Potent anti-tumor immunity generated by a CD40-targeted adenoviral vaccine

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Abstract

In situ delivery of tumor-associated antigen (TAA) genes into dendritic cells (DC) has great potential as a generally applicable tumor vaccination approach. Although adenoviruses (Ad) are an attractive vaccine vehicle in this regard, Ad-mediated transduction of DC is hampered by the lack of expression of the Ad receptor CAR on the DC cell surface. DC activation also requires interaction of CD40 with its ligand CD40L to generate protective T cell-mediated tumor immunity. Therefore, to create a strategy to target Ad to DC in vivo, we constructed a bispecific adaptor molecule with the CAR ectodomain linked to the CD40L extracellular domain via a trimerization motif (CFm40L). By targeting Ad to CD40 using CFm40L, we enhanced both transduction and maturation of cultured bone marrow-derived DCs. Moreover, we improved transduction efficiency of DCs in lymph node (LN) and splenic cell suspensions in vitro and in skin and vaccination site-draining LNs in vivo. Furthermore, CD40 targeting improved the induction of specific CD8+ T cells along with therapeutic efficacy in a mouse model of melanoma. Taken together, our findings support the use of CD40-targeted Ad vectors encoding full-length TAA for in vivo targeting of DC and high-efficacy induction of anti-tumor immunity.
Introduction

Melanoma vaccines based on ex vivo prepared autologous Dendritic Cells (DC) have been reported to induce specific T cell responses which in some cases were associated with improved survival (1-3). In spite of its first clinical successes, serious draw-backs are inherent to this approach. The ex vivo culture and antigen-loading of DC according to cGMP guidelines and on a per-patient basis are laborious, time-consuming, and introduce inter-donor variability that may complicate the interpretation of clinical outcome. Furthermore, ex vivo modified DC migrate poorly in vivo, affecting the efficacy of the vaccine (4). In vivo targeting of tumor-associated antigens (TAA) and/or activating agents to DC presents an attractive alternative (5). The approaches used for in vivo targeting of antigens to DC range from viral or liposomal targeting to the conjugation of antigens directly to DC-binding ligands or mAbs (5, 6), (7-12). Targeting of DC receptors such as DC-SIGN (13), DEC-205 (8) and CD11c (9) required the incorporation of additional DC maturation stimuli such as CD40L or Toll-Like Receptor-Ligands (TLR-L) to induce potent anti-tumor immune responses. Alternatively, DC-targeting motifs that simultaneously effect maturation induction may be used, such as the TLR4-binding Extra Domain A of fibronectin (14) or CD40L. Indeed, our in vitro and in situ studies have shown that the genetic targeting of antigens to the CD40 receptor efficiently and selectively delivers antigens to both human and murine DC and simultaneously induces their stable maturation, obviating the need for additional maturation stimuli for proper T cell activation (15-17). Genetic targeting of TAA ensures durable presentation through MHC-I to specific cytotoxic T lymphocytes (CTL) (18). Adenoviruses (Ad) are attractive vaccine vehicles as they are easily grown to high titers, exhibit low cytotoxicity, have a large cloning capacity, and display high-efficiency gene transfer, even to non-dividing target cells (19, 20). Moreover, modification of the capsid hexon motif has been shown to abolish the neutralizing effects of pre-existent humoral immunity to the commonly used Ad type-5 (Ad5) vector, thus
widening its therapeutic window and opening the possibility to use it for both priming and boosting (21). Unfortunately, DC are relatively resistant to infection with Ad5 due to their lack of expression of the primary Ad5 receptor, Coxsackie-Adenovirus Receptor (CAR) (22). We previously showed that this limitation could be overcome by re-targeting Ad5 to CD40 using bispecific adaptor molecules that simultaneously neutralized the Ad5 fiber knob and agonistically bound CD40 on the DC surface (16, 23). This approach facilitated the induction of T cell-mediated immune responses against non-self transgene products (17, 24) and protected mice from tumor growth through vaccination with ex vivo modified DC (17). However, the capacity of this approach to induce effective immune responses against poorly immunogenic TAA by targeted delivery to DC in vivo, being its most promising application, has not yet been evaluated.

