

ORIGINAL RESEARCH

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Proof of concept study with an HER-2 mimotope anticancer vaccine deduced from a novel AAV-mimotope library platform

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ABSTRACT

Background: Anticancer vaccines could represent a valuable complementary strategy to established therapies, especially in settings of early stage and minimal residual disease. HER-2 is an important target for immunotherapy and addressed by the monoclonal antibody trastuzumab. We have previously generated HER-2 mimotope peptides from phage display libraries. The synthesized peptides were coupled to carriers and applied for epitope-specific induction of trastuzumab-like IgG. For simplification and to avoid methodological limitations of synthesis and coupling chemistry, we herewith present a novel and optimized approach by using adeno-associated viruses (AAV) as effective and high-density mimotope-display system, which can be directly used for vaccination. **Methods:** An AAV capsid display library was constructed by genetically incorporating random peptides in a plasmid encoding the wild-type AAV2 capsid protein. AAV clones, expressing peptides specifically reactive to trastuzumab, were employed to immunize BALB/c mice. Antibody titers against human HER-2 were determined, and the isotype composition and functional properties of these were tested. Finally, prophylactically immunized mice were challenged with human HER-2 transfected mouse D2F2/E2 cells. **Results:** HER-2 mimotope AAV-vaccines induced antibodies specific to human HER-2. Two clones were selected for immunization of mice, which were subsequently grafted D2F2/E2 cells. Both mimotope AAV clones delayed the growth of tumors significantly, as compared to controls. **Conclusion:** In this study, a novel mimotope AAV-based platform was created allowing the isolation of mimotopes, which can be directly used as anticancer vaccines. The example of trastuzumab AAV-mimotopes demonstrates that this vaccine strategy could help to establish active immunotherapy for breast-cancer patients.

ARTICLE HISTORY

Received 27 January 2016
Revised 18 March 2016
Accepted 21 March 2016

KEYWORDS

AAV; adeno-associated virus; cancer vaccine; HER-2; mimotope


Introduction

Ever since the first promising vaccination experiments against cancer by the New York surgeon William B. Coley (1862–1936), who injected inoperable sarcoma patients with bacteria and thereby accomplished a cure rate of around 10%,¹ cancer research has been aiming to develop active immunotherapies against cancer. The “Coley’s toxin” vaccine was evidently unspecific and clinical effects were likely due to the response of macrophages with the release of pro-inflammatory cytokines such as IL-12.² Nevertheless, such adjuvant therapies may certainly help to overcome tumor-induced immunosuppression and may initiate an active immune reaction against the malignant cells.³

Current targeted anticancer immunotherapies are directed against tumor-associated antigens, which are highly overexpressed on malignant cells, but scarcely expressed in normal tissue.⁴

Candidate targets comprise growth factor receptors⁵ that are important during embryonic development⁶ and silenced in adults, but overexpressed again in some cancers.⁷ The human epidermal growth factor receptor-2 (HER-2) is a ligand-less receptor tyrosine kinase typically amplified in breast, gastric and esophageal cancer.⁸ Via homo- or hetero-dimerization with related molecules, HER-2 mediates proliferative and anti-apoptotic signals, ultimately leading to unfavorable courses of the disease.⁹ Therefore, HER-2 belongs to the most prominent targets for specific anticancer therapies, proven many times by the clinically used anti-HER-2 monoclonal antibodies trastuzumab (Herceptin®, Roche) or pertuzumab (Perjeta®, Roche).^{10–11} As the serum half-life of these monoclonal antibodies is around 2–4 weeks, depending on the dosage^{12–13} ($t_{1/2}$ = 16.4 d for trastuzumab¹⁴ and 18 d for pertuzumab¹⁵), repetitive applications in tri-weekly intervals are necessary.¹⁶ Even after

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 Supplementary data of this article can be accessed on the [publisher's website](#).

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being chimerized or humanized, these antibodies are recognized as foreign proteins by the immune system and severe side effects may occur during treatments due to hypersensitivity reactions.¹⁷⁻²⁰ Hence, these therapies have to be applied under strict medical supervision and often under cortisol or anti-histamine premedication,²¹ making them laborious for patients as well as oncologists. Last but not the least, they are expensive for the health-care system and may encourage development of class-based treatment. Consequently, turning passive immunotherapies with monoclonal antibodies into active vaccines triggering the patient's own immune system to produce antibodies of the same specificity, would be highly desirable and could overcome many of the aforementioned obstacles.²²

However, HER-2, due to its pivotal role in ductal morphogenesis of the human mammary gland²³ and its involvement in repair mechanisms of myocardial cells,²⁴⁻²⁵ is a self-antigen. Therefore, immunization strategies against HER-2 have to vanquish the patient's self-tolerance. Small B-cell epitope mimicking peptides, so called "mimotopes," could be an interesting option to break tolerance, as they do not share consensus sequence with the mimicked epitopes, but possess the same structure due to similar amino acid charges.²⁶ Thus, immunization with mimotopes induces antibodies that recognize the natural antigen only via molecular mimicry. In previous studies, our group selected mimotopes from phage display libraries and demonstrated their high specificity and immunogenicity in mice.²⁶⁻³² As it is not possible to use filamentous phages for vaccinating patients, the mimotope peptides had to be produced synthetically and to reconstitute their immunogenicity, chemical coupling to antigenic carriers such as keyhole limpet hemocyanin²⁸ or tetanus toxoid³³ was necessary. During this process substantial loss of the mimicry potential of the epitope was observed.²⁶ Therefore, suitable vehicles are called for, which guarantee safe and stable display while maintaining immunogenicity. We propose in this study AAV as novel mimotope display systems, as they could be proven to induce strong humoral³⁴ and cellular³⁵ immune responses.³⁶

