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Evaluation of recombinant adenovirus vectors and adjuvanted protein as a heterologous prime-boost strategy using HER2 as a model antigen

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ABSTRACT

Induction of strong antigen-specific cell-mediated and humoral responses are critical to developing a successful therapeutic vaccine. Herein, using HER2 as a model antigen, we aim to evaluate a therapeutic vaccine protocol that elicits anti-tumor antibody and cytotoxic T cells to HER2/neu antigen. Replication-competent (ΔPS AdV) and non-replicating recombinant adenoviral vectors (AdV) expressing a rat HER2/*neu* (ErbB2) oncogene, were generated and compared for four different doses and over four time points for their ability to induce antigen-specific T and B cell responses in mice. Although ΔPS AdV:Her2 vector was shown to induce more durable antigen-specific $CD8^+$ T cell responses, overall, the AdV:Her2 vector induced broader T and B cell responses. Hence the AdV:Her2 vector was used to evaluate a heterologous prime-boost vaccination regimen using rat HER2 protein encapsulated in archaeosomes composed of a semi-synthetic glycolipid (sulfated S-lactosylarchaeol, SLA; and lactosylarchaeol, LA) (SLA/LA:HER2enc) or admixed with archaeosomes composed of SLA alone (SLA:HER2adm). We first tested AdV:Her2 using a prime-boost approach with SLA/LA:HER2enc, and thereafter evaluated a sub-optimal AdV:Her2 dose in a heterologous prime-boost approach with SLA:HER2adm. A single administration of AdV:Her2 alone induced strong cell-mediated immune responses, whereas SLA/LA:HER2enc alone induced strong antigen-specific IgG titers. In mice primed with a suboptimal dose of AdV:Her2, strong $CD8^+$ T-cell responses were observed after a single dose which were not further augmented by protein boost. AdV:Her2 induced $CD4^+$ specific T-cell responses were augmented by SLA:HER2adm. Homologous vaccination using SLA:HER2adm induced strong antigen-specific antibody responses. However, the overall magnitude of the responses was similar with three doses of SLA:HER2adm or Ad:HER2 prime followed by two doses of SLA:HER2adm.

We demonstrate that AdV:Her2 is capable of inducing strong antigen-specific $CD8^+$ T cell responses, even at a low dose, and that these responses can be broadened to include antigen-specific antibody responses by boosting with SLA adjuvanted proteins without compromising CD8 T cell responses elicited by AdV priming.

1. Introduction

Breast cancer is the most prevalent cancer in women worldwide with a high yearly incidence (~1.15 million newly diagnosed) and early death rate (~465,000) [1]. Human epidermal growth factor-2 (HER2; also known as ErbB2), a member of transmembrane tyrosine kinase receptors, was identified as an oncogene activated by a point mutation in chemically-induced rat neuroblastomas [2]. It was later found to be overexpressed by up to 25% in some human breast carcinomas, and has been traditionally associated with an aggressive cancer phenotype and with poor prognosis of those patients [3–5]. Consequently, HER2 oncoprotein has been an active target for development of therapeutic agents and cancer research [6]. Passive immunotherapeutic strategies with HER2-directed recombinant humanized monoclonal antibodies (mAbs) such as tratuzumab, pertuzumab and bevacizumab have significantly improved prognosis of HER2-positive breast cancers [7]. Despite the clinical success of passive HER2 immunotherapy, patients often relapse from these therapies likely due to acquired resistance. Thus

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new therapies such as those based on active immunotherapy strategies such as cancer vaccines and checkpoint inhibitors are needed [8]. The key advantage of cancer vaccines in such therapies is the potential for a specific, sustained immune response after completion of passive treatment, negating the need for multiple treatments/infusions typical of passive immunotherapy.

Cancer vaccines generally target un-mutated self-proteins that are aberrantly overexpressed in cancers such as HER2. Therapeutic cancer vaccines have had limited success in human clinical studies likely due to their inability to induce immune responses strong enough to break immunological tolerance to these self-proteins and overcome tumor-mediated immune suppression. Various strategies have been employed to enhance the poor immunogenicity of these tumor associated antigens including the use of strong antigen delivery platforms such as viral vectors which have inherent adjuvanticity through virally-derived pathogen associated molecular patterns [9,10], the use of adjuvants to augment immune responses to the vaccine antigen(s) and the concomitant use of immunomodulatory agents such as cytokines and immune checkpoint inhibitor antibodies [11,12].

Viral vector-based vaccines are known for their efficient antigen delivery through high transgene expression *in vitro* and *in vivo* allowing enhanced vaccine immunogenicity and efficacy. Adenovirus, a non -enveloped double stranded DNA virus, has several features that have attracted their use for vaccines, including infecting both dividing and non-dividing cells, high transgene expression efficacy, ability to grow in high titers, and lack of integration into host genome platforms [13,14]. Adenovirus-based vectors are one of the most commonly used antigen delivery platforms for therapeutic vaccines in human clinical and pre-clinical studies and have been shown to induce potent T- and B-cell responses to a wide range of transgenes [14–16]. Conventional 1st generation Ad5 vectors usually have the E1 region (genes involved in viral replication) deleted to ensure replication deficiency of the virus allowing it to be safely used as a gene delivery tool, and the E3 gene deleted (ΔE1/E3) to accommodate larger recombinant transgenes (up to 8 Kb). These replication-incompetent vectors have limited pathogenicity; however some replication-competent (ΔE3) adenovirus currently being tested may have the benefit of vaccine vector genome replication *in vivo*, resulting in a more potent long-term immune response [17]. An Ad5 vector based on a replication-competent platform, with a deletion in the protease (PS) gene necessary for viral assembly, has been described previously [18,19]. The adenoviral vector replicates its DNA normally in infected cells but fails to form infectious particles.

One limitation of adenovirus is that adenoviral vectors are inherently immunogenic and hence develop strong neutralizing anti-viral immune responses which can limit transgene-specific responses following primary immunization. As such these vectors are not effective for repeated dosing which is a necessity in therapeutic vaccinations. Thus, effective booster vaccination regimens that can boost and sustain vaccine-specific immune responses are needed. Adjuvanted protein antigens is a feasible approach for boosting immune responses primed by viral vector based vaccines. Liposomal vesicles composed of total polar lipids (TPL) unique to the *Archaea* domain (archaeosomes), or semi-synthetic glycerolipids have been shown to exhibit strong adjuvant activity across multiple antigens [20–22]. However, TPL formulations and semi-synthetic phosphor-archaeol–based lipids are relatively complex formulations, demand higher costs to manufacture, and may be prone to chemical and enzymatic degradation. Recently, a novel adjuvant formulation comprising of a simple semi-synthetic glycerolipid sulfated S-lactosylarchaeol (SLA), when used alone or mixed with uncharged glycolipid (lactosylarchaeol, LA) has been shown to strongly enhance antigen-specific humoral and cell-mediated immunogenicity against multiple antigens [22,23]. Indeed, when used as adjuvant with entrapped ovalbumin (OVA) or hepatitis B surface antigen (HBsAg), SLA archaeosomes induced as good as or better immune responses to multiple other adjuvants including TLR3/4/9 agonists, oil-in-water and water-in-oil emulsions and aluminum hydroxide [24].

Herein, we compare replication-incompetent (ΔE1/ΔE3) adenoviral vector expressing rat HER2 (herein referred to as AdV:Her2) to a replication-competent non-disseminating adenoviral vector expressing rat HER2 (herein referred to as ΔPS AdV:Her2) for their ability to prime immune responses against rat HER2 protein and assess the immunogenicity of prime boost vaccination strategies using recombinant AdV to prime and HER2 protein to boost using SLA archaeosomes (encapsulated or admixed) as adjuvant. There is >95% homology between rat and mouse HER2 proteins [25] thus; the rat Her2 is used as a surrogate self-antigen in these studies.

2. Materials & methods

2.1. Animals

All experiments were carried out using 6–8 week-old female BALB/c mice (Charles River Laboratory; Saint-Constant, QC, Canada), and performed in strict accordance with the guidelines and regulations set by the Canadian Council of Animal Care (CCAC) and the Institutional Animal Care and Use Committee at the National Research Council of Canada. Animals were monitored for any clinical signs of distress using approved institutional humane endpoints and guidelines throughout the study.

