


ORIGINAL ARTICLE

Cancer immunotherapy using artificial adjuvant vector cells to deliver NY-ESO-1 antigen to dendritic cells in situ

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Abstract

NY-ESO-1 is a cancer/testis antigen expressed in various cancer types. However, the induction of NY-ESO-1-specific CTLs through vaccines is somewhat difficult. Thus, we developed a new type of artificial adjuvant vector cell (aAVC-NY-ESO-1) expressing a CD1d-NKT cell ligand complex and a tumor-associated antigen, NY-ESO-1. First, we determined the activation of invariant natural killer T (iNKT) and natural killer (NK) cell responses by aAVC-NY-ESO-1. We then showed that the NY-ESO-1-specific CTL response was successfully elicited through aAVC-NY-ESO-1 therapy. After injection of aAVC-NY-ESO-1, we found that dendritic cells (DCs) in situ expressed high levels of costimulatory molecules and produced interleukin-12 (IL-12), indicating that DCs undergo maturation in vivo. Furthermore, the NY-ESO-1 antigen from aAVC-NY-ESO-1 was delivered to the DCs in vivo, and it was presented on MHC class I molecules. The cross-presentation of the NY-ESO-1 antigen was absent in conventional DC-deficient mice, suggesting a host DC-mediated CTL response. Thus, this strategy helps generate sufficient CD8⁺ NY-ESO-1-specific CTLs along with iNKT and NK cell activation, resulting in a strong antitumor effect. Furthermore, we established a human DC-transferred *NOD/Shi-scid/IL-2γc^{null}* immunodeficient mouse model and showed that the NY-ESO-1 antigen from aAVC-NY-ESO-1 was cross-presented to antigen-specific CTLs through human DCs. Taken together, these data suggest that aAVC-NY-ESO-1 has potential for harnessing innate and adaptive immunity against NY-ESO-1-expressing malignancies.

KEYWORDS

cancer, cytotoxic T cell, dendritic cell, immunotherapy, NKT cell

Abbreviations: α-GalCer, α-galactosylceramide; aAVC, artificial adjuvant vector cell; aAVC-NY-ESO-1, NY-ESO-1-expressing aAVCs; APC, antigen-presenting cell; BM, bone marrow; cDC, conventional dendritic cell; CFSE, carboxyfluorescein succinimidyl ester; CFA, complete Freund's adjuvant; DC, dendritic cell; DC-NOG, DC-transferred; DT, diphtheria toxin; ELISPOT, enzyme-linked immune absorbent spot; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; imDC, immature DC; iNKT, invariant natural killer T; NK, natural killer; NOD, NOD/Shi-scid/IL-2Rγ^{null}; PBLs, peripheral blood leukocytes; PE, phycoerythrin; RT, room temperature; SFCs, spot forming cells; TBS-T, TBS-Tween 20; TCR, T cell receptor; zDC-DTR, Zbtb46- diphtheria toxin receptor.

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1 | INTRODUCTION

Cancer vaccine efficacy depends on the generation of robust tumor antigen-specific CD8⁺ and CD4⁺ T-cell responses. Most cancer vaccines target tumor antigens derived from self-antigens that are overexpressed in malignant cells, but have low expression in normal tissues, such as cancer-testis antigens, oncofetal antigens, and melanosomal antigens.^{1,2} Although antitumor immune responses could be spontaneously elicited against certain self/tumor antigens, the generation of antitumor CD8⁺ CTLs with high avidity is required.^{3,4} NY-ESO-1 is an intracellular cancer/testis antigen expressed in a wide range of tumor types, including 10%–50% of metastatic melanomas and lung, breast, prostate, thyroid, and ovarian cancers^{5–7} as well as 70%–80% of synovial cell sarcomas.^{8,9} Because the NY-ESO-1 antigen is highly immunogenic, it can trigger marked cellular and humoral immune responses in a high percentage of patients with advanced NY-ESO-1-expressing cancers.⁹ To induce NY-ESO-1 responses in vivo, a variety of NY-ESO-1-based vaccines, such as HLA class I- and/or class II-binding peptides, recombinant viruses, DNA, and recombinant proteins, with or without adjuvants, have been explored.^{10–15} Using these strategies, although CD4⁺ T cells are well-elicited, it is somewhat difficult to detect the induction of CTL for NY-ESO-1.^{10,13,16,17}

Recently, the antitumor effects of NK cells of the innate immunity have been reevaluated,^{18,19} and NK cells have been recognized as effector cells in cancer immunotherapies. These cells are particularly effective against HLA-deficient tumor cells, which are not targeted by CD8⁺ CTLs. Moreover, they show strong Ab-dependent cellular cytotoxicity.¹⁹ Furthermore, new cancer immunotherapy methods using NK cells, such as CAR-NK, immune checkpoint blockade for NK cells, and anti-NKG2A Ab, have been developed.^{20–22} Additionally, α -GalCer acts as an immunological adjuvant by activating iNKT cells.^{23,24} Invariant natural killer T cells recognize synthetic and natural glycolipids presented by CD1d, a nonclassical MHC molecule found on APCs. Intravenously injected α -GalCer rapidly activates iNKT cells in the red pulp of the spleen and the liver, thus leading to systemic IFN- γ and IL-4 responses.^{25,26} Furthermore, in both animal and clinical studies using autologous or syngeneic α -GalCer-loaded CD1d⁺ cells (eg, DCs or tumor cells), IFN- γ production by activated iNKT cells was greater than that induced by free α -GalCer.^{27–29} In therapeutic strategies using α -GalCer-loaded CD1d⁺ cells, an adjunctive effect on the NK cell-mediated antitumor activity is apparent.^{28–31} A recent study using α -GalCer-pulsed GM-CSF plus IL-2 cultured cells showed NK cell activation and clinical response in advanced lung cancer patients.³² Natural killer cell activation in addition to CTL induction should be ideal for preventing tumor cells from escaping immune surveillance.

Cancer vaccine approaches based on synthetic long peptides, antigen RNA, or DNA are useful with the added advantage of multiple antigen delivery to APCs over peptide or protein therapy.^{33–36} Particularly, anti-DEC205Ab therapy reportedly targets in vivo DCs. Similar to the DC targeting therapy,^{37,38} we previously reported α -GalCer-loaded, OVA, or WT1 antigen mRNA-transfected allogeneic NIH3T3 fibroblasts,^{39,40} called aAVCs, as a cellular therapy

platform. Such in vivo DC-targeting therapies, including aAVC and anti-DEC205Ab, are more effective than ex vivo DC therapy in promoting prominent CD8⁺ T cell responses. Herein, in an attempt to combine NK cell activation and induction of CTL for NY-ESO-1, we established a new therapeutic cancer vaccine, aAVC-NY-ESO-1, and challenged the induction of not only NK cells but also CTL against NY-ESO-1-expressing tumor cells. We also elucidated the mechanism of aAVC-NY-ESO-1 particularly through DCs in situ.