AAV are small (25 nm), non-enveloped single-stranded DNA viruses³⁷⁻³⁸ which belong to the genus *Dependovirus*—they need a helper virus to facilitate their replication. Appropriate helper viruses for AAV are adenoviruses or herpes simplex virus.³⁸ As AAV are non-pathogenic, they were long-neglected in medical research,³⁸ a fact that suddenly reversed when Hermonat and Muzyczka used an AAV-based vector to express foreign genes in mammalian tissue culture cells in 1984.³⁹ Within the last 30 y, AAV were established as vectors for gene therapy and their experimental use ranges from the cardiovascular system⁴⁰ over neurodegenerative disorders⁴¹ to bone defects, cartilage lesions or rheumatoid arthritis⁴² as well as infectious diseases.³⁶ In parallel, AAV or their self-assembled capsid proteins alone as virus-like particles (AAVLP) were employed as a display system for vaccines,³⁶ because the virus serotype 2 (AAV2) tolerates the insertion of small peptides into its VP3 capsid protein.⁴³

With regards to oncology, promising results for AAVLP-based vaccines were obtained especially against the tumorigenic virus Human Papilloma Virus (HPV). Nieto et al. could demonstrate that upon prophylactic vaccination with AAVLP displaying the L2 epitope of HPV, high titers of antibodies were

induced in mice and rabbits, resulting in neutralization of infections with several HPV types in a pseudovirion infection assay.⁴⁴

Especially intriguing, however, would be a combination of the high-density display AAV system with a library allowing the selection of mimotopes from a repertoire of peptides with an antibody of interest. We addressed here whether in this way AAV mimotope vaccines could be created that at the same time would be useful as immunogen, using HER-2 as a model target.

Results

Selection of HER-2 mimotopes from an AAV library using trastuzumab

AAV-mimotope clones (schematic representation in Fig. 1A, amino acid sequences depicted in Table 1) were selected based on their ability to bind to the monoclonal anti-HER-2 antibody trastuzumab. Extensive description of the selection algorithm and the results can be found in the Supplementary section.

Immunization with HER-2 mimotope AAV clones

AAV-mimotope clones were next selected based on their ability to induce HER-2 specific antibodies. After four rounds of immunization without any observed local or systemic side effects, HER-2 specific antibodies were detectable in sera of immunized mice, with significantly higher levels compared to naïve mice in 5 out of 7 tested mimotope groups. When compared to wtAAV immunized animals, mice from 3 mimotope groups, DMD4 ($p < 0.05$), DMD6 ($p < 0.001$) and DDD19 ($p < 0.001$), displayed significantly higher levels of HER-2 specific IgG antibodies (Fig. 1B). To prove the specificity of the induced antibodies, immunohistochemical stainings were performed with HER2-overexpressing and non-expressing tumor cells. As depicted in Fig. 1C, staining with IgG from sera of immunized mice showed a membrane specific pattern in HER-2 transfected D2F2/E2 cells only, whereas the parental cell line D2F2, negative for human HER-2, remained unstained. Specificity was tested using purified IgG antibodies in ELISA against rHER-2, but also against two other known tumor-associated antigens, EGFR and CEA, or against BSA for control purposes. The HER-2 mimotope clones DMD4 and DMD6 induced specific anti-HER-2 antibodies (Fig. 2A), which reacted significantly higher compared to antibodies purified from naive mice ($p < 0.001$), or antibodies purified from the DMD1 or DMD2 groups ($p < 0.001$ for both clones). Only background reactivity against control proteins sEGFR, sCEA or BSA was measured for all treatment groups (Fig. 2A).

Epitope specificity is particularly essential for cancer immunotherapy, because antibodies against HER-2 can act either tumor-promoting or -inhibiting, even when directed against the same molecule.^{26,45} Thus, the second line of screening was done by means of a tetrazolium-based cell proliferation assay to exclude mimotopes that induce antibodies either with insufficient tumoricidal effects or favoring tumor growth. Here, purified antibodies from sera of immunized mice were employed for incubation of HER-2 overexpressing BT474 cells. After 72 h, cell viability was measured (Fig. 2B). Clones DMD1,