2.2. Generation of AdV:Her2 and ΔPS AdV:Her2 vectors

The rat HER2/*neu* (ErbB2) gene was synthesized by GenScript (Piscataway, NJ, USA) and cloned into pShuttle-CMV5-Cuo vector [26]. The non-replicating and non-disseminating AdV (ΔE1, ΔE3; 1st generation) encoding the rat HER2 (AdV:Her2), also known as *neu* in rats, antigens were made by homologous recombination in bacteria using the AdEasy plasmid system (Agilent Technologies, Santa Clara, CA, USA). The replicating but non-disseminating adenoviral vector (ΔPS AdV) based on human AdV5 with a deletion of the protease (PS) gene, but expressing E1a, has been described previously [18] (Fig. 1A). The ΔPS AdV:Her2 was generated by replacing the C:U gene in transfer vector of pAdVatorCMV5-Cuo-IRES-E1a with rat HER2 gene. The resulting plasmid was linearized with *Pme*I and co-transformed into bacteria with an adenoviral plasmid deleted for protease gene to generate by homologous recombination a plasmid encoding the complete genome of the ΔPS E1+ adenovirus encoding the HER2 gene. The latter plasmid was then digested with *Pac*I and transformed into HEK293A-CymR-PS (a HEK293A cell line that stably expresses the adenoviral protease). Ad-CMV-ErbB2 (1st generation) and Ad5-ΔPS-CMV-ErbB2-IRES-E1a (Human adenovirus serotype 5; replicating but non-disseminating adenovirus) were amplified using SF-BMAd-R [27] and SF-BMAd-PS cells (a cell line derived from SF-BMAd-R cells that stably expresses the protease of adenovirus) respectively and purified by ultracentrifugation on CsCl gradients as described previously [19].

2.3. Western blot assays

Western blot analysis to determine rat HER2 protein expression by AdV:Her2 and ΔPS AdV:Her2 was performed after infection of A549 cells. Briefly, 10^6 human lung carcinoma cells (A-549) (ATCC number CCL-185) were infected at a multiplicity of infection (MOI) of 1, 3 and 50 infectious particles per cell and incubated for 24 hr, 48 hr and 72 hr followed by lysis of the cells. A-549 cell supernatants and cell lysis were resolved on a SDS-PAGE gel under

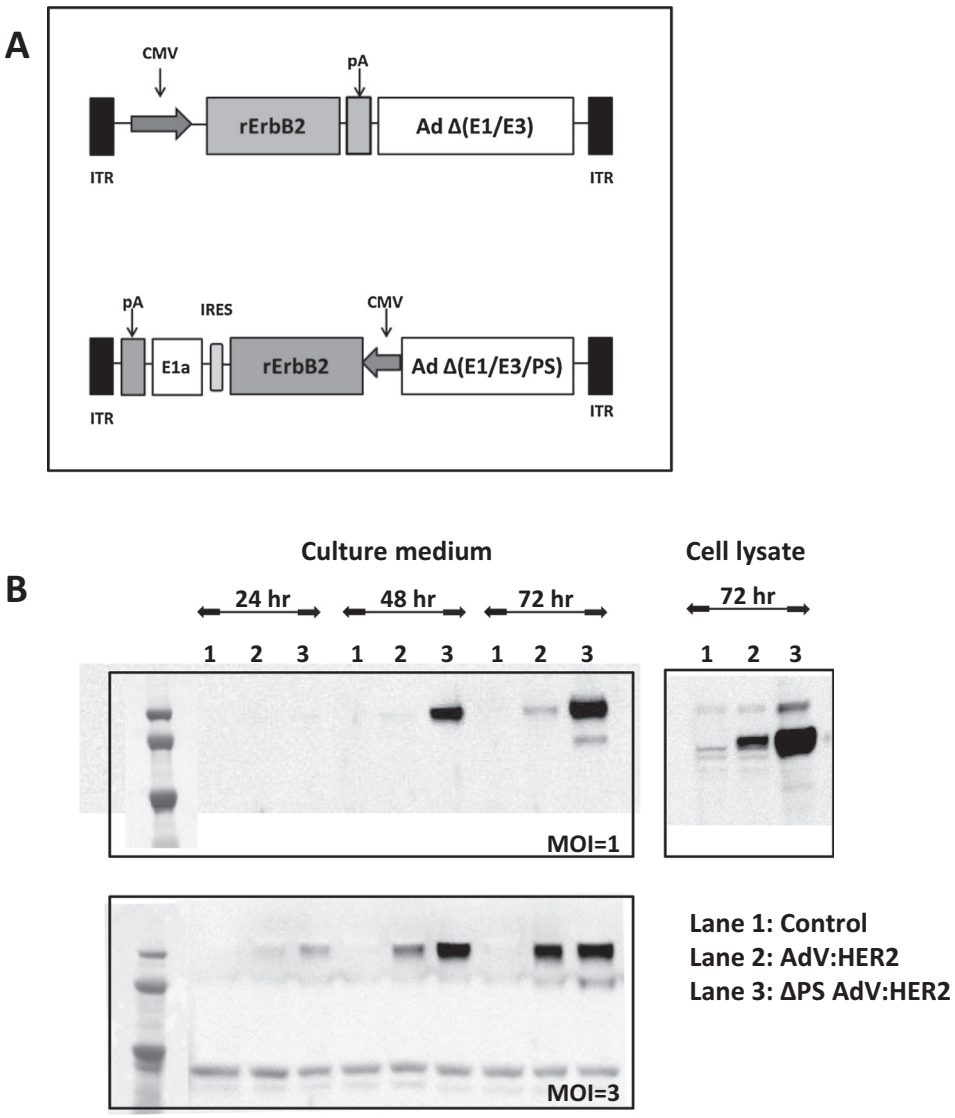


Fig. 1. Construction and expression of recombinant AdV:Her2 (ErbB2). A) The 1st generation AdV:Her2 lacks the E1 and E3 regions (upper schematic), and the rat ErbB2 cDNA is inserted in the E1 region and controlled by the CMV promoter. ΔPS AdV:Her2 (lower schematic) lacks the E1/E3 region as well as the protease (PS) gene, but includes the E1a gene and the rat ErbB2 cDNA. Expression is controlled by the CMV promoter in a discistronic configuration (co-expression of ErbB2 and Ad E1a through the use of an internal ribosome entry site (IRES). B) Western blot analysis of rat HER2 protein expression in A549 cells infected with recombinant AdV:Her2 at an MOI = 1 (upper panel) and 3 (lower panel). The presence of HER2 was analyzed in the culture medium and in cell lysate. Mock-infected cells were used as a control.

reducing condition, followed by transfer onto polyvinylidene difluoride (PVDF) membranes, which was then blocked with 5% dry non-fat milk in phosphate buffered saline with tween 20 (PBS-T) buffer (1 × PBS and, 0.05% Tween 20). The membrane was incubated overnight at 4 °C with anti-ErbB2 antibody (Abcam, Toronto, Canada; 131490; dilution 1/500), washed, and incubated with HRP-conjugated anti-rabbit antibody (GE Healthcare, Mississauga, Canada; dilution 1/5000).

2.4. Antigens, peptides and adjuvants

Rat HER2 extracellular ligand-binding domain (ECD) protein was purchased from Sino Biological Inc. (Beijing, China) and used as protein antigen either encapsulated or admixed with archaeosomes. Synthetic peptides used in immune assays were: p66 (TYVPANASL) a dominant rat $CD8^+$ HER2 epitope in H-$2K^d$ mice [28], or p169 (DMVLWKDVFRKNNQL), a $CD4^+$ T-cell HER2 extracellular domain epitope [29]. Short AdV peptides with immunodominant epitopes for AdV hexon and DNA-binding proteins: KYSPSNVKI (p486-494), FALSNAEDL (p419-427), and LPKLTPFAL (p413-421) respectively, previously shown to have strong responses [30,31]. Peptides were custom synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). Lactosylarchaeol (LA; *β*-d-Gal*p*-(1,4)-*β*-d-Glc*p*-(1,1)-archaeol) and sulfated lactosylarchaeol (SLA; 6′-sulfate-*β*-d-Gal*p*-(1,4)-*β*-d-Glc*p*-(1,1)-archaeol) were synthesized as reported previously [32,33]. For encapsulation, SLA/LA archaeosome were prepared by adding antigen to a dried lipid film, suspending and sonicating until desired size was obtained followed by purification and centrifugation to remove untrapped HER2 protein antigen (SLA/LA:HER2enc). Percent antigen entrapment was quantified by SDS-PAGE [34]. For admixed formulations, antigen solution with a specific antigen:lipid ratio was added to pre-formed empty archaeosomes (SLA:HER2adm) before immunization.