Melanoma antigens such as tyrosinase-related proteins 1 and 2 (TRP-1/-2] and gp100, are non-mutated auto-antigens (25, 26) to which T cell tolerance prevails (27), eliciting only low-avidity CD8\(^+\) T cells due to thymic or extrathymic deletion of high-avidity autoreactive T cell clones (28). Targeted in vivo delivery of TAA to DC may break this tolerance (5). Moreover, agonistic binding of CD40 has been pinpointed as a vital mode of DC maturation to facilitate the induction of TAA-specific CTL (29), thus providing a clear rationale for CD40-mediated DC targeting of Ad-based tumor vaccines. Here, we show that in vivo delivery through the skin of a CD40-targeted Ad5 vector leads to selective in vivo transduction of DC in the skin and injection site-draining lymph nodes (LN), resulting in increased CD8\(^+\) T cell responsiveness and enhanced therapeutic efficacy in the murine B16F10 melanoma model.
Materials and Methods

Animals and cell lines

Five- to eight-week old C57Bl/6 mice (H-2K^b) (Harlan Netherlands B.V.) were used for this study which was approved by the institutional experimental animal ethics committee. The murine melanoma cell line B16F10 (H-2K^b), kindly provided to us in 2003 by Dr. John Haanen, Netherlands Cancer Institute, The Netherlands) was maintained in IMDM (BioWhittaker) supplemented with 10% heat inactivated fetal calf serum (Hyclone Laboratories), 100 I.E./ml sodium penicillin, 100 μg/ml streptomycin sulfate, 2mM L glutamine, and 0.01 mM 2-mercaptoethanol, i.e., complete medium. TRP2 expression in B16F10 cells was confirmed on western blot in 2008, prior to its use in the here described experiments; at that time its characteristic ability to form melanotic lung metastases was also confirmed, as well as prevention of their outgrowth by vaccination with an adenovirus encoding the H-2K^b-restricted TRP2_{aa180-188} epitope (see below).

Peptides

The H-2K^b-binding peptides SVYDFFVWL (TRP2_{aa180-188}, derived from the murine melanosomal protein TRP2), ICPMYARV (βgal_{aa497-504}, derived from Escherichia coli β-galactosidase) and the H2-D^b-binding peptide KVPRNQDWL (gp100_{25-33} derived from the human melanosomal protein gp100 (30) were purchased from Leiden University Medical Center, The Netherlands.

Ad vectors

The following replication defective, E1-substituted and E3-deleted Ad5 vectors were used: 1) Ad-GFP, encoding enhanced green fluorescent protein (eGFP) (31), 2) Ad-gp100, encoding full-length human gp100 (32), and 3) Ad-GFP-TRP2_{aa180-188}, encoding the
imunodominant H-2K^b-binding epitope TRP2_{aa180-188} fused to eGFP, under the control of the cytomegalovirus (CMV) promoter (33). These Ad vectors were propagated, titered and stored as described previously (34).

**Recombinant CFm40L adaptor protein**

A recombinant molecular adaptor protein consisting of the soluble ectodomain of CAR linked to the ectodomain of mouse CD40 ligand via a trimerization motif (CFm40L) was constructed, produced and purified as described previously (23). CD40-Ad-GFP-TRP2_{aa180-188}, CD40-Ad-gp100 or CD40-Ad-GFP (i.e. CD40-targeted Ad) were prepared by pre-incubating 3.125\mu g of CFm40L with 60 \times 10^6 viral particles (vp) (600vp/cell) of Ad-GFP-TRP2_{aa180-188}, Ad-gp100 or Ad-GFP for 30 min in 10-20\mu l of serum free medium at room temperature (RT).

**Bone marrow-derived DC, skin DC and Ad infections**

Bone Marrow-derived DC (BMDC) were generated as described previously by Samsom et al. (35) and were used for Ad infections. Murine ears were collected from sacrificed C57BL/6 mice and washed in 70% alcohol. The dorsal and ventral halves of the ears were separated mechanically and dorsal halves were cultured in 24-well plates in RPMI complete medium for 48 h at 37°C. Migrated DC were harvested and used for Ad infections as described below.

DC were seeded at a density of 0.5 \times 10^6 cells/well in a 24-well plate in 500\mu l serum free medium and were transduced with CD40-targeted- or untargeted-Ad-GFP or Ad-GFP-TRP2_{aa180-188} at 37°C for 2 h at a multiplicity of infection (MOI) of 600 based on vp. After 2 h of incubation, 2 ml of serum containing IMDM was added. Transduced DC were harvested after 48h, washed repeatedly and used for either flow cytometric analysis or vaccination.
Vaccination and vaccination site excision

Mice were vaccinated as indicated with Ad-transduced BMDC (0.5x10^6/mice), CD40-targeted- or untargeted-Ad (dose:10x10^6 iu/mouse of Ad-GFP-TRP2_{aa180-188} [vp/iu=51] or 1x10^9 iu/mouse/vaccination of Ad-gp100 [vp/iu=31]). Vaccines were suspended in sterile PBS in a total volume of 50μl and were injected via the intradermal route (i.d.) on the flank using a MicroFine insulin syringe with a 29G needle (BD Biosciences). In some experiments, the vaccination site was excised at 4h post i.d. vaccination and the excision wound was closed using surgical staples and glue (3M Nederland BV, Netherlands).