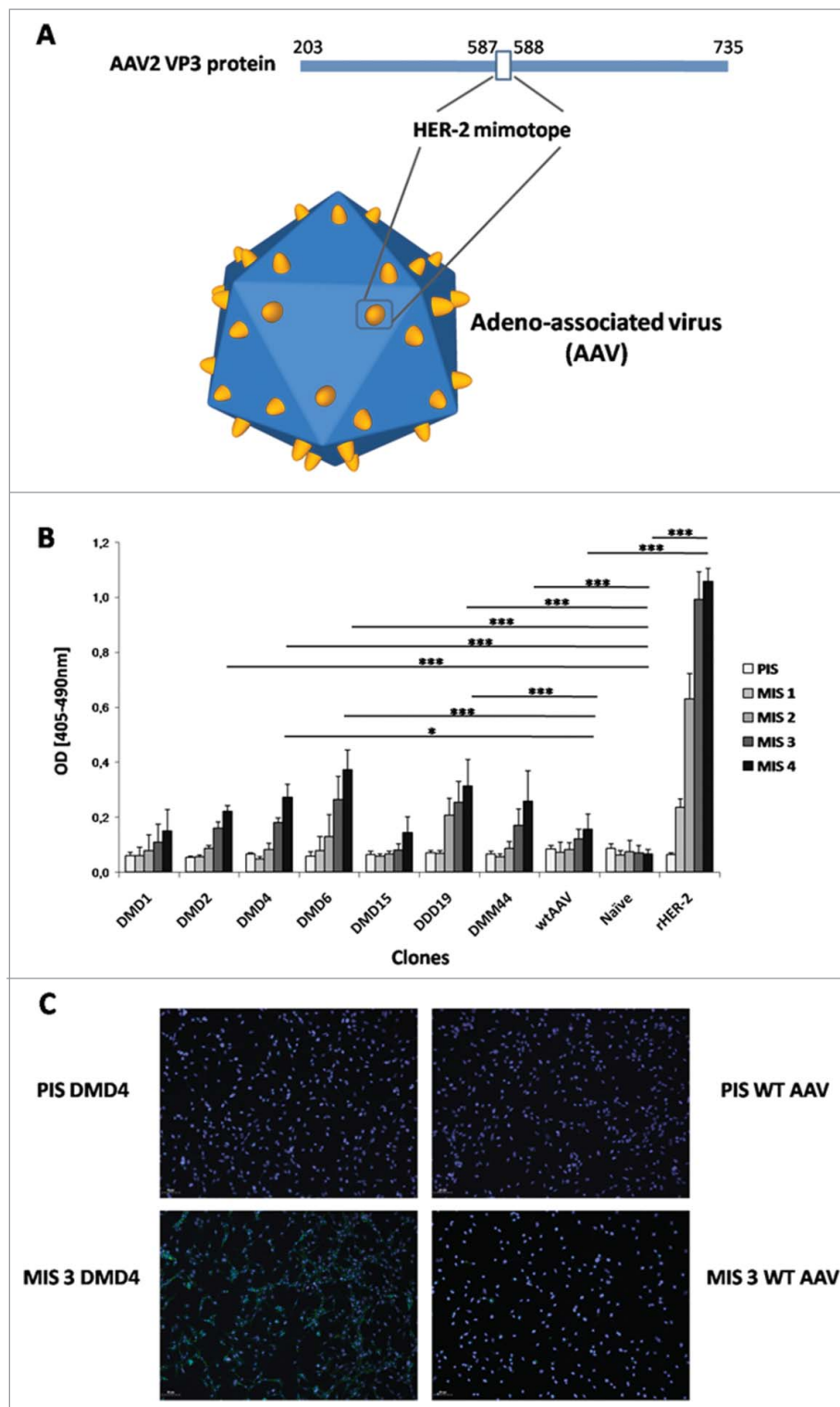


Figure 1. HER-2 mimotope AAV model and immunogenicity evaluation. (A) Model of the AAV vector displaying HER-2 specific mimotopes fused to its capsid protein VP3. Mimotopes are inserted between amino acid positions 587 and 588, which resemble a peak in the capsid, for optimal display. (B) AAV vectors displaying HER-2 mimotopes are able to induce HER-2 specific antibodies. Screening of sera from mice, immunized with AAV-displaying HER-2 mimotopes and Al(OH)₃ as adjuvant, against the extracellular domain of human HER-2. Depicted are means (n = 2/mouse serum) & SD; values of MIS4 were statistically analyzed (One-way ANOVA, Tukey's post-test). (C) Antibodies induced by AAV clone DMD4 recognize HER-2 on the surface of cancer cells. Immunofluorescence staining of D2F2/E2 cells, overexpressing human HER-2 with sera of mice immunized with AAV clone DMD4 (PIS = Pre-Immune Serum, MIS 3 = MIS after three immunizations). After immunization with the HER-2 specific mimotope, membrane specific staining can be seen. Pre-Immune sera and serum of mice immunized with wtAAV alone do not show any specific staining on D2F2/E2 cells.

DMD4 and DMD6 mediated growth inhibition; DMD2, DDD19 and DMM44 had only minor effects on tumor growth, but also antibodies purified from wtAAV immunized or

mice showed tumor growth inhibition to some extent. Also antibodies induced by rHER-2, which are not restricted to the trastuzumab epitope and thus a mix of tumor-promoting and

Table 1. Amino acid sequences of tested mimotopes.

Clone ID	Amino acid sequence
DMD1	RLVPVGLERGTVDWV
DMD2	TRWQKGLALGSGDMA
DMD4	QVSHWVSLAEGSFG
DMD6	LSHTSGRVEGSVLL
DMD15	LDSTSLAGGPYEAIE
DDD19	HVVMNWMREEFVEEF
DMM44	SWASGMAVGSVSFEE

-inhibiting ones, were not able to mediate significant growth inhibition. Upon statistical evaluation, only antibodies induced by clones DMD1 and DMD6 were able to reach significance when compared to untreated cells ($p < 0.01$ for DMD1 and $p < 0.05$ for DMD6).

Finally, antibodies induced by clone DMD15 were tumor-promoting (Fig. S1). This effect, however, was drastic as BT474 cells showed 4-fold faster proliferation compared to untreated cells ($p < 0.01$) and underlines the importance of tumor cell proliferation inhibition assays as part of the screening procedure of novel anticancer vaccines.

Analyzing the subclass immune responses after vaccination of BALB/c mice

Clones DMD4 and DMD6 had performed best overall throughout the screening steps and were thus chosen for monitoring the IgA, IgG1, IgG2a, IgG2b and IgE responses during a further immunization experiment in BALB/c mice.

Both DMD4 and DMD6 induced negligible levels of HER-2 specific IgA (Fig. 3A), whereas DMD4, less DMD6, induced IgG1 against HER-2 ($p < 0.05$ for DMD4 compared to the naive group, Fig. 3B). Neither DMD4 nor DMD6 induced large extents of HER-2 specific IgG2a and IgG2b (Figs. 3C–D). However, IgG2a levels in DMD4 immunized mice were significantly elevated after four rounds of immunization compared to the animals of the naive group ($p < 0.001$; Fig. 3C, right panel). Interestingly, this effect was not observed, when the DMD4 group was compared to the group immunized with wtAAV, as this group also showed elevated levels of IgG2a antibodies.

