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RESEARCH ARTICLE

Tumor vaccine based on extracellular vesicles derived from γδ-T cells exerts dual antitumor activities

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Abstract

γδ-T cells are innate-like T cells with dual antitumor activities. They can directly eradicate tumor cells and function as immunostimulatory cells to promote antitumor immunity. Previous studies have demonstrated that small extracellular vesicles (EVs) derived from γδ-T cells (γδ-T-EVs) inherited the dual antitumor activities from their parental cells. However, it remains unknown whether γδ-T-EVs can be designed as tumors vaccine to improve therapeutic efficacy. Here, we found that γδ-T-EVs had immune adjuvant effects on antigen-presenting cells, as revealed by enhanced expression of antigen-presenting and co-stimulatory molecules, secretion of pro-inflammatory cytokines and antigen-presenting ability of DCs after γδ-T-EVs treatment. The γδ-T-EVs-based vaccine was designed by loading tumor-associated antigens (TAAs) into γδ-T-EVs. Compared with γδ-T-EVs, the γδ-T-EVs-based vaccine effectively promoted more tumor-specific T-cell responses. In addition, the vaccine regimen preserved direct antitumor effects and induced tumor cell apoptosis. Interestingly, the allogeneic γδ-T-EVs-based vaccine showed comparable preventive and therapeutic antitumor effects to their autologous counterparts, indicating a better way of centralization and standardization in clinical practice. Furthermore, the allogeneic γδ-T-EVs-based vaccine displayed advantages over the DC-EVs-based vaccine through their dual antitumor activities. This study provides a proof-of-concept for using the allogeneic γδ-T-EVs-based vaccine in cancer control.

KEYWORDS
extracellular vesicle, immunotherapy, tumor, vaccine, γδ-T cells

1 | INTRODUCTION

Tumor vaccine plays a critical role in cancer therapy because they can amplify antigen-specific antitumor responses (Hollingsworth & Jansen, 2019). To date, dendritic cells (DCs)-based tumor vaccine has been widely investigated. However, their clinical application is impeded due to the high cost and difficulty in preparing personalized DCs (Smith & Khanna, 2015). In addition, the antitumor efficacies of cell-based vaccines can be attenuated by immunosuppressive tumor microenvironment (Pitt et al., 2016). Recently, cell-free nanoparticles (NPs)-based vaccine has attracted great attention in cancer therapy (Smith et al., 2015). NPs can protect vaccine components from degradation to improve stability, prolong bioavailability and enhance antigen uptake by antigen-presenting cell (APC). NPs size influences the uptake and specificity of NPs-based vaccines. For example,

smaller NPs (20–200 nm) can be directly drained into lymph nodes and readily taken by resident DCs (Manolova et al., 2008). Furthermore, smaller NPs are more capable of promoting APC functions and inducing stronger antigen-specific immunity than larger NPs (Joshi et al., 2013). Therefore, small NPs have great potential in vaccine development.

Small extracellular vesicles (EVs; 20–200 nm), such as endosome-derived exosomes, can shuttle lipids, proteins, and nucleic acids in intercellular communication, thus influencing the tumorigenesis and therapeutic outcomes of tumors (Viaud et al., 2010; Wang et al., 2021). EVs can be used to develop tumor vaccines (Tan et al., 2010). So far, tumor-derived EVs (TEVs) and DCs-derived EVs (DC-EVs) have been mainly investigated (Pitt et al., 2016; Viaud et al., 2010; Naseri et al., 2020). TEVs are rich reservoirs of tumor-associated antigens (TAAs) and can function as tumor vaccines after being loaded with immune adjuvants (Xia et al., 2022). Administration of TEVs-based vaccine induced broad antitumor immune responses and showed higher efficacy than tumor lysates (Naseri et al., 2020). However, TEVs are also found to promote tumor development and metastasis, which could limit the clinical application of TEVs-based vaccines due to safety concerns (Azmi et al., 2013; Sun et al., 2021; Zhang & Yu, 2019). Compared to TEVs, immune cells-derived EVs have a better safety profile because of their natural immunosurveillance property (Veerman et al., 2019). DC-EVs carried potent antigen-presenting and co-stimulatory molecules. After being loaded with immunogens, the DC-EVs-based vaccine can directly induce antigen-specific immune responses or be taken by recipient APCs to promote antitumor immunity indirectly (Viaud et al., 2010). However, the therapeutic efficacy of DC-EVs-based tumor vaccine has been unsatisfactory in clinical trials so far (Xia et al., 2022). The heterogeneity of ex vivo expanded DCs may partially account for the poor therapeutic outcomes, as EVs derived from immature DCs (iDCs) have immune-tolerant activities (Morse et al., 2005). In addition, the difficulties in large-scale ex vivo expansion of human DCs may also limit the preparation of DC-EVs for clinical use. Therefore, it is worth exploring other novel and more accessible EVs-based vaccines.

Human $\gamma\delta$-T cell is a minor T cell population that can act as innate-like T cells with direct antitumor activities against many different types of tumors (Mu et al., 2022; Silva-Santos & Mensurado, 2019; Xiang & Tu, 2017). $\gamma\delta$-T cells also function as antigen-presenting cells (APCs) to induce antigen-specific T-cell responses (Brandes et al., 2009). More interestingly, $\gamma\delta$-T cells have immune adjuvant effects to induce APC functions and synergize CD4 T follicular helper cells to indirectly promote specific cellular and humoral immune responses (Chen et al., 2018; Petrasca & Doherty, 2014). Recently, we found that $\gamma\delta$-T cells derived EVs ($\gamma\delta$-T-EVs) displayed dual antitumor activities by carrying cytotoxic and immunostimulatory molecules, which could expand the pre-existing tumor-specific T cells (Wang et al., 2020, 2022). However, whether $\gamma\delta$-T-EVs can be designed as a tumor vaccine to further amplify antitumor immunity remains unknown.

In the present study, we found that $\gamma\delta$-T-EVs had immune adjuvant effects on APCs, which was mediated by the IFN-γ carried on $\gamma\delta$-T-EVs. Using in vitro, immunodeficient, and humanized mouse models of Epstein-Barr virus (EBV)-associated tumors, we further investigated the efficacies of the $\gamma\delta$-T-EVs-based vaccine developed by loading with TAAs. We found that the vaccine regimen induced more tumor-specific T-cell responses and preserved the direct killing activity of $\gamma\delta$-T-EVs against tumor cells. Interestingly, the allogeneic $\gamma\delta$-T-EVs-based vaccine was comparable with its autologous counterpart in the induction of tumor-specific T-cell responses and tumor control. Moreover, the allogeneic $\gamma\delta$-T-EVs-based vaccine showed more advantages over the DC-EVs-based vaccine by exerting dual antitumor activities. Our study provides a robust preclinical proof of principle for a novel and potent strategy using the allogeneic $\gamma\delta$-T-EVs-based vaccine in cancer therapy.

2 | RESULTS

2.1 | $\gamma\delta$-T-EVs-based vaccine efficiently induces tumor-specific T cells

Similar to our previous studies, EVs were isolated from different donor-derived $\gamma\delta$-T cells through differential ultracentrifugation (Wang et al., 2020, 2022). Generally, 4.1 ± 0.3 μg $\gamma\delta$-T-EVs proteins could be obtained from 10^6 cells ($N = 4$). To prepare the vaccine regimen, $\gamma\delta$-T-EVs indirectly loaded with TAAs ($\gamma\delta$-T-EVs (TAAs)) were isolated from the conditioned medium of TAAs-pulsed $\gamma\delta$-T cells (Bu et al., 2015; Damo et al., 2015). Characterization analysis revealed that the antigen-loading procedures did not change the size distribution and expression of EVs markers on $\gamma\delta$-T-EVs (Figure 1a,b). In addition, the $\gamma\delta$-T-EVs (TAAs) contained tumor antigens, including EBV antigens such as Epstein–Barr nuclear antigen 1 (EBNA1) and latent membrane protein 2a (LMP2a) (Figure 1c), which are critical therapeutic targets of EBV-associated tumors (Cui & Snapper, 2021). These data indicate that TAAs can be loaded on $\gamma\delta$-T-EVs using an indirect-antigen loading strategy.

The efficacy of $\gamma\delta$-T-EVs-based vaccine in the induction of tumor-specific T cells was further examined. Human peripheral blood mononuclear cells (huPBMCs) were treated with the vaccine regimens. After seven days, the cells were restimulated with tumor peptides or irrelevant HIV p17 peptide pools. The results showed that $\gamma\delta$-T-EVs (TAAs) induced more EBNA1- and LMP2a-specific CD4 and CD8 T cells compared with $\gamma\delta$-T-EVs, TAAs or $\gamma\delta$-T-EVs + free TAAs. Furthermore, the cells treated with $\gamma\delta$-T-EVs-based vaccine did not respond to irrelevant HIV p17 peptides, confirming the antigen specificity of these T-cell responses (Figure 1d–g). Taken together, these data indicate that $\gamma\delta$-T-EVs can be designed as tumor vaccines to amplify tumor-specific T-cell immunity.

