A Hexon and Fiber-modified Adenovirus Expressing CD40L Improves the Antigen Presentation Capacity of Dendritic Cells

Lina Liljenfeldt, Di Yu, Liye Chen, Magnus Essand, and Sara M. Mangsbo

Summary: CD40 ligand (CD40L), a strong stimulator of Th1 immune responses, acts via dendritic cells to trigger T-cell activation. AdCD40L therapy introduces the *CD40L* gene into the tumor microenvironment with an adenoviral vector and has shown promising results in experimental tumor models, dogs, and patients (phase I-II trials). The transduction efficiency of AdCD40L is dependent on the expression of CAR (coxsackie/adenovirus adhesion receptor), which is commonly downregulated on tumor cells. To enhance transduction efficiency, and therefore the therapeutic efficacy, a double-modified adenovirus was developed. The double-modified Ad5PTDf35(*mCD40L*) had a protein transduction domain (PTD) inserted into the hexon protein and the virus fiber is switched from serotype 5 to serotype 35. These modifications enable transduction of a wider range of cell types. In comparison with Ad5(*mCD40L*), Ad5PTDf35(*mCD40L*) showed increased transduction capacity on a variety of murine cells. Furthermore, antigen presentation was improved after transduction with Ad5PTDf35(*mCD40L*). This was demonstrated in an antigen presentation assay, both in vitro and in vivo, in which transduced dendritic cells were loaded with suboptimal concentrations of the human gp100 peptide and allowed to interact with gp100-specific transgenic T cells (pmel). Finally, Ad5PTDf35(*mCD40L*) could delay tumor growth in a murine cancer model at a particle load, wherein therapeutic efficacy of the Ad5(*mCD40L*) vector was lost. Hence, the Ad5PTDf35(*CD40L*) vector holds great promise as a second-generation immune stimulatory therapy, as it not only targets tumor cells but also antigen-presenting cells that are, among other cells, present in the tumor microenvironment.

Key Words: CD40, CD40L, PTD, adenovirus, local immunotherapy, antigen presentation

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CD40L (CD154) is expressed on activated T cells and promotes dendritic cell (DC) activation by triggering the CD40 receptor and is therefore a strong stimulator of Th1 immune responses. In *CD40L* gene therapy, the *CD40L* gene is introduced into the tumor microenvironment with an adenoviral vector of serotype 5 (Ad5), and we have over the past years demonstrated promising results in experimental tumor models,[1–4] dogs with melanoma[5,6] and in bladder cancer patients (phase I-II trial).[7] For efficient Ad5 transduction, high expression of coxsackievirus-adenovirus receptor (CAR) is required on the target cells. CAR enables viral attachment and infection and has been demonstrated to be a component of tight junctions[8]; however, the cellular function of CAR remains largely unknown. Unfortunately, CAR is often downregulated on human tumor cells.[9–12] Transduction of DCs with Ad5 is limited and the expression of CAR on DCs is nonexisting.[13]

We have recently showed an increased transduction capacity on a wide spectrum of human primary cell types with an Ad5PTDf35 vector, including DCs.[14] This new vector has the protein transduction domain (PTD) from the HIV-1 Tat protein inserted into the hexon variable region 5 (HVR5) of the virus capsid and the fiber is switched from serotype 5 to serotype 35. In this study, we compare the Ad5(*mCD40L*) vector with this new double-modified adenovirus vector and demonstrate increased transduction efficiency on a variety of murine cell types. Locally delivered double-modified AdCD40L therapy has the possibility to transduce all cell types in the tumor microenvironment, including DCs. DCs are excellent at presenting antigen to T cells and an antitumor response is dependent of this. We therefore investigated the ability of transduced DCs to prime gp100-specific Tg T cells in vitro and in vivo. In both assays we demonstrate an improvement in antigen presentation with increasing transduction efficiency. Furthermore, the double-modified vector could prolong the survival of tumor-bearing animals at a particle load where the Ad5(*mCD40L*) vector had no therapeutic effect.

MATERIALS AND METHODS

Cell Lines and Culture

The mouse bladder carcinoma cell line MB49 and the mouse melanoma cell line B16-F10 are derived from C57BL/6 mice and were kind gifts from Dr K. Esuvaranathan (National University Hospital, Singapore) and Dr A. Dimberg (Uppsala University, Sweden), respectively. MB49 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.1% sodium pyruvate. B16-F10 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% sodium pyruvate. The D1 cell line[15] is a growth factor-dependent immature splenic mouse DC line derived from C57BL/6 mice and was cultured in IMDM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% β-mercaptoethanol, and 20 ng/mL recombinant murine GM-CSF (Nordic Biosite; Stockholm, Sweden).

Splenocytes from C57BL/6 mice were cultured in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, 1% HEPES, 1% β-mercaptoethanol, and 0.1% sodium pyruvate. Medium and supplements were purchased from Life Technologies (Paisley, UK).

