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Novel fusion cells derived from tumor cells expressing the heterologous α-galactose epitope and dendritic cells effectively target cancer

Fengzhen Mo [a,b,c,1], Dabing Xue [a,b,1], Siliang Duan [a,b], Aiqun Liu [b], Xiaomei Yang [a,b], Xiaoqiong Hou [a,b], Xiaoling Lu [a,b,d,*]

[a] School of Preclinical Medicine, Guangxi Medical University, Nanning, Guangxi 530021, China
[b] Intenational Nanobody Research Center of Guangxi, Guangxi Medical University, Nanning, Guangxi 530021, China
[c] Pharmaceutical College, Guangxi Medical University, Nanning, Guangxi 530021, China
[d] National Center for International Research of Biological Targeting Diagnosis and Therapy, Guangxi Medical University, Nanning, Guangxi 530021, China

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ABSTRACT

Tumor cells/dendritic cells (DCs) fusion cells (tumor/DC) represent a promising immunotherapeutic strategy but are still under performed in clinical trials for cancer treatment. To further boost their anticancer efficacy, here we developed a novel design for fusing dendritic cells with MDA-MB-231 cells expressing the heterologous α-galactose (α-gal) epitope and assessed its anticancer activities both *in vitro* and *in vivo*. The high expression of α-gal in MDA-MB-231 (Gal^+)/DC correlated with enhanced DC activation. When applied to T cells, MDA-MB-231 (Gal^+)/DC significantly stimulated T-cell proliferation and activation, promoted productions of cytokines IL-2 and IFN-γ, and enhanced T-cell-mediated cytotoxicity against MDA-MB-231 cells. MDA-MB-231 (Gal^+)/DC inhibited proliferation and promoted apoptosis of tumor cells *in vivo*, prolonged mouse survival, and significantly boosted anticancer immunity by increasing $CD4^+$ and $CD8^+$ T cells systemically and elevating serum levels of cytokines and IgG. These results suggested that fusing dendritic cells with tumor cells expressing the heterologous α-gal epitope provides a novel therapeutic strategy for cancer treatment.

1. Introduction

Breast cancer is a leading cause of cancer-related deaths and a major health threat among women worldwide [1]. The traditional therapeutic modalities for breast cancer include surgical resection combined with hormonal therapy, chemotherapy, and/or radiation therapy, depending on the stage and molecular characteristics of the tumor [2,3]. Although these strategies have achieved great success in targeting the disease at an early stage and significantly reducing cancer mortality, they have failed to control breast cancer of an advanced and/or metastatic nature.

Cancer immunotherapy, achieved through either active administration of a cancer vaccine or passive transfer of cancer-specific antibodies, immunomodulators, or immune cells, is under intensive development. There are ongoing clinical trials employing immunotherapy for the treatment of various cancers including breast cancer, particularly after the initial success against melanoma, lung cancer, and prostate cancer [4-6]. Compared to other immunotherapeutic approaches, cancer vaccines offer the advantages of high specificity, low or no toxicity, and induction of immune memory to prevent tumor relapse. Thus, immunotherapy is considered an ideal modality, either alone or combined with other approaches for cancer treatment [7]. Dendritic cells are a critical component in both innate and acquired immunity and possess a potent capability of processing/presenting antigens to T cells thus orchestrating T-cell-mediated regulatory (procancer) and/or effector (anticancer) functions. Therefore, they are widely exploited as a vehicle to deliver tumor-specific antigens [8]. Upon antigen processing and presentation, dendritic cells display characteristic phenotypes and functions such as upregulating the expression of DC activation and maturation markers (major histocompatibility complex class II (MHC-II) molecules, CD80 and CD86), secreting high levels of interleukin (IL)-12p70, stimulating the production of interferon gamma (IFN-γ) from $CD4^+$ and $CD8^+$ T cells, and activating the cytotoxicity of T cells [9]. Limited by the availability of known tumor-specific peptides, the idea of generating fusion cells (FCs) from tumor cells and dendritic cells was proposed which allows a broad array of tumor-specific antigens to be presented on MHC-I and -II molecules of dendritic cells

* Corresponding author at: Intenational Nanobody Research Center of Guangxi, Guangxi Medical University, Nanning, Guangxi 530021, China.
E-mail address: luxiaoling@gxmu.edu.cn (X. Lu).
[1] These authors contributed equally to this work.

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[10]. Although well tolerated, the clinical response conferred by tumor/DC fusion cells are suboptimal [11,12]. Current research has focused on developing novel strategies to further improve the efficacy of tumor/DC fusion cells.

Galα1-3Galβ1-4GlcNAc-R (α-gal) is a carbohydrate epitope naturally synthesized in nonprimate mammals, prosimians, and New World monkeys but not in humans due to silencing of the α1,3galactosyltransferase (α1,3GT) gene, the enzyme required for the synthesis of α-gal [13,14]. However, the anti-gal antibody that specifically recognizes α-gal is the most abundant immunoglobulin in the human circulation [13]. Although anti-gal presents a significant immune barrier for transplanting pig organs into humans, it is exploited in cancer immunotherapy to boost the recognition, targeting, and processing of autologous tumor antigens by dendritic cells, thus inducing strong immune responses [15-17].

In this study, we combined the immune-boosting α-gal/anti-gal system with tumor/DC fusion cells. We hypothesized that fusing tumor cells expressing the heterologous α-gal epitope with dendritic cells would generate a cancer vaccine with enhanced immunogenicity and superior anticancer activity. To test our hypothesis, we used human breast adenocarcinoma MDA-MB-231 cells as model system and examined the anticancer activities of the novel cancer vaccine both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cells and animals

Human embryonic kidney 293 T, human hepatocellular carcinoma HepG2, and human mammary adenocarcinoma MDA-MB-231 cells were purchased from the American Type Culture Collection at 2015 (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μg/mL streptomycin (Sigma-Aldrich) in a sterile incubator at 37 °C infused with 5% CO_2. To stably express α-gal in HepG2 or MDA-MB-231 cells, the cDNA for the α-1, 3-GT gene was cloned into the lentiviral vector pLVX-Puro. The reconstructed α-1, 3-GT-expressing lentiviral plasmid or empty lentiviral plasmid vector was transfected into 293 T cells to produce the respective lentivirus. MDA-MB-231 cells were infected with lentivirus stably expressing α-1, 3-GT and then cultured in complete DMEM containing 0.35 μg/mL puromycin. Cells were selected with puromycin and were designated as MDA-MB-231(Gal^+) cells. As controls, cells infected with control lentivirus were referred to as MDA-MB-231(Gal^-) cells, Western blotting and flow cytometry analysis was used to detect the expression of α-1,3-GT in MDA-MB-231(Gal-) cells and MDA-MB-231(Gal+), The results have been published [18].

Female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice of 3 to 4 weeks age and 18 to 22 g in body weight were purchased from Vitalriver (Beijing, China) and housed under specific pathogen-free conditions. All animal protocols were approved by the Institutional Animal Care and Use Committee (Guangxi Medical University, Nanning, China). All experimental protocols were approved, and the methods were carried out according to the relevant guidelines and regulations.