2.5. Immunization

AdV vectors or SLA-adjuvanted protein were administered into mice via bilateral intramuscular injections (i.m.) into the quadri-

ceps muscle in a total volume of 50 μL per side. Mice received either single (day 0), double (day 0 and 21) or triple (day 0, 21 and 35) immunizations. Serum and splenocytes collected at 1-week post immunizations were used for quantifying humoral and cell-mediated immune responses respectively.

2.6. Cytotoxic T lymphocyte (CTL) assay

HER2-specific $CD8^+$ T cell mediated cytotoxic activity was measured using an *in vivo* CTL killing assay which has been described previously [35]. Briefly, a mixture of TYVPANASL peptide-pulsed (labelled with 2 μM carboxyflourescein diacetate succinimidyl ester; CFSE) and non-peptide-pulsed CFSE (labelled with 0.2 μM CFSE) naive BALB/c splenocytes was mixed at 1:1 ratio and injected into immune mice. 24 hr post cell injection, spleens were removed from recipient mice and analyzed by flow cytometry for loss of peptide pulsed target cells relative to unpulsed non-target control cells, and the percent specific lysis (%) was calculated as: $100 \times (1 - [\text{ratio of cells recovered from naive mice/ratio of cells recovered from infected mice}])$.

2.7. IFN-γ ELISPOT assay

This assay was performed using an IFN-γ enzyme linked immunospot (ELISPOT) mouse assay kit (MabTech Inc., Cincinnati, OH, USA) in accordance with the manufacturer's instructions. Briefly, splenocytes from mice were plated at a final cell density of 4×10^5 per well into ELISPOT plates pre-coated with an anti-IFN-γ antibody (Mabtech Inc.), co-cultured with 10 μg/mL of $CD8^+$ or $CD4^+$ antigen-specific peptides (p66 = TYVPANASL, p169 = DMVLWKDVFRKNNQL respectively), or rat HER2 ECD protein (5 μg/mL) for 20 hr at 37°C, 5% CO_2. Cells were also incubated without any stimulants to measure background responses. The plates were then incubated, washed and developed according to the manufacturer's instructions. AEC substrate (Becton Dickenson, Franklin Lakes, NJ, USA) was used to visualize the spots. IFN-γ-specific spot-forming units (SFU) were counted using an automated BioSys ELISpot plate-reader (BioSys GmbH, Karben, Germany).

2.8. Rat HER-2 specific antibody ELISA

Serum anti-HER2 antibody titers from immunized mice were measured by a direct ELISA using 96-well plate. ELISA plates were coated overnight at room temperature with 100 μL of 0.5 μg/mL rat HER2 protein in PBS per well (Sino Biological Inc. China). Plates were washed with PBS/0.05% Tween20 (PBS-T; Sigma-Aldrich, St. Louis, MO, USA) and then blocked for 1 hr at 37 °C with 200 μL 10% fetal bovine serum (Thermo Fisher Scientific, Waltman, MA, USA) in PBS buffer. After plates were washed five times with PBS-T, serial dilutions of sera were added for 1 hr at 37 °C, and then incubated with the 100 μL HRP-conjugated goat anti-mouse IgG (1:4000; Southern Biotech, Birmingham, AL USA) for 1 hr at 37 °C, washed. ELISA plates were developed with OPD substrate (Sigma Aldrich) for 30 min at room temperature (RT) in the dark. The reaction was stopped with 50 μL/well of 4 N H_2SO_4. Bound IgG antibodies were detected spectrophotometrically at 450 nm. Titers for IgG in serum were defined as the dilution that resulted in an absorbance value (OD 450) of 0.2 and were calculated using XLfit software (ID Business Solutions, Guildford, UK).

2.9. Statistical analysis

Data were analyzed using GraphPad Prism® (GraphPad Software, San Diego, CA). Statistical significance of the difference between groups was calculated by 1-way analysis of variance (ANOVA) or 2-way ANOVA followed by post-hoc analysis using Tukey's (comparison between all groups) multiple comparison tests. Antibody titers and cytokine levels were log transformed prior to statistical analysis. For all analyses, differences were considered to be not significant with $p > 0.05$.

3. Results

3.1. Kinetics and magnitude of HER2 protein expression by 1st generation AdV and ΔPS Ad vectors

Fig. 1A depicts a cartoon of human Ad5 based replication defective 1st generation AdV and replicating but non-disseminating ΔPS adenoviral vectors expressing rat HER2. The latter vector can replicate because it expresses the E1a region of adenovirus, but cannot form infectious particles because of the absence of the PS gene. [19]. A549 human lung carcinoma cell line was infected with these vectors at multiplicity of infection (MOI) of 1, 3 and 50 to assess the kinetics and the magnitude of transgene expression. Level of protein expression was measured by Western blot analysis at 24, 48 and 72 hr in cell supernatant and at 72 hr in cell lysates. The ΔPS AdV:Her2 showed faster kinetics and greater magnitude of transgene expression compared to AdV:Her2 vector which was evident at MOI of 1 and 3 (Fig. 1B). Infection with ΔPS AdV:Her2 at MOI = 50 induced cytopathic effect (CPE) starting at 48 hr (data not shown). The level of transgene expression was also assessed in 72 hr cell lysates at MOI = 1 where ΔPS AdV:Her2 showed greater magnitude of transgene expression compered to AdV:Her2 (Fig. 1B).

3.2. Dose response of AdV:Her2 and ΔPS AdV:Her2 vectors for priming HER2 specific immune responses in mice

AdV:Her2 and ΔPS AdV:Her2 vectors were tested at doses of 10^5 (four-log lower), 10^7 (2-log lower) and 10^9 infectious units (IFU) to assess their ability to prime HER2 specific T- and B-cell responses in mice 3 weeks post immunization. The magnitude and the functionality of $CD8^+$ T cell responses were measured using ELISPOT and *in vivo* CTL assays respectively using a dominant CTL-eliciting $CD8^+$ T cell epitope [28]. Dose dependent increase in IFN-γ levels and CTL activity were seen with both AdV:Her2 and ΔPS AdV:Her2 vectors with the lowest dose tested (10^5 IFU) inducing significantly lower $CD8^+$ responses compared to the two higher doses (10^7 and 10^9 IFU) tested ($p < 0.05$). Overall, there was no significant difference between the 2 vectors in their ability to prime cytotoxic $CD8^+$ T cell responses (Fig. 2A). The antigen-specific $CD4^+$ T cell responses were measured by ELISPOT assays using either whole HER2 ECD protein or HER2 peptides containing known $CD4^+$ epitopes. Overall much lower $CD4^+$ responses were seen compared to the $CD8^+$ responses with both AdV vectors and there was no difference between the two vectors (Fig. 2B). For both vectors there were minimal responses to HER2 ECD protein. In order to compare the 2 vectors for their ability to stimulate antibody responses, rat HER2 specific total IgG was measured. As shown in Fig. 2C, an overall dose dependent increase in HER2 specific antibody responses was seen with both AdV vectors; however, overall significantly greater Ab titers were induced with AdV:Her2 compared to ΔPS AdV:Her2 at 10^9 dose.

3.3. Dose-dependence and longevity of immune response induced by AdV:Her2 and ΔPS AdV:Her2

Since there was no detectable difference in cell-mediated immune response between the AdV:Her2 and ΔPS AdV:Her2 at 3 weeks post single immunization, we next determined whether the difference in vectors would impact the longevity of the

