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Vaccination with antigen-transfected, NKT cell ligand-loaded, human cells elicits robust in situ immune responses by dendritic cells

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**Running title**: Innate and adaptive tumor immunity elicited by aAVCs

**Key words**: α-galactosylceramide, mRNA, dendritic cells, NKT cells, cross-presentation

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**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Abbreviations**: α-GalCer, α-galactosylceramide; aAVC, artificial adjuvant vector cells; OT-I, CD8 T cells from OVA T cell receptor transgenic mice

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Abstract

Both innate and adaptive immunity are crucial for cancer immunosurveillance, but precise therapeutic equations to restore immunosurveillance in cancer patients have yet to be developed. In murine models, α-galactosylceramide (α-GalCer)-loaded, tumor antigen-expressing syngeneic or allogeneic cells can act as cellular adjuvants, linking the innate and adaptive immune systems. In the current study, we established human artificial adjuvant vector cells (aAVC) consisting of human HEK293 embryonic kidney cells stably transfected with the NKT immune cell receptor CD1d, loaded with the CD1d ligand α-GalCer and then transfected with antigen-encoding mRNA. When administered to mice or dogs, these aAVCs activated invariant NKT (iNKT) cells elicited antigen-specific T cell responses with no adverse events. In parallel experiments, using NOD/SCID/IL-2rγnull immunodeficient (hDC-NOG) mouse model, we also showed that the human melanoma antigen, MART-1 expressed by mRNA transfected aAVCs can be cross-presented to antigen-specific T cells by human dendritic cells. Antigen-specific T cell responses elicited and expanded by aAVC were verified as functional in tumor immunity. Our results support the clinical development of aAVC to harness innate and adaptive immunity for effective cancer immunotherapy.
Introduction

Numerous studies using irradiated autologous and allogeneic tumor cells, as well as tumor-associated antigen (TAA) proteins, TAA-derived peptides or DNA encoding the TAA have highlighted the therapeutic potential of cancer vaccines (1-3). Recently, immunotherapeutic strategies have mostly shifted from use of a single class I TAA peptides to long or multiple peptides or to whole protein antigens in order to stimulate responses to a range of tumor antigen epitopes (4-6). Transfection of dendritic cells (DCs) with mRNA encoding TAA induces high levels of antigen protein expression in a reproducible manner, and is available in good manufacturing practice (GMP) grade for use in patients (7, 8). A crucial regulatory advantage of this mode of immunization is that mRNA transfection does not constitute gene therapy because the mRNA is not integrated into the patient’s genome. Furthermore, this approach is not dependent on particular haplotypes as peptides derived from translated proteins are loaded into multiple MHC molecules. DCs transfected with TAA mRNA have already been shown to be effective in generating a robust cytotoxic T lymphocyte (CTL) response and antitumor immunity in both mice (9, 10) and humans (11-13). Several groups have attempted to improve MHC class I antigen presentation by administering DCs co-transfected with mRNA encoding TAA and mRNA encoding a variety of potent adjuvants, such as OX40 ligand (14), GM-CSF (15) or IL-12 (16).

Adjuvants are a crucial component of successful immunotherapy. Several types of adjuvants have been intensively studied including aluminum salt-based adjuvants, oil-water emulsion-based adjuvants such as montanide ISA, granulocyte-macrophage colony-stimulating factor (GM-CSF) and Toll-like receptor (TLR) ligands, such as
Imiquimod (TLR7) and monophosphoryl lipid A (MPL), a low toxicity LPS-derived TLR-4 agonist derived from salmon (17, 18). DCs, as “nature’s adjuvants”, play a pivotal role in determining the character and magnitude of an immune response, and dying tumor cells are known to be selectively taken up by DCs and can be cross-presented to CD8+ T cells. The ex vivo loading of autologous patient DC with tumor specific antigens (ex vivo DC strategy) is one of the most promising current immunotherapeutic strategies (1, 2). Recently, Sipuleucel-T (Provenge, Dendreon Corp.), an autologous DC vaccine for patients with prostate cancer that uses prostatic acid phosphatase fused with GM-CSF, demonstrated biologic activity and prolonged median survival in Phase III vaccine trials and became the first antigen-specific, cell-based immunotherapy to receive US Food and Drug Administration (FDA) approval (19). This promising initial outcome with autologous cellular therapy will require long-term follow up, but may herald a new era of prolonged overall cancer survival resulting from tumor antigen-specific T cell responses. On the other hand, ex vivo DC therapy requires the generation of large numbers of DCs from individual patients and the quality of DCs will likely depend on the patient’s condition at the time of venipuncture to harvest DC precursors and the cytokine combination used for in vitro DC maturation.

In vivo targeting of DCs has been attempted using chimeras of an anti-DC specific C-type lectin receptor (CLR) antibody fused to a selected antigen. DEC205 was the first and most studied CLR for antigen targeting purposes, but other DC receptors targeted by cancer vaccines include the mannose receptor (MR), DC-SIGN and CLEC9A (20-23). The efficiency of in vivo DC-targeted therapy depends on the quality of antibody-conjugated antigen and the adjuvant. In the current study, we evaluate another in vivo DC
targeting strategy that exploits the pro-inflammatory potential of dying cells together with the adjuvant activity of invariant natural killer T (iNKT) cells.