2.2. Preparation of dendritic cells and T cells from human peripheral circulation

All protocols involving the use of human samples were approved by the Institutional Review Board (Guangxi Medical University). Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors through density gradient centrifugation and cultured in RPMI-1640 medium (Gibco) containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin [19]. After 2 h, cells in suspension were transferred to a new dish and cultured in RPMI-1640 medium containing 100 U/mL recombinant human IL-2 (R&D, Minneapolis, MN, USA), while the adherent cells were cultured in RPMI-1640 medium containing 1000 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; R&D) and 500 U/mL rhIL-4 (R&D) to induce differentiation into dendritic cells [20]. Half of the medium was replaced every 3 days.

2.3. Generation of MDA-MB-231(Gal^+)/DC fusion cells

MDA-MB-231 cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich), and dendritic cells were labeled with PKH26 (Sigma-Aldrich) on the 5th day of culture according to the manufacturer's instructions. The two cell types were then mixed at a 1:1 tumor/DC ratio, exposed to polyethylene glycol (PEG; 50% (w/v); Sigma-Aldrich) that was preheated to 40 °C and transferred to a water bath at 40 °C for 3 min. The reaction was stopped by the addition of 40 mL of phosphate-buffered saline (PBS). After centrifugation at 352 × *g* for 10 min, the fusion cells was suspended in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and cultured at 37 °C for 48 h.

2.4. Flow cytometry

To characterize the phenotypes of fusion cells, on day 2 after the fusion reaction, cells were stained with phycoerythrin (PE)-conjugated anti-CD80, PE-conjugated anti-CD86, or PE-conjugated anti- MHC-II antibody (eBiosciences, San Diego, CA, USA) at 4 °C in the dark for 30 min, washed in PBS, and analyzed by flow cytometry.

T cells were labeled with CFSE and seeded into a 24-well plate to analyze proliferation. After incubation with MDA-MB-231(Gal^+) cells, dendritic cells alone, a mixture of MDA-MB-231(Gal^+) cells and dendritic cells (MDA-MB-231(Gal^+) cells+DC), MDA-MB-231 (Gal^-)/DC, or MDA-MB-231(Gal^+)/DC were incubated at 37 °C for 5 days. Then, CFSE-labeled T cells were purified using nylon wool chromatography (Seebio, Shanghai, China) and analyzed by flow cytometry.

To assess T-cell activation, cells were seeded into a 24-well plate and incubated with MDA-MB-231(Gal^+) cells, dendritic cells alone, a mixture of MDA-MB-231(Gal^+) cells and dendritic cells [MDA-MB-231(Gal^+)+DC], MDA-MB-231(Gal^-)/DC, or MDA-MB-231(Gal^+)/DC at 37 °C for 5 days. T cells were then purified using nylon wool chromatography (Seebio) and stained with PE-conjugated anti-CD69 or PE-conjugated anti-CD25 antibody (eBiosciences) at 4 °C in the dark for 30 min, washed in PBS, and analyzed by flow cytometry.

To examine T-cell-mediated cytolysis against tumor cells, T cells (effector cells) were incubated with MDA-MB-231(Gal^+) cells, dendritic cells alone, a mixture of MDA-MB-231(Gal^+) cells and dendritic cells (MDA-MB-231(Gal^+) cells+DC), MDA-MB-231 (Gal^-)/DC, or MDA-MB-231(Gal^+)/DC at a 10:1 ratio and 37 °C for 5 days. Subsequently, the effector T cells were purified using nylon wool chromatography, and incubated with PKH26-labeled MDA-MB-231 cells (target cells) at an effector cell to target cell (E:T) ratio of 10:1, 20:1, 40:1 or the indicated PKH26-labeled tumor cells at an E:T ratio of 40:1. After incubation at 37 °C for 6 h, the target cells were collected, stained with 7-aminoactinomycin D (7-AAD; eBiosciences) according to the manufacturer's instructions, and analyzed by flow cytometry.

To quantify $CD4^+$ and $CD8^+$ T cells in the peripheral circulation and tumors from mice, PBMCs were isolated from the peripheral

blood and a single-cell suspension was prepared from tumor tissues as described previously [21]. The PBMCs or cell suspensions from the tumor tissues were then incubated with PE-conjugated anti-CD3 together with FITC-conjugated anti-CD4 or FITC-conjugated anti-CD8 antibody (eBiosciences) at 4 °C in the dark for 30 min, washed in PBS, and analyzed by flow cytometry.

All flow cytometry experiments were performed on a FACS Calibur cytometer (Beckman Coulter, Brea, CA, USA) and data were analyzed using FlowJo software (Version 10.0, Ashland, OR, USA) and Expo32 ADC (Beckman Coulter).

2.5. Enzyme-linked immunosorbent assay (ELISA)

To examine the secretion of IL-12p70 from the fusion cells, cells were seeded into a 96-well plate at 5×10^5 cells/well and cultured for 7 days. The conditioned medium was collected from each well and examined using an IL-12p70 ELISA kit (eBiosciences) according to the manufacturer's instructions.

To measure the production of IL-2 and IFN-γ from T lymphocytes in response to the MDA-MB-231(Gal$^+$)/DC fusion cells, T cells were seeded into a 24-well plate and incubated with MDA-MB-231 (Gal$^+$) cells, dendritic cells alone, a mixture of MDA-MB-231(Gal$^+$) cells and DC (MDA-MB-231(Gal$^+$) cells+DC), MDA-MB-231(Gal$^-$)/DC, or MDA-MB-231(Gal$^+$)/DC at 37 °C for 5 days. After purification using nylon wool chromatography, T cells were seeded into a 48-well plate and cultured at 37 °C for another 5 days. The levels of IL-2 and IFN-γ in the conditioned medium were measured using the corresponding ELISA kits (eBiosciences) according to the manufacturer's instructions.

The serum levels of IL-2, IL-12, IFN-γ, and total IgG from mice were measured using the corresponding ELISA kits (eBiosciences) as per manufacturer's instructions.

2.6. In vivo xenograft tumor model

To establish the *in vivo* xenograft tumor model, MDA-MB-231 cells were injected subcutaneously into the right inguinal region (2×10^6 cells/mouse) of NOD/SCID mice. When the tumor grew to approximately 0.5 mm in diameter, the humanized immune system was established by tail vein injection of 200 μL of fresh human serum together with an intraperitoneal injection of 1×10^7 freshly isolated PBMCs every 5 days, for a total of six injections. After the establishment of the humanized immune system, mice were randomly divided into various groups (N = 6/group). Fusion cells (1×10^6/injection) were administered through the tail vein every 7 days, for a total of five injections. The length (L) and width (W) of each tumor was measured every 7 days, and the tumor volume (V) was calculated as $V = L \times W^2 \times 0.5$. Mice survival were monitored and the mouse survival was determined using Kaplan–Meier analysis.