IFN-γ intracellular staining

The IFN-γ intracellular staining (ICS) assay was carried out to detect TRP2_{aa180-188} and gp100_{25-33} specific T cells in mouse PBMC or splenocytes, as described previously (34).

Isolation and Ad transduction of murine LN cells and splenocytes

LN and spleen were harvested from mice and single cell suspensions were made by chopping the tissues with scissors and gently pressing them through 100μm filters (BD Biosciences). Finally, the LN and spleen cells were washed twice in serum free medium, counted, and seeded at a density of 1x10^6 cells/well of 24-well plates in 500μl serum free medium. Cells were infected with Ad-GFP or CD40-Ad-GFP at an MOI of 300 (vp, calculated based on the DC numbers) for 2h at 37°C. After infection, 2 ml of IMDM complete medium was added and the cells were cultured for 2 days. Subsequently, cells were harvested, washed, labeled with PE-labeled anti-MHC II (eBioscience) and/or anti-B220, and/or APC-labeled anti-CD11c and appropriate isotype control antibodies (BD Biosciences), and subjected to flowcytometric analysis.
**In vitro antigen presentation assay**

BMDC or DC in LN suspensions were transduced with CD40-targeted- or untargeted-Ad vectors encoding GFP or TRP2<sub>aa180-188</sub> fused to GFP. Transgene-expressing DC were plated in round-bottom 96 well plates (Greiner Bio-one) with splenocytes containing previously primed TRP2<sub>aa180-188</sub>-specific CD8<sup>+</sup> T cells, isolated from mice vaccinated with Ad-GFP-TRP2<sub>aa180-188</sub>, at a ratio of 1:10. Splenocytes stimulated with DC loaded with TRP2<sub>aa180-188</sub> or βgal<sub>aa497-504</sub> peptides were used as controls. After overnight stimulation, the percentage of CD8<sup>+</sup> T cell producing intracellular IFN-γ was determined by flow cytometry as described previously (34).

**Imaging and quantification of transgene expression in vivo**

Transgenic GFP expression levels at the site of vaccination and in vaccination site draining LN were measured 24h post-injection of Ad vectors, using a IVIS lumina II bioluminescent imager (Xenogen). Images and measurements of transgene expression were captured for 10 seconds and analyzed using Living Image software (Xenogen). The GFP expression was imaged and digitally displayed as a pseudocolor overlay on the gray scale image of the animal or excised LN. Regions of interest (ROI) were drawn around the transgene expression site digitally on the displayed images and quantified as photons/sec/cm²/sr.

**Tumor inoculation and measurements**

Syngeneic B16F10 cells (3x10<sup>5</sup> suspended in 50µl PBS) were implanted subcutaneously on the flank. Twice a week, two bisecting diameters of each tumor were measured with a digital slide calliper until the end of the experiment. The reported tumor volumes were calculated using the formula (0.4)x(\(a\)^2), with "a" as the larger and "b" as the
smaller diameter (36). When tumors reached the maximal allowable volume according to Dutch national regulations, mice were sacrificed.

_Tissue fixation and immunofluorescence microscopy_

Skin biopsies from the site of vaccination and the vaccination site draining LN (axillary, identified by injection of Evans Blue dye [Sigma]) were fixed in 4% paraformaldehyde and 10% sucrose in PBS at 4°C for 30 min (37). Fixed tissues were embedded in Tissue-Tec OCT (Sakura Fineteck, The Netherlands) above liquid nitrogen. 10 μm-Cryosections were cut from frozen tissue blocks and mounted on poly-L-lysine-coated slides. Slides were dried at RT overnight, fixed in acetone at 4°C for 10 min and then stored at -20°C until use.

Slides were brought to RT and placed in PBS for 5 min to remove OCT; endogenous biotin was blocked (DAKO Corporation) and slides were stained with biotinylated anti-IA/IE or anti-CD11c mAbs (BD Pharmingen). Specific binding of the antibody was detected using Cy3-conjugated streptavidine (Invitrogen). DAPI (Molecular probes) was used to stain nuclei and slides were mounted using mounting medium (Immunoconcepts NA Ltd). Staining was evaluated by fluorescence microscopy (Nikon instruments BV, The Netherlands) using appropriate filters.