iNKT cells recognize synthetic and natural glycolipids presented by CD1d, a non-classical MHC molecule found on APCs. When activated by iNKT cell ligands, such as α-galactosylceramide (α-GalCer), iNKT cells produce IFN-γ and IL-4. In both animal and clinical studies using α-GalCer-loaded DCs, it has been clearly shown that IFN-γ production by iNKT cells correlates with anti-tumor effects (24, 25). We and others previously reported that α-GalCer behaves as an immunological adjuvant by activating iNKT cells, which in turn induce DC maturation (26, 27). DCs matured by iNKT cells rapidly express IL-12, resulting in a Th1 polarized immune response. Many studies have demonstrated that co-administration of soluble or cell-associated antigen plus α-GalCer leads to Th1-type CD4+ T cell responses and CTLs. (26-31). In developing our immunotherapeutic strategy, we have already shown that α-GalCer-loaded tumor cells or α-GalCer-loaded, ova or trp2 antigen mRNA-transfected allogeneic fibroblasts can efficiently generate antigen-specific CTLs (32, 33). In these systems, DCs present antigen in vivo in two ways that lead to antitumor effects. The first is cross-presentation of tumor-associated antigen to T cells and the second is presentation of glycolipid to iNKT cells (34, 35). Important factors for effective generation of CTLs through iNKT cell-licensed host DCs are adequate α-GalCer loading, CD1d expression and the types of vector cells used (35, 36).

In the current study, we have further expanded this immunotherapy model to investigate whether mRNA-transfected human vector cells loaded with α-GalCer can
generate innate and adaptive immunity to tumor antigens in canine and human DC-
transferred NOD/Shi-scid/IL-2γcnull (NOG) immunodeficient mice.
Materials and Methods

Animals and cell lines

Pathogen-free male beagles were purchased from Kitayama Labs (Nagano, Japan) at 1 year of age and were maintained under specific pathogen-free conditions and studied in compliance with Yamaguchi University institutional guidelines. Pathogen-free C57BL/6 (B6) mice were purchased from CLEA Japan (Tokyo, Japan) at 6-8 weeks of age. iNKT cell-deficient, Jα18−/− mice were kindly provided by Dr. Taniguchi (RIKEN). OT-I TCR transgenic mice were generously provided by Dr. W.R. Heath (Walter and Eliza Hall Institute, Victoria, Australia) and Ly5.1 congenic OT-1 mice were generated by cross/backcross breeding of OT-1 with B6. Ly5.1 mice and screening for the presence of Vβ2 and Ly5.1 and absence of Ly5.2 expression by flow cytometry. CD11c-diphtheria toxin receptor (DTR) transgenic (CD11c-DTR/GFP) mice were generously provided by Dr. D.R. Littman (New York University, New York, NY). NOD/Shi-scid/IL-2Rγnull (NOG) mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions and studied in compliance with our institutional guidelines. Human melanoma cell line 624 MEL (HLA-A0201+/MART-1+) and the HLA-A2+ T cell clone derived from tumor infiltrating lymphocytes (JKF6-TIL) specific for the MART-1 Ag were kindly provided by Dr. S.A. Rosenberg (National Institutes of Health (NIH), Bethesda, MD). 624 MEL was directly obtained from the Surgery Branch, National Cancer Institute (NCI), NIH where the cell line was established. The cell line has been routinely tested for HLA-A2 and melanoma antigen expression at the Surgery Branch. HLA-A2 expression was tested by FACS and genotyping. Melanoma specific antigen expression was tested by
examining the reactivity of T-cell lines that recognize such antigens as MART-1 and gp100. The TIL clone, JKF6-TIL was also established in the Surgery Branch (NCI, NIH) and tested for HLA-A2-restricted MART-1 peptide reactivity. After receipt of the 624 MEL and JKF6-TIL cell lines, we have used the same protocols to test them routinely and before use (the most recent testing was 3/6/2012 and 1/26/2011, respectively). EL4, EG7, and HEK293 cell lines were obtained from the American Type Culture Collection (Rockville, MD). NIH3T3 cells, which were derived from outbred NIH/Swiss mice, were obtained from the RIKEN CELL BANK (Ibaraki, JAPAN) (the last tested time was 7/17/2012). These cell lines were maintained and treated according to the supplier's recommendations. CIRA0201 is an EBV transformed cell line expressing HLA-A*0201 that was established and kindly provided by Dr. M. Takiguchi (Kumamoto University, Kumamoto, Japan) (37). The CIRA0201 cell line has been maintained in RPMI-1640 medium supplemented with 5% FCS and 0.15 mg/mL of hygromycin B and routinely tested for HLA-A2 using flow cytometry (the last tested time was 1/26/2011). To introduce human CD1d into HEK293 cells, pCMV6-XLA4/hCD1d (OriGene Technologies Inc., Rockville, MD) and the pCAG-puromycin resistance gene (provided by Dr. M. Murakami, Osaka University, Osaka, Japan) were cotransfected into HEK293 cells and selected by puromycin. After one week, MX-hCD1d-transfected HEK293 cells were subsequently sorted based on the expression of hCD1d by FACS Aria Sorter. Murine NIH3T3 cells expressing high levels of murine CD1d (CD1d-NIH3T3) were generated by retrovirus transduction, as previously described (33).

_In vitro transcription (IVT) of RNAs_


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Enhanced GFP (EGFP) in a pSP64 Poly(A) vector was excised with HindIII and BamHI and re-cloned into the pGEM-4Z vector (Promega, Madison, WI) (33). The OVA plasmid used for this study was previously described (33). The expression plasmid for MART-1 (pcDNA3 (+)-MART-1) was isolated in our laboratory (38). For IVT, these plasmids were linearized by restriction digestion (BamHI for EGFP and OVA and NotI for MART-1), purified by QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany), and used as a template. The RNAs were generated under a T7 promoter sequence on the vectors by using mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Austin, TX). The template DNAs were then digested with DNase I from the kit. IVT RNAs were then purified by RNeasy Mini/Midi Kits (QIAGEN, Valencia, CA) and eluted in water. RNA integrity was verified by agarose gel electrophoresis under denaturing conditions and the concentration was determined by spectrophotometry.

