Targeting the antigen encoded by adenoviral vectors to the DEC205 receptor modulates the cellular and humoral immune response

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Abstract

Replication-defective adenoviral vectors have emerged as promising vaccine candidates for diseases relying on strong CD8+ T-cell responses for protection. In this study, we modified a non-replicative adenoviral vector to selectively deliver, in situ, an encoded ovalbumin (OVA) model antigen to dendritic cells (DCs). Efficient uptake and presentation of OVA was achieved through fusion of the antigen to a single-chain antibody directed against DEC205, an endocytic receptor expressed on DCs. The immunogenicity of the vaccine was thereby enhanced as demonstrated by elevated antibody levels and increased T-cell responses after low-dose vaccination with 10⁷ viral particles compared with a non-targeted control. Nevertheless, after immunization with higher doses of the targeted vaccine, the capacity of vaccine-induced CD8+ T cells to produce the cytokine IL-2 was diminished and the CD8+ T-cell response was dominated by an effector memory phenotype (CD62L−/CD127+) in contrast to the effector phenotype (CD62L−/CD127−) observed after non-targeted antigen delivery. Interestingly, the protective capacity of the non-targeted vaccine was superior to that of the targeted vaccine in an antigen-specific vaccinia virus infection as well as in a tumor challenge model. In the latter, the low dose of the DC-targeted vaccine also conferred partial protection from tumor growth, demonstrating dose-dependent effects of the DC-targeting on the quality of the vaccine-induced immune response. Significant differences could be observed in regard to the antibody pattern, the functional and phenotypic T-cell repertoire, and to the protective capacity.

Keywords: adenoviral vector, DC-targeting, vaccination

Introduction

Recombinant replication-defective adenoviral vectors are promising vaccine candidates that have been shown to provide protective immunity against viral infections in mice and non-human primates, including acute infections caused by the respiratory syncytial virus (1) or influenza (2, 3) and chronic infections caused by Simian immunodeficiency virus, HIV or SHIV (4, 5). Since the STEP clinical study of a prophylactic HIV vaccine based on a trivalent adenoviral vector vaccine failed to provide any evidence of protection (6, 7), further optimization of the vaccines might be necessary to enhance the breadth and strength of the humoral and cellular responses. One approach that had been reported to enhance the immunogenicity of vaccines is the selective delivery of antigen to dendritic cells (DCs), which are critical in the initiation and regulation of adaptive immune responses. Monoclonal antibodies to several DC antigen-uptake receptors including DEC205, Dectin-1, Langerin or Clec9 were generated and coupled to antigens resulting in enhanced antigen uptake and presentation in vitro as well as in vivo (8–12). However, injection of DC-targeted antigens in the absence of adjuvants induces initial T-cell proliferation, but this is not followed by strong CD4+ and CD8+ effector T-cell responses because of peripheral deletion, tolerance and/or induction of regulatory T cells (13–17). The immunogenicity of protein vaccines targeted to DCs via antibodies against DEC205 were analyzed in a variety of antigen models in mice, including HIV, EBV or tumor antigens, and demonstrated to be
enhanced in comparison with non-targeted proteins if delivered together with maturation stimuli, such as TLR ligands or CD40-binding antibodies. In particular, the cellular immune responses were improved as revealed by robust numbers of antigen-specific CD4+ and CD8+ T cells, which are usually poorly induced by ‘classical’ protein vaccination (18–20).

More recent vaccine trials have focused on the use of genetic vaccine candidates, like plasmid DNA or viral vectors, which allow the vaccine recipient to express the antigen in situ. The selective delivery of the antigen to DCs via single-chain antibodies (scAb), directed against DC-restricted uptake receptors such as DEC205 has been demonstrated to enhance the efficacy of genetic vaccines. Thus, DNA vaccines, and vaccines using recombinant Newcastle disease virus (NDV) as a vector, that encode such scAb-antigen fusion proteins have been demonstrated to enhance antigen presentation by DCs in vivo leading to improved immunogenicity (21–23). We have now developed a vaccine that uses replication-defective adenovirus (Ad) as a vector and that targets an encoded ovalbumin (OVA) protein to murine DCs in situ. The impact on the activation of antigen-specific T cells by DCs was evaluated in vivo, before comparative immunogenicity studies were performed side-by-side with adenoviral vectors expressing a non-targeting control scAb fused with OVA. The quality and magnitude of humoral and cellular immune response were analyzed dose dependently and the protective capacity was assessed in an antigen-expressing vaccinia virus infection as well as in an OVA-specific tumor model.