2 | MATERIALS AND METHODS

2.1 | Animals and cell lines

Pathogen-free C57BL/6 (B6) mice were purchased from Charles River Japan at 6–8 weeks of age. zDC-DTR mice were purchased from Jackson Laboratory. NOG mice were purchased from the Central Institute for Experimental Animals. All mice were maintained under specific pathogen-free conditions and studied in compliance with institutional guidelines. HEK293 cells were purchased from ATCC. The NIH3T3 cell line was obtained from RIKEN Cell Bank. The B16 and MO4 cell lines were received from Dr RM Steinman (Rockefeller University). B16-NY-ESO-1 cells were established by transfection of a human NY-ESO-1 expression vector into B16 cells. Murine NKT hybridoma 1B6 cells were kindly provided by Dr M Kronenberg (La Jolla Institute for Immunology).

2.2 | Reagents

Human IL-2 was purchased from Shionogi & Co., Ltd. Recombinant human GM-CSF and IL-4 were from R&D Systems. α -Galactosylceramide was from Funakoshi. α -Galactosylceramide and vehicle (0.4% DMSO) were diluted in PBS. Peptivator NY-ESO-1 was from Miltenyi Biotec. Complete Freund's adjuvant was from Difco Laboratories. OVA SIINFEKL peptide was from MBL.

2.3 | In vitro transcription of RNAs

Murine CD1d and human CD1d and OVA plasmids for this study have been previously described.⁴⁰ The cDNA for NY-ESO-1 was purchased from OriGene Technologies. Plasmid construction was carried out by Takara Bio, and a fragment of the sequence from base 54 to 596 of accession no. NM_001327 with the CCACC sequence (Kozak sequence) added to the 5'-end was inserted into the *HindIII* and *EcoRI* sites of the pGEM-4Z vector. Before in vitro transcription, the NY-ESO-1 plasmid was linearized with *EcoRI* and purified as described above. In vitro transcription was carried out using the mMessage mMachine T7 Ultra Kit (Ambion) according to the manufacturer's instructions. RNA was purified using the RNeasy Mini/Midi Kit (Qiagen), and the integrity was verified by denaturing agarose gel electrophoresis.

2.4 | Preparation of aAVCs

The serum-free cultured HEK293 (sfHEK293) cell line was established. For α -GalCer loading, NIH3T3 cells (for mouse) or sfHEK293 cells (for human) were cultured for 48 hours in the presence of 500 ng/mL α -GalCer and then washed three times before electroporation with Ag mRNA together with murine CD1d mRNA. RNA electroporation of NIH3T3 cells was carried out as previously described.⁴⁰ Briefly, cells were resuspended in OptiMEM at a concentration of 5×10^7 cells/mL. RNA (final concentration, 50–500 μ g/mL) was transferred to a cuvette (Harvard Apparatus), and the cell suspension was added and pulsed in an ECM 830 Square Wave Electroporation System (Harvard Apparatus). The pulse condition was a single 500 V, 3 ms square pulse. Immediately after electroporation, the cells were transferred to a culture medium and cultured in the presence of 500 ng/mL α -GalCer. Transfected cells were analyzed by western blotting for NY-ESO-1 protein, ELISA (ITEA) for OVA protein, and flow cytometry for CD1d surface expression.

2.5 | Western blot analysis

To quantify NY-ESO-1 protein levels by western blot analysis, 2×10^6 aAVC-NY-ESO-1 cells were lysed in 150 μ L Laemmli sample buffer (Bio-Rad). Samples were boiled at 95°C for 5 minutes, and 15 μ L of samples, as well as molecular weight marker (Bio-Rad), were loaded and separated on a polyacrylamide gel. Protein was then electrotransferred onto a PVDF membrane. The membranes were blocked in TBS-T containing 5% skim milk and then incubated with the primary anti-NY-ESO-1 Ab (clone, E978; Santa Cruz Biotechnology, #sc-53869) at 4°C with gentle shaking overnight. The membranes were washed with TBS-T for 10 minutes three times and incubated with HRP-conjugated goat anti-mouse Ab (R&D Systems) at RT with gentle shaking for 1 hour. Chemiluminescence images were acquired using a luminescence image analyzer (LAS 1000) and Image Gauge software (Fujifilm Co.). Following chemiluminescence imaging, a bright-field image of the PVDF membrane was obtained. Protein levels were measured using Image Gauge software, and image processing (resizing, cropping, and merging) was undertaken using Adobe Photoshop (Adobe Systems).

2.6 | Sample processing and flow cytometry

Mononuclear cells from the spleen and lungs of mice were isolated as previously described.^{41,42} Briefly, splenocytes were obtained by pressing the spleen through a 70- μ m cell strainer or were digested with collagenase D (Roche); erythrocytes were lysed with ACK lysing buffer (Gibco), followed by two washes with RPMI medium. For the isolation of lung mononuclear cells, tissues were digested with collagenase D (Roche), layered on Percoll gradients (40/60%) (Amersham Pharmacia Biotec), and centrifuged for 20 minutes at 900 g. The cells

were then washed with PBS, and erythrocytes were lysed with ACK lysing buffer (Gibco), followed by two washes with RPMI medium.

2.6.1 | Natural killer/iNKT cells

After preparation, spleen cells were incubated with anti-CD16/CD32 (BioLegend) in FACS buffer (PBS with 2% heat-inactivated FBS) for Fc-blockade and then stained with surface Abs, including CD1d-dimer/ α -GalCer, anti-TCRV β -FITC, anti-NK1.1-PE/Cy7, and anti-CD69-PE, as well as viability dye for 30 minutes on ice. For IFN- γ intracellular staining, after incubation with all surface Abs, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) and stained with anti-IFN- γ -PE for 30 minutes at RT. After washing, cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences).

2.6.2 | Dendritic cells

Spleen cells obtained by collagenase D digestion were incubated with anti-CD16/CD32 (BioLegend) in FACS buffer (PBS with 2% heat-inactivated FBS) for Fc-blockade and then stained with the surface Abs anti-CD11b-FITC, anti-CD8-APC, anti-CD11c-PE/Cy7, anti-I-AE/K-PB, and anti-CD86 biotin and viability dye for 30 minutes on ice, followed by streptavidin-PE. For IL-12 intracellular staining, splenic CD11c⁺ cells were isolated using CD11c MACS beads at 4 hours after aAVC-NY-ESO-1 immunization and then cultured for 2 hours in the presence of GolgiPlug. After Fc-blockade, cells were stained with anti-CD8-APC, anti-CD11c-PE/Cy7, anti-I-AE/K-PB, and viability dye for 30 minutes on ice and then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), followed by staining with anti-IL-12p40/p70-PE for 30 minutes at RT. After washing, cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences).

2.7 | Tumor experiments

To test the adjuvant effects on NK cells, C57BL/6 mice were injected with 3×10^5 B16 cells intravenously at 3 hours before administration of aAVC-NY-ESO-1. Mice were killed 14 days after tumor inoculation, the lungs were removed, and individual surface lung metastases were counted using a microscope. For the vaccination model, C57BL/6 mice were immunized with 5×10^5 aAVC-NY-ESO-1 i.v. and inoculated with 1×10^5 B16 or B16-NY-ESO-1 s.c. 2 weeks later. In some experiments, C57BL/6 mice were immunized with 5×10^5 aAVC-OVA i.v. or CFA/OVA SIINFEKL peptide (100 μ g) s.c. and inoculated with 1×10^5 MO4 s.c. 2 weeks later. For the therapeutic model, C57BL/6 mice were inoculated with 2×10^5 B16-NY-ESO-1 s.c. and then treated with or without 5×10^5 aAVC-NY-ESO-1 i.v. on day 10. In some experiments, C57BL/6 mice were inoculated with 5×10^5 MO4 s.c. and then treated with or without 5×10^5 aAVC-OVA iv or

CFA/OVA SIINFEKL peptide (100 µg) s.c. on day 10. Tumor growth was monitored by measuring three perpendicular diameters. Tumor volume was calculated according to the formula $V = L \times W^2 \times 0.52$, where V is the volume, L is the length, and W is the width.