**Preparation of artificial adjuvant vector cells (aAVC)**

To load α-GalCer, CD1d-HEK293 cells were cultured for 48 hours in the presence of 500 ng/mL of α-GalCer and then washed three times before transfection. IVT RNAs were transfected into CD1d-HEK293 with TransMessenger transfection kit (QIAGEN) following the manufacturer’s instructions. Briefly, the ratio of mRNA, enhancer solution and transmessenger reagent was 1: 2: 4 for performing lipofection. α-GalCer-loaded CD1d-HEK293 cells were transfected for 4 hours and then cultures were replenished with DMEM containing 10% fetal bovine serum for 2 hours for OVA mRNA and 20 hours for EGFP or MART-1 mRNA. Transfected cells were analyzed by ELISA (Morinaga, Yokohama, JAPAN) for OVA and flow cytometry for EGFP. To quantify the MART-1
protein by western blot analysis, 2x10^6 aAVC-MART-1 cells were lysed in 150 μL of sample buffer. Anti-MART-1 Ab-3 (NeoMarkers, Fremont, CA) and anti-mouse IgG HRP (Sigma-Aldrich, St. Louis, MO) were used for detection and protein expression was measured by a luminescence image analyzer, LAS 1000 (Fujifilm Co., Tokyo, Japan).

**Assays for evaluating in vivo cross-presentation**

To prepare the CD1d-HEK293/Gal-ova cells, CD1d-HEK293 transfectants that had been loaded with or without α-GalCer for 2 days were transfected with OVA mRNA. To analyze OVA presentation, mice were adoptively transferred with 2 x 10^6 CFSE-labeled OT-I cells and immunized on the following day with or without 5 x 10^5 CD1d-HEK293/Gal-ova. OT-1 cell proliferation in the spleen was monitored by dilution of CSFE 3 days later. In some experiments, CD11c-DTR mice that had been treated with diphtheria toxin (DT) (Sigma-Aldrich) were used to assess host DC presentation of cell-associated antigens to T cells (39).

**Statistical analysis**

Differences in *in vitro* data were analyzed using Mann-Whitney U-test. P<0.05 was considered statistically significant.
Results

Optimization of iNKT cell activation by human cell lines transfected with antigen-encoding mRNA

We previously showed that even CD1d expressing tumor cells or allogeneic fibroblasts that lack costimulatory molecules were able to present α-GalCer to primary iNKT cells (32, 33, 35). To develop the human cell therapy system, we screened 11 human fibroblasts and 4 non-fibroblast cell lines for efficacy of mRNA transfection, cell growth, and the capacity to expand iNKT cells after loading with α-GalCer (data not shown) and found that the human embryonic kidney cell line, HEK293, was optimal. HEK293 cells analyzed by flow-cytometry do not express CD80, CD86 or HLA-DR (data not shown). We could demonstrate stable, high-level expression of human CD1d by the human CD1d HEK293 transfectants (Fig. 1A), and that CD1d-HEK293 cells loaded with α-GalCer [hereafter referred to artificial adjuvant vector cells, (aAVC) ] could stimulate production of IFN-γ by iNKT cells in vitro (Fig. 1B). Furthermore, B6 mice injected i.v. with aAVC had more CD1d-dimer+ iNKT cells in spleen as compared to untreated mice (Fig. 1C). In addition, we observed the upregulation of CD25, an activation marker on NKT cells, as well as IFN-γ production by iNKT cells in mice given CD1d-HEK293/Gal cells but not mice given CD1d-HEK 293 cells (Fig. 1C).

In the current study, we modified the previous Ribomax (Promega) in vitro transcription protocol (33) by switching to MESSAGE mMACHINE (Ambion), which gives a higher yield of capped polyA+ mRNA. High efficiency transfection of EGFP mRNA was observed (Fig. 1D) and significant amounts of OVA protein were produced in HEK293 and CD1d-HEK293 cells (Fig. 1E). In quantitative studies, we verified a ten-
fold increase in the amounts of OVA protein produced by the transfectants, compared to the previous protocol (data not shown). Importantly, CD1d expression on HEK293 cells did not disturb transfection/expression of OVA mRNA (Fig. 1E).

**In vivo cross-presentation of antigen is dependent on in situ DCs**

OT-I cells did not recognize OVA peptide antigen *in vitro* when it was pulsed on human HEK293 cells, due to the xenogeneic MHC disparity (data not shown). Previously we demonstrated that when transferred intravenously into mice, allogeneic cells loaded with α-GalCer (CD1d<sup>hi</sup>-NIH3T3/Gal-ova) were mainly captured by splenic CD8<sup>+</sup> DCs. Furthermore, these DCs could cross-present OVA antigen derived from the CD1d<sup>hi</sup>-NIH3T3/Gal-ova cells *in vivo* (33). Therefore, we tested whether human aAVCs-ova were also cross-presented by resident DCs. When CFSE-α-GalCer-loaded, OVA mRNA-transfected aAVC cells (aAVC-ova) were injected into mice, they were captured by *in situ* splenic CD11c<sup>+</sup>CD8<sup>+</sup>DCs (Fig. 2A, right panel), leading to increased DC maturation as assessed by expression of CD80 (Fig. 2B). As shown in Fig. 2C, we did not detect the DC maturation in mice given unloaded CD1d-HEK293 cells; moreover, DC maturation was not induced in aAVC injected, iNKT cell-deficient, J<sup>α</sup>18 KO mice (Fig. 2C). These results indicate that iNKT cells are required for DC maturation *in situ* in our system.