Methods

Adenoviral vector vaccines

To generate adenoviral vectors expressing the single-chain fusion proteins, scDEC-OVA or scGLL117-OVA, the expression cassettes of DEC-OVA and GLL117-OVA, previously described (21), were cloned into pShuttle plasmid. E1- and E3-deleted, replication-defective adenoviruses with the corresponding expression cassettes (Ad-DEC-OVA, Ad-GLL117-OVA) were generated by the AdEasy-system (24), as well as the previously described control vectors Ad-GFP (25) and Ad-AGM-OVA (26). Transgene expression was confirmed for all viral vectors, before purification with the Vivapure AdenoPak kit (Sartorius, Göttingen, Germany), in accordance with the manufacturer’s protocol. Purified particles were quantified by optical density measurements and the TCID50 of the vectors were determined on 293 cells. The ratios of particle to infectious particle were comparable for all vectors and were in the range of 100:1. The adenoviral vector preparations were also tested for endotoxin levels with the LAL quantification assay (Cambrex Bio Science, Verviers, Belgium), confirming that the dose used for immunization of mice contained <0.1 EU (Endotoxin Units).

Cell culture media and reagents

The 293 cells, 293T cells, CV-1, CHOneo cells and CHODEC205 cells were cultured in DMEM containing 10% FCS and 1% penicillin/streptomycin. RPMI 1640 supplemented with 10% FCS, 2mM l-glutamine, 10mM HEPES, 50μM β-mercaptoethanol and 1% antibiotic/antimycotic (all Gibco, Karlsruhe, Germany) was used for the leukocyte cultures.

Transgene expression analysis

To confirm equal expression levels and protein secretion, 293T cells were infected with 1 × 10⁸ particles of either Ad-DEC-OVA or Ad-GLL117-OVA and supernatants were collected after 48 h. Western blot analyses were performed as described previously (26). The binding capacity of the single-chain antibodies to the DEC205 receptor were analyzed with DEC205-expressing CHO (CHODEC205) and control CHO (CHOneo) cells (23). Cells (1 × 10⁷) were incubated for 30 min at 4°C with the above-mentioned infection supernatants to allow binding of the single-chain antibodies. The cells were pelleted in a 96-well U-bottom plate at 2000 r.p.m. for 3 min and then washed twice with 200 μl of PBS/BSA (0.5% BSA). Bound DEC-OVA was stained with rabbit-anti-OVA for 30 min at 4°C, followed by two washing steps and FITC-conjugated anti-rabbit-immunoglobulin (BD Bioscience, Heidelberg, Germany) was used for the detection (30 min, 4°C). The cells were resuspended in PBS/BSA (0.5%) and analyzed by flow cytometry using a FACScalibur (BD Bioscience).

In vitro T-cell proliferation assay

CD11c+ DCs were isolated from spleens of C57BL/6 mice. In brief, single-cell suspensions were prepared with 400U ml⁻¹ collagenase D (Roche) for 25 min at 37°C, before CD11c+ cells were isolated with magnetic beads carrying anti-CD11c antibodies (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer’s protocol. Enriched DCs were incubated for 12 h with the above-mentioned infection supernatants allowing antigen uptake. The next day CD8+ T cells were prepared from lymph nodes and spleen of OT-1 transgenic mice using the CD8- T-cell Isolation Kit (Miltenyi Biotech). The isolated T cells were labeled with 5 μM Carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes®, Invitrogen, Darmstadt, Germany) for 8 min at reverse transcription and then cultured with antigen-experienced DCs for 96 h. CFSE intensity of OT-I cells, detected by OVA-specific tetramers (Sanquin, Amsterdam, the Netherlands), were analyzed by flow cytometry.

Animals and immunizations

CD45.2+ OT-I mice were bred in the central animal facility of the Ruhr-University Bochum, whereas CD45.1+ OT-I and OT-II mice were kept in the animal facility of the Rockefeller University. C57BL/6 mice (6–8-week-old female) were purchased from Janvier or Charles River and housed in accordance with the national law and institutional guidelines. All vaccines were diluted in PBS and injected subcutaneously in both hind foot pads on day 0. Serum samples were collected at day 14 and day 35, whereas the cellular immune response was analyzed on day 14 or day 28. The intra-nasal challenge infection with OVA-expressing vaccinia virus (VV-OVA) was applied between days 38 and 42 and the tumor challenge was performed on day 36.

T-cell transfer and in vivo antigen presentation

To assess the antigen uptake and presentation by DCs in vivo, C57BL/6 mice were immunized with different doses (10⁷, 10⁸ and 10⁹ viral particles) of either Ad-DEC-OVA or
Vaccinia challenge

Ad-GL117-OVA. Four days later, CD8⁺ or CD4⁺ T cells were prepared from spleen and lymph nodes of OT-I or OT-II mice, respectively, by negative selection using hybridoma supernatants directed against MHC-II, F4/80, B220, NK1.1 and CD4 or CD8 followed by goat anti-rat Dynabeads (Dynal®, Invitrogen, Darmstadt, Germany). Purified OT-I and OT-II cells, 10⁵ cells ml⁻¹, were labeled with 5 µM CFSE (Molecular Probes®, Invitrogen, Darmstadt, Germany). About 2 x 10⁶ labeled OT-I or OT-II cells were injected intravenously into immunized animals. Three days later, single-cell suspensions were obtained from draining lymph nodes (popliteal and inguinal) and spleen. Cells were stained with anti-CD4-PacificBlue (PB), anti-CD45.2-allophycoerythrin (APC) and anti-CD8-Alexa 700 (all antibodies, BD Bioscience). The CFSE intensity of OT-I and OT-II cells was then evaluated by multicolor flow cytometry using the BD LSR II cytometer.

