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A novel Chimpanzee serotype-based adenoviral vector as delivery tool for cancer vaccines

Daniela Peruzzi[a], Sridhar Dharmapuri[a], Agostino Cirillo[a], Bruno Ercole Bruni[a], Alfredo Nicosia[b], Riccardo Cortese[b], Stefano Colloca[b], Gennaro Ciliberto[a], Nicola La Monica[a], Luigi Aurisicchio[a,*]

[a] Oncology/Functional Dept., IRBM – Merck Research Labs – via Pontina Km30.6, 00040 Pomezia, Rome, Italy
[b] Okairòs, Via dei Castelli Romani 22, 00040 Pomezia, Rome, Italy

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ABSTRACT

The use of adenovirus (Ad) as vaccine vectors is hindered by pre-existing immunity to human Ads in most of the human population. In order to overcome this limitation, uncommon alternative Ad serotypes need to be utilized. In this study, an E1–E3 deleted recombinant Ad based on the chimpanzee serotype 3 (ChAd3) was engineered to express human carcinoembryonic antigen (CEA) protein or rat *neu* extracellular/transmembrane domains (ECD.TM). ChAd3 vectors were tested in CEA transgenic (CEA.Tg) and BALB/NeuT mice, which show immunologic tolerance to these antigens. ChAd3 is capable of inducing an immune response comparable to that of hAd5 serotype-based vectors, thus breaking tolerance to tumor associated antigens (TAAs) and achieving anti-tumor effects. Of importance is that ChAd3 can overcome hAd5 pre-existing immunity and work in conjunction with DNA electroporation (DNA-EP) and other Ad vaccines based on common human serotypes.

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1. Introduction

The adenoviruses (Ads) comprise a large family of double-stranded DNA viruses found in amphibians, avians and mammals, which have a nonenveloped icosahedral capsid structure [1–4]. Ad vectors have been developed for gene therapy purposes with the aim of treating inherited or acquired diseases [5]. However, the high immunogenicity of E1-deleted Ad recombinants excluded their successful use for sustained gene therapy and led to their development as vaccine carriers [6]. Ad vaccines have been shown to induce the highest B- and $CD8^+$ T-cell responses in experimental animals, including rodents [7,8], canines [9], and primates [10–12] and have been tested in human clinical trials for antigens of HIV-1 [13]. Ad vectors are being evaluated in additional clinical trials using DNA vaccine priming regimens followed by Ad vector booster immunizations [14].

The Ad vectors used for the treatment of many diseases are predominantly derived from human serotype Ad2 (hAd2) and Ad5 (hAd5) although several issues still hamper this gene delivery system. These include the inability to transduce certain target cell populations, an acute Ad mediated toxicity *in vivo*, and most importantly the high prevalence of hAd5 vector neutralizing antibodies (VNAs) within the human population which decrease gene transfer efficiency or blunt vaccine potency. In fact, subgroup C (which includes hAd2 and hAd5) adenoviral infection is endemic in the human population and consequently, the majority of humans seroconvert within the first 5 years of life as the result of natural infections. In the United States, depending on the age of the study population and the sensitivity of the assay, 40–60% of humans carry readily detectable VNAs to hAd5 virus [8]. Seroprevalence rates to hAd5 virus are markedly higher in human populations from developing countries [8]. In animal models, including non-human primates (NHPs), VNAs generated upon pre-exposure to hAd5 virus have been shown previously to strongly impair the B- and T-cell responses to the transgene product of a vaccine based on an E1-deleted Ad of the same serotype [15–17]. A similar impairment was observed in human volunteers in a clinical trial with a hAd5 vaccine to HIV-1 [18].

In order to overcome these problems, different strategies are being pursued. These include immunosuppression [19,20], artificial envelopment with lipid bilayers [21], use of different human Ad serotypes [22,23], retargeting of human vectors by masking the adenoviral fiber knob to ablate binding to the coxsackievirus adenovirus receptor (CAR) [24], targeting specific cell receptor by genetic modification of hAd fiber [25,26] and vector encapsulation into microparticles [27]. Vectors based on human non-subgroup C adenovirus have been previously reported [22]. Among them, hAd35 has been proposed as an alternative to hAd5 for vaccine delivery because of its low seroprevalence: comparison of hAd5- and hAd35-based vectors in mice and non-human primates showed a lower

* Corresponding author. Tel.: +39 06 91093233; fax: +39 06 91093654.
E-mail address: luigi_aurisicchio@merck.com (L. Aurisicchio).

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immunological potency of the latter vector [28,29]. Similarly, two other vectors based on the rare serotypes hAd24 and hAd34 induced lower CMI than hAd5 in non-human primates [30]. Additionally, use of non-human Ads as vectors to elude hAd immunity has been proposed (reviewed in [31]). Several non-human Ads are naturally non-pathogenic to humans and have been well characterized and unlike human Ads, it is expected that immunity to non-human adenoviruses will not be prevalent in the human population. It has been shown that pre-existing humoral immunity in humans did not cross-neutralize ovine [32], chimpanzee [33–36], canine [37,38] or porcine Ads [39].

These strategies are particularly relevant for the use of Ad vectors as cancer vaccines. In fact, one of the major obstacles to achieving a tumor-specific immune response is that of overcoming central and/or peripheral T cell tolerance against TAAs and inducing CTLs that could effectively eradicate disseminated tumor metastases and subsequently maintain a long lasting immunological memory, preventing tumor recurrence [40]. Ad have been shown to be efficacious vaccine vectors able to break immune tolerance to target antigens in human TAA transgenic mice [41–44] as well as in non-human primates [45]. Increasingly, combinations of Ad vectors with heterologous modalities of immunization, such as *in vivo* electroporation of plasmid DNA (DNA-EP) induce superior immune responses compared to single modality vaccines [41,45,46].

