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Membrane-bound HSP70-engineered myeloma cell-derived exosomes stimulate more efficient CD8$^+$ CTL- and NK-mediated antitumour immunity than exosomes released from heat-shocked tumour cells expressing cytoplasmic HSP70

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Abstract

Exosomes (EXO) derived from tumour cells have been used to stimulate antitumour immune responses, but only resulting in prophylatic immunity. Tumour-derived heat shock protein 70 (HSP70) molecules are molecular chaperones with a broad repertoire of tumour antigen peptides capable of stimulating dendritic cell (DC) maturation and T-cell immune responses. To enhance EXO-based antitumour immunity, we generated an engineered myeloma cell line J558$_{HSP}$ expressing endogenous P1A tumour antigen and transgenic form of membrane-bound HSP70 and heat-shocked J558$_{HS}$ expressing cytoplasmic HSP70, and purified EXO$_{HSP}$ and EXO$_{HS}$ from J558$_{HSP}$ and J558$_{HS}$ tumour cell culture supernatants by ultracentrifugation. We found that EXO$_{HSP}$ were able to more efficiently stimulate maturation of DCs with up-regulation of Ia^b, CD40, CD80 and inflammatory cytokines than EXO$_{HS}$ after overnight incubation of immature bone-marrow-derived DCs (5×10^6 cells) with EXO (100 μg), respectively. We also i.v. immunized BALB/c mice with EXO (30 μg/mouse) and assessed P1A-specific T-cell responses after immunization. We demonstrate that EXO$_{HSP}$ are able to stimulate type 1 CD4$^+$ helper T (Th1) cell responses, and more efficient P1A-specific CD8$^+$ cytotoxic T lymphocyte (CTL) responses and antitumour immunity than EXO$_{HS}$. In addition, we further elucidate that EXO$_{HSP}$-stimulated antitumour immunity is mediated by both P1A-specific CD8$^+$ CTL and non-P1A-specific natural killer (NK) responses. Therefore, membrane-bound HSP70-expressing tumour cell-released EXO may represent a more effective EXO-based vaccine in induction of antitumour immunity.

Keywords: tumour exosome • membrane-bound HSP70 • DC maturation • CTL • NK • antitumour immunity

Introduction

One general characteristic of tumour cells is releasing or shedding membrane vesicular, nowadays, called exosomes (EXO), which was initially described by Taylor *et al.* 25 years ago [1]. Recently, tumour derived EXO have attracted much attention as a source of tumour antigens (Ag) for vaccines [2–4]. EXO are small (~100 nm in diameter), membrane-bound vesicles of the endocytic pathway that are externalized by a variety of cell types. They are formed by the fusion of multivesicular bodies with the plasma membrane, followed by exocytosis [5, 6]. Such EXO display a discrete set of proteins involved in antigen presentation, that is, major histocompatibility complex class I and II (MHC-I and MHC-II), costimulatory (CD80,CD86) and tetraspan molecules (CD63, CD82) and are selectively enriched

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in molecules potentially involved in effector cell targeting, that is, CD11b, lactadherin and CD9 molecules [7, 8]. These tumour-derived EXO isolated from malignant effusions can transfer tumour Ags to dendritic cells (DCs) and induce tumour-specific cytotoxic T lymphocyte (CTL) responses and antitumour immunity [9–11]. It has been reported that EXO need the host DC as an adjuvant for stimulation of $CD8^+$ CTL responses [12, 13]. Zitvogel *et al.* first demonstrated eradication of tumours by EXO vaccination in animal models [10]. Subsequently, EXO-based vaccines have been confirmed to stimulate $CD8^+$ CTL responses and induce antitumour immunity [9, 14–16]. However, its efficiency is less effective because it only induces either prophylatic antitumour immunity in animal models or very weak antitumour immune responses in clinical trials [17, 18].

Heat shock protein (HSP) molecules are stress-induced molecular chaperones that function to facilitate presentation of endogenous antigenic peptides [19] leading to potent adjuvant effect on stimulation of DC maturation and enhanced $CD8^+$ CTL responses [20]. Tumour-derived HSP have thus been used as adjuvant in cancer vaccines [21]. It has been demonstrated that enhanced expression of cytoplasmic HSP in EXO derived from heat-shocked tumour cells induced more efficient $CD8^+$ CTL responses and antitumour immunity than EXO derived from untreated tumour cells [22, 23]. It has also been shown that expression of membrane-bound exosomal HSP stimulated cytolytic activity of natural killer (NK) cells [24]. Within HSP family, HSP70, the peptides of which can be quickly loaded onto MHC I and II complexes of DCs, can exhibit potent adjuvant effect in stimulation of the host immune responses and antitumour immunity [25–27].

In this study, we compared the efficiency of stimulation of T-cell responses and antitumour immunity between membrane-bound HSP70-expressing EXO derived from HSP70-engineered tumour cells engineered and cytoplasmic HSP70-expressing EXO derived from heat-shocked tumour cells. We first transfected a myeloma cell line J558 expressing its tumour Ag P1A [28] with pcDNAHSP70 vector expressing membrane-bound HSP70 and the control vector pcDNAneo without HSP70 expression, respectively [28]. We also incubated J558 tumour cells at 42°C for 1 hr for heat shock treatment to generate heat-shocked J558 ($J558_{HS}$) tumour cells. We then purified EXO_{HSP}, EXOneo and EXO_{HS} from transfected $J558_{HSP}$ and J558neo and heat-shocked $J558_{HS}$ cell culture supernatants, respectively. To assess the antitumour immunity derived from EXO vaccination, we immunized wild-type BALB/c mice with membrane-bound HSP70-expressing EXO_{HSP} or control EXOneo or cytoplasmic HSP70-expressing EXO_{HS}. We demonstrate that EXO_{HSP} vaccination is able to more efficiently induce DC maturation leading to stimulation of type 1 helper $CD4^+$ T (Th1) and more efficient $CD8^+$ T-cell responses and immunity against J558 tumour cells than EXP_{HS} and EXOneo vaccination. We also elucidate that the antitumour immunity resulted from EXO_{HSP} vaccination is mediated by both $CD8^+$ CTL and NK cells.

Materials and methods

Reagents, cell lines and animals

The myeloma cell line J558 expressing tumour antigen P1A was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). Biotin-labelled or fluorenscein isothiocyanate (FITC)-labelled antibodies (Abs) specific for H-$2K^d$ (SF-1.1), Ia^d (39–10-8), CD11c (HL3), CD40 (3/23), CD54 (3E2) and CD80 (16–10A1) as well as FITC-conjugated avidin were all obtained from BD Pharmingen, Inc. (Mississauga, Ontario, Canada). The Abs specific for LAMP-1 (1D4B) and AIP1 (49/AIP1) were obtained from BD Biosciences (Mississauga, Ontario, Canada). The anti-galectin Ab (M3/38) was obtained from BioLegend (San Diego, CA, USA). The anti-HSP70 Ab (BRM-22) was obtained from SIGMA (Oakville, Ontario, Canada). The anti-P1A Ab [28] was obtained from Dr. Yang Liu, The Ohio State University Medical Center (Columbus, OH, USA). The anti-CD8 and anti-NK Abs were purified from ascites of hybridoma cell line 3.155 and PK136 obtained from ATCC. The P1A peptide (EILPYLGWLVFA) of J558 tumour cells and an irrelevant peptide (YPHFMPTNL) of mouse cytomegalovirus [28] both specific for H-$2L^d$ were synthesized by Multiple Peptide Systems (San Diego, CA, USA). Cytokine ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). Female wild-type BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, MA, USA). Mice were treated according to animal care committee guidelines of the University of Saskatchewan.