2.8 | Enzyme-linked immune absorbent spot assay

The ELISPOT assays for murine IFN- γ -secreting CD8⁺ T cells were carried out as previously described.³⁹ Briefly, 96-well filtration plates (Millipore) were coated with rat anti-mouse IFN- γ (R4-6A2; BD Biosciences) at 10 µg/mL overnight. CD8⁺ T cells were isolated from the spleen of naïve control mice and aAVC-NY-ESO-1-treated mice on day 7 using CD8 MACS beads (Miltenyi Biotec). Splenic DCs were isolated from naïve mice with CD11c MACS beads (Miltenyi Biotec) and used as APCs. Additionally, 5×10^5 CD8⁺ T cells were cocultured with 1×10^5 DCs pulsed with or without a Peptivator NY-ESO-1 (Miltenyi Biotec) for 24 hours. After culture, the plates were washed and incubated with biotinylated anti-mouse IFN- γ (XMG1.2; BD Biosciences) (2 µg/mL) for 2 hours, and spots were developed with streptavidin-HRP (BD Biosciences) and stable DAB substrate (Research Genetics). Interferon- γ spot forming cells were quantified through microscopy. To study the role of cDCs in the generation of antigen-specific CD8⁺ T cells, we undertook BM chimera experiments. C57BL/6J mice were lethally irradiated and transfected with 1×10^7 BM cells from WT mice or zDC-DTR mice. Three months later, the mice were treated with DT (Sigma) (20 ng/g; days -2, -1, 0, +1, +2, and +4) to delete in vivo cDCs and were then immunized with aAVC-NY-ESO-1 at day 0. NY-ESO-1-specific CD8⁺ T cells were analyzed by ELISPOT assay as described above on day 7.

2.9 | Isolation of human PBMCs

Human PBMCs were obtained from healthy blood donors and separated by Ficoll-Hypaque (Amersham Pharmacia Biotech) density centrifugation. The PBMCs and, in some cases, CD14⁺ monocytes purified through magnetic bead separation (Miltenyi Biotec) were washed three times with PBS and stored using serum-free freezing medium Cellbanker2 (JUJI Field) in liquid nitrogen until use. All studies were approved by the Institutional Review Board of RIKEN.

2.10 | In vitro generation of human iNKT cell lines

To prepare NKT cell lines, PBMCs from healthy HLA-A2⁺ donors were pulsed with α -GalCer (100 ng/mL) in the presence of 100 U/mL IL-2. After 10-14 days, human iNKT cells were stained using FITC-labeled anti-V α 24 mAb and selected with anti-FITC magnetic beads (Miltenyi Biotec). Human iNKT cells were maintained in the presence of 100 U/mL IL-2, 5 ng/mL IL-7, as well as 10 ng/mL IL-15 and stimulated with mouse BM-DCs pulsed with α -GalCer every other week.

2.11 | Generation of human DCs

CD14⁺ cells from healthy HLA-A2⁺ donors were isolated using magnetic beads (Miltenyi Biotec) and used for the generation of imDCs. Monocytes were cultured in the presence of GM-CSF (100 ng/mL) and IL-4 (25 ng/mL) for 4 days to generate imDCs.

2.12 | Preparation of TCR-transduced PBLs

T cells from healthy HLA-A2⁺ donors were transduced with a retroviral vector carrying the anti-NY-ESO-1 TCR gene (1G4 TCR), as previously described.^{43,44} In brief, retroviral supernatants were prepared using the pMSGV 1G4 furin 2-A vector provided by Dr Hanada and Steven A. Rosenberg (NIH); Plat-GP was provided by Dr T. Kitamura (Tokyo University). Peripheral blood leukocytes were stimulated in vitro at 10^6 cells/mL with 50 ng/mL anti-CD3 mAb OKT3 (Janssen Pharmaceutical) in DMEM (Invitrogen) supplemented with 10% FCS-containing DMEM (Sigma-Aldrich) and 100 IU IL-2. Two days later, TCR-encoding retrovirus-containing supernatant was rapidly thawed, diluted 1:1 in 20% DMEM, and added to 24-well plates that had been coated with 25 µg/mL retromectin (Takara Bio) overnight. The supernatant was spin-loaded onto plates by centrifuging for 2 hours at 2000 g and 32°C. The stimulated PBLs were added to retrovirus-loaded plates. Plates were centrifuged at 1000 g at 32°C for 10 minutes and incubated overnight at 37°C with 5% CO₂. The next day, PBLs were transferred to freshly prepared retrovirus-coated 24-well plates. The following day, transduced PBLs were washed, resuspended in fresh TCR media, transferred to flasks, and incubated at 37°C, 5% CO₂ for 5 days, and then sorted.

2.13 | Cytokine ELISA

The NKT cell-stimulating capacity of mouse aAVC-NY-ESO-1 was examined by coculturing mouse aAVCs (1×10^4 cells/96 wells) with V α 14 iNKT hybridoma 1B6 (1×10^5 cells/96 wells) for 24 hours, then mouse IL-2 production was measured. Alternatively, human aAVCs (1×10^4 cells/96 wells) were cocultured with human iNKT cells (1×10^5 cells/96 wells) for 24 hours, and human IFN- γ production was determined.

2.14 | In vivo human DC cross-presentation of aAVC-NY-ESO-1

The human DC cross-presentation assay was carried out as previously described.⁴⁵ Approximately 3×10^6 CFSE-labeled NY-ESO-1 TCR-transfected T cells were adoptively transferred to NOG mice. Three hours later, the mice were injected with immature monocyte-derived DCs (1×10^6 cells) and NKT cells (1×10^6 cells) from the same donor, with or without aAVC-NY-ESO-1 (1×10^6 cells). Five

days later, lungs were harvested from NOG mice, and the proliferation of NY-ESO-1 TCR-T cells was analyzed based on CFSE dilution using flow cytometry.

2.15 | Statistical analyses

Statistical significance was assigned when *P* values were less than .05 using StatMate (3B Scientific). The number of animals (*n*), median values, and statistical comparison groups are included in the figure legends. All *P* values were calculated using Tukey's test for three or more groups or the Mann-Whitney test for two groups.

3 | RESULTS

3.1 | Establishment of aAVC-NY-ESO-1 as single cells expressing the α -GalCer-CD1d complex and NY-ESO-1 antigen

To establish the NY-ESO-1-expressing aAVCs, we cotransfected CD1d mRNA and NY-ESO-1 mRNA into NIH3T3 cells, then loaded them with α -GalCer (hereafter referred to aAVC-NY-ESO-1) (Figure 1A). We verified that they expressed NY-ESO-1 protein ($2.64 \pm 0.46 \mu\text{g}/10^6$ cells) (Figure 1B) and surface CD1d highly (Figure 1C). We also confirmed α -GalCer presentation in vitro using aAVC-NY-ESO-1 for mouse iNKT hybridoma by measuring IL-2 production (Figure 1D).