In this study, we describe the use of an E1–E3 deleted recombinant Ad vector obtained from a novel Chimpanzee adenovirus type 3 (ChAd3) belonging to subgroup C adenoviruses (Colloca et al., in preparation), for the development of cancer vaccines directed against two model TAAs: carcinoembryonic antigen (CEA) and HER-2/*neu*, recognized as optimal targets for therapeutic intervention. CEA is an oncofetal membrane antigen, member of the immunoglobulin gene superfamily that is expressed in normal gastrointestinal tissue and is overexpressed by a high percentage of human colon, pancreatic, breast, and lung adenocarcinomas [47]. HER-2/*neu* oncoprotein is a tyrosine kinase receptor overexpressed in several human tumors and associated with poor prognosis [48,49].

Here, we show that ChAd3-based vaccines are able to induce an immune response comparable to hAd5 serotype-based vectors, break tolerance to tumor antigens, overcome Ad5 pre-existing immunity and achieve anti-tumor effects. Our findings may have significant impact on the development of Ad-based cancer vaccines.

2. Materials and methods

2.1. Chimpanzee adenovirus 3

Isolation, expansion and cloning of ChAd3 is described elsewhere (Colloca et al., in preparation). Briefly, ChAd3 was isolated from chimpanzee stool specimens and expanded on 293 and A549 cells. ChAd3 belongs to subgroup C Ads and has a viral genome of 37,741 bp, homologous to Ad5 with a E3 region of 4400 bp. ChAd3 has low seroprevalence in human sera (8% and 33% with a titer >200 for ChAd3 and hAd5, respectively) and a capability of insertion of 9000 bp deleting the region E1–E3 (Colloca et al., in preparation).

2.2. Plasmid constructs and adenovirus vectors

Plasmid pV1J/CEA and hAd5/CEA carry CEA codon optimized cDNA and have been previously described [44]. Generation of hAd5/*neu*.ECD.TM has been described elsewhere [50].

For ChAd3 vectors, shuttle plasmids containing codon optimized human CEA and rat *neu* ECD.TM cDNAs [44,50] driven by hCMV promoter were generated and the expression cassette was inserted by homologous recombination in ChAd3 backbone, E1–E3 deleted (Colloca et al., in preparation). Briefly, CEA and rat *neu* ECD.TM expression cassettes were excised from polyMRK-CEAopt and polyMRK-rat *neu* ECD.TM with SspI/AscI and were recombined to SnaBI linearized pChAd3-EGFP to replace the EGFP expressing cassette through homology of hCMV and bGH polyA, using BJ5183 *E. coli* cells. The resulting plasmids pChAd3-CEA and pChAd3_rat*neu*ECD.TM were cut with PmeI, to release the adenovirus ITRs and transfected in Per.C6 cells (human retinoblastoma cell line), which provide E1 of hAd5 virus. Viruses were expanded through serial passages on Per.C6 cells, purified by CsCl gradient centrifugation and the viral particle concentration was determined by spectrophotometry. *In vitro* and *in vivo* CEA transgene expression was tested by ELISA (Direct Elisa CEA Kit, DBC-Diagnostics Biochem Canada Inc.). Rat *neu*_ECD.TM expression was verified by FACS. The amount of transducing particles of hAd5 or ChAd3 preparations were determined by limiting dilution on Per.C6 cells.

2.3. Peptides

Lyophilized peptides were purchased from Bio-Synthesis (Lewisville, TX) and resuspended in DMSO at 40 mg/ml. Pools of CEA peptides of 15 aa overlapping by 11 residues were assembled as previously described [44]. Particularly, in this study we utilized CEA pool D (53 peptides, each at 0.769 mg/ml in DMSO), encompassing the protein from residue 481 to 703. Peptides and pools were stored at −80 °C.

2.4. Mice and immunizations

C57BL/6 mice transgenic for human CEA (CEA.Tg, H-2^b) were kindly provided by Dr Primus (Vanderbilt University). Female BALB/c mice (H-2^d) transgenic for the transforming *neu*T oncogene (*neuT*$^+$/*neuT*$^-$) under the transcriptional control of the MMTV promoter [51] and transgene negative (*neuT*$^-$/*neuT*$^-$) were bred under specific pathogen-free conditions by Charles River Breeding Laboratories (Calco, Italy) and were kindly provided by Dr G. Forni (University of Torino, Italy). For all experiments, mice were anaesthesized with Isoflurane (Schering Plough, New Augusta, USA) and immunized by injecting the indicated vp of hAd5 or ChAd3 vectors in quadriceps muscle. CEA.Tg mice were vaccinated with DNA by injecting a total amount of 50 μg of pV1J-CEA-LTB plasmid DNA into one quadriceps muscle (50 μg in 50 μl of physiological solution) followed by electroporation (EP) as previously described [44]. At the end of the treatment period and before necropsy, mice were euthanized by compressed CO_2 gas in cylinder as indicated in the American Veterinary Medical Association (AVMA) Panel on Euthanasia and according to the guidelines described in [52]. The experiments were conducted according to EU Directive EC86/609 on the protection of animals used for experimental and other scientific purposes, which was ratified by Italian Legislation with DL no. 116/92 on 19 February, 1992

2.5. Antibodies detection and titration

Sera for antibody titration were obtained by retro-orbital bleeding. ELISA assays were performed using highly purified CEA protein or rat*neu*ECD-hFc as previously described [44,50]. Anti-CEA and anti-rat HER-2/*neu* titers were calculated by plotting serum dilution vs. optical density values and the Michaelis–Menten curve was fitted to the data using Kaleidagraph (v3.5, Synergy Software).