In order to monitor *in vivo* antigen presentation, C57BL/6 mice were injected with CFSE-labeled OT-I cells and on the following day received aAVC-ova cells. OT-I proliferation was measured 3 days later by dilution of CFSE fluorescence intensity. *In vivo* aAVC-ova treatment resulted in OT-I cell proliferation despite the xenogeneic mismatch of MHC class I (Fig. 2D). In order to determine if host DCs presented OVA...
peptide to the OT-I cells, we used CD11c-DTR/GFP mice treated with diphtheria toxin (DT) to ablate host CD11c+DCs in vivo. (39) In these mice, there was little proliferation of OT-I cells, demonstrating the essential role of host DCs in antigen cross-presentation in mice given aAVC-ova cells.

To evaluate the antitumor immune response elicited by OVA-expressing aAVC cells, we administered EL4 or EG7 (EL4-expressing ova) tumor cells subcutaneously into mice that received OT-I cells i.v. 1 day prior. A week after the tumor inoculation, the mice were given either aAVC-ova or nothing. As shown in Fig. 2E, both tumors grew robustly in untreated mice or mice given only OT-I cells. However, treatment with aAVC-ova dramatically inhibited growth of the EG7 tumor, which expresses OVA antigen, but not the parental EL4, which does not.

Before applying our system to human studies, we felt it would be important to compare the immunological responses of mice given CD1d-expressing allogeneic vector cells versus syngeneic DCs. To demonstrate the antigen specific T cell response in an allogeneic setting, we used allogeneic murine aAVC (CD1dhi-NIH3T3 cells loaded with α-GalCer and then transfected with ova mRNA, i.e., CD1d-NIH3T3/Gal-ova mRNA) as previously reported (33). We compared the T cell responses in mice given these murine aAVC to those in mice given DC/Gal transfected with ova mRNA. We found that the T cell response in mice given the aAVC was higher than in mice given DC/mRNA and DC/mRNA/Gal (Fig. 2F). Also, we verified that an injection of CD1d-NIH3T3-ova alone did not induce DC maturation (data not shown) or T cell responses in vivo (Fig. 2F). The T cell response in mice given aAVC could not be seen in Jα18 KO mice (Fig. 2G), indicating that the T cell response in aAVC-administered mice depends on NKT cells.
aAVCs elicit antigen uptake, maturation and antigen presentation by human dendritic cells in a DC-NOG mouse model

We next tested whether human DCs can phagocytize aAVC by co-culturing CFSE-labeled aAVC with immature DCs (imDCs) (1:1), or imDCs plus human iNKT cells (1:1:1) for 12 hours and testing for the presence of CFSE within CD11c$^+$ cells. ImDCs or imDCs plus iNKT cells without added aAVC served as negative controls. Approximately 5% of human imDCs phagocytized CFSE-labeled live aAVCs in cultures containing DCs plus aAVC (DC+CD1d-HEK293/Gal). This percentage increased 4 fold in cultures that also included iNKT cells (DC+CD1d-HEK293/Gal+NKT) (Fig. 3A). Moreover, the frequency of mature DCs increased in cultures in which both antigen aAVC and iNKT cells were present (Fig. 3B). On the other hand, we noticed some differences when we assessed the IFN-$\gamma$ iNKT cell response in each group. By this criterion, iNKT cells were activated only by DC plus CD1d-HEK293/Gal, and very weakly or not at all in other groups (Fig. 3C). Thus, iNKT cells cannot be fully activated by DCs alone or by DC plus CD1d-HEK293 cells (Fig. 3C).

aAVCs transfected with mRNA encoding the human melanoma antigen, MART-1 (aAVC-MART1) were prepared, and these aAVCs expressed 18.9±7.4 μg of MART-1 protein/5x10$^5$ cells as assessed by Western blot (Fig. 4A). NOG mice lack B, T, NK and iNKT cells and do not reject adoptively transferred human cells. After optimizing the number of imDCs that needed to be transferred into NOG mice, we established a robust system (hDC-NOG) for evaluating the ability of imDCs in situ to phagocytize and present tumor-specific antigen derived from aAVCs to antigen-specific human T cells.
(Fig. 4B). Human imDCs and iNKT cells from HLA-A2\(^+\) donors were adoptively transferred into NOG mice with or without aAVC-MART1. Ten hours later, HLA-A2 matched, but otherwise allogeneic JKF6-TILs specific for MART-1 antigen were adoptively transferred into the NOG mice (Fig. 4B).

CD86 expression by human DCs in NOG mice that received aAVCs and iNKT cells was increased (Fig. 4C). The antigen presenting activity of the DCs was then evaluated by analyzing CFSE dilution of the transferred HLA-A2\(^+\)JKF6-TIL cells by flow cytometry (Fig. 4D). JKF6-TIL did not proliferate in NOG mice transferred with either TIL plus aAVC or with TIL plus aAVC and imDCs (Fig. 4D, left), indicating that these T cells could not be stimulated by aAVC alone or by aAVC together with DCs. Significant JKF6-TIL proliferation was only observed in the mixed transfer group (TIL+DC+iNKT+aAVC), suggesting that the human DCs were capable of phagocytosis and cross-presentation to antigen-specific T cells in an HLA-A2 dependent manner (Fig. 4D, right). We interpret these findings to indicate that \(\alpha\)-GalCer on aAVCs leads to activation of iNKT cells followed by antigen uptake of the aAVCs by imDCs and presentation by iNKT-licensed mature DCs.

**Vaccination with aAVCs results in antigen-specific T cell responses**

Next, we evaluated whether tumor antigen-specific T cell responses could be generated in an autologous setting (Fig. 5A). T cells from healthy donors were transduced with a retroviral vector carrying the MART-1 DMF5 TCR gene (DMF5TCR) (40). We tested the functional capacity of the transduced T cells by co-culturing them with CIRA0201 cells pulsed with MART-1 peptide (A2-CIR/pep) or nothing (A2-CIR). Only cultures
with A2-CIR/pep had detectable levels of IFN-γ in the supernatants (Fig. 5B).