3.2 | Enhancement of innate lymphocyte-mediated antitumor effects induced by injection of aAVC-NY-ESO-1

Lung metastasis is reportedly suitable for evaluating NK cell-mediated antitumor effect.^{30,39,42} When B6 mice were injected intravenously with aAVC-NY-ESO-1, CD1d dimer⁺ iNKT cells in the spleen expressed CD69 and produced IFN- γ compared to those in untreated mice (Figure 1E, upper panel). Simultaneously, NK cells expressed CD69 and produced IFN- γ (Figure 1E, lower panel). These results indicate that an NK cell-adjunctive effect was observed soon after the activation of iNKT cells by aAVC-NY-ESO-1.

To assess the antitumor effects of innate immune cells in response to aAVC-NY-ESO-1, we used a lung metastasis model wherein mice were administered B16 melanoma cells intravenously and injected 3 hours later with or without aAVC-NY-ESO-1. C57BL/6 mice readily developed lung metastases (Figure 1F). However, this was not observed in aAVC-NY-ESO-1-administered mice (Figure 1F). C57BL/6 mice pretreated with an NK-depleting anti-asialo-GM1 Ab did not show resistance to tumor metastases (Figure 1F). These results indicate that activation of innate lymphocytes by aAVC-NY-ESO-1 is sufficient to inhibit lung metastases in vivo.

3.3 | In situ DC maturation after aAVC-NY-ESO-1 treatment

The maturation of DCs is a critical step in the link between innate and adaptive immunity. Herein, we examined whether DC maturation can be achieved in vivo after aAVC-NY-ESO-1 treatment. Carboxyfluorescein succinimidyl ester-labeled aAVC-NY-ESO-1 were transferred to B6 mice (Figure 2A). As shown in Figure 2B, CD8⁺ DCs took up the debris of aAVCs in situ 12 hours later. We also detected that both CD8a⁺ and CD8a⁻ subsets of host DCs highly expressed CD86 after 12 hours (Figure 2C). As an indicator of CD8⁺ DC function, IL-12 production was observed (Figure 2D).

3.4 | Generation of CTLs after treatment with aAVC-NY-ESO-1

To assess the generation of an NY-ESO-1-specific T cell response, we immunized C57BL/6 mice with aAVC-NY-ESO-1 and analyzed the T cell response 1 week later. For this, we isolated CD8⁺ T cells from the spleens of immunized mice and then cocultured them with splenic DCs from naïve mice, with or without the NY-ESO-1 peptide pool (Figure 3A). We detected the NY-ESO-1-specific T cell response in aAVC-NY-ESO-1-immunized mice but not in control mice (Figure 3B). To examine the cross-presenting activity of cDCs in aAVC-NY-ESO-1-immunized mice, we established BM chimera mice by transferring BM of either zDC-DTR or WT mice. The BM chimera mice were treated with DT daily from day -2 to day 2 and every other day thereafter. One week later, CD8⁺ T cell responses were determined via IFN- γ ELISPOT assay. Anti-NY-ESO-1-specific CD8⁺ T cell responses were detected in WT BM chimera mice but not in BM chimera from zDC-DTR mice (Figure 3C). These findings indicated that the CD8a⁺ DCs that engulfed aAVC-NY-ESO-1 were activated and able to cross-present the NY-ESO-1 peptide to CTLs. Taken together, aAVC-NY-ESO-1 therapy can deliver tumor antigens to DCs in vivo and promote NY-ESO-1 antigen cross-presentation by cDCs.

3.5 | Prophylactic and therapeutic effects of aAVC-NY-ESO-1 against NY-ESO-1-expressing tumors

A subcutaneous tumor model is reportedly suitable for evaluating CTL-mediated antitumor effect.^{30,39,40,42,45} We initially compared aAVC-OVA to the conventional approach; that is, peptide therapy using MO4 (OVA-expressing B16 melanoma) in the prophylactic model and therapeutic model (Figure 4A,B). As shown in Figure 4A,4B, we detected the antitumor effect to be higher in mice given aAVC-OVA than in mice given OVA plus CFA. To assess NY-ESO-1 antigen-specific T cell-mediated antitumor immunity, we established a stable NY-ESO-1-expressing B16 melanoma cell line (B16-NY-ESO-1). To trigger an antigen-specific T cell-mediated antitumor response in the prophylactic model, we vaccinated C57BL/6 mice with aAVC-NY-ESO-1. Two weeks later, the mice were challenged with B16-NY-ESO-1 or parental B16 cells (Figure 4C). As