2.6. Cytokine intracellular staining

The detection of peripheral immune response was measured as previously described [53]. Briefly, PBMCs were resuspended

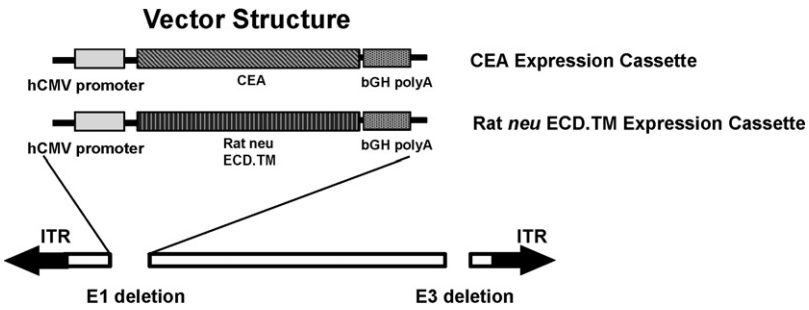

Fig. 1. Schematic representation of the vectors. CEA and rat *neu* ECD.TM expression cassettes were inserted by homologous recombination in E1–E3 deleted hAd5 and ChAd3 vectors as indicated.

in 0.6 ml RPMI, 10% FCS and incubated at 37 °C for 12–16 h with the pool D of CEA peptides or rat *neu* 15.3 (TYVPANASL, identified in a previous study [43] at 5 μg/ml final concentration of each peptide) and brefeldin A (1 μg/ml; BD Pharmingen). Staphylococcus enterotoxin B (SEB) at 10 μg/ml and DMSO were used as positive and background control. Cells were then washed and stained with surface antibodies. After washing, cells were fixed, permeabilized and incubated with the IFNγ-FITC antibodies (BD Pharmingen), fixed with formaldehyde 1% in PBS and analyzed on a FACS-Calibur flow cytometer, using CellQuest software (Becton Dickinson).

2.7. Pre-immunity to hAd5 and neutralization studies

Mice received one or two injections of a E1-deleted hAd5 vector (H14, Q-Biogene–Montreal, hereafter referred to as hAd5wt) and 2–4 weeks later neutralizing antibody titer vs. hAd5 was determined as previously described [54]. Briefly, 2×10^6 to 1.5×10^7 viral particles of hAd5 expressing secreted alkaline phosphatase (hAd5-SEAP) were diluted in 100 μl of complete cell medium and added to an equal volume of mouse serum diluted in complete medium. Each serum sample was tested at various dilutions. Samples were pre-incubated for 1 h at 37 °C and then added to 293 cells seeded into 96 wells (3×10^4 cells/well). The inoculum was then removed and cells re-fed with fresh medium. 24 h later, 50 μl of medium was removed and SEAP activity was measured by a chemiluminescent assay (Phospha-Light System™, Applied Biosystems, Bedford, MA) according to manufacturer instructions. The neutralization titer is defined as the dilution of serum giving a 50% reduction of the SEAP activity observed in the positive control with the virus alone.

2.8. Statistics

Student's *t*-test or Log-Rank test were performed where indicated. p value < 0.05 were considered significant.

3. Results

3.1. Construction of ChAd3 vectors

E1–E3 deleted, replication-defective ChAd3 vectors containing codon optimized CEA sequence and rat *neu* ECD.TM cDNAs were constructed by homologous recombination. As shown in Fig. 1, the transgenes were cloned in a cassette driven by the human CMV promoter (hCMV) and with a bovine growth hormone (bGH) polyadenylation signal at the 3′-end. Vectors were rescued and expanded in Per.C6 cells. The vp/pfu ratio, a parameter that determines the relative infectivity level of different Ad viruses and preparations, was similar to hAd5 preparations (from 20 to 35).

3.2. hAd5 and ChAd3 express similar levels of CEA

To verify the capability of ChAd3 to infect human cells and express the antigen of interest, HeLa cells were infected at different ratio vp:cell with ChAd3-CEA and hAd5-CEA. CEA is a GPI-anchored protein, normally shed in biological fluids and for this reason is easily detectable [55]. Forty-eight hours later, the supernatant of the infected cells was collected and CEA protein was quantified by ELISA. As shown in Fig. 2A ChAd3-CEA was able to infect cells and protein secretion levels were comparable with those of hAd5-CEA infected cells.

We next assessed the infectivity of the vectors and CEA expression *in vivo*. Groups of CEA.Tg were injected intramuscularly with three doses (10^{10}, 10^9 and 10^8 vp/mouse) of either of the two adenoviral vectors. Three days later, CEA expression was measured in the serum and we found that levels of the secreted protein by ChAd3-CEA were roughly 3–4-fold lower than with hAd5-CEA vector (Fig. 2B).