OVA-specific antibody ELISA

Blood was taken retro-orbitally and serum was collected after centrifugation for 5 min at 5000 rpm in a table top centrifuge. The ELISAs were performed as previously described (26). Horseradish peroxidase-coupled antibodies against mouse IgG1 or IgG2a antibodies (BD Bioscience) were used for the detection. Luminescence was analyzed in a microplate luminometer with Simplicity software (ORION-96; Berthold, Bad Wildbad, Germany).

Tetramer and intracellular cytokine staining

Splenocytes were collected at indicated time points. After red blood cell lysis, 1 x 10⁷ cells were plated in 96-well round-bottom plates (Nunc) for each staining. For the tetramer staining, cells were washed once and incubated with 2 µl of APC-labeled tetramers (Sanquin), specific for the OVA-derived peptide SIINFEKL, in total volume of 100 µl PBS/BSA/azide for 40 min at room temperature. In a second step, surface staining was performed with anti-CD8-PerCP, anti-CD127-FITC and anti-CD62L-PE (all BD Bioscience). A second step, surface staining was performed with anti-CD3-PacificBlue (PB), anti-CD45.2-allophycoerythrin (APC) and anti-CD8-Alexa 700 (all antibodies, BD Bioscience). The CFSE intensity of OT-I and OT-II cells was then evaluated by multicolor flow cytometry using the BD LSR II cytometer.

Vaccinia challenge

Animals were anesthetized with xylazine/ketamin and 5 x 10⁴ or 1 x 10⁵ PFU of VV-OVA were applied intra-nasally in 50 µl of PBS. The weight loss was monitored daily. Six days post-infection animals were sacrificed via isofluran inhalation. Lungs were homogenized in 2 ml PBS and stored at –80°C prior to virus titration or DNA isolation for quantitative PCR (qPCR). Virus titrations were performed as described previously (23). Briefly, lung homogenates were used to infect CV-1 cells. Two days after infection, cells were stained with 0.1% crystal violet solution to count number of vaccinia virus-induced plaques. For qPCR, DNA was isolated from lung homogenates using the DNA blood Kit (Qiagen) according to the manufacturer’s protocol and quantified by the Cubit system (Invitrogen). Viral DNAs were amplified with vaccinia-specific primers in the presence of SYBR green (Sigma, Munich, Germany). Genome copy numbers were calculated using cloned plasmids as standards and expressed as copies per total microgram of DNA.

B16-OVA melanoma challenge

Five weeks after the vaccination, 2 x 10⁶ B16-OVA cells were inoculated at the right flank of the mice. The volume of the tumor was determined every 3 days and mice were sacrificed when the volume reached 4000 mm³ or when there was any sign of ulceration of the tumor.

Results

Characterization of the viral vector constructs

Adenoviral vectors encoding a DC-targeted OVA, Ad-DEC-OVA, and a control virus expressing non-targeted OVA, Ad-GL117-OVA, were generated by using the well-established Ad-Easy system (24). After purification, transgene expression of the vectors was analyzed 2 days after infection of 293 cells. Comparable amounts of the fusion proteins DEC-OVA and GL117-OVA, respectively, were detected in the supernatants of infected cells by an OVA-specific western blot analysis with an expected size of 75 kD (Fig. 1A). To ensure the antibody binding specificity, the supernatants of infected cells were incubated with DEC205-expressing CHO cells (CHODEC205) or parental CHO cells (CHOneo). Bound single-chain molecules were visualized with an FITC-labeled OVA-specific antibody and subsequent flow cytometric analysis (Fig. 1B). As revealed by the shift to higher fluorescence intensities only supernatants of Ad-DEC-OVA-infected cells contained fusion proteins that bound specifically to the DEC205-receptor on CHOneo cells, but not to the control cells. The incubation with supernatants of Ad-GL117-OVA- or Ad-GFP-infected cells did not show any binding to both cell lines. The specific binding to DEC205 was also confirmed on DEC205⁺ B220⁻ and DEC205⁺ CD11c⁺ cells derived from a mixed culture of spleen and lymph node cells (Supplementary Figure 1, available at International Immunology Online). In contrast, GL117-OVA and parental OVA did not show detectable levels of binding. To address the capacity of DCs to activate antigen-specific T cells after delivering the DEC-targeted antigen, spleen-derived DCs were incubated with the supernatants of vector-transduced cells for 24 h. After antigen uptake and processing, DCs were co-cultured with CFSE-labeled TCR-transgenic OT-I lymphocytes and antigen presentation was indirectly measured by proliferation of activated CD8⁺ T cells (Fig. 1C). Although comparable amounts of OVA were present in these supernatants, the incubation with
targeted antigen led to stronger T-cell activation by DCs as demonstrated by the fact that >80% of the transgenic T cells started to proliferate. Thus, we showed that Ad-DEC-OVA and Ad-GL117-OVA express comparable levels of antigens and that DEC205 targeting increased binding to DCs and DC-induced activation of OVA-specific T cells in vitro. In addition, both adenoviral vectors showed comparable in vitro infection efficiencies and should thus only differ in the specificity of the encoded scAb fused to OVA antigen.