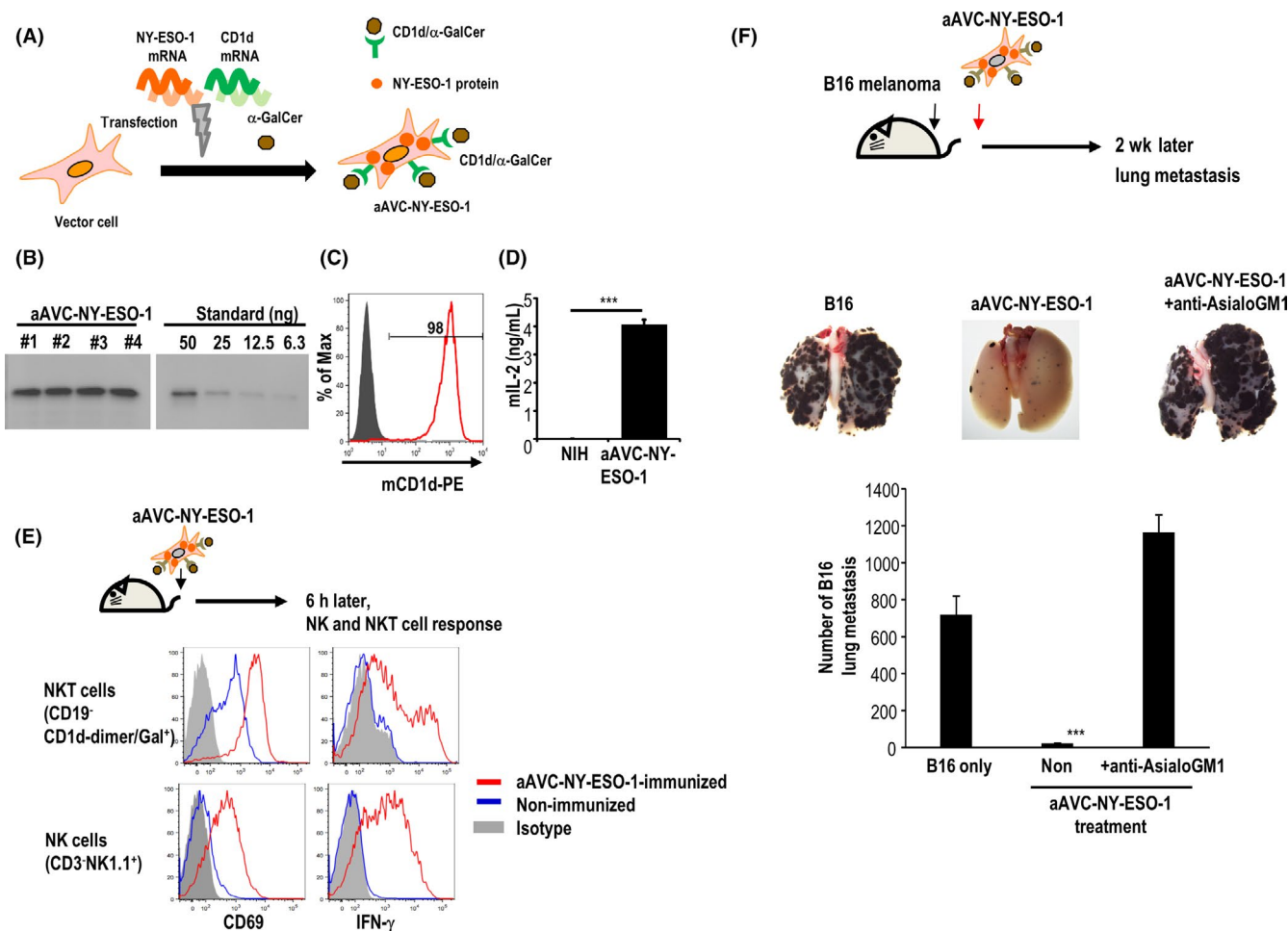


FIGURE 1 Activation of innate immunity by NY-ESO-1-expressing artificial adjuvant cells (aAVC-NY-ESO-1). A, aAVC-NY-ESO-1 cells were established by loading NIH3T3 vector cells with α -galactosylceramide (α -GalCer) and coelectroporation with NY-ESO-1 and murine CD1d mRNA. B-D, NY-ESO-1 protein levels and CD1d surface expression were assessed using western blotting (B) and flow cytometry (C), respectively (red, aAVC-NY-ESO-1; bold, NIH3T3; shaded, isotype). D, α -GalCer presentation by aAVC-NY-ESO-1. aAVC-NY-ESO-1 were cocultured with the V α 14 invariant natural killer T (iNKT) cell hybridoma 1.2 for 24 h, and interleukin-2 (IL-2) production in the culture supernatant was evaluated by ELISA ($n = 4$; mean \pm SEM) *** $P < .001$ (Mann-Whitney). E, iNKT and natural killer (NK) cell activation. Mice were injected with 5×10^5 aAVC-NY-ESO-1 i.v. Splens were removed 6 h later, and iNKT as well as NK cells were analyzed by staining with CD19-antigen presenting cells (APC) and CD1d-dimer⁺ phycoerythrin (PE) or CD3-FITC and NK1.1-APC. These cells were simultaneously stained with either surface CD69-PE or intracellular interferon- γ -PE and evaluated through flow cytometry ($n = 4$). F, Antitumor immunity by innate lymphocytes. Mice were injected with 3×10^5 B16 melanoma cells i.v. and, 3 h later, were inoculated with 5×10^5 aAVC-NY-ESO-1. The number of lung metastases was determined 14 d later ($n = 5$ per group, mean \pm SEM). In some experiments, NK cells were depleted using anti-asialo GM1 Ab. *** $P < .001$ (Tukey's test) (aAVC-immunized vs others)

shown in Figure 4C, mice vaccinated with aAVC-NY-ESO-1 responded efficiently to B16-NY-ESO-1, but not B16 cells, whereas both B16 and B16-NY-ESO-1 grew in nonimmunized mice. These results indicated an apparent antigen-specific antitumor effect. In the therapeutic model, mice were inoculated with B16-NY-ESO-1 s.c. and injected with aAVC-NY-ESO-1 on day 10. As shown in Figure 4D, aAVC-NY-ESO-1-treated mice showed clear protection against tumor growth.

3.6 | Human aAVC-NY-ESO-1 elicits antigen-specific T cells through human DCs in a DC-NOG mouse model

We examined whether human aAVC-NY-ESO-1 generates both innate and adaptive immunity. Using sfHEK293 cells as carrier cells,

which were established under serum-free culture conditions, we transfected CD1d and NY-ESO-1 mRNA to establish human aAVC-NY-ESO-1. We confirmed NY-ESO-1 protein levels using western blot analysis (Figure 5A, left panel) and human CD1d surface expression using flow cytometry (Figure 5A, right panel). To assess α -GalCer presentation by human aAVC-NY-ESO-1, two protocols were used as previously described. First, human NKT cells were cocultured with aAVC-NY-ESO-1 in vitro. Interferon- γ production was detected in the supernatant, but not in that of NKT cells alone (Figure 5B). We also evaluated the iNKT cell response in vivo (Figure 5C). We previously reported that α -GalCer-loaded human CD1d⁺ cells, including DCs, can stimulate murine iNKT cells in the spleen.⁴⁶ When aAVC-NY-ESO-1 was given to WT mice, we detected an increased number of NKT cells in the spleen (Figure 5C).