Generation of engineered $J558_{HSP}$ cell line

Twenty million J558 tumour cells were resuspended in 0.7 ml phosphate-buffered saline (PBS) and mixed with 0.3 ml PBS containing 10 μg pcDNA-HSP70 DNA expressing membrane-bound inducible HSP70 or the control pcDNAneo DNA [29]. Tumour cells were transfected with these DNA using a Bio-Rad gene pulser (Bio-Rad Lab, Mississauga, Ontario, Canada) with parameters of 250 V and 125 μF capacitance as previously described [29]. Transfected cells were selected for growth in medium containing G418 (4 mg/ml). The selected $J558_{HSP}$ and the control J558neo clones maintained in culture medium containing 10% foetal calf serum (FCS) and G418 (0.5 mg/ml) were subjected to flow cytometric analysis and production of tumour-released EXO.

Purification of tumour cell-released EXO

J558 tumour cells were exposed to 42°C in a water bath for 1 hr for heat shock treatment to induce HSP70 expression [30] and termed $J558_{HS}$. EXO were isolated from tumour cells as described previously [16, 31]. Briefly, the supernatants of $J558_{HS}$ cells cultured for 4 hrs in FCS-free AIM-V medium to avoid contamination of FCS-derived EXO and the supernatants of $J558_{HSP}$ or J558neo cells cultured overnight in AIM-V medium containing G418 (0.5 mg/ml) were subjected to four successive centrifugations at $300 \times g$ for 5 min. to remove cells, $1200 \times g$ for 20 min. and $10,000 \times g$ for 30 min. to remove cellular debris and $100,000 \times g$ for 1 hr to pellet EXO. The EXO pellets were washed twice in a large volume of PBS and recovered by centrifugation at $100,000 \times g$ for 1 hr. The amount of exosomal proteins recovered was measured using Bradford assay (Bio-Rad, Richmond, CA, USA). EXO derived from $J558_{HSP}$, J558neo and $J558_{HS}$ tumour cells were termed as EXO_{HSP}, EXOneo and EXO_{HS}, respectively.

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Tumour cell lysate preparation

Live J558 tumour cell lysates were prepared as previously described [32]. Briefly, tumour cells were lysed using extraction buffer containing 125 mM tris(hydroxymethyl)aminomethane, 0.05% sodium dodecyl sulphate and 10% β-mercaptoethanol. Cell extracts were harvested. To remove cellular debris, cell extracts were centrifuged at 1000 × *g* for 5 min. The supernatant containing tumour cell lysate protein was freezed at –80°C until use.

Electron microscopy

EXO were fixed in 4% paraformaldehyde. The pellets were then loaded onto carbon-coated formvar grids. After incubation in a moist atmosphere for 20 min., the samples were washed twice in PBS and then fixed for 5 min. in 1% glutaraldehyde. After three washes, the EXO sample-loaded grids were stained for 10 min. with saturated aqueous uranyl. EXO samples were then examined with a Zeiss EM10C (Carl Zeiss Canada Ltd., Montreal, Canada) electron microscope at 60 kV.

Western blot analysis

EXO samples (30 μg/each) were loaded onto 12.5% acrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked by incubation for 2 hrs at room temperature with OYSSEY blocking buffer (LI-COR Bioscience, Lincoln, NE, USA), and immunoblotted with various antibodies at 4°C for overnight. After three washes with PBS, the membrane was further incubated with goat anti-rabbit/antimouse IRDye^{R}800CW and scanned using ODYSSEY instrument according to manufacturer's instruction (LI-COR Bioscience).

Flow cytometric analysis of tumour cells and tumour cell-released EXO

Tumour cells and tumour cell-derived EXO were analysed by flow cytometry as previously described [13, 33]. Briefly, J558, J558neo and J558$_{HSP}$ tumour cells (1 × 10^6 cells) were stained with a panel of biotin-labelled Abs and followed with FITC-avidin. To check apoptosis formation, J558, irradiated (9000 rad) J558, engineered J558$_{HSP}$ and heat-shocked J559$_{HS}$ tumour cells were stained with FITC-annexin V (BD Pharmingen, Inc.) on ice for 30 min., washed with PBS for two times, and then analysed by flow cytometry. EXO such as EXO$_{HSP,}$ EXOneo and EXO$_{HS}$ (30 μg/300 μl PBS) were incubated with a panel of FITC-Abs on ice for 30 min., and then directly analysed by flow cytometry.

Stimulation of immature DCs

The generation of bone marrow derived immature DCs from wild-type BALB/c mice in presence of GM-CSF (1 ng/ml) has been previously described [34]. To assess stimulatory effect, immature DCs (5 × 10^6 cells) were incubated with EXO$_{HSP}$ or EXOneo or EXO$_{HS}$ (100 μg) for overnight, then stained with a panel of Abs, and analysed by flow cytometry.

T-cell proliferation assays

BALB/c mice were i.v. immunized with EXOneo, EXO$_{HS}$ and EXO$_{HSP}$ (30 μg/mouse), respectively. The mice injected with PBS as control. In *in vitro* T-cell proliferation assay, 6 days after immunization, CD4$^+$ T cells were purified from splenocytes of the immunized mice by passage through nylon wool columns followed with CD8-microbeads for removal of CD8$^+$ T cells [35]. CD4$^+$ T cells (0.2 × 10^6 cells/well) and its two-fold dilutions were then incubated with irradiated (4000 rad) J558 tumour cells at a cell ratio of 4:1 in presence of interleukin (IL)-2 (10 U/ml) in 96-well U-bottom plate for 3 days [36]. For measurement of T-cell proliferation, ^{3}H-thymidine incorporation was assessed by liquid scintillation counting. For assessment of cytokine secretion, T-cell supernatants were harvested for measurement of IL-4 and interferon (IFN)-γ secretion by cytokine ELISA kits [35]. In *in vitro* mixed T lymphocyte reaction assay, irradiated (4000 rad) EXO$_{HSP}$- or EXOneo- or EXO$_{HS}$-DCs (1 × 10^5 cells per well) and its two-fold dilutions were co-cultured in 96-well plates with a constant number of allogeneic C57BL/6 naïve T cells (2 × 10^5 cells per well). After incubation for 3 days, T-cell proliferation was measured by adding 1 μCi ^{3}H-thymidine (1 mCi/ml, Amersham, Baie D'Urfe, Canada) to each well. After incubation for overnight, the levels of ^{3}H-thymidine incorporation into cellular DNA were determined by liquid scintillation counting [37]. In *in vivo* T-cell proliferation assay, 6 days after immunization, the tail blood samples or the splenocytes were harvested and stained with PE-conjugated H-2L^d/P1A peptide tetramer (ProImmune, Inc., Springfield, VA, USA) and FITC-conjugated anti-CD8 (Beckman Coulter, Mississauga, Ontario, Canada) for 30 min. at room temperature. The erythrocytes were then lysed using lysis/fixed buffer (Beckman Coulter). The cells were then analysed by flow cytometry [35].

Cytotoxicity assay

The *in vivo* cytotoxicity assay was performed as previously described [35]. C57BL/6 splenocytes were harvested from naive mouse spleens and incubated with either high (3.0 μM, CFSE^{high}) or low (0.6 μM, CFSE^{low}) concentrations of CFSE, to generate differentially labelled target cells. The CFSE^{high} cells were pulsed with P1A peptide, whereas the CFSE^{low} cells were pulsed with an irrelevant control peptide and served as internal control. These peptide-pulsed target cells were i.v. injected at 1:1 ratio into the above mice 6 days after immunization. Sixteen hours later, the spleens of immunized mice were removed and residual CFSE^{high} and CFSE^{low} target cells remaining in the recipients' spleens were analysed by flow cytometry [35]. In NK cytotoxicity assay, spleen T cells of mice i.v. immunized with EXO$_{HSP}$ (30 μg/mouse) at different times (2, 4 and 6 days) after immunization were prepared by passing splenocytes through nylon wool columns, and red cells were then lysed using 0.84% ammonium chloride. These T cells were used as the source of effector NK cells. (^{51}Cr)-chromate-labelled J558 tumour cells were used as target cells [38]. Ten thousand labelled target cells per well were mixed with effector cells at various effector/target cell ratios in triplicate in a 96-well plate with V bottom and were incubated for 6 hrs. Percentage of specific lysis was calculated as: 100 × ([experimental CPM – spontaneous CPM]/[maximal CPM – spontaneous CPM]). Spontaneous CPM of target cells was released in absence of effector cells, whereas maximal CPM was released by adding 1% Triton X-100 to wells in the experiment.

