Enhancing the anti-leukemia immunity of leukemia-derived exosome-based vaccine by downregulation of PD-L1 expression

Fang Huang[1], Zhichao Li[1], Wenhao Zhang[1], Jiaqi Li[1], and Siguo Hao[1]

[1]Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine

July 11, 2021

Abstract

Cell-released nanovesicles can induce anti-leukemia immunity. Leukemia cell-derived exosomes (LEXs) are promising anti-tumor vaccine components for cancer immunotherapy. Nonetheless, LEX-based vaccines show modest potency in vivo, likely due to the presence of immunosuppressive PD-L1 proteins in the exosomes. We hypothesized that targeting exosomal PD-L1 could optimize LEX-based vaccines. To test this hypothesis, we compared the capacity of exosomes derived from PD-L1-silenced leukemia cells (LEXPD-L1si) and non-modified exosomes to induce anti-leukemia immunity.Lentivirus-mediated PD-L1 shRNA was used to downregulate PD-L1 expression in parental leukemia cells and LEXs. LEXPD-L1si were characterized by electron microscopy, western blotting, and flow cytometry, and their anti-leukemia immune effects were tested on immune cells and in animal models.In the present study, lentivirus-mediated PD-L1 shRNA successfully downregulated PD-L1 expression in parental leukemia cells and in LEXs. LEXPD-L1si induced better DC maturation and subsequently enhanced T-cell activation, as compared with non-modified LEXs. Consistently, immunization with LEXPD-L1si induced greater T-cell proliferation and Th1 cytokine release. LEXPD-L1si was a more potent inducer of antigen-specific cytotoxic lymphocyte (CTL) response. Finally, we vaccinated DBA/2 mice with exosome formulations to test their ability to induce both protective and therapeutic anti-tumor CTL responses in vivo. Vaccination with LEXPD-L1si strongly inhibited tumor growth and prolonged survival. Downregulation of exosomal PD-L1 expression in LEXs effectively induce more potent anti-leukemia immunity. Therefore our strategy for optimizing LEX-based vaccine has a potential application in leukemia immunotherapy.

Introduction

Leukemia is a hematologic malignancy that seriously threatens human health[1,2]. Despite recent advances in conventional chemotherapy, numerous patients still experience severe toxicity, including infections, gastrointestinal reactions, and cerebral hemorrhage[3]. Moreover, up to half of the patients may be insensitive to, or unfit for, chemotherapy[4]. Thus, the 5-year overall survival rates for acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) are approximately 40% and 27%, respectively[5,6]. Elderly patients with acute leukemia generally have worsened prognosis and show a 5-year overall survival rate of less than 20%[7]. These findings highlight the need for alternative treatment strategies in patients ineligible for intensive chemotherapy and those with recurrent or refractory disease[8].

Recent studies examining the interactions between leukemia cells and the immune system have yielded immunotherapeutic approaches that can be used to improve prognosis and survival in patients with leukemia[9]. A well-known immunotherapeutic approach that is entering clinical application is CD19-chimeric antigen receptor (CAR) T-cell therapy, targeted to kill CD19-positive lymphoblastic leukemia cells via gene-modification. Although CD19-CAR T-cell therapy is promising, it still shows off-target effects and has a high proportion of side effects, with a high disease-recurrence [10,11]. Additionally, due to the lack of highly specific antigens in AML cells, using CAR-T therapy in patients with AML may increase the risk of hematopoietic stem cells and off-target toxicity[12]. Therefore, using CAR-T for the treatment of patients

with leukemia does not meet expectation[11]. Alternatively, the direct and sustained activation of tumor-specific T cells in vivo via local inoculation of anti-leukemia vaccines carrying leukemia-associated antigens shows a superior therapeutic spectrum and safety profile[13].

Exosomes, which are bioactive vesicles released by eukaryotic cells and have a diameter of 30–130 nm, carry various information components derived from their parental cells[14]. Tumor cell-derived exosomes carry tumor-associated antigens, which can be used in novel tumor vaccines to stimulate the priming of T cells in immunotherapy[15]. Our previous studies have shown that similar to other tumor cells, leukemic cells can release significant quantities of exosomes that harbor native tumor-associated antigens derived from their parental cells. These findings indicate that leukemia cell-derived exosomes (LEX) can be utilized in an anti-leukemia vaccine for targeted elimination of leukemia cells[16-19]. Additionally, LEX-based vaccines are relatively more stable than cell-based vaccines and can cross the blood-brain barrier non-invasively[20]. These characteristics of LEX vaccines are useful in clinical applications. Conversely, unmodified tumor exosomes (TEX) show poor immunogenicity and can promote immune tolerance, thereby substantially compromising their therapeutic performance[21]. However, the immunogenicity of LEX-based leukemia vaccines requires optimization. The unsatisfactory immune response induced by LEX can be attributed to two factors. First, deficiencies in immune-stimulating factors, such as adhesion and co-stimulatory molecules, compromise the immunogenicity of exosome-derived vaccines[16]. Second, high levels of immunosuppressive factors in TEXs mediate immune tolerance[22,23]. Among these immunosuppressive factors, programmed cell death protein-ligand 1 (PD-L1), one of immune checkpoint molecules that interacts with programmed cell death protein-1 (PD-1), is expressed on the surface of the tumor cells and on TEXs[24,22]. Exosomal PD-L1, which is resistant to anti-PD-L1 therapy, can transmit immunosuppressive signals to T cells and anti-apoptotic signals to tumor cells, inducing local and systemic immunosuppression and promotion of tumor growth[22]. Our previous study showed that PD-L1 is highly enriched in LEX, thus creating a barrier for therapeutic vaccination against leukemia.

To overcome these limitations and improve the immunogenicity of LEX-based vaccines, we downregulated exosomal expression of PD-L1. For this, we used a lentiviral vector containing PD-L1 small hairpin RNA (shRNA) to silence PD-L1 expression in L1210 leukemia cells. Then, we isolated and analyzed exosomes derived from these genetically engineered parental cells. Finally, we investigated the anti-leukemia efficacy of exosomes derived from PD-L1 silenced leukemia cells ($LEX_{PD\text{-}L1si}$).

2. Materials and Methods

2.1 Reagents,

RPMI-1640 medium, fetal bovine serum (FBS), and serum-free medium AIM-V were purchased from Invitrogen (Shanghai, China). Recombinant mouse IL-2 protein was purchased from Abcam (Shanghai, China). Recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF), recombinant human interleukin (rhIL)-4, and lipopolysaccharide (LPS) were purchased from PeproTech (Shanghai, China). Rabbit anti-mouse shock protein 70 (HSP70), TSG101, and CD63 and CD63 antibodies were obtained from Cell Signaling Technology (Shanghai, China). Rabbit anti-mouse antibodies PD-L1 were purchased from Abcam (Shanghai, China). PE-labeled anti-MHC Ia/Ib, PE-cyanine7 conjugated anti-CD80, and APC-labeled anti-CD86 were purchased from eBioscience (Shanghai, China). EasySep Mouse $CD4^+$and $CD8^+$ T cell isolation kits were purchased from Stem Cell Technologies (Vancouver, Canada). Aldehyde/sulfate latex beads were purchased from Invitrogen(Shanghai, China).