_Statistical analysis_

Data was analyzed using one-way ANOVA with Bonferroni correction using GraphPad-Prism software.
Results

Enhanced in vitro transduction and maturation of BMDC by CD40-Ad and enhanced prophylactic vaccination efficacy

BMDC were more efficiently transduced by CD40-Ad than by untargeted-Ad as determined by GFP transgene expression (Fig. 1A) and simultaneously underwent maturation as determined by expression levels of the DC maturation markers MHC-II and CD86 (Fig. 1B). To compare the anti-tumor efficacy of CD40-Ad and untargeted-Ad, we used the aggressive and poorly immunogenic murine B16F10 melanoma model (38). Mice were vaccinated with syngeneic BMDC transduced in vitro by CD40-targeted- or untargeted-Ad-GFP-TRP2aa180-188. Animals were subsequently inoculated s.c. with B16F10 cells on the distant flank (see Fig. 1C for a schedule of the experiment). BMDC transduced by CD40-Ad-GFP-TRP2aa180-188 exhibited superior vaccination efficacy over BMDC transduced with Ad-GFP-TRP2aa180-188 (Fig. 1D,E) and induced stronger TRP2aa180-188-specific CD8+ T cell responses in peripheral blood (Fig. 1D).

Enhanced DC transduction and MHC-I mediated presentation of transgenic antigens through CD40-targeted transduction of DC in LN suspensions

To ascertain the ability of CD40-Ad to target and transduce tissue-derived DC, we performed in vitro experiments with skin-derived DC as well as with LN and spleen suspensions. Although transduction levels were low, CD40-mediated Ad infection significantly improved the transduction efficiency of CD11c+ DC migrated from murine ear skin explants (Fig. 2A). As CD40 expression is not exclusively restricted to DC, we evaluated the tropism of CD40-Ad in single-cell suspensions of murine LN and spleen. CD40-Ad very efficiently and
selectively transduced DC, identified as CD11c and MHC II double positive cells, but not B220+ B cells, in both LN and spleen single-cell suspensions (Fig. 2A).

To test whether DC in LN suspensions, transduced by CD40-Ad, presented endogenously synthesized and processed transgene-encoded antigens more efficiently than untargeted-Ad-transduced DC, we assessed the \textit{in vitro} antigen presentation efficiency of LN samples transduced either by CD40-targeted- or untargeted-Ad-GFP-TRP2\textsubscript{aa180-188}. Data presented in Fig. 2B show that LNDC transduction with a CD40-Ad vector encoding TRP2\textsubscript{aa180-188} and GFP led to the activation of higher rates of specific IFN-γ producing CD8+ T cells in TRP2\textsubscript{aa180-188}-primed splenocytes than transduction with the untargeted vector, although this did not reach significance, likely due to background reactivity e.g. against GFP.

\textit{CD40 targeting of Ad in vivo dramatically reduces total transgene expression levels but results in more selective DC transduction}

Re-targeting Ad to CD40 may reduce undue toxicity of Ad by blocking the naturally broad tropism of Ad vectors. To this end we assessed the \textit{in vivo} GFP transgene expression at the site of vaccination and in excised draining LN, using bioluminescent imaging. Total transgene expression levels were significantly lower at the site of vaccination in mice injected with CD40-Ad compared to untargeted-Ad at 24h post-injection (Fig. 3A,B). Immunohistochemical and fluorescence microscopic examination revealed sparse transduction of DC at the skin injection site (Fig. 3C). Comparable reduction in total transgene expression levels (Fig. 4A,B) but more selective transduction of DC was also observed in the vaccination site-draining LN (Fig. 4C). This indicates a drastic reduction in the number of transduced cells resulting from an effective block of the natural tropism of the
Ad vector by the CFm40L retargeting adaptor protein. In contrast, injection of untargeted-Ad led to massive transduction of larger cells, both in skin and in LN, which, by their morphological features, their lack of CD11c expression, and their localization, most likely represented fibroblasts and/or macrophages (see Sfig. 1A). Note that assessment of co-localization of MHC class II and low-level GFP expression in the skin (as a measure of DC transduction) was further complicated by autofluorescence of hair follicles and their close proximity to surrounding DC (exemplified in Sfig. 1B; hair follicles indicated by asterisks in Fig. 3). Although rare and at low levels, and thus hard to demonstrate immunohistochemically, the targeted transduction of cutaneous DC by CD40-Ad was supported by surgical removal of the dermal vaccination site 4h post-vaccination with CD40-targeted or untargeted-Ad-GFP-TRP2<sub>aa180-188</sub>. This surgical intervention almost completely abrogated the priming of TRP2<sub>aa180-188</sub>-specific CD8<sup>+</sup> T cells (Sfig. 2A) as well as anti-tumor efficacy (Sfig. 2B) in mice vaccinated with CD40-Ad-GFP-TRP2<sub>aa180-188</sub>, but not in mice vaccinated with untargeted-Ad-GFP-TRP2<sub>aa180-188</sub>. This indicates rapid and selective transduction of cutaneous DC by CD40-targeted Ad vectors, followed by their migration to draining LN to prime an effective anti-tumor response. In contrast, untargeted-Ad vectors may depend more on later cross-priming events and/or transduction of DC in the LN for their anti-tumor efficacy.