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Bone marrow DCs (BMDCs) were obtained from the femur and tibia bone of male wild-type (wt) C57BL/6 and *CD40*$^{-/-}$ mice from The Jackson Laboratory (B6.129P2-CD40^{tm1Kik}/J) by exposing the bone marrow and flushing out the cells with a sterile syringe. Bone marrow cells were passed through a cell strainer (BD Biosciences, Franklin Lakes, NJ) and plated on nontreated Petri dishes. After 3 days of culture, the medium was refreshed and after additional 3 days the nonadherent DCs were harvested and transferred to tissue culture dishes for culture for 2 days before further experiments. The BMDCs were cultured in IMDM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% HEPES, 50 μM β-mercaptoethanol, and 20 ng/mL recombinant murine GM-CSF. All cells were cultured at 37°C and 5% CO_2 and were free from mycoplasma.

Production of Recombinant Adenovirus

The Ad5(*mCD40L*) and Ad5(Mock) vectors were constructed and produced as previously described.[3] Briefly, 2 replication-deficient, E1/E3-deleted, human adenoviruses type 5 were produced using the Ad-Easy system. The Ad5(*mCD40L*) virus encodes the murine CD40L molecule, and Ad5(Mock) is a noncoding control virus. The construction of the double-modified vectors Ad5PTDf35(*mCD40L*) and Ad5PTDf35(Mock) are based on the Ad-Easy system by recombination of pSh(*mCD40L*) and pSh(Mock) with pAd5PTDf35(ΔE3), which has recently been described.[14] Adenoviruses were produced by 4 rounds of infection of 911 cells, purified by a discontinuous cesium chloride (CsCl) gradient and dialyzed against a buffer containing 10 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, and 4% sucrose. As the viruses are modified, the infectious virus titer will be dependent on the cell line chosen for titration. To avoid this, the virus titers for all viruses were determined by quantitative polymerase chain reaction, by establishing encapsidated virus genomes (evg) as described earlier.[16]

Transduction of Murine Cells

Different concentrations of virus, from 625 evg/cell to 20,000 evg/cell, were tested on different murine cells. Forty-eight hours after transduction the cells were stained for CD40L (clone MR1; Biolegend, San Diego, CA) and the percentage of CD40L-positive cells was evaluated by flow cytometry (FACSCanto II; BD Biosciences). Data analysis was performed using FlowJo software (Treestar Inc., Ashland, OR).

Antigen Presentation Assay In Vitro

D1 cells were transduced with 20,000 evg/cell, and 24 hours after transduction the cells were washed and the short H-2D^b restricted hgp100 peptide, KVPRNQDWL, (Proimmune, UK) was added at 0.025 ng/mL to 25,000 D1 cells/well and incubated for 2 hours before being washed 3–4 times; 100,000 CFSE-labeled (Life Technologies) pmel-1 splenocytes were added to the D1 cells. Thy1.1$^+$ CD8$^+$ T cells from a pmel-1 mouse carry a TCR that recognizes the hgp100 peptide in H-2D^b. The cells were incubated for 72 hours, supernatant was saved, and the cells were stained for the markers Thy1.1 and CD8 (antibodies from Biolegend). Percentage of proliferating cells was evaluated by flow cytometry (FACSCanto II; BD Biosciences). Data analysis was performed using FlowJo software (Treestar Inc.). Supernatant from the cells were used in a mouse interferon (IFN)-γ enzyme-linked immunosorbent assay (ELISA), wherein the procedure was carried out according to the manufacturer's protocol (Biolegend).

IL-12p40 ELISA on BMDCs and D1 Cells

Supernatant was collected from transduced and untransduced D1 cells and BMDCs. The supernatant was analyzed for its concentration of IL-12p40 by coating 96-well plates with an IL-12p40 antibody (clone C15.6; Biolegend) at a concentration of 1 μg/mL. After an overnight coating and washing of the plates the samples were added, as well as an IL-12p40 standard titration curve (Biolegend) was derived. The plates were incubated and washed before a biotinylated antibody anti-IL-12p40 (clone C17.8; Biolegend) was added at a concentration of 1 μg/mL. After incubation and washing, Avidin/HPR (Dako, Glostrup, Denmark) was added to the wells. After the subsequent incubation and additional washes, TMB (Dako) was added. Finally, the reaction was stopped using 1M H_2SO_4, and the absorbance was read at 450 nm (Emax precision microplate reader, Molecular devices).

Antigen Presentation Assay In Vivo

6×10^6 D1 cells were transduced with different vectors at a concentration of 20,000 evg/cell and 24 hours later the D1 cells were loaded with 1 μg/mL hgp100 peptide for 24 hours, followed by 2 washes. 10×10^6 splenocytes from pmel-1 mice (approximately 15% CD8$^+$ cells) were injected intravenously 24 hours after D1 transduction and the D1 cells (6.4×10^5 cells/mouse) were injected intravenously 24 hours after the splenocytes injection. After additional 72 hours the mice were killed and spleen and inguinal lymph nodes were analyzed for Thy1.1, Vβ-13, CD8, and CD3.

Animal Models

Female C57BL/6 mice were obtained from Taconic M&B (Bomholt, Denmark). 250,000 MB49 cells in PBS were injected subcutaneously, and the mice received 3 local intratumoral treatments of Ad5(*mCD40L*), Ad5(Mock), Ad5PTDf35(*mCD40L*), or Ad5PTDf35(Mock) (5×10^9 evg) on day 7, 10, and 13 after tumor instillation. Tumors were measured continuously throughout the experiments and the tumor size was calculated by the ellipsoid volume formula:

$$\text{Volume} = 4/3 \times \pi \times \text{r(length)} \times \text{r(width)} \times \text{r(depth)}.$$

Mice were killed if the tumor exceeded 1 cm^3 or if ulcers developed.