2.3 Cell lines and animals

The murine acute leukemia cell lines, L1210 and p388, were purchased from the Shanghai Institute for Biological Science (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). DBA/2 female mice (6–8 weeks old) were purchased from the Shanghai Laboratory Animal Center,and were housed in a specific pathogen-free, regularly controlled animal house at 18-22 in a 12 h light/dark cycle and fed standard chow and water ad libitum. All procedures involving animals were approved by the Ethics Committee of Xinhua Hospital Affiliated with the Shanghai Jiaotong University

School of Medicine.

2.4 Generation of bone marrow-derived DCs

DBA/2 mice were sacrificed, and dendritic cells (DCs) were generated from bone marrow-derived precursors as previously described and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 u/mL penicillin, 100 mg/mL streptomycin, 10 ng/mL rmG-MSF, and 10 ng/mL rmIL-4. Following cell culture for 6 days, DCs were collected and incubated with PBS, LEX, LEX_{GFP}, $LEX_{PD\text{-}L1si,}$ or LPS in complete medium containing 10% exosome-free FBS for 24 h. Then DCs and the supernatants were collected and stored for flow cytometry and ELISA analysis.

2.5 Lentivirus vector construction and cell infection

Three pairs of self-complementary oligonucleotides carrying shRNA sequences targeting mouse PD-L1 were synthesized at the Shanghai Hanbio Co., Ltd (Shanghai, China). A scrambled shRNA sequence was used as negative control. Oligonucleotides encoding PD-L1 shRNAs and scrambled shRNA were introduced into lentiviral frame plasmids, pHBLV-U6-Scramble-Zsgreen (Shanghai Hanbio Co., Ltd, Shanghai, China). The recombinant plasmid DNAs were then transfected into*Escherichia coli* for construction of the recombinant plasmid. After confirming successful ligation, 293T cells were co-transfected with the recombinant lentiviral vector (10 μg), pSPAX2 vector (10 μg), and pMD2G vector (10 μg) topack the vector. The harvested lentiviruses were titered as previously described[25].The targeted cell line was transduced using previously described protocols[17]. To evaluate the efficiency of interference, RT-PCR and western blotting were used to detect the mRNA and protein expression levels of PD-L1 in the targeted cell line. Of the three lentiviral vectors containing PD-L1 shRNA, the vector containing PD-L1 shRNA3 showed the highest interference efficiency for PD-L1 mRNA and protein expression, and was, therefore, selected for use in further procedures. The shRNA3 sequence targeting mouse PD-L1 was as follows: Top strand: CCGGGAAGCAAAGTGATACACATCTCAAGAGAATGTGTATCACTTTGCTTCTTTTTTTG; Bottom strand: AATTCAAAAAAGAAGCAAAGTGATACACATTCTCTTGAGATGTGTATCACTT TGCTTC. The scramble shRNA sequence used as negative control was as follows: Top strand: GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTGG AAA-3; Bottom strand: AGCTTTTCCAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGT GACACGTTCGGAGAAGGG.

L1210 cells were pre-cultured for 24 hours in AIM-V medium without FBS to avoid contamination with serum exosomes. Cell-culture supernatant was collected and used for exosome extraction. Leukemia-cell-derived exosomes were accumulated using continuous centrifugation. The harvested exosome pellets were washed twice with glacial phosphate-buffered saline (PBS) and re-concentrated using ultra-centrifugation (at 100,000 g for 1 hour). Exosomes derived from non-modified L1210 cells were designated as LEX. Exosomes derived from L1210 cells transfected with the lentiviral vector containing the scrambled shRNA sequence and PD-L1 shRNA were designated as LEX_{GFP} and $LEX_{PD\text{-}L1si}$, respectively. Exosome morphology and typical exosomal proteins (HSP70, TSG101 and CD63) were identified using transmission electron microscopy (Philips CM12) and western blotting analysis as described previously[16,17].

2.6 Flow cytometry

To quantify the expression levels of PD-L1 on the exosomal surface, 30 μg of exosomes were first incubated with aldehyde/sulfate latex beads at 4°C overnight; the reaction was then blocked by the addition of 100 mmol/L glycine. Exosome-loaded latex beads were washed twice in PBS containing 1% fetal taurine, and were then stained with either a specific antibody against mouse PD-L1 or an isotype control. Fluorescence intensity of exosome-loaded latex beads was analyzed using a BD FACScan TM flow cytometer.

To analyze the effects of the exosomes on the phenotype of bone-marrow-derived DCs, DCs were incubated with PBS, LEX, LEX_{GFP}, $LEX_{PD\text{-}L1si,}$ or LPS for 24 hours. Afterwards, DCs were collected and stained with PE-cyanine7-labeled anti-CD80, PE-labeled anti-MHC Ia/Ib, and APC-labeled anti-CD86 antibodies, and then analyzed using FACScan.

2.7 T cell proliferation assay

Splenic T cells were collected from 6-8 week-old female DBA/2 mice immunized seven days earlier. The isolated splenic T cells (1 x 10^5 cells/well) were then co-cultured for 72 hours with irradiated L1210 (1 x 10^4 cells/well) or p388 cells (1 x 10^4 cells/well; used as controls) in the presence of PHA (20 μg/ml) at 37@C and 5% CO_2. Then, [^{3}H] thymidine (0.5 μCi per well) was added to the cultures and allowed to incubate for an additional 16 hours. Subsequently, the cells were harvested, and [^{3}H]-thymidine uptake was detected by MicroBeta counter (Beckman Coulter, Krefeld, Germany).

2.8 Cytotoxicity assay

For the CTL assay, single-cell suspensions of splenocytes were collected from 6-8 week-old female DBA/2 mice immunized seven days earlier. The CD8$^+$ T cells were then isolated from splenocytes using an EasySep mouse CD8$^+$ T cell Isolation Kit, and re-stimulated with irradiated L1210 cells and mouse IL-2 ((100 μg/mL)) or 7 days; the resulting effector cells were then harvested. L1210 cells (used as specific target cells) or p388 cells (serving as controls) were seeded in a 96-well plate at 1 $\times$ 10^4 cells/well. The magnitude of cytotoxic response at different effector/target (E / T) ratios was evaluated by a lactate dehydrogenase (LDH) release assay, and percentage of specific lysis was calculated as follows: (experimental LDH release - effector cells - target spontaneous LDH release)/(target maximum LDH release) x 100.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Cytokine (IL-12p70, TNF-α, IFN-γ, and IL-2) levels secreted by immune cells were detected using an ELISA kit following the manufacturer's instructions. The concentrations of these cytokines were determined according to a standard curve.