As for IgE, again DMD4 induced significant levels of HER-2 specific IgE, as compared to naive ($p < 0.001$) and wtAAV immunized animals ($p < 0.01$), but vaccination with DMD6 did not lead to a significant IgE anti HER-2 immune response (Fig. 3E).

The positive control group, being vaccinated four times with the extracellular domain of HER-2, showed significant antibody levels of HER-2 specific IgG1, IgG2a, IgG2b and IgE isotypes, but not IgA (Figs. 3A–E).

To determine the effect of different adjuvant agents on the elicited immune response, a cohort of BALB/c mice was immunized with the HER-2 mimotope AAV clone DMD2, alongside several different adjuvants, being either in clinical use or in clinical trials. DMD2 plus aluminum-hydroxide (Alum), monophosphoryl-Lipid A (MPL), or a combination of both, induced higher levels of IgG specific for HER-2 as compared to ODN-1826 or Alum plus ODN-1826 (Fig. S2A). This IgG response seems to be constituted predominantly by IgG1

antibodies (Fig. S2B). As for all other investigated isotypes (IgG2a, IgG2b and IgM), no differences were observable (Figs. S2C–E).

The memory effects of the mimotope-AAV vaccine were also investigated: the immune response in immunized mice remained stable until day 137 and was not dependent on the type of adjuvant used (Fig. S3).

Tumor graft trial

Immunized mice were grafted with 2×10^6 D2F2/E2 cells, overexpressing human HER-2. In all mice, tumors could be detected after 5 d. In control groups (naive mice and mice immunized with wtAAV), tumors grew exponentially. In clear contrast, the tumors in mice vaccinated with either rHER-2, or HER-2 mimotope AAV clones DMD4 or DMD6 grew significantly slower ($p < 0.01$ for DMD4 compared to naive mice; $p < 0.05$ compared to wtAAV treated animals; $p < 0.05$ for DMD6 compared to naive as well as wtAAV immunized mice; Fig. 4A). The mean tumor volumes at day 12 of the trial and standard errors of the mean were as follows: DMD4 = $139, 27 \text{ mm}^3 \pm 40, 45$; DMD6 = $156, 13 \text{ mm}^3 \pm 54, 99$; wtAAV = $289, 61 \text{ mm}^3 \pm 67, 91$; rHER-2 = $124, 11 \text{ mm}^3 \pm 51, 11$ and naive mice = $309, 09 \text{ mm}^3 \pm 128, 59$, respectively.

When the first mouse reached a tumor volume larger than 300 mm^3 , the experiment was terminated and all mice were euthanized. Fig. 4B shows representative pictures of explanted tumors and Fig. 4C displays a graph of the tumor weights in grams, where mice of the rHER-2 and DMD6 immunized groups presented the lowest tumor weights.

Thus, vaccination with both tested vaccines, DMD4 and DMD6, distinctly showed a protective effect in BALB/c mice, comparable to vaccination with human HER-2.

Discussion

Although HER-2 is highly successfully targeted by passive immunotherapies in clinical oncology, no active anticancer vaccine against HER-2 is in clinical use yet. However, an active anti-HER-2 vaccine could be complementary to passive immunotherapy and especially relevant in a prophylactic setting in high-risk families, in early stage and in minimal residual disease, or even to protect the next generation.⁴⁶ Anticancer-vaccines that lead to apoptosis of cancer cells may besides the antibody-mediated effects⁴⁷ also activate the T-cellular branch via antigen crosspresentation and recruitment of CD8⁺^{48,49} depending on the induced isotype.⁵⁰

Moreover, we propose that anticancer vaccines, especially in combination with immune checkpoint-inhibitors may be another breakthrough in oncology. Overcoming the strong immunosuppressive tumor environment may also be supported by using a similar, but not identical antigen for immunization, such as a mimotope.

Mimotopes are small epitope-mimicking peptides that via molecular mimicry are able to induce epitope-specific antibodies. Hence, mimotopes for clinically approved antibodies could be effective tools to induce the desired epitope specificity in an active vaccination approach.^{26-32,51} However, the small