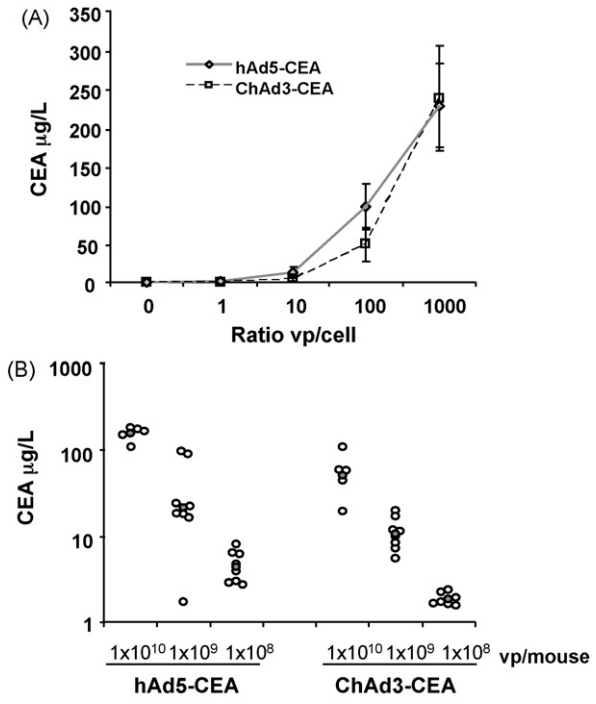

Fig. 2. ChAd3 and hAd5 show comparable expression of CEA *in vitro* and *in vivo*. (A) HeLa cells were infected at the indicated ratio vp/cell. 48 h later secreted CEA was measured in the supernatant by ELISA. Grey diamonds and white square symbols show average CEA expression mediated by hAd5-CEA and ChAd3-CEA, respectively. (B) CEA.Tg mice were injected i.m. with the indicated amount (vp) of either hAd5 or ChAd3. Three days later, mice were bled and circulating CEA levels were measured by ELISA. Grey dots indicate the geometric mean of the group.

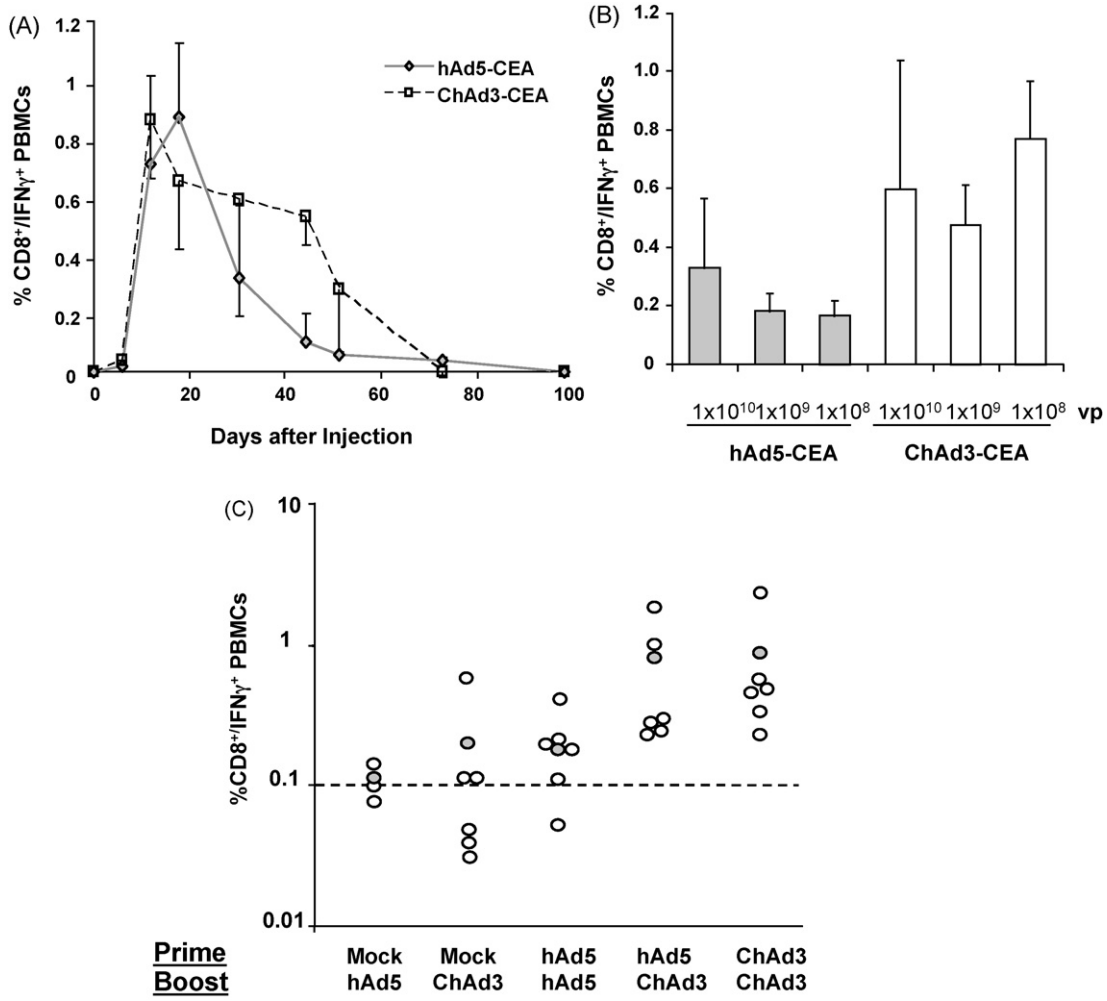

Fig. 3. Characterization of the immunogenic properties of ChAd3-CEA. (A) Kinetics of the immune response. Groups of 6–8 CEA.Tg mice were immunized with 1×10^{10} vp of either hAd5-CEA or ChAd3-CEA vector. At the indicated time point, mice were bled and PBMCs were stimulated with a pool of peptides covering the C-term of CEA (pool D) and analyzed by intracellular staining for IFNγ. Group average with standard deviations (error bar) is shown. (B) Vaccine potency of ChAd3-CEA. Groups of 6 CEA.Tg mice were vaccinated with the indicated vp of either hAd5-CEA or ChAd3-CEA vector. Thirty days after the injection, PBMCs were analyzed for CEA-specific response by intracellular staining for IFNγ. Histograms and error bars show group average and standard deviations, respectively. (C) hAd5 and ChAd3 vector mixed modality. Groups of 6 CEA.Tg mice were immunized either with 10^8 vp of hAd5-CEA or ChAd3-CEA vector or mock injected. Thirty days later, mice were boosted with the indicated vector and 2 weeks later, PBMCs were analyzed for CEA-specific response by intracellular staining for IFNγ. White circles indicate the immune response measured per single animal; gray dots represent the group geometric mean. Mice with responses above 0.1% $CD8^+/IFN\gamma^+$ (dashed line) are considered responders. Student's *t*-test, $p = 0.05$ for hAd5/hAd5 vs. hAd5/ChAd3 or ChAd3/ChAd3 groups.