2.10 Assessment of $LEX_{PD\text{-}L1si}$ efficacy in vivo

To evaluate the protective effect of exosome vaccines, 100 μL PBS (blank control), 10 μg LEX, 10 μg LEX_{GFP}, or 10 μg $LEX_{PD\text{-}L1si}$ was injected subcutaneously (s.c.) into the inner side into the inner side of the right hind limbs of DBA/2 6-8 week old female mice on Day 0 . Immunization was boosted twice on Days 7 and 14. On Day 21, the immunized mice were challenged with L1210 cells (0.5 x 10^6 cells/mouse) injected s.c. into the lateral thigh. The survival rate of the mice was recorded every 2 days after tumor inoculation; tumor size, calculated as length $\times$ width^2 xπ /6, was also measured every 2 days.

To evaluate the therapeutic immune effect of our exosome-based vaccine, we pre-established a tumor-bearing mouse model by s.c. inoculating L1210 cells (0.5 x 10^6 cells/mouse) into the lateral part of the right thigh on Day 0. Then, 10 μg LEX, 10 μg LEX_{GFP}, or 10 μg $LEX_{PD\text{-}L1si}$ was injected s.c. into the inner side of the right thigh on Day 5. An identical treatment regimen was performed on Days 10 and 15, and PBS was used as blank control. Tumor-bearing mice were monitored every 2 days to evaluate the therapeutic efficacy of the vaccines. Survival rate and tumor size were recorded as described above.

2.11 Statistical analysis

All experiments were performed in triplicate. Data are presented as mean ± SD or SEM. The log-rank test was used to analyze survival data, and differences between the two groups were analyzed by Student's t-test. Statistical significance was determined at $p < 0.05$.

3. Results

3.1 Gene-mediated PD-L1 blockade in leukemia cells decreases PD-L1 expression in LEX

L1210 cells were transduced with a scramble or PD-L1 shRNA-incorporated lentivirus. Stable transduction of L1210 cells with PD-L1 shRNA-modified lentiviral vector efficiently silenced PD-L1 expression in L1210 cells (Figure 1a, b). We analyzed the properties of exosomes derived from PD-L1-silenced L1210 cells ($LEX_{PD\text{-}L1si}$). As shown in Fig. 1c, electron microscopy illustrated that characteristics of $LEX_{PD\text{-}L1si}$ were consistent with previously reported exosomal morphologic characteristics[26]. Western blotting showed that expression of specific exosomal markers Hsp70, TSG101, and CD63 was abundant in all types of exosomes

derived from L1210 cells (Fig. 1d). Western blotting and flow cytometry also indicated that PD-L1 expression in $LEX_{PD\text{-}L1si}$ was significantly downregulated compared with those of non-modified LEX and LEX obtained from GFP-transduced L1210 cells (LEX_{GFP}); this expression pattern was consistent with that of parental cells (Fig. 1d,e).

3.2 $LEX_{PD\text{-}L1si}$ efficiently promotes maturation and function of dendritic cells while limiting PD-L1 induction on DC surface

DCs are indispensable for antigen presentation during T-cell priming, which is critical for anti-leukemia immunity. Therefore, we explored the influence of $LEX_{PD\text{-}L1si}$ on DC phenotype and function. The expression of CD86, CD80 and MHC-II on DCs is essential for antigen presentation and T cell activation. It has been shown that immature DCs (imDCs) express a relatively low level of CD86, CD80 and MHC-II, and secrete a scant amount of IL-12p70 and TNF-α. Following incubation with the three types of exosomes (10 μg/ml) for 24 hours, DC surface expression of CD86, CD80 and MHC-II, was markedly upregulated. Stimulation with $LEX_{PD\text{-}L1si}$ exerted the most significant effects on upregulating CD86, CD80 and MHC-II expression on the DC surface. DC produced pro-inflammatory factors, IL-12p70 and TNF-α, act as essential elements to block T cells to differentiate into effectors. It has been shown that DCs in the $LEX_{PD\text{-}L1si}$-stimulated group secreted significantly more IL-12p70 and TNF-α compared with secretion levels of DCs in the LEX- and LEX_{GFP}-stimulated groups. Mixed lymphocyte reaction (MLR) assay showed that $LEX_{PD\text{-}L1si}$-stimulated-DCs acted as more potent inducers of T-cell proliferation than LEX- or LEX_{GFP}-treated DCs at stimulator/responder ratios of 1:5 and 1:20. These results suggest that $LEX_{PD\text{-}L1si}$ promoted maturation and function of dendritic cells more efficiently than the other two exosomes.

3.3 $LEX_{PD\text{-}L1si}$ promotes T-cell activation and antigen-specific CTL response

It has been proved that exosomal PD-L1 suppresses T cell activation and function[24]. In our study, we examined whether $LEX_{PD\text{-}L1si}$ could reverse T-cell tolerance and immunosuppression, and restore anti-tumor immunity. After pre-labeling our exosomes with CFSE, we co-cultured them with splenic T cells for 2–12 hours, and then analyzed exosome internalization efficiency by T cells. After 8 hours of co-incubation, CFSE-positive T cells were observed using confocal fluorescence microscopy (Fig. 3a). Furthermore, exosomal uptake efficiency was detected by flow cytometry at different time points. As shown in Fig. 3b, CFSE-positive T cells ($8.1 \pm 2.7\%$) were confirmed as early as 2 hours after incubation, and the percentage of CFSE-positive T cells reached a plateau at 12 hours after incubation.Therefore, these results implies that besides influencing T cell activation through DC maturation, exosomes could directly act on T cells.

After revealing the favorable exosomal uptake efficiency by T cells, we then focus on the effects of $LEX_{PD\text{-}L1si}$ on T-cell activation and function by analyzing splenic T cells obtained from mice immunized with LEX, LEX_{GFP}, or $LEX_{PD\text{-}L1si}$. As shown in Fig. 3c, immunization with LEX, LEX_{GFP}, or $LEX_{PD\text{-}L1si}$, promoted T-cell expansion in response to challenge, with L1210 cells used as specific targets. Immunization with $LEX_{PD\text{-}L1si}$ exerted the strongest effects on boosting T-cell proliferation ($p < 0.05$). We also measured cytokine production in splenic $CD4^+$ T cells, which is indicative of $CD4^+$ T-cell activation. The results of our ELISA assay showed that splenic $CD4^+$ T cells obtained from mice immunized with $LEX_{PD\text{-}L1si}$ secreted the highest levels of IFN-γ and IL-2 compared with those secreted by $CD4^+$ T-cells obtained from mouse immnuized with LEX and LEX_{GFP} groups (Fig. 3d and 3e). To further assess the CTL activity elicited by the optimized vaccine, we analyzed splenic $CD8^+$ T cells obtained from mice immunized with LEX, LEX_{GFP}, or $LEX_{PD\text{-}L1si}$. Tumor-specific CTL response in $CD8^+$ T cells was evaluated by a LDH release assay. Notably, $LEX_{PD\text{-}Lsi}$ immunized $CD8^+$ T cells exhibited the highest lysis rate in response to L1210 cells ($p < 0.05$). However, the CTL response induced by $LEX_{PD\text{-}L1si}$ did not show significant eradicating activities against p388 cells, indicating that superior CTL activity induced by $LEX_{PD\text{-}L1si}$ is likely tumor-specific.