**Antigen presentation after adenoviral vector immunization**

We next determined if antigen presentation and subsequent T-cell activation in vivo is also affected following immunization with adenoviral vector targeting the encoded OVA to DCs. Female C57BL/6 mice received graded doses (10^7 to 10^9 viral particles) of either Ad-DEC-OVA or Ad-GL117-OVA via the foot pads. Four days later, 10^6 CFSE-labeled TCR-transgenic CD8+ T cells (OT-I) or CD4+ T cells (OT-II) were adoptively transferred into the vaccinated mice. If OVA antigen was being presented, these T cells would be activated and start to proliferate. Three days later, this was measured by CFSE-dilution in expanding donor CD45.2-CD8+ or CD4+ cells, respectively, both in the draining lymph nodes as well as in the spleen (Fig. 2A and B). Adenoviral vector-mediated targeting of the encoded OVA to DCs in situ resulted in robust T-cell activation with just 10^7 Ad-DEC-OVA viral particles inducing higher percentages of expanding OT-I T cells in the lymph nodes than 10^9 viral particles of the control Ad-GL117-OVA (Fig. 2A and B; left). The enhanced presentation after DEC-targeted vaccination was also observed in the spleen, but to a lesser extent (Fig. 2B).

Higher doses of the vaccines were needed to induce antigen-specific activation of transgenic CD4+ T cells (OT-II). Proliferation of CD4+ T cells was detected only after immunization with the highest dose of Ad-DEC-OVA, but not with the non-targeted vaccine (Fig. 2A and B; right). Again, proliferating cells were mainly found in the lymph nodes and to a lesser extent in the spleen (Fig. 2B).

DEC205+ CD11c+ DCs are known to play a critical role in the uptake and presentation of DEC205-targeted antigen. To verify if these cells were involved, CD11c+ and CD11c-cells were isolated from lymph nodes and spleens of immunized mice and then co-cultured with OT-I T cells in vitro. C57BL/6 mice were vaccinated with 10^9 viral particles of either Ad-DEC-OVA or Ad-GL117-OVA 1 day or 4 days before cells were isolated. As early as 1 day following injection of Ad-DEC-OVA, CD11c+ DCs that were isolated from the lymph nodes and that
Adenoviral vector targeting encoded antigens to DCs

with Ad-GL117-OVA could hardly stimulate OT-I cells in this assay. No antigen-specific proliferation could be observed in co-culture of CD11c+ and OT-I T cells (Supplementary Figure 2, available at International Immunology Online).

Influence of DC targeting on the humoral immune response

In order to determine the immunogenicity of adenoviral vector vaccines encoding DC-targeted antigens, female C57BL/6 mice received a low dose (10⁷ viral particles) or a high dose (10⁸ viral particles) of either Ad-DEC-OVA or Ad-GL117-OVA, respectively. Serum antibody levels were analyzed 2 and 5 weeks after immunization and characterized in IgG1- and IgG2c-specific ELISAs (Fig. 3). Targeting the antigen to DCs dramatically enhanced the IgG1 response, so that immunization with either dose of Ad-DEC-OVA induced significant IgG1 antibody levels as early as 2 weeks after vaccination, which could not be observed after immunization with Ad-GL117-OVA (Fig. 3A). Remarkably, Ad-DEC-OVA outperformed the non-targeted vaccine even at a 100-fold lower dose. Although the overall IgG1 responses were higher at 5 weeks after immunization, the ranking between the groups did not change (Fig. 3A).

In sharp contrast, the highest IgG2c antibody levels at both time points were detected in sera from mice which received the high dose of the non-targeted vaccine, Ad-GL117-OVA (Fig. 3B). At day 14, the antibody levels of these animals were significantly higher than the levels detected in control animals (Ad-GFP) or in animals vaccinated with the low dose of either vector. Nevertheless, the targeted vaccine also elicited antibody levels that were significantly different from the background. As observed for the IgG1 subtype, the IgG2c antibody levels were higher at the later time point without changing the differences between the groups. At this point, the high dose of Ad-GL117-OVA even elicited significantly higher antibody levels than the high dose of Ad-DEC-OVA. Both low dose regimens induced only marginal antibody levels (Fig. 3B).