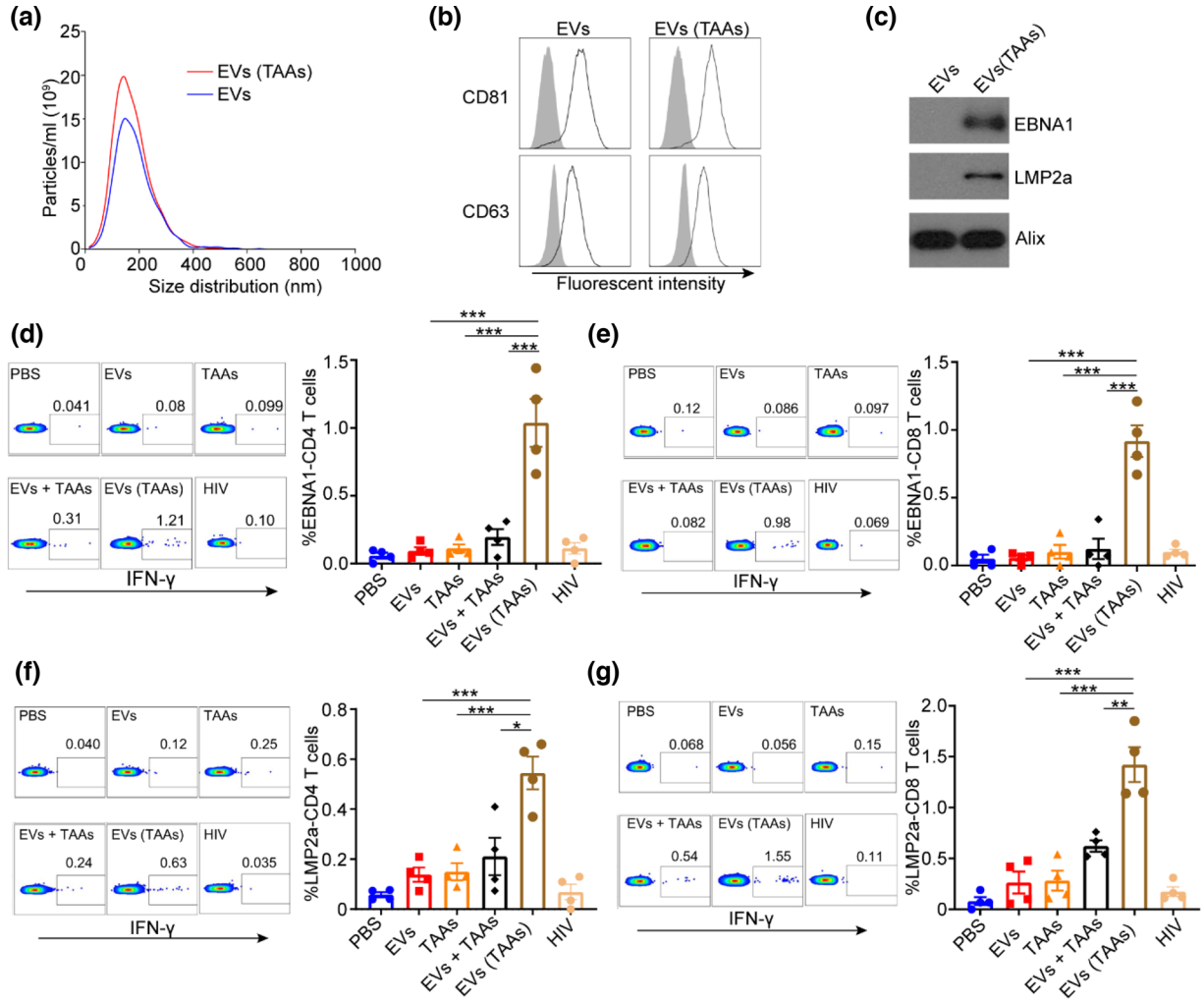

FIGURE 1 γδ-T-EVs-based vaccine efficiently induces tumor-specific T-cell responses. (a) Size distributions; (b) Expression of EVs markers CD63 and CD81, Gray histograms represent isotype control; (c) Expression of Alix, EBNA1, and LMP2a proteins in γδ-T-EVs and γδ-T-EVs (TAAs). (d–g) Equivalent amounts of PBS, TAAs, γδ-T-EVs, γδ-T-EVs + free TAAs, or γδ-T-EVs (TAAs) were used to treat huPBMCs. After 7 days, the cells were restimulated with peptide pools and subjected to detecting intracellular IFN-γ in T cells. (D,E) Induction of EBNA1-specific CD4 and CD8 T cells, (F,G) induction of LMP2a-specific CD4 and CD8 T cells by γδ-T-EVs (TAAs) and corresponding controls. Representative data were shown as mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. EVs: γδ-T-EVs; HIV: huPBMCs pretreated with γδ-T-EVs (TAAs) were restimulated with HIV p17 peptide pool.

2.2 | γδ-T-EVs-based vaccine displays immune adjuvant effects on antigen-presenting cells

To determine whether recipient APCs could influence the efficacy of γδ-T-EVs-based vaccine, we used anti-HLA-DR microbeads to deplete the APCs in huPBMCs as previously described (Walker et al., 2009). As shown in Figure 2a, HLA-DR^{+} cells-depleted huPBMCs had much fewer antigen-specific T cells induced by γδ-T-EVs-based vaccine than complete huPBMCs, suggesting that the efficacy of γδ-T-EVs-based vaccine depended on recipient APCs. Thus, we hypothesized that γδ-T-EVs-based vaccine could mobilize APCs to induce antigen-specific T-cell responses.

To confirm this hypothesis, we treated purified CD3^{+} T cells with the preparations in the absence or presence of immature DCs (iDCs). γδ-T-EVs (TAAs) cocultured with iDCs increased the efficacy of γδ-T-EVs (TAAs) to induce tumor-specific T-cell responses (Figure 2b). Although γδ-T-EVs + free TAAs also showed a trend in the augment of tumor-specific T cells in the presence of iDCs, its efficacy was significantly lower than γδ-T-EVs preloaded with TAAs (γδ-T-EVs (TAAs)) (Figure 2b). Hence, co-delivery of antigen and γδ-T-EVs to recipient iDCs is important to promote tumor-specific T-cell responses. Taken together, these findings demonstrate that γδ-T-EVs-based vaccine can induce tumor-specific T cells through APCs.

To elucidate the effects of γδ-T-EVs on APCs, iDCs were cultured with TAAs, γδ-T-EVs, or γδ-T-EVs (TAAs) for 48 h. Compared with TAAs, both of γδ-T-EVs and γδ-T-EVs (TAAs) promoted the expression of APC functional molecules, including MHC-II, CD83, CD86 and CD40 (Figure 2c). In addition, the secretion of pro-inflammatory cytokine IL-6 and TNF-α from

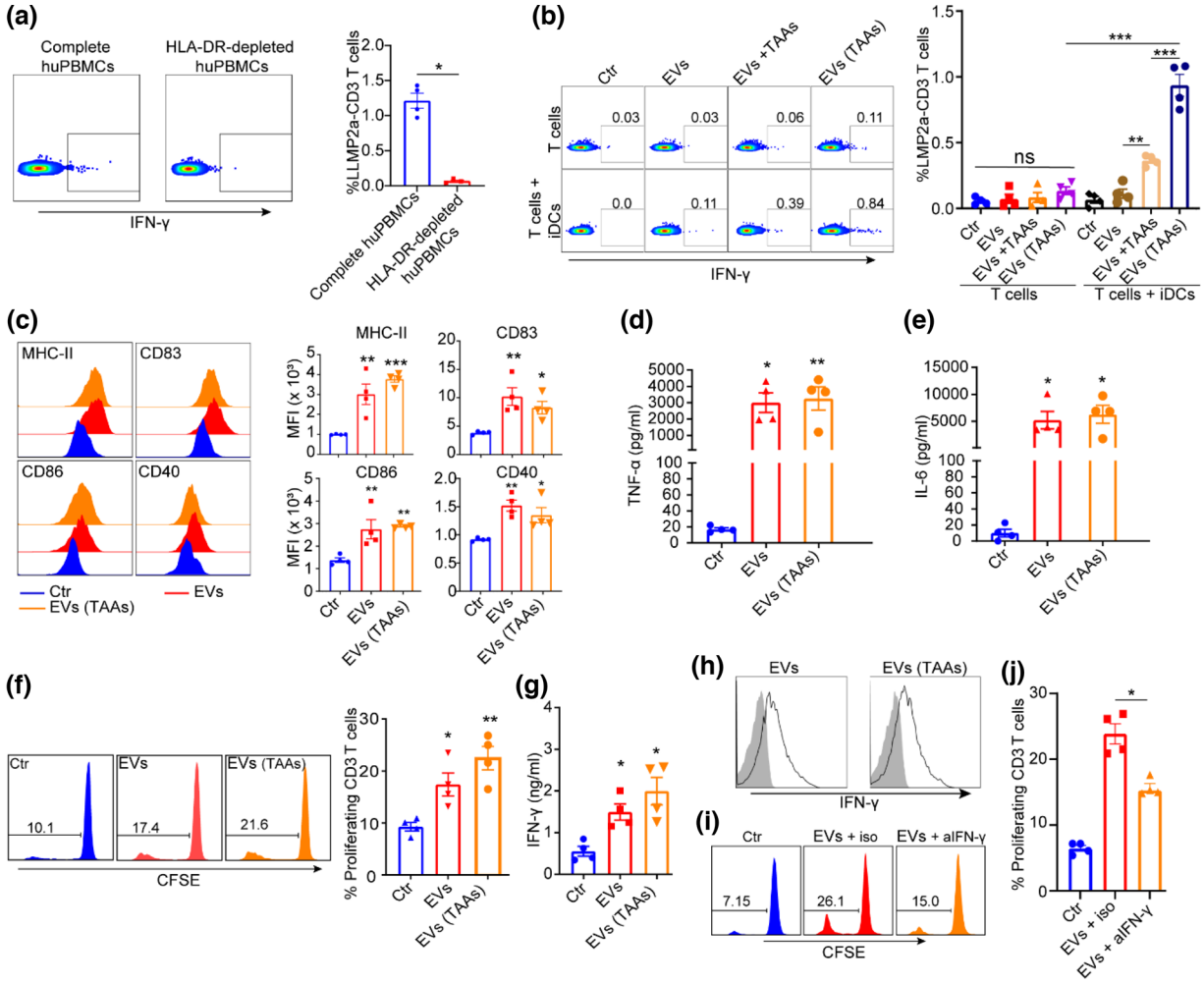

FIGURE 2 γδ-T-EVs-based vaccine displays immune adjuvant effects on antigen-presenting cells. (a) Completed huPBMCs or HLA-DR$^+$ cell-depleted huPBMCs were treated with γδ-T-EVs (TAAs) for seven days, then LMP2a-specific T cells were detected. (b) Purified CD3 T cells were treated with γδ-T-EVs or γδ-T-EVs (TAAs) in the absence or presence of iDCs. TAAs treatment was also used as a control (Ctr). Seven days later, LMP2a-specific T cells were detected. (c) iDCs were treated with γδ-T-EVs or γδ-T-EVs (TAAs) for 48 h. TAAs was used as control (Ctr). The APC functional makers (MHC-II, CD83, CD86, and CD40) were detected by flow cytometry. (d-e) Secretion of TNF-α and IL-6 from iDCs after 48 h treatment with γδ-T-EVs or γδ-T-EVs (TAAs). (f) iDCs were treated with γδ-T-EVs, or γδ-T-EVs (TAAs) for 48 h, then cocultured with CFSE-stained allogeneic CD3 T cells. Seven days later, proliferating T cells were determined by CFSE dilution using flow cytometry. (g) Secretion of IFN-γ in the coculture system of pretreated iDCs with allogeneic CD3 T cells. (h) Expression of IFN-γ on γδ-T-EVs or γδ-T-EVs (TAAs). (I-J) iDCs were treated with γδ-T-EVs, or γδ-T-EVs (TAAs), in the presence of neutralizing anti-IFN-γ antibody or isotype control. 48 h later, the pretreated iDCs were cocultured with CFSE-stained allogeneic CD3 T cells. After seven days, proliferating T cells were determined. Representative data were shown as mean ± SEM from three independent experiments. MFI: median fluorescence intensity. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

iDCs were significantly increased after being treated with γδ-T-EVs or γδ-T-EVs (TAAs) (Figure 2d,e). Moreover, iDCs pretreated with γδ-T-EVs or γδ-T-EVs (TAAs) had higher antigen-presenting abilities, as revealed by promoting more proliferation (Figure 2f) and IFN-γ secretion (Figure 2g) of allogenic CD3$^+$ T cell in a mixed lymphocyte reaction (MLR) assay. In addition, we found that γδ-T-EVs-based vaccines also activated B cells to increase their antigen presenting function in terms of enhancing MHC-II and CD83 expressions on B cells (Figure S1A,B), and effectively induce antigen-specific T cells in the presence of these B cells in vitro (Figure S1C). These data suggested that γδ-T-EVs could promote APC functions of human B cells. In addition, the effects of γδ-T-EVs (TAAs) on iDCs were comparable to γδ-T-EVs (Figure 2c–g), suggesting that the antigen loading procedures did not hamper the adjuvant effects of γδ-T-EVs (TAAs).