3.4 $LEX_{PD\text{-}L1si}$ exerts immuno-protective effects against tumor challenge in vivo

Next, we evaluated the immuno-protective effects of $LEX_{PD\text{-}L1si}$ in our mouse model. DBA/2 mice were vaccinated thrice with either PBS, LEX, LEX_{GFP}, or $LEX_{PD\text{-}L1si}$ at 7-day intervals. On Day 7 after the last vaccination, the mice were subcutaneously challenged with L1210 cells (Fig. 4a). Tumor growth and

survival rate were monitored daily during the 24-day observation period. As shown in Fig. 4b, vaccination with LEX and LEX_{GFP} showed moderate inhibitory effects on tumor growth compared with those in the PBS-treated group, whereas immunization with $LEX_{PD\text{-}L1si}$ performed substantially better than that with LEX or LEX_{GFP} in delaying tumor growth. Accordingly, the survival of tumor-challenged mice was prolonged most significantly by $LEX_{PD\text{-}L1si}$ vaccination. As shown in Fig. 4c, mice in the PBS-treated group died at 24 days after the tumor challenge. The mean survival time (MST) of mice in the PBS-treated group was 20 days. The MST of mice immunized with LEX or LEX_{GFP} was approximately 26-28 days post tumor challenge. However, MST was prolonged to 32 days post tumor challenge by vaccination with $LEX_{PD\text{-}L1si}$. These results indicate that $LEX_{PD\text{-}L1si}$ induced a stronger protective immune response against leukemia cells than that induced by LEX or LEX_{GFP}.

3.5 $LEX_{PD\text{-}L1si}$ induces a robust therapeutic effect against leukemia cells in vivo

Next, we examined whether $LEX_{PD\text{-}L1si}$ could induce therapeutic anti-tumor effects against established tumors in vivo. For this, $5x10^5$ L1210 cells were pre-inoculated subcutaneously into each mouse on Day 0. Then, tumor-bearing mice were injected with different formulations on Days 5, 10, and 15 (Fig. 5a). Tumor mass was measured for 20 days after tumor inoculation. Our results indicate that all the exosome formulations examined in our present study significantly inhibited the growth of pre-established tumors. $LEX_{PD\text{-}L1si}$ inhibited tumor growth more effectively than LEX and LEX_{GFP} (Fig. 5b). That is, 30% of the mice in the $LEX_{PD\text{-}L1si}$-treated group were alive at 6 weeks after tumor inoculation, whereas mice in the PBS group died in 24 days, and mice in the LEX or LEX_{GFP} groups all died within 36 days (Fig. 5c). These results suggest that $LEX_{PD\text{-}L1si}$prolonged the survival of tumor-bearing mice more efficiently than LEX or LEX_{GFP}.

4. Discussion

Exosomes derived from tumor cells, including leukemia cells, are a rich source of tumor antigens; these antigens originate from parental cells and reflect the tumor content and activities of these parental cells[27,28]. TEX carrying tumor-associated antigens can act as potent inducers of the immune response[29]. Therefore, TEXs were expected to be a promising cell-free anti-cancer vaccine. However, studies using animal models and clinical trials have shown that treatment with non-modified TEXs does not induce an effective anti-tumor CTL response that specifically eliminates tumor cells. Therefore, improving the efficacy of TEX-based tumor vaccines remains a challenge. Emerging evidence has shown that TEXs are enriched in immunosuppressive factors that inhibit the immune response and even facilitate tumor evasion, thereby impeding the utility of TEXs in immunotherapy[30,31]. In our previous studies, we confirmed that LEXs are enriched in immunosuppressive factors such as TGF-β1 and PD-L1[17], which is similar to the immunosuppressive-factor content in other tumor-derived exosomes. To improve the immunogenicity of LEX-based vaccines, we also modulated exosomal components using genetic modification of parental tumor cells. We found that exosomes obtained from TGF-β1-silenced leukemia cells induced a more potent anti-tumor immune response than that induced by non-modified LEXs[17]. In our current study, we show that exosomes from PD-L1-silenced leukemia cells robustly promoted DC maturation and function, induced T-cell activation, and facilitated an effective and antigen-specific CTL response.

PD-L1 is a typical immune checkpoint molecule that is highly expressed in tumor cells. PD-L1 inhibits T-cell anti-tumor activities by binding to the PD-1 receptor on the surface of activated T cells, thereby playing a critical role in tumor immunosuppression[32]. Tumor-derived exosomes also carry PD-L1 on their surface; exosomal surface-membrane topology is the same as that of their parental cells[30]. TEXs, which carry PD-L1 on their surface, are responsible for suppressing T-cell function and decreasing the frequencies of TILs[30,33]. Moreover, TEXs enriched in PD-L1 can migrate to PD-L1-negative tumor and immune cells, thereby augmenting both local and systemic immunosuppression and even promoting tumor growth by engaging with PD-1[34]. For these reasons, blockade of exosomal PD-L1 may be a novel therapeutic strategy for improving anti-tumor immunity and inhibiting tumor evasion. However, evidences have been shown that exosomal PD-L1 resist to the already approved antibodies to block the PD-L1/PD-1[30]. For example, Yu at al have demonstrated that the TRAMP-C2 prostate cancer model is resistant to current anti-PD-L1/PD-1

antibody. In contrast, genetic blockade of *PD-L1* had a striking effect[35]. Similarly, the MC38 murine colon carcinoma model shows only partial responsiveness to anti-PD-L1 therapy,while deletion of the*PD-L1* exhibited a more potent effect[30]. The reason of exosomal PD-L1 resistance to current anti-PD-L1/PD-1 antibody blockade was still unclear. It is possible that how PD-L1 is presented on the TEX makes it less responsive to the current antibodies. Besides, It is also possible that exosomal PD-L1 may be produced at high enough levels that it can compete with the delivered antibody. In our study, we aimed to block the immunosuppressive effects of exosomal PD-L1 by downregulating exosomal PD-L1 expression through genetic blockade of *PD-L1* in parental cells. Our results indicate that LEXs derived from PD-L1-silenced leukemia cells expressed a significantly lower level of PD-L1 than non-modified LEXs, demonstrating that artificially modulating exosomal expression of PD-L1 via genetic modification is a feasible and straightforward strategy. $LEX_{PD\text{-}L1si}$ also expressed the typical exosomal markers and morphologic characteristics, indicating that genetic modification of parental cells did not affect exosomal biological properties.