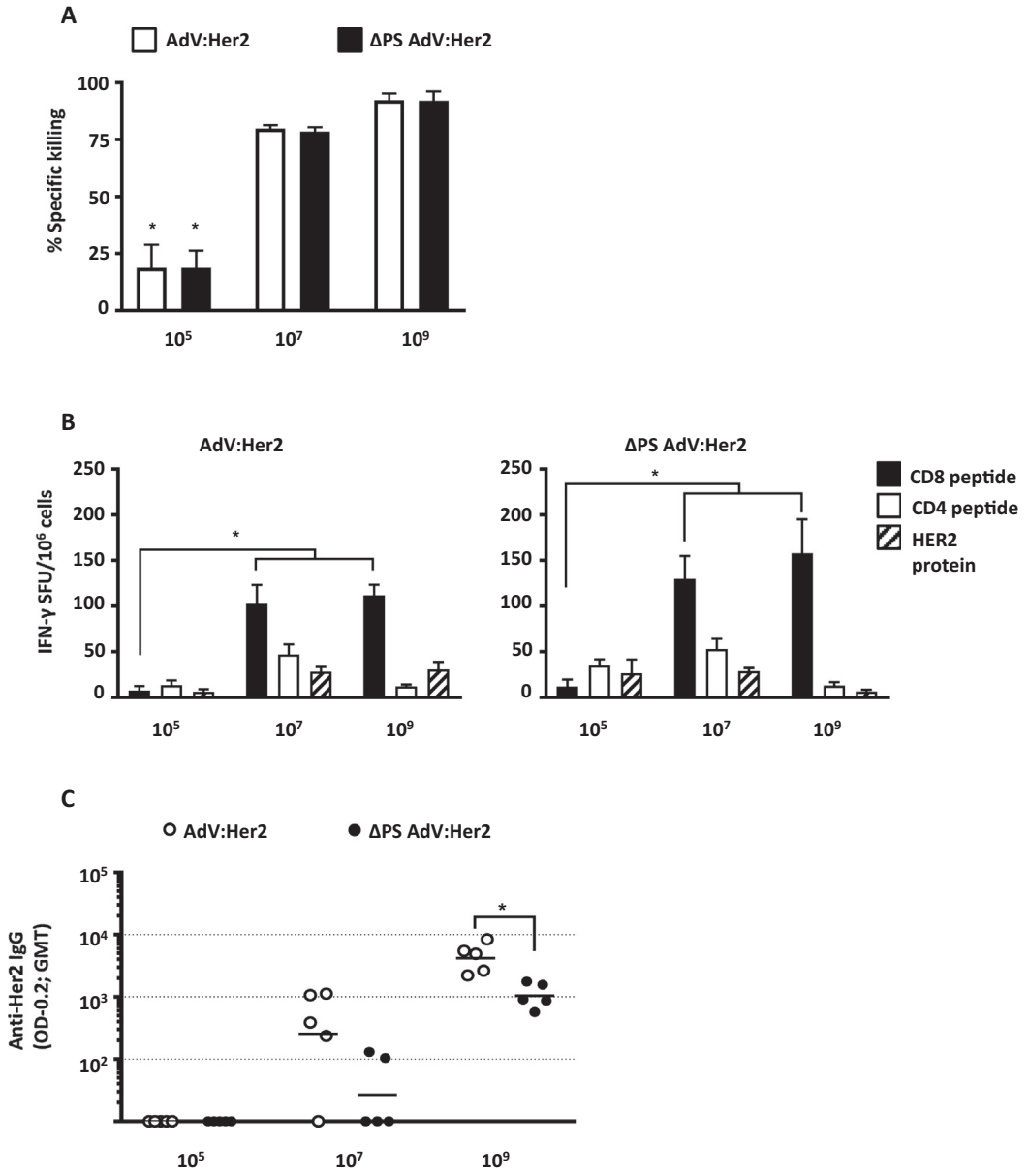


Fig. 2. Dose response of AdV:Her2 vs. ΔPS AdV:Her2: Immune responses at 3 weeks post-immunization. Female BALB/c mice (n = 5/group) were injected on day 0 with 10^5, 10^7 or 10^9 IFU of AdV:Her2 or ΔPS AdV:Her2 by bilateral i.m. injection into quadriceps muscles. Immune responses were evaluated 3 weeks later. A) Cytotoxic $CD8^+$ T cell responses were measured using an *in vivo* CTL assay. The bars represent the mean specific lysis ± SEM. *$p < 0.001$ of responses with 10^5 IFU compared to 10^7 or 10^9 IFU with the same vector. B) Splenocytes were used to quantify IFN-γ secreting T-cells by ELISPOT in response to stimulation with $CD8^+$ or $CD4^+$ peptides as well as rat HER2 protein. Bars represent the number of IFN-γ spot forming units (SFU) per 10^6 splenocytes ± SEM, and data represent the background subtracted values. C) HER2 specific total IgG in serum was quantified using ELISA assay. The dots indicate the titers of individual animals. The bars represent the geometric means of individual groups; *$p < 0.001$ for groups compared to each other by one-way ANOVA followed by Tukey's multiple comparison test.

immune responses following a single immunization at four levels: 10^6, 10^7, 10^8 and 10^9 IFU.

There was a dose dependent increase in cytotoxic $CD8^+$ T cell responses with both AdV vectors at all time points tested (Fig. 3A). With AdV:Her2, there was a decline in cytotoxic $CD8^+$ T cell responses over the 28 week period with all doses tested. For example, at 28 weeks, $CD8^+$ T cell responses were significantly lower at all doses tested when compared to the responses seen at 3 week post-prime ($p < 0.05$) (Fig. 3A; upper panel). In contrast, with the ΔPS AdV:Her2, although there was a significant decline in the cytotoxic $CD8^+$ T cell responses by the 28 week period at the 2 lower doses tested; the responses remained high and not significantly different ($p > 0.05$) from levels seen at 3 weeks post prime with the higher doses (10^8 and 10^9 IFU) (Fig. 3A; lower panel), likely in response to the stronger viral gene expression resulting in more durable T-cell responses. There was no significant difference in the overall cytotoxic $CD8^+$ T cell responses between the two vectors at any of the time-points or the dose levels tested.

HER2 antigen specific IFN-γ secreting T cells were detected in splenocytes by IFN-γ ELISPOT assays (Fig. 3B). Overall, as was seen at the 3 week time-point, a greater magnitude of transgene specific $CD8^+$ responses were seen compared to $CD4^+$ responses with both AdV groups at all time-points tested. The magnitude of CD4 responses detected upon ex vivo stimulation of splenocytes with whole HER2 protein was lower compared to stimulation with the CD4 peptides. This is likely due to the suboptimal presentation of $CD4^+$ peptides with the use of whole proteins compared to when peptides are used. Peptides spanning adenovirus hexon and DNA-binding proteins [30] were used to test the responses against viral proteins. ΔPS AdV:Her2 induced potent responses against Ad viral

proteins which were significantly higher ($p < 0.001$) compared to the responses seen with AdV:Her2 at the 3 lowest dose levels at all four time-points tested, confirming stronger *in vivo* viral gene expression with the ΔPS AdV:Her2 as anticipated (Fig. 3C).

As shown in Fig. 3D, a dose dependent increase in HER2 specific antibody responses was seen with both vectors. However, overall greater Ab titers were induced by AdV:Her2 compared to ΔPS AdV:Her2 at all time points with all doses tested. The titers were significantly different with 10^8 and 10^9 IFU doses at 3, 6 and 12 weeks post immunization and with 10^8 IFU dose at 28 week post immunization ($p < 0.05$).

3.4. Prime-boost regimen with AdV:Her2 and SLA/LA archaeosome encapsulated HER2

Comparison of AdV:Her2 and ΔPS AdV:Her2 vectors showed that both vectors were capable of priming equally strong $CD8^+$ T cell responses; however, AdV:Her2 vector was significantly superior to the ΔPS vector for inducing transgene-specific antibody responses (Fig. 3D) and induced lower level of anti-vector immunity (Fig. 3C). As such, the AdV:Her2 was selected for subsequent studies. Since priming with AdV also induces vector-specific immunity, a heterologous boosting strategy is required to boost and sustain the AdV primed transgene-specific immune responses. Since archaeosome adjuvants have previously been shown to augment immune responses against a wide variety of antigen in mice and break immunological tolerance to self-antigens [20–22], we evaluated the ability of SLA-archaeosome with 2 μg encapsulated rat HER2 (SLA/LA:HER2enc) to boost AdV:Her2 primed immune responses in mice. Mice receiving either one or two immunizations with 2×10^9 IFU AdV:Her2 had >95% cytotoxic $CD8^+$ killing of HER2-specific target cells, whereas there was <2% antigen specific $CD8^+$ killing of target cells observed in mice receiving either one or two immunizations of SLA/LA:HER2enc (Fig. 4A). Similarly, mice receiving either one or two immunizations with AdV:Her2 showed HER2-specific T cell IFN-γ responses by ELISPOT assay whereas there were no detectable T-cell responses seen in mice receiving single or double dose of SLA/LA:HER2enc (Fig. 4B).

A single immunization with AdV:Her2 induced significantly higher HER2-specific total IgG compared to a single immunization with SLA/LA:HER2enc (Fig. 4C). As expected there was no increase in HER2-specific IgG titers with homologous AdV:Her2 boost but homologous SLA/LA:HER2enc significantly increased HER2-specific antibody titers ($p < 0.001$). Ad primed humoral responses were significantly increased following SLA/LA:HER2enc boost compared to homologous AdV:HER2 prime/boost ($p < 0.001$). Overall, the strongest humoral responses were seen with the heterologous prime-boost regimen using AdV:Her2 and SLA/LA:HER2enc, which was significantly greater than homologous prime-boost using SLA/LA:HER2enc or AdV:Her2 ($p < 0.05$ and $p < 0.001$ respectively) (Fig. 4C).