The underlying mechanism of γδ-T-EVs-based vaccine induced adjuvant effects on DCs was further determined. Similar to their parental cells, γδ-T-EVs expressed robust IFN-γ (Figure 2h), which is a well-known cytokine to promote APC functions (Castiello et al., 2011). The blockage of IFN-γ using neutralizing antibody inhibited the antigen-presenting abilities of iDCs enhanced by γδ-T-EVs (Figure 2i and j), indicating that the carried IFN-γ mediated the immune adjuvant effects of γδ-T-EVs. In addition, CD40L expression was also identified on γδ-T-EVs (Figure S2A), and blockage of CD40L by using anti-CD40L blocking antibody could significantly reduce the adjuvant effect of γδ-T-EVs on promoting CD3 T cell proliferation (Figure S2B).

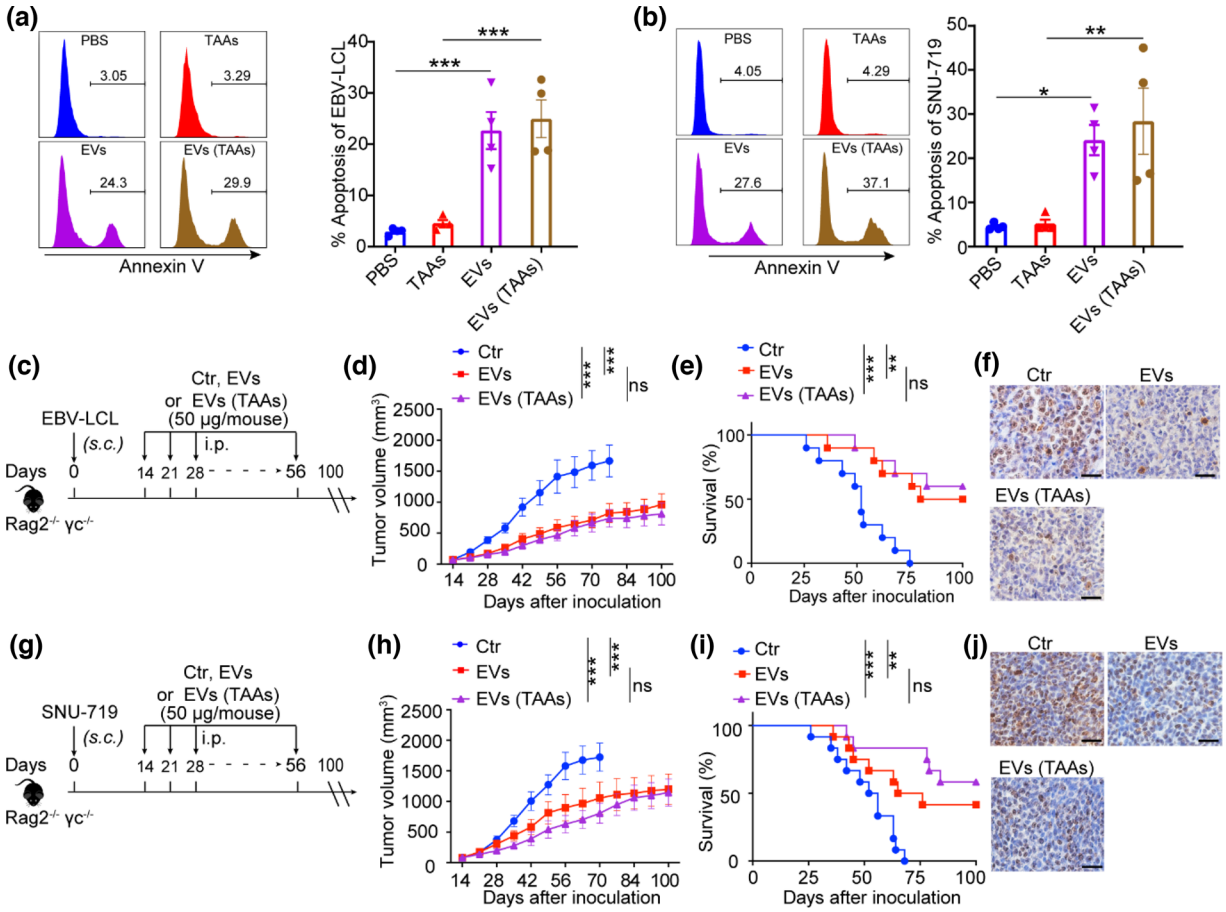

FIGURE 3 γδ-T-EVs-based vaccine preserves direct antitumor activities both in vitro and in vivo. (a) Apoptosis of EBV-LCL and (b) SNU-719 cells were determined after cultured with γδ-T-EVs or γδ-T-EVs (TAAs) for 18–24 h. An equivalent amount of PBS or TAAs was used as a control. (c) EBV-LCL cells were injected *s.c.* in Rag2$^{-/-}$γc$^{-/-}$ mice. After 14 days, mice with subcutaneous tumors were treated with γδ-T-EVs, or γδ-T-EVs (TAAs) at indicated (n = 10). TAAs was used as control (Ctr). The tumor volume (d) and mice survival (e) were determined at the indicated time. (f) Histochemical analysis of human Ki-67 in tumor tissues at the endpoints, scale bar = 25 μm. (g) Mice with subcutaneous SNU-719 tumors were treated with γδ-T-EVs, or γδ-T-EVs (TAAs) as indicated ($n = 12$). TAAs was used as control (Ctr). The tumor volume (h) and mice survival (i) were determined at the indicated time. (j) Histochemical analysis of human Ki-67 in tumor tissues at the endpoints, scale bar = 25 μm. For the bar graphs, representative data were shown as mean ± SEM from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns: not significant.

Therefore, both IFN-γ and CD40L carried on γδ-T-EVs mediated their immune adjuvant effects. Taken together, these results demonstrate that γδ-T-EVs-based vaccine can promote tumor-specific T-cell responses by delivering antigens and serving as an immune adjuvant on APCs.

2.3 | γδ-T-EVs-based vaccine preserves direct antitumor activities in vitro and in vivo

Our previous studies identified that γδ-T-EVs selectively induced tumor cell apoptosis through Fas/FasL or DR5/TRAIL pathways (Wang et al., 2020). We then determined whether the γδ-T-EVs-based vaccine inherited this direct antitumor property. As shown in Figure 3a and b, both γδ-T-EVs and γδ-T-EVs (TAAs) induced more tumor cell apoptosis compared with TAAs. No significant differences were found between γδ-T-EVs or γδ-T-EVs (TAAs) (Figure 3a,b). In addition, inhibitory effects of γδ-T-EVs or γδ-T-EVs (TAAs) on tumor cell proliferation were also identified (Figure S3)

To determine the direct antitumor effects of γδ-T-EVs-based vaccine in vivo, EBV-induced B cell lymphoma-bearing Rag2$^{-/-}$γc$^{-/-}$ mice were treated with γδ-T-EVs, or γδ-T-EVs (TAAs) (Figure 3c). Mice treated with γδ-T-EVs or γδ-T-EVs (TAAs) effectively inhibited tumor growth and prolonged mice survival when compared with TAAs. No significant differences in their antitumor effects were observed between γδ-T-EVs or γδ-T-EVs (TAAs) (Figure 3d,e). The tumor cells in mice treated with γδ-T-EVs or γδ-T-EVs (TAAs) were found with lower proliferative capability because immunophenotypic analysis found fewer Ki-67 positive cells in residual tumors (Figure 3f).

Another tumor model based on EBV-associated gastric carcinoma cells was also used to validate the direct antitumor activities of γδ-T-EVs-based vaccine in vivo (Figure 3g). Similarly, smaller tumor volume and longer survival of tumor-bearing mice were found after being treated with γδ-T-EVs or γδ-T-EVs (TAAs) than TAAs. Consistently, no significant differences were found between γδ-T-EVs or γδ-T-EVs (TAAs) (Figure 3h,i). The tumor cells in mice treated with γδ-T-EVs or γδ-T-EVs (TAAs) were also found with lower proliferative capability, as fewer Ki-67 positive cells were found in residual tumors by histological analysis (Figure 3j). Taken together, our data demonstrate that γδ-T-EVs-based vaccine preserves their direct antitumor activities both in vitro and in vivo.

2.4 | γδ-T-EVs-based vaccine inhibits tumor progression in humanized mice

Humanized mice harboring functional human immune cells (e.g., B cells, T cells and antigen-presenting cells) are especially appropriate for preclinical modeling of vaccine therapy (Akkina, 2013). Previously, we established protocols to generate humanized mice with stable reconstitution of functional huPBMCs (Tu et al., 2011; Wang et al., 2020; Xiang et al., 2014). To determine the therapeutic efficacy of γδ-T-EVs-based vaccine in humanized mice, tumor models were built as we described before through *s.c.* inoculation of autologous EBV-LCL in the humanized mice (Wang et al., 2020; Xiang et al., 2014). Mice that had developed subcutaneous tumors were treated with γδ-T-EVs or γδ-T-EVs (TAAs) as indicated (Figure 4a). TAAs was used as control. γδ-T-EVs or γδ-T-EVs (TAAs) inhibited tumor growth and prolonged mice survival compared with control group (Figure 4b,c). The tumor cells in mice treated with γδ-T-EVs or γδ-T-EVs-based (TAAs) were also found with lower proliferative capability, as fewer Ki-67 positive cells were identified in corresponding residual tumors by histological analysis (Figure 4d). Interestingly, different from their comparable therapeutic efficacies in immunodeficient mice (Figure 3c–j), γδ-T-EVs (TAAs) had higher therapeutic efficacy than γδ-T-EVs in humanized mice (Figure 4b–d). Similar to those obtained from in vitro experiments, γδ-T-EVs and γδ-T-EVs-based (TAAs) also enhanced the expressions of MHC-II and CD83 on human B cells (Figure S4A,B) and induced the secretion of pro-inflammatory cytokines such as IL-12, IL-6 and TNF-α in EBV-induced B cell lymphoma-bearing humanized mice (Figure S4C–E). In contrast, γδ-T-EVs (TAAs) induced more tumor-specific T cells than γδ-T-EVs or TAAs (Figure 4e,f) in vivo. These data suggest that γδ-T-EVs-based vaccines can induce antigen-specific immune responses by mobilizing different APC populations, such as DCs and B cells. Since human B cells, rather than human DCs, were reconstituted stably in our humanized mouse model (Tu et al., 2011), the induction of tumor-specific T cells by γδ-T-EVs (TAAs) in this model might probably be mediated by human B cells with increased APC functions.