Tumor antigen-pulsed dendritic cells (DC), capable of triggering antigen-specific T-cell activation, play an essential role in the initiation and modulation of anti-tumor immune responses[36]. The maturation status of DCs determines their immunological potency (enhancing anti-tumor immunity or promoting immunologic tolerance). Although immature dendritic cells can participate in antigen uptake and processing, they can not provide the signals required for the initiation of T cell response. By contrast, mature DCs induced by external signals can migrate to secondary lymphoid organs, and upregulate immunogenicity to initiate T cell response[37]. Moreover, DC-based anti-tumor immune responses can be regulated by TEXs[38]. PD-L1 on TEXs mediates suppression of DC maturation and blockage of DC-regulated T-cell activation, thereby promoting tumor immune escape[38]. Our results indicate that stimulation with $LEX_{PD\text{-}L1si}$partially reversed DC tolerance by promoting DC maturation and pro-inflammatory factors production, thereby enhancing the capacity of DCs to stimulate T-cell activation. These results suggest that depleting PD-L1 from LEXs may be a potential strategy for enhancing the immunological potency of DCs.

T cells play a crucial role in TEX-induced anti-tumor immunity. Previous studies have shown that directly combining T-cell PD-1 receptors with their corresponding PD-L1 ligands on tumor cells or TEXs can down-regulate the amplitude of T cell activation and induce T-cell dysfunction, leading to tumor-cell immune escape[39]. In our present study, we show that LEX was efficiently uptaken and internalized by T cells in vitro, demonstrating that the highly biologically-active membrane-form of PD-L1 on LEXs can exert a direct suppressive effect on T-cell activation.

Furthermore, $LEX_{PD\text{-}L1si}$ outperformed non-modified LEX in inducing T-cell proliferation and promoting the secretion of Th1 cytokines in an antigen-specific manner. These results suggest that downregulation of PD-L1 expression on exosomal surfaces can effectively reverse the negative immune effects of LEXs on T cells and promote T-cell activation. Moreover, $LEX_{PD\text{-}L1si}$ effectively induced a CTL response. Compared with non-modified LEX, $LEX_{PD\text{-}L1si}$ potentiated a stronger antigen-specific cytotoxic response, which directly contributed to inhibition of leukemia-cell growth. Having shown the effectiveness of $LEX_{PD\text{-}L1si}$ in promoting the function of DCs and T cells, we examined the anti-leukemia effects of $LEX_{PD\text{-}L1si}$ in vivo. Our results show that $LEX_{PD\text{-}L1si}$ effectively attenuated tumor growth and prolonged the survival time of L1210 cell-bearing mice. Our findings indicate that vaccination with $LEX_{PD\text{-}L1si}$ induced a potent systemic immune response against leukemia in vivo.

5. Conclusions

In summary, we extensively characterized $LEX_{PD\text{-}L1si}$, and showed that $LEX_{PD\text{-}L1si}$ can be developed into an effective LEX-based vaccine for inducing anti-leukemia immunity via hyper-activation of DCs and T cells. This study offers a novel strategy for optimizing the immunogenicity of LEX-based tumor vaccines.

Declarations

Ethics approval and consent to participate

All animal experiments were conducted according to the guidelines of the Ethics Committee of Xinhua

Hospital Affiliated to the Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Availability of data and materials

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

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by the National Natural Science Foundation of China (Grant No. 81470314, 81873435).

Authors' contributions

Fang Huang performed the experiments and prepared the manuscript. Zhichao Li analyzed the data. Wenhao Zhang and Jiaqi Li reviewed the data. Siguo Hao designed the study. All authors read and approved the manuscript.

Acknowledgements

Not applicable.

References

1. Greaves M (2016) Leukaemia 'firsts' in cancer research and treatment. Nature reviews Cancer 16 (3):163-172. doi:10.1038/nrc.2016.3

2. Malard F, Mohty M (2020) Acute lymphoblastic leukaemia. Lancet 395 (10230):1146-1162. doi:10.1016/S0140-6736(19)33018-1

3. Karol SE, Alexander TB, Budhraja A, Pounds SB, Canavera K, Wang L, Wolf J, Klco JM, Mead PE, Das Gupta S, Kim SY, Salem AH, Palenski T, Lacayo NJ, Pui CH, Opferman JT, Rubnitz JE (2020) Venetoclax in combination with cytarabine with or without idarubicin in children with relapsed or refractory acute myeloid leukaemia: a phase 1, dose-escalation study. The Lancet Oncology 21 (4):551-560. doi:10.1016/S1470-2045(20)30060-7

4. Borlenghi E, Pagani C, Zappasodi P, Bernardi M, Basilico C, Cairoli R, Fracchiolla N, Todisco E, Turrini M, Cattaneo C, Da Via M, Ciceri F, Passamonti F, Mancini V, Sciume M, Cerqui E, Rossi G (2020) Validation of the "fitness criteria" for the treatment of older patients with acute myeloid leukemia: A multicenter study on a series of 699 patients by the Network Rete Ematologica Lombarda (REL). Journal of geriatric oncology. doi:10.1016/j.jgo.2020.10.004

5. Pandey R, Ramdas B, Wan C, Sandusky G, Mohseni M, Zhang C, Kapur R (2019) SHP2 inhibition reduces leukemogenesis in models of combined genetic and epigenetic mutations. The Journal of clinical investigation 129 (12):5468-5473. doi:10.1172/JCI130520

6. Gavralidis A, Brunner AM (2020) Novel Therapies in the Treatment of Adult Acute Lymphoblastic Leukemia. Current hematologic malignancy reports 15 (4):294-304. doi:10.1007/s11899-020-00591-4

7. Miller KC, Al-Kali A, Shah MV, Hogan WJ, Elliott MA, Begna KH, Gangat N, Patnaik MM, Viswanatha DS, He R, Greipp PT, Sproat LZ, Foran JM, Litzow MR, Alkhateeb HB (2019) Elderly acute

lymphoblastic leukemia: a Mayo Clinic study of 124 patients. Leukemia & lymphoma 60 (4):990-999. doi:10.1080/10428194.2018.1509318

8. Bollard CM, Stevenson FK (2016) Introduction to a review series on advances in cell-based immune therapeutics in hematology. Blood 127 (26):3293. doi:10.1182/blood-2016-05-713255

9. Vago L, Gojo I (2020) Immune escape and immunotherapy of acute myeloid leukemia. The Journal of clinical investigation 130 (4):1552-1564. doi:10.1172/JCI129204

10. Roselli E, Faramand R, Davila ML (2021) Insight into next-generation CAR therapeutics: designing CAR T cells to improve clinical outcomes. The Journal of clinical investigation 131 (2). doi:10.1172/JCI142030

11. Shah NN, Fry TJ (2019) Mechanisms of resistance to CAR T cell therapy. Nature reviews Clinical oncology 16 (6):372-385. doi:10.1038/s41571-019-0184-6

12. Cummins KD, Gill S (2019) Will CAR T cell therapy have a role in AML? Promises and pitfalls. Seminars in hematology 56 (2):155-163. doi:10.1053/j.seminhematol.2018.08.008

13. Avigan D, Rosenblatt J (2018) Vaccine therapy in hematologic malignancies. Blood 131 (24):2640-2650. doi:10.1182/blood-2017-11-785873

14. Whiteside TL (2016) Tumor-Derived Exosomes and Their Role in Cancer Progression. Advances in clinical chemistry 74:103-141. doi:10.1016/bs.acc.2015.12.005

15. Seo N, Akiyoshi K, Shiku H (2018) Exosome-mediated regulation of tumor immunology. Cancer science 109 (10):2998-3004. doi:10.1111/cas.13735

16. Hu W, Huang F, Ning L, Hao J, Wan J, Hao S (2020) Enhanced immunogenicity of leukemia-derived exosomes via transfection with lentiviral vectors encoding costimulatory molecules. Cell Oncol (Dordr) 43 (5):889-900. doi:10.1007/s13402-020-00535-3

17. Huang F, Wan J, Hao S, Deng X, Chen L, Ma L (2017) TGF-beta1-silenced leukemia cell-derived exosomes target dendritic cells to induce potent anti-leukemic immunity in a mouse model. Cancer immunology, immunotherapy : CII 66 (10):1321-1331.