3.5. Heterologous prime-boost study using a suboptimal AdV:Her2 prime and single or double boost approach with SLA archaeosome admixed with rat HER2 protein

We assessed the immunogenicity of suboptimal AdV:Her2 as a priming vaccine vector in a heterologous prime-boost regimen with HER2 protein admixed with SLA. The efficacy of antigen encapsulation within archaeosome lipid vesicles is typically low (5–40%) which results in low antigen entrapment, increased cost and varied amounts of archaeal lipid in the final formulation. To overcome this, we have developed a novel archaeosome formulation whereby empty pre-formed archaeosomes are admixed with antigen prior to injection thereby resulting in no antigen loss during formulation process, a better defined final formulation and retaining strong immunostimulatory activity [36]. Therefore 10 μg rat HER2 protein antigen was admixed with pre-formed empty SLA archaeosomes and evaluated as boosting strategy (SLA:HER2adm). In order to assess the full potential of the SLA adjuvant, AdV:Her2 was used at a suboptimal dose of 10^5 IFU followed by a single (on day 21) or double booster (day 21 and 35) immunization using SLA:HER2adm. As shown in Fig. 5A, a single dose of AdV:Her2 induced significantly higher cytotoxic $CD8^+$ T cell responses compared to a single or multiple doses of SLA:HER2adm ($p < 0.001$). The Ad primed cytotoxic $CD8^+$ T cell responses were not further boosted by SLA:HER2adm boost ($p < 0.05$). The greatest magnitude of cytotoxic $CD8^+$ T cell responses induced by AdV:Her2 were seen at day 28 post dose which declined ~ 50% by day 42post dose (Fig. 5A). Similarly, a single dose of AdV:Her2 also induced significant IFNγ $CD8^+$ T cell responses ($p < 0.001$) which increased by day 28 post prime and remained steady at day 42 post prime (Fig. 5B). There was no significant increase in AdV:Her2 primed IFNγ $CD8^+$ T cell responses following a single or double booster

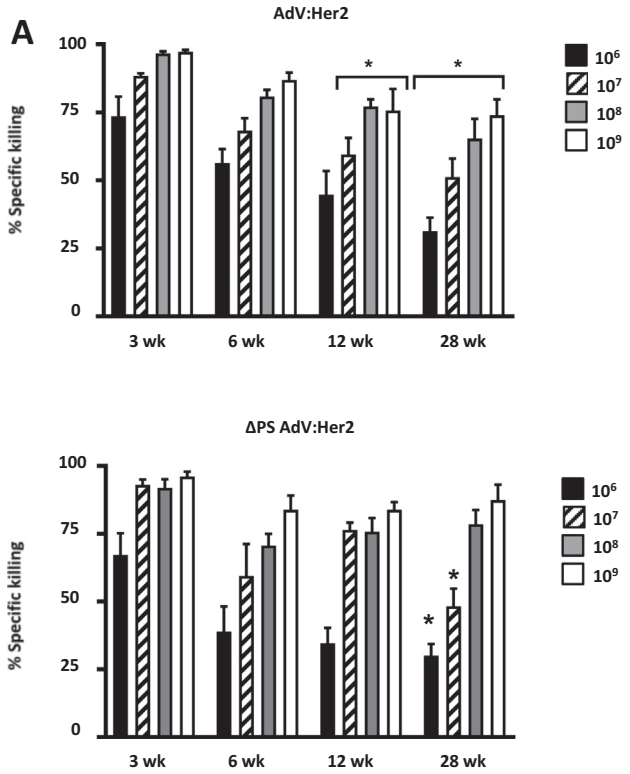


Fig. 3. Dose response and longevity of immune response with AdV:Her2 vs. ΔPS AdV:Her2. Female BALB/c mice ($n = 20$/group) were immunized with either AdV:Her2 or ΔPS AdV:Her2 at 4 doses: 10^6, 10^7, 10^8 and 10^9 IFU. Serum and spleens were harvested and analyzed at 3, 6, 12, or 28 weeks post immunization. A) Cytotoxic $CD8^+$ responses measured using *in vivo* CTL assay for AdV:Her2 (upper panel) and ΔPS AdV:Her2 (lower panel). Data is grouped by time point/dose and the bars represent the group ($n = 5$/group) mean for % specific killing ± SEM. * $p < 0.05$. B) Detection of T cell responses by IFN-γ ELISPOT assay. Splenocytes ($n = 5$/group/-time point) were stimulated with $CD8^+$ and $CD4^+$ HER2 epitope peptides [10 μg/mL], and rat HER2 protein [5 μg/mL]. Data are presented as IFN-γ SFU/10^6 cells with background subtracted. For each stimulant, the bars from left to right represent escalating AdV doses from lowest to highest dose. The left panels represent AdV:Her2; and the right panel represent ΔPS AdV:Her2. C) Anti-vector T cell responses measured by IFN-γ ELISPOT post-stimulation with the relevant synthetic peptides directed against the hexon and DNA binding protein of adenovirus [10 μg/mL]. *$p < 0.001$, comparing the two vectors for each dose. Each bars represent the mean number of IFN-γ SFU/10^6 cells ± SEM, background subtracted, for each of the four doses tested (10^6, 10^7, 10^8, 10^9 IFU). D) HER2 specific humoral responses using ELISA. The dots indicate the titers of individual animals, and the bars represent the geometric means of the groups; *$p < 0.05$ when comparing the two vectors receiving the same dose.