The superior therapeutic efficacy of γδ-T-EVs (TAAs) in humanized mice was likely attributed to the antitumor immune responses induced by γδ-T-EVs (TAAs), especially T-cell responses, because the only difference between humanized mice and immunodeficient mice was that humanized mice had been reconstituted with functional human immune cells. Therefore, a cytotoxicity assay was further used to determine the killing effect of tumor-specific T cells induced by γδ-T-EVs-based vaccine. Data showed that the γδ-T-EVs (TAAs)-induced T cells killed more autologous EBV-LCL cells, compared with the γδ-T-EVs-induced T cells (Figure 4g). In addition, preloading of tumor peptides on autologous EBV-LCL resulted in higher cytotoxic effects of γδ-T-EVs (TAAs)-induced T cells than those EBV-LCL preloaded with irrelevant HIV p17 peptide (Figure 4h). Taken together, these data indicate that γδ-T-EVs-based vaccine can induce antigen-specific T cells to eradicate tumor cells.

2.5 | Allogeneic γδ-T-EVs-based vaccine mobilizes recipient APCs to induce tumor-specific T-cell responses

Since many cancer patients are immunocompromised either because of cancer or anti-cancer therapy, it is challenging to obtain enough patient γδ-T cells and expand them *ex vivo* to prepare autologous γδ-T-EVs in clinical practice. In addition, collecting γδ-T cells and preparing γδ-T-EVs from allogeneic healthy donors are much easier and have advantages in standardization and centralization. Therefore, we further determined the antitumor potentials of allogeneic γδ-T-EVs-based vaccine. As shown in Figure 5a and b, autologous and allogeneic γδ-T-EVs or γδ-T-EVs (TAAs) promoted APC functions, as revealed by upregulation of MHC-II and CD83 compared with TAAs treatment. The secretion of pro-inflammatory cytokines (Figure 5c,d) and antigen-presenting abilities of iDCs (Figure 5e) were also comparable after being treated with autologous versus allogeneic γδ-T-EVs or γδ-T-EVs-based vaccine. These data demonstrate that allogeneic γδ-T-EVs-based vaccine has similar immune adjuvant effects with autologous counterparts.

The efficacy of autologous versus allogeneic γδ-T-EVs-based vaccine in the induction of tumor-specific T cells was further compared. Compared with γδ-T-EVs, both autologous and allogeneic γδ-T-EVs (TAAs) induced more tumor-specific T cells, in which the levels of tumor-specific T cells were comparable (Figure 5f,g). Similarly, depletion of HLA-DR$^+$ APCs from huPBMCs prevented the induction of tumor-specific T cells by both autologous and allogeneic γδ-T-EVs (TAAs) (Figure 5h), suggesting that recipient APCs are indispensable for the functions of both autologous and allogeneic γδ-T-EVs-based vaccines. These results

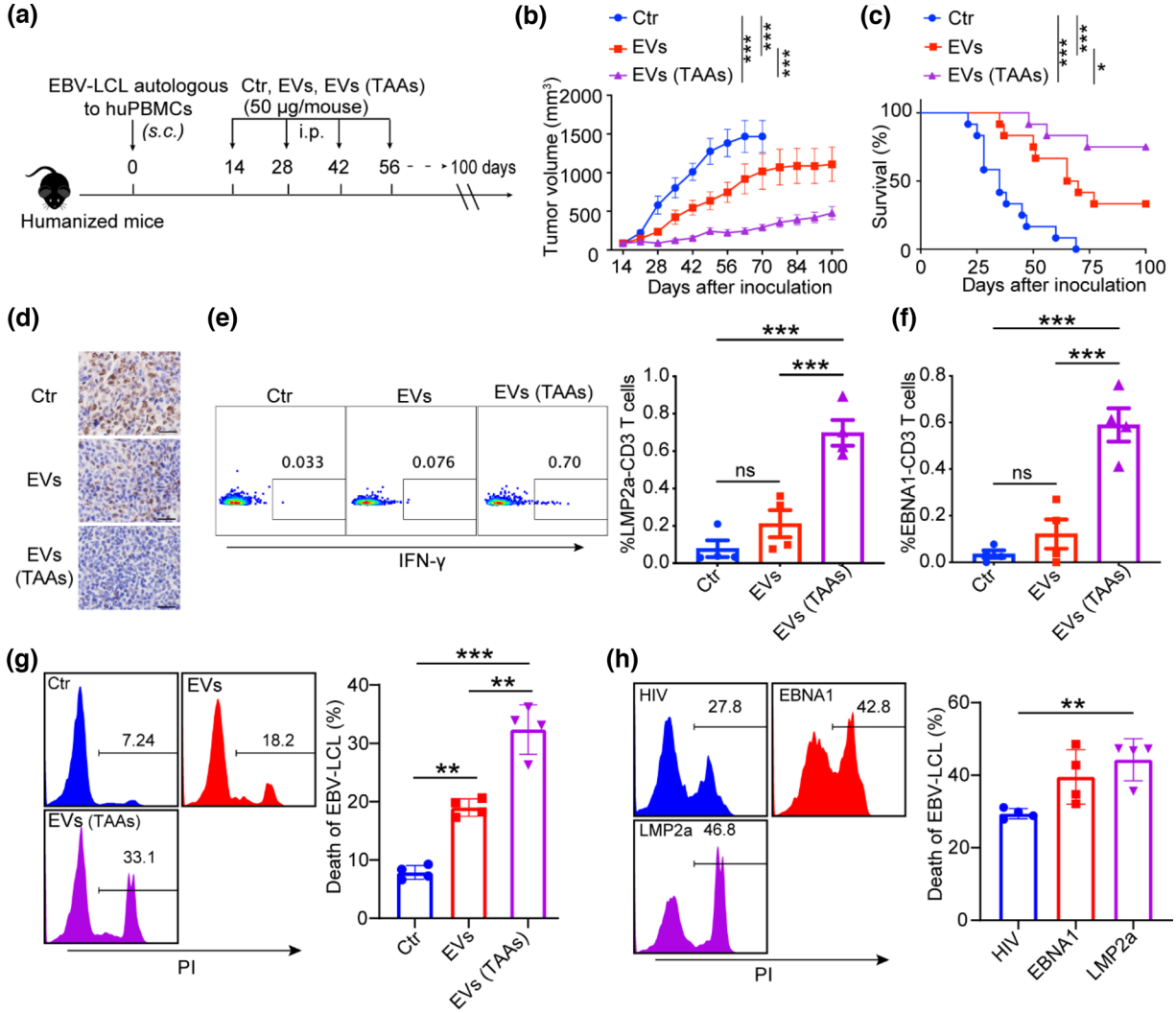

FIGURE 4 γδ-T-EVs-based vaccine inhibits tumor progression in humanized mice. (a) Tumor models were established by injection *s.c.* of autologous EBV-LCL cells in humanizedmice. Then, γδ-T-EVs or γδ-T-EVs (TAAs) were injected into humanized mice *i.p.* as indicated (n = 12). TAAs was used as control (Ctr). The tumor volume (b) and mice survival (c) were determined at the indicated time. (d) Representative histochemical analysis of human Ki-67 in tumor tissues at the endpoints, scale bar = 25 μm. (e-f) TAAs, γδ-T-EVs or γδ-T-EVs (TAAs) were injected into EBV-induced B cell lymphoma-bearing humanized mice biweekly for two doses ($n = 4$). Seven days post the booster injection, the percentage of LMP2a- and EBNA1-specific T cells in peripheral blood nucleated cells were detected. (g) Death of EBV-LCL cells after 6 h cocultured with autologous T cells induced by γδ-T-EVs or γδ-T-EVs (TAAs). EBV-LCL cells alone were used as control (Ctr). (h) Death of EBV-LCL cells with preloading of EBV peptides after 6 h cocultured with autologous T cells induced by γδ-T-EVs (TAAs). EBV-LCL cells preloaded with HIV p17 peptides were used as control (Ctr). For the bar graphs, representative data were shown as mean ± SEM from two to three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns: not significant.

indicate that the allogeneic γδ-T-EVs-based vaccine is comparable with its autologous counterpart in mobilizing recipient APCs and inducing tumor-specific T-cell responses.

2.6 | Allogeneic γδ-T-EVs-based vaccine inhibits tumor progression in humanized mice

The antitumor efficacies of allogeneic versus autologous γδ-T-EVs-based vaccine in humanized mice were further compared. As indicated in Figure 6a, EBV-induced B cell lymphoma-bearing humanized mice were treated with allogeneic or autologous γδ-T-EVs (TAAs). PBS, TAAs, or γδ-T-EVs was used as controls. Mice treated with γδ-T-EVs had more negligible tumor growth and more prolonged mice survival than either PBS or TAAs treatment. After being loaded with tumor antigens, either autologous or allogeneic γδ-T-EVs (TAAs) showed better antitumor efficacies than γδ-T-EVs (Figure 6b,c). In addition, allogeneic γδ-T-EVs (TAAs) were not inferior to autologous γδ-T-EVs (TAAs) in tumor control (Figure 6b,c). The tumor cells in mice treated with either autologous or allogeneic γδ-T-EVs (TAAs) were also found with lower proliferative capability, as fewer Ki-67 positive cells