2.7. Immunohistochemistry (IHC)

Tumor tissues isolated from the mice were fixed in 4% paraformaldehyde and processed into paraffin-embedded tissue sections. The proliferation and apoptosis within the tumor tissues were examined by IHC using the anti-Ki67 antibody (Boster, Wuhan, China) and a terminal-deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) kit (Roche, Basle, Switzerland), respectively, according to the manufacturer's instructions.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Comparison between the experimental group and the control group was performed using the one-way analysis of variance test. Bonferroni's test was used for multiple comparisons. Mouse survival was plotted using the Kaplan–Meier method and analyzed using the log-rank test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Preparation and characterization of MDA-MB-231 (Gal$^+$)/DC fusion cells

We tested our hypothesis that inducing the expression of heterologous α-gal in a tumor/DC fusion cell will combine the anti-gal-mediated immunity with dendritic cells presenting a broad range of tumor antigens further boosting the anticancer effects. We stably expressed α-1, 3-GT in MDA-MB-231 cells (MDA-MB-231(Gal$^+$)) while MDA-MB-231(Gal$^-$) cells were used as controls. We labeled MDA-MB-231(Gal$^+$) cells and dendritic cells with CFSE (green fluorescence) and PKH26 (red fluorescence), respectively, to monitor the fusion efficiency. Following the PEG-mediated fusion reaction, we observed that a large number of fusion cells were positive for both green and red fluorescence, suggesting that they represented a fusion between MDA-MB-231 (Gal$^+$) cells and dendritic cells (Fig. 1A). Quantification by flow cytometry showed that the fusion efficacy was approximately 60% for both MDA-MB-231(Gal$^+$)/DC and MDA-MB-231(Gal$^-$)/DC fusion cells (Fig. 1B–C).

To characterize the functional status of the MDA-MB-231(Gal$^+$)/DC fusion cells, we examined the expression levels of several DC activation/maturation markers, including MHC-II, CD80, and CD86 and the production of IL-12p70. The results showed MDA-MB-231(Gal$^+$)/DC secreted a significantly higher level of IL-12p70 than MDA-MB-231(Gal$^+$) alone, dendritic cells alone, MDA-MB-231(Gal$^+$)+DC, or MDA-MB-231(Gal$^-$)/DC (Fig. 1D).The expression levels of CD80, CD86 and MHC-II were significantly upregulated in MDA-MB-231(Gal$^+$)/DC (Fig. 1E–F).

3.2. MDA-MB-231 (Gal$^+$)/DC stimulated in vitro proliferation, activation of T cells

To explore the functional activities of MDA-MB-231(Gal$^+$)/DC, we focused on their effects on the proliferation, activation, and cytokine production of T cells. Upon incubation with MDA-MB-231(Gal$^+$)/DC, T-cell proliferation was significantly increased, when compared to incubation with MDA-MB-231(Gal$^+$) or dendritic cells alone, MDA-MB-231(Gal$^+$)+DC, or MDA-MB-231(Gal$^-$)/DC (Fig. 2A–B). Similar effects were also observed on the expression levels of T-cell activation markers CD25 (Fig. 2C-D) and CD69 (Fig. 2E–F) as well as the secretion of proinflammatory cytokines IL-2 and IFN-γ (Fig. 3A-B, $P < 0.01$, $P < 0.001$), suggesting that MDA-MB-231(Gal+)/DC possessed superior activities to stimulate T cells.

3.3. MDA-MB-231 (Gal$^+$)/DC enhanced in vitro T-cell-mediated cytotoxicity against MDA-MB-231 cells

Given that MDA-MB-231(Gal$^+$)/DC were associated with higher antigen-presentation features and significantly activated T cells, we examined their effects on T-cell-mediated cytolysis against tumor cells. For this purpose, we stimulated T cells with MDA-MB-231(Gal$^+$)/DC or other controls for 5 days, purified T cells from the cell mixture, and then applied the isolated T cells to the parental MDA-MB-231 cells. As shown in Fig. 3C, at different E:T ratios, T cells stimulated by MDA-MB-231(Gal$^+$)/DC displayed the highest cytolytic activity on MDA-MB-231 cells compared to those stimulated with MDA-MB-231(Gal$^+$) alone, dendritic cells alone, MDA-MB-231(Gal$^+$)+DC, or MDA-MB-231(Gal$^-$)/DC ($P < 0.05$),