Intradermally delivered untargeted-Ad vectors preferentially transduced large cells in the marginal LN sinuses, most likely representing macrophages (see Sfig. 1A). To confirm the uptake of untargeted-Ad vectors by macrophages, regional macrophages were depleted by injection of clodronate-containing liposomes prior to vaccination of mice with the CD40-targeted- or untargeted-Ad-GFP-TRP2<sub>aa180-188</sub> vector. Specific T cell responses in peripheral blood were significantly increased by local depletion of macrophages for the untargeted, but not the CD40-targeted vector (Sfig. 2C). This indicates that CD40 targeting of Ad prevents scavenging by macrophages that might otherwise interfere with subsequent T cell activation.
Nevertheless, even in the presence of Ad-binding macrophages, small numbers of DC may be transduced by untargeted Ad and thus effect direct CTL priming.

_**CD40-targeted intradermal delivery significantly improves the anti-tumor efficacy of an Ad5 vector encoding a full-length weakly immunogenic tumor antigen**_

We next tested the anti-tumor efficacy of intradermally injected Ad vaccines. Direct _in vivo_ delivery of untargeted-Ad-GFP-TRP2_{aa180-188} induced potent TRP2_{aa180-188} specific CD8\(^+\) T cell responses and conferred prophylactic protection against tumor outgrowth, neither of which was improved by CD40 retargeting of the employed Ad5 vector (Fig. 5A-C). These results indicate that i.d. delivered Ad vectors encoding an epitope of a self antigen linked to an immunogenic non-self protein (in this case GFP) can break tolerance for this epitope and induce potent cell-mediated anti-tumor immunity, relying on cross-priming without further requirement of redirected targeting to professional antigen-presenting cells (APC). Of note, this was further supported by the observation that the anti-tumor efficacy of the i.d. delivered untargeted-Ad vector was superior over that afforded by _ex vivo_ Ad-transduced and i.d. injected BMDC (Fig. 1D). To assess the effect of CD40 targeting on the induction of immunity against weakly immunogenic TAA, subsequent experiments (see Fig. 6A for the followed experimental schedule) were conducted with an Ad vector encoding a full-length TAA of clinical relevance, i.e. gp100. Importantly, CD40 targeting of Ad-gp100 significantly enhanced the induction of a gp100\(_{25-33}\) specific CD8\(^+\) T cell response (Fig. 6B) and accordingly also enhanced the anti-tumor efficacy, both prophylactically (not shown) and, more importantly, therapeutically (Fig. 6C). This also translated into an improved survival of tumor-bearing animals receiving a CD40-Ad-gp100 vaccine (Fig. 6D). These results thus
clearly demonstrate the enhanced anti-tumor efficacy afforded by CD40-mediated *in vivo* targeting to DC of Ad vaccines encoding weakly immunogenic full-length TAA.
Discussion

Direct \textit{in vivo} delivery of full-length melanoma antigen genes such as TRP2 or gp100 offers a standardized off-the-shelf vaccination approach, but does not induce protective immunity even with strongly immunogenic viral vehicles (30, 39-42). We therefore investigated the suitability of CD40-targeted Ad vectors to selectively deliver open reading frames encoding a Trp2-derived epitope or full-length gp100 to DC \textit{in vivo}, and evaluated their therapeutic efficacy in the murine B16F10 melanoma model. Our results demonstrate that the direct \textit{in vivo} administration of CD40-Ad significantly blocks the otherwise predominant transduction of non-APC and leads to more selective transduction of CD11c\(^+\) DC in skin and draining LN. Consequently, a significantly improved therapeutic anti-tumor efficacy was observed for an intradermally delivered Ad-gp100 vaccine through CD40 targeting.