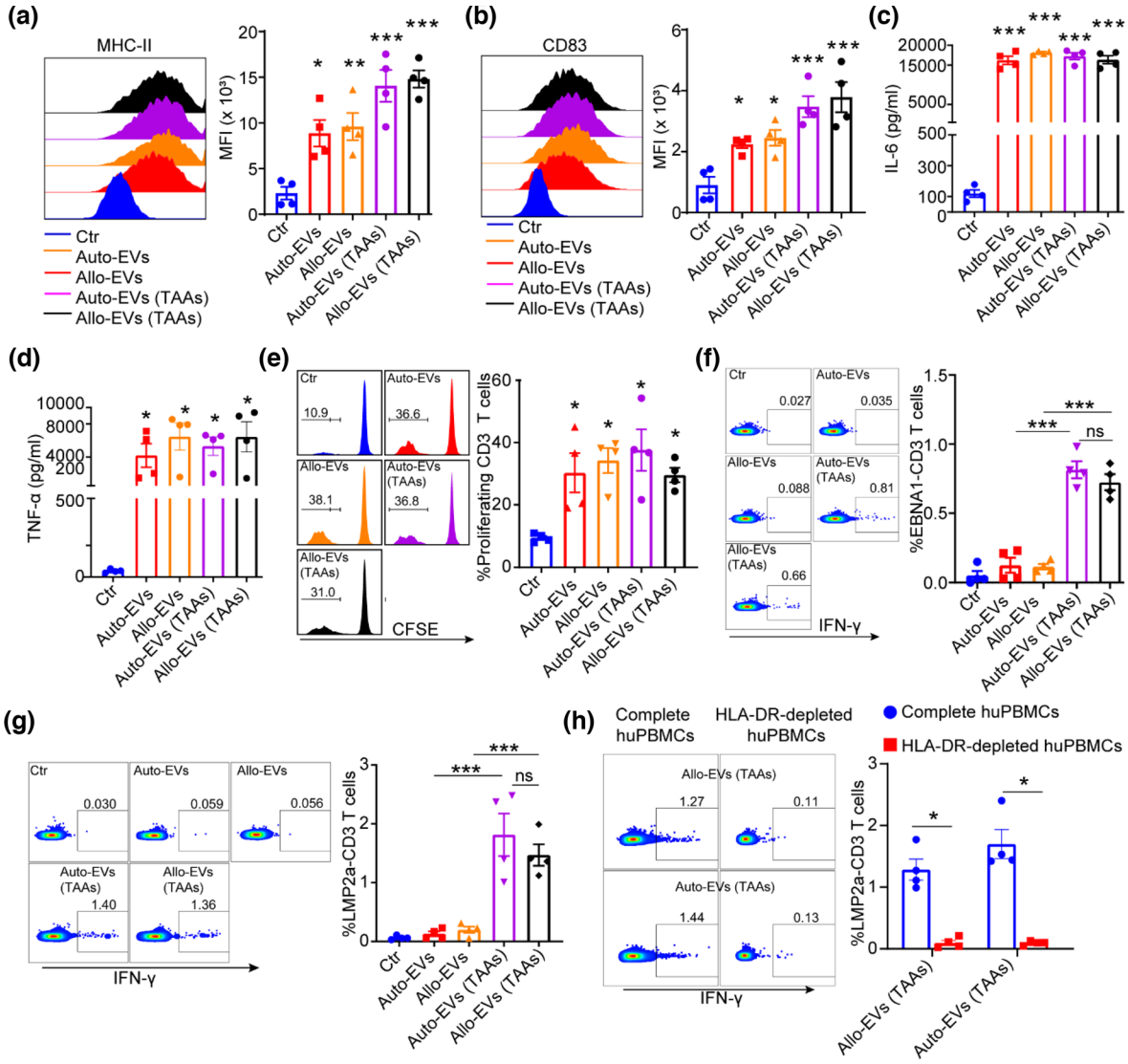

FIGURE 5 Allogeneic γδ-T-EVs-based vaccine promotes antigen-presenting cells to induce tumor-specific T-cell responses. Expression of MHC-II (a) and CD83 (b) on iDCs after treated with autologous, allogeneic γδ-T-EVs or γδ-T-EVs (TAAs) for 48 h. TAAs was used as control (Ctr). (c,d) Secretion of IL-6 and TNF-α from iDCs after treatment. (e) iDCs were pretreated with autologous, allogeneic γδ-T-EVs or γδ-T-EVs (TAAs) for 48 h, then cocultured with CFSE-stained allogeneic CD3 T cells. Seven days later, proliferating T cells were determined by CFSE dilution using flow cytometry. (F,G) Autologous, allogeneic γδ-T-EVs or γδ-T-EVs (TAAs) were used to treat huPBMCs. After 7 days, the cells were restimulated with EBNA1 (f) or LMP2a (g) peptide pools and subjected to detecting intracellular IFN-γ in T cells. (h) Completed huPBMCs or HLA-DR$^+$ cell-depleted huPBMCs were treated with the autologous or allogeneic γδ-T-EVs (TAAs) for seven days, then the LMP2a-specific T cells were detected. For the bar graphs, representative data were shown as mean ± SEM from three independent experiments. MFI: median fluorescence intensity. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns: not significant.

were found in corresponding residual tumors (Figure 6d). Considering the advantages of allogeneic EVs in clinical application, these data suggest the great translational potentials of the allogeneic γδ-T-EVs-based vaccine in cancer therapy.

To evaluate whether human T cells are involved in the antitumor activities of allogeneic γδ-T-EVs-based vaccine, humanized mice reconstituted with complete huPBMCs, or CD3 T cell-depleted huPBMCs were used for tumor establishment and treatment (Figure 6e). Consistently, treatment of complete huPBMCs-reconstituted humanized mice with allogeneic γδ-T-EVs (TAAs) significantly suppressed tumor growth and prolonged mice survival when compared with TAAs (Figure 6f,g). However, the therapeutic efficacy was decreased in the humanized mice reconstituted with CD3 T cell-depleted huPBMCs. Moreover, after being treated with allogeneic γδ-T-EVs (TAAs), the tumor cells in mice reconstituted with CD3 T cell-depleted huPBMCs had increased proliferative capability than those in the mice reconstituted with complete huPBMCs, as more Ki-67 positive cells were found in corresponding residual tumors (Figure 6h). Therefore, these results indicate that allogeneic γδ-T-EVs-based vaccine controls tumor progression in humanized mice.

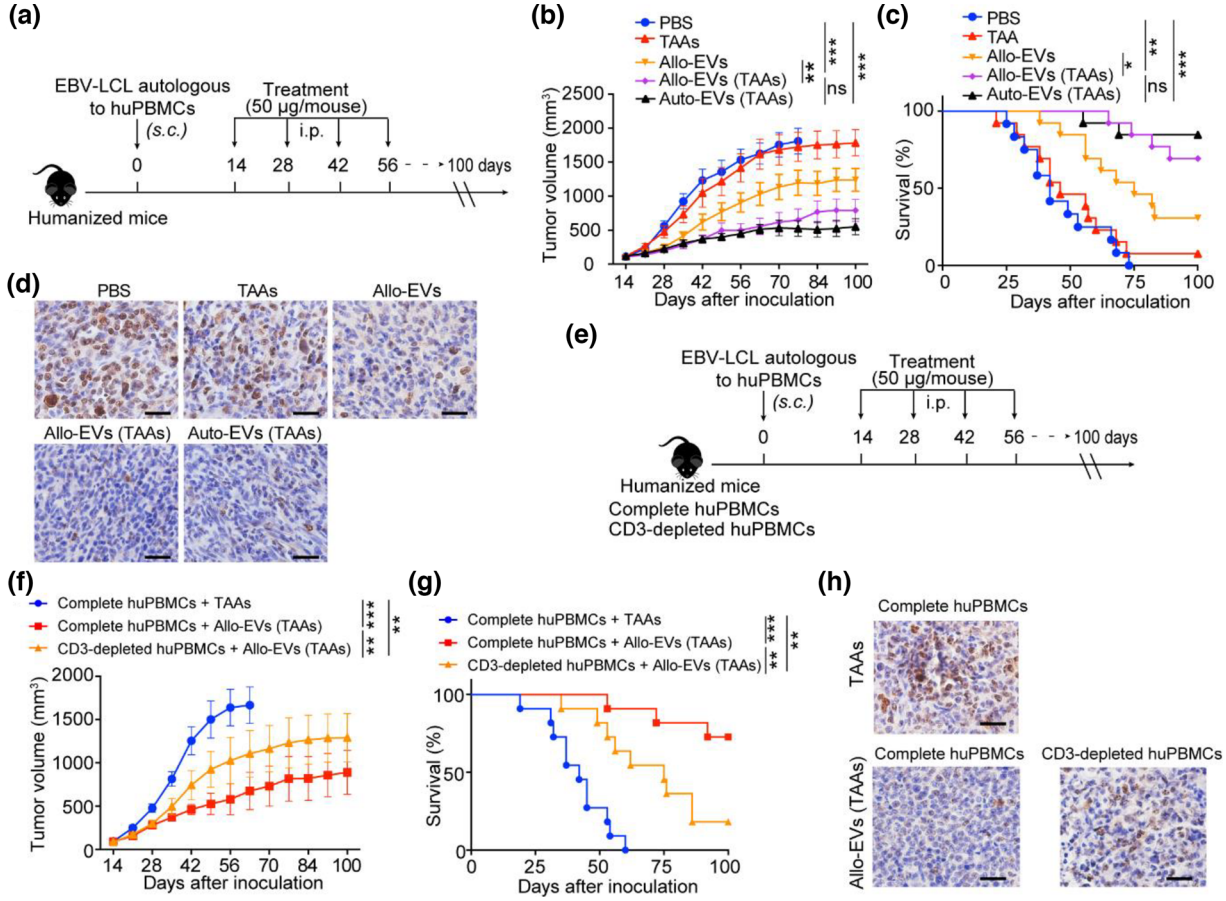

FIGURE 6 Allogeneic γδ-T-EVs-based vaccine inhibits tumor progression in humanized mice. (a) Tumor models were established by injection *s.c.* of autologous EBV-LCL in humanizedmice. Autologous or allogeneic γδ-T-EVs (TAAs) were injected into humanized mice *i.p.* at the indicated time. PBS, TAAs, or γδ-T-EVs was used as control ($n = 12$ or 13). Tumor volume (b) and mice survival (c) was calculated as indicated. (d) Histochemical analysis of human Ki-67 in tumor tissues at the endpoints, scale bar = 25 μm. (e) Tumor models were established by injection of autologous EBV-LCL in humanized mice reconstituted with complete huPBMCs or CD3 T cell-depleted huPBMCs. TAAs or allogeneic γδ-T-EVs (TAAs) were injected intraperitoneally into humanized mice at the indicated time ($n = 11$). The tumor volume (f) and mice survival (g) were calculated as indicated. (h) Histochemical analysis of human Ki-67 in tumor tissues at the endpoints, scale bar = 25 μm. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns: not significant; Auto-: autologous; Allo-: allogeneic.

2.7 | Allogeneic γδ-T-EVs-based vaccine prevents tumor development in humanized mice

To determine whether the allogeneic γδ-T-EVs-based vaccine can prevent tumor development, humanized mice were injected with PBS, TAAs, allogeneic γδ-T-EVs or γδ-T-EVs (TAAs) *i.p.* at day 21 and 7 before tumor cell inoculation. Autologous γδ-T-EVs (TAAs) also served as a control (Figure 7a). One week after the booster injection, the mice were challenged with autologous EBV-LCL cells (Figure 7a). Rapid development of tumor was observed in all the mice immunized with PBS, TAAs or γδ-T-EVs. However, only 25% and 12.5% of mice had developed tumors after being vaccinated with allogeneic and autologous γδ-T-EVs (TAAs), respectively (Figure 7b). In Vivo Imaging analysis was also used to validate the tumor development of each group at 6 weeks after tumor cell inoculation (Figure 7c). In addition, immunization with allogeneic or autologous γδ-T-EVs (TAAs) effectively inhibited tumor growth (Figure 7d) and prolonged mice survival (Figure 7e). The preventive efficacies of allogeneic and autologous γδ-T-EVs (TAAs) had no significant differences (Figure 7b–e). Thus, our data demonstrate that allogeneic γδ-T-EVs-based vaccine has a preventive effect against tumor development.