18. Yao Y, Wang C, Wei W, Shen C, Deng X, Chen L, Ma L, Hao S (2014) Dendritic cells pulsed with leukemia cell-derived exosomes more efficiently induce antileukemic immunities. PloS one 9 (3):e91463. doi:10.1371/journal.pone.0091463

19. Yao Y, Chen L, Wei W, Deng X, Ma L, Hao S (2013) Tumor cell-derived exosome-targeted dendritic cells stimulate stronger CD8+ CTL responses and antitumor immunities. Biochemical and biophysical research communications 436 (1):60-65. doi:10.1016/j.bbrc.2013.05.058

20. Saint-Pol J, Gosselet F, Duban-Deweer S, Pottiez G, Karamanos Y (2020) Targeting and Crossing the Blood-Brain Barrier with Extracellular Vesicles. Cells 9 (4). doi:10.3390/cells9040851

21. Srivastava A, Rathore S, Munshi A, Ramesh R (2021) Extracellular Vesicles in Oncology: from Immune Suppression to Immunotherapy. The AAPS journal 23 (2):30. doi:10.1208/s12248-021-00554-4

22. Yin Z, Yu M, Ma T, Zhang C, Huang S, Karimzadeh MR, Momtazi-Borojeni AA, Chen S (2021) Mechanisms underlying low-clinical responses to PD-1/PD-L1 blocking antibodies in immunotherapy of cancer: a key role of exosomal PD-L1. Journal for immunotherapy of cancer 9 (1). doi:10.1136/jitc-2020-001698

23. Teixeira AF, Ten Dijke P, Zhu HJ (2020) On-Target Anti-TGF-beta Therapies Are Not Succeeding in Clinical Cancer Treatments: What Are Remaining Challenges? Frontiers in cell and developmental biology 8:605. doi:10.3389/fcell.2020.00605

24. Ayala-Mar S, Donoso-Quezada J, Gonzalez-Valdez J (2021) Clinical Implications of Exosomal PD-L1 in Cancer Immunotherapy. Journal of immunology research 2021:8839978.

25. He X, He Q, Yu W, Huang J, Yang M, Chen W, Han W (2021) Optimized protocol for high-titer lentivirus production and transduction of primary fibroblasts. Journal of basic microbiology. doi:10.1002/jobm.202100008

26. Yamaguchi T, Kawamoto E, Gaowa A, Park EJ, Shimaoka M (2021) Remodeling of Bone Marrow Niches and Roles of Exosomes in Leukemia. International journal of molecular sciences 22 (4). doi:10.3390/ijms22041881

27. Khalife J, Sanchez JF, Pichiorri F (2020) Extracellular Vesicles in Hematological Malignancies: From Biomarkers to Therapeutic Tools. Diagnostics (Basel) 10 (12). doi:10.3390/diagnostics10121065

28. Gholipour E, Sarvarian P, Samadi P, Talebi M, Movassaghpour A, Motavalli R, Hojjat-Farsangi M, Yousefi M (2020) Exosome: From leukemia progression to a novel therapeutic approach in leukemia treatment. Biofactors 46 (5):698-715. doi:10.1002/biof.1669

29. Droste M, Thakur BK, Eliceiri BP (2020) Tumor-Derived Extracellular Vesicles and the Immune System-Lessons From Immune-Competent Mouse-Tumor Models. Frontiers in immunology 11:606859. doi:10.3389/fimmu.2020.606859

30. Chen G, Huang AC, Zhang W, Zhang G, Wu M, Xu W, Yu Z, Yang J, Wang B, Sun H, Xia H, Man Q, Zhong W, Antelo LF, Wu B, Xiong X, Liu X, Guan L, Li T, Liu S, Yang R, Lu Y, Dong L, McGettigan S, Somasundaram R, Radhakrishnan R, Mills G, Kim J, Chen YH, Dong H, Zhao Y, Karakousis GC, Mitchell TC, Schuchter LM, Herlyn M, Wherry EJ, Xu X, Guo W (2018) Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. Nature 560 (7718):382-386. doi:10.1038/s41586-018-0392-8

31. Robbins PD, Morelli AE (2014) Regulation of immune responses by extracellular vesicles. Nature reviews Immunology 14 (3):195-208. doi:10.1038/nri3622

32. Xu-Monette ZY, Zhang M, Li J, Young KH (2017) PD-1/PD-L1 Blockade: Have We Found the Key to Unleash the Antitumor Immune Response? Frontiers in immunology 8:1597. doi:10.3389/fimmu.2017.01597

33. Poggio M, Hu T, Pai CC, Chu B, Belair CD, Chang A, Montabana E, Lang UE, Fu Q, Fong L, Blelloch R (2019) Suppression of Exosomal PD-L1 Induces Systemic Anti-tumor Immunity and Memory. Cell 177 (2):414-427 e413. doi:10.1016/j.cell.2019.02.016

34. Morrissey SM, Yan J (2020) Exosomal PD-L1: Roles in Tumor Progression and Immunotherapy. Trends in cancer 6 (7):550-558.