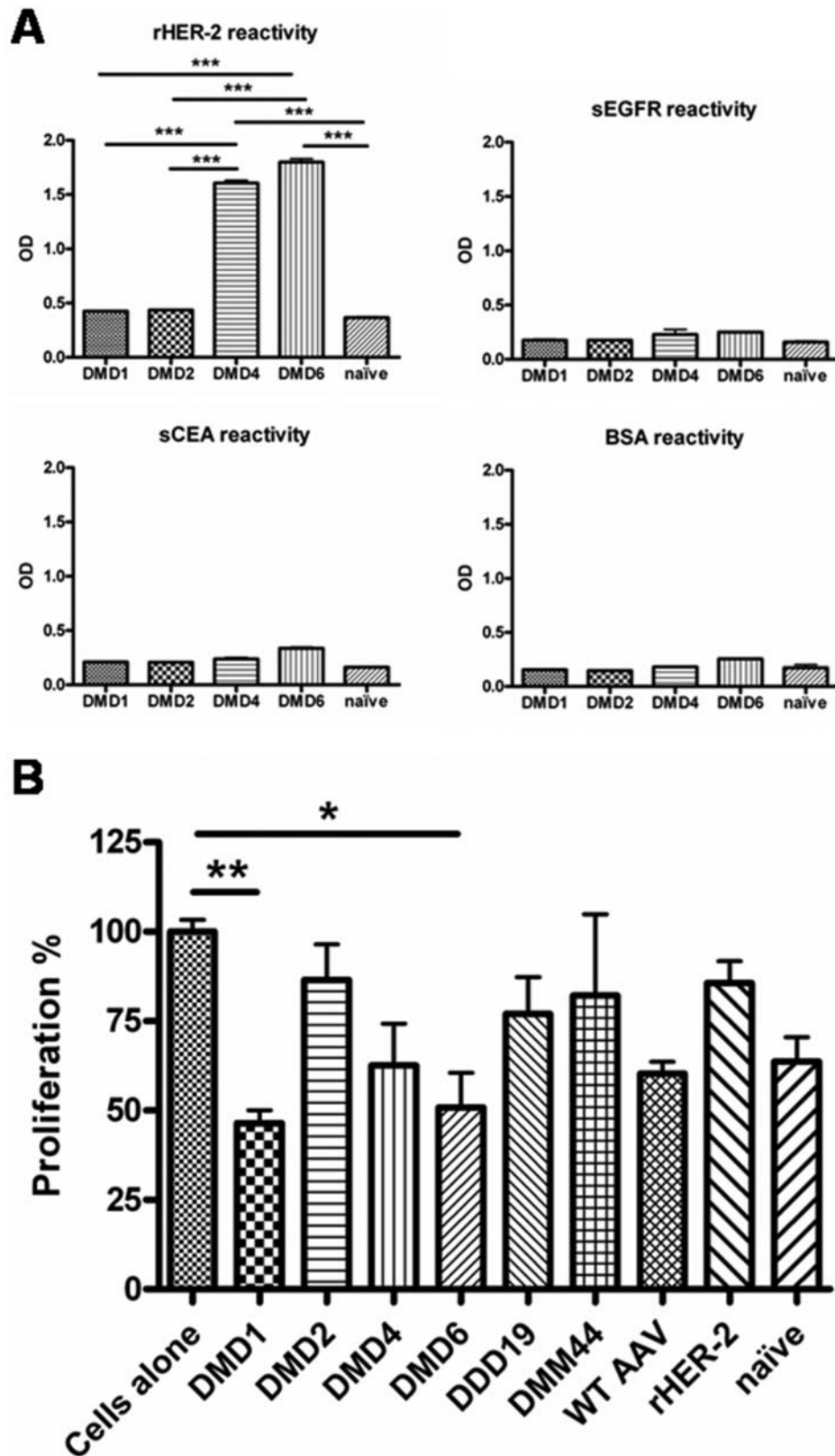


Figure 2. Specificity and functionality testing of antibodies purified from sera of immunized mice. (A) AAV-mimotope induced antibodies recognize HER-2, but not tumor-associated antigens EGFR, CEA or control protein BSA. Antibodies ($c = 1 \mu\text{g/mL}$) purified from sera of naïve mice and mice immunized with candidate particles were screened for their reactivity toward known tumor antigens HER-2, EGFR, CEA as well as the control protein BSA in ELISA. Antibodies induced by mimotopes DMD4 and DMD6 show significant higher IgG reactivity toward rHER-2 than naïve mice ($p < 0.001$) and mice immunized with candidates DMD1 ($p < 0.001$) and DMD2 ($p < 0.001$). Toward all other antigens only background reactivity is visible. Bars represent mean values & SD ($n = 2$); One-way ANOVA, Tukey's post-test. (B) Purified antibodies of immunized mice to different degrees inhibit growth of human HER-2 overexpressing mammary carcinoma cells. Tetrazolium-based proliferation assays with HER-2 overexpressing BT474 cells are depicted upon incubation for 72 h with purified antibodies ($c = 1 \mu\text{g/mL}$) of sera from mice immunized with candidate particles. Bars displaying mean values & SD, analyzed by means of Kruskal–Wallis test plus Dunns post-test.