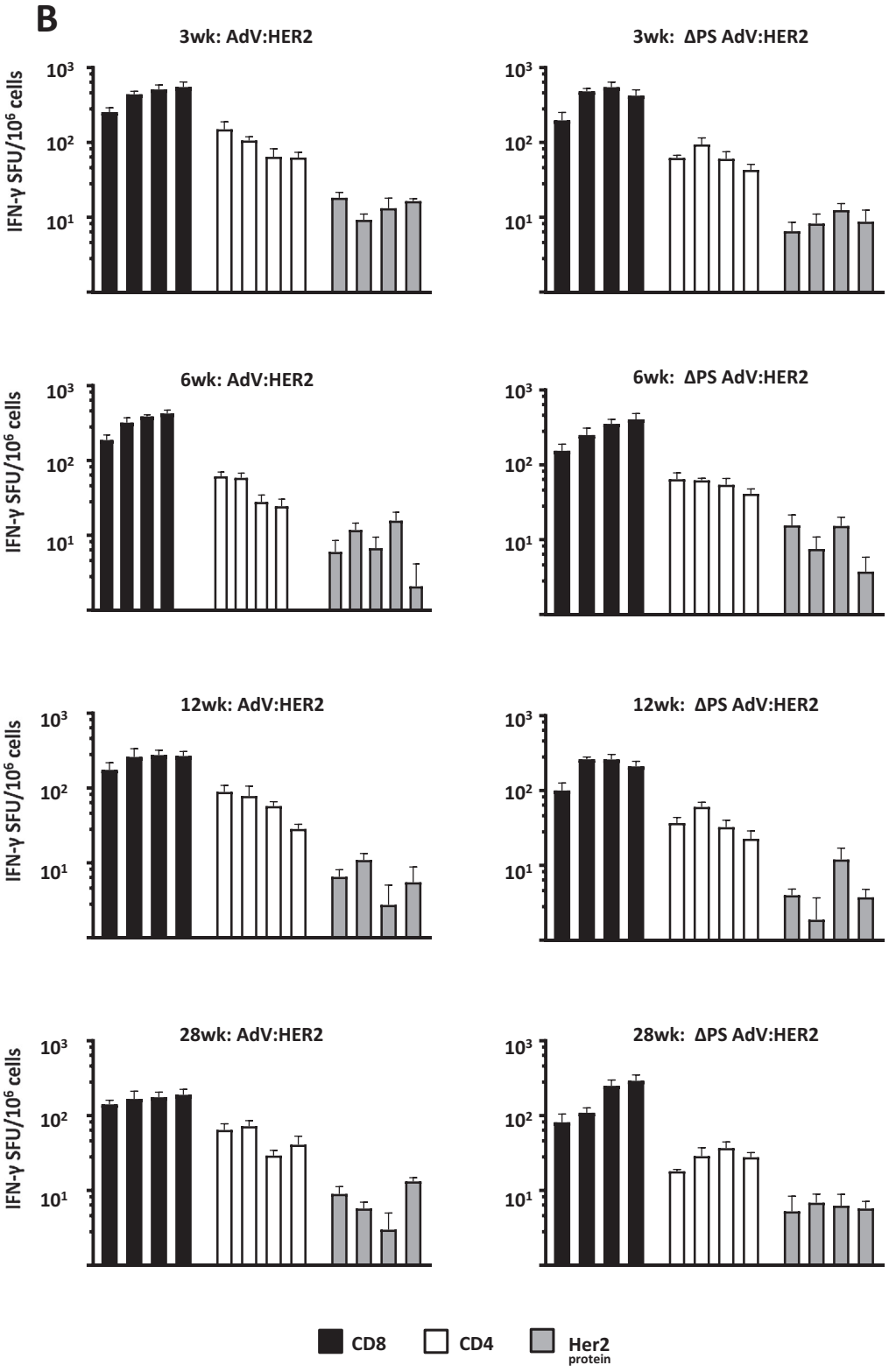
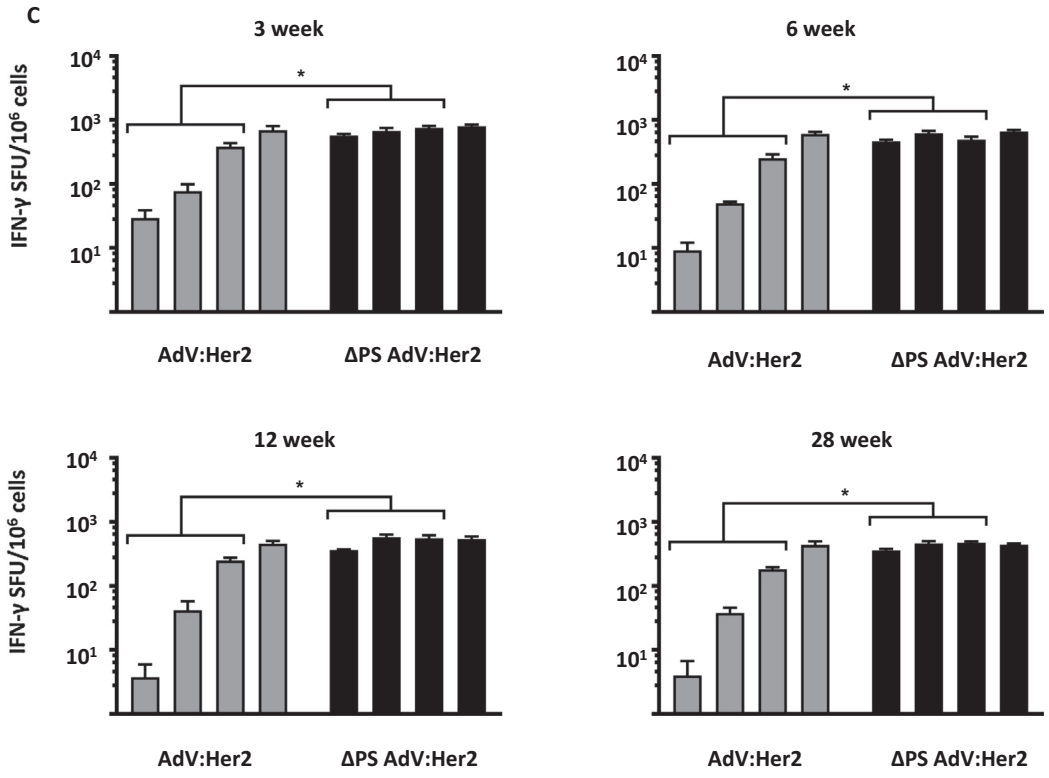


Fig. 3 (*continued*)

immunizations using SLA:HER2adm (day 28 vs. day 42 $p > 0.05$). However, SLA:HER2adm boost significantly enhanced AdV:Her2 primed IFNγ $CD4^+$ T cell responses following a single ($p < 0.05$) and double ($p < 0.001$) immunization when tested using $CD4^+$ peptide as stimulant. Likewise, when IFN-γ T-cell responses to rat HER2 protein were evaluated, SLA:HER2adm significantly enhanced both AdV:Her2 and SLA:HER2adm primed responses.

As would be expected, no detectable HER2-specific IgG responses were seen at day 7 post single dose of AdV:Her2 or SLA:HER2adm (*data not shown*). Mice primed with SLA:HER2adm



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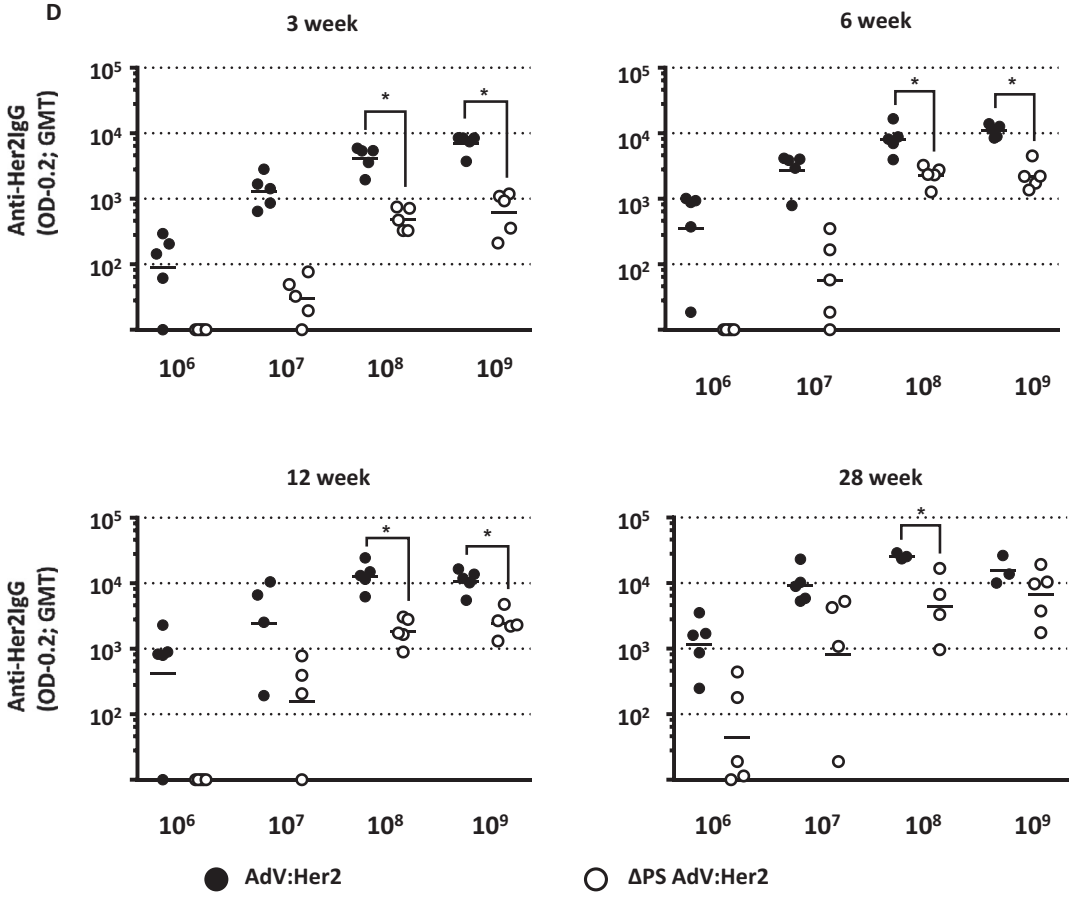