Transduced or non-transduced T cells were then adoptively transferred into NOG mice. Three hours later syngeneic imDCs were injected i.v. followed 3 hours later by iNKT cells with or without aAVCs (Fig. 5A). The transferred MART-1 TCR gene-transduced T cells only expanded robustly in the mixed transfer group (CFSE-T+DC+iNKT+aAVCs) (Fig. 5C & 5D). This proliferation occurred despite the HLA mismatch between aAVC and T cells, suggesting cross-presentation of MART-1 antigen by the human DCs in situ. Interestingly, there was little in situ proliferation of MART-1 TCR gene-transduced T cells in mice injected with an HLA-matched B lymphoblastoid cell line presenting the MART-1 peptide (CSFE-T+A2-CIR/pep) (Fig. 5C and 5D), indicating that autologous DC and iNKT cells are required for full T cell activation in this model. Also, we demonstrated that the T cell response in DC and iNKT-transferred NOG mice given CD1d-HEK293-MART-1 [aAVC-MART-1(Gal-)] alone was not be enhanced (Fig. 5E).

In order to evaluate the feasibility of using this system for immunotherapy in patients with low numbers of iNKT cells, we tested PBMCs containing a low number of iNKT cells (iNKT=0.02+/−0.01). For the PBMCs, the frequency of MART-1 TCR gene-transduced T cells was kept low (1/1000). In these experiments, proliferation of MART-1-specific T cells occurred in co-cultures containing both DCs and aAVCs more than in cultures with IL-2 alone or IL-2 plus peptide (Fig. 5F, left). The MART-1-specific T cells maintained their antigen specificity and functioned as cytotoxic T cells as assessed by a flow cytometric CTL assay (Fig. 5F, right).

We then evaluated whether aAVC-MART-1 cells could elicit an antitumor immune response in vivo. NOG Mice were first given human HLA-A2+ 624 melanoma cells...
subcutaneously. Two weeks later, the mice received MART-1 TCR gene-transduced T cells, DCs and iNKT cells from two different healthy donors (B65 and B119) with or without aAVC-MART-1 (Fig. 5G). As shown in Fig. 5H, treatment with aAVC-MART-1 inhibited the growth of the 624 MEL tumor for 50 days. There was no inhibition of tumor growth in the group without aAVC-MART-1.

**aAVCs activate iNKT cells in vivo in canine models**

We next evaluated aAVCs as potential vaccines in a preclinical safety and adverse event monitoring study using beagles. In these studies, we used 30 Gy-irradiated aAVCs. Two doses of aAVCs were given to 3 dogs per group; a low dose consisting of $5 \times 10^6$ cells and a high dose of $5 \times 10^7$ cells. The numbers of PBMC iNKT, CD4$^+$ T and CD8$^+$ T cells in the recipients were monitored by flow cytometry. We found that the frequency of CD4$^+$ T and CD8$^+$ T cells did not change over 28 days of monitoring (Fig. 6A middle and right panel). Previously, we reported that canine iNKT cells could be detected using mouse CD1d-dimer/$\alpha$-GalCer (41). The number of canine iNKT cells is generally lower than that in humans and mice, which we verified in the current study. The frequency of iNKT cells in the beagles was $0.018 \pm 0.009$ % of the total lymphocyte population in peripheral blood, i.e., $0.036 \pm 0.018$ % of CD3$^+$ T cells. Therefore, we used a previously established method for detecting low numbers of iNKT cells in human cancer patients by coculturing PBMCs with 100 ng/mL $\alpha$-GalCer-loaded murine DCs (42). Using this approach, we could detect canine iNKT cells and follow their kinetics after the injection of aAVCs (Fig. 6A left and 6B). The number of iNKT cells increased from day 7 to day 14, but went back to control level one month later (Fig. 6A left and 6B).
We also evaluated iNKT cell activation in aAVC-treated dogs using an IFN-γ ELISPOT assay (Fig. 6C) (24, 28, 32). The number of IFN-γ producing cells in aAVCs-ova immunized dogs increased at 1 week after both the low and high dose aAVC treatment. These data further indicate that aAVCs stimulate iNKT cell proliferation. Most importantly, all dogs in groups receiving both doses of aAVC were monitored from the time of aAVC immunization until 1 to 4 weeks post-immunization and they experienced no adverse effects were noted (Table 1). We have additionally verified the safety of this therapy in the dog after three injections of aAVCs (data not shown).

**Immunization of dogs with aAVC-ova leads to antigen-specific T cell responses**

We next tested whether antigen specific T cell immunity can be generated in dogs after immunization with aAVC-ova. Serum was collected from the dogs at different time points after immunization with aAVC-ova, and IL-12 levels were found to be elevated 2 and 6 hours after treatment, suggesting that DC maturation in situ occurs early after aAVC immunization (Fig. 6D). Fourteen days after an immunization, we restimulated PBMC from immunized dogs with or without OVA protein-transduced canine DCs for 36 hours and measured IFN-γ secretion by ELISPOT. The number of OVA-specific IFN-γ secreting CD8⁺T cells was elevated with both doses of aAVCs (Fig. 6E). Thus, our preclinical study demonstrated that aAVCs could safely and effectively generate an antigen-specific immune response in dogs.
Discussion

In this study, we evaluated the efficacy of human adjuvant vector cells for use as cancer vaccines in dogs and immunodeficient mice using human materials. We used allogeneic human cells, the CD1d-HEK293 cell line loaded α-GalCer and transfected with mRNA encoding tumor antigen as artificial adjuvant vector cells. Our approach uses antigen-expressing vector cells expressing two antigens, which allows for host DC cross-presentation of tumor antigens to naïve T cells while simultaneously presenting iNKT cell ligand to iNKT cells (34, 35). This strategy induces activation of tumor antigen-specific CTLs as well as iNKT cells. An additional benefit of this strategy is the flexibility of mRNA transduction, which uses mRNA derived from tumor cell lines or tumors from third party patients and encodes for tumor antigens without the need for HLA matching. Furthermore, we have found in other studies that transfer of allogeneic cells expressing cell-associated tumor antigen and iNKT cell ligand induces not only effector T cells, but also long-term T cell memory without iNKT cell anergy (manuscript in preparation).