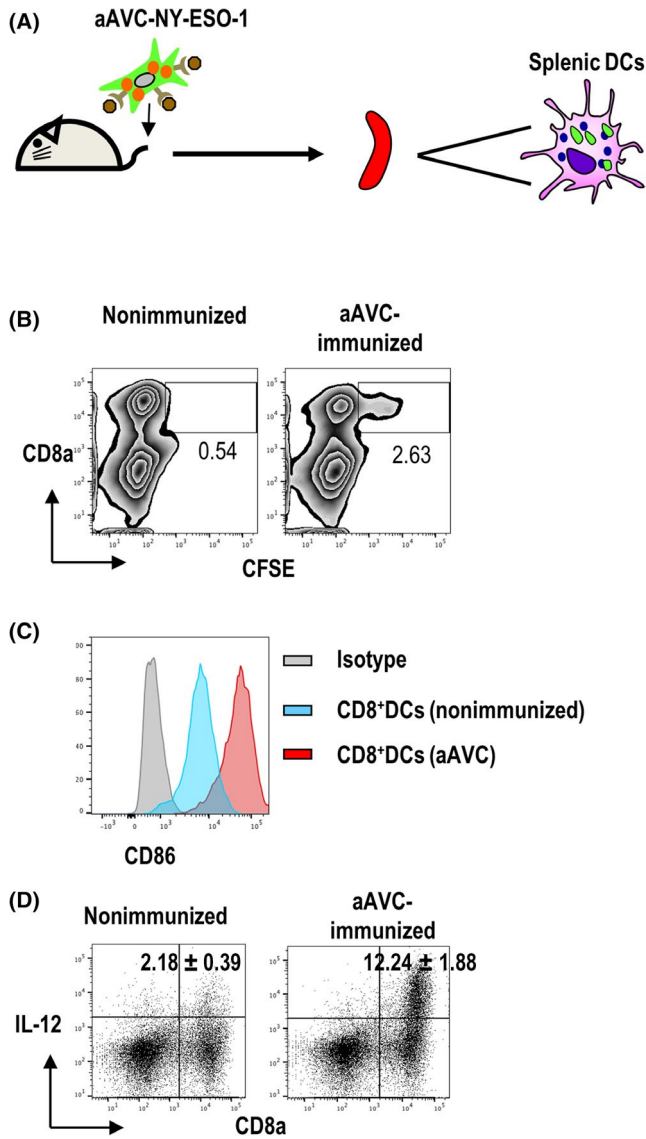


FIGURE 2 NY-ESO-1-expressing artificial adjuvant cells (aAVC-NY-ESO-1) lead to the functional maturation of dendritic cells (DCs) in situ. A, Three million carboxyfluorescein succinimidyl ester (CFSE)-labeled aAVC-NY-ESO-1 were injected i.v. into C57BL/6 mice. Twelve hours later, splenic DCs were analyzed. B, Uptake of CFSE⁺ aAVCs by murine CD11c⁺ splenic DCs was measured based on CFSE⁺ gating on CD11c⁺ class II⁺ by flow cytometry. C, DCs were analyzed for the upregulation of CD86 expression after gating on CD11c⁺ at 12 h using CD11c-antigen presenting cells and CD86-phycoerythrin (isotype control, shaded; nonimmunized mice, blue; aAVC-immunized mice, red). Data are representative of three independent experiments ($n = 3$ per group). D, Interleukin-12 (IL-12) production by conventional DCs in vivo was evaluated through intracellular staining ($n = 3$ per group, mean \pm SEM)

Next, to assess iNKT cell-stimulated DC function and the induction of T cell immunity, we used NOG mice that lack B, T, NK, and iNKT cells, thus not rejecting adoptively transferred human cells. As previously reported,⁴⁵ we established a robust system for evaluating

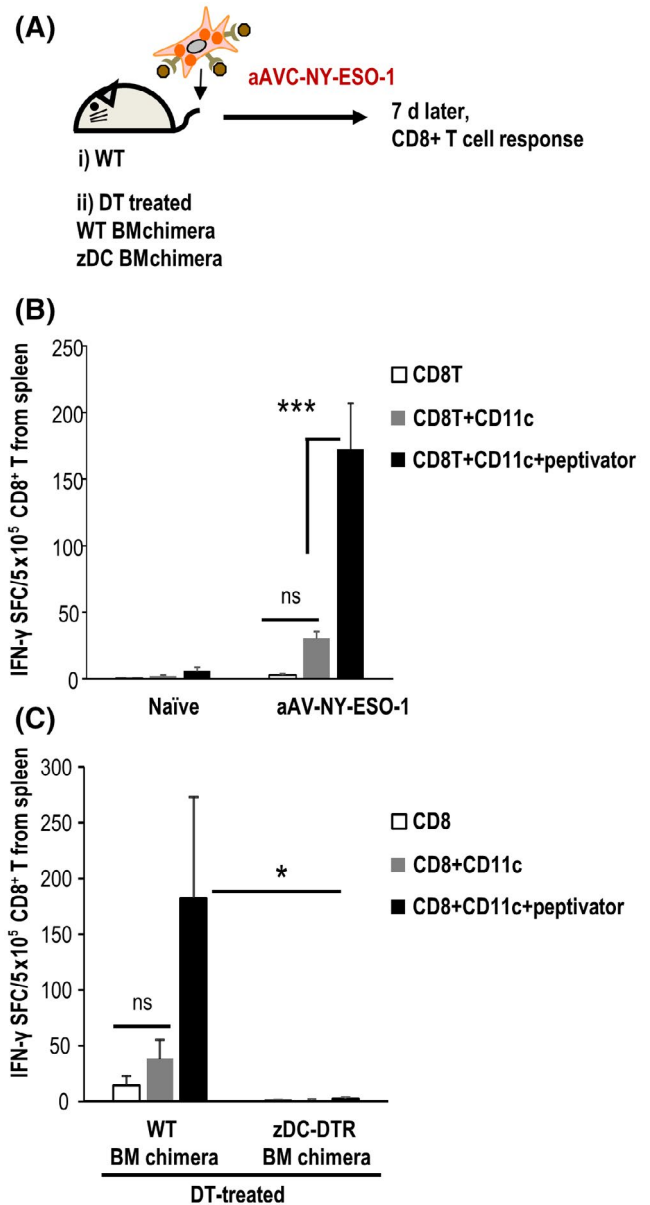


FIGURE 3 NY-ESO-1-specific CD8⁺ T cell response induced by NY-ESO-1-expressing artificial adjuvant cells (aAVC-NY-ESO-1). A, Mice were immunized with 5×10^5 aAVC-NY-ESO-1 i.v. One week later, NY-ESO-1-specific CD8⁺ T cells were analyzed by interferon- γ (IFN- γ) enzyme-linked immune absorbent spot (ELISPOT) assay. CD8⁺ T cells were obtained from the spleen and cultured with splenic CD11c⁺ dendritic cells (DCs) from naïve mice in the presence or absence of the NY-ESO-1 peptide pool for 24 h. B, NY-ESO-1-specific CD8⁺ T cell response in aAVC-NY-ESO-1-immunized mice and nonimmunized mice ($n = 5$ per group, mean \pm SEM). *** $P < .001$ (aAVC-NY-ESO-1 CD8T+CD11c+peptivator vs others, Tukey's test). C, NY-ESO-1-specific CD8⁺ T cell response in conventional DC (cDC)-depleted mice. To test the role of cDCs in the generation of NY-ESO-1-specific CD8⁺ T cells, WT bone marrow (BM) or zDC-DTR BM chimera mice were treated with diphtheria toxin (DT) and then immunized with aAVC-NY-ESO-1 ($n = 4$ per group, mean \pm SEM). * $P < .05$ (WT BM chimera vs zDC-DTR BM chimera, Mann-Whitney). ns, not significant; SFC, spot forming cell