2.8 | Allogeneic γδ-T-EVs-based vaccine displays advantages over DC-EVs-based vaccine through dual antitumor activities

As allogeneic DC-EVs also have adjuvant effects and can be used as a nanocarrier in tumor vaccine (Hiltbrunner et al., 2016; Larssen et al., 2019), we then compared the efficacies of allogeneic γδ-T-EVs- with DC-EVs-based vaccines. Both vaccines were prepared by pulsing parental cells with TAAs before EVs isolation. γδ-T-EVs- and DC-EVs-based vaccines had similar size

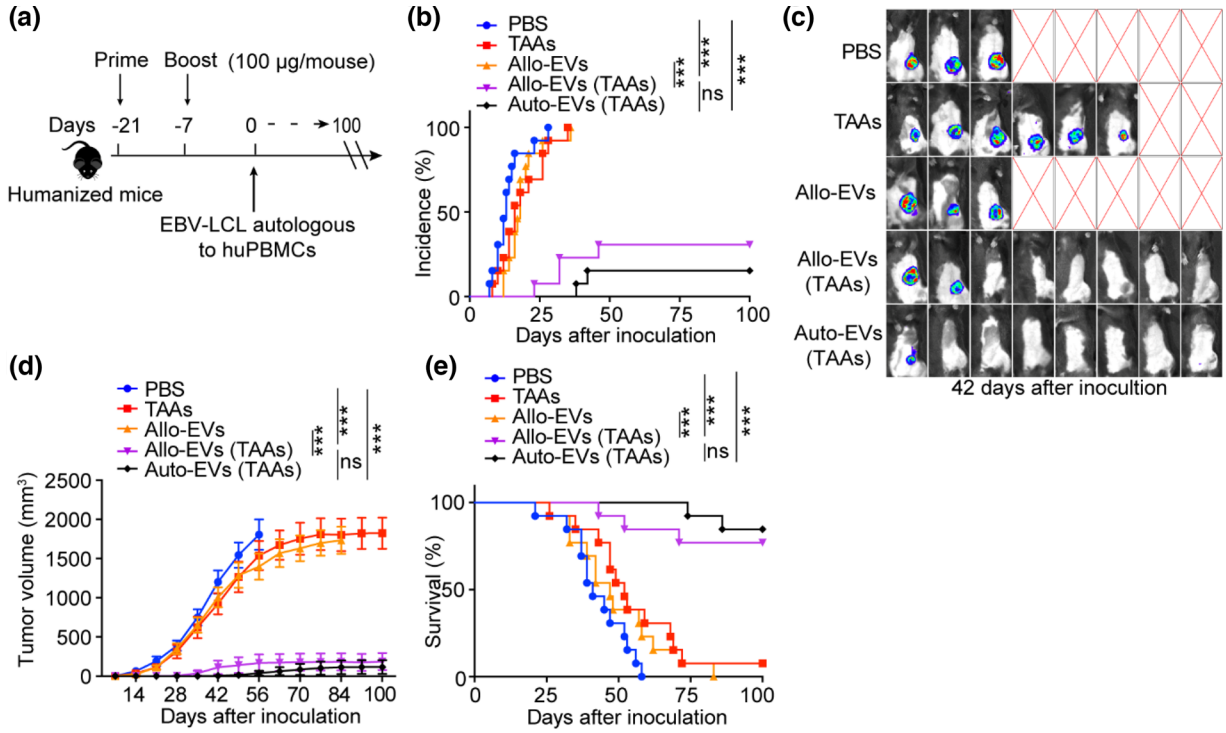

FIGURE 7 Allogeneic γδ-T-EVs-based vaccine prevents tumor development in humanized mice. (a) Humanized mice were primed and boosted using the autologous or allogeneic γδ-T-EVs-based vaccine as indicated, then EGFP-expressing EBV-LCL cells were subcutaneously injected 1 week later ($n = 13$). An equivalent amount of PBS, TAAs, or γδ-T-EVs was used as a control. (b) Tumor incidence, (c) whole-body fluorescence images of mice 42 days after tumor cell inoculation, (d) tumor volume, and (e) mice survival were determined as indicated. ***$p < 0.001$. ns: not significant; Auto-: autologous; Allo-: allogeneic.

distribution profiles with peaks ~160 nm (Figure S5). In addition, γδ-T-EVs and γδ-T-EVs (TAAs) carried robust IFN-γ, while DC-EVs and DC-EVs (TAAs) did not (Figure S6). Allogeneic γδ-T-EVs and γδ-T-EVs (TAAs) induced more pro-inflammatory cytokines from iDCs than either DC-EVs or DC-EVs (TAAs) (Figure 8a,b). In an MLR assay, allogeneic γδ-T-EVs- and γδ-T-EVs (TAAs)-pretreated iDCs also promoted more T cell proliferation and IFN-γ secretion than the DC-EVs counterparts (Figure 8c,d). These data suggest that the allogeneic γδ-T-EVs-based vaccine has higher immune adjuvant effects than the DC-EVs-based vaccine.

The vaccine efficacies of allogeneic DC-EVs- and γδ-T-EVs-based vaccines were further compared. TAAs, allogeneic γδ-T-EVs, γδ-T-EVs (TAAs), DC-EVs, or DC-EVs (TAAs) were used to treat huPBMCs. Compared with TAAs or the allogeneic EVs, both allogeneic γδ-T-EVs (TAAs) and DC-EVs (TAAs) induced more tumor-specific T cells (Figure 8e). However, allogeneic γδ-T-EVs (TAAs) were more effective to induce tumor-specific T cells than allogeneic DC-EVs (TAAs) (Figure 8e). More importantly, γδ-T-EVs and γδ-T-EVs (TAAs) could directly induce tumor cell apoptosis, while DC-EVs or DC-EVs (TAAs) did not (Figure 8f). These data suggest that γδ-T-EVs-based vaccine has advantages over DC-EVs-based vaccine by displaying dual antitumor activities.

The therapeutic efficacies of allogeneic γδ-T-EVs- versus DC-EVs-based vaccines in humanized mice were further compared. As indicated in Figure 8g, EBV-induced B cell lymphoma-bearing humanized mice were treated with allogeneic γδ-T-EVs (TAAs) or DC-EVs (TAAs), respectively. TAAs or the allogeneic EVs were used as controls. Mice treated with allogeneic γδ-T-EVs (TAAs) or DC-EVs (TAAs) had smaller tumor sizes and more prolonged mice survival than TAAs. However, allogeneic γδ-T-EVs (TAAs) exerted higher therapeutic effects than allogeneic DC-EVs (TAAs) (Figure 8h–i). The proliferation of tumor cells in residual tumors also supported these findings (Figure 8j). Thus, our results demonstrate that the allogeneic γδ-T-EVs-based vaccine displays advantages over allogeneic DC-EVs-based vaccine in inhibiting tumor progression in humanized mice.

3 | DISCUSSION

In the present study, we found that γδ-T-EVs could serve as nanocarriers to deliver TAAs and function as an immune adjuvant to induce antigen-specific T-cell responses. In addition, the γδ-T-EVs-based vaccine preserved direct killing activity against tumors. Administration of γδ-T-EVs-based vaccine effectively controlled different tumors in vivo. More importantly, allogeneic γδ-T-EVs-based vaccine also had robust therapeutic effects and displayed advantages over the allogeneic DC-EVs-based vaccine