Animal studies

To examine whether EXO$_{HSP}$ or DCs with uptake of EXO can induce protective antitumour immunity, wild-type BALB/c mice ($n = 8$) were s.c. injected with EXOneo, EXO$_{HS}$ and EXO$_{HSP}$ (30 μg/mouse) or DCs with uptake of EXOneo, EXO$_{HS}$ and EXO$_{HSP}$ (1 × 10^6 cells per mouse), respectively. The

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immunized mice were s.c. challenged with 0.5×10^6 J558 tumour cells 6 days later. We previously demonstrated that i.p. injection of anti-CD8 (3.155) and anti-NK (PK136) Ab (0.5 mg/mouse) leads to *in vivo* depletion of more than 95% $CD8^+$ T and NK cells in mouse spleens by flow cytometric analysis [38]. To assess whether $CD8^+$ T or NK cells were involved in EXO-induced antitumour immunity, BALB/c mice ($n = 8$) were i.p. injected with anti-CD8 or anti-NK Ab (0.5 mg/mouse) 1 day before immunization, and the treatment was repeated every 3 days for a total of three time [38]. The Ab-treated mice were first immunized by s.c. injection of EXOneo, EXO_{HS} and EXO_{HSP} (30 μg/mouse), respectively, and then s.c. challenged with 0.5×10^6 J558 tumour cells 6 days after the immunization. The tumour growth was monitored daily. For humanitarian reasons, all mice with tumours that achieved a size of 1.5 cm in diameter were killed. Log-rank test and Graphpad Prism software (Graphpad Software, Inc., San Diego, CA, USA) were used to compare the mouse survival data [39].

Results

Phenotypical characterization of engineered and heat-shocked J558 tumour cells

The original J558 tumour cells expressed H-$2K^d$, CD54 and P1A molecules, but not Ia^d and HSP70 (Fig. 1A). In addition to the above molecules expressed on J558 tumour cells, the engineered $J558_{HSP}$ cells transfected with pcDNAHSP70 displayed cellular surface HSP70 expression, whereas the heat-shocked $J558_{HS}$ tumour cells did not express membrane-bound HSP70. The control J558neo tumour cells transfected with the control vector pcDNA expressed a similar pattern of cellular surface molecules as the original J558 (data not shown). The above molecules expressed on $J558_{HSP}$ were stable since a long-term culturing $J558_{HSP}$ cell line had a similar phenotype as the originally generated one (data not shown). Heat-shocked $J558_{HS}$ cells were generated by culturing J558 tumour cells at 42°C for 1 hr [22, 23]. As shown in Fig. 1(B), the original J558, transfected $J558_{HSP}$ and heat-shocked $J558_{SH}$ tumour cells were mostly live cells without staining of annexin V (early apoptosis marker) [22, 23], whereas irradiated J558 tumour cells (95%) became apoptosis.

Phenotypical characterization of J558 tumour cell-released EXO

EXO derived from J558, J558neo and $J558_{HSP}$ tumour cells were purified from tumour cell culture supernatants by differential ultracentrifugation. The yields of J558-released EXO, J558neo-released EXOneo and $J558_{HSP}$-released EXO_{HSP} were 1.0 μg/10^6 cells/24 hrs, 2.6 μg/10^6 cells/24 hrs and 2.5 μg/10^6 cells/24 hrs, respectively, indicating that tumour cells with DNA transfection secrete more EXO than the original tumour cells. The yield of $J558_{HS}$-released EXO_{HS} was 0.94 μg/10^6 cells/24 hrs similar to J558-released EXO. These EXO were then subjected to electron microscopic, Western blot and flow cytometric analysis. As shown in Fig. 1(C), EXO derived from either J558neo or $J558_{HSP}$ or $J558_{HS}$ had typical exosomal characteristic of 'saucer' or round shape with a diameter between 50 and 90 nm [40]. We also confirmed that EXO-associated proteins including LAMP-1, Alix/AIP1 and H-$2K^d$ were abundant by Western blot analysis (Fig. 1D) [41], whereas galectin, a protein found in the endoplasmic reticulum [42] was only detectable in tumour cell lysates but absent in the EXO samples, indicating that there is no contamination of other vesicles in EXO preparation. Both EXO_{HS} and EXP_{HSP} contained HSP70 by Western blot analysis (Fig. 1D). EXO were then stained with a panel of FITC-Abs and analysed by flow cytometry. As shown in Fig. 1(E), EXO (50–90 nm in diameter) were detectable by flow cytometry even though they were much smaller than microbeads (5 μm in diameter). $J558_{HSP}$-derived EXO_{HSP}, J558neo-derived EXOneo and $J558_{HS}$-released EXO_{HS} all displayed expression of molecules (H-$2K^d$, CD54 and P1A), but in much less extent than $J558_{HSP}$, $J558_{HS}$ and J558neo tumour cells (Fig. 1F). In addition, EXO_{HSP} did, but EXO_{HS} did not display surface HSP70 expression by flow cytometric analysis, indicating that EXO_{HSP} and EXO_{HS} express membrane-bound and cytoplasmic HSP70, respectively.

Immature DC uptake J558 tumour cell-released EXO

We previously established a protocol for *in vitro* generation of immature DCs by culturing bone marrow cells in low amount of GM-CSF, but no IL-4 [34] and demonstrated that immature DCs can uptake mature DC-released EXO [39]. To assess the phenotype of immature bone marrow-derived DCs generated in presence of low amount of GM-CSF, we performed flow cytometric analysis. As shown in Fig. 2(A), DCs cultured in presence of low GM-CSF (1 ng/ml) expressed CD11c and CD54, but no Ia^d, CD40 and CD80 [41], indicating that they are immature DCs. To assess uptake of EXO, immature DCs were incubated with CFSE-labelled J558 tumour cell-released EXO (EXO_{CFSE}) for different times and then analysed by flow cytometry and confocal fluorescence microscopic analysis. As shown in Fig. 2(B), immature DCs displayed CFSE expression after incubation with EXO_{CFSE}, indicating that immature DC uptake J558 tumour cell-released EXO. As shown in Fig. 2(C) and (D), the amount of immature DCs with acquired CFSE increased with the time of incubation with EXO_{CFSE} and reached a maximal level after a 4-hr incubation.

EXO_{HSP} efficiently stimulate DC maturation

It has been demonstrated that HSP70 can stimulate DC maturation [20]. To assess whether EXO_{HSP} were also able to stimulate DC maturation, we analysed immature DCs cultured with EXO_{HSP} expressing membrane-bound HSP70 or EXO_{HS} expressing cytoplasmic HSP70 or EXOneo without HSP70 expression. EXOneo-stimulated