3.3. Immunogenicity of ChAd3 and hAd5 is comparable

To assess whether ChAd3 was capable of breaking the immunologic tolerance to CEA in CEA.Tg mice and to measure the kinetics of the immune response, PBMCs were analyzed at different time points by intracellular staining (ICS) for the frequency of CEA-specific IFNγ producing $CD8^+$ cells, 2 weeks after a single treatment of mice with 10^{10} vp of either hAd5-CEA of ChAd3-CEA vaccines. Both vectors were able to elicit a significant immune response that reached a peak level between 14 and 21 days post-injection (Fig. 3A) and decreased to basal level 75–100 days after the treatment, with apparently slower kinetics than those observed with ChAd3-CEA vector.

To better characterize the immunogenic potential of ChAd3 with respect to hAd5, we immunized CEA.Tg mice with escalating doses of each virus. One month after the injection, ChAd3 elicited higher responses, especially at the lower dose (10^8 vp, Fig. 3B).

To measure the effect of heterologous vs. homologous Ad-based vaccination, CEA.Tg mice were vaccinated 1 month apart with different combinations (hAd5/hAd5; ChAd3/ChAd3; hAd5/ChAd3) at low doses (10^8 vp/mouse) that did not induce measurable NVAs. As shown in Fig. 3C, hAd5 prime/boost vaccination induced a apparently low immune response (geomean: 0.2% CEA-specific $CD8^+IFN\gamma^+$ PBMCs; 4/6 responder mice) compared to ChAd3/ChAd3 and hAd5/ChAd3 regimens (geomean: 0.8% and 0.9%, respectively; 100% responders).

Taken together, these data show that ChAd3 has similar potency compared to hAd5 in eliciting CEA-specific immune response.

3.4. ChAd3 vaccine overcomes hAd5 pre-existing immunity

The impact of pre-existing immunity to hAd5 was tested in mice previously exposed to high dose wild type hAd5 (1×10^{10} vp). One month later, all animals developed high titer NVAs (titer ≈1/1000 to 1/2000) and were treated with one injection of hAd5 or ChAd3-CEA expressing vectors (see scheme in Fig. 4A). CEA-specific T cell immune response was unaffected in animals vaccinated with ChAd3-CEA (see Figs. 4A and 3 for reference) but completely abolished in mice receiving the hAd5-CEA treatment. The effect of anti-hAd5 immunity in the "recall" phase was also evaluated