The transgenic pmel-1 mice are kept in breeding by our group and were originally obtained from the Jackson Laboratory (Maine). Pmel-1 mice are on a C57BL/6 background and express a TCR specific for the human melanoma gp100 peptide (epitope 25-33) presented on MHC class I, H-2D^b.[17] The T cells also recognize the corresponding murine gp100 epitope.

Female C57BL/6 mice from Taconic M&B (Bomholt) were injected intradermally with 1×10^8 evg and 15 μg CFSE in a total volume of 20 μL in the right flank under anesthesia with isoflurane. Eighty-four hours after injection, the draining inguinal lymph node and the nondraining inguinal lymph node was analyzed for migrated CFSE$^+$ cells within the DC and macrophage population (CD11c^{high} and CD11b$^+$ cells, respectively) by flow cytometry. In addition, inguinal lymph nodes were imaged by 2-photon microscopy (Zeiss 710; Carl Zeiss, Germany). Lymph nodes

were fixated in 4% PFA and CFSE $^+$ migrated cells were 2-photon excited at 780 nm and visualized in green and collagen by second harmonic generation, visualized here as blue.

All animal experiments were approved by the regional ethical committee in Uppsala, Sweden, Dnr: C148/12 and C215/12 with the addition of N170/13.

Statistical Analyses

All statistical analyses were performed using the Graph Pad Prism 5.0 (Graph Pad Software Inc., San Diego, CA). The Mann-Whitney test was used for comparison of the Ad5(*mCD40L*) and the Ad5PTDf35(*mCD40L*) vectors. The Log-rank test was used to compare the Kaplan-Maier survival curves. *P*-values < 0.05 were considered significant (see figure legend texts for more information).

RESULTS

Increased Transduction Efficacy With Double-modified Vector

With *CD40L* gene therapy we introduce the *CD40L* gene into the tumor microenvironment, and a good therapeutic effect is partly dependent on transduction efficacy and also on the subsequent transgene expression. As the transduction efficiency of Ad5 vectors can be limited and recent papers have demonstrated increased transduction ability with modified adenoviruses, we wanted to evaluate if transduction could be improved on murine cell types as a means to improve the subsequent immune activation. To start with, 4 different adenoviral vectors were compared side by side at different concentrations (evg/cell). The 4 vectors that were evaluated were: a regular adenovirus of serotype 5 (Ad5), an adenovirus of serotype 5 in which the fiber was switched to one of serotype 35 (Ad5f35), an adenovirus of serotype 5 with the insertion of a PTD motif into the hexons (Ad5PTD), and finally an adenovirus of serotype 5 with both the fiber switch into serotype 35 and the insertion of the PTD motif (Ad5PTDf35). All vectors carry the *green fluorescent protein (GFP)* transgene and GFP expression was evaluated 48 hours after transduction. The transduction efficacy was greatly improved with the double-modified virus on both the murine bladder cancer cell line (MB49) (Fig. 1A) and a growth factor-dependent murine DC line (D1 cells) (Fig. 1C), whereas the transduction efficacy of the 4 viruses were equal on the murine melanoma cell line (B16-F10) in the highest concentration but improved somewhat for the lower concentrations with the double-modified vector (Fig. 1B). To ensure that the viruses did not kill the DCs, a death marker was added to the transduction test on the D1 cells. The amount of dead cells was below 10% for all viruses at each concentration and was determined as tolerable (Fig. 1D).

Improved Expression of CD40L With Double-modified Vector

A double-modified vector carrying the gene for *murine CD40L* was created [Ad5PTDf35(*mCD40L*)] as well as an empty double-modified vector was created as control [Ad5PTDf35(Mock)] (Fig. 2). The transduction rate of these new vectors was compared with the adenovirus serotype 5 vectors that have been used in our group for over several years [Ad5(*mCD40L*) and Ad5(Mock)]. The expression of CD40L significantly improved with the double-modified virus on MB49 cells, D1 cells, and splenocytes (Figs. 3A, C, D). Both CD40L-expressing viruses showed good transduction rate of the B16-F10 cell line (Fig. 3B). Transduction efficacy of murine BMDCs derived from wt and *CD40*$^{-/-}$ mice was also improved compared with the Ad5(*mCD40L*) vector (Fig. 3E), and expression of CD86 was enhanced on *CD40L*-transduced BMDCs irrespective of transgene expression (data not shown). Supernatant from the transduced BMDCs was collected, and the concentration of IL-12p40 was found to be highly elevated in the supernatant from the Ad5PTDf35(*mCD40L*)-transduced wt BMDCs (Fig. 3F) but not in *CD40*$^{-/-}$ BMDC cultures.