35. Yu P, Steel JC, Zhang M, Morris JC, Waitz R, Fasso M, Allison JP, Waldmann TA (2012) Simultaneous inhibition of two regulatory T-cell subsets enhanced Interleukin-15 efficacy in a prostate tumor model. Proceedings of the National Academy of Sciences of the United States of America 109 (16):6187-6192. doi:10.1073/pnas.1203479109

36. Markiewicz MA, Kast WM (2004) Progress in the development of immunotherapy of cancer using ex vivo-generated dendritic cells expressing multiple tumor antigen epitopes. Cancer investigation 22 (3):417-434. doi:10.1081/cnv-200029072

37. Lutz MB, Backer RA, Clausen BE (2021) Revisiting Current Concepts on the Tolerogenicity of Steady-State Dendritic Cell Subsets and Their Maturation Stages. J Immunol 206 (8):1681-1689. doi:10.4049/jimmunol.2001315

38. Ning Y, Shen K, Wu Q, Sun X, Bai Y, Xie Y, Pan J, Qi C (2018) Tumor exosomes block dendritic cells maturation to decrease the T cell immune response. Immunology letters 199:36-43. doi:10.1016/j.imlet.2018.05.002

39. Tang Y, Zhang P, Wang Y, Wang J, Su M, Zhou L, Zhou J, Xiong W, Zeng Z, Zhou Y, Nie S, Liao Q (2020) The Biogenesis, Biology, and Clinical Significance of Exosomal PD-L1 in Cancer. Frontiers in immunology 11:604. doi:10.3389/fimmu.2020.00604

Figure legends:

Figure 1. Characterization of $LEX_{PD\text{-}L1si}$ (a) Comparison of PD-L1 protein expression in L1210 cells, L1210 cells transduced with control vector ($L1210_{GFP}$), and L1210 cells transduced with PD-L1 shRNA modified vector ($L1210_{PD\text{-}L1si}$), as assessed using western blotting. (b) Representative images show flow cytometric analysis of PD-L1 expression on L1210, $L1210_{GFP}$, and $L1210_{PD\text{-}L1si}$cells. (c) Exosomes derived from $L1210_{PD\text{-}L1si}$ cells were visualized by electron microscopy and are visible as dimpled micro-vesicles, ranging between 40 and 100 nm. Scale bar is 100 nm. (d) Expression pattern of PD-L1, and typical exosome markers HSP70, TSG101 and CD631, in exosome preparations. (e) Membrane-bound PD-L1 protein levels in LEX, LEX_{GFP}, and $LEX_{PD\text{-}L1si}$were measured using flow cytometry. All experiments were performed in triplicate. One representative experiment is shown.

Figure 2. Co-incubation with $LEX_{PD\text{-}L1si}$effectively promotes phenotypic and functional maturation of DCs. Bone marrow-derived DCs (BMDCs) were co-incubated with 30 μg LEX, LEX_{GFP}, or $LEX_{PD\text{-}L1si}$ for 24 h. BMDCs stimulated with PBS were used as negative controls, while BMDCs stimulated with LPS (1 ug/ml) were used as positive controls. (a) Expression levels of CD86, CD80 and MHC-II (b) IL-12p70 (c) and TNF-α secretion level the supernatant of each group of exosomes co-incubated with DCs, as measured by ELISA. (d) The effect of each type of exosome on the capacity of DCs from 8-week-old DBA/2 female mice to stimulate proliferation of allogeneic T-lymphocytes. $*p < 0.05$ and $**p < 0.01$ denote statistically-significant differences. Data are representative of three independent experiments and are expressed as mean ± SEM.

Figure 3. $LEX_{PD\text{-}L1si}$ effectively promotes T-cell activation and antigen-specific CTL response. (a) First, 10^5/ml splenic T cells were incubated with 20 μg CFSE-labeled $LEX_{PD\text{-}L1si}$ for 8 h. CFSE-positive T cells were then detected using confocal fluorescence microscopy. splenic T cells incubated with 20 μg CFSE-unstained $LEX_{PD\text{-}L1si}$ was used as a negative control.(b) Splenic T cells were co-incubated with CFSE-labeled $LEX_{PD\text{-}L1si}$ for 1–12 hours. Time-dependent curve of percentage of CFSE-positive T cells was constructed based on flow cytometry data. (c) DBA/2 mice were immunized subcutaneously with 100 μL PBS or 10 μg each exosome type three times at 1-week intervals. At Day 7 after the last immunization, splenic T cells obtained from immunized mice were co-incubated with irradiated L1210 cells or p388 cells for 72 h. T-cell proliferation was evaluated using^{3}H thymidine incorporation. (d) and (e) IFN-γ and IL-2 secretion levels in splenic $CD4^+$ T-cells isolated from immunized mice in each group were detected by ELISA. (f) Splenic $CD8^+$ T cells obtained from immunized mice were re-stimulated with irradiated L1210 (4000 rad) cells in the presence of mIL-2 in vitro for 7 days. The separated viable $CD8^+$ T cells served as effector cells. L1210 or p388 cells served as target cells, and were mixed with effector cells at different ratios. Data were obtained using three independent experiments and are expressed as mean ± SD. $*p < 0.05$ and $**p < 0.01$ denote statistically-significant differences.

Figure 4. $LEX_{PD\text{-}L1si}$ immunization induces potent anti-leukemia preventive immunity. (a) Female DBA/2 mice, 6-8 week old, were immunized with 10 μg of each exosome type, or injected with 100 μl PBS, on day 0 (prime), 7 (booster I), and 14 (booster II). On day 21, mice were challenged with 5 x 10^5 L1210 cells subcutaneously. Each group contained 10 mice. (b) Tumor volume was measured using calipers after the tumor challenge every 2 days. (c) Survival rate of immunized mice for up to 50 days after tumor challenge. All experiments were performed in triplicate. $*p < 0.05$ and $**p < 0.01$ denote statistically-significant differences.

Figure 5. $LEX_{PD\text{-}L1si}$ immunization induces robust anti-leukemia therapeutic immunity. (a) Female DBA/2 mice, 6-8 week old, were subcutaneously inoculated with 5 x 10^5 L1210 cells on Day 0, and were then vaccinated with 10 μg each exosome type, or injected with 100 μl PBS, on Day 5 (prime), 10 (booster I), and 15 (booster II). Each group contained 10 mice. (b) Tumor volume was measured using calipers from Day 6 to Day 22. (c) Survival rate of tumor-bearing mice was recorded from Day 6 to Day 50. $*p< 0.05$ and $**p < 0.01$ denote statistically-significant differences. All experiments were performed in triplicate. $*p < 0.05$ and $**p < 0.01$ denote statistically-significant differences.

Fig.1, Fang Huang, et al.

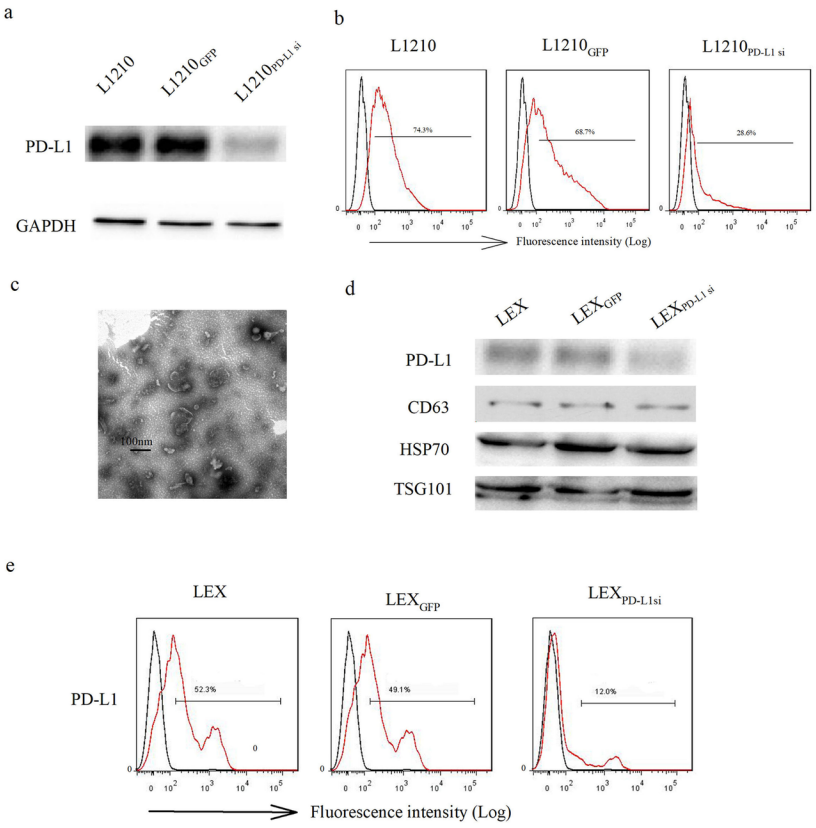

Fig.2, Fang Huang, et al.