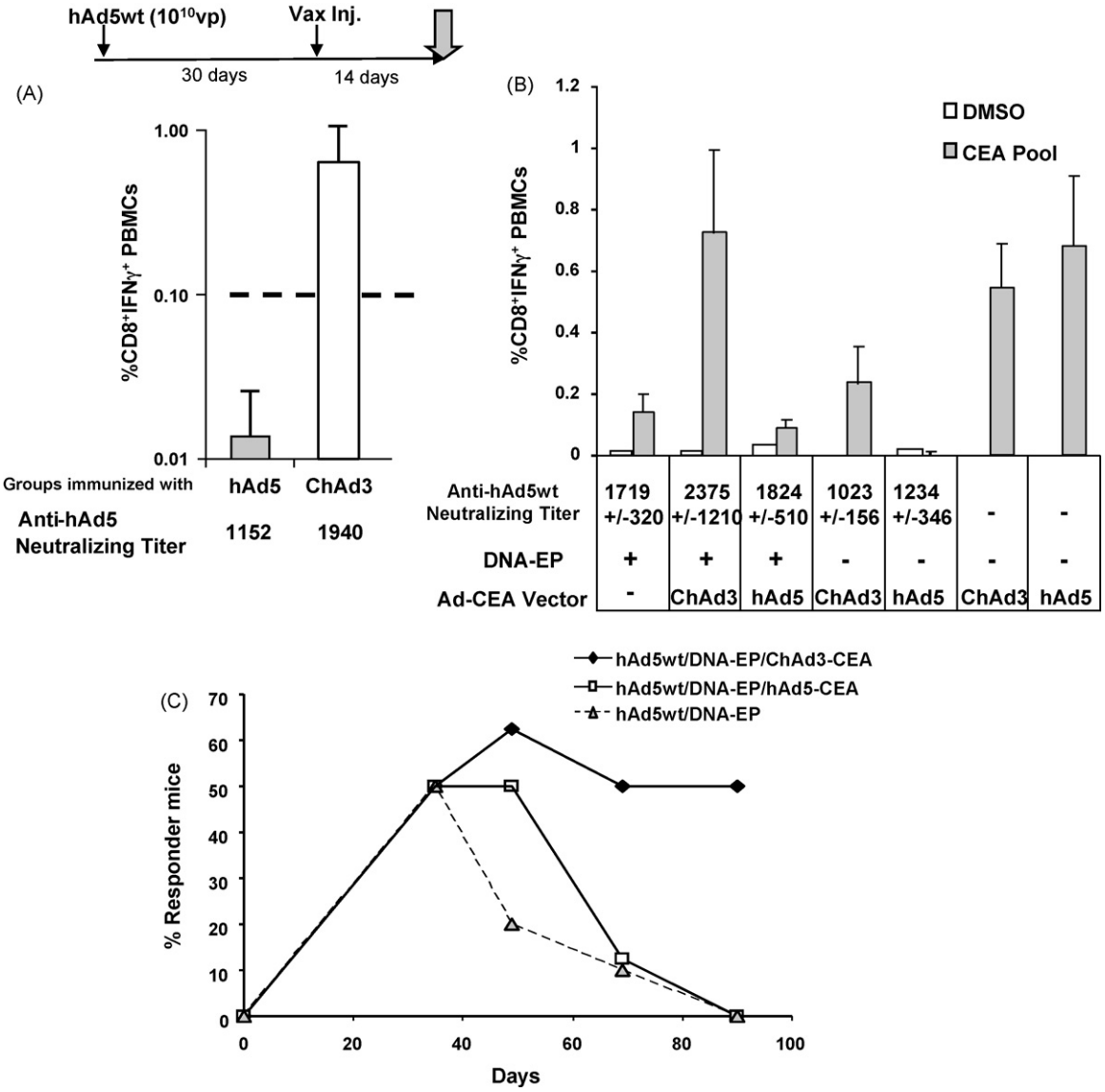

Fig. 4. ChAd3-CEA vaccine overcomes hAd5 immunity. (A) Top scheme. Groups of CEA.Tg mice were pre-injected with 10^{10} vp of wild type hAd5 and immunized either with hAd5-CEA or ChAd3-CEA (10^{10} vp). The average (±SD) anti-hAd5 titer of the group is shown. Two weeks later, the immune response against CEA was measured by intracellular staining for IFNγ. (B) Pre-exposure to wt hAd5 does not impair ChAd3 vaccine potency. Groups of 10 CEA.Tg mice received 4 weekly DNA-EP with pV1J-CEA or left untreated (mock). Two weeks after, animals were injected with 10^9 vp of either of the two vectors and the immune response against CEA was measured 14 days later by intracellular staining for IFNγ. (C) Kinetics of immune response. Treated mice were bled at the indicated time points and PBMCs were analyzed by intracellular staining for IFNγ. Mice with a CEA-specific response >0.1% $CD8^+/IFN\gamma^+$ were considered responders.

following previous treatments with DNA-EP. CEA.Tg mice with pre-existing immunity received 4 weekly DNA-EP treatments, 2 weeks later were injected with the Ad vectors and the immune response was measured thereafter. As shown in Fig. 4B, anti-hAd5 antibodies completely blunted the "priming" and "boosting" capability of regimens containing hAd5. In contrast, poor or no reduction was observed in mice that received ChAd3 vector. Similarly, the duration of the response in DNA-EP/ChAd3-CEA treated animals was not affected by pre-existing immunity (Fig. 4C). These data indicate that antibodies against hAd5 are unable to impair ChAd3 immunization potency both in a homologous and heterologous vaccination setting.

3.5. ChAd3 vaccine confers anti-tumor effects in BALB/NeuT mice with anti-hAd5 immunity

To further extend our observations to another TAA/mouse model, we utilized the BALB/NeuT mice. This is one of the most suitable models for vaccine evaluation since female mice develop autochthonous, spontaneous and synchronous breast cancer in each of their 10 mammary glands [51] and the anti-tumor response is highly correlated with the immune response against rat *neu* [50].

Two weekly injections of hAd5_*neu*.ECD.TM at week 8 and 9 were sufficient to induce a strong *neu*-specific $CD8^+$ T-cell response (mean ≈1%) as measured by IFNγ intracellular staining at week 11. This response persisted at significant levels ($CD8^+/IFN\gamma^+ > 0.1\%$) for at least another 3 weeks (week 14, see Fig. 5A). At both time points, at least 80% of the mice responded to the vaccination. Two similarly spaced weekly injections of ChAd3_*neu*.ECD.TM induced a slightly lower T-cell response than hAd5_*neu*.ECD.TM injections. In this case, about 80% of the mice responded at the first time point and 60% persisted at the second. PBMCs collected from saline treated mice (controls) did not show any IFNγ producing T cells (data not shown).

To determine whether neutralizing antibodies to hAd5 cross-reacted with ChAd3 in this mouse model, groups of mice were immunized as previously described with two injections of either