Improved Antigen Presentation by Ad5PTDf35(*mCD40L*)-transduced DCs

With local administration of *CD40L* gene therapy, the vector, if equipped with a broad cell tropism, should in theory be able to infect any cell type it encounters, including DCs. Therefore, the ability of transduced D1 cells to present antigen to T cells was investigated in an antigen presentation assay. Both transduced (mock or transgene) and untransduced D1 cells were loaded with the short H-2D^b restricted hgp100 peptide at a suboptimal peptide concentration and allowed to interact with CFSE-labeled splenocytes from a pmel-1 mouse. The proliferation of T cells was determined by flow cytometry after 72 hours. The proliferation of the T cells encountering Ad5PTDf35(*mCD40L*)-transduced D1 cells increased significantly compared with the Ad5(*mCD40L*) transduced cells at both peptide concentrations (Fig. 4A). Supernatant from the cocultures were collected and analyzed for IFN-γ and the concentration was significantly increased in the cultures in which D1 cells had been transduced with Ad5PTDf35(*mCD40L*) (Fig. 4B). The supernatant from transduced D1 cells was also analyzed for the concentration of IL-12p40, and a significant increase in IL-12p40 could be found in the supernatant of Ad5PTDf35(*mCD40L*)-transduced D1 cells (Fig. 4C).

An antigen presentation assay was also performed in vivo, in which splenocytes from a pmel-1 mouse were injected intravenously into a wt C57BL/6 mouse, followed by an intravenous injection of hgp100 peptide-loaded, transduced, or untransduced D1 cells. Four days later the spleen and inguinal lymph nodes were collected and the percentage of hgp100-specific T cells was determined in the CD8 $^+$ T-cell population. In both spleen and lymph nodes the highest percentage of antigen-specific T cells was found in the animals who had been injected with Ad5PTDf35(*mCD40L*)-transduced D1 cells (Fig. 4D).

Migration of Transduced Cells to Draining Lymph Node

To study the ability of transduced cells to migrate to lymph nodes we injected virus together with CFSE intradermally in the right flank and after 84 hours the draining inguinal lymph nodes were analyzed for migrated CD11c^{high} and CD11b $^+$ cells. A slight increase in migrated cells could be noticed within both cell populations when comparing Ad5PTDf35(Mock) and Ad5PTDf35(*mCD40L*) (Figs. 5A, B). Images of whole inguinal lymph nodes were obtained with a 2-photon microscopy, which further could demonstrate migration of CFSE $^+$ cells when injected with the immunostimulatory *CD40L* gene (Fig. 5D), in comparison with a Mock vector (Fig. 5C). However, in this experiment, the percentage of migrated cells was similar for

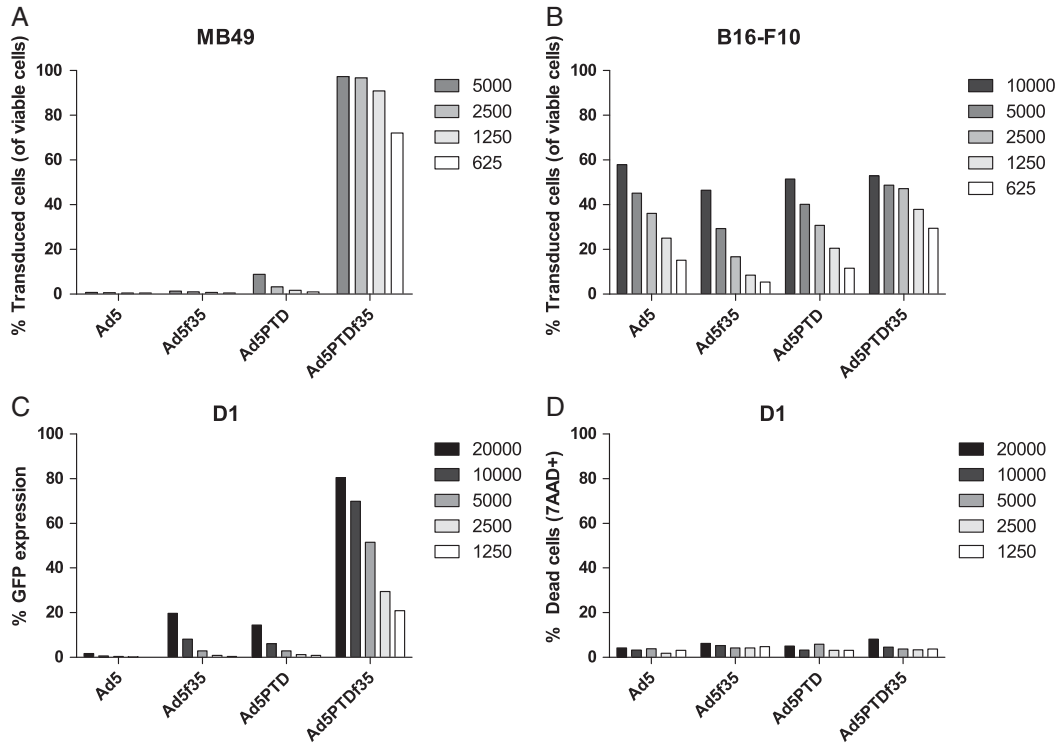

FIGURE 1. Enhanced transduction efficacy with double-modified vector. The transduction efficacy of 4 different GFP expressing viruses was evaluated on (A) MB49 cells, (B) B16-F10 cells, and (C) D1 cells at different concentrations (evg/cell). D, A dead cell marker (7AAD) was added to the D1 cells to determine the percentage of dead cells at different virus concentrations.

the Ad5(*mCD40L*) and the Ad5PTDf35(*mCD40L*) virus (data not shown).