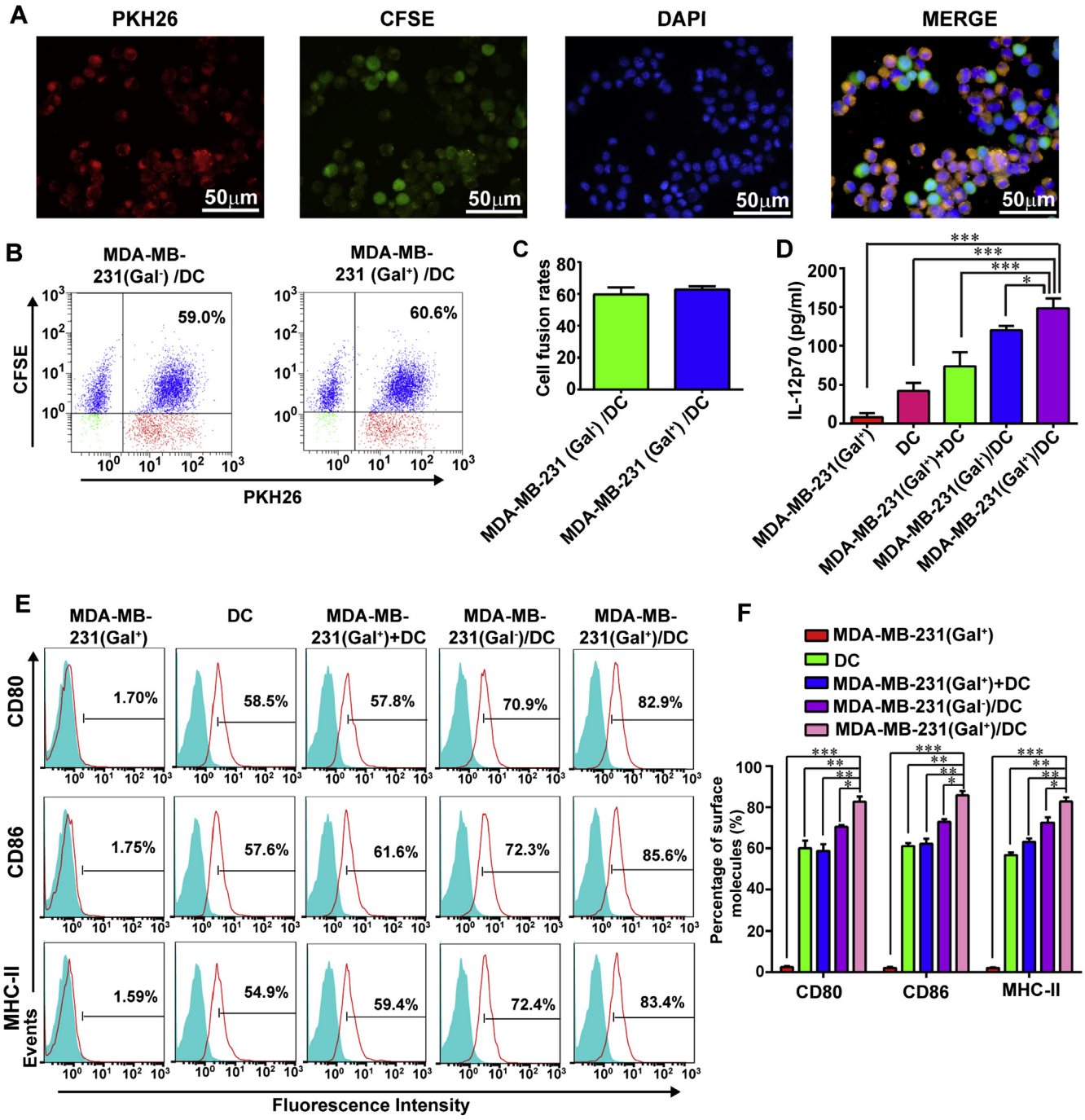

Fig. 1. MDA-MB-231(Gal$^+$)/DC fusion cells display characteristics of enhanced antigen presentation. (A) Identification of MDA-MB-231(Gal$^+$)/DC fusion cells. CFSE-labeled MDA-MB-231 cells (green) were fused with PKH26-labeled dendritic cells (red), the cells were stained with DAPI (blue).Fluorescence microscopic images are shown with a representative image from each individual channel and a merged image. scale bar, 50 μm. (B) Fusion efficiency of MDA-MB-231(Gal$^+$)/DC fusion cells or MDA-MB-231(Gal$^-$)/DC fusion cells as measured by flow cytometry on CFSE$^+$PKH26$^+$ cells. (C) Quantitative analysis. There was no significant difference between the two groups. (D) Secretion of IL-12p70 from the indicated cells measured by ELISA. (E-F) Expression levels of MHC-II, CD80, and CD86 on the indicated cells by flow cytometry. Data are presented as the mean±standard deviation from at least three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$. MDA-MB-231(Gal$^+$), MDA-MB-231 cells expressing heterologous α-gal; DC, dendritic cells; MDA-MB-231(Gal$^+$)+DC, the mixture of MDA-MB-231(Gal$^+$) cells and dendritic cells; MDA-MB-231(Gal$^-$)/DC, MDA-MB-231(Gal-)/DC fusion cells; MDA-MB-231(Gal$^+$)/DC, MDA-MB-231(Gal$^+$)/DC fusion cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggesting that MDA-MB-231(Gal$^+$)/DC enhanced the *in vitro* anticancer activities of T cells. Furthermore, we investigated the specificity of MDA-MB-231(Gal$^+$)/DC and discovered that T cells stimulated with MDA-MB-231(Gal$^+$)/DC were most effective in killing MDA-MB-231 cells. In contrast, the cytolysis of these T cells against other cancer cells, including HepG2 (Gal$^+$), HepG2, and A549 cells, was significantly reduced and there was minimal cytolysis against the noncancerous H293T cells, indicating that MDA-MB-231(Gal+)/DC induced highly specific anticancer activities of T cells (Fig. 3D).

3.4. MDA-MB-231 (Gal$^+$)/DC exhibited substantial antitumor activities in vivo

The promising results we achieved in the *in vitro* experiments prompted us to examine the *in vivo* anticancer effects of

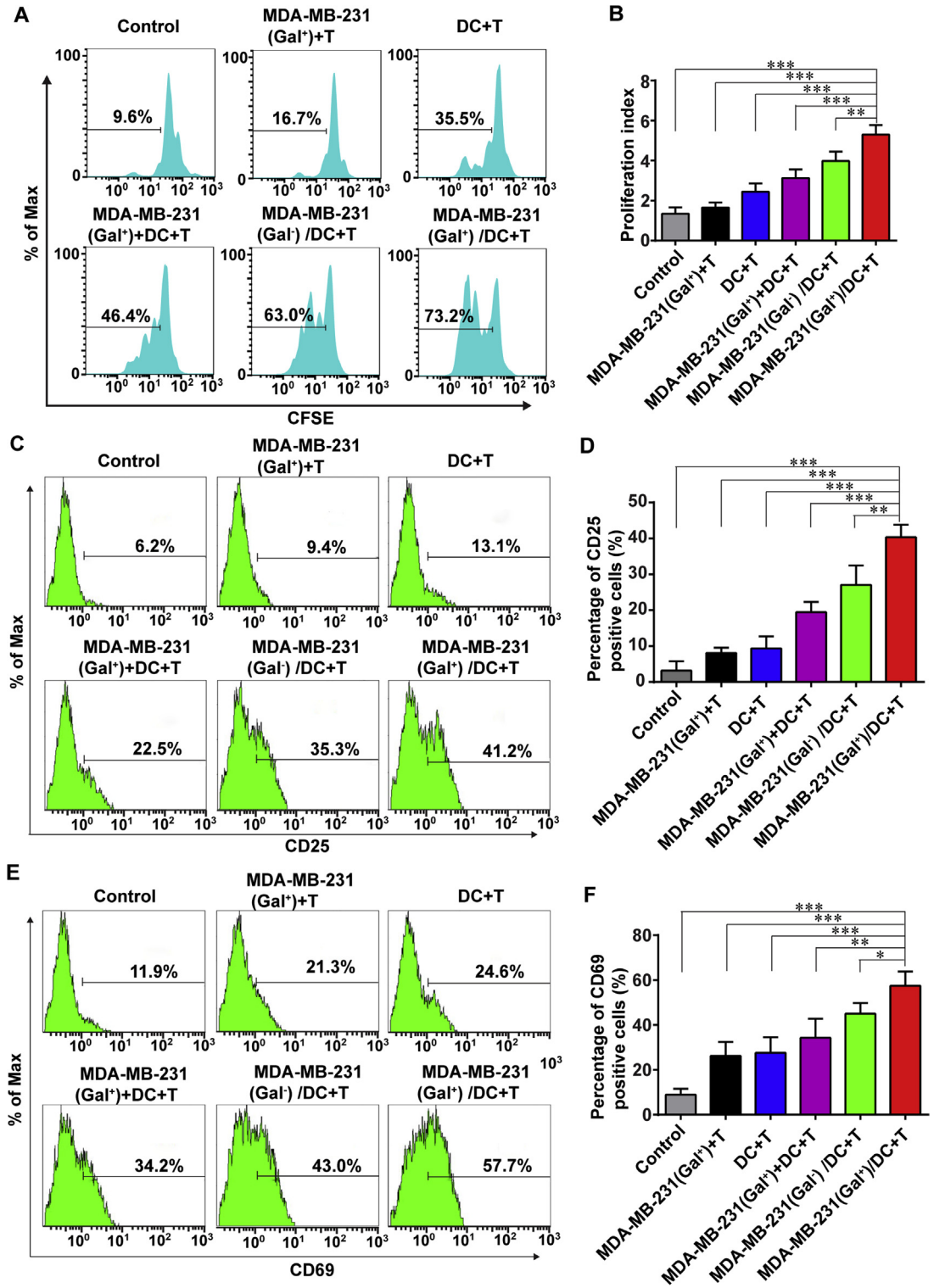

Fig. 2. MDA-MB-231(Gal$^+$)/DC fusion cells stimulated T-cell activation. T cells labeled with or without CFSE were incubated with the indicated cells and then purified. (A) T cells proliferation were examined by flow cytometry. (B) The proliferation index was analyzed using ModFit LT. Expression levels of CD25 (C-D) and CD69 (E-F) were examined by flow cytometry and presented as the percentage of CD25-positive cells or CD69-positive cells from each group. Data are presented as histograms and expressed as the mean±SD of individual groups of cells from three separate experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