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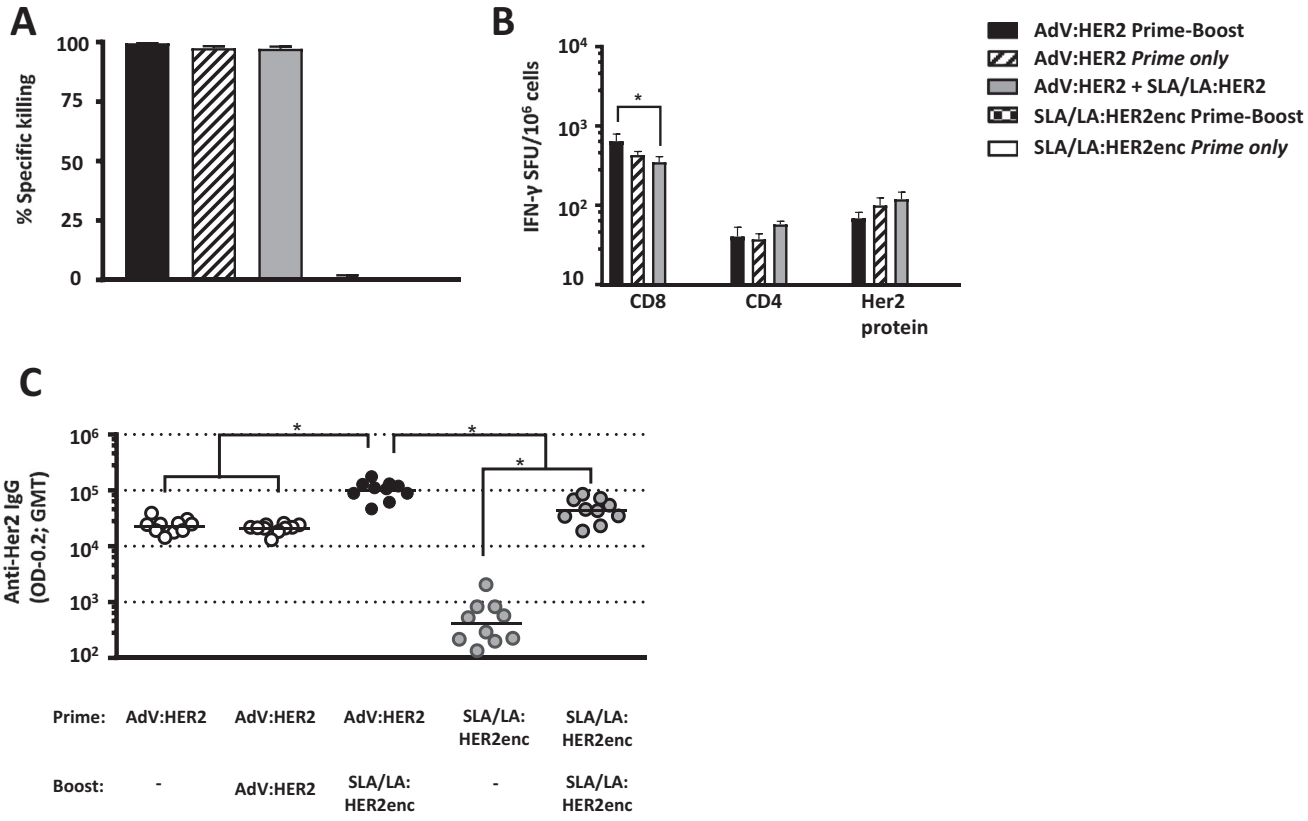

Fig. 4. Immunogenicity of heterologous prime-boost using AdV:Her2 (2×10^9 IFU/mouse) and 2 μg HER2 encapsulated in SLA/LA archaeosomes. Female BALB/c mice (n = 10/group) were either given either a single dose of AdV:Her2 (prime only; $2x10^9$ IFU/mouse) or SLA/LA:HER2enc (prime only) or were boosted at 3 weeks post prime with homologous or heterologous vaccine formulation. A) Cytotoxic $CD8^+$ T cell responses were measured using an *in vivo* CTL assay. The bars represent the mean specific lysis ± SEM. *$p < 0.05$. B) Splenocytes were used to quantify IFN-γ secreting T-cells by ELISPOT in response to stimulation with $CD8^+$, $CD4^+$ peptides as well as rat HER2 protein. Bars represent the number of IFN-γ SFU/10^6 splenocytes ± SEM. Data represent the background subtracted values. *$p < 0.05$. C) Serum collected 1 week post-boost (day 28) was analyzed for anti-Her2 IgG titers by ELISA. Grouped data is presented as geometric mean titer; *$p < 0.05$ for various groups when compared to all groups using a two-way ANOVA.

and boosted once with the same formulation had significantly higher HER2 specific IgG titres compared to mice primed with AdV:Her2 and boosted once with SLA:HER2adm ($p < 0.05$). Responses were further increased if mice received a double boost of SLA:HER2adm, regardless of whether prime was with AdV:Her2 or SLA:HER2adm ($p < 0.001$) (Fig. 5C).

4. Discussion

Passive immunotherapy with anti-HER2 agents such as mAbs and tyrosine kinase inhibitors have been approved by FDA for treatment and have improved outcomes [37], some toxicity and resistance to these drugs has been reported in some patients [38]. Poor $CD4^+$ T helper type 1 (T_h1) responses following HER2-targeted antibody therapy strongly correlated with recurrence [39], suggesting the need to develop vaccines which can induce broad immune responses including T_h1 responses needed to improve therapeutic efficacy [40].

In the present study, we chose HER2 as a model antigen to evaluate a heterologous prime-boost strategy consisting of Ad5 based viral vector and adjuvant protein for inducing balanced HER2-specific immune responses consisting of humoral and $CD4^+$/$CD8^+$ T cell responses. Most adenoviral vectors used are replication-incompetent, following deletion of E1 and E3 viral genes, limiting their pathogenicity. Recombinant AdV capable of replicating its genomic DNA, due to the presence E1a gene, but incapable of making infectious particles through deletion of an essential protease gene (PS) has been described previously [19]. Comparing the two vectors, they both induced similar magnitude of $CD8^+$ and $CD4^+$ T cell responses; however, the ΔPS AdV:Her2 vector induced more durable $CD8^+$ T cell responses with the higher two doses. The ability of ΔPS AdV vector to induce more durable $CD8^+$ T cells responses may be reflective in their ability of greater viral gene expression. We have previously reported on immunization using these replicating non-disseminating (ΔPS, ΔE1/E3) AdV vectors with other tumor models [18,41]. This work along with other published studies have shown the increased magnitude and duration of transgene expression by the replicating vectors resulting in superior transgene specific antibody responses [42,43]. Herein, we wanted to examine the potency of ΔPS AdV vectors to induce both cell mediated and antibody responses and compare them to more conventional replication incompetent AdV using rat HER2 as the transgene.

Although both AdV:Her2 and ΔPS AdV:Her2 were able to induce strong cell mediated immune responses of similar magnitude, AdV:Her2 vector was superior to ΔPS AdV:Her2 for induction of HER2-specific humoral responses. Contrary to this, a previous study by Crosby et al. [43] had shown that an Ad5-based viral vector with intact E1 gene (facilitating the replication of the viral genome) but deficient in viral protein PIIa, essential for making infectious particles, induced significantly greater antibody responses to influenza hemagglutinin protein compared to E1 deleted replication incompetent AdV vector [43]. This difference between the two studies however, may be attributed to multitude of reasons including the nature of the antigen, differences in the