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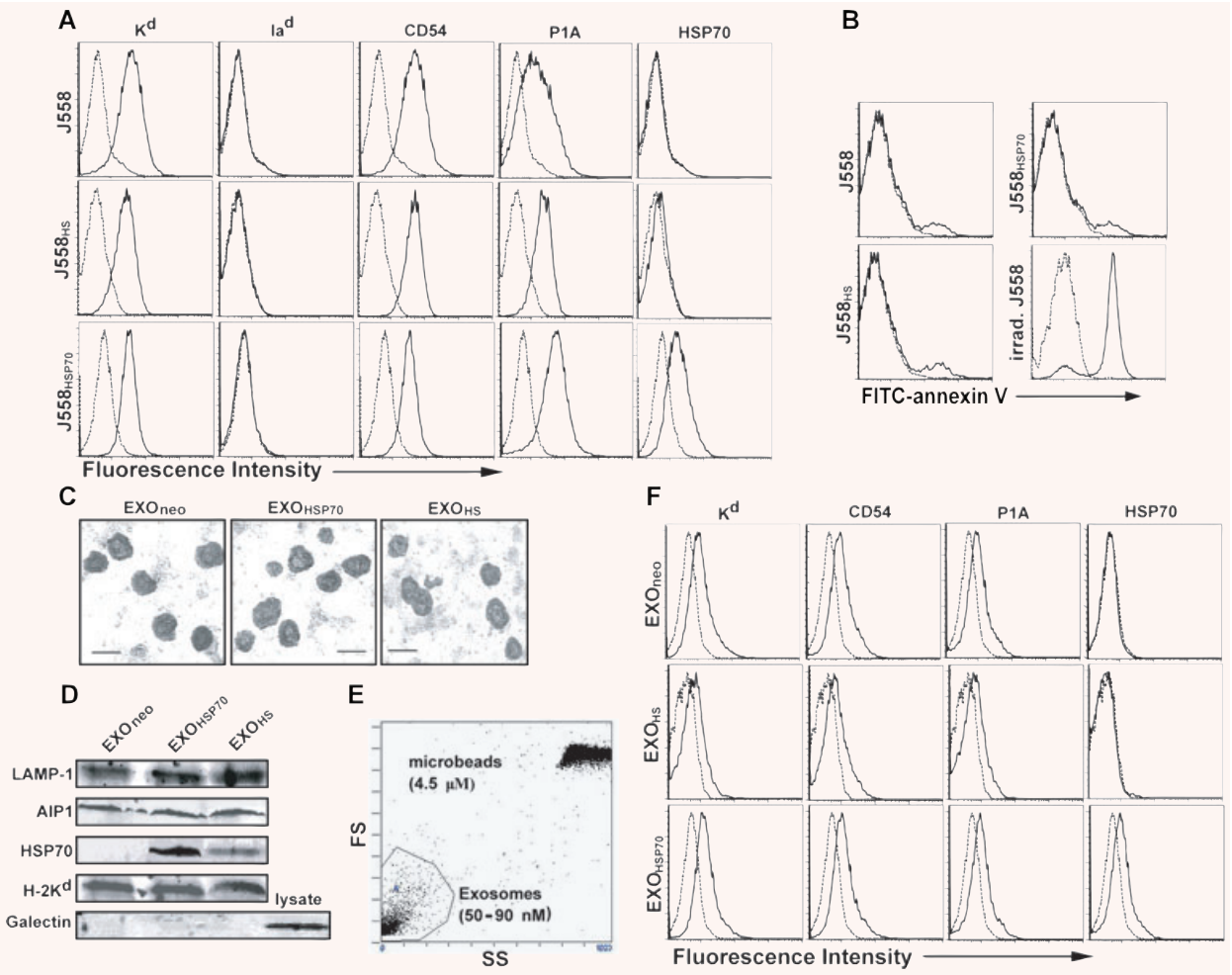

Fig. 1 Phenotypic analysis of $J558_{HSP}$-released EXO. (**A**) $J558_{HSP}$, $J558_{HS}$ and J558 tumour cells were stained with a panel of Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines), and analysed by flow cytometry. (**B**) $J558_{HSP}$, $J558_{HS}$ and J558 tumour cells as well as irradiated J558 tumour cells were stained with FITC-annexin V (solid lines) or FITC anti-rat IgG (dotted lines) and analysed by flow cytometry. (**C**) Electron micrograph of EXO. The image shows small cup-shaped vesicles of 60–100 nm in diameter, which are representative of EXO released by tumour cells. Bar, 100 nm. (**D**) Western blot analysis of tumour cell-released EXO and tumour cell lysates was performed with a panel of antibodies. (**E**) Flow cytometric analysis of EXO. EXO were grouped for analysis of expression of surface molecules. (**F**) EXOneo, EXO_{HS} and EXO_{HSP} released from J558neo, $J558_{HS}$ and $J558_{HSP}$ tumour cells were stained with a panel of FITC antibodies (solid lines) or isotype-matched irrelevant FITC antibodies (dotted lines), and analysed by flow cytometry. One representative experiment of two is displayed.

DCs still showed a similar pattern of the expression of the above molecules on immature DCs (Fig. 3A), indicating that EXOneo does not modulate DC maturation. Interestingly, we found that EXO_{HS}- and EXO_{HSP}-stimulated DCs up-regulated expression of Ia^d, CD40 and CD80, indicating that both EXO_{HS} and EXO_{HSP} are able to stimulate DC maturation. However, the up-regulation of the above molecules stimulated by the later was more than the former, indicating that EXO_{HSP} is a stronger stimulator for DC maturation than EXO_{HS}. In addition, EXO_{HSP}-stimulated mature DCs also secreted more amount of inflammatory cytokines such as IL-1β (1.8 ng/ml/10^6 cells/24 hrs), IL-12 (1.1 ng/ml/10^6 cells/24 hrs), IFN-γ (0.8 ng/ml/10^6 cells/24 hrs) and tumour necrosis factor (TNF)-α (0.9 ng/ml/10^6 cells/24 hrs) (Fig. 3B) than EXO_{HS}-stimulated DCs. Since EXO_{HSP} harboured the above immune molecules, they may have potent effect in stimulation of T-cell immune responses [43]. We first assessed whether DCs with uptake of EXO by incubation of immature DCs with EXO for overnight stimulate allogeneic T-cell proliferation in a mixed T lymphocyte reaction assay. As shown in Fig. 3C, DCs with uptake of EXO_{HSP} (DC + EXO_{HSP}) stimulated the strongest allogeneic T-cell proliferation

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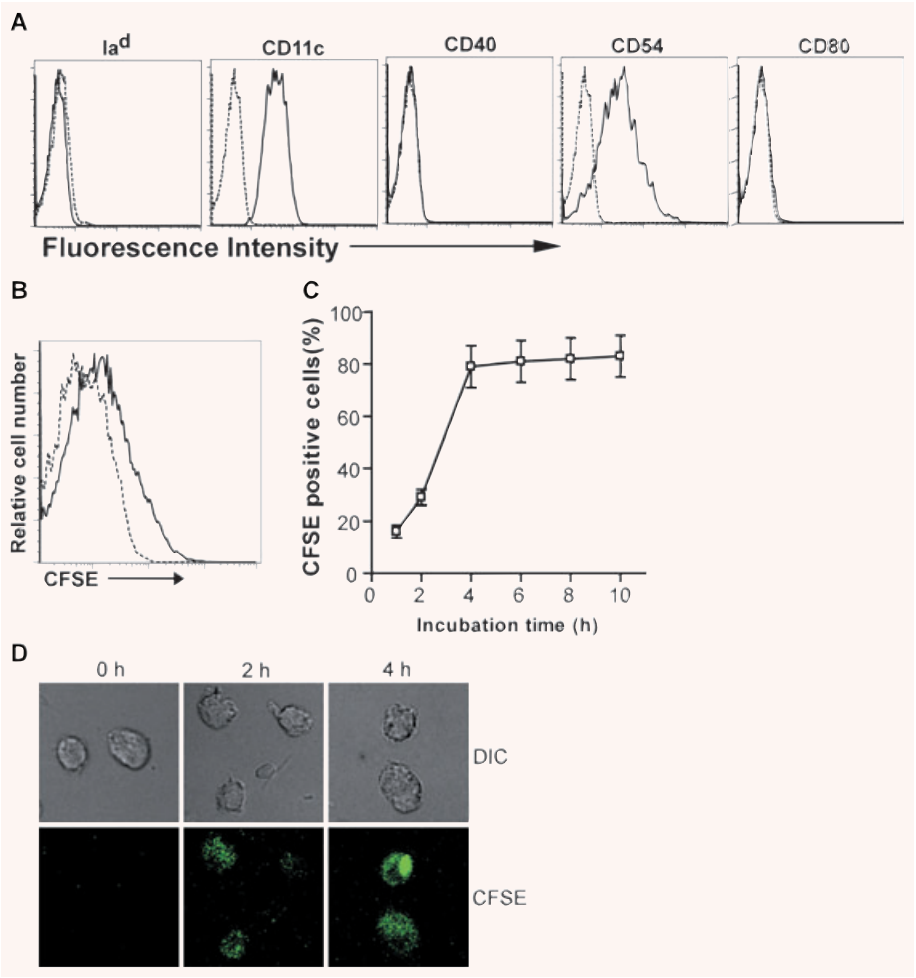