Delayed Tumor Growth With Double-modified Vector

The Ad5(*mCD40L*) vector was compared with the Ad5PTDf35(*mCD40L*) vector with respect to in vivo anti-tumor efficacy. This was performed in our bladder cancer model where the Ad5(*mCD40L*) vector has previously been assessed. Three intratumorally delivered injections were given with 3 days apart at a concentration of 5×10^9 evg/mice, a dose approximately 10 times lower than we have assessed earlier with the unmodified virus. The Ad5PTDf35(*mCD40L*) vector was able to inhibit tumor growth (Fig. 5A). Survival of the animals was prolonged with the Ad5PTDf35(*mCD40L*) vector, in comparison with Ad5(*mCD40L*) ($P = 0.0031$) and Ad5(Mock) ($P < 0.01$) (Fig. 5B).

DISCUSSION

Local therapy with AdCD40L is dependent on a good transduction efficacy to get an efficient expression of CD40L in the tumor microenvironment. The interaction of CD40L with CD40 on DCs causes an activation and maturation of the DCs, leading to antigen presentation and an induction of Th1 immune responses.[18,19] An expression of CD40L in the tumor microenvironment can also cause

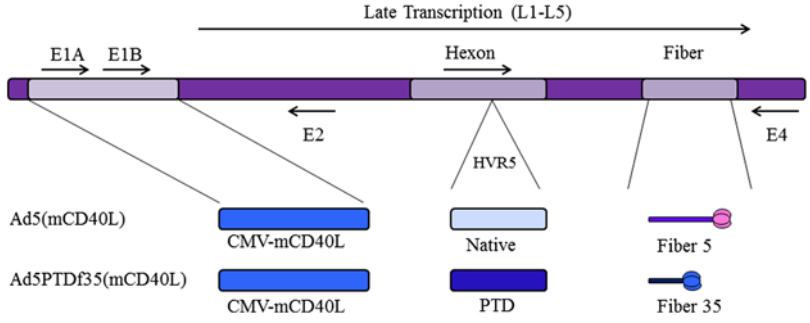

FIGURE 2. Schematic illustration of Ad5(*mCD40L*) and Ad5PTDf35(*mCD40L*). Both vectors are replication deficient and the mCD40L expression is controlled by the CMV promoter. The Ad5(*mCD40L*) is native, whereas the Ad5PTDf35(*mCD40L*) vector has a protein transduction domain (PTD) from the HIV-1 Tat protein inserted into the hexon variable region 5 (HVR5) of the virus capsid and the fiber is switched from serotype 5 to serotype 35.