MDA-MB-231(Gal$^+$)/DC. First, we established MDA-MB-231 xenograft tumors in NOD/SCID mice with humanized immune system in mice. The mice were treated with MDA-MB-231(Gal$^+$)/DC or with other control cells including vehicle control (PBS), MDA-MB-231(Gal$^+$) alone, dendritic cells alone, the mixture of MDA-MB-231 (Gal$^+$) and DC cells, or MDA-MB-231(Gal$^-$)/DC. As shown in Fig. 4, tumor growth was most robustly suppressed in mice receiving MDA-MB-231(Gal$^+$)/DC when compared to mice treated with the other cells (Fig. 4A). The delay and inhibition of tumor growth also translated to mouse survival: the median survival time for mice

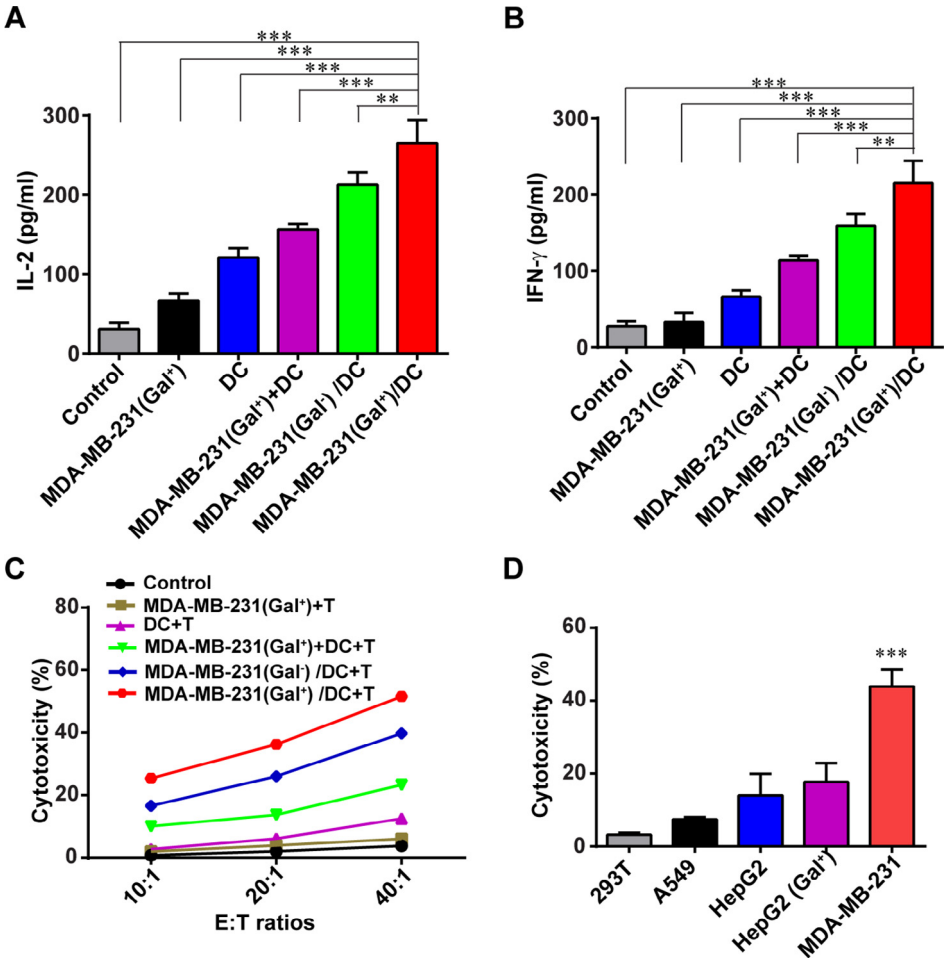

Fig. 3. MDA-MB-231(Gal$^+$)/DC fusion cells promoted T cell-mediated cytolysis against tumor cells. Secretion of IL-2 and IFN-γ into the conditioned medium by T cells was examined by ELISA. T cells (effector cells) were incubated with the indicated cells, and subsequently incubated with PKH26-labeled MDA-MB-231 cells (target cells) at the different ratios for 6 h. Apoptosis of tumor cells was determined by staining with 7-aminoactinomycin D (7-AAD) followed by flow cytometry, the percentage of 7-AAD$^+$ cells was presented as cytotoxicity (%). E: T, effector cells to target cells. Data are presented as the mean±SD from at three independent experiments. (A) The levels of IL-2. (B) The levels of IFN-γ. (C) Cytotoxicity to the target cells at the different ratios. (D) Cytotoxicity of T cells stimulated by MDA-MB-231(Gal$^+$)/DC fusion cells was compared for the indicated target cells, the ratio of T cells to target cells was 40:1. $^{**}P < 0.01$, $^{***}P < 0.001$.

treated with MDA-MB-231(Gal$^+$)/DC was 112 days, longer than that with MDA-MB-231(Gal$^-$)/DC (102 days), MDA-MB-231(Gal+) +DC (88 days), DC alone (70 days), MDA-MB-231(Gal$^+$) cells alone (65 days), or PBS (57 days). (Fig. 4B).

To examine whether the suppression of tumor growth was associated with any changes in tumor cell viability, we examined the proliferative and apoptotic activities within the tumor tissue by Ki67 IHC staining and TUNEL assays, respectively. Our data showed that treatment with MDA-MB-231(Gal$^+$)/DC resulted in the lowest percentage of Ki67$^+$ cells and the highest percentage of TUNEL$^+$ cells compared to mice treated with other groups (Fig. 4C–F), suggesting that MDA-MB-231(Gal$^+$)/DC effectively inhibited tumor growth through directly and/or indirectly targeting the proliferation/apoptosis of tumor cells.

3.5. MDA-MB-231 (Gal$^+$)/DC fusion cell elevated post-treatment systemic immunity

To assess the effects of MDA-MB-231(Gal$^+$)/DC on the immune system, we measured the percentages of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells in both the peripheral circulation and the tumor tissues. Our data showed that both cell populations were significantly increased in the peripheral circulation and tumor tissues in response to the treatment with MDA-MB-231(Gal$^+$)/DC when compared to the other groups (Fig. 5). Furthermore, treatment with MDA-MB-231(Gal$^+$)/DC also significantly elevated the serum levels of IL-12, IL-2, IFN-γ and total IgG compared to treatments with the other cells (Fig. 6).

4. Discussion

Here we presented evidence supporting our novel hypothesis that ectopically expressing α-gal in MDA-MB-231(Gal$^+$) tumor cells augmented the immunogenicity of a tumor/DC fusion cell vaccine by stimulating the activation/maturation of dendritic cells, elevating the production of IL-12, and inducing the proliferation, activation, and cytokine production of T cells. This translated into superior cytotoxicity specific for MDA-MB-231 tumor cells but not for other tumor cells or normal cells. More importantly, the *in vivo* administration of α-gal-expressing MDA-MB-231(Gal$^+$)/DC into tumor-bearing mice strongly activated systemic immune responses, inhibited tumor growth, and prolonged mouse survival.