Fig. 2 Immature DCs uptake J558-released EXO. (**A**) Flow cytometric analysis of immature DCs with a panel of antibodies (solid lines) or isotype-matched irrelevant antibodies (dotted lines). (**B**) Immature DCs (5×10^6 cells) were incubated with J558 tumour cell-released EXO (dotted line) or CFSE-labelled J558 tumour cell-released EXO_{CFSE} (100 μg) (solid line) for 4 hrs and analysed by flow cytometry. (**C** and **D**) Immature DCs (5×10^6 cells) were incubated with CFSE-labelled J558 tumour cell-released EXO_{CFSE} (100 μg) (solid line) for different times and analysed by (**C**) flow cytometry and (**D**) confocal fluorescence microscopy under differential interference contrast. One representative experiment of two is displayed.

than DCs with uptake of EXOneo (DC + EXOneo) and EXO_{HS} (DC + EXO_{HS}) ($P < 0.05$). We then assessed whether DCs with uptake of EXO by incubation of immature DCs with EXO for overnight induce P1A-specific antitumour immunity. As shown in Fig. 3(D), DCs with uptake of EXOneo (DC + EXOneo), EXO_{HS} (DC + EXO_{HS}) and EXO_{HSP} (DC + EXO_{HSP}) were able to stimulate P1A-specific antitumour immunity to protect one of eight, three of eight and eight of eight mice from tumour growth after the immunized mice were challenged with J558 tumour cells, respectively, indicating that EXO_{HSP}-treated DCs are the most immunogenic among these three types of DCs.

EXO_{HSP} stimulate type 1 $CD4^+$ T-cell responses

To assess whether EXO_{HSP} can stimulate $CD4^+$ T-cell responses, we performed *in vitro* T-cell proliferation assay by using $CD4^+$ T cells derived from EXO-immunized mouse spleens. As shown in Fig. 4(A), EXO_{HSP} immunization could mount a more efficient $CD4^+$ T-cell proliferation compared to that of EXO_{HS}-immunized group ($P < 0.05$). To assess the type of $CD4^+$ T-cell response, we measured the cytokine secretion of $CD4^+$ T cells derived from EXO_{HSP}-immunized mice by ELISA. $CD4^+$ T cells derived from EXO_{HSP} immunized mice secreted IL-2 (1.4 ng/ml/10^6 cells/24 hrs) and IFN-γ (1.1 ng/ml/10^6 cells/24 hrs), but not IL-4 (Fig. 4B), indicating that EXO_{HSP} stimulate type 1 $CD4^+$ helper T (Th1) cell responses.

EXO_{HSP} stimulate stronger $CD8^+$ effector CTL responses than EXO_{HS}

To assess the stimulatory effect of EXO_{HSP} on *in vivo* $CD8^+$ T-cell responses, we performed PE-H-2L^d/P1A peptide tetramer staining assay [43]. As shown in Fig. 4(C), PBS-treatment group did not stimulate any P1A-specific (tetramer$^+$) $CD8^+$ T-cell responses. However, EXO_{HSP} immunization was able to induce 0.64% tetramer$^+$ $CD8^+$ T cells of the total $CD8^+$ T-cell population, whereas

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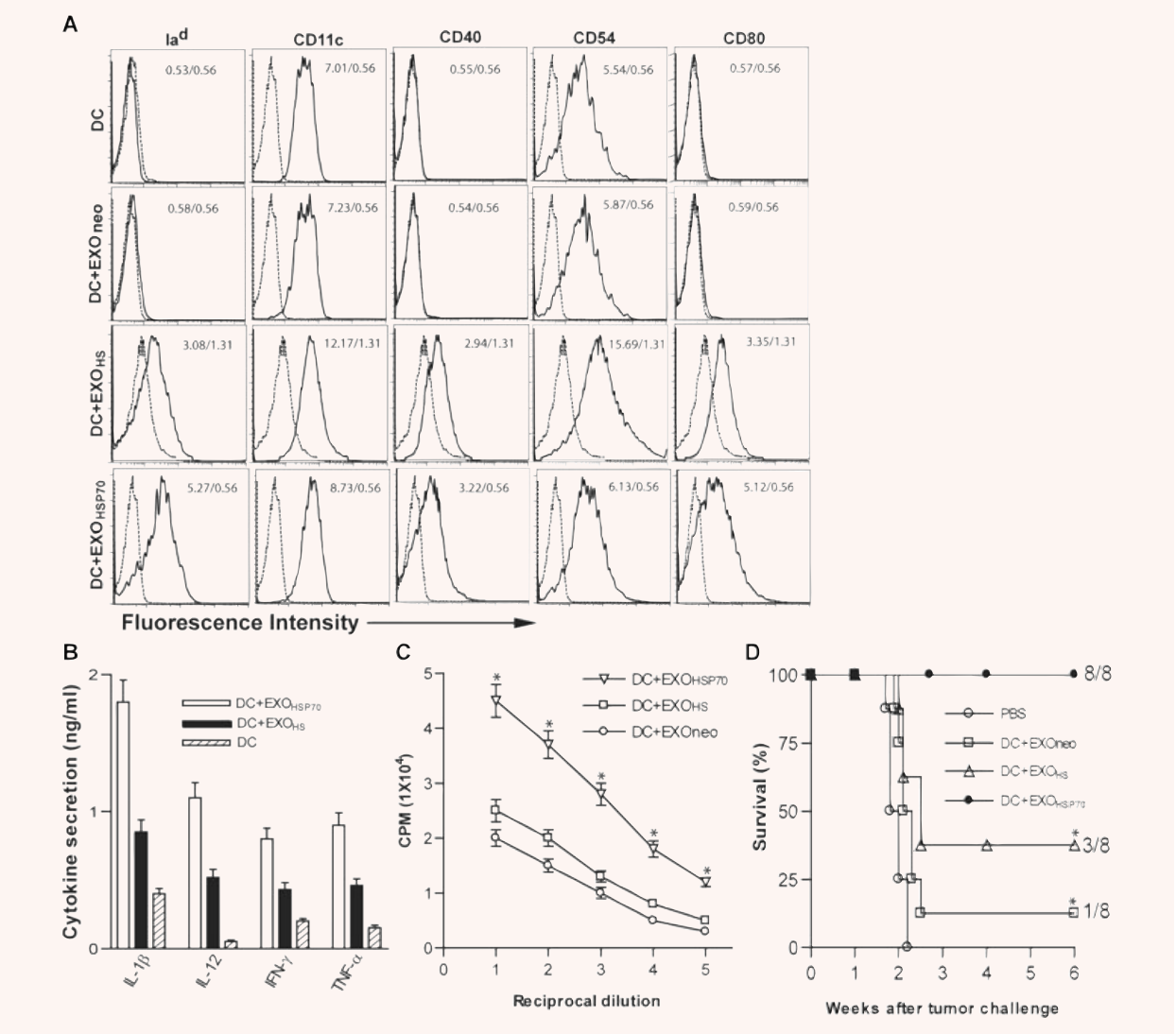

