Gene Therapy (2017) **24**, 92–103

www.nature.com/gt

OPEN

ORIGINAL ARTICLE

Activation of myeloid and endothelial cells by CD40L gene therapy supports T-cell expansion and migration into the tumor microenvironment

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CD40 is an interesting target in cancer immunotherapy due to its ability to stimulate T-helper 1 immunity via maturation of dendritic cells and to drive M2 to M1 macrophage differentiation. Pancreatic cancer has a high M2 content that has shown responsive to anti-CD40 agonist therapy and CD40 may thus be a suitable target for immune activation in these patients. In this study, a novel oncolytic adenovirus armed with a trimerized membrane-bound extracellular CD40L (TMZ-CD40L) was evaluated as a treatment of pancreatic cancer. Further, the CD40L mechanisms of action were elucidated in cancer models. The results demonstrated that the virus transferring TMZ-CD40L had oncolytic capacity in pancreatic cancer cells and could control tumor progression. TMZ-CD40L was a potent stimulator of human myeloid cells and T-cell responses. Further, CD40L-mediated stimulation increased tumor-infiltrating T cells *in vivo*, which may be due to a direct activation of endothelial cells to upregulate receptors for lymphocyte attachment and transmigration. In conclusion, CD40L-mediated gene therapy is an interesting concept for the treatment of tumors with high levels of M2 macrophages, such as pancreatic cancer, and an oncolytic virus as carrier of CD40L may further boost tumor killing and immune activation.

Gene Therapy (2017) **24,** 92–103; doi:10.1038/gt.2016.80

INTRODUCTION

CD40 has lately gained interest as a target in cancer immunotherapy. Its natural ligand, CD40 ligand (CD40L, CD154), activates the immune system and tilts the pro-tumor response into an anti-tumor response.[1,2] CD40L-expressing T-helper (Th) cells are supporting the maturation of dendritic cells (DCs) that then promotes the activation of cytotoxic T cells.[3] Clinical trials using recombinant soluble CD40L or anti-CD40 agonistic antibodies have shown promising results in various cancers including pancreatic cancer.[4–7] For example, patients with advanced disease were treated with standard of care gemcitabine combined with anti-CD40 antibody infusion. Metabolic responses were noted in both primary and metastatic lesions in most patients. However, biopsies did not reveal T-cell infiltrates but rather the presence of macrophages. In a murine model of pancreatic cancer, anti-CD40 antibody therapy was not dependent on T cells even if those cells were activated in the periphery.[4] Pancreatic cancer has shown resistance to checkpoint blockade using antibodies that interrupt CTLA-4 or PD-1/PD-L1 signaling,[8,9] which may be explained by the high content of macrophages in the tumor lesions, whereas T cells are confined in the stroma unable to migrate into the tumor parenchyma. Nevertheless, recent studies support that CD40-mediated therapies may induce tumor immunity in pancreatic cancer via activation of myeloid cells, and that such stimulation can overcome the resistance to checkpoint blockade.[10,11]

Previously, we have shown promising results in mice[1,2,12] dogs[13,14] and humans[15,16] using a replication-deficient adenovirus carrying the *CD40L* gene (AdCD40L). CD40L is predominantly produced as a membrane-bound protein that trimerizes upon binding to CD40 but can be cleaved and released as a soluble monomer.[3,17,18] Trimerized CD40L is a more potent activator than its soluble monomeric form, and soluble CD40L may instead promote the suppressive capacity of myeloid-derived suppressor cells in cancer patients.[19] To optimize CD40L gene therapy, we present herein a trimerized membrane-bound isoleucine zipper CD40L (TMZ-CD40L). TMZ-CD40L was inserted into an oncolytic adenovirus to further enhance and prolong transgene expression. In this study, the ability of this virus to infect and subsequently kill pancreatic cancer cells, as well as its capacity to activate the immune system, were evaluated. Finally, the effect of CD40L gene therapy on endothelial cells was investigated to describe a mechanism of action for increased tumor-infiltrating T cells post CD40-mediated therapy.

RESULTS

Trimerized membrane-bound CD40L is retained on the cell surface

The TMZ-CD40L molecule was cloned to trimerize in cells to increase its stability on the cell surface as well as to maintain high signaling capacity (Figure 1a). Transfection of 293 cells with a plasmid containing TMZ-CD40L showed that TMZ-CD40L is

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Received 24 June 2016; revised 30 October 2016; accepted 11 November 2016; accepted article preview online 1 December 2016; advance online publication, 5 January 2017

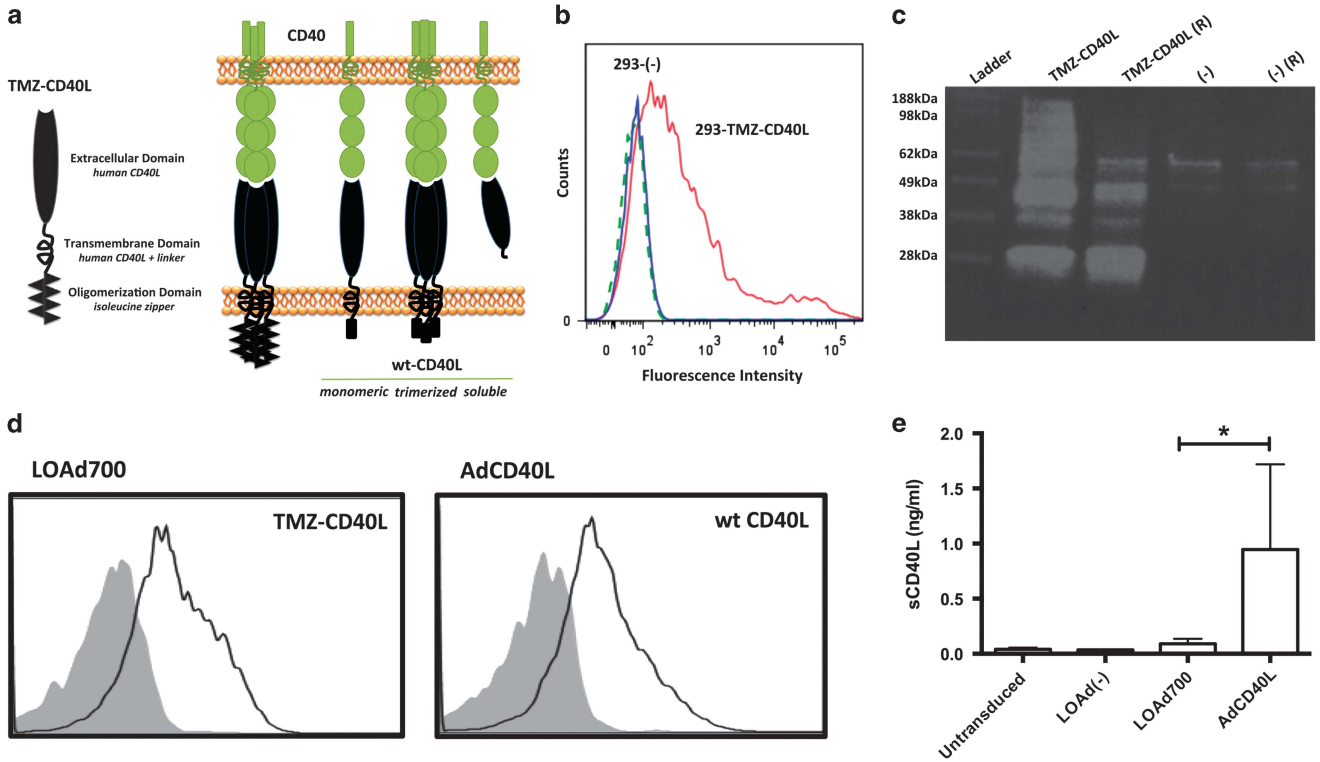


Figure 1. TMZ-CD40L is membrane-bound post expression. (**a**) Schematic figure of TMZ-CD40L compared with wild-type CD40L. (**b**) 293 cells were transfected with a TMZ-CD40L-containing plasmid (LP-700; red line), empty plasmid (LP(−); blue line) or untransfected (green broken line) and analyzed day 2 by flow cytometry. (**c**) 293 cells transfected with LP-700 or LP(−) were lysed at day 2 post transfection and the protein suspension analyzed by gel electrophoresis and western blot to detect TMZ-CD40L. Samples boiled in reducing sample buffer (R) or not boiled but mixed with a non-reducing sample buffer before gel electrophoresis. (**d**) Pancreatic cancer cell lines were untransduced or transduced with LOAd viruses transferring TMZ-CD40L (LOAd700), empty virus LOAd(−) or with AdCD40L-expressing wild-type CD40L. At day 2, the cells were analyzed for CD40L expression using flow cytometry. Black line demonstrates CD40L expression in LOAd700- or AdCD40L-transduced cells compared with LOAd(−) cells (filled curve). A representative figure of transduced PaCa3 cells is shown. (**e**) The supernatants were collected from the cell cultures in **d** and the presence of soluble CD40L (sCD40L) was determined by ELISA. The difference of sCD40L levels between the groups was determined by the Student's *t*-test with Welsh correction ($P = 0.02$) and the error graphs show s.e.m.

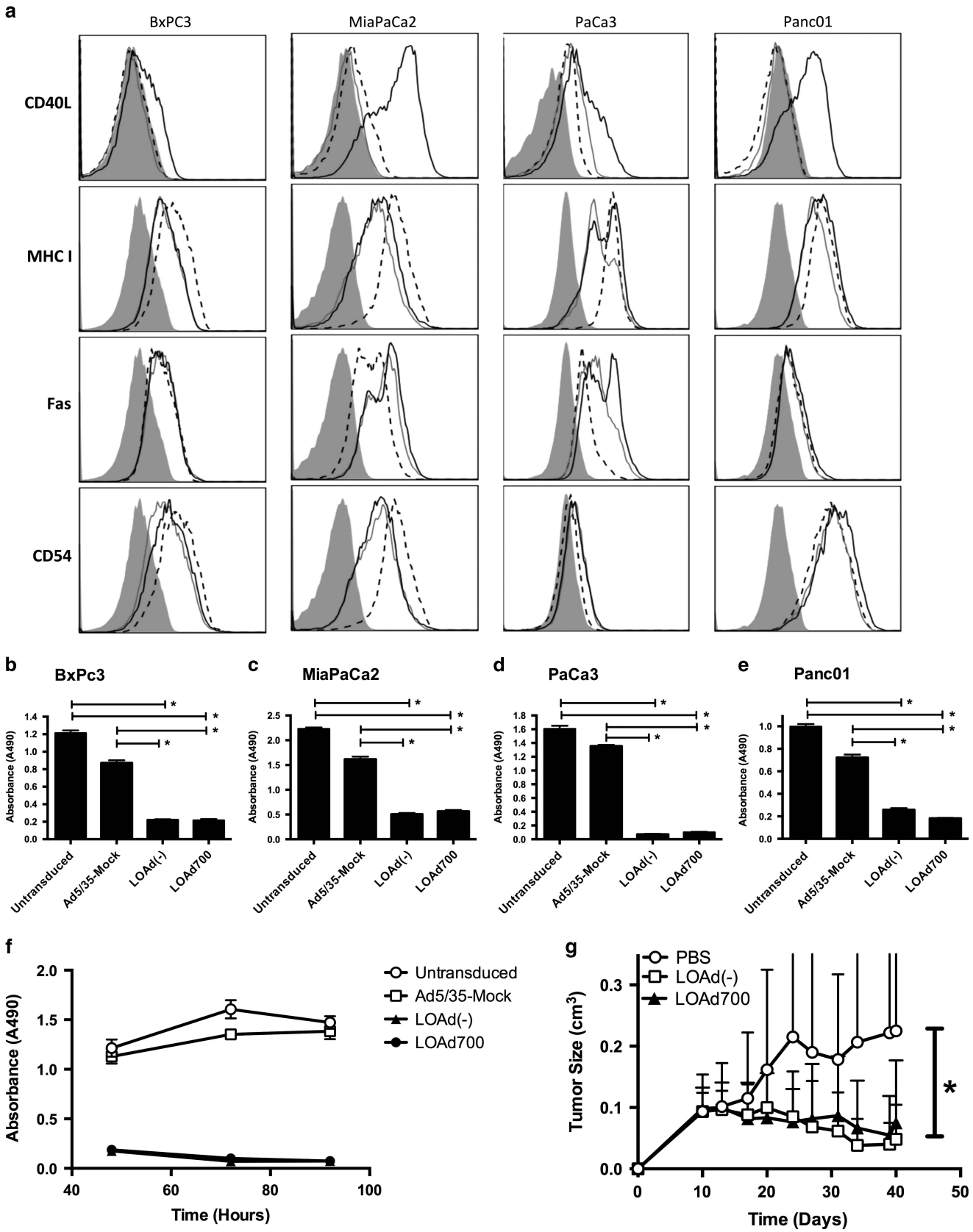
expressed, translated and displayed on the cell surface (Figure 1b). Oligomerized TMZ-CD40L was detected in cell lysates by western blot and in a reducing environment oligomers dissociated into monomers of TMZ-CD40L (31 kDa) as expected (Figure 1c). TMZ-CD40L was transferred to the LOAd adenovirus backbone creating LOAd700 and used to transduce a panel of pancreatic cancer cell lines. In Figure 1d, the membrane-bound expression of TMZ-CD40L after LOAd700 infected of PaCa3 was comparable to the CD40L expression after transduction with an adenovirus transferring wild-type human *CD40L* (AdCD40L). Wild-type CD40L is released to the supernatant upon AdCD40L cell transduction, whereas the TMZ-CD40L is not released post infection by LOAd700 (Figure 1e). The difference of detected sCD40L in these two groups was significant ($P = 0.0165$).

LOAd700 is an efficient inducer of oncolysis *in vitro* and *in vivo*

To demonstrate the effect of TMZ-CD40L on pancreatic tumor cells, cell lines was infected with LOAd700. The infected cells expressed CD40L, whereas uninfected or cells infected with empty LOAd virus (LOAd(−)) did not (Figure 2a). Most pancreatic cancer cell lines were positive for molecules important for T-cell-mediated recognition and killing independently of virus infection (Figure 2a) but lacked CD40 (data not shown). The infected cell lines were further analyzed for viability. At 48 h post infection, the LOAd viruses had efficiently reduced tumor cell viability (Figures 2b–e). Over time, the viability was reduced even further as shown in PaCa3 cells (Figure 2f). The oncolytic effect is also prominent *in vivo*, as LOAd700 or LOAd(−) could control tumor growth of human Panc01 cells (Figure 2g). In these immunodeficient mice, the TMZ-CD40L transgene has no function, as human CD40L does not cross-react with murine CD40 ([20]; Figure 3) and the model lacks functional T cells. However, if murine splenocytes are co-cultured with murine pancreatic tumor cells (Panc02) transduced with a LOAd virus expressing the murine version of TMZ-CD40L (mLOAd700), the splenocytes produce cytokines such as tumor necrosis factor-α and interleukin 12 (IL12), which is not noted upon transduction with a virus lacking CD40L (Figure 3).

TMZ-CD40L-expressing tumor cells promote M1 macrophages

As macrophage stimulation seems crucial for anti-tumor responses in pancreatic cancer,[4] the pancreatic cancer cell line MiaPaCa2 was co-cultured with human monocytes after being infected with the LOAd viruses or left uninfected. Co-culture of tumor cell lines with monocytes commonly promotes differentiation of the monocytes into M2 macrophages that are CD163 positive. In our co-cultures, no significant difference between the groups at 3 or 5 days of culture was noticed (Figure 4a). However, an increase of highly MHC class II-positive cells in the co-cultures with LOAd700-infected tumor cells compared with uninfected cells was seen but it did not reach significance (Figure 4b). However, similarly to the murine splenocytes, 3 days post co-culture initiation, the immunostimulatory cytokine IL12 was increased in the LOAd700 group ($P = 0.0465$), whereas IL6 was decreased ($P = 0.0059$; Figure 4c). At day 5, IL12 was normalized, whereas IL6 and IL8 were decreased in the LOAd700 group ($P = 0.0418$, $P = 0.0026$, respectively) and IL8 in the LOAd(−) group


a
BxPC3
MiaPaCa2
PaCa3
Panc01
CD40L
MHC I
Fas
CD54
b
BxPc3
c
MiaPaCa2
d
PaCa3
e
Panc01
Absorbance (A490)
Untransduced
Ad5/35-Mock
LOAd(-)
LOAd700
f
Absorbance (A490)
Time (Hours)
Untransduced
Ad5/35-Mock
LOAd(-)
LOAd700
g
PBS
LOAd(-)
LOAd700
Tumor Size (cm3)
Time (Days)

lacking TMZ-CD40L ($P=0.0062$). To determine the *in vivo* role of macrophage activation, the Panc01 human xenograft model was utilized, as the LOAd viruses efficiently infect human tumor cells, whereas they do not infect murine tumor cells *in vivo* due to the lack of the entry receptor CD46.[21] Tumor-bearing mice were treated by a single intratumoral injection with mLOAd700 carrying the murine TMZ-CD40L, LOAd(−) lacking transgenes or phosphate-buffered saline (PBS). After 48 h, before the oncolysis exerted effect, the mice were killed and the tumors were dissected for flow cytometry. The tumor sizes at this time point were similar (Figure 4d). However, the M1/M2 ratio determined by the ratio of CD11b+F4/80+CD206− (M1) versus CD11b+F4/80+CD206+ (M2)[22] was significantly improved in the mLOAd700 group compared with PBS ($P=0.05$). The virus backbone may have some effect on its own, as mLOAd700 was not significantly different from LOAd(−) empty control virus (Figure 4e).

TMZ-CD40L activates human DCs and promotes expansion of T cells

DCs were differentiated from CD14+ monocytes sorted from five blood donors using granulocyte–macrophage colony-stimulating factor and IL4. The immature DCs (CD14− and CD1a+) were infected with the LOAd700, the empty LOAd(−) or left uninfected. The maturation marker CD83 was strongly expressed after LOAd700 infection (Figure 5a, $P=0.0079$). Although MHC class II expression was stable, the costimulatory molecule CD86 was increased on both. However, the increase of CD86 was significantly higher for LOAd700-infected DCs compared with LOAd(−)-infected ($P=0.0397$). CD70, a molecule important to stimulate activated T cells via CD27, was increased in LOAd700-infected DCs. All DCs expressed IL6 and IL10 receptors, but infected cells tended to have decreased levels of both receptors. IL6 was increased in the LOAd700-infected DCs (Figure 5b; $P=0.0079$), whereas the

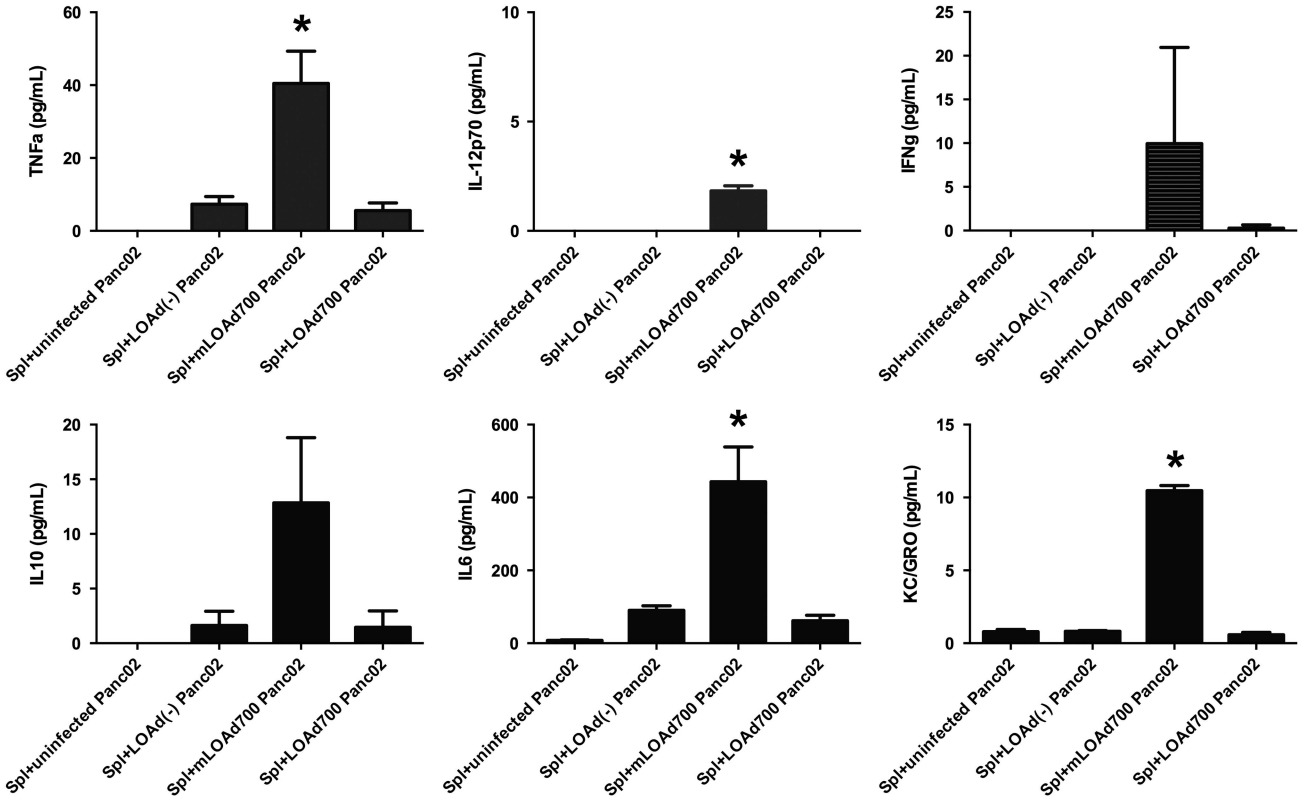
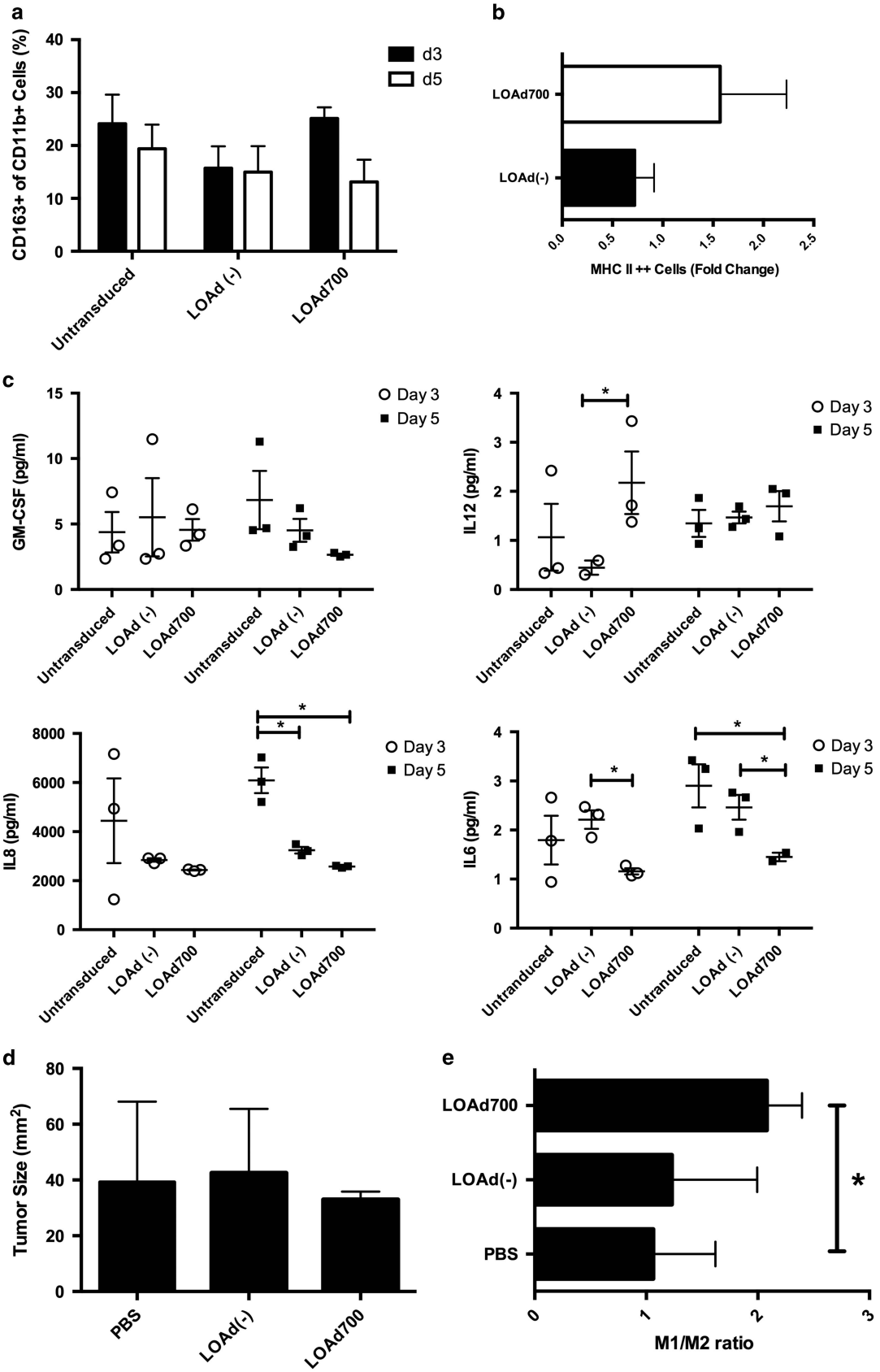


Figure 3. Murine CD40L activates splenocyte co-cultures. Splenocytes from mice were co-cultured with murine Panc02 tumor cells (10:1) infected with LOAd(−), mLOAd700 with murine TMZ-CD40L, LOAd700 with human TMZ-CD40L or left uninfected. After 3 days culture, supernatants were collected and analyzed by Meso Scale. Background from unstimulated splenocytes is withdrawn. The experiment show duplicate samples and statistical differences were calculated using one-way analysis of variance with Dunnett's multicomparison test against control virus group (LOAd(−)) at a 95% confident interval.

Figure 2. LOAd viruses exhibit high oncolytic capacity *in vitro* and *in vivo*. (**a**) Pancreatic cancer cell lines were infected with LOAd700 (black line), LOAd(−) (gray line) or left uninfected (broken line). After 48 h, the cells were collected and analyzed by flow cytometry for different cell surface markers. Filled histogram shows isotype control. (**b**–**e**) The cell viability of infected cells was determined by MTS assay at 48 h post infection. LOAd700- and LOAd(−)-infected cells had significantly poorer survival; BxPc3 (**b**; $P=0.03$, $P=0.03$, respectively), MiaPaCa2 (**c**; $P=0.03$, $P=0.03$, respectively), PaCa3 (**d**; $P=0.0286$, $P=0.0286$, respectively) and Panc01 (**e**; $P=0.03$, $P=0.03$, respectively). (**f**) Oncolysis was increased over time as demonstrated using PaCa3 cells. (**g**) Human Panc01 cells were grown in immunodeficient Nu/Nu mice ($n=6$ per group). Palpable tumors were injected 6×, 5 days apart with LOAd700 or LOAd(−) at a dose of 4x10e9 IU per treatment or with PBS as a negative control. Difference between LOAd700 and LOAd(−) to PBS group was calculated at day 40 using Student's *t*-test with Welsh correction. In **g**, multicomparison analysis of variance revealed a significant difference of LOAd(−) and LOAd700 from PBS at time points day 24, 34, 39 and 40. Error bars represent s.e.m. The experiments were repeated with similar results.


a
CD163+ of CD11b+ Cells (%)
d3
d5
Untransduced
LOAd (-)
LOAd700
b
LOAd700
LOAd(-)
MHC II ++ Cells (Fold Change)
c
GM-CSF (pg/ml)
IL12 (pg/ml)
IL8 (pg/ml)
IL6 (pg/ml)
Day 3
Day 5
Untransduced
LOAd (-)
LOAd700
d
Tumor Size (mm2)
PBS
LOAd(-)
LOAd700
e
LOAd700
LOAd(-)
PBS
M1/M2 ratio

empty LOAd(−) virus promoted production of the inhibitory cytokine IL10 ($P = 0.0079$). Further, LOAd700 induced a significant production of Th1-promoting cytokines IL12 ($P = 0.0079$), IL21 ($P = 0.0079$), interferon-γ (IFNγ; $P = 0.0317$) and tumor necrosis factor-α ($P = 0.0079$).

Next, the functional capacity of LOAd700-activated DCs was investigated to understand the ability of LOAd700 to induce antigen-specific T-cell responses. We utilized the robust model in which cytomegalovirus (CMV) peptide-pulsed activated DCs are used to promote expansion of CMV-specific T cells. CMV+ blood donors were used to prepare immature DCs. The DCs were infected with LOAd700 or empty LOAd(−) virus, left unstimulated or stimulated with tumor necrosis factor-α and PolyIC as a positive control. The DCs were pulsed with CMVpp65 peptides and co-cultured with autologous peripheral blood mononuclear cells for 11 days. Thereafter, the cultures were analyzed for total number of lymphocytes and the presence of CMV-specific T cells. LOAd700-transduced DCs had as good capacity as the positive control to induce expansion of CMV-specific T cells (Figure 6a). However, a high peptide dose expanded lymphocytes in all groups, which demonstrates an intrinsic immunogenic property of the peptides (Figure 6b). Nevertheless, only a low dose of peptides was required in LOAd700-activated or positive control-activated DCs to expand CMV-specific T cells.

CD40L activates endothelial cells

The effect of TMZ-CD40L on endothelial cell expression of attachment and transmigration receptors was investigated. Human umbilical vein endothelial cells were infected with virus and at 48 h post infection LOAd700 increased the expression of VCAM-1 ($P = 0.0022$), ICAM-1 ($P = 0.0053$) and E-Selectin ($P = 0.0084$; Figure 7a). To evaluate whether CD40L could induce T-cell infiltration *in vivo* we utilized an Ad5 virus (mAdCD40L) to transfer murine CD40L into the tumor since Ad5 viruses have better *in vivo* uptake in mice than LOAd 5/35 virus. *In vitro*, mAdCD40L could induce upregulation of E-selectin, ICAM-I and CX3CL-1 (fractalkine) as shown by PCR (Figure 7b), indicating a similar effect of mAdCD40L and LOAd700. To demonstrate that T cells are indeed migrating to the tumor upon CD40L stimulation the transgenic Thy1.1 pmel model was utilized in which *in vitro* pre-activated gp100-specific, *in vitro* activated (gp100+IL2) Thy.1.1 + T cells were infused into mice with growing B16F10 tumors that express gp100. Tumor-bearing mice were treated twice with mAdCD40L or PBS as a negative control. Thereafter, gp100-specific T cells were injected intraperitoneal After 3 days, Thy1.1+ pmel T cells were detected in tumor biopsies of mice treated with T cells alone while they were lacking in mice receiving PBS or mAdCD40L alone (Figure 7c). Of note, there was a significant increase of pmel T cells in the tumors even if the number is low that were pre-treated with mAdCD40L and the CD8 cells including T cells (both Thy1.1 positive and naturally occurring Th1.1 negative) in mAdCD40L-treated tumors were active as shown by positive CD107a staining of tumors treated with mAdCD40L with or without pmel tumors (Figure 7d). mAdCD40L therapy reduced the growth of B16 cells (Figure 7e, $P = 0.0069$) and enhanced survival of the AdCD40L-treated mice (Figure 7f, $P = 0.009$).

DISCUSSION

CD40L has been utilized in various forms in cancer immunotherapy.[4–6,15,16,23,24] The results have been encouraging and some patients showed complete and/or long-term responses. Herein, we have developed a novel immunostimulatory gene therapy that utilizes an oncolytic adenovirus to deliver TMZ-CD40L into the tumor microenvironment. TMZ-CD40L was developed to optimize the treatment and reduce systemic CD40L toxicity. TMZ-CD40L was expressed and displayed on the cell surface upon gene transfer, whereas it was not released into the supernatants compared to wild-type CD40L. Hence, TMZ-CD40L is retained locally, which may reduce *in vivo* toxicity, whereas the stimulatory capacity at the tumor site is still optimal. TMZ-CD40L gene therapy using the LOAd adenovirus system demonstrated high capacity to stimulate myeloid cells in our preclinical models as shown by phenotypic and functional assays such as cytokine release. Further, LOAd700-activated DCs could drive the expansion of antigen-specific T cells. The effect of LOAd700 is likely due both to TMZ-CD40L and the virus *per se*, as the virus will stimulate Toll-like receptors (TLRs) such as TLR9 as well as IFN regulatory factor family-3 protein (IRF3).[25,26] IRF3 induces type I IFNs and it has previously been shown that either TLR stimulation or type I IFNs can potentiate CD40 signaling to significantly increase cellular immune responses.[27,28] One mechanism seems to be upregulation of CD70 on DCs, which stimulates CD27+ effector T cells.[27] Hence, the combination of CD40 stimulation via TMZ-CD40L and type I IFNs induced by viral DNA can explain the higher frequency of CD70 in our experiments. In previous work, we have blocked the effect of CD40L in human[29] and murine[1] experimental models of myeloid cell activation to confirm that the demonstrated effects are not due to the virus alone.

CD40 stimulation may support the recruitment of lymphocytes to the tumor by activation of the endothelium.[30] Our findings support that CD40L induced a higher expression of receptors important for T-cell attachment, rolling and migration. Previously, we have treated patients with urinary bladder cancer with AdCD40L infusions and the bladders were thereafter infiltrated by T cells.[15] CD40L stimulation may induce on site proliferation and/or migration of systemic T cells to the tumor site. To evaluate whether T cells indeed migrated to the tumor in an enhanced manner post CD40L stimulation, we utilized the pmel model,[31] as there are unfortunately no good similar models for pancreatic cancer. Pre-treatment of the tumors with mAdCD40L intratumoral injections significantly enhanced the localization of the infused tumor-targeting T cells to the tumors. The increased infiltration can partly be explained by an activated endothelium but it is also possible that CD40L expression in the tumor upregulate expression of chemokines.[32] Nevertheless, drugs that modify the tumor vasculature also lead to enhanced lymphocyte infiltration such as the tyrosine kinase inhibitor sunitinib.[33]

Figure 4. CD40L promotes M2 to M1 macrophages and IL12 production. Human monocytes were co-cultured with MiaPaCa2 pancreatic cancer tumor cells infected with LOAd700, LOAd(−) or uninfected cells. Cells and supernatants were collected and analyzed by flow cytometry and Meso Scale at day 3 and 5. The experiment was repeated using three different healthy donor-derived monocytes and the figures show pooled data. CD11b+ myeloid cells were gated and the CD163 (M2) macrophages were compared as % positive cells (**a**) or the MHC-II++ cells were analyzed as fold change from co-cultures with uninfected cells (**b**, **c**) granulocyte–macrophage colony-stimulating factor, IL12, IL8 and IL6 levels are shown in the figure. Immunodeficient Nu/Nu mice lacking T lymphocytes but with sustained myeloid cells were implanted with human Panc01 cells. Palpable tumors were injected with mLOAd700, LOAd(−) using 1×10^9 infectious units (IU) per mouse (intratumoral injection; $n = 3$/group). (**d**) At 48 h post injection, the tumors were measured, and the murine tumor biopsies analyzed by flow cytometry for F4/80+CD11b+CD206− (M1) or F4/80+CD11b+CD206+ (M2) macrophages (**e**). Error bars represent s.e.m. and statistical significant differences were calculated using Student's *t*-test.

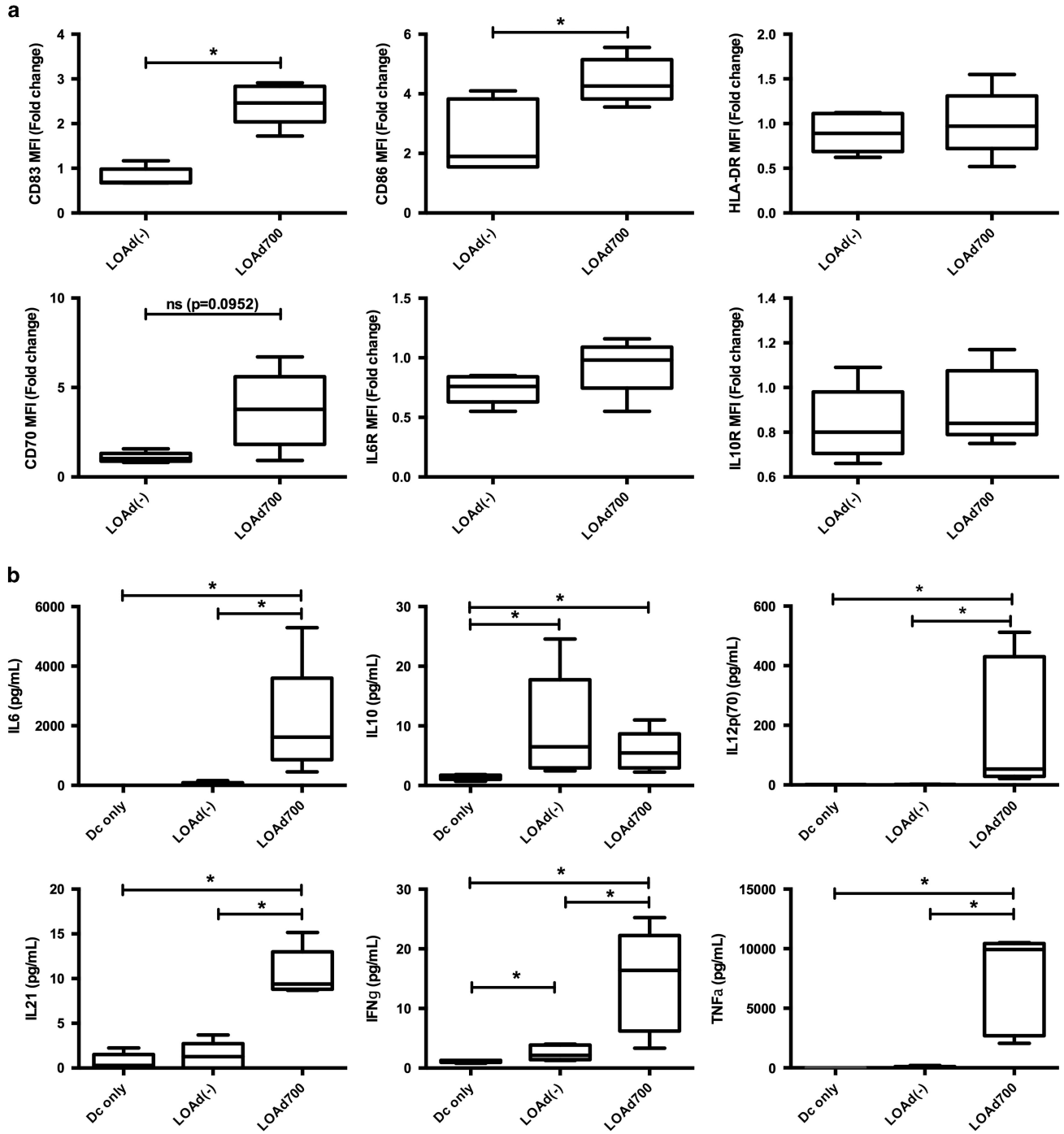


Figure 5. LOAd700 induces DC differentiation and maturation. (**a**) DCs were infected with LOAd700, LOAd(−) (50 f.f.u. per cell) or left uninfected. After 48 h, the CD1a+ DCs were evaluated for maturation markers by flow cytometry. The fold increase of respective marker compared with uninfected DCs is shown in the figure. (**b**) The supernatants were analyzed for cytokine production using Luminex. The experiment was repeated using DCs from five different donors and the results are pooled in the figures. The Error bars represent s.e.m. and significant differences were calculated using Mann–Whitney.

LOAd700 transfers the TMZ-CD40L gene, but it also has oncolytic capacity as shown in a panel of pancreatic cancer cell lines. Replication is restricted to cells with hyper-phosphorylated retinoblastoma proteins, which is a reality in most tumors.[34,35] Oncolysis can increase the therapeutic efficacy because of the increased tumor cell killing, but since adenoviruses are very immunogenic continuous spreading of the virus is limited.[36] Hence, the major efficacy of LOAd700 is likely due to induction of tumor immunity.

In conclusion, TMZ-CD40L is a potent stimulator both myeloid and T cells that remains oligomerized in the cell membrane, which may be a great benefit from both efficacy and toxicity concerns. Introduction of CD40L into the tumor microenvironment enhances level of tumor-infiltrating T cells, which may be due to

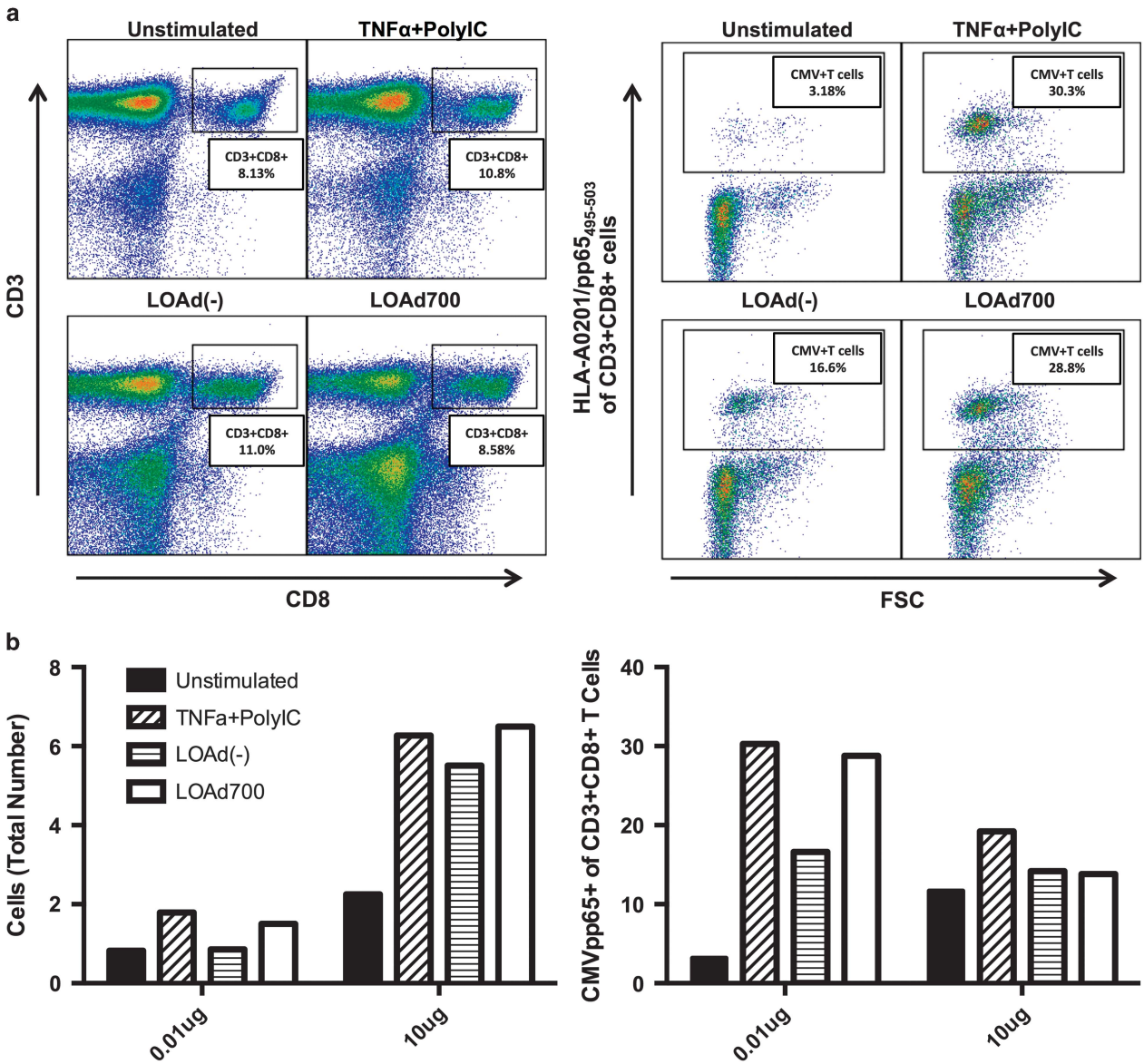


Figure 6. LOAd700-stimulated DCs expand antigen-specific T cells and NK cells. Peripheral blood mononuclear cells expanded using DCs from CMV-positive blood donors ($n = 3$). (**a**) Expansion of CD3+, CD8+ and double-positive T lymphocytes of all cultured cells are shown in the left panel, and the percentage of CMV-specific T cells of all gated CD3+CD8+ T cells against forward scatter are shown in the right panel. (**b**) The left panel demonstrates the total number of cells in the co-cultures using 0.01 or 10ug pp65 CMV peptides. The right panel shows percentage of CMV-specific CD3+CD8+ T cells in the co-cultures using 0.01 or 10 μg pp65 CMV peptides as calculated by multiplying the % cells with the manual count of cells in the cultures. Representative figures are shown.

the upregulation of receptors involved in lymphocyte transmigration on CD40L-stimulated endothelial cells. The LOAd700 virus or other CD40 stimulating therapies may be interesting options for pancreatic cancer both a monotherapy and in combinations with other cancer therapeutics. Clinical evaluation is warranted.

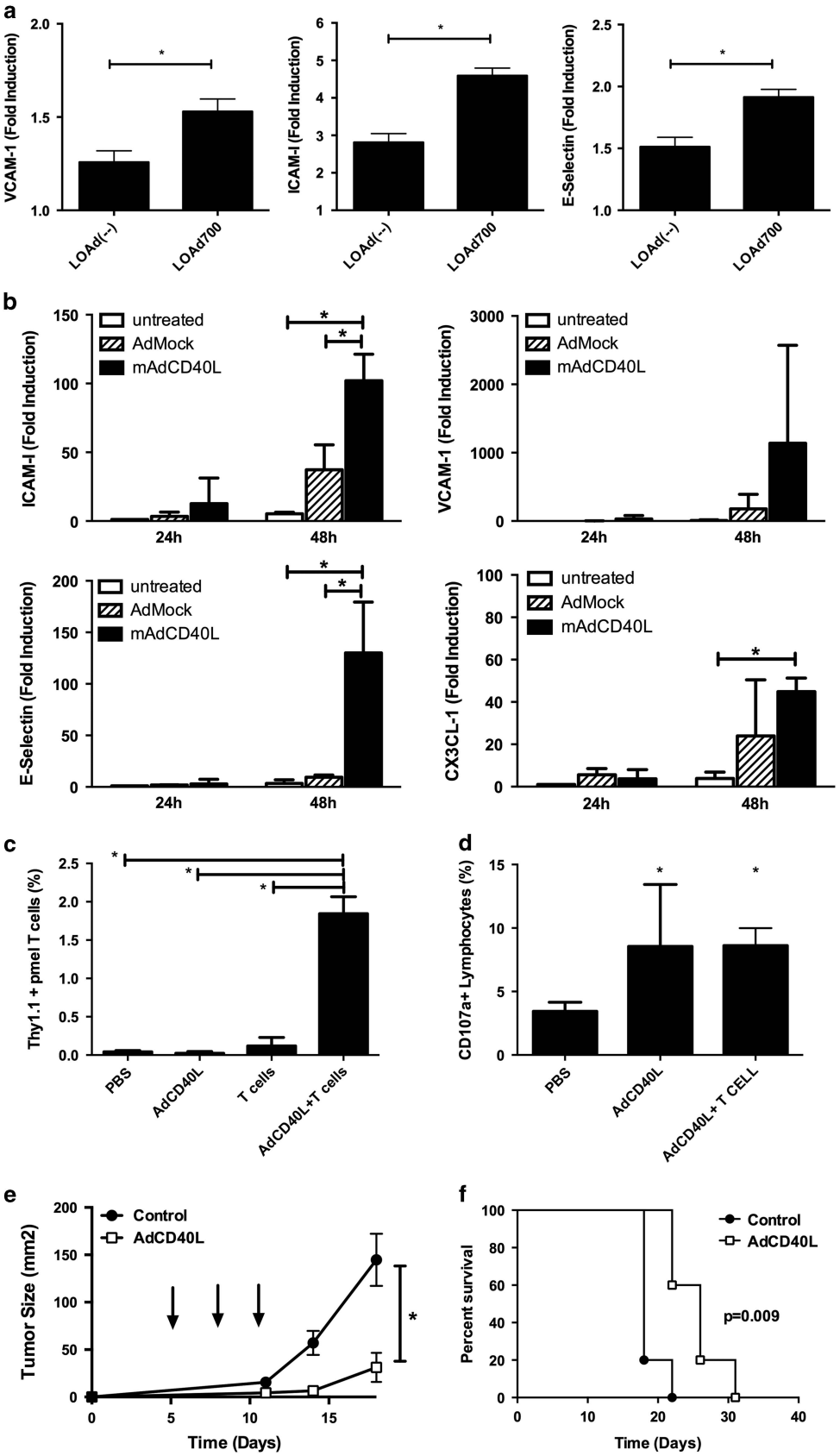
MATERIALS AND METHODS

TMZ-CD40L

The gene sequence of TMZ-CD40L was constructed using the free pDraw32 software by AcaClone and then synthesized with a CMV promoter upstream of the 5′-gene and adenovirus-flanking regions (also containing the restriction sites 5′-*Ssp*I and 3′-*Sca*I) for homologous recombination into adenoviral vectors at both ends. The gene fragments were synthesized and sub-cloned into a pUC57-Kan plasmid (LP-700) at GenScript Inc (Piscataway Township, NJ, USA). A control virus lacking the transgene was used as a negative control (LP(−)). Plasmids were transformed into bacteria and purified using a MAXI kit (Qiagen Inc, Valencia, CA, USA). 293 cells (0.75×10^6 per group) were resuspended in medium and seeded in six-well plates and cultured overnight. The next day, 5 μg plasmid was mixed into a suspension of 8 μl polyethylenimine in 100 μl OptiMEM (Life Technologies, Carlsbad, CA, USA) and incubated for 20 min. The plasmid suspension was then used to transfect 293 cells that were further cultured for analyses.

Flow cytometry. Transfected 293 cells were cultured for 24–48 h and stained with antibodies (CD40L, MHC-I, Fas, CD54; BioLegend, San Diego, CA, USA; antibody clones are shown in Supplementary Information), washed (0.5% bovine serum albumin in PBS) and fixed (1% paraformaldehyde/PBS). Cells were analyzed by flow cytometry using BD FACSCanto 2 (BD Biosciences, San Jose, CA, USA) and data were evaluated in Flow Jo (Tree Star, Ashland, OR, USA).

Gel electrophoresis and western blot. Lysates were prepared from cells transfected with plasmids. Cells were washed in ice cold PBS and


a
VCAM-1 (Fold Induction)
ICAM-I (Fold Induction)
E-Selectin (Fold Induction)
LOAd(-)
LOAd700
b
untreated
AdMock
mAdCD40L
ICAM-I (Fold Induction)
VCAM-1 (Fold Induction)
E-Selectin (Fold Induction)
CX3CL-1 (Fold Induction)
24h
48h
c
Thy1.1 + pmel T cells (%)
PBS
AdCD40L
T cells
AdCD40L+T cells
d
CD107a+ Lymphocytes (%)
PBS
AdCD40L
AdCD40L+T CELL
e
Control
AdCD40L
Tumor Size (mm2)
Time (Days)
f
Control
AdCD40L
Percent survival
p=0.009
Time (Days)

resuspended in lysis buffer containing M-PER mammalian protein extraction reagent supplemented with 1% Halt phosphatase inhibitor cocktail and 1% Protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). An amount of 10 μg of reduced or non-reduced samples was loaded onto pre-cast gels from Bio-Rad Laboratories Inc (Hercules, CA). Reduced conditions were achieved by addition of β − mercaptoethanol. The samples were incubated for 5 min at 95 °C before loading. The gel was run for 50 min at 110 V and then transferred to a nitrocellulose membrane using gel transfer stacks in an iBlot (Life Technologies). The membranes were blocked (10% bovine serum albumin in PBS) for 1 h at room temperature followed by overnight incubation at 4 °C with αCD154 Antibody (H215; 1:500; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). After washing (0.05% Tween in PBS), the membrane was incubated 1 h at room temperature using secondary antibody Goat anti-rabbit IgG-HRP (1:2000; Life Technologies). The membrane was developed by Clarity Western ECL Substrate and exposing it using V3 Western Workflow (Bio-Rad Laboratories Inc). The image was prepared as a TIF file using Adobe Photoshop Elements 11 (Adobe Systems, San Jose, CA, USA).

Virus production and infection

The ICOVIR-15 virus[34] was modified by a series of homologous recombination steps to construct the LOAd viruses. The Ad5 fiber was replaced by a chimeric 5/35 fiber with the tail from Ad5 and shaft and knob from Ad35.[37] Next, the E3A ONYX-304.323 deletion was introduced which eliminates the genes 6.7 K/gp19K.[38] Thereafter, the transgene cassette was added after the fiber 5/35. In brief, the plasmids LP-700 containing CMV promoter and the TMZ-CD40L gene and LP(−) with no transgene cassette were digested with *Ssp*I and *Sca*I enzymes and the band was isolated by standard gel electrophoresis and purification. The gene cassettes were inserted into an adenoviral backbone plasmid by homologous recombination. LOAd(−) is the LOAd virus without a transgene in the gene expression cassette while LOAd700 includes a gene encoding for TMZ-CD40L (Figure 1a). Ad5/35-Mock is a replication-deficient (E1/E3 deleted) adenovirus 5/35 virus. A replication defective serotype 5 adenovirus (E1/E3 deleted) was used to express wild-type human (AdCD40L) or murine CD40L (mAdCD40L). AdMock is the equivalent virus without transgenes.[2] Viruses were produced by transfection of 293 cells (American Type Culture Collection, Manassas, VA) with backbone virus plasmids followed by expansion in A549 cells (American Type Culture Collection). The virus supernatants were purified by CsCl gradient centrifugation and diluted in 20 mM TRIS, 25 mM NaCl and 2.5% glycerol. Viable virus titer was measured in a fluorescent-forming units (f.f.u.) assay.[39] 293 cells were grown in Dulbecco's modified Eagle medium (1×) GlutaMAX-1 supplemented with 10% fetal bovine serum, 1% sodium pyruvate and 1% penicillin streptomycin (PeSt). A549 cells were grown in Roswell Park Memorial Institute 1640 medium supplement with 10% fetal bovine serum, 1% PeSt, 1% HEPES and 0.1% sodium pyruvate. All culture medium components were purchased from Life Technologies. Upon transduction, cells were washed with serum-free medium and centrifuged. The supernatant was discarded and the cells mixed in the remaining medium (~200 μl). Virus was thawed and added to the cells (25–100 f.f.u. per cell) and the cell/virus suspensions were incubated for 2 h, at 37C, 5% CO_2. Medium with serum was added and the cells plated and further incubated for transgene expression and functional assays.

Phenotypic and viability testing of pancreatic cancer cell lines

The pancreatic cell lines MiaPaca2, Panc01, PaCa3 and BxPC3 were a kind gift from Dr Rainer Heuchel (Karolinska Institute, Stockholm, Sweden). MiaPaca2, Panc01 and PaCa3 were grown in Dulbecco's modified Eagle medium, whereas BxPC3 was grown in Roswell Park Memorial Institute medium as described above. The cell lines were infected with viruses at 100 f.f.u. per cell (viability) or 25 f.f.u. per cell (phenotype). A total of 10 000 infected cells in a volume of 100 μl were plated in 96-well plates in triplicates. The viability of the cells was measured by MTS Cell Titer Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA). For phenotypic evaluation, the cell lines were cultured for 48 h and stained with antibodies (BioLegend). Cells were analyzed by flow cytometry as stated above. All cell lines were subjected to short random repeat analysis profiling at Uppsala University Genome Center and identity confirmed. Mycoplasma testing is routinely performed on thawed cell lines at our laboratory.

DCs and CMV-specific T cells

Blood from consenting donors was obtained from the Blood Bank at Uppsala University Hospital. Peripheral blood mononuclear cells were obtained by Ficoll-Paque gradient centrifugation (GE Health Care, Little Chalfont, UK) of five healthy donor buffy coats. Monocytes were isolated by CD14+ magnetic bead separation (Miltenyi Biotec, Bergisch Gladbach, Germany) and cryopreserved (−80 °C). After thawing, the CD14+ cells were differentiated into immature DC by culturing them in medium containing granulocyte, granulocyte–macrophage colony-stimulating factor (150 ng ml^{-1}) and IL4 (50 ng ml^{-1}; Gentaur, Brussels, Belgium). After 6 days, the immature DCs were infected with virus (50 f.f.u. per cell) and 48 h later cells were collected for flow cytometry. DCs were stained with antibodies (BioLegend) and analyzed by flow cytometry as described above. DCs generated from CMV-positive healthy blood donors ($n = 3$) were infected with virus (50 f.f.u. per cell) or stimulated with tumor necrosis factor-α (40 ng ml^{-1}) and PolyIC (30 μg ml^{-1}; Sigma-Aldrich, Saint Louis, MO, USA). After 24 h, the DCs were pulsed with CMV $pp65_{495–503}$ NLVPMVATV peptide (0.01 or 10 μg; GenScript) together with β2 microglobulin (1 ng; Sigma-Aldrich) for 4 h and then mixed in a ratio of 1:10 with autologous CD14 peripheral blood mononuclear cells. After 11 days of co-culture, the cells were collected and stained for flow cytometry with antibodies (CD3 and CD8; BioLegend) and Tetramer/PE-HLA-A*02:01 CMVpp65 (NLVPMVATV), negative iTag Tetramer/PE-HLA-A*02:01, both from MBL International (Woburn, MA, USA, USA). Cells were analyzed by flow cytometry as described above.

Human umbilical vein endothelial cells

Human umbilical vein endothelial cells were infected with virus (50–200 f.f.u. per cell). After 24–48 h, cells were collected for flow cytometry or PCR.

Flow cytometry. Cells were stained with antibodies (BioLegend) and analyzed by flow cytometry as described above.

Quantitative PCR. RNA was prepared from the cells using Qiagen RNeasy Minikit accordingly to protocols of the manufacturer (Qiagen Inc). Complementary DNA was acquired with SuperScript II/III (Thermo Fisher Scientific) and random primers. Quantitative real-time PCR was performed using SYBR Green (Thermo Fisher Scientific; primers are shown in Supplementary Information), 0.25 μM forward and reverse primer and 0.2 μl complementary DNA per reaction with human hprt serving as an internal control. All reactions were run in triplicates. For gene expression analysis, relative