plasma of leukemia patients have higher TGF- β 1 levels than exosomes from healthy donors [31]. Moreover, leukemia exosome-associated TGF- β 1 had been reported to suppress the cytotoxic activity of NK and T cells [12, 32]. In our study, we also proved that TGF- β 1 was abundant on the membrane surface of non-manipulated leukemia cell-derived exosomes, which was consistent with the previous researches. Moreover, we demonstrated that exosomal TGF- β 1 could be downregulated through shRNA-mediated gene silencing of parental cells, suggesting the feasibility and conveniences of artificially modulating the protein composition in exosomes via genetic modifications. Previous studies had reported that TEXs were predominantly internalized by DCs and directly influenced the maturation and function of DCs [7, 33]. Yang et al. reported that TEX exerted an inhibitory effect on DC maturation and might even potentially predispose DCs towards a tolerogenic phenotype via TGF- β 1, indicating that downregulating exosomal TGF- β 1 might dramatically reverse the immunosuppressive effect in DCs [13]. As DCs are the most important antigen presenting cells (APC), responsible for initiating both CD4⁺ and CD8⁺ T-cells against tumors [34], we examined the effect of LEX_{TGF- β 1si} on DCs *in vitro*. As expected, we found that leukemia-cell-released exosomes could be uptaken by DCs similar to other TEXs; therefore, a LEX-carried antigen profile can transfer to DCs and may influence the phenotype of DCs. Furthermore, it has been observed that LEX_{TGF- β 1si} stimulation could partially turn over the tolerance of DC by promoting its phenotypical and functional

maturation and by enhancing the stimulatory capacity of T-cell proliferation. These results suggested that TGF- β 1 may be the key immunosuppressive factor in leukemia cell-derived exosomes impeding the maturation and activation of DCs. Depletion of TGF- β 1 from TEX could be effective for enhancing the function of DCs, thus mediating antitumor immunity.

A plethora of evidence showed that CD4⁺ T-cells and CD8⁺ T-cells both played unique roles in specific antitumor immunity [35]. Therefore, inducing effective antigen-specific CD4⁺ T-cells and CTL response is the key to antitumor vaccines. However, LEXs alone are less effective in activating T cells or worse yet, may delay or even suppress T-cell activation, which partially results from exosomal TGF- β 1 [36]. In our study, LEX_{TGF- β 1si} more efficiently induced CD4⁺ T-cell proliferation and promoted Th1 cytokine secretion in an antigen-specific manner. This effect may be mediated by LEX_{TGF- β 1si} stimulated-DCs, which are more effectively activated and matured. Increased Th1 cytokines may result in a more potent CTL response. As expected, it has been observed that antigen-specific CTL activity was most significantly enhanced in mice that were immunized with LEX_{TGF- β 1si}. Despite of DC-mediated CTL activation, exosomes can be taken up by CD8⁺ T-cells. Moreover, the internalized exosomes can directly induce specific cytotoxic activity of CD8⁺ T-cells via exosomal MHC molecules [37]. Therefore, the enhanced CTL response may be directly mediated by LEX_{TGF- β 1si} through attenuating the TGF- β 1 signaling pathway in CD8⁺ T-cells or indirectly mediated by higher levels of Th1 cytokines induced by LEX_{TGF- β 1si} stimulated DCs.

A previous study has reported that NK cells were also involved in TEX induced anti-tumor immunity [21]. However, exosomal TGF- β 1 can be transferred to NK cells, therefore impair the function of NK cells through downregulation of the NKG2D expression. In this study, the LEX_{TGF- β 1si} immunization enhanced the cytotoxicity of NK cells remarkably; indicating that downregulation of exosomal TGF- β 1 can remove the negative effect of TEX on NK function to some extent.

Based on the LEX_{TGF- β 1si} exerted positive effect on immune cells, we investigated the contribution of LEX_{TGF- β 1si} to anti-tumor immunity in animal models. In our study, application in LEX_{TGF- β 1si} in mice as preventive and therapeutic tumor vaccines have both shown superiority in inhibiting tumor growth as well as in prolonging survival. These data indicate that LEX_{TGF- β 1si} can more efficiently induce anti-leukemia immunity compared to non-modified LEX. However, a majority of mice immunized with LEX_{TGF- β 1si} failed to acquire long-term survival during our observation period, suggesting that downregulation of TGF- β 1 alone in TEX-based tumor vaccines may still not be sufficient to inhibit tumor progression *in vivo*. Therefore, further optimization of TEX-based vaccines is still required. Apart from exosomal TGF- β 1, a variety of factors may participate in interfering with an effective anti-leukemia immune response and in mediating the compromising efficacy of vaccines. Current studies have demonstrated that several immunosuppressive factors such as Fas ligand, TRAIL, and heat shock protein 72 (HSP72) can exist in TEXs [38, 39], thus exerting a negative effect on the antitumor response. For example, the exosome-carried Fas ligand and TRAIL can mediate T-cell apoptosis [38]. Membrane associated HSP72 can facilitate a suppressive effect of myeloid-derived suppressor cells [39]. Therefore, downregulating these factors in LEX-based vaccine formulations may facilitate a specific anti-tumor response. Furthermore, the deficiencies of MHC complexes, co-stimulatory factors, and adhesion molecule signals in TEXs may also negatively influence the immunogenicity of TEX-based vaccines as these factors could augment the magnitude of the specific immune response [40-43]. However, due to the complexity of TEX composition and numerous factors that are involved in tumor immunity, clinical applications of the TEX-based vaccines are far more than the inhibiting tumor growth in animal models. Consequently, TEX-based vaccines still have a long way to go.

Conclusion

In summary, this study demonstrated that exosomes from TGF- β 1 silenced leukemia cells contained less TGF- β 1 amount and could more efficiently promote DC activation than

exosomes from non-modified leukemia cells. Furthermore, immunization with LEX_{TGF- β 1si} induced more potent CD4⁺ proliferation, Th1 cytokines secretion, specific CTL response, and NK cell cytotoxicity. Importantly, LEX_{TGF- β 1si} can more effectively retard tumor growth and prolong survival in mice as preventive and therapeutic vaccines compared to non-manipulated LEX. Therefore, our study provides a novel and promising strategy for optimizing TEX-based vaccines.

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The datasets used during the current study can be obtained from the corresponding author upon reasonable request.

All animal experiments were conducted according to the guidelines of the Ethics Committee of Xinhua Hospital Affiliated to the Shanghai Jiaotong University School of Medicine, Shanghai, China.

Disclosure Statement

The authors declare that they have no competing interests.

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