As the most potent antigen-presenting cells (APCs) and also controlling both immune activation and tolerance, dendritic cells have become an ideal vehicle for generating a cancer vaccine. For cancer therapy, a DC vaccine is expected to induce anticancer immunity by expanding effector T cells including CD4$^+$ T helper 1 (Th1) and CD8$^+$ cytolytic T lymphocytes (CTLs), inhibiting

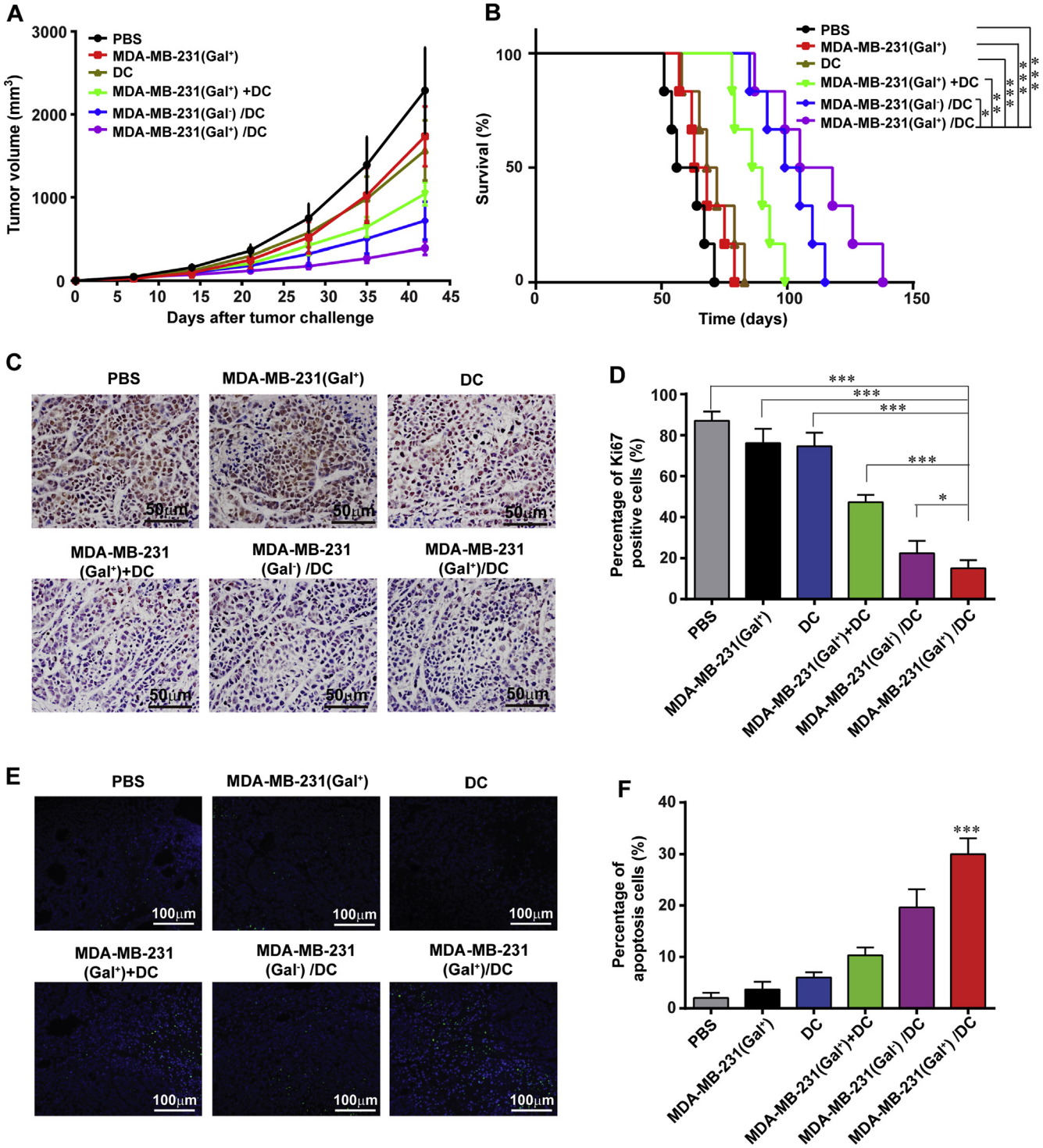

Fig. 4. MDA-MB-231(Gal+)/DC fusion cells vaccine exhibited certain anticancer activities *in vivo*. MDA-MB-231 cells were subcutaneously injected into the right inguinal region of NOD/SCID mice. The mice (n = 6 mice per group) were treated with PBS, MDA-MB-231(Gal$^+$), DC, MDA-MB-231(Gal$^+$)+DC, MDA-MB-231(Gal$^-$)/DC vaccine, MDA-MB-231(Gal$^+$)/DC vaccine weekly for five weeks. (A) Tumor volumes. (B) Mouse survival was determined using Kaplan–Meier analysis. (C) Proliferation within the tumor tissue was examined by IHC staining for Ki67 (400 × magnification). (D) Quantitative analysis of the percentage of Ki67-positive cells within each image. (E) Apoptosis within the tumor tissue was examined by the TUNEL assay (200 × magnification). (F) Quantitative analysis of the percentage of TUNEL-positive cells within each image. Ki67-positive cells and apoptotic tumor cell were counted in 5 randomly selected fields of tumor thin sections. Data are presented as representative images or expreseed as mean±SD of each group of tumors. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to other groups.

immunosuppressive regulatory T (Treg) cells, and generating immune memory to prevent cancer relapse [22]. However, cancers through interactions with multiple components of the microenvironment develop various mechanisms to evade host immune attack such as maintaining dendritic cells at an immature, functionally impaired state with minimal capability for antigen presentation. This may account for the challenges experienced in clinical trials of DC-based vaccines [23,24]. Therefore, strategies to endow DC-based vaccines with a stronger ability for presenting antigens and activating effector T cells are highly desired. Anti-gal is a