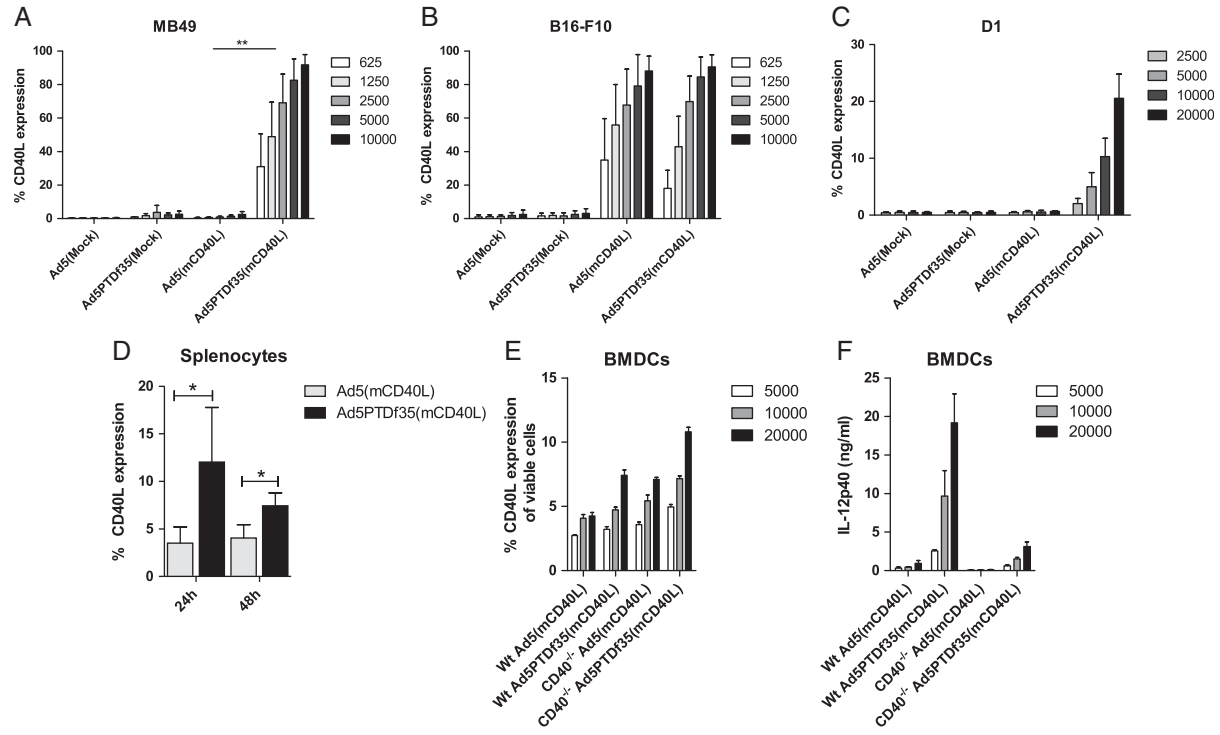

FIGURE 3. Enhanced mCD40L expression on murine cell types with Ad5PTDf35(*mCD40L*). Comparison of our well-used Ad5(*mCD40L*) versus the double-modified vector [Ad5PTDf35(*mCD40L*)], both carrying the murine *CD40L* gene. Mock viruses of each kind were used as controls. CD40L expression was determined 48 hours after transduction on (A) MB49 cells, (B) B16-F10 cells, and (C) D1 cells at different concentrations (evg/cell). D, Splenocytes, activated with αCD3, αCD28, and IL-2 were transduced with 20,000 evg/cell of either Ad5(*mCD40L*) or Ad5PTDf35(*mCD40L*) and CD40L expression was evaluated at 24 and 48 hours. E, BMDCs from wt and *CD40KO*$^{-/-}$ mice were transduced with either Ad5(*mCD40L*) or Ad5PTDf35(*mCD40L*) at 20,000, 10,000, or 5000 evg/cell and CD40L expression was evaluated after 48 hours. F, The supernatant of transduced cells in (E) where evaluated for their IL-12p40 secretion by enzyme-linked immunosorbent assay (ELISA). $*P<0.05$, $**P<0.01$ with the Mann-Whitney test.

apoptosis of CD40$^+$ tumors[20] with subsequent antigen release allowing for T-cell priming. However, Ad5 displays a limited cell tropism. To determine if a different vector could be beneficial for delivery of an immunostimulatory gene we compared our previously used Ad5 vector with 3 modified vectors using GFP expression as read-out. The vector showing the greatest transduction efficacy was a double-modified vector of serotype 5, wherein the fiber was switched to a fiber from adenovirus serotype 35 and with an insertion of PTD into the hexon hyper variable region 5 of the virus capsid. We have previously demonstrated that this double-modified vector increases transduction efficacy on a number of human primary cells and that it can efficiently be used to deliver antigens into human DCs.[14] All nucleated human cells express CD46, which is the primary receptor for an adenovirus with fiber from serotype 35; however, the expression of CD46 in mouse is limited to the testis[21] and can therefore not be the way of entry into the cell. Our hypothesis for the high transduction efficiency of the double-modified virus is that the shorter fiber (f35) facilitates the PTD motif to come closer to the cell and therefore can enable an entry of the virus. This is supported by the fact that neither of the single-modified viruses had an improved transduction efficiency on the tested cell lines. Thus, a double-modified vector carrying the murine *CD40L* was created, Ad5PTDf35(*mCD40L*), and a comparison of this new vector with Ad5(*mCD40L*) was carried out on different murine cells, demonstrating an increased, or equally good, transgene expression by all evaluated murine cells, including splenocytes, murine BMDCs, and D1 cells. We know from previous results that human DCs that are transduced to express CD40L upregulate their secretion of IL-12.[22] Herein, we demonstrate that Ad5PTDf35(*mCD40L*) transduced BMDCs and D1 cells increased their IL-12p40 secretion, which is an indication of maturation and activation. As the modification of the virus leads to increased transgene expression, but not increased cell death, we wished to establish the role of the transgene expression for immune activation. The viral transduction increased CD86 expression on a nontransgene-related manner as CD86 expression on CD40L-expressing BMDCs was similar on wt and *CD40*$^{-/-}$ BMDCs. However, increased IL-12p40 secretion was dependent on CD40L/CD40 interaction demonstrating that the virus could be armed to increase its immunostimulatory capacity and that immune activation was not only due to viral encounter.

In addition, an antigen presentation assay showed that DCs transduced with Ad5PTDf35(*mCD40L*) and loaded with the short hgp100 peptide significantly increased the amount of proliferating T cells carrying a TCR specific for hgp100, both in vitro and in vivo. Although only $<20\%$ of the D1 cells expressed CD40L 48 hours after transduction, the CD40L-expressing cells can interact with neighboring, untransduced cells and induce maturation. Of note,