The ratio of IgG2c/IgG1 in these ELISAs indicated that targeting of the antigen to DCs rather altered the predominant subtype than the magnitude of the antibody response. Thus, Ad-DEC-OVA immunization seemed to favorably induce IgG1 antibodies and the non-targeted antigen led to higher IgG2c responses (Fig. 3C). The strongest bias was observed for the low-dose immunization with Ad-DEC-OVA, which induced strong IgG1 but poor IgG2c responses.

Effect of DC targeting on the cellular immune response

Next, we immunized female C57BL/6 as described above and antigen-specific CD8+ T cells were monitored by tetramer and ICS. For the latter, splenocytes were restimulated with OVA-specific peptide (SIINFEKL) for 6 h and analyzed for the expression of the degranulation marker CD107a and IFN-γ production (26). This type of

stimulated the proliferation of OT-I T-cells at a DC-T cell ratio of 1:1. The capacity to stimulate T-cell proliferation increased, when DCs were isolated 4 days after immunization with the targeted antigen allowing T-cell proliferation to be observed at DC-T cell ratio of 1:3 with lymph node-derived CD11c+ cells and at a 1:1 ratio with spleen-derived DCs (Fig. 2C). In contrast, DCs isolated either 1 or 4 days after immunization

Fig. 2. In vivo antigen presentation and T-cell activation. (A) To assess the antigen presentation by DCs in vivo, C57BL/6 mice were immunized with different doses (10⁷, 10⁸, and 10⁹ viral particles) of either Ad-DEC-OVA or Ad-GL117-OVA s.c. in the foot pads. Four days after immunization, 2 × 10⁶ CFSE-labeled OT-I CD8+ T cells or OT-II CD4+ T cells were adoptively transferred and their proliferation capacity determined after 3 days by CFSE dilution. Analyzed were lymphocytes from spleen and pooled lymph nodes (popliteal and inguinal) after gating on CD45.2– donor T cells. Representative histograms of lymph node cells for the different groups are shown and percentages of proliferating cells are indicated for the marked region. (B) Mean values and standard error of the means (SEM) for three animals per group are shown for OT-I CD8+ T cells (left) and OT-II CD4+ T cells (right), respectively. Percentages of proliferating cells in the lymph nodes (white bars) and spleens (black bars) are indicated; data are out of two to three independent experiments. (C) Animals were immunized with 1 × 10⁸ viral particles of either Ad-DEC-OVA or Ad-GL117-OVA on days -4 and -1, respectively. CD11c+ cells were isolated from lymph nodes (popliteal and inguinal) and spleen from immunized animals and co-cultured in vitro with CFSE-labeled OT-I T cells for 3 days before the proliferation capacity was analyzed by CFSE dilution. Lymph node and spleen-derived CD11c+ cells were pooled from three individual mice and mixed with OT-I cells in different ratios (DC-T-cells: 1:1, 1:3, 1:9), where the total amount of DCs was adjusted by adding DCs from naive animals.
Fig. 3. Humoral immune responses. C57BL/6 mice were immunized with a low (10^7 viral particles) or high dose (10^9 viral particles) of either Ad-DEC-OVA or Ad-GL117-OVA s.c. in the foot pads. Negative control animals were immunized with 10^9 viral particles of Ad-GFP. Serum antibody levels of the subclasses IgG1 (A) and IgG2c (B) were measured in an OVA-antibody ELISA on the days 14 (white bars) and 35 (black bars) post-immunization. Serum dilutions used in this assay were 1:50. Each bar represents the mean and SEM of five animals per group. Log_{10} transformed relative light units (RLU) are shown. The IgG2c/IgG1 ratio of the log_{10}-transformed RLUs are shown as mean and SEM in (C). [*P < 0.05, **P < 0.01, ***P < 0.001; one-way analysis of variance (ANOVA), Tukey post-test].
antigen-specific T cells could be induced by immunization with the low dose of Ad-DEC-OVA, but not with the same dose of Ad-GL117-OVA (Fig. 4A). At the high dose, the mean percentage of CD107a+IFN-γ+/CD8+ T cells did not differ significantly after immunization with either the targeted or non-targeted vaccine (5.3% versus 3.8%) (Fig. 4B).