The efficacy of \textit{ex vivo} loaded DC, which are often used as cancer vaccines (43), may be improved upon by transduction with CD40-Ad (17, 44). This approach is however wrought with issues concerning logistics and consistency; \textit{in vivo} targeting of antigens to DC would instead allow for a more standardized vaccination methodology (5). Targeting proteins or antigenic peptides to cutaneous DC \textit{in vivo} through their conjugation to antibodies against e.g. DEC-205, DCIR2, or MHC-II has led to T cell anergy and the induction of Tregs, unless CpG or agonistic anti-CD40 were admixed to ensure long-term memory and effective anti-tumor responses (8) (45). The CD40 receptor represents an attractive DC-targeting motif in itself, as it plays an important role in the induction of DC maturation and priming of cytotoxic T cells (46). Indeed, we previously reported the selective \textit{in situ} transduction of DC and the simultaneous enhancement of their (tumor) antigen-specific CD8\(^+\) T cell stimulatory capacity by CD40-Ad in a human skin explant model (15) and in human melanoma-draining lymph node suspensions (47). Such demonstrations of selective DC transduction in more complex tissue microenvironments are important as CD40 expression is not exclusively restricted to
DC but is also found in macrophages, B cells and epithelial cells, which are poorly equipped to prime an effective CTL response. DC in single-cell suspensions of murine LN and spleen were also selectively and more efficiently transduced by CD40-Ad than by untargeted-Ad, and displayed enhanced presentation of the transferred gene products in the context of MHC class I. Similarly, CD46- or CD80/CD86-targeted Ad vectors have been reported to selectively transduce DC despite high expression levels of these receptors on e.g. monocytes and B cells (48). These differences in infection susceptibility may at least in part be attributed to the presence or absence of active endocytic pathways (49).

Our *in vivo* bioluminescent imaging data clearly showed that *in vivo* retargeting of Ad to CD40 almost completely ablated its natural tropism in both murine skin and skin-draining LN. Re-targeting may thus reduce unwanted cytopathic side effects associated with the clinical use of Ad vectors. This reduction in overall transgenic antigen load was accompanied by a more selective delivery of the transgene to CD11c⁺ DC in both skin and draining LN. Moreover, removal of the skin vaccination site after 4h effectively blocked the induction of specific CD8⁺ T cells and reduced associated anti-tumor efficacy, implying a vital role for cutaneous DC transduction by CD40-Ad in this regard.

We observed a consistent association between the level of CD8⁺ T cell activation and anti-tumor efficacy. Indeed, previous studies with CD40-Ad-transduced DC vaccines demonstrated a complete dependence of the observed anti-tumor efficacy on CD8⁺ T cells and less so on CD4⁺ T cells, presumably due to the CD40-mediated activation of the transduced DC which might have substituted in part for the natural role of Th cells in this regard. Consistent with this notion, CD40-targeted dermal delivery of full-length human gp100, which is 80% homologous to murine gp100 at the amino acid level (42), afforded a clear advantage both in terms of CD8⁺ T cell priming and of prophylactic or therapeutic anti-tumor efficacy. The high homology of human gp100 to its murine counterpart may not have allowed
for the presence of non-self Th epitopes supporting Th activation and subsequent DC activation and CD8\(^+\) T cell priming. As such, it represented a relevant model for the generation of an anti-tumor response against weakly immunogenic TAA and clearly demonstrated the superior efficacy afforded by \textit{in vivo} CD40-targeted Ad vaccines under such conditions of prevailing self tolerance. These findings underline the advantage of collateral DC activation by CD40-targeted delivery of TAA. Indeed, even though additional DC activation stimuli were not included in the present study, our findings are comparable with those reported by Johnson \textit{et al.}(8), who repeatedly vaccinated B16F10 tumor-bearing mice with the gp100 antigen fused to a single-chain Fv specific for DEC-205 combined with TLR-L to ensure DC activation.

As the induced melanoma specific immune response by CD40-Ad delayed rather than abolished tumor growth, we tested whether the anti-tumor efficacy of CD40-Ad could be further improved by interference with T cell suppression, either by blocking the inhibitory receptor cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or by depleting suppressive regulatory T cells (Tregs) using anti-CD25. While single administration of either anti-CTLA4 or anti-CD25 delayed tumor growth, neither Treg depletion prior to vaccination, nor CTLA-4 blockade during vaccination, further enhanced the anti-tumor efficacy of CD40-Ad (see SFig. 3A,B). These observations suggest that CD40-Ad transduction activates DC to such a level that they override the immunosuppressive effects of CTLA4 and/or Tregs on the induction of autoreactive CD8\(^-\) T cells and are in keeping with our recently reported finding of decreased Treg rates and enhanced expansion of melanoma-specific CD8\(^+\) T cells from human melanoma draining LN suspensions transduced by CD40-Ad-MART-1 (47).