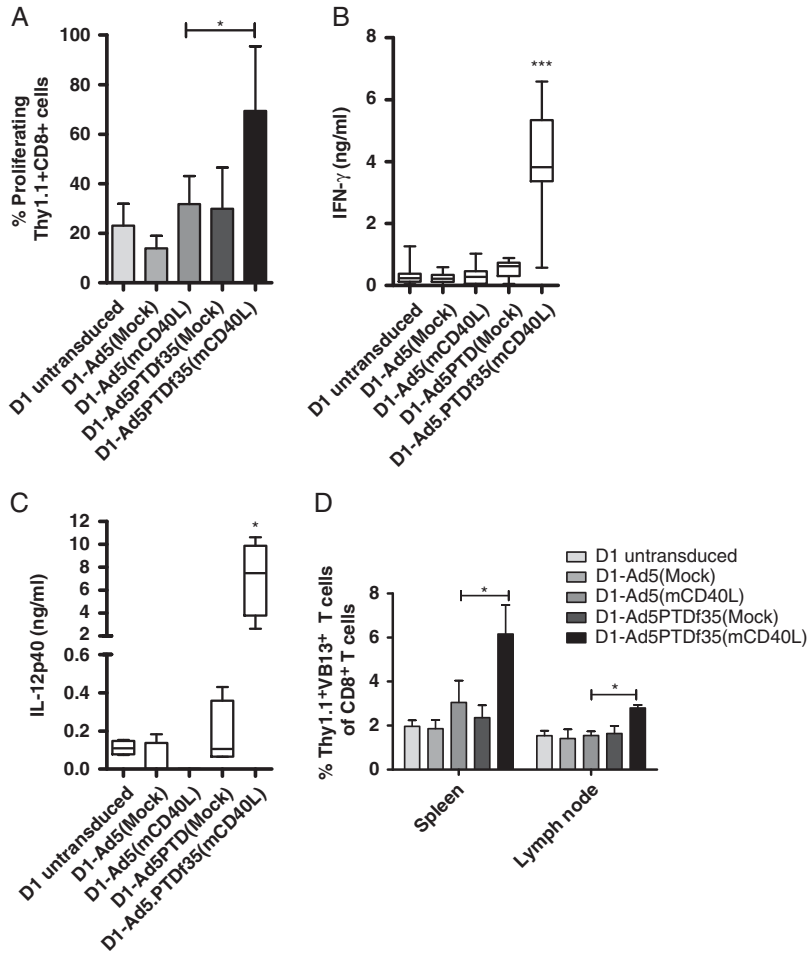

FIGURE 4. Improved antigen presentation on [Ad5PTDf35(*mCD40L*)] transduced DCs. Antigen presentation assay in vitro. A, The percentage of proliferating gp100-specific T cells after interaction with transduced [Ad5(Mock), Ad5(*mCD40L*), Ad5PTDf35(Mock) or Ad5PTDf35(*mCD40L*)] or untransduced, hgp100 loaded, D1 cells. The peptide was added at a concentration of 0.025 ng/mL and residual peptides were washed of before adding the T cells. B, Supernatant from the cocultures was collected and analyzed for IFN-γ. C, IL-12p40 enzyme-linked immunosorbent assay (ELISA) on supernatant from transduced and untransduced D1 cells. Supernatant was collected 48 hours after transduction. D, Antigen presentation assay in vivo. Splenocytes from pmel-1 mice were intravenously injected into wt C57BL/6 mice and after 24 hours transduced and hgp100 peptide-loaded D1 cells were intravenously injected. Four days later the animals were killed, and spleen and lymph nodes were collected and analyzed for the percentage of hgp100-specific T cells of the $CD8^+$ T-cell population. $*P<0.05$, $***P<0.001$ with the Mann-Whitney test.

transduction of human immature monocyte-derived DCs or monocytes with double-modified virus containing *GFP* as a reporter gene demonstrates close to 80% transgene expression with as low as 1000 evg/cell.[14] This indicates that the double-modified vector could be beneficial in the expansion of tumor-specific T cells by transducing feeder cells.

Previous studies with intratumoral injections of *CD40L*-transduced DCs have demonstrated tumor regression in murine models.[23,24] We believe that a CD40L-expressing virus that efficiently can target antigen-presenting cells will have the ability to affect the lymph node activity when intratumorally transduced APCs migrate to the lymph node with engulfed tumor antigens and a transgene expression. Subsequently, this will lead to improved T-cell priming and a more efficient antitumor response. In an in vivo migration assay we could show that CFSE-stained $CD11c^{high}$ and $CD11b^+$ cells did migrate to a lymph node after injection of CD40L-expressing virus, although no benefit could be demonstrated for the double-modified virus.

To assess antitumor responses using the double-modified vector expressing CD40L, we applied our previously used experimental bladder cancer model and compared therapeutic efficacy to the Ad5(*mCD40L*) virus. This was performed with lower viral particle load compared with our previous published data, to be able to perform the comparison. The double-modified virus delayed tumor growth and prolonged overall survival of mice and was superior to the Ad5(*mCD40L*) virus at the same particle load.

In conclusion, the Ad5PTDf35(*mCD40L*) vector holds great promise as a second-generation immune stimulatory therapy as it not only targets tumor cells but also improves antigen presentation by DC transduction. The virus could either be used to efficiently "arm" in vitro-cultured DCs or feeder cells or it could be used locally in the tumor microenvironment to deliver both antigens as well as immune stimulants and thereby increase the immunogenicity of a tumor.