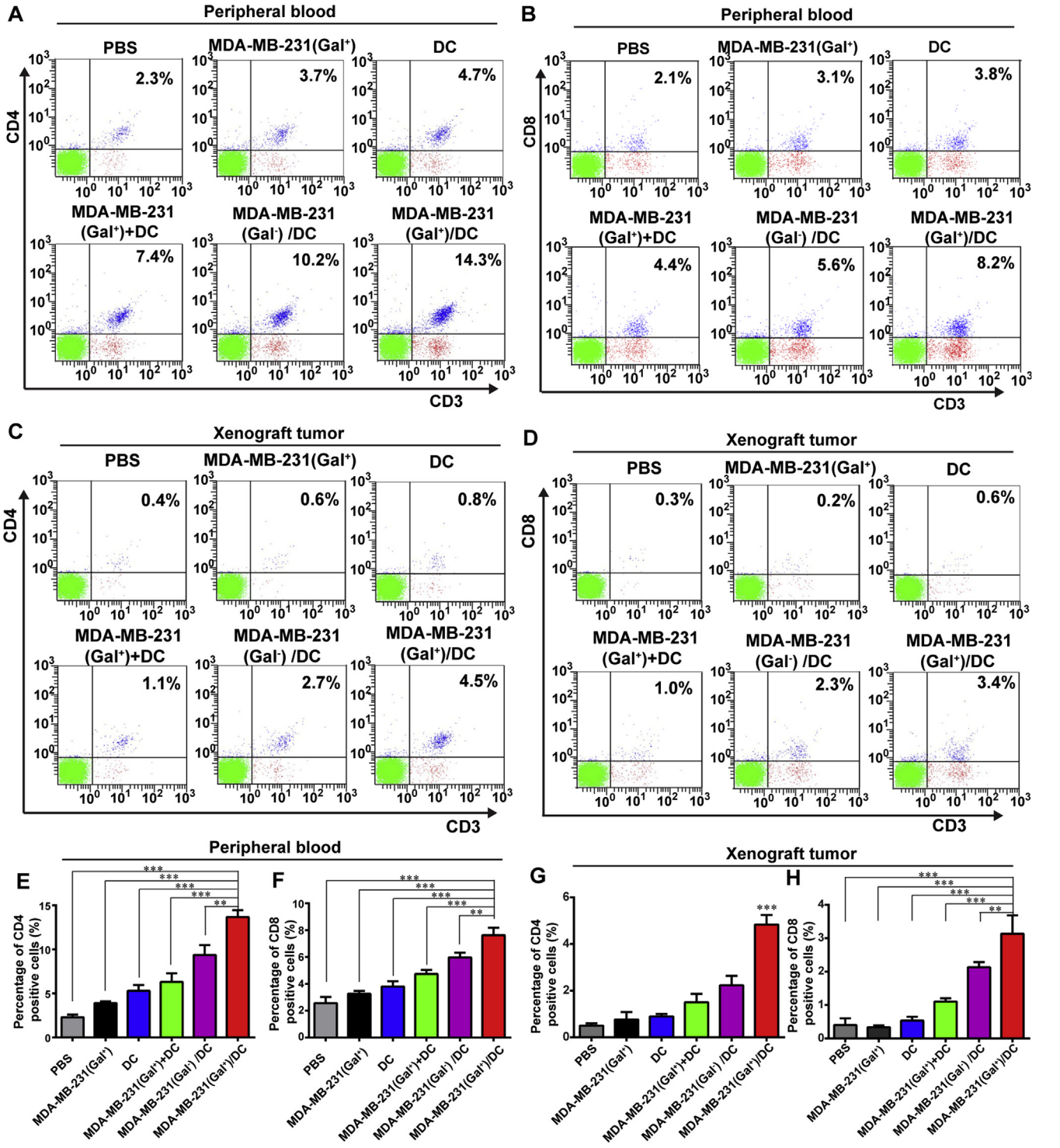

Fig. 5. MDA-MB-231(Gal+)/DC fusion cells boost the systemic immunity in tumor-bearing mice. Peripheral blood and tumor tissues were collected from NOD/SCID mice bearing MDA-MB-231 xenograft tumors at 1 week after the last treatment with the indicated cells. The percentages of $CD4^+CD3^+$ and $CD8^+CD3^+$ T cells in the peripheral circulation (A and B) and within tumor tissues (C and D) were examined by flow cytometry and compared between different groups (E to H). Data are presented as representative flow cytometry charts or expressed as the mean±SD of each group (n = 5) of cells from three separate experiments. The percentage of $CD4^+CD3^+$ and $CD8^+CD3^+$ T cells in the blood/tumor of mice treated with MDA-MB-231(Gal^+)/DC fusion vaccine was higher than that of other control groups. $^{**}P < 0.01$, $^{***}P < 0.001$.

polyclonal antibody that accounts for approximately 1% of the circulating IgG in humans [25]. Although polyclonal in nature, anti-gal exhibits a high specificity for α-gal but not other carbohydrate epitopes expressed on mammalian cells [26-28]. The strong specificity between anti-gal and α-gal, together with the abundance of anti-gal antibody and the absence of α-gal in humans, not only significantly thwarts the xenotransplantation of organs from pigs to humans but also presents a unique tool that may benefit cancer immunotherapy. When introduced into the human body, α-gal complexes with anti-gal, the Fc portion of the latter binding to the Fcγ receptor of APCs such as dendritic cells or macrophages, and stimulates the phagocytosis of APCs [29-31]. Exploiting this

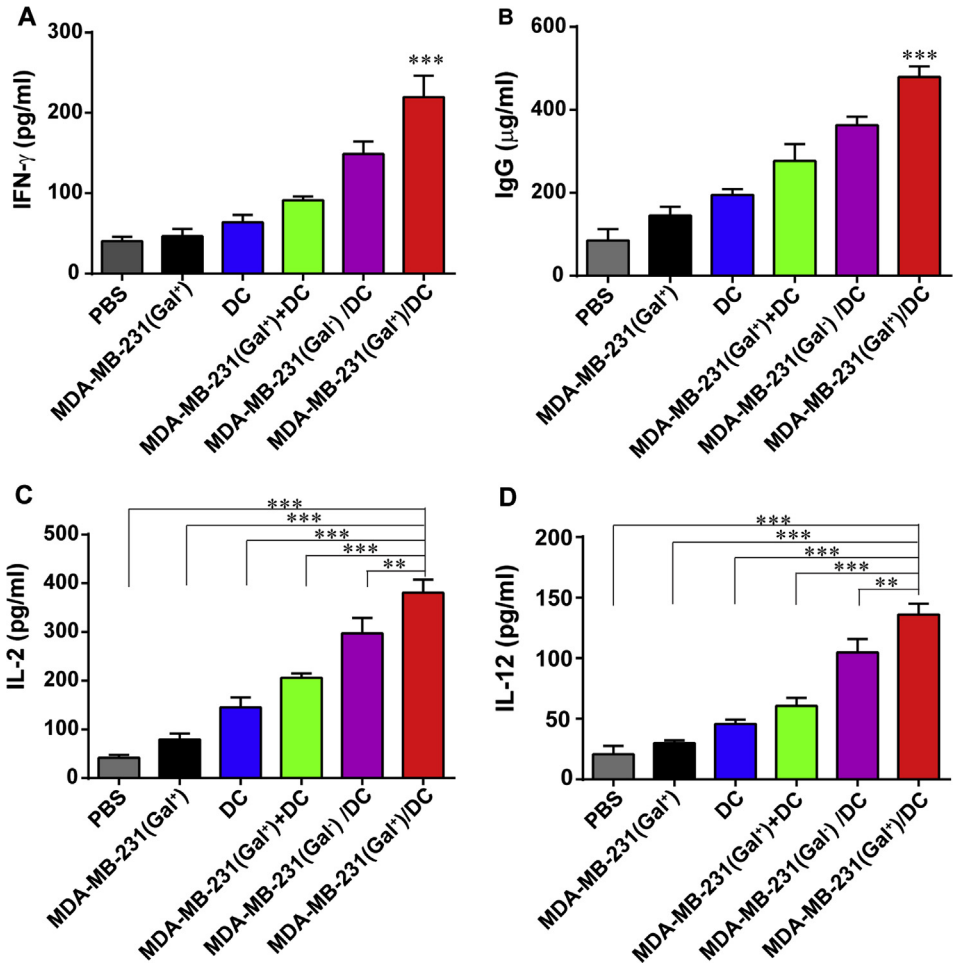

Fig. 6. Treatment with MDA-MB-231 (Gal^+) /DC fusion cells promoted the secretion of cytokine in serum. Serum was collected from the blood of each group mice (n = 3) and the levels of IFN-γ, IgG, IL-2 and IL-12 were examined by ELISA. Data are expressed as the mean±SD of each group from three separate experiments. The levels of cytokines IFN-γ, IgG, IL-2 and IL-12 in mice treated with MDA-MB-231(Gal^+)/DC fusion vaccine were higher than those of other control groups MDA-MB-231 (Gal^-) /DC, MDA-MB-231 (Gal^+)+DC, DC, MDA-MB-231 (Gal^+) and PBS. $^{**}P < 0.01$, $^{***}P < 0.001$.