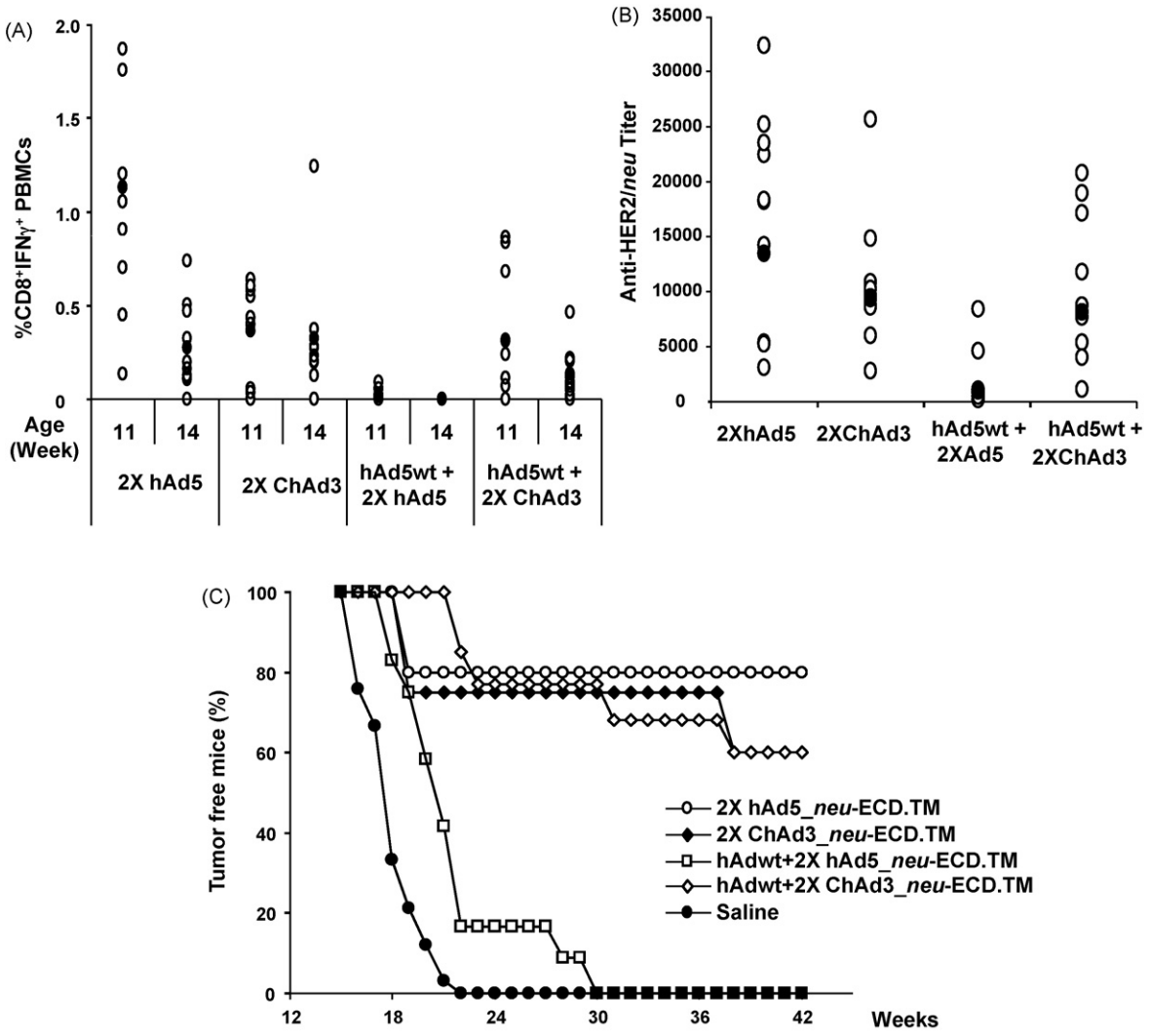

Fig. 5. ChAd3-_neu.ECD.TM immunogenic properties and anti-tumor effects. (A) T-cell responses. Groups of 10 BALB/NeuT mice were vaccinated at the 8th and 9th week of age. The immune response against rat *neu* 15.3 epitope was analyzed at week 11 and 14. Open circles indicate the response of individual mice, filled circle denotes the group mean. A mouse with T-cell reactivity of at least 0.1% was considered a responder. Student's *t*-test at week 11, $p < 0.05$ for hAd5wt+hAd5 vs. hAd5 alone or hAd5wt+ChAd3 or ChAd3 alone. (B) Antibody titer. Anti-rat HER-2/*neu* titers were determined as described in materials and methods. At least 10 mice were included in each group. Open circles indicate the response of individual mice, filled circle denotes the group mean. Student's *t*-test, $p < 0.0003$ for hAd5wt+hAd5 vs. hAd5 alone or hAd5wt+ChAd3 or ChAd3 alone. (C) Anti-tumor effect. Mice were immunized as described in the text and tumor development followed from week 15 onwards. At least 10 mice were included in each group. Percentage of tumor-free mice is shown. Group symbols are shown in graph legend. Log-Rank test, hAd5wt+ChAd3-ECD.TM vs. hAd5wt+hAd5-ECD.TM: $p = 6.27 \times 10^{-06}$.

hAd5_*neu*.ECD.TM or ChAd3_*neu*.ECD.TM. Two weeks prior to the vaccination, mice were injected with 10^{10} particles of wild type hAd5 to induce neutralizing antibodies. Following administration of the vaccine, the T-cell response was analyzed. Mice that received hAd5 wild type prior to hAd5_*neu*.ECD.TM showed no detectable *neu*-specific T cell response by IFNγ intracellular staining (Fig. 5A). The mean response dropped from 1% to undetectable, suggesting that the vector had been neutralized by pre-existing anti-hAd5 humoral responses. In contrast, no such significant drop in immunogenicity was observed in the ChAd3_*neu*.ECD.TM immunized mice. 50–70% of the mice were responders at both time points. Similar results were obtained for antibody response against rat HER2-*neu*, also strongly affected by pre-exposure to wt hAd5.

Finally, to assess if ChAd3 vaccine could confer therapeutic anticancer effects, tumors were palpated from week 15 onwards. Two hAd5 injections resulted in 80% of the mice remaining tumor free until week 42 (Fig. 5C). Similarly, data obtained with two ChAd3 injections showed that 70% of the mice were tumor free for up to 42 weeks. Control mice developed 10 tumors by week 25. The groups that received a pre-immunization of wt hAd5 were also evaluated. The presence of neutralizing antibodies drastically reduced the efficacy of hAd5_*neu*.ECD.TM. By week 30, none of the mice were tumor free. On the other hand, pre-existing immunity to hAd5 did not have any impact on the anti-tumor effect of ChAd3_*neu*ECD.TM immunization, as 60% mice were tumor free with or without pre-exposure to hAd5. These data confirmed that anti-hAd5 humoral immunity do not cross-react with ChAd3 and therefore, the new vector is an effective substitute for hAd5 in mice.