Fig. 3 J558$_{HSP}$-released EXO$_{HSP}$ stimulate DC maturation. (**A**) Flow cytometric analysis of EXO-treated DCs. Immature DCs with or without incubation of EXOneo, EXO$_{HS}$ and EXO$_{HSP}$ were stained with a panel of antibodies (solid lines) or isotype-matched irrelevant antibodies (dotted lines), and analysed by flow cytometry. Mean fluorescence intensity of solid line / mean fluorescence intensity of dotted line was presented in each panel. (**B**) Cytokine secretion. Culture supernatants of DCs alone (DC), DC with EXO$_{HS}$ (DC + EXO$_{HS}$) and DC with EXO$_{HSP}$ (DC + EXO$_{HSP}$) were tested using cytokine ELISA kits. Values represent the mean of triplicates from three experiments. (**C**) Mixed T lymphocyte reaction assay. Irradiated DCs including DC + EXO$_{HS}$ and DC + EXO$_{HSP}$ and their two-fold dilutions were co-cultured in 96-well plates with a constant number of allogeneic C57BL/6 naïve T cells. After 3 days, T-cell proliferation was measured by adding 1 μCi ^{3}H-thymidine to each well in an overnight ^{3}H-thymidine uptake assay. The levels of ^{3}H-thymidine incorporation into cellular DNA were determined by liquid scintillation counting. (**D**) BALB/c mice were s.c. vaccinated with irradiated (4000 rad) DC + EXOneo, DC + EXO$_{HS}$ and DC + EXO$_{HSP}$ (0.5×10^6 cells per mouse). 6 days after immunization, the immunized mice were s.c. inoculated with J558 tumour cells (0.5×10^6 cells per mouse). Animal mortality and tumour growth were monitored daily for up to 60 days. *, $P < 0.05$ *versus* cohorts in EXO$_{HSP}$ group (log-rank test). One representative experiment of two is displayed.

EXO$_{HS}$ and EXOneo immunization only stimulated 0.34% and 0.22% tetramer$^+$ CD8$^+$ T cells of the total CD8$^+$ T-cell population at day 6 after immunization ($P < 0.05$), indicating that EXO$_{HSP}$ expressing membrane-bound HSP70 can activate more efficient P1A-specific CD8$^+$ T-cell responses *in vivo* than EXO$_{HS}$ expressing cytoplasmic HSP70 and EXOneo without HSP70 expression, respectively. To confirm that P1A-specific (tetramer$^+$) CD8$^+$ T cells detected in peripheral blood represent the systemic condition, we

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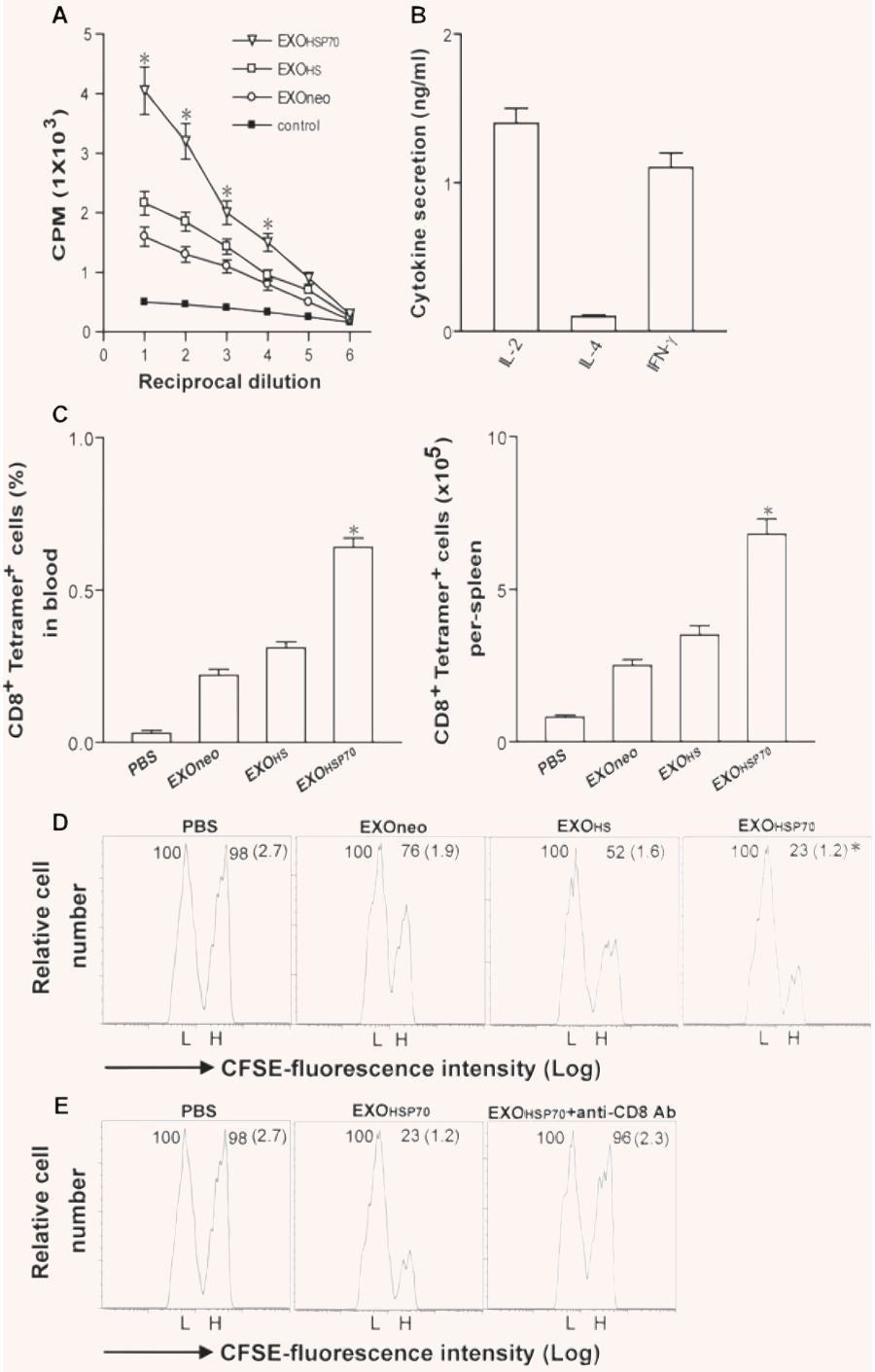

Fig. 4 J558$_{HSP}$-released EXO$_{HSP}$ stimulate T-cell responses. (**A**) A T-cell proliferation assay. Splenic CD4$^+$ T cells from EXOneo- or EXO$_{HS}$- or EXO$_{HSP}$-immunized mice were incubated with irradiated J558 tumour cells for 3 days. ^{3}H-thymidine incorporation was assessed by liquid scintillation counting. *, $P < 0.05$ *versus* cohorts in EXOneo or EXO$_{HS}$ group (Student's t-test). (**B**) Cytokine secretion. The culture supernatants of CD4$^+$ T cells derived from EXO$_{HSP}$-immunized mice were tested using cytokine ELISA kits. Values represent the mean of triplicates from three experiments. (**C**) Tetramer staining assay. 6 days after immunization of mice with EXOneo, EXO$_{HS}$ and EXO$_{HSP}$, the tail blood and splenocyte samples were taken from the immunized mice, stained with PE-H-2L^d/P1A peptide tetramer and FITC anti-CD8 Ab and analysed by flow cytomery. The value in each panel represents the percentage of tetramer$^+$ CD8$^+$ T cells *versus* the total blood CD8$^+$ T-cell population or the total tetramer$^+$ CD8$^+$ T cells per spleen. *, $P < 0.05$ *versus* cohorts in EXOneo or EXO$_{HS}$ group (Student's t-test). (**D** and **E**) In *in vivo* cytotoxicity assay. BALB/c splenocytes were harvested from naive mouse spleens and incubated with either high (3.0 μM, CFSE^{high}) or low (0.6 μM, CFSE^{low}) concentrations of CFSE, to generate differentially labelled target cells. The CFSE^{high} cells were pulsed with P1A peptide, whereas the CFSE^{low} cells were pulsed with the control peptide and served as internal controls. These peptide-pulsed target cells were i.v. injected at 1:1 ratio into (**D**) the above immunized mice 6 days after immunization of EXOneo, EXO$_{HS}$ and EXO$_{HSP}$, respectively, or into (**E**) the mice immunized with EXO$_{HSP}$ but also treated with anti-CD8 Ab to deplete CD8$^+$ T cells. 16 hrs later, the spleens of immunized mice were removed and the percentages of the residual P1A-specific CFSE^{high} (H) and control CFSE^{low} (L) target cells remaining in the recipients' spleens were analysed by flow cytometry. The value in each panel represents the percentage of CFSE^{high} *versus* CFSE^{low} target cells remaining in the spleen. The value in parenthesis represents the standard deviation. *, $P < 0.05$ *versus* cohorts in EXOneo or EXO$_{HS}$ group (Student's t-test). One representative experiment of three is shown.