Cellular immunotherapy using allogeneic tumor cells has been found to have a good safety profile (2, 3) and in some studies where DCs are targeted, generated a CTL response (43, 44). Several platforms using modified allogeneic cells have been introduced. GVAX immunotherapy, in which tumor cells are genetically engineered to produce GM-CSF, delivers tumor antigens to endogenous DCs in an immunostimulatory context (45, 46). Our aAVCs express both tumor antigen and iNKT cell ligand and serve as vector cells. The result of our current preclinical study verified that these cells were safe and effective in generating an immune response. To stringently evaluate safety, we
used repeated injections of aAVC in our studies. To evaluate the antigen-specific T cell response in an allogeneic setting, we used a murine allogeneic system, i.e., allogeneic murine aAVC(CD1d-NIH3T3/Gal-ova mRNA) (33). We observed an elevated OVA antigen-specific T cell response by boosting with aAVC-ova 2 week after the initial vaccination under the allogeneic setting (data not shown), suggesting that it was not blocked by an allogeneic response by the recipient. Since boosting is very important in any effective immunotherapy strategy, we continue to search optimal conditions to enhance boosting effects.

NOG mice have almost no functional endogenous immune system. NOG or NOD/SCID/IL-2rγnull (NSG) mice that have their immune systems reconstituted with engrafted human CD34+ stem cells (“humanized mice”) can be used to test potential therapeutics that modulate human immunity and viral infection (47, 48). In the current study, to evaluate the ability of human DCs to cross-present tumor antigen and α-GalCer, we developed a model in which immature human DCs were transferred into NOG mice (hDC-NOG) to generate immature DCs in the steady state as shown in Figure 4. In vivo DCs remain immature in the steady state and these cells then drives T cells toward tolerance in an antigen-specific manner. However, we showed that immature human DCs retain their phagocytic activity after transfer into NOG mice and after their maturation induced by activated iNKT cells, they can cross-present tumor antigen and activate naïve T cells.

Translational studies using large animals, e.g., dogs or monkeys, serve as an important research intermediary so that discoveries at the bench can ultimately be used in human. Continued development of our aAVC cancer vaccine as an immunotherapeutic
required testing in a large animal so that relevant toxicity endpoints could be assessed and efficacy of treatment could be confirmed. The results of a dose-escalation and multiple-dose design of aAVC delivery to beagles in this study established a robust safety profile for this agent. Furthermore, physical examination, blood chemistry analysis, and autoantibody tests shown in Table 1, as well as tissue biopsies from liver, lung and other organs from dogs given high dose-aAVCs confirmed the safety of this approach (data not shown).

The biological and immunological systems in dogs and humans share many features, therefore canines trials of immunotherapies provide a significant rationale for continued development of treatment modalities (49). These various kinds of novel cancer treatments will ultimately become available not only for humans, but also for the treatment of pet dogs. In the current study, we verified the low percentage of iNKT cells in canine PBMCs, however our data showed that canine iNKT cells responded well to human aAVCs. We also found that human PBMC, in which there are only a small number of iNKT cells can respond to aAVC in vitro (data not shown ). We previously demonstrated in a phase I/II study that an increase in IFN-γ producing iNKT cells in PBMCs from lung cancer patients, even in patients with low starting numbers of iNKT cells, was associated with prolonged median survival time (25). Furthermore, iNKT cells from cancer patients with low numbers of iNKT cells were capable of activation by DC/Gal (50). Our data in this report together with the previous clinical studies encourage further evaluation of aAVC immunotherapy for cancer patients.

The novelty of our cancer vaccine strategy is the presence of α-GalCer in combination with tumor antigen on aAVCs, targeting DCs for antigen uptake and
maturation. Once NK cells and T cells are activated following vaccination with aAVCs, iNKT cell activation is no longer required. Thus, the ultimate killing of the tumor cells could be mediated by NK cells and T cells, and not dependent on the expression of CD1d. This mechanism expands the potential use of this vaccine to target various types of cancer, including CD1d-low or -deficient cancer cells in patients. Our current study has demonstrated that aAVCs harness the innate and adaptive immune systems and could prove clinically beneficial in the development of immunotherapies against malignant diseases.

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References

Figure legends

Figure 1. Determination of the optimal conditions of human vector cells

(A) The human CD1d gene was transfected into HEK293 cells, and CD1dhi cells (CD1d-HEK293) were selected to a purity >98% using a FACS Aria. (B) To show iNKT cell ligand-presenting activity, 1x10⁴ CD1d-HEK293 cells loaded with or without α-GalCer for 48 hours were cocultured with 1x10⁵ human iNKT cell lines established from three different healthy donors for 24 hours. IFN-γ in the supernatant was measured by ELISA (BD Bioscience). (C) Mice were injected i.v. with 5x10⁵ aAVCs. Spleens were removed 2 days later, and the frequency of iNKT cells was analyzed by CD1d-dimer+ staining (24, 28). Also, the activation of iNKT cells was evaluated by CD25 expression and IFN-γ production after restimulation with α-GalCer for 12 hours. Data are representative of two independent experiments with 4 mice per group (mean ± SEM). (D) HEK293 cells were transfected with 10 μg EGFP mRNA or nothing and evaluated for EGFP expression by flow cytometry. The percentage of EGFP positive cells is indicated. Data are representative of 3 independent experiments. (E) HEK293 or CD1d-HEK293 cells were transfected with 10 μg of full length OVA mRNA. Levels of OVA protein in cell lysates were measured by ELISA (ITEA Inc.). Data shown are mean ± S.E.M. of 4 independent experiments.