4. Discussion

Cancer immune therapy and its translation to the clinic are strictly dependent on efficient vaccine technology, delivery systems and evaluation in appropriate pre-clinical models. CEA.Tg mice express CEA with a tissue distribution similar to that of humans [56] and provide a particularly relevant model to critically verify the potential efficacy of a human CEA-based vaccine [57]. Similarly, BALB/NeuT mice allow assessment of active cancer immunotherapy against HER-2/*neu*. Here, overexpression of the activated (V664E) form of the rat HER-2/*neu* oncogene under the control of the mouse

mammary tumor virus promoter, leads to the progressive development of invasive mammary adenocarcinoma in all ten mammary glands [51]. We have shown that DNA-EP technology and particularly hAd5 carrier, along with codon usage optimization [44] and antigen engineering [41,42], lead to breakage of immune tolerance and hamper cancer progression in these models [43,50].

The exceptional immunogenicity of replication-defective hAd5 vectors is probably due to a number of properties: (i) the high levels of transgene expression driven in most vectors by the potent cytomegalovirus promoter, (ii) the activation of the innate immune system, (iii) the transduction of immature dendritic cells driving their maturation into antigen-presenting cells, and (iv) a sufficiently long duration of antigen presentation due to lack of apoptosis induction of the transduced cells. However, pre-existing anti-adenovirus immunity and, in particular, neutralizing antibodies which reduce cell uptake of the adenoviral vectors can significantly dampen vaccine responses and represent a major limitation to the successful use of common serotypes of adenovirus.

To circumvent the impairment of vaccine efficacy by pre-existing neutralizing antibodies, we developed vaccine vectors based on Ads that had been isolated from chimpanzees. One of these, ChAd3 has close sequence homology with the hAd5 virus and thus belongs to subgroup C of the *Adenovirideae* (Colloca et al., in preparation).

In this study, we show that ChAd3-CEA and hAd5-CEA have similar infectivity potency *in vitro* and *in vivo* (Fig. 2). The primary receptor responsible for attachment of all Ad serotypes, except those from group B that use CD46 [22], is the CAR [58]. Recent studies have shown that Ad can also use other receptors, such as the major histocompatibility complex I (MHCI) and heparin sulfate glycosaminoglycans [59]. Gene transfer efficacy mediated by hAd5 and ChAd3 has been investigated in a large panel of human primary cells of different tissue origin and found to be similar (Colloca et al., in preparation). This suggested that both vectors utilize the same entry mechanisms, at least in *in vitro* cultured cells. However, expression of CEA was apparently lower in ChAd3-CEA i.m. injected mice (Fig. 2B), suggesting that this vector may have different properties and/or tropism in transducing mouse tissues compared to hAd5 [60,61].

Importantly, ChAd3-CEA was able to break tolerance in CEA.Tg mice inducing a potent antigen-specific $CD8^+$ response with kinetics comparable to that of hAd5-CEA (Fig. 3A and B), thus indicating that this vector could infect the cells instrumental for antigen expression and induction of the immune response via direct or indirect presentation of the antigen. ChAd3_*neu* ECD.TM showed similar immunogenic properties in BALB/NeuT mice (Fig. 5A and B). ChAd3-CEA vector also showed good efficacy in boosting hAd5-CEA elicited immune response in a heterologous type of vaccination (Fig. 3C). This feature is important, since regimens consisting of rare serotype Ad vectors combined to hAd5 have recently proven able to elicit augmented magnitude, breadth and polyfunctionality of cellular immune responses and superior protective efficacy as compared with the homologous hAd5 regimen against HIV-1 [62].

The ChAd3 vector was developed to overcome pre-existing serotype-specific neutralizing antibodies to hAd5, shown to interfere with the efficacy of the homologous vaccine carrier. This limitation could only in part be circumvented by increasing the vaccine dose, which may result in severe vector-mediated toxicity. Indeed, cross-neutralization of the ChAd3-mediated immune response did not occur in animals with high titer hAd5 pre-existing immunity (Figs. 4A and 5B) and equally the Ad boosting effect as a consequence of DNA-EP priming was not impaired (Fig. 4B and C).

Finally, we showed that ChAd3_*neu* ECD.TM had an anti-tumor effect comparable to the corresponding hAd5 vector, and most important of all, that ChAd3 vaccine efficacy was not blunted by the presence of hAd5 neutralizing antibodies (Fig. 5C) and was associated to the measured HER-2/*neu*-specific immune response.

Recently, Chimpanzee Ad vectors such as AdC7, AdC68 and AdC1 have proven particularly effective tools as vaccines against a variety of viral antigens and diseases, such as influenza caused by H5N1 strains [63], human immunodeficiency virus type 1 in NHPs [33], rabies virus [36] and severe acute respiratory syndrome mediated by SARS-Coronavirus [35]. Of note, in these models the organism recognizes the antigen as an exogenous protein and consequently the elicited immune response is generally strong and effective against the target pathogen. On the other hand, choosing appropriate preclinical models in which TAAs are recognized as self-protein is imperative to better predict the possible outcome of a cancer vaccine in human patients.

Here we report for the first time that novel chimp Ad vectors, such as ChAd3, can be employed as potent cancer vaccine tools and may prove particularly useful as vaccine vectors for populations in the developing world with high levels of pre-existing anti-hAd5 immunity. Additionally, ChAd3 vectors could be utilized together with hAd5, other common hAd vectors and DNA-EP in heterologous prime-boost regimens.

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