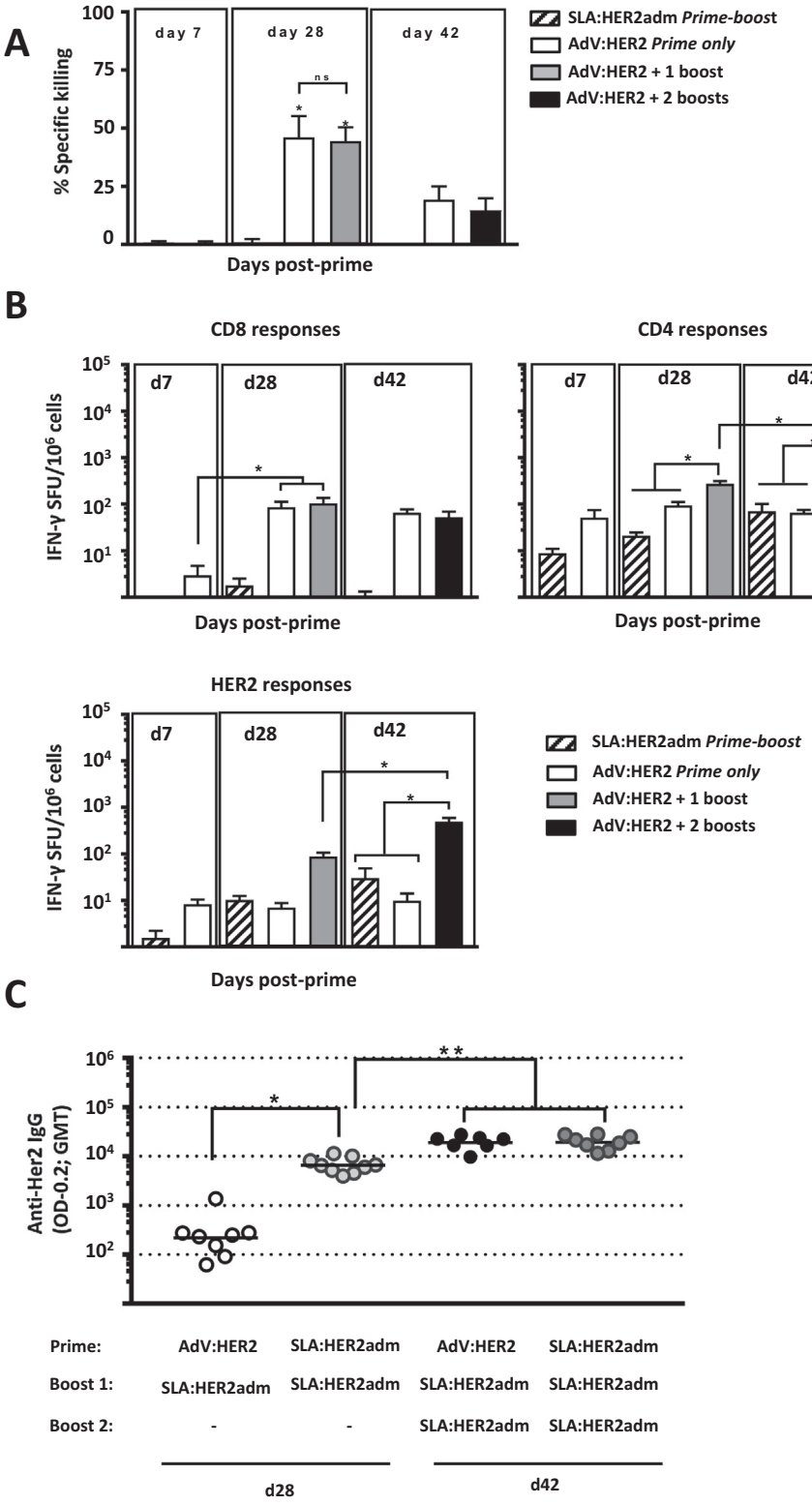

Fig. 5. Immunogenicity of heterologous prime-boost using AdV:Her2 (10^5 IFU/mouse) and 10 μg HER2 admixed with SLA archaeosomes (SLA:HER2adm): BALB/c mice ($n = 20$/group) were primed with AdV:Her2 (10^5 IFU) or SLA:HER2adm on day 0. Some groups were boosted on day 21 and day 35 with SLA:HER2adm. Spleen and serum samples ($n = 5$/group/time point) were collected and analyzed at: day 7 (1 week post prime), day 28 (1 week post-first boost), and day 42 (1 week post-second boost). A) Cytotoxic $CD8^+$ T cell responses using *in vivo* CTL assay. The figure shows the % specific killing pre-boost (day 7), post-boost 1 (day 28) and post-boost 2 (day 42). In SLA:HER2adm immunized groups, minimal levels compared to background were detected at all time points. Bars represent the mean killing ± SEM. $^*p < 0.001$, when compared to each group to the other at each time point using 2-way ANOVA. B) ELISPOT assay: Impact of vaccination and boost on the magnitude of HER2 specific IFN-γ secreting T-cells. The graphs represent mean IFN-γ SFU/10^6 cells ± SEM, background subtracted, using an immunodominant $CD8^+$ T cell epitope (graph 1), immunodominant $CD4^+$ T cell epitope (graph 2) or using rat HER2 ECD protein (graph 3). * $p < 0.05$, comparing all groups within each time point or with groups at other time points. C) ELISA data: Analysis of sera antibody for specific anti-HER2 IgG responses in mice immunized with AdV:Her2 and boosted with SLA:HER2adm, at 1 week post first boost (day 28) and 1 week post second boost (day 42). Each dot represents a single animal, and bars show the geometric mean titers. All groups were compared using a one-way ANOVA Tukey's multiple comparison test. *, $p < 0.05$; **, $p < 0.001$.

source of animals, environmental factors etc. The difference may also be attributed to the vector itself; overall cloning strategy including the promoter structure, transgene position and orientation which could all impact the degree of transgene expression. Several other studies have also reported the use of replication-competent Ad5-based vaccines in therapeutic cancer vaccines [44] or in infectious disease vaccines [45] and have demonstrated that the immune responses specific to Ad-encoded transgenes can have a significant impact on the efficacy of the vaccine [46,47].

Immunogenicity of AdV leading to induction of vector-specific immune responses render them sub- optimal for use in booster immunizations [48], and the use of doses that are high enough to overcome the impact of pre-existing anti-vector immunity may be too toxic. Hence heterologous prime boosting strategies which combine live heterologous viral vectors, DNA/RNA, and adjuvanted protein immunization have been used to boost Ad vector induced immune responses both in pre-clinical and clinical settings [49,50]. A number of studies have previously demonstrated the strong immunostimulatory effects of a novel adjuvant formulation comprising archaeosomes composed of SLA with or without LA [22–24]. Therefor we evaluated the use of archeaosome composed of SLA/LA encapsulating the HER2 antigen (SLA/LA:HER2enc) or archeaosome composed of SLA only admixed with HER2 (SLA:HER2adm) antigen as a boosting strategy for AdV induced HER-2 specific immune responses. When used at a high dose of 2×10^9 IFU, which was the highest dose tested in this study, a single administration of AdV:Her2 induced potent cytotoxic $CD8^+$ T cell responses (>95% killing) and strong HER2-specific IFN-γ T cell responses that were not further boosted by SLA/LA:HER2enc. Although SLA/LA:HER2enc was capable of inducing strong antigen-specific antibody responses, the level of antigen-specific T cell responses were low. This was somewhat surprising since archaeosomes have previously been shown to induce strong cellular responses against multiple antigens [24,36,51]. The low T cell responses may be attributed to the dose of HER2 antigen used which may have been sub-optimal for inducing strong T cell responses. Studies have shown that based on the nature of antigens and formulations, dose levels of each antigen may vary in inducing specific responses [36]. Further studies optimizing the dose of encapsulated HER2 protein or peptide antigen will be needed to determine the optimal cell-mediated immune responses.

Although archaeosomes have been shown to be potent adjuvants [22], the efficiency of antigen entrapment has typically been low (5–40%) resulting in loss of antigen, thereby increasing cost, laborious formulation procedure, but also lack consistency between batches with various ratios of archaeal lipid to antigen present. We use an admixed alternative, which has also been shown to induce strong humoral and cell-mediated immune responses [36]. When used at a sub-optimal dose of 10^5 IFU, AdV:Her2 was still capable of inducing strong $CD8^+$ T cell responses which could not be further boosted by 10 μg HER2 admixed with SLA, demonstrating the high innate immunogenicity of AdV. However, boosting with 10 μg HER2 admixed with SLA, in a heterologous prime-boost approach, augmented Ad-induced HER2-specific $CD4^+$ T cell responses as well as antibody responses. Overall, our results showed that the greatest magnitude and breadth of HER2-specific immune responses were seen with AdV:Her2 prime/SLA:HER2adm heterologous prime-boost strategy. The strong adjuvanticity seen in this study associated with the use of protein admixed with SLA is in line with other studies reports in mice using SLA. Homologous prime-boost or prime-boost/boost with 10 μg HER2 admixed with SLA induced antigen-specific antibody and $CD4^+$ responses but no detectable antigen-specific $CD8^+$ responses. Although recent studies have shown antigen encapsulated within SLA/LA archaeosomes [36] or admixed with SLA [23] to induce strong T and B cell responses against multiple antigens, the lack of $CD8^+$ T cell responses in our study with SLA/LA or SLA archaeosomes may be due to multiple reasons including the nature of the antigen and the antigen dose.

In summary, our results demonstrate that AdV:Her2 is capable of inducing strong antigen-specific $CD8^+$ T cell responses, even at a low dose. These responses can be broadened to include antigen-specific antibody responses by boosting with SLA adjuvanted proteins without compromising CD8 T cell responses elicited by AdV priming. Although not a true self-antigen rat HER2 has >95% homology to the mouse homologue [25] and hence is a good surrogate for a self-antigen. Thus, this immunization strategy has the potential to break immunological tolerance to self-antigens in the context of cancer and maybe of use in developing therapeutic vaccines targeting HER2 antigen. This needs to be validated using appropriate tumor models. We aim to use the rat HER2 transgenic BALB-neuT mouse model where mice are tolerant to rat HER2 and develop spontaneous mammary tumors [52] to evaluate the anti-tumor efficacy of AdV:Her2/SLA adjuvanted HER2 protein in prophylactic and therapeutic settings.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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