Figure 2. Antigen-presenting activity of DCs in situ in mice immunized with mRNA transfected aAVC

(A) Ten million CFSE -labeled aAVCs were injected i.v. into C57BL/6 mice. Twelve hours later, the uptake of CFSE⁺ aAVCs by murine CD11c⁺ splenic DCs was measured.
by expression of CFSE after gating on CD11c using CD8a-PE and CD11c-APC by flow cytometry (left, C57BL/6 mice control and right, aAVC-injected mice). (B) DCs were analyzed for upregulation of CD80 after gating on CD11c⁺ at 12 hours using CD11c-APC and CD80-PE (isotype control, shaded; wild type mice, dot; aAVC-immunized mice, bold). Data are representative of three independent experiments with 3 mice per group (mean ± SEM). (C) The maturation of DCs was assessed by analysis of upregulation of CD40 and CD80 12 hours after an injection of aAVC, CD1d-HEK293 or nothing into WT or Jα18 deficient mice (Jα18KO). Data are representative of 2 independent experiments with 4 mice per group. (D) The antigen presenting activity of DCs was evaluated using transferred OVA-transgenic CD8⁺ T cells (OT-I cells). C57BL/6 mice were given 2x10⁶ CFSE-labeled OT-I cells and then immunized 24 hours later with 5x10⁵ aAVC-ova. In some experiments, diphtheria toxin (DT)-treated CD11c-DT receptor/GFP transgenic mice (DC-depleted mice) were used as recipients. Proliferation of the OT-I cells (CD45.1⁺CD8⁺) in spleen was measured 3 days later by dilution of the CFSE label. Data are representative of two independent experiments with 4 mice per group. The percentage indicates the ratio of divided OT-I cells in each group (mean ± SEM) (E) 2x10⁵ of either EL4 or EG7 cells were injected into C57BL/6 mice that had been given 1x10⁶ OT-I cells or nothing. One group of mice was then given 5x10⁵ aAVC-ova intravenously at day 7. Tumor size was evaluated at the indicated time points (n=5-7 per group). Arrow indicates the time of therapy with aAVC-ova. (F, G) OVA-specific CD8⁺ T cell responses were assessed a week after an injection with murine CD1d-NIH-ova mRNA, CD1d-NIH/Gal-ova mRNA, DC transfected with ova mRNA or DC/Gal transfected with ova mRNA in WT mice (F). T cell responses in CD1d-NIH/Gal-ova
mRNA-immunized WT or Jα18KO mice were assessed using Kb/OVA tetramer-PE and CD8-APC (G). Data are representative of two independent experiments with 4 mice per group (mean ± SEM). (*P<0.05 CD1dNIH/Gal-ova mRNA vs DC-ova mRNA or DC/Gal-ova mRNA) (*P<0.01 WT vs Jα18KO mice)

**Figure 3. Phagocytic capacity and maturation status of human DCs in response to aAVCs**

(A) Uptake of aAVC by human immature DCs was analyzed 12 hours after *in vitro* culture. 6 groups were tested; 1x10^5 DCs (DC only), DC plus 1x10^5 iNKT cells (DC+iNKT), DC plus 1x10^5 CD1d-HEK293 (DC+ CD1d-HEK293), DC plus CD1d-HEK293 and iNKT cells (DC+ CD1d-HEK293+iNKT), DC plus CD1d-HEK293/Gal (DC+ CD1d-HEK293/Gal), and DC plus CD1d-HEK293/Gal and iNKT (DC+ CD1d-HEK293/Gal+iNKT). (B) Simultaneously, the human DCs were also evaluated for their maturation state as indicated by the expression of CD86 after gating on CD11c⁺ using CD11c-APC and CD86-PE (isotype control, shaded). Data are representative of 4 independent experiments. (C) The NKT cell response was assessed in different cultures as shown in (A) and (B). The supernatants were harvested after culture for 24 hours and IFN-γ levels were measured by IFN-γ EILSA. Data are representative of four independent experiments (mean ± SEM). (*P<0.001 iNKT+DC+CD1d-HEL293/Gal vs other groups)

**Figure 4. The antigen presenting capacity of human dendritic cells in response to aAVCs**
(A) Recombinant MART-1 protein (left) and MART-1 protein expression by aAVCs-MART-1 (right). For quantification purposes, cell lysates were serially diluted (1/250, 1/500, 1/1000) and analyzed by immunoblotting as in Methods. (B) The treatment protocol for Figure 4C and 4D is shown. (C) 2x10^6 imDCs generated in vitro from human CD14^+ monocytes using GM-CSF and IL-4 for 4 days were transferred into NOG mice and 3 hours later, 2x10^6 iNKT cells and 2x10^6 CFSE-labeled aAVC or nothing were transferred. The upper panels illustrate the phagocytosis of CFSE-labeled aAVCs by the human CD11c^+ cells in NOG mice (left; imDC alone and right imDC plus iNKT cells plus aAVC). CD86 expression by these DCs is shown in the lower panel. (D) After following the same protocol as in (C), 10 hours later 2x10^6 CFSE-labeled HLA-A2^+ JKF6 cells (stable melanoma responding TIL cells) were adoptively transferred. Five days later, JKF6 cell proliferation (CD8^+HLA-A2/MART1-tetramer^+) was evaluated by dilution of CFSE. Data are representative of 3 independent experiments. The percentage indicates the ratio of divided JKF6 cells in each group (mean ± SEM).