In summary, the present data show that CD40-targeted Ad vectors can be used to efficiently and selectively deliver TAA to DC \textit{in vivo} and to enhance their TAA-specific CD8\(^+\) T cell stimulatory capacity. Most importantly, CD40 targeting significantly improved
the therapeutic anti-tumor efficacy of an Ad vector encoding full-length gp100 in the B16F10 melanoma model. Combined with our previous observations in human skin explant and LN models, these data make a strong case for the clinical development of CD40-Ad vectors for the \textit{in vivo} targeting and transduction of cutaneous DC for cancer immunotherapy.

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References


Legends to the figures

Figure 1. Targeting Ad to CD40 enhances \textit{in vitro} transduction and maturation of Bone Marrow Derived DC (BMDC) and enhances their prophylactic vaccination efficacy. A) The percentages of CD11c$^+$ BMDC expressing the transgene GFP and B) mean fluorescence intensity of DC maturation markers MHC II and CD86 after transduction with Ad-GFP alone or complexed to CFm40L (CD40-Ad-GFP) are shown. Untransduced BMDC (medium) were included as a negative control. Means±SEM (n=3) C) Mice were vaccinated i.d. with BMDC transduced \textit{ex vivo} with Ad-GFP-TRP2$_{aa180-188}$, CD40-Ad-GFP-TRP2$_{aa180-188}$, Ad-GFP or CD40-Ad-GFP (0.5x10$^6$DC/mice). On day 0 TRP2$_{aa180-188}$ specific CD8$^+$ T cells in peripheral blood were measured and B16F10 melanoma cells were injected s.c. on the flank. Tumor volumes were measured on the indicated days. D) Percentage IFN-$\gamma$ producing CD8$^+$ T cells in peripheral blood and E) kinetics of B16F10 tumor growth are shown. The results are representative of two independent experiments with n=6/group. Means±SEM (n=6). *P<0.05, **P<0.01, ***P<0.001.

Figure 2. Targeting Ad to CD40 selectively enhances the transduction of skin, Lymph Node (LN) and splenic DC and improves MHC-I mediated presentation of transgenic antigens. (A) Percentages of CD11c$^+$ skin-emigrated DC, and MHC II$^+$ and CD11c$^+$ DC and B220$^+$ B cells in LN and spleen single-cell suspensions, expressing GFP after \textit{in vitro} transduction with Ad-GFP or CD40-Ad-GFP. B) LNDC were transduced \textit{in vitro} with Ad-GFP-TRP2$_{aa180-188}$ or CD40-Ad-GFP-TRP2$_{aa180-188}$ and used to stimulate splenocytes from Ad-GFP-TRP2$_{aa180-188}$ immunized mice to compare antigen presentation ability. LNDC pulsed with either TRP2$_{aa180-188}$ or $\beta$gal$_{aa497-504}$ were included as positive and negative control, respectively. The percentage IFN-$\gamma$-producing cells within CD8$^+$ cells was determined by flowcytometry after intracellular
staining. Mean±SEM are shown from three independent experiments. *P<0.05, **P<0.01, ***P<0.001.

Figure 3. CD40 targeting of Ad drastically reduces total transgene expression levels in the skin. A) GFP expression in the skin of mice i.d. injected with PBS, Ad-GFP (10x10^9 iu/mouse, vp/iu=11) or CD40-Ad-GFP. Gray scale mouse images and pseudo color GFP signals were overlaid. B) Quantified skin GFP expression in photons/second/cm^2/steradian (p/s/cm^2/sr). **P<0.01. C) Skin biopsies from the Ad injection site were analyzed by immunofluorescent staining. Transduced cells were detected by GFP (green) and dendritic cells by anti-MHC II (red). Cell nuclei were visualized using DAPI (blue) (magnification 400x). Double positive DC and autofluorescent hair follicles are marked with arrow heads and asterisks, respectively. Representative data of three independent experiments (n=2-3/group) is shown.

Figure 4. CD40 targeting of Ad in vivo significantly reduces transgene expression levels but results in more selective DC transduction in the vaccination site draining lymph nodes (LN). A) GFP transgene expression was measured in the vaccination site draining auxiliary LN using a bioluminescence imager at 24h post intradermal administration of Ad-GFP or CD40-Ad-GFP (10x10^9 iu/mouse). B) Quantified GFP signals in the LN in p/s/cm^2/sr. ***P<0.001. C) Transduced cells in the LN were detected by GFP expression (green) and DC by anti-CD11c (red). Cell nuclei were visualized using DAPI (blue, magnification 400x). Double positive DC are marked with arrow heads. Representative data of three independent experiments (n=2-3/group) is shown.