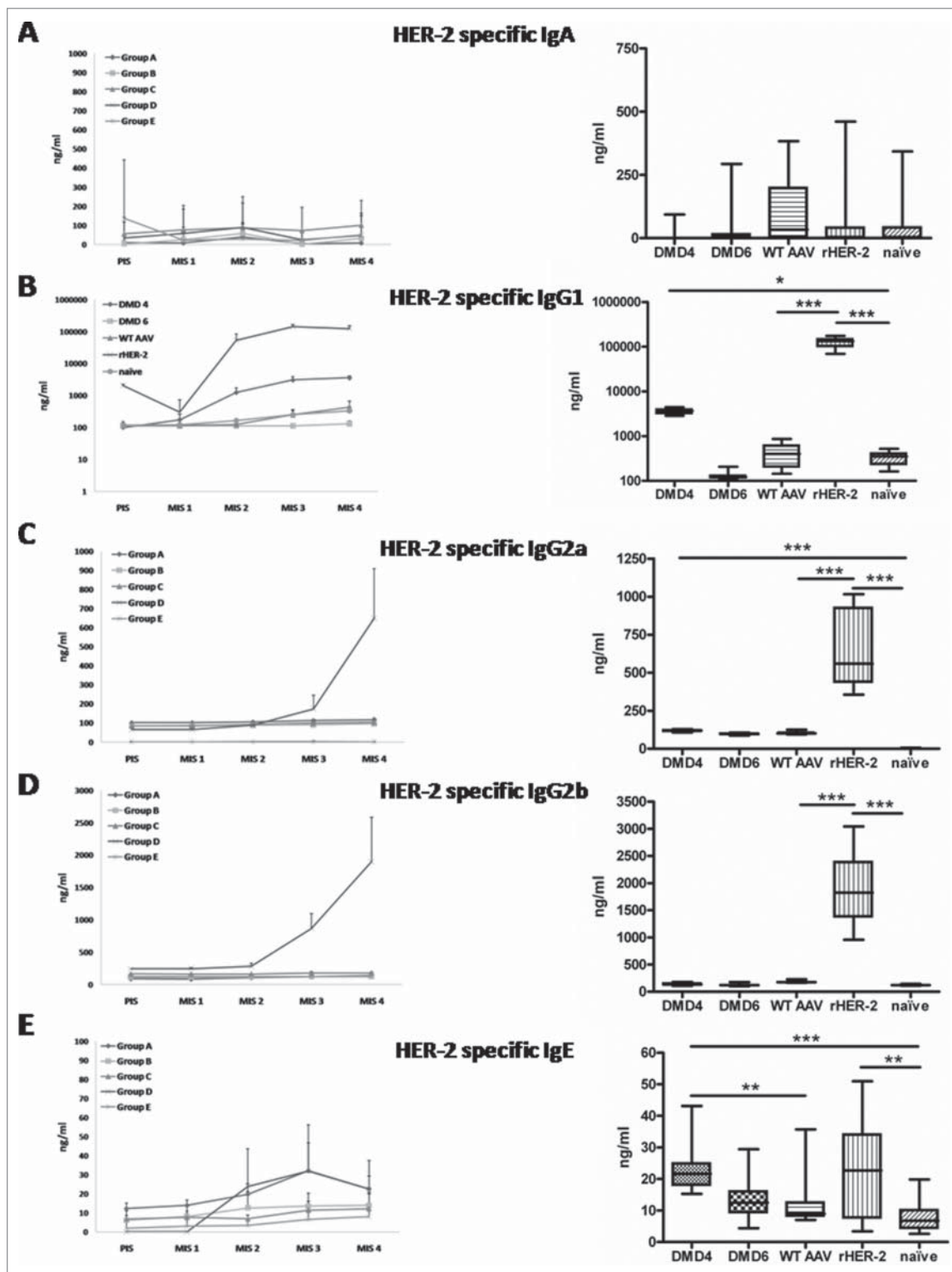


Figure 3. Subclass analysis of HER-2 specific antibodies induced by selected mimotope AAV clones DMD4 and DMD6 by ELISA. Left Panel: a-e: IgA, IgG1, IgG2a, IgG2b or IgE antibody determination before (PIS) and after each immunization (MIS 1-4). Right panel: Antibody levels after four immunization rounds (MIS 4); Box and whiskers plot, displaying minimum and maximum values. Each serum was measured in duplicate, eight mice per group \pm SD; Kruskal–Wallis test, Dunns post-test.

mimotope peptides need to be fixed to an immunogenic carrier to elicit an immune response.^{28,33}

Up to now this has been a methodological problem in mimotope vaccine production when translating phage-displayed mimotopes to a vaccine, involving peptide synthesis and chemical coupling to an immunogenic carrier. Our study presents an

AAV library that enables selection of mimotope AAV clones, which can be used without further modifications as a high-density display system for vaccination (Fig. 1A). Selections of the AAV library with trastuzumab (Herceptin®) rendered HER-2 mimotopes, which triggered antibody formation against the same epitope as trastuzumab. Seven mimotopes were used for