**Figure 5. Human T cell activation by in situ DCs in mice immunized with iNKT cells and aAVCs.**

(A) The treatment protocol is shown. A retroviral vector carrying the MART-1-TCR gene (DMF5) was transduced into human T cells from HLA-A2^+ healthy donors as described in Methods (40). These T cells (2x10^6) were injected into NOG mice and 3 hours later, 2x10^6 imDCs were transferred, then the mice were given i.v. 2x10^6 iNKT cells and 2x10^6 aAVC-MART-1 cells 3 hours later. (B) Human IFN-γ secretion was measured from the supernatant after co-culturing 1x10^5 MART-1 TCR-transduced T...
cells with 1x10^4 HLA-A2-positive target cells (CIRA0201) pulsed or not with MART-1 peptide. (C-E) As shown in (A), autologous MART-1-TCR-transduced CD8^+ T cells or non-transduced CD8^+ T cells were transferred to NOG mice. Five days later, the proliferation of T cells (CD8^+ 6B11^+) was evaluated by flow cytometry. Histograms from one representative donor (C) and individual data from 3 donors (D) are depicted. T cell responses were assessed in NOG mice by injection with CD1d-HEK293 cells transfected with MART-1 mRNA, i.e, aAVC-MART-1(Gal-) or with aAVC-MART-1 (Gal+) (E).

(F) 1x10^6 PBMCs from HLA-A2^+ donors were mixed with 1x10^7 TCR-gene transduced T cells from the same donors (1000:1) and cultured in the presence of IL-2, IL-2 plus peptide, or IL-2 plus aAVC plus imDCs for 7 days. Left panels, the fold increase in cell number (post-culture/pre-culture) was evaluated. Right panels, CD8^+ T cells were sorted and tested for cytotoxicity against CIRA0201 or CIRA0201/peptide (E/T=1). (G) The treatment protocol is shown. MART-1-TCR-transduced T cells, iNKT cells and DCs were prepared from 2 HLA-A2^+ donors (B65 and B119). 5x10^6 624 MEL were subcutaneously inoculated to NOG mice. Two weeks later, MART-1 TCR-transduced T cells, iNKT cells and DCs together with or without aAVCs were administered i.v. to mice on the same day. Transferred cells were from the same donors in each study. (H) Tumor size was evaluated at indicated the time points in each group.

**Figure 6. Immunization with aAVC-ova leads to iNKT cell responses in dogs**

(A) Two doses of aAVCs (low dose; 5x10^6, high dose; 5x10^7) were administered to 3 dogs. The frequency of CD4^+ T cells and CD8^+ T cells among total CD3^+ T cells was assessed by flow cytometry. The frequency of iNKT cells among total CD3^+ T cells was
evaluated as previously described (42). iNKT cells were stained with murine CD1d-dimer/Gal and canine CD3 Ab as previously reported (41). (B) Representative data of iNKT cell frequency in dogs injected with a high dose of aAVC as assessed by flow cytometry. (C) On day 7 after the vaccine, expanded PBMCs were restimulated with α-GalCer (100 ng/mL) for 16 hours and then tested for IFN-γ secretion by IFN-γ-ELISPOT. Data shown are mean ± S.E.M. of 3 dogs per each group. (*P<0.05, pre versus 1 week) (D) Serum IL-12 was measured by ELISA (R&D Systems) in aAVC-injected dogs at the indicated time points after immunization with aAVC-ova. Left panel is the representative data of high dose (○) and low dose (■) aAVC injected groups and the right panel shows the mean ± S.E.M.. (The control is the data of six unimmunized dogs.) (E) Seven days after immunization with aAVC-ova, T cell responses to OVA were evaluated by canine IFN-γ-ELISPOT. CD8+T cells were isolated from PBMC of immunized dogs using rat anti-dog CD8-PE (Serotec) and PE-magnetic beads (Miltenyi) and then restimulated with or without OVA protein–transfected canine DCs for 36 hours before the ELISPOT assay. Data shown are mean ± S.E.M. of 3 dogs per each group. (*P<0.05, -OVA versus +OVA)
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Table 1. Analysis of adverse events in canine
Fig. 2

A

SSC

CD11c

CD8

Non-immunized

CFSE-aAVC-ova

B

CD80

CD8

C

Immunization

Recipient

aAVC

aAVC

Jx18KO

WT

WT

CD1dHEK-293

WT

non

isotype

CFSE

D

WT-mice

DC-depleted mice

aAVC-ova

(i.v.)

E

EL4

n=5

EG7

n=7

EL4+OT-1

n=5

EG7+OT-1

n=7

EL4+OT-1+aAVC-ova

n=5

EG7+OT-1+aAVC-ova

n=7

Days after tumor inoculation

F

CD1d-NIH-ova mRNA

Kb/OVA-tetramer

DC-ova mRNA

CD1d-NIH/Gal-ova mRNA

DC-Gal-ova mRNA

CD8

Total no. of CD8^+ OVA-tet^+ cells in spleen (x10^6)

G

Total no. of CD8^+ OVA-tet^+ cells in spleen (x10^6)

WT

Jx18KO

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Fig. 3

A

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<th>CD11c⁺</th>
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B

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C

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* Indicates significant difference.
Fig. 4

A. MART-1

B. Uptake of aAVC by DCs

NOG mice

T cell response

C. DC

CD11c

CFSE

CD86

D. A2^+TIL +DC +aAVC-MART-1

A2^+TIL +DC +aAVC-MART-1+inKT

A2^+TIL only

A2^+TIL +DC +inKT

A2^+TIL only
Vaccination with antigen-transfected, NKT cell ligand-loaded, human cells elicits robust in situ immune responses by dendritic cells

Kanako Shimizu, Takuya Mizuno, Jun Shinga, et al.

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