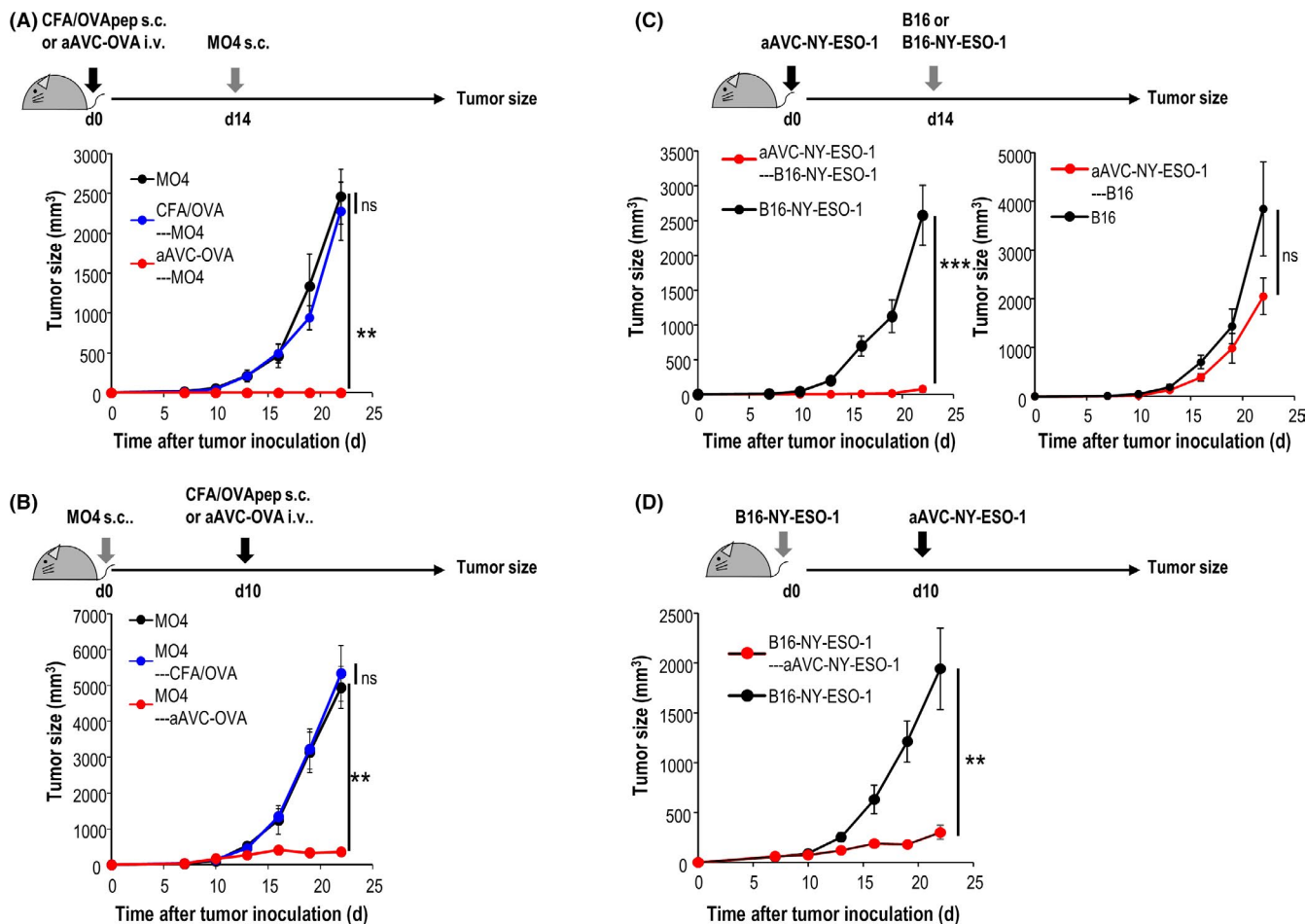


FIGURE 4 Antitumor response induced by NY-ESO-1-expressing artificial adjuvant cells (aAVC-NY-ESO-1). Antitumor response was evaluated in a prophylactic and a therapeutic vaccination model. **A**, Mice were immunized with 5×10^5 aAVC-OVA i.v. or OVA peptide plus complete Freund's adjuvant (CFA) and challenged with 1×10^5 MO4 s.c. at day 14 (d14). ($n = 5-6$ per group, mean \pm SEM). * $P < .001$. ns, not significant (Mann-Whitney). MO4, black; CFA/OVApep, blue; aAVC-OVA, red. **B**, Mice were inoculated with 5×10^5 MO4 s.c. and treated with 5×10^5 aAVC-OVA or OVA peptide plus CFA on d10. Tumor size was measured at the indicated time points ($n = 5-6$ per group, mean \pm SEM). ** $P < .01$ (Mann-Whitney). MO4, black; CFA/OVApep, blue; aAVC-OVA, red. **C**, **D**, Mice were immunized with 5×10^5 aAVC-NY-ESO-1 i.v. and challenged with 1×10^5 NY-ESO-1-expressing B16 (B16-NY-ESO-1) (**C**, left) or B16 (**C**, right) s.c. at d14 ($n = 7-8$ per group, mean \pm SEM). * $P < .001$. ns, not significant (Mann-Whitney). B16 or B16-NY-ESO-1, black; aAVC-NY-ESO-1, red. **D**, Mice were inoculated with 2×10^5 B16-NY-ESO-1 s.c. and treated with 5×10^5 aAVC-NY-ESO-1 on d10. Tumor size was measured at the indicated time points ($n = 6-7$ per group, mean \pm SEM). ** $P < .01$ (Mann-Whitney). B16 or B16-NY-ESO-1, black; aAVC-NY-ESO-1, red

DC function by administering human monocyte-derived imDCs from HLA-A2⁺ healthy donors into NOG mice. To evaluate the cross-presentation of NY-ESO-1 by human DCs, NY-ESO-1-specific TCR-expressing T cells from the same donors were generated by transduction with a retroviral vector carrying the NY-ESO-1 TCR gene (1G4 TCR).⁴⁴ Invariant NKT cells from the same donor were also prepared.

Transduced T cells were adoptively transferred into NOG mice. Three hours later, immature DCs and iNKT cells, with or without aAVC-NY-ESO-1, were inoculated into the same mice intravenously (Figure 5D), and lung mononuclear cells were analyzed on day 5. Only the NY-ESO-1 TCR gene-transduced T cells in the mixed transfer group (CFSE-T+DC+iNKT+aAVCs) expanded robustly in vivo (Figure 5D). These results indicate that DCs are essential for T cell activation following vaccination with human aAVC-NY-ESO-1.

4 | DISCUSSION

Cancer vaccines are an immunotherapy approach currently explored for the prophylactic or therapeutic treatment of cancer. Although diverse strategies have been applied, there is a paucity of vaccines that can safely elicit robust innate and adaptive responses for cancer immune surveillance. Based on basic and preclinical studies using the aAVC platform, we recently completed the phase I trial of aAVC-WT1 for AML patients (UMIN000028083). Herein, as a next step, we present evidence that aAVC-NY-ESO-1 significantly enhanced the immunogenicity of NY-ESO-1 in DCs in situ. In particular, the subsequent maturation of aAVC-NY-ESO-1-captured DCs resulted in efficient targeting of full-length NY-ESO-1 proteins in the MHC-I pathway for cross-presentation to antigen-specific CD8⁺ T cells by fully matured DCs. Thus, our current study