mechanism, Rossi et al ectopically expressed α-gal in B16 melanoma cells (B16αGal) and irradiated these cells to generate a whole-cell vaccine [32]. When administered to mice with pre-existing xenografts or pulmonary metastasis derived from α-gal-negative B16, B16αGal significantly inhibited the xenograft growth as well as pulmonary metastasis thus improving mouse survival. At the cellular level, spleen mononuclear cells from mice treated with B16αGal specifically induced the activation (as detected by CD25 and CD69 expression) and production of tumor necrosis factor (TNF-α) of $CD8^+$ T cells, which potently suppressed pulmonary metastasis [32] demonstrating the anticancer efficacy of the α-gal/anti-gal system. Furthermore, Rossi et al. have shown that sensitizing mice with an irradiated whole-cell B16αGal vaccine afforded long-term protection against xenograft growth or pulmonary metastasis of α-gal-negative B16 cells associated with a superior capability to expand cytotoxic T cells [33]. Similarly, α-gal-mediated immunogenicity has been examined in the context of lymphoma [17,34], hepatocellular carcinoma [35], ovarian cancer [36], pancreatic carcinoma [15,37,38], and breast cancer corroborating the anticancer promise of vaccines expressing the heterologous α-Gal gene.

In this study, we first introduced the stable expression of α-gal in human breast adenocarcinoma MDA-MB-231 cells, which were subsequently used to generate a tumor/DC fusion cells. The expression of α-Gal in MDA-MB-231 cells did not affect the fusion efficiency between MDA-MB-231 cells and dendritic cells. CD80, CD86 and MHC molecules were important molecules on the surface of mature dendritic cells and played an important role in the activation of T cells and the proliferation of T cell. MDA-MB-231 (Gal^+)/DC were associated with higher levels of antigen-presenting MHC-II and, CD80, and CD86 when compared to derive from MDA-MB-231(Gal^-) as well as dendritic cells and other controls. IL-12 is a pleiotropic proinflammatory cytokine that critically controls the differentiation of naive T cells to Th1 cells, stimulates the cytotoxicity of $CD8^+$ T cells and NK cells, and promotes the production of proinflammatory cytokines such as IFN-γ and TNF-α from effector cells [39-41]. Consistent with the upregulation of these markers, we observed that MDA-MB-231(Gal^+)/DC more potently stimulated T-cell proliferation, activation, and production of proinflammatory cytokines *in vitro*. The fusion cells expanded both $CD4^+$ and $CD8^+$ populations *in vivo* within the peripheral circulation and tumor tissues and elevated the serum levels of IL-12, IL-2, IFN-γ, and total IgG. Functionally, T cells stimulated with MB-231(Gal^+)/DC displayed a higher *in vitro* cytotoxicity against the parental MDA-MB-231 cells when compared to MB-231(Gal^-)/DC and other controls. Also, administration of MB-231(Gal^+)/DC into mice bearing pre-existing MDA-MB-231 xenografts inhibited tumor growth and prolonged mouse survival. To the best of our

knowledge, this is the first study showing that the introduction of α-gal into a tumor/DC fusion cell could substantially promote the immunogenicity and the anticancer potency of the vaccine.

Studies on tumor/DC fusion cells have reported attractive anticancer activities in an *in vitro* cell culture system or experimental rodent cancer models [42-47]. However, the suboptimal responses achieved in clinical trials emphasize the drastic differences between human and rodent immune systems. To improve the translation potential of findings from the rodent system into clinical applications, humanized mice have been developed and are being widely used in basic research on immunotherapy [48]. Using this strategy, we established a humanized immune system in NOD/SCID mice bearing pre-existing xenografts by engrafting the mice with freshly isolated human serum and PBMCs. The human serum provided abundant anti-gal IgG to enable the opsonization of the α-gal-coated tumor /DC fusion cells and the hematopoietic PBMC cells differentiated into various immune components. In this humanized model system, we showed that MDA-MB-231(Gal^+) / DC significantly activated the anticancer immune response, inhibited tumor growth, and, more importantly, prolonged the mouse survival. The observations in this study may thus represent a higher level of translation potential for the fusion cells.

In this study, the expression of α-gal was achieved through the resumption of the α-gal biosynthetic enzyme α1,3GT by viral transduction [18]. We realized that this is probably not a valid approach in clinical practice, since primary tumor cells isolated from patients may not undergo active proliferation. Galili et al. have reported the use of a mixture containing neuraminidase, α1, 3GT, and uridine diphosphate galactose to synthesize α-Gal on the membrane preparation from ovarian carcinoma cells [16]. Manches et al. have applied the same reaction to express α-gal epitopes on intact lymphoma cells and have shown that this method effectively loads a large amount of α-gal epitopes (>10^6/lymphoma cell) and induces the phagocytosis of autologous tumor cells modified with these epitopes by APCs. Our study presented the proof-of-principle evidence that expressing α-Gal epitopes on breast cancer cells substantially enhances the immunogenicity and induces the anticancer immunity of a tumor/DC fusion cell. Further studies should be carried out to develop a simple yet efficient method for expressing abundant α-Gal epitopes on a tumor/DC fusion cell either by biochemical synthesis or by transgene expression delivered by vectors that can target nonproliferative cells, such as a lentiviral vector.

A study was performed on 11 patients with a variety of tumors including 3 pancreatic adenocarcinomas, 2 colon cancers, 2 pancreatic neuroendocrine tumors, and 1 each of prostate cancer, renal cell cancer, ovarian cancer, and mucinous appendiceal cancer. The tumors were not amenable to surgical resection or other treatments. Intratumoral injections of α-gal glycolipids in these patients showed no toxicity or autoimmunity with an unexpectedly long survival in 2 patients with pancreatic adenocarcinoma, although disease progression was observed at different time points in all patients [49]. This study suggests that the immune reaction derived from the α-gal/anti-gal system is safe and potentially effective in eliminating α-gal-labeled antigens. Consistent with the results of this study, we observed that the administration of the MDA-MB-231(Gal^+)/DC fusion cell into tumor-bearing mice with a humanized immune system did not produce any obvious toxic reactions and elicited prolonged survival.

In summary, we provided evidence that expression of heterologous α-gal epitopes on the MDA-MB-231/DC fusion cell significantly boosted the immunogenicity of the fusion cell to activate effector T cells and enhance their cytotoxicity against MDA-MB-231 cells. Using NOD/SCID mice with an introduced humanized immune system, we demonstrated the *in vivo* anticancer potential of this novel strategy. Future studies should explore its ability to target cancer metastasis, to generate immune memory for preventing cancer relapse, and possibly more robust anticancer activities in combination with other therapeutic regimens.

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Conflict of interest

All authors declare no conflict of interest

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