Figure 5. Direct in vivo delivery of Ad-GFP-TRP2 aa180-188 induces potent TRP2 aa180-188 specific CD8^+ T cell responses and confers protection against B16F10 tumor growth, neither of which is improved upon by CD40 retargeting. A) Experimental scheme; 3x10^5 B16F10
cells were injected s.c. on the flank. Tumor volumes were measured on the indicated days. B) IFN-γ producing CD8+ T cells in blood and C) kinetics of tumor growth in mice vaccinated with Ad-GFP-TRP2aa180-188, CD40-Ad-GFP-TRP2aa180-188, Ad-GFP or CD40-Ad-GFP (10x10^6 iu/mice) are shown. The results are representative of three independent experiments with n=6 per group. Means±SEM (n=6). *P<0.05.

Figure 6. CD40-targeted delivery of Ad5-gp100 enhances its therapeutic efficacy. A) B16F10 melanoma cells (3x10^5/mouse) were injected s.c. on the flank. Mice were vaccinated on the distant flank with CFm40L, Ad-GFP, Ad-gp100 or CD40-Ad-gp100 (i.d., 1x10^9 iu/mouse) once tumors of 3mm diameter were established; booster vaccine was given on day 9. Blood gp100 specific CD8+ T cells were measured on d.14. B) IFN-γ producing CD8+ T cells in peripheral blood were determined by flowcytometry after in vitro stimulation with gp10025-33 peptide. C) Growth of B16.F10 melanomas over time and D) the percentages of surviving mice are shown. Results are representative of two independent experiments with 4-6 mice per group. Means±SEM (n=6). *P<0.05, **P<0.01, ***P<0.001.
Figure 2

Skin DC

<table>
<thead>
<tr>
<th>Condition</th>
<th>% of GFP⁺ / CD11c⁺ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40-Ad.GFP</td>
<td>5⁺</td>
</tr>
<tr>
<td>Ad.GFP</td>
<td>0⁺</td>
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<tr>
<td>Medium</td>
<td>0⁺</td>
</tr>
<tr>
<td>GFP⁺ of MHC⁺ cells</td>
<td>25⁺</td>
</tr>
<tr>
<td>GFP⁺ of B220⁺ cells</td>
<td>15⁺</td>
</tr>
<tr>
<td>GFP⁺ of MHC⁺CD11c⁺ cells</td>
<td>15⁺</td>
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<tr>
<td><strong>NS</strong></td>
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Lymph node cells

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<th>Condition</th>
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<tr>
<td>CD40-Ad.GFP</td>
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<tr>
<td>Ad.GFP</td>
<td>15⁺</td>
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<tr>
<td>Medium</td>
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Splenocytes

<table>
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<th>Condition</th>
<th>%IFN-γ⁺/CD8⁺ cells</th>
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<td>Ad.GFP</td>
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<tr>
<td>LNDC</td>
<td>0.25</td>
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</tbody>
</table>

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 4

A

Ad-GFP
CD40-Ad-GFP
PBS

B

Avg Radiance (p/s/cm²/lan)

PBS
Ad-GFP
CD40-Ad-GFP

***

C

Ad-GFP

CD11c

GFP

CD40-Ad-GFP
Figure 5

A

Blood for IFN-γ
+ 3*10^5 B16F10 injection s.c

Day -14 -7 0 3 7 9 11 15
Vaccinations

Tumor monitoring

B

%IFN-γ+ within CD8+ T cells

Ad-GFP
CD40-Ad-GFP
CD40-Ad-GFP-TRP2
CD40-Ad-GFP-TRP2

C

Tumor volume (mm^3)

Days post tumor inoculation

Ad-GFP
CD40-Ad-GFP
Ad-GFP-TRP2
CD40-Ad-GFP-TRP2
Figure 6

A

3^10^5 B16F10 injection s.c

Visible tumor

Tumor monitoring

Day

0

3

6

9

12

15

18

20

Vaccinations

Blood for IFN-γ

C

Tumor volume (mm^3)

1000

800

600

400

200

0

Ad-GFP

CFm40L

Ad-gp100

CD40-Ad-gp100

Days post tumor inoculation

D

Percent survival

100

75

50

25

0

Ad-GFP

CFm40L

Ad-gp100

CD40-Ad-gp100

Days post tumor inoculation
Potent anti-tumor immunity generated by a CD40-targeted adenoviral vaccine

Basav N Hangalapura, Dinja Oosterhoff, Jan de Groot, et al.

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