To address the polyfunctionality of these CD8+ T cells, we further analyzed the expression of the proliferation-inducing cytokine IL-2. In this study, the highest percentages of CD8+ cells positive for all three proteins were found in animals that received the high dose of Ad-GL117-OVA, although the percentages of IFN-γ-producing cells were higher in the Ad-DEC-OVA-treated animals (Fig. 4B). The pie charts indicate that only a minor fraction of OVA-specific T cells were able to produce IL-2 after immunization with the high dose of Ad-DEC-OVA (~3%), whereas this fraction was significantly higher after Ad-GL117-OVA treatment (~12%) (Fig. 4B). Interestingly, this observation was dose dependent because the low dose of Ad-DEC-OVA induced a functional T-cell repertoire similar to the one observed after delivering the high dose of the non-targeted vaccine. In that case, around 17% of all induced CD8+ T cells were positive for all three proteins. Furthermore, the T-cell activation by the low dose of Ad-GL117-OVA is negligible. This is supported by the observation that the small number of antigen-specific cells is dominated by monofunctional cells that show up-regulation of CD107a, but no IFN-γ production (Fig. 4A). Since the transfer studies revealed the highest T-cell activation in the lymph nodes, we also analyzed this population by ICS and could not find significant differences relative to responses detected in the spleen (data not shown).

Since IL-2 production is considered to be different between various subtypes of memory T cells, we analyzed the phenotypic expression of the early memory markers CD127 and CD62L on OVA-specific T cells detected by tetramer staining. For this analysis, animals were vaccinated with the high dose of either Ad-DEC-OVA or Ad-GL117-OVA to guarantee a substantial amount of tetramer-positive cells. In accordance with their lower capacity to produce IL-2, vaccine-induced OVA-specific CD8+ T cells displayed a more effector memory phenotype (TEM; CD127hiCD62Llow) if the antigen was targeted to DCs (~50% of all Tet+CD8+). In contrast, the CD8+ T-cell response after vaccination with Ad-GL117-OVA was dominated by effector T cells (TEFF; CD127loCD62Llo) (Fig. 5). Typically for adenoviral vector vaccines, both of the vaccines induced only marginal numbers of T cells of the central memory subset (T CM; CD127hiCD62Lhi), whereas it was still significantly higher for the non-targeted vaccine. Therefore, the phenotypic analysis supported the observation of functional

**Fig. 4.** Cellular immune responses. C57BL/6 mice were immunized with a low (10^7 viral particles) or high dose (10^9 viral particles) of either Ad-DEC-OVA (black bars) or Ad-GL117-OVA (white bars) s.c. in the foot pads. Negative control animals were immunized with 10^9 viral particles of Ad-GFP. Antigen-specific CD8+ T-cell responses were analyzed 14 days after the immunization by intracellular staining for the cytokines IFN-γ and IL-2. The degranulation marker CD107a was additionally analyzed to estimate the cytotoxic potential. The percentages of the different subpopulations among the total CD8+ cells are shown for the low dose (A) and the high dose (B). Mean values and SEM represent 7 mice per group out of two combined, independent experiments. The pie charts represent the relative contributions of single (1+), double (2+) or triple (3+) positive cells to the total amount of antigen-specific cells detected by ICS. (*P < 0.05, one-way ANOVA, Tukey post-test).
Vaccine-induced protection

Since we detected phenotypic and functional differences in the CD8^{+} T-cell responses, we addressed the impact of these alterations on the protection from a viral challenge.
these group lost less weight compared not only to the control (Ad-GFP) but also to the other vaccinated animals. This difference reached statistical significance compared with the Ad-GFP-treated animals as early as 3 days post-infection and to the other vaccinated animals at the days 5 and 6. Although the weight loss was most prominent in the Ad-GFP-treated animals, no other group reached a statistically significant improvement in regard to the disease progression (Fig. 6A). In accordance with the weight loss, the viral load in the lung was only significantly reduced in the animals that were immunized with the high dose of Ad-GL117-OVA resulting in 83% reduction compared with control animals. In this stringent challenge model, the differences in viral loads between the vaccinated groups did not reach statistical significance. Nevertheless, immunization with the low dose of Ad-GL117-OVA could reduce the viral burden by 46%, whereas immunization with Ad-DEC-OVA reduced the viral load by 53% at the low dose and 74% at the high dose. The protection was provided in an antigen-specific manner, since no impact on weight loss and viral replication was found after challenging the animals receiving the high dose of either Ad-DEC-OVA or Ad-GL117-OVA with a HIVgag-expressing vaccinia virus (Supplementary Figure 3, available at International Immunology Online). Furthermore, CD4+ cell depletion before challenging the mice did not alter the protective capacity of the vaccines indicating that CD8+ T cells were likely the main mediator of protection in this setup. Additionally, no reduction of viral loads was observed for the vaccinated animals after depletion of CD8+ T cells (Supplementary Figure 3, available at International Immunology Online).