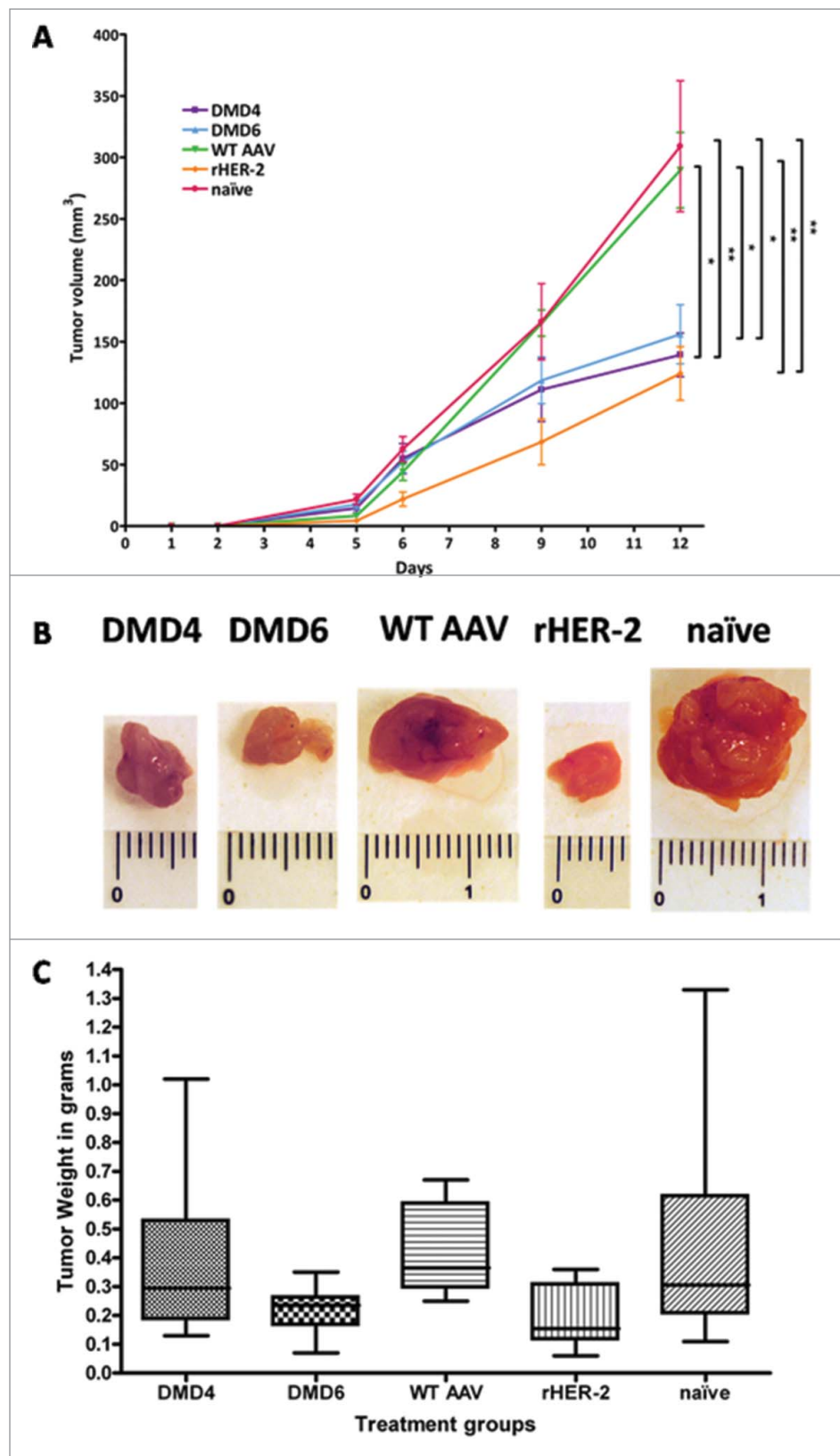


Figure 4. Readout of tumor graft trial. (A) Mice immunized with either rHER-2, DMD4 or DMD6 particles display significantly slower and smaller tumor growth compared to naïve mice and mice immunized with wtAAV only. Tumor growth curve of grafted HER-2 overexpressing D2F2/E2 cells; at day 12, DMD4 immunized mice had significantly smaller tumors compared to those of the naïve ($p < 0.01$) and of the wtAAV group ($p < 0.05$). DMD6 treated mice also developed significantly smaller tumors ($p < 0.05$ compared to naïve mice, $p < 0.05$ compared to wtAAV). Mice immunized with rHER-2 also developed significantly smaller tumors compared to both wtAAV treated ($p < 0.01$) or naïve mice ($p < 0.01$). Tumor size at day 12 was analyzed by One-way ANOVA alongside Tukey's post-test; displayed are mean tumor volume values \pm standard error of the mean (SEM). (B) Representative macroscopic pictures of explanted tumors. (C) Tumors of mice immunized with mimotope DMD6 or rHER-2 had significantly lower weight compared to tumors of animals receiving wtAAV. Diagram displaying weight of explanted tumors in grams; Box and whiskers plot, whiskers displaying minimum and maximum values (eight mice/group). Analysis by means of Kruskal–Wallis test and Dunns post-test.

immunizing mice and optimal candidates classified with respect to (i) antibody induction capacity (Fig. 1B), (ii) specificity of the induced antibodies (Fig. 2A) and (iii) growth inhibitory potential when applied to HER-2 overexpressing cancer cells (Fig. 2B). In spite of the fact that we used trastuzumab for mimotope selections, clone DMD15 induced antibodies which acted tumor-promoting (Fig. S1), underlining the importance of our screening algorithm. Based on their good performance, two candidate molecules, DMD4 and DMD6, were chosen for prophylactic vaccination in mice prior to subcutaneous tumor grafting with HER-2 overexpressing syngeneic D2F2/E2 cells. Both immunized groups showed significantly slower tumor growth compared to control groups (Fig. 4A).

In order to elucidate the type of the induced humoral immune response, sera of mice were taken after each immunization round and screened in ELISA for HER-2 specific antibodies. As can be seen in Fig. 3, the immune response is mainly mediated via IgG1 antibodies and to some extent via IgE, whereas IgA and IgG2b levels were not significantly elevated. This might be explained by the fact, that BALB/c mice show a genetic bias toward Th2 immunity.⁵²⁻⁵³ Independent of the employed adjuvant, ranging from the typical Th2 adjuvant Al(OH₃) (Alum)⁵⁴ to Th1 adjuvants such as MPL⁵⁵ or ODN,⁵⁶ the immune response was stable and all adjuvants triggered mostly IgG1 antibodies (Fig. S2). However, groups receiving AAV alongside Alum or MPL (or in combination) had higher IgG levels of HER-2 specific antibodies. Based on these results, Alum, the adjuvant most used in clinical practice, was selected for all further immunizations.

Taken together, these results indicate that from our AAV library platform potent HER-2 mimotope AAV vaccines can be generated and directly used for vaccination. These HER-2 vaccines did not cause any local or systemic side effects in the immunized mice. Our observations are in line with the clinical experience from the use of AAV for gene therapy (e.g., in inherited retinal disorders and hemophilia B), where those vectors displayed favorable safety profiles (reviewed in^{57,58}). However, vaccination approaches using viral vectors are often considered dangerous due to their content of viral DNA. In this regard, AAV exhibit significant advantages, as they are non-pathogenic and inherently replication defective,⁵⁹ as they need concomitant helper viruses to cause an infection.³⁸ Therefore, usage of adeno-associated virus-like particles (AAVLP), consisting only of the virus capsid as display system instead of the whole virus, will enhance safety. In a previous study, AAVLP had no anaphylactogenic potency even when employed in an allergy mouse model where high levels of antibodies including IgE and IgG1 were induced.⁶⁰

Thus, the concept of AAVLP-based vaccines is of high interest in the field of cancer vaccines, with a great potential for prophylactic vaccination of high-risk patients, or those with minimal residual disease.