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also measured the tetramer$^+$CD8$^+$ T cells in mouse spleens. The pattern of P1A-specific CD8$^+$ T cells in different groups of mouse spleens was similar to that seen in mouse blood samples. To assess whether EXO can also stimulate CD8$^+$ T-cell differentiation into CTL effectors *in vivo*, we adoptively transferred P1A peptide-pulsed splenocytes that had been strongly labelled with CFSE (CFSE^{high}) as well as the control peptide-pulsed splenocytes that had been weakly labelled with CFSE (CFSE^{low}) into the recipient mice that had been vaccinated with EXO$_{HSP}$, EXO$_{HS}$ and EXOneo, respectively. We then assessed loss of P1A-specific CFSE^{high} target cells in the recipient mice, which represent the killing activity of P1A-specific effector CD8$^+$ CTLs in the recipient mice. As shown in Fig. 4(D), little CFSE^{high} target cells loss (2%) was observed in mice immunized with PBS. As expected, there was a substantial loss (24% and 48%) of CFSE^{high} cells in mice immunized with EXOneo and EXO$_{HS}$, indicating that EXO$_{HS}$ and EXOneo can stimulate CD8$^+$ T-cell differentiation into CTL effectors *in vivo*. However, there was a more substantial loss (77%) of CFSE^{high} cells in mice immunized with EXO$_{HSP}$ ($P < 0.05$), indicating that EXO$_{HSP}$ stimulate stronger CD8$^+$ T-cell differentiation into CTL effectors than EXO$_{HS}$ and EXOneo, respectively. To confirm that loss of CFSE^{high} cells in mice immunized with EXO$_{HSP}$ is due to P1A-specific effector CD8$^+$ CTLs in the recipient mice, we depleted CD8$^+$ T cells using neutralizing anti-CD8 antibody treatment before adoptive transfer of P1A peptide-pulsed splenocytes. As shown in Fig. 4(E), little CFSE^{high} target cells loss (4%) was seen in EXO$_{HSP}$-immunized mice with CD8$^+$ T-cell depletion, confirming that EXO$_{HSP}$ efficiently stimulates P1A-specific effector CD8$^+$ CTL responses.

EXO$_{HSP}$ induce stronger antitumour immunity than EXO$_{HS}$

To investigate the antitumour immunity derived from EXO$_{HSP}$ vaccination, BALB/c mice were s.c. immunized with EXO$_{HSP}$, EXOneo, EXO$_{HS}$ and PBS, respectively. 6 days after the immunization, the immunized mice were s.c. challenged with J558 tumour cells. As shown in Fig. 5(A), all the mice injected with PBS died of tumour within 14 days after tumour cell challenge. EXO$_{HS}$ and EXOneo vaccine protected four of eight (50%) and two of eight (25%) mice from tumour growth, respectively, and the rest of four or six tumour-bearing mice had significantly delayed tumour growth compared to the control group of mice treated with PBS ($P < 0.05$). However, EXO$_{HSP}$ immunization protected all eight of eight (100%) mice from tumour growth, indicating that EXO$_{HSP}$ expressing membrane-bound HSP70 can also induce more efficient antitumour immunity than EXO$_{HS}$ expressing cytoplasmic HSP70 and the control EXOneo without HSP70 expression.

The EXO$_{HSP}$-induced antitumour immunity is mediated by CD8$^+$ CTL and NK cells

Since CD8$^+$ T cells have been defined as effector T cells with cytotoxic activity to tumour or virally infected cells [44] and the membrane-bound HSP70 molecules are identified as a target structure for cytolytic attack mediated by NK cells [45], we assessed whether CD8$^+$ T and NK cells are involved in EXO$_{HSP}$-induced antitumour immunity. We first performed a kinetic study on CD8$^+$ CTL and NK cell killing activity derived from EXO$_{HSP}$ vaccination. The killing activity of NK derived from naïve BALB/c mice was 8% (data not shown). As shown in Fig. 5(B) and (C), P1A-specific CD8$^+$ CTL responses (0.64%) and non-P1A-specific NK killing activity (38%) was peaked at day 6 and day 2 after vaccination. At day 6 after immunization, NK killing activity was still 20%, indicating that both CD8$^+$ CTL and NK cells may be involved in the antitumour immunity seen at day 6 after EXO$_{HSP}$ vaccination (Fig. 5A). We then treated BALB/c mice with either anti-CD8 or anti-NK Ab, immunized the mice with EXOneo or EXO$_{HS}$ or EXO$_{HSP}$ and then challenged the immunized mice with J558 tumour cells. We found that the EXOneo-, EXO$_{HS}$- and EXO$_{HSP}$-immunized mice with treatment of anti-CD8 Ab completely lost the protective antitumour immunity, since all (eight of eight) immunized mice died of tumours within these three mouse groups with treatment of anti-CD8 Ab (Fig. 5D, E and F), indicating that EXOneo-, EXO$_{HS}$- and EXO$_{HSP}$-induced antitumour immunities are mainly mediated by CD8$^+$ T cells. When mice were treated with anti-NK Ab, 25% (two of eight) of EXOneo-immunized mice were protected against tumour cell challenge (Fig. 5D), indicating that NK cell responses are not involved in EXOneo-induced antitumour immunity. However, 38% (three of eight) of EXO$_{HS}$-immunized mice were tumour free after treatment of anti-NK Ab, compared to six of eight (76%) immune protection in EXO$_{HSP}$-immunized mice with anti-NK Ab treatment (Fig. 5E and F), indicating that (*i*) NK cell responses are involved in both EXO$_{HS}$- and EXO$_{HSP}$-induced antitumour immunity and (*ii*) EXO$_{HSP}$-vaccination stimulates a stronger NK activity than EXO$_{HS}$-vaccination.

Discussion

In recent years, EXO research has attracted more attention by the finding that human and mouse tumour cells constitutively release membrane vesicles, similar to DC-derived EXO in their morphology, density and expression of certain membrane markers [10]. To enhance immunogenicity of tumour cell-released EXO, tumour cells have been engineered to express various immunologically related genes for use in EXO production. For example, Xiu *et al.* genetically engineered tumour cells to express superantigen Staphylococcal enterotoxin A [46]. Chen *et al.* generated IL-2-expressing EXO released from transgene IL-2-engineered tumour cells [47]. They demonstrated that all these modified EXO were able to stimulate more efficient T-cell responses and antitumour immunity. Furthermore, EXO derived from IL-18 transgene engineered human tumour cells expressing carcinoembryonic Ag were able to more efficiently stimulate carcinoembryonic Ag specific CTL responses [36]. In addition, EXO derived from heat-shocked tumour cells displayed enhanced expression of HSP by Western