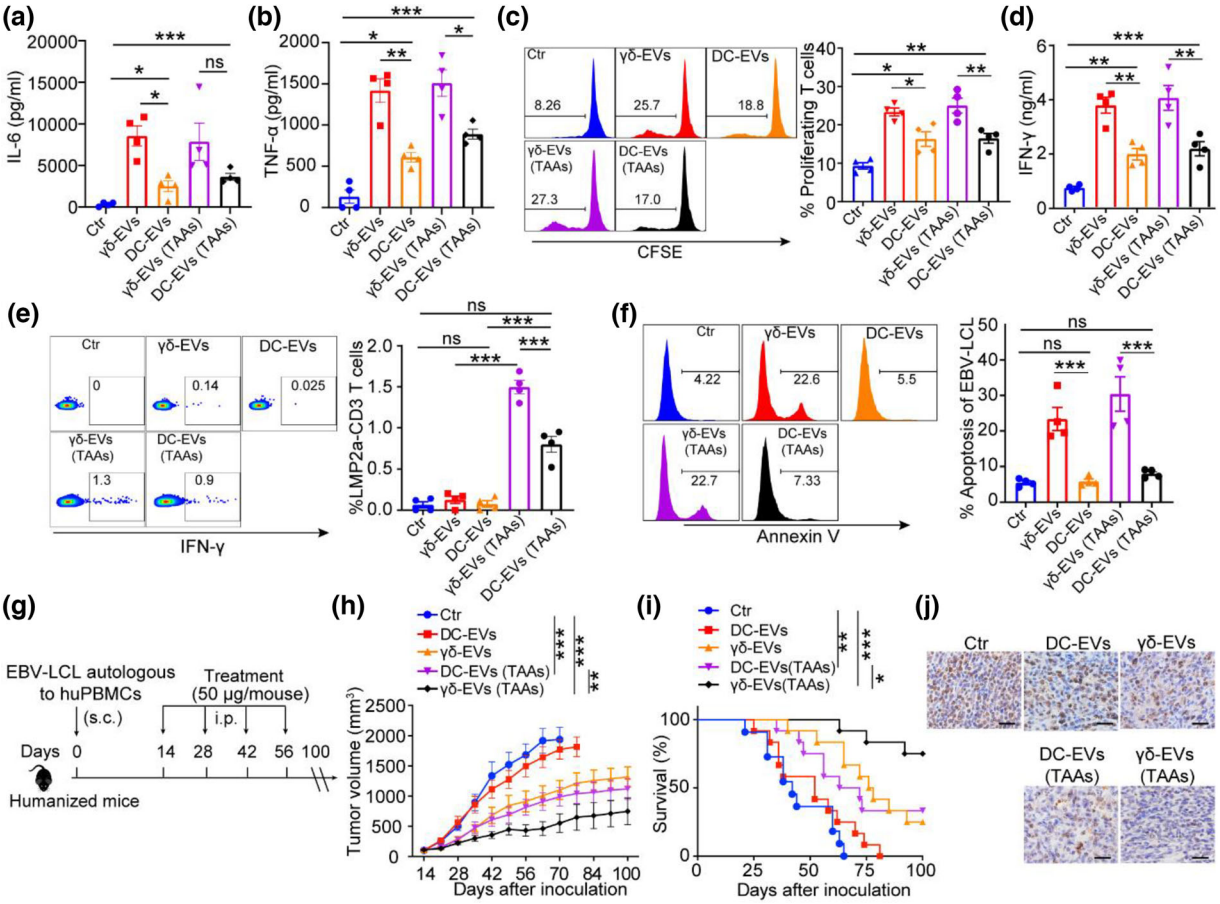

FIGURE 8 Allogeneic γδ-T-EVs-based vaccine has advantages over DC-EVs-based vaccine by displaying dual antitumor activities. (a-b) Secretion of TNF-α and IL-6 from iDCs after 48 h treatment with allogeneic γδ-T-EVs, DC-EVs, γδ-T-EVs (TAAs), or DC-EVs (TAAs). TAAs was used as control (Ctr). (c) iDCs were treated with allogeneic γδ-T-EVs, DC-EVs, γδ-T-EVs (TAAs), or DC-EVs (TAAs) for 48 h, and TAAs were used as control (Ctr). Then, the pretreated-iDCs were cocultured with CFSE-stained allogeneic CD3 T cells. Seven days later, proliferating CD3 T cells were determined by CFSE dilution using flow cytometry. (d) Secretion of IFN-γ in the coculture system of pretreated iDCs with allogeneic CD3 T cells. (e) Allogeneic γδ-T-EVs- or DC-EVs-based vaccines were used to treat huPBMCs. After seven days, the cells were restimulated with LMP2a peptide pools and subjected to detecting intracellular IFN-γ in T cells. (f) Apoptosis of EBV-LCL cells after being cultured with allogeneic γδ-T-EVs- or DC-EVs-based vaccine for 18–24 h. (g) Tumor models were established by injection *s.c.* of EBV-LCL cells in humanizedmice. Then, allogeneic γδ-T-EVs-based or DC-EVs-based vaccines were injected into humanized mice *i.p.* at the indicated time ($n = 11$ or 12). TAAs was used as control (Ctr). The tumor volume (h) and mice survival (i) were calculated as indicated. (j) Histochemical analysis of human Ki-67 in tumor tissues at the endpoints, scale bar = 25 μm. For the bar graphs, representative data were shown as mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns: not significant.

by exerting dual antitumor activities. Therefore, the application of γδ-T-EVs-based vaccine has excellent potential in cancer control.

As a bridge between innate and adaptive immune systems, γδ-T cells play critical roles in the regulation of APCs function (Ismaili et al., 2002; Petrasca & Doherty, 2014). Here, to the best of our knowledge, we demonstrated for the first time that γδ-T cells could promote APC functions in a cell-free manner through EVs, which was mediated by the IFN-γ and CD40L carried by γδ-T-EVs. Compared with γδ-T-EVs + free TAAs, γδ-T-EVs (TAAs) showed high efficacy in inducing tumor-specific T-cell responses. As demonstrated in previous studies, the delivery efficacy of TAAs to APCs could be increased after loading the TAAs on nanoparticles, resulting in more immune responses (Hou et al., 2020; Lindenbergh & Stoorvogel, 2018; Morishita et al., 2016). Since γδ-T-EVs (TAAs) not only deliver TAAs to DCs, but also simultaneously serve as an immune adjuvant, therefore the co-delivery of TAAs and adjuvant by γδ-T-EVs (TAAs) induced more immune responses than TAAs or γδ-T-EVs + free TAAs. The adjuvant effects of γδ-T-EVs are potent enough without the need for additional adjuvants for γδ-T-EVs-based vaccine, in which the manufacturing processes are generally different from TEVs-based or DC-EVs-based vaccine (Naseri et al., 2020; Xia et al., 2022). Therefore, preparing the γδ-T-EVs-based vaccine could be more convenient since preloading the immune adjuvant is unnecessary. Moreover, compared with TEVs-based vaccine, γδ-T-EVs-based vaccine may have a better safety profile due to their nature in tumor surveillance (Wang et al., 2020, 2022), while TEVs can drive tumorigenesis (Whiteside, 2016).

Preparing personalized vaccines for cancer patients is very difficult in clinical practice since the procedures are time- and labor-consuming. In addition, the γδ-T cells of cancer patients are difficult to be expanded ex vivo for EVs production due to the

immune dysregulation caused by cancer or anti-cancer therapy (e.g., radiotherapy and chemotherapy). In contrast, it is much easier to prepare γδ-T cells from healthy donors in large-scale using the protocols modified by our or others' groups (Alexander et al., 2008; Bonneville & Scotet, 2006; Kouakanou et al., 2020; Qin et al., 2012, 2009; Peters et al., 2022; Tu et al., 2011), which can ensure the production of γδ-T-EVs for clinical application. We found that allogeneic γδ-T-EVs also had strong immune adjuvant effects by inducing bystander activation of recipient APCs. When serving as a nanocarrier of tumor antigens, allogeneic γδ-T-EVs are comparable to their autologous counterparts in the induction of tumor-specific immune responses and tumor control. More importantly, the allogeneic γδ-T-EVs-based vaccine not only showed potent therapeutic antitumor effects, but also effectively prevented tumor development. After two doses of immunization with γδ-T-EVs-based vaccine, most mice were protected from tumor development and survived longer. Therefore, γδ-T-EVs-based vaccine has a wide range of application scenarios in clinical practice for preventing and treating tumors. Some previous studies showed that allogenic and syngeneic DC-based vaccines had similar antitumor efficacies, supporting our findings (Hiltbrunner et al., 2016; Larssen et al., 2019). Considering that preparing γδ-T-EVs from healthy donors has advantages in quality control, standardization and centralization, the allogeneic γδ-T-EVs-based vaccine could be used as an "off-the-shelf" approach in cancer control.

DC-EVs-based vaccines have been widely investigated for cancer immunotherapy and demonstrated with promising therapeutic potentials in preclinical models. However, their therapeutic efficacies were limited in clinical trials (Besse et al., 2016; Escudier et al., 2005; Morse et al., 2005). The heterogeneity of ex vivo expanded DCs may partially account for the poor therapeutic outcomes of DC-EVs-based vaccines since the EVs derived from immature DCs exerted immunoinhibitory activities (Morse et al., 2005; Song et al., 2016). On the contrary, homogeneous and immunostimulatory human γδ-T cells can be expanded in large-scale by phosphoantigens (Alexander et al., 2008; Bonneville & Scotet, 2006; Kouakanou et al., 2020; Qin et al., 2009; Tu et al., 2011; Wang et al., 2020; Xiang et al., 2014,), which ensure the preparation of γδ-T-EVs-based vaccine for clinical use. Similar to our findings, allogeneic DC-EVs were demonstrated with noticeable adjuvant effects (Larssen et al., 2019). We found that natural γδ-T-EVs had higher adjuvant effects than DC-EVs and induced tumor-specific T-cell responses more effectively after antigen loading. Besides, the γδ-T-EVs-based vaccine preserved direct antitumor activities, while the DC-EVs-based vaccine could not directly kill tumor cells. Therefore, γδ-T-EVs-based vaccine has advantages over DC-EVs-based vaccine via their dual antitumor activities. However, the comparison of γδ-T-EVs with DC-EVs in tumor vaccines warrants further investigation since the immunostimulatory effects of DC-EVs can be improved with many strategies, such as treating DCs with different maturation agents or adjuvants (Andre et al., 2004; Larssen et al., 2019; Tkach et al., 2017) before EVs isolation. Therefore, although we showed that the γδ-T-EVs-based vaccine is more effective than the DC-EVs-based vaccine here, the DC-EVs-based vaccine could be competitive under other contexts.

In summary, our study provides a strong preclinical proof-of-concept to use γδ-T-EVs-based vaccine in cancer control. As a cell-free method, γδ-T-EVs-based vaccine has dual antitumor activities. More importantly, the allogeneic γδ-T-EVs-based vaccine also has robust antitumor effects. Therefore, this approach has promising translational potential. Our work supports the extension of this approach into clinical trials for cancer control.

4 | METHODS

4.1 | Cell culture

EBV-LCL cell lines were established using huPBMCs isolated from healthy donors provided by Hong Kong Red Cross as described before (Xiang et al., 2014). Briefly, huPBMCs were treated with EBV-containing supernatants of B95.8EbfaV-GFP or B95-8 cell line (Speck & Longnecker, 1999) (kindly provided by Diane Hayward, Johns Hopkins University, Baltimore) and cultured with 15% fetal bovine serum (FBS)-RPMI-1640 medium supplemented with cyclosporine-A. The EBV-positive gastric carcinoma SNU-719 cell line (Korean cell line bank) was cultured in 10% FBS-RPMI-1640 medium. Practically, γδ-T cells/DCs were cultured for EVs preparation, and EBV-LCL cell lines were induced immediately after huPBMCs isolation. The remaining huPBMCs and autologous transformed EBV-LCL cells were frozen and subjected to establishing tumor-bearing humanized mouse models. All the research protocols and animal work in the present study were approved by The Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster and the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong.

4.2 | Preparation of γδ-T-EVs and γδ-T-EVs-based vaccine

γδ-T cells were expanded for EVs isolation through modified procedures from previous studies (Qin et al., 2011; Wang et al., 2020, 2022; Xiang et al., 2014). Briefly, huPBMCs were isolated from buffy coats of healthy donors from the Hong Kong Red Cross. The huPBMCs were activated with 9 μg/mL pamidronate at day 0 and 3, and cultured in 10% FBS-RPMI-1640 medium. Human recombinant interleukin-2 (IL-2; Invitrogen) was added from day 3 in a final concentration of 200 IU/mL. After 14–20

days, γδ-T-EVs were prepared using protocols modified from our previous studies (Wang et al., 2020, 2022). γδ-T cells were conditioned in an EVs-free culture medium supplemented with 200 IU/mL IL-2, in which the FBS-derived EVs were removed in advance by ultracentrifugation for 18 h at 100 000 × *g* (SW32Ti rotor, Beckman). About 48 h later, the conditioned medium was harvested for EVs isolation.

To prepare the γδ-T-EVs-based vaccine, γδ-T cells were pulsed with 500 μg/mL TAAs, which were tumor lysates of EBV-LCL cells obtained via freeze-thaw. About 24 h later, γδ-T cells were washed extensively with PBS and cultured in an EVs-free culture medium supplemented with 200 IU/mL IL-2. About 48 h later, the conditioned medium was harvested and subjected to EVs isolation. In the functional assays, an equivalent amount of free TAAs to that in the γδ-T-EVs (TAAs) was used as control after quantification via the detection of EBV antigens by ELISA.