To confirm the protective capacity of the vaccines in a second challenge model, B16-OVA melanoma cells were inoculated 5 weeks after the immunization and the tumor growth as well as the survival of the animals were documented (Fig. 7). As seen in the virus infection model, Ad-GL117-OVA at the high dose provided the best protection against tumor progression (Fig. 7A) and the mice showed prolonged survival after the challenge (Fig. 7C). In addition, the mean tumor volume of these animals at day 21, the day the last animal of the control group reached the end-point criteria, was significantly lower than in any other vaccine group (Fig. 7B). Interestingly, the two doses of the DC-targeted vaccine were comparably effective in protection from tumor growth, by which the animals of both groups had significantly smaller tumors than animals that had received the low dose of Ad-GL117-OVA and were completely unprotected (Fig. 7B). In accordance, both groups treated with Ad-DEC-OVA showed significantly prolonged survival (Fig. 7C). Taken together with the different CD8+ T-cell responses, the effects of the DC-targeting on the vaccine-induced immune response seemed to be dose dependent. The better protection by Ad-GL117-OVA might be also partially dependent on the higher IgG2a antibody levels, since the IgG2a levels correlated inversely with the tumor size (data not shown).

Discussion

Recombinant adenoviral vectors have emerged as promising candidate for the development of viral vector-based vaccines. The high immunogenicity of adenoviral vectors has
been demonstrated with a large variety of different antigens and comprised cellular as well as humoral immune responses against the encoded pathogenic protein (1–5). The central idea of using viral vectors is based on the endogenous antigen expression and the induction of cellular responses comparable of those induced by live-attenuated vaccines. Nevertheless, this approach failed to induce protection against HIV in a human clinical phase IIb study, although the vaccine recipients developed substantial CD8⁺ T-cell responses (6, 7). These findings demonstrate the necessity of further optimization of either the quantity or even the quality of the vaccine-induced T-cell response.

Since targeting the encoded antigen to DCs had improved the immunogenicity of DNA- or NDV-based vaccines (23, 28), we addressed the influence of DEC205 targeting in the context of adenoviral vectors. Using the adoptive transfer of TCR-transgenic cells, we could demonstrate that targeting the encoded OVA to DCs results in enhanced activation of antigen-specific CD4⁺ and CD8⁺ T cells indicated by stronger proliferative responses. This observation is most probably due to enhanced antigen presentation on MHC class I molecules by CD11c⁺ DCs. This is supported by the finding that CD11c⁺ cells, but not CD11c⁻ cells, isolated from draining lymph nodes of Ad-DEC-OVA-immunized mice had a higher capacity to stimulate the proliferation of OT-I T cells than cells isolated from Ad-GL117-OVA-treated animals. This is in line with a report of vaccination with plasmid DNA expressing DEC205-targeted OVA, which induced strong activation of transgenic CD8⁺ T cells even at very low concentrations (23). In the quoted study, the T-cell activation was mediated by CD11c⁺ cells and was abrogated when DEC205 (−/−) knockout mice or CD11c-DTR mice, in which the CD11c⁺ cells had been depleted, were used. Since we took advantage of the same expression cassette, it is unlikely that the uptake and the presentation will be highly different once the protein is synthesized and secreted by the transduced cell. Although higher doses are needed, Ad-DEC-OVA also induced stronger activation of transgenic CD4⁺ cells, which is in accordance with the effects observed for the DNA vaccine. Taken together, these results demonstrate that DC-targeting enhanced the capacity to stimulate transgenic T cells in vivo most probably due to enhanced antigen uptake and presentation via MHC class I and II by DEC205-CD11c⁺ cells.

Next, we had to analyze the immunogenicity of the two vectors in the context of a non-transgenic, naive immune status. Similar to other gene-based vaccine candidates, including DNA vaccines (22, 23) or recombinant NDV expressing DEC-targeted HIVgag (28), Ad-DEC-OVA induced elevated antibody levels compared with the non-targeted vector in this study. Maamary et al. (28) detected an increase in the amount

Fig. 7. B16-OVA melanoma challenge. C57BL/6 mice were immunized with a low (10⁷ viral particles) or high dose (10⁹ viral particles) of either Ad-DEC-OVA or Ad-GL117-OVA s.c. in the foot pads. Negative control animals were immunized with 10⁹ viral particles of Ad-GFP. On day 36, 2 × 10⁶ B16-OVA cells were inoculated in the right flank and the tumor volume was determined every 3 days. (A) The mean tumor volumes and the SEM are shown for each group over the first 21 days. The numbers indicate the number of animals that were alive at day 21 in relation to the starting number in this group. (B) A statistical analysis of the tumor volumes of the vaccinated groups was performed at day 21. (*P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA, Tukey post-test). (C) Survival of the animals were plotted as a Kaplan–Meier curve for each group. Mice were sacrificed when the volume reached 4000 mm³ or when there was any sign of ulceration of the tumor.
of total IgG using the DC-targeted antigen expressed by NDV, whereas Nchinda et al. (23) further analyzed the distribution of the subtypes and showed a dominant induction of IgG1 antibodies by the targeted vaccine. In our study, we also observed predominantly antibodies of the IgG1 subtype after immunization with Ad-DEC-OVA, whereas animals of the non-targeted group showed significantly higher IgG2c responses. This is in line with our previous results that immunization with adenoviral vectors preferentially leads to IgG2a/c responses (1, 26). Therefore, DC-targeting of the antigen does not only influence the quantity of the antibody response but more drastically altered their functionality, which depends on different Fc-receptor binding and activation by the different subclasses.