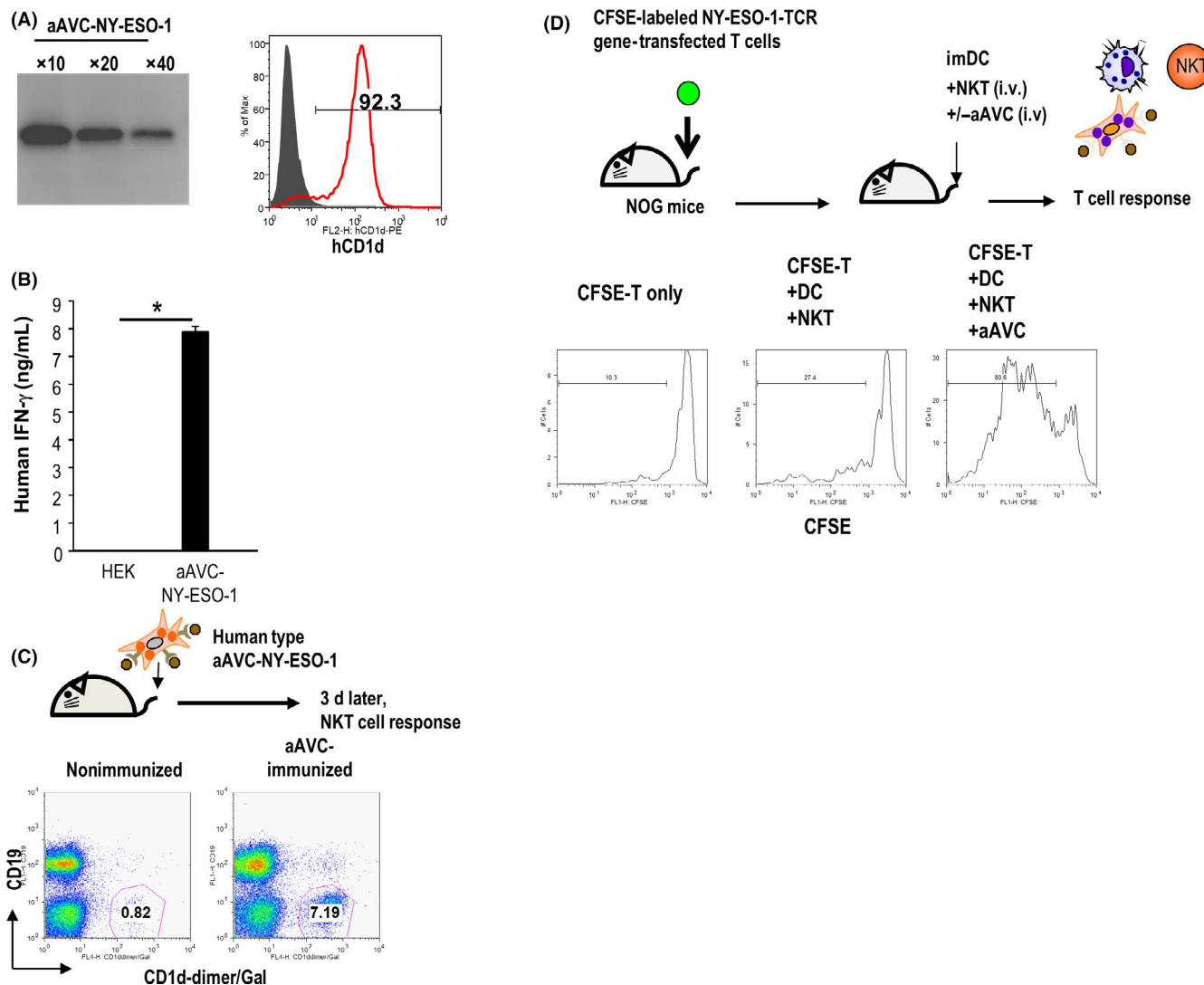


FIGURE 5 Human T cell activation by dendritic cells in NOG mice immunized with human NY-ESO-1-expressing artificial adjuvant cells (aAVC-NY-ESO-1). A, Human aAVC-NY-ESO-1 were established by loading HEK293sf cells with α -galactosylceramide (α -GalCer) and coelectroporation with NY-ESO-1 as well as human CD1d mRNA. Recombinant NY-ESO-1 protein expression in aAVC-NY-ESO-1 (left) and hCD1d expression (right). B, α -GalCer presentation by human aAVC-NY-ESO-1. Human natural killer T (NKT) cells were cocultured with HEK293sf or aAVC-NY-ESO-1 for 24 h. Interferon- γ (IFN- γ) production was analyzed by ELISA ($n = 4$ per group, mean \pm SEM). * $P < .05$ (Mann-Whitney). C, Activation of NKT cells by human type aAVC-NY-ESO-1 in C57BL/6 mice. Mice were immunized with 5×10^5 aAVC-NY-ESO-1 i.v. Three days later, the frequency of invariant NKT (iNKT) cells in the spleen was analyzed by flow cytometry. D, CD8⁺ T cell activation in humanized mice. NY-ESO-1 TCR-T cells, NKT cells, and dendritic cells (DCs) were prepared from the same HLA-A2⁺ healthy donor. The treatment protocol is shown. These T cells (NY-ESO-1 TCR-T) were labeled with carboxyfluorescein succinimidyl ester (CFSE) and injected into NOG mice. Three hours later, monocyte-derived immature DCs (imDC) and iNKT cells were transferred, together with or without aAVC-NY-ESO-1. Five days later, the proliferation of NY-ESO-1 TCR-T cells was evaluated by flow cytometry. Histograms from one representative donor are shown ($n = 3$)

showed that aAVC-NY-ESO-1 harnessed both the innate (iNKT/NK cells) and adaptive (CTL) immune responses. We observed strong antigen-specific CTL induction through mouse and human DCs in situ. Treatment with aAVC-NY-ESO-1 enhanced strong cross-presentation to T cells by murine DCs in tumor-bearing mice as well as by human DCs in preclinical DC-NOG mice.

Demonstrating NY-ESO-1 antigen cross-presentation by human DCs to T cells was a crucial step in our preclinical study. We used the immature human DC-transferred NOG model (hDC-NOG) in the

current work. In the transfer of human PBMCs into NOG animals, stable engraftment sometimes fails 2–4 weeks later due to xenogeneic graft-versus-host disease.⁴⁷ Therefore, we assessed the immune response within 1 week after the transfer study. As shown in Figure 5B, NKT cells did not directly stimulate T cells, even in the presence of DCs in vivo. This indicated that NKT cells did not activate CTLs through cell-to-cell interactions. However, together with aAVC-NY-ESO-1, they drove the generation of antigen-specific effector T cells, indicating tumor antigen cross-presentation by DCs in hDC-NOG mice.

In cancer, NY-ESO-1 expression is a consequence of epigenetic events that involve the tightly controlled recruitment and sequential interaction of histone deacetylases, histone methyltransferases, DNA methyltransferases, and transcription factors. There are several emerging strategies utilizing epigenetic drugs against NY-ESO-1⁺ cancers. Cartron et al reported the restoration of NY-ESO-1 gene expression in glioma cell lines by epigenetic drugs and the presentation of NY-ESO-1 peptide on HLA-A*02:01 in a drug-treated glioma cell line.⁴⁸ Griffiths et al reported an increase in NY-ESO-1 mRNA levels in AML blasts of DNMT1 inhibitor decitabine-treated AML patients.⁴⁹ They also reported an NY-ESO-1-specific immune response in the phase I trial of a combined decitabine and NY-ESO-1 vaccine (CDX-1401+Poly-ICLC) for myelodysplastic syndrome.⁵⁰ Recently, the focus of immunotherapy research has shifted toward combination therapy. As both innate and adaptive immunity were stimulated by aAVC-NY-ESO-1, epigenetic-targeting drugs could represent another promising option for combination with aAVC therapy against NY-ESO-1-expressing tumor cells to enhance more antitumor immunity, particularly against aggressive types of cancers or advanced stages.

Overall, we showed aAVC-NY-ESO1 therapy, as an innovative immunotherapy approach, to induce multiple functions. The current findings highlight the therapeutic potential of aAVC-NY-ESO-1 fully against NY-ESO1-expressing malignant diseases. This therapy overcomes the key challenges in the quest of making the best use of the immune system as one of the new techniques of cancer immunotherapy.

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DISCLOSURE

Shin-ichiro Fujii received research fund and honoraria from Astellas Pharma Inc. Kanako Shimizu received honoraria from Astellas Pharma Inc. The other authors declare no competing financial interests.

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