4.3 | Preparation of DC-EVs and DC-EVs-based vaccine

To prepare iDCs, human $CD14^+$ cells were isolated from huPBMCs using magnetic beads (Miltenyi Biotec) and incubated for 6 days in 10%-FBS-RPMI medium supplemented with 10 ng/mL IL-4 and 50 ng/mL GM-CSF (Peprotech) as we did before (Wang et al., 2020; Wu et al., 2011). iDCs cells were pulsed with or without 500 μg/mL TAAs for 10 h. Then, 0.5 μg/mL lipopolysaccharide was added. 14 h later, the cells were washed extensively with PBS and cultured in EVs-free 10% FBS-RPMI culture medium. 48 h later, the conditioned medium was harvested for EVs isolation.

4.4 | EVs isolation and characterization

EVs were isolated by differential ultracentrifugation at 4°C as described before (Pei et al., 2020; Wang et al., 2021, 2022). The conditioned medium was centrifuged at 300 x *g* for 10 min, 2000 x *g* for 10 min and 10,000 x *g* for 30 min. Then, the supernatant was filtered using 0.22-μm syringe filter and ultracentrifuged at 100,000 x *g* for 70 min twice (SW32Ti rotor, Beckman). EVs were dissolved using PBS. The protein concentration of EVs was determined by a BCA Protein Assay Kit (Pierce, Bonn). The size distribution of EVs was determined through nanoparticle tracking analysis. For flow cytometry analysis, EVs were conjugated with 4-μm aldehyde/sulfate latex beads overnight (Thery et al., 2006) and incubated with glycine to block remaining binding sites. The EVs-loaded beads were stained with antibodies and corresponding isotype controls (Thery et al., 2006).

For Western blot analysis, proteins from cellular lysates or EVs were obtained by lysis in radioimmunoprecipitation assay buffer in the presence of protease inhibitor cocktail (Thermo Fisher Scientific) and separated by SDS-electrophoresis on 8%–12% gels. Subsequently, proteins were transferred onto membranes and blocked with 5% nonfat milk. Membranes were incubated with anti-Alix (Abcam), anti-EBNA1(Santa Cruz) or anti-LMP2a antibodies overnight (Abcam). After incubation with corresponding secondary horseradish peroxidase (HRP)–conjugated antibodies, chemiluminescence signals were detected with Immobilon Classico Western HRP substrate (Millipore).

4.5 | Induction of tumor antigen-specific T cells in vitro

Completed huPBMCs or huPBMCs with $HLA\text{-}DR^+$ cells depletion by anti-HLA-DR MicroBeads (Miltenyi) from EBV-seropositive donors were incubated with 20 μg/mL EVs preparations and cultured in RPMI-1640 medium supplemented with 10% human AB serum. In some experiments, purified T cells were treated with EVs preparations and cultured in the presence of iDCs or primary B cells. After seven days, the cells were restimulated with 1 μg/mL corresponding or irrelevant peptide pools for 6 h, with the addition of 10 μg/mL brefeldin A (BFA, Sigma-Aldrich) 2 h later. The antigen-specific cells were detected on flow cytometry by staining T cell markers and IFN-γ (Biolegend).

4.6 | Determination of APC functions

To determine the effects of EVs-based vaccine on APC functions, Primary B cells or iDCs were cultured with 20 μg/mL EVs or EVs-based vaccine. In some experiments, neutralizing anti-IFN-γ, anti-CD40L antibodies or isotype antibody was added. 48 h later, the cells were collected to detect functional markers (e.g., CD40, CD83, CD86, and MHC-II), cell culture supernatant was also harvested to detect the secretion of IL-6 and TNF-α or mixed lymphocyte reaction (MLR) was performed to determine the functional activity of DCs as described before (Leung et al., 2013). Briefly, allogeneic CD3 T cells were isolated from huPBMCs using a Pan T cell isolation kit (Miltenyi Biotec) and stained with Carboxyfluorescein succinimidyl ester (CFSE). Then, the EVs preparations-pretreated iDCs were cocultured with allogenic CD3 T cells in a ratio of 1:10. Five days later, T cell proliferation was measured by flow cytometry. The culture supernatant of MLR assay was also collected and subjected to detecting the secretion IFN-γ.

4.7 | Cell apoptosis and proliferation assay

EBV-LCL or SNU-719 cells were treated with 30 μg/mL EVs or EVs-based vaccine. 18–24 h later, the cell apoptosis was evaluated through annexin V expression, and the cell proliferation was assessed through Ki67 expression (Biolegend).

4.8 | Cytotoxicity assay

As effector cells, T cells were negatively selected from γδ-T-EVs or γδ-T-EVs-based vaccine pretreated huPBMCs using Pan T cell isolation kit (Miltenyi). The cytotoxic activities of T cells were determined against the following targets: (i) autologous EBV-LCL, (ii)autologous EBV-LCL pulsed with 1 μg/mL EBNA1, LMP2a or HIV p17 peptides pools. Effector and target cells were cocultured at a ratio of 10:1. To distinguish both cell populations in detection, target cells were stained with CFSE before coculturing. After 6 h, the cells were stained with propidium iodide (PI) and then analyzed by flow cytometry. The death of target cells was measured as the percentage of PI^+ cells in the CFSE-positive population.

4.9 | Establishment and treatment of EBV-associated tumors in $Rag2^{-/-}\gamma c^{-/-}$ and humanized mice

$Rag2^{-/-}\gamma c^{-/-}$ mice were maintained in the Laboratory Animal Unit of the University of Hong Kong. Four to 6-week-old $Rag2^{-/-}\gamma c^{-/-}$ mice were used to build humanized mice by reconstitution with EBV-seropositive CD3 T cells-depleted huPBMCs or complete huPBMCs using the method we described previously (Pei et al., 2020; Wang et al., 2020; Xiang et al., 2014). After huPBMCs injection, symptoms of graft-versus-host disease (e.g. weight loss, diarrhea) were monitored daily. Generally, the weight loss occurred from day 1–10 day and became tolerance 15–20 days after the huPBMCs injection. Four weeks after huPBMCs reconstitution, the mice were referred to "humanized" mice as they had a functional human peripheral immune system (Tu et al., 2011; Xiang et al., 2014). $Rag2^{-/-}\gamma c^{-/-}$ mice or humanized mice were implanted s.c. with SNU-719 or EBV-LCL cells to establish EBV-associated tumor models. For humanized mouse model, EBV-LCL cells were autologous to the huPBMCs. Almost all tumor cells-injected mice developed tumors if no treatment was given. Mice with palpable tumors were randomly grouped for therapeutic experiments. Mice were age- and gender-matched, and there were no evident differences in tumor volume among the groups before treatment. The mice were i.p. injected with autologous, allogenic EVs or EVs-based vaccine as indicated. Tumor volume, tumor incidence and animal survival were monitored and calculated at indicated time. Tumor volume was determined by a digital caliper and calculated as length x $[\text{width}]^2$ x 0.52. Comply with the regulation of the University of Hong Kong, mice bearing subcutaneous tumors with diameters reaching 17 mm were euthanized (Wang et al., 2020, 2022). Tumor tissues were harvested for immunohistochemical analysis.

4.10 | Preventive effects of the γδ-T-EVs-based vaccine against EBV-associated tumors in humanized mice

Humanized mice were established as indicated above. The mice were vaccinated with γδ-T-EVs or γδ-T-EVs (TAAs). Two weeks later, animals were boosted with the same preparations. Seven days after booster vaccination, EBV-LCL cells (1×10^6 per mouse) were inoculated into the mice. Tumor incidence, tumor volume and mice survival were calculated at the indicated time. Tumor development was also validated by an IVIS Spectrum in vivo imaging system (Caliper Life Sciences).

4.11 | Detection of tumor-specific T cells ex vivo

About 50 μg/mouse γδ-T-EVs or γδ-T-EVs (TAAs) were injected (i.p.) into EBV-induced B cell lymphoma-bearing humanized mice biweekly for two doses. Equivalent amount of TAAs was also used as control. Seven days post the booster injection, the peripheral blood nucleated cells were isolated and subjected to peptide restimulation to detect EBNA1- or LMP2a-specific T cells.

4.12 | Histological and immunohistochemical analysis

Tumor tissues were fixed with 10% formalin and embedded in paraffin for sectioning. The sections were subjected to immunohistochemistry staining. Ki67 was detected by immunohistochemistry using an anti-human Ki67 antibody (Abcam, UK) and visualized by a diaminobenzidine detection kit (Maixin, China).

4.13 | Flow cytometric analysis

Surface staining of cells was performed using the following antibodies: anti-CD63 (H5C6), anti-CD3 (HIT3a), anti-CD4 (RPA-T4), anti-CD8 (SK1), anti-MHC-II (Tü39), anti-CD83 (HB15e), anti-CD86 (GL-1) and anti-CD40 (5C3). For the intracellular staining, cells were fixed, permeabilized and followed by staining with anti-IFN-γ (B27) or isotype control as described before (Ni et al., 2018; Zheng et al., 2011). All samples were detected using a FACSLSR II Flow Cytometer (BD, USA) and analyzed with FlowJo software (Tree Star, USA).

4.14 | Cytokine secretion assay

The concentrations of cytokines in the culture supernatants were detected and analyzed with LEGENDplex human Cytokine detection kit (BioLegend).

5 | STATISTICS

Quantitative data were expressed as mean ± SEM. The comparison of the two groups was Mann-conducted by the Whitney U test. One-way analysis of variance (ANOVA) with Bonferroni correction was used to compare multiple groups. Two-way ANOVA analysis was used to determine the effects of multiple variables and corresponding interactions. Animal survival and tumor incidence were analyzed via the Kaplan-Meier log-rank test. Statistical significance was calculated using two-tailed tests, and $P < 0.05$ was considered significant. The main text or figure legends describe specific sample sizes, P values, and other details for each test.

AUTHOR CONTRIBUTIONS

Xiwei Wang: Conceptualization; data curation; formal analysis; investigation; methodology; validation; writing—original draft; writing—review & editing. **Yanmei Zhang**: Data curation; methodology; software. **Yuet Chung**: Data curation; methodology. **Chloe Ran Tu**: Methodology; writing—original draft. **Wenyue Zhang**: Data curation; methodology. **Xiaofeng Mu**: Methodology. **Manni Wang**: Methodology. **Godfrey Chi-Fung Chan**: Methodology. **Wing-hang Leung**: Methodology. **Yu-lung Lau**: Methodology; writing—original draft. **Yinping Liu**: Formal analysis; funding acquisition; investigation; methodology; project administration; supervision; visualization; writing—original draft; writing—review & editing. **Wenwei Tu**: Conceptualization; formal analysis; funding acquisition; project administration; supervision; validation; visualization; writing—original draft; writing—review & editing.

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CONFLICT OF INTEREST STATEMENT

W.T., Y.L., and X.W. are inventors on a filed provisional application entitled "Compositions and Methods of Gamma-Delta T Cell Extracellular Vesicle-Based Tumor Vaccines" with USPTO on 17 February 2023 (application no. 63/485734).
The other authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data supporting this article's findings are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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