It was shown that IgG2a had a better ratio of binding to activating or inhibitory Fc-receptors than IgG1 in the mouse model, which was beneficial in regard to tumor clearance (29). Further evidence that DC targeting might alter the quality of the antibody response is presented in a recent non-human primate study, in which the authors used DC-targeted HIV protein followed by a recombinant New York vaccinia virus in a heterologous prime-boost immunization. Interestingly, the avidity of antibodies induced by DEC-targeted proteins seems to be lower than of the one induced by non-targeted proteins, although the overall antibody response was enhanced (30). This might be explained by DEC205-mediated uptake by all DEC205+ germinal center B cells and impairment of affinity maturation if these cells receive T cell help independent of the B-cell receptor (BCR) affinity (31). Since we confirmed binding of our fusion proteins to DC205+B220+ cells, it will be interesting to see in future experiments whether class switching might be influenced in a similar way.

Although CD8+ T-cell activation was significantly enhanced by DC targeting in the TCR-transgenic transfer model, this was only slightly reflected in naive C57BL/6 mice. In this study, the antigen-specific CD8+ T-cell response was only significantly enhanced by DC targeting if the low dose of both vectors was compared. The percentage of cytokine-producing, antigen-specific CD8+ T cells did not differ significantly after vaccination with the high dose. This seemed to be in accordance with findings from the DNA vaccine study, where the overall cellular response in the higher doses was not enhanced by DC-targeting, but a lower amount of DNA was needed to induce a CD8+ T-cell response, demonstrated by ICS (23). Interestingly, although both adenoviral vector vaccines induced comparable amounts of IFN-γ-producing CD8+ T cells, these differ significantly in their ability to produce IL-2. Since it was restricted to immunization with the high dose of Ad-DEC-OVA, this might display a kind of partial exhaustion of T cells primed by strong antigenic stimulation as it could be observed in models of chronic infections, e.g., lymphocytic choriomeningitis virus (32). Generally, T cells induced by adenoviral vectors had a phenotype of partial exhaustion indicated by low IL-2 production after antigenic re-stimulation (33), but here it was further enhanced by DC targeting. Further characteristics of adenovirus-induced CD8+ T cells are low expression levels of CD62L and CD127 (33), two marker molecules that allow an early distinction between CD8+ memory populations (34, 35). In this study, we confirmed the majority of CD8+ T cells after immunization with Ad-GL117-OVA to be of effector (T_{eff}) or effector memory (T_{em}) phenotype and only a very minor part shows characteristics of central memory (T_{cm}) cells. Interestingly, the DC targeting seems to have an influence on the differentiation into distinct CD8+ sub-population indicated by significant differences in the ratio of T_{eff}, T_{em} and T_{cm}. In contrast to the non-targeted antigen, Ad-DEC-OVA mainly induced CD8+ T cells of the T_{em} subtype and lower numbers of T_{eff} and central memory T cells, which is in accordance with their different ability to produce IL-2 (35, 36). In correlation with the induction of higher numbers of T_{eff} cells, Ad-GL117-OVA treatment mediated better protection against an antigen-expressing vaccinia virus challenge than the immunization with the DEC205-targeted antigen. This lineage relationship was described in an earlier study, where antigen-specific CD8+ T cells were separated according to their expression of CD62L and CD127 and then transferred into naive mice, which were then challenged with a vaccinia virus. It was demonstrated that (CD62L/CD127+) CD8+ T cells were superior in protecting against the vaccinia challenge (36). The stronger protective capacity of the CD8+ T cells induced by the non-targeted vaccine was confirmed in the OVA-specific tumor challenge. Additionally, the CD8+ T-cell responses induced by the low dose of Ad-DEC-OVA could also provide partial protection against disease progression in this model, supporting the hypothesis of a dose-dependent benefit of DEC205 targeting. These functional differences in the T-cell responses evoked by DC targeting were not reported for the DNA or NDV vaccine, where in both cases the immunogenicity and efficacy were enhanced by DC targeting. Both studies describe rather quantitative than qualitative changes in the immune response (23, 28). Although all three approaches delivered the antigen by the same scAb to DCs, the strong T-cell activation observed after DC-targeted adenoviral vector immunization did not result in protective responses. As mentioned previously, this might be due to stronger T-cell exhaustion as a consequence of ‘over-activation’ by antigen-presenting T cells, as described in chronic infections. Nevertheless, the efficient T-cell activation after low dose vaccination with adenoviral vectors encoding DEC205-targeted antigens might be an interesting approach for vaccination protocols usually resulting in low antigen expression levels, like atroscopic oral spray immunization (37). However, co-stimulatory signals may be needed to avoid the risk of inducing T-cell exhaustion rather than effector and memory T cells.

**Supplementary data**

Supplementary data are available at International Immunology Online.

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