Materials and methods

Cell lines

The human BT474 cell line is a human ductal mammary carcinoma cell line, first described by E. Lasfargues and W.G. Coutinho in 1978,⁶¹ distributed by ATCC (American Type Culture

Collection; Cat-No: BT474 or HTB-20TM, respectively), and was a kind gift of Prof Thomas Grunt from the Institute of Cancer Research of the Medical University of Vienna. BT474 cells were authenticated by short tandem repeat profiling (PowerPlex[®] 16 Loci Service, LGC Standards).

Mouse mammary carcinoma cells D2F2/E2 are derived from the D2F2 cell line⁶² by stable transfection of human HER-2⁶³ and were kindly provided by Prof Wei-Zen Wei (Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan, USA).

AAV particle production and candidate selection

Methods for production of the AAV library and selection of trastuzumab specific AAV clones are described in detail within the supplementary information. In short, an AAV capsid library was produced by transfection of HEK293T cells with a plasmid library encoding for AAV and containing a random peptide insertion of 15 amino acids with a diversity of 10⁹ between amino acid position 587 and 588 of the capsid protein VP3 (see schematic Fig. 1A). Resulting AAV capsids with different peptide insertions were selected for this study based on their reactivity toward trastuzumab (for detailed information see Supplemental Materials and Results).

Animals

For immunization and tumor graft experiments, 6–8 weeks old female BALB/c mice were obtained from Charles river and kept based on authorization of the Animal Ethics Committee of the Medical University according to the Austrian, European Union and FELASA guidelines for animal care and protection (GZ: BWMF-66.009/0003-II/3b/2011).

Immunization trials

Mice were immunized in immunization experiments according to the scheme in Fig. S4A; respective groups received subcutaneously 10 μ g of either AAV displaying HER-2 mimotopes or wild type AAV (wtAAV) adsorbed to 50 μ L AlOH₃ (Imject \rightarrow Alum Adjuvant, Pierce Protein Biology Products, Thermo Scientific; Cat-No: 77161) + 50 μ L PBS. Mice were immunized four times in two-week intervals. Serum samples were taken prior to the first immunization (PIS = pre immune serum) and 10 d after each immunization round (MIS1-4 = mouse immune serum 1-4).

For evaluation of the effects of different adjuvants, only one candidate clone, DMD2, was used; the time points of immunization and serum sampling were scheduled as depicted in Fig. S4B. In this trial, the long-term effects of the AAV based mimotope vaccine were also evaluated. Hence, multiple blood samples were taken after the last round of immunization (MIS3-7) and the immune response was monitored until day 137 (Fig. S4B).

Tumor graft experiments

2 \times 10⁶ D2F2/E2 cells were grafted subcutaneously (Day 0) in immunized mice (see scheme in Fig. S4C). Mice were observed regularly for tumor growth as well as tumor-related side effects such as

ceasing to ingest food or weight loss, which did not occur in a single mouse during the whole study. Tumor size was monitored by caliper measurement and tumor volume was calculated by means of the following formula: $V \text{ (mm}^3\text{)} = d^2 \text{ (mm}^2\text{)} \times D \text{ (mm)}/2$, where d stands for the smallest and D for the largest diameter of the tumor. Endpoint of the tumor graft trial was defined as when the tumor of the first mouse reached 300 mm^3 . Then, all mice were sacrificed and tumors were taken for further analysis.

Data handling and statistics

ELISAs measuring antibody responses (Fig. 1B) and specificity of induced antibodies (Fig. 2A) were done in duplicates and data of MIS4 were analyzed by means of one-way ANOVA with Tukey's post-test. EZ4U[®] cell viability assays (Fig. 2B) were calculated using Kruskal–Wallis test due to the non-Gaussian distribution of the values in connection with Dunns post-test. Cell viability evaluation in Fig. S3 was analyzed using the Mann–Whitney test. Subclass analysis of induced anti-HER-2 antibodies (Fig. 3) in MIS4 was analyzed again by Kruskal–Wallis test in connection with Dunns post-test. In the tumor graft trial, size of mouse tumors was measured by caliper (Fig. 4A) and values of day 12 were analyzed by means of one-way ANOVA with Tukey's post-test. Tumor weight (Fig. 4B) was analyzed using Kruskal–Wallis test in connection with Dunns post-test. For all experiments significance was accepted at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). All statistic calculations were performed using GraphPad Prism 4 Software (GraphPad).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors would like to express their gratitude to all members of the Jensen-Jarolim lab for critical and fruitful discussions, as well as to Anna Willensdorfer and Erika Bajna for excellent technical support. Moreover, we thank Amelie Wein for proofreading our manuscript.

Funding

This work was supported by MediGene AG (Martinsried, Germany) and Biomed Int. R + D GmbH (Vienna, Austria), and in part by the Austrian Science Fund (FWF) grants P23398-B11 and W1205-B09. Erika Jensen-Jarolim is shareholder of Biomed Int. R+D GmbH. The study was funded by grants from Medigene AG to Erika Jensen-Jarolim.

Notes

A patent has been filed by Medigene and Biomedical International R+D GmbH, Vienna, Austria: "Anti-HER-2 vaccine based upon AAV derived multimeric structures," WO2013/037961. Markus Hörer, Mirko Ritter and Kerstin Pino Tossi are inventors of the AAV related patent application WO 2008/145400 "Mutated structural protein of a parvovirus" and related patent applications. Kerstin Pino Tossi is an employee of Medigene AG.

This does not alter our adherence to all the journal's policies on sharing data and materials.

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