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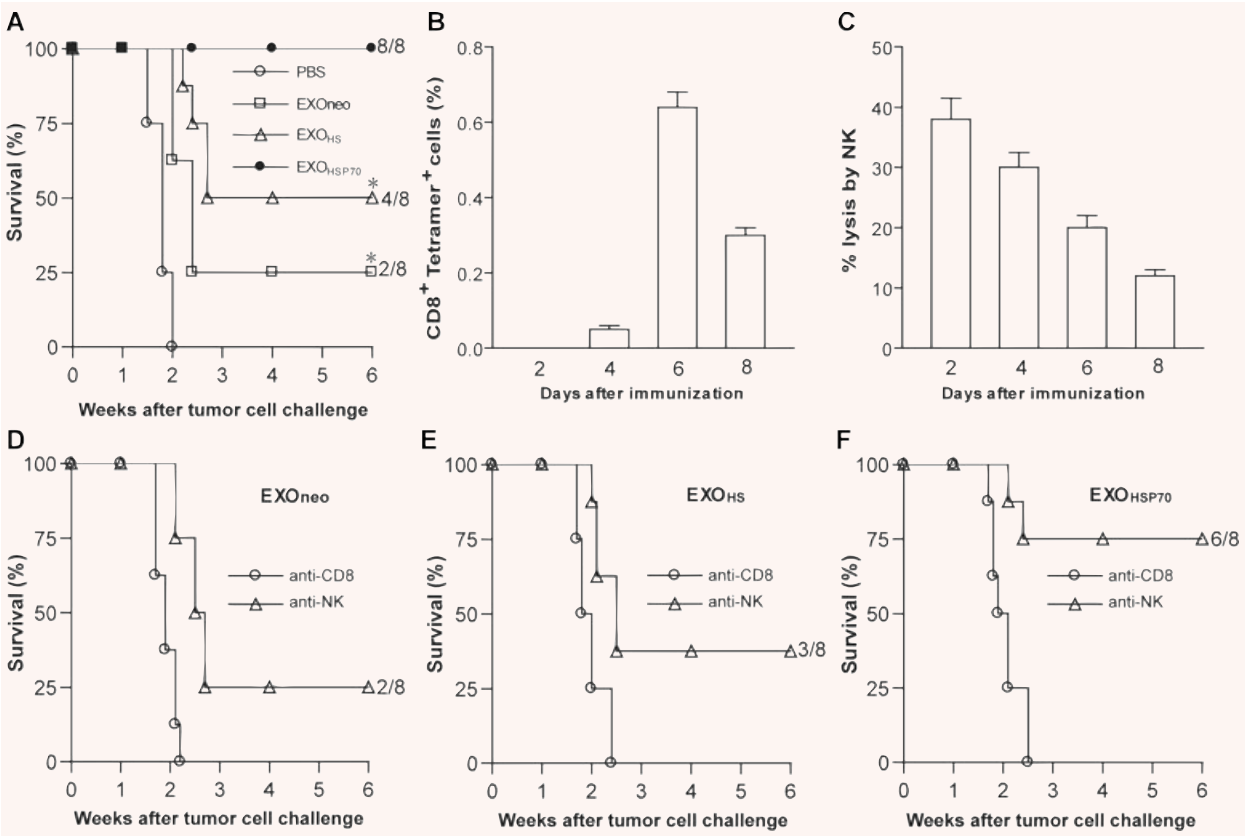

Fig. 5 Animal studies. (**A**) BALB/c mice were s.c. vaccinated with EXOneo, EXO$_{HS}$ and EXO$_{HSP}$ or PBS. 6 days after immunization, the immunized mice were s.c. inoculated with J558 tumour cells. Animal mortality and tumour growth were monitored daily for up to 60 days. *, $P < 0.05$ *versus* cohorts in EXO$_{HSP}$ group (log-rank test). (**B**) Kinetic tetramer staining assay. 2 to 8 days after immunization of mice with EXO$_{HSP}$, the tail blood samples were taken from the immunized mice, stained with PE-H-2L^d/P1A peptide tetramer and FITC anti-CD8 Ab and analysed by flow cytometry. The value in each panel represents the percentage of tetramer$^+$ CD8$^+$ T cells *versus* the total blood CD8$^+$ T-cell population. (**C**) Kinetic study of NK activity. 2 to 8 days after immunization of mice with EXO$_{HSP}$, the mouse spleen T cells were tested for NK killing activity to J558 tumour cells. BALB/c mice with treatment of anti-CD8 or anti-NK Ab were s.c. vaccinated with either (**D**) EXOneo or (**E**) EXO$_{HS}$ (**F**) EXO$_{HSP}$. 6 days after immunization, the immunized mice were s.c. inoculated with J558 tumour cells. Animal mortality and tumour growth were monitored daily for up to 6 weeks. One representative experiment of three is shown.

blotting analysis induced DC maturation and more efficient CD8$^+$ CTL responses than EXO derived from untreated tumour cells [22, 48]. However, whether these enhanced HSP are cytoplasmic soluble ones or membrane-bound cellular surface ones is unclear. It has been reported that DCs secrete pro-inflammatory cytokines in responses to soluble HSP protein through a CD14-dependent signalling pathway [23, 49], whereas membrane-bound HSP70 molecules are identified as a target structure for cytolytic attack mediated by NK cells [45]. In this study, we generated HSP70-expressing EXO$_{HSP}$ derived from engineered J558$_{HSP}$ tumour cell line expressing membrane-bound transgene HSP70 and assessed the efficiency of tumour-specific CD8$^+$ CTL responses induced by EXO$_{HSP}$. For the first time, we demonstrated that EXO$_{HSP}$ expressing membrane-bound HSP70 were able to stimulate tumour-specific CD4$^+$ Th1 cell responses possibly due to IL-12 secretion of EXO$_{HSP}$-activated mature DCs. In addition, we also demonstrated that EXO$_{HSP}$ expressing membrane-bound HSP70 were able to stimulate more efficient tumour-specific CD8$^+$ CTL responses and antitumour immunity than EXO$_{HS}$ expressing cytoplasmic HSP70 and EXOneo derived from the control J558neo tumour cells without HSP70 expression. Furthermore, we elucidated that EXO$_{HSP}$-induced antitumour immunity is mediated by both CD8$^+$ T and NK cells, indicating that EXO$_{HSP}$ vaccination can not only stimulate the P1A tumour Ag-specific T cell, but also the non-P1A-specific NK cell responses.

It has been previously reported that EXO may need the host DCs as an adjuvant for induction of immune responses based only upon *in vitro* experiments [12, 50]. We have recently demonstrated

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that DC- or tumour cell-derived immunogenic EXO exhibiting efficient T-cell responses completely lost their stimulatory effects in induction of $CD8^+$ T responses in diphtheria toxin (DT)-treated DT receptor transgenic mice with the host DC deficiency [13], indicating that EXO need the host DCs for delivery of their stimulatory effect to $CD8^+$ CTL responses *in vivo*. In this study, we demonstrated that EXO_{HSP} expressing membrane-bound HSP70 were able to stimulate maturation of DCs with up-regulation of Ia^b, costimulatory molecules (CD40 and CD80) and inflammatory cytokines (IL-1β, TNF-α, IFN-γ and IL-12). Therefore, it is conceivable that the enhanced $CD8^+$ CTL responses and antitumour immunity induced by EXO_{HSP} may be derived from the adjuvant effect of membrane-bound expression of HSP70. This is because HSP70 can trigger a danger signal by binding to DC receptors such as Toll-like receptor 4 [49, 51] and CD14 [23] through NF-κB to up-regulate costimulatory molecules of DCs and induce DC maturation and secretion of pro-inflammatory cytokines [52] leading to stimulation of both $CD8^+$ CTL and NK cell responses. Thus, the dual role of HSP70 as antigenic peptide chaperone [53] and danger signal [52] thus makes them especially critical in EXO-based antitumour vaccine.

Taken together, our data showed that EXO derived from tumour cells engineered to express membrane-bound HSP70 can induce DC maturation and stimulate $CD4^+$ Th1, $CD8^+$ CTL and NK cell responses leading to more efficient antitumour immunity. Therefore, membrane-bound HSP70-expressing EXO may represent a more effective EXO-based vaccine in induction of antitumour immunity.

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