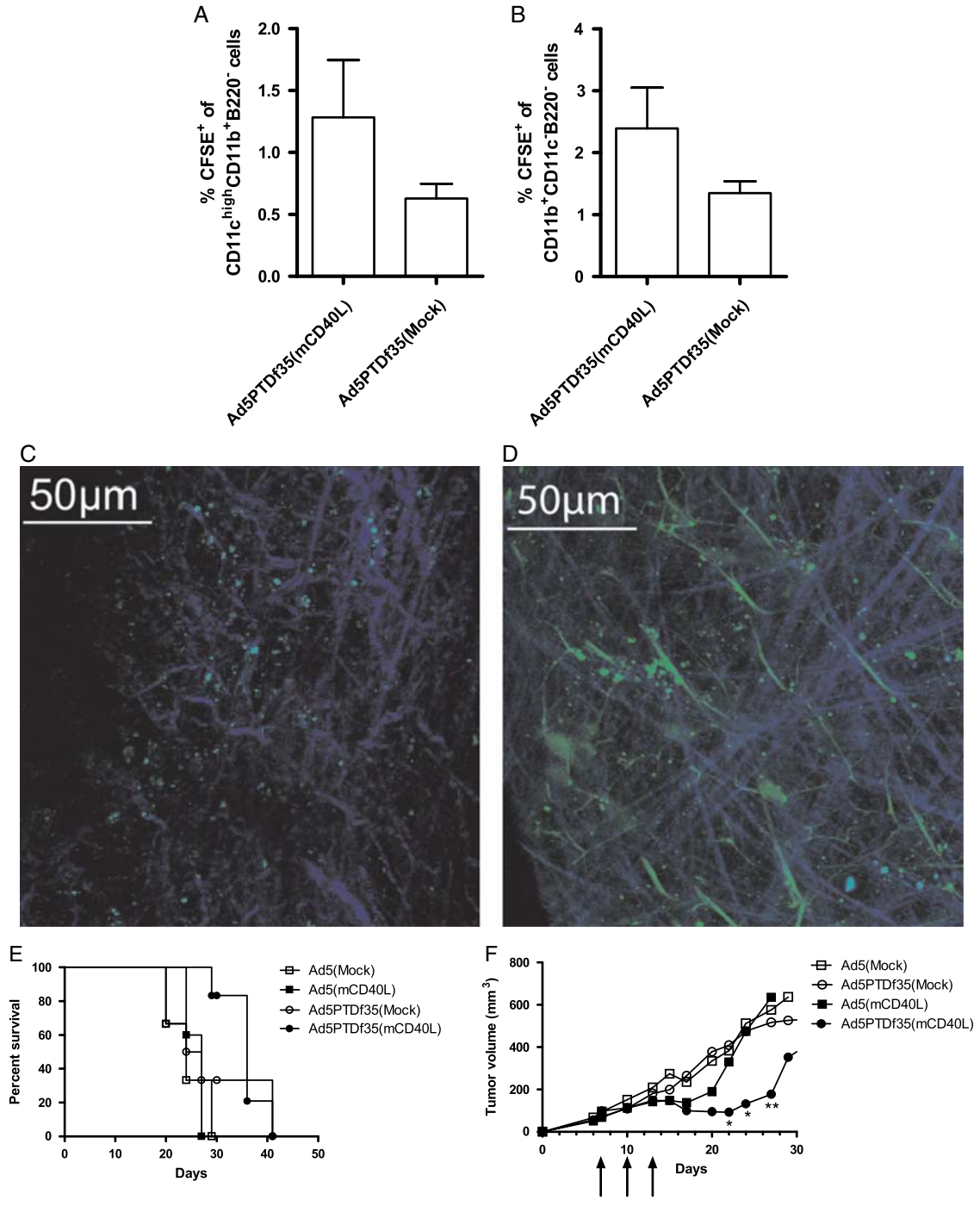

FIGURE 5. Treatment with Ad5PTDf35(*mCD40L*) results in migrating myeloid cells and delayed tumor growth. In an in vivo migration study, female C57BL/6 mice were injected intradermally on the right flank with 1×10^8 evg and 15 µg CFSE in PBS. Eighty-four hours after injection the right inguinal lymph node was analyzed for $CFSE^+$ cells within the (A) $CD11c^{high}$ and (B) $CD11b^+$ population (mean ± SEM, n = 4). In addition, whole inguinal lymph nodes were imaged by 2-photon microscopy, showing migrating cells in green (780 nm) and collagen in blue (second harmonic) in (C) Ad5f35PTD(Mock)-injected or (D) Ad5PTDf35(*mCD40L*)-injected animals. Further, Ad5(*mCD40L*) and Ad5PTDf35(*mCD40L*) vectors were compared on established SC MB49 tumors on female C57BL/6 mice. Three treatments were given intratumorally with 3 days apart at a virus load of 5×10^9 evg. Figures demonstrate the tumor growth and survival over 30 days. E, Tumor growth was measured and followed on all animals in the treatment groups Ad5(Mock) (n = 3), Ad5PTDf35(Mock) (n = 5), Ad5(*mCD40L*) (n = 5), and Ad5PTDf35(*mCD40L*) (n = 5). Arrows demonstrate days of treatment. F, Survival curve of the animals in (E). $^*P < 0.05$, $^{**}P < 0.01$ with the Mann-Whitney test and demonstrates differences between Ad5(*mCD40L*) and Ad5PTDf35(*mCD40L*) at indicated time-points. Curve comparison of Ad5(*mCD40L*) and Ad5PTDf35(*mCD40L*) gave $P < 0.01$ with the log-rank test.

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

D.Y. and M.E. have filed a US patent related to the Tat-PTD modification. S.M.M. has a royalty agreement with Alligator Bioscience. The project was financed by research money from Uppsala University hospital. and by FP7 MCA-ITN 317445 (S.M.M.).

The remaining authors have declared there are no financial conflicts of interest with regard to this work.

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