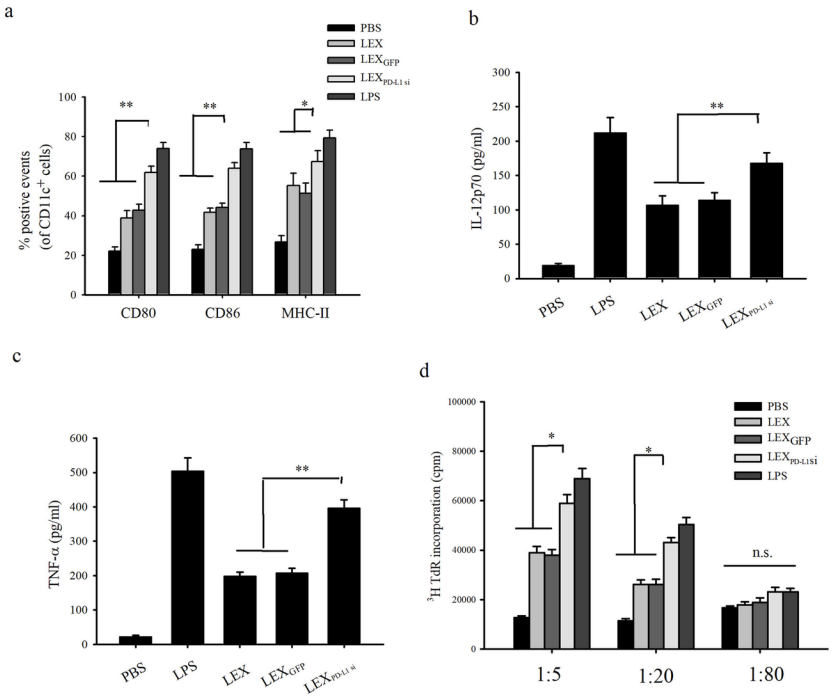

Fig.3, Fang Huang, et al.

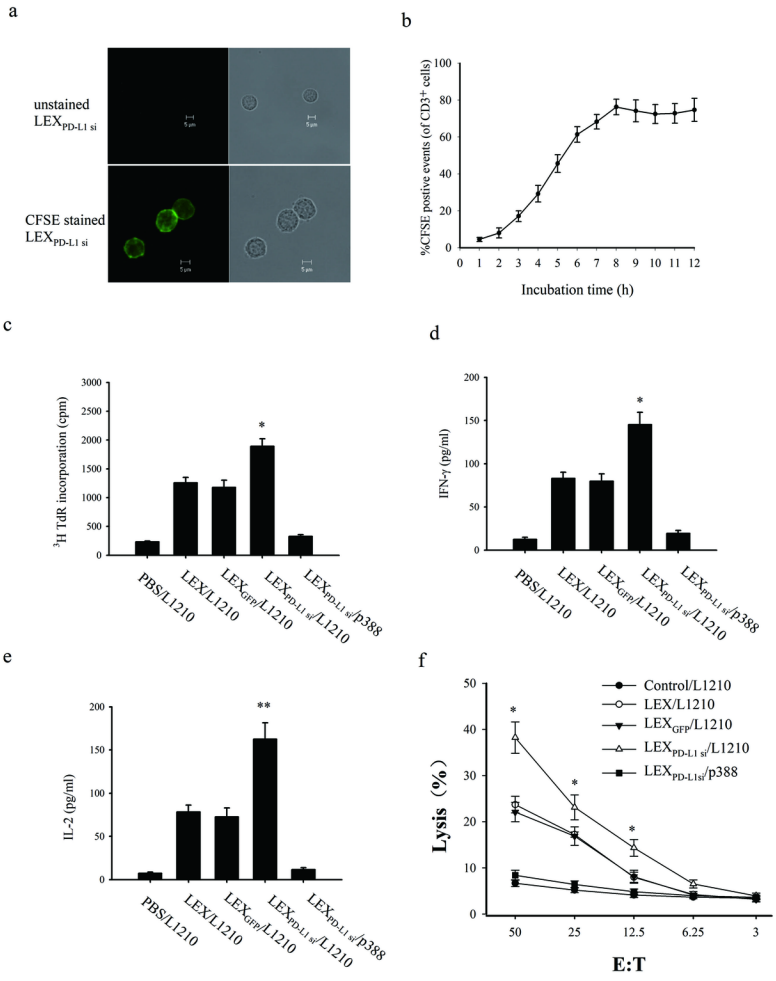

Fig.4, Fang Huang, et al.

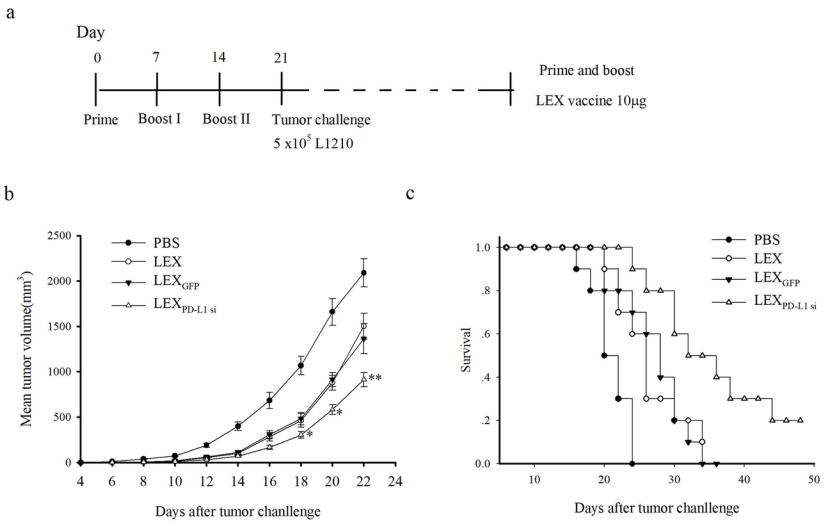

Fig.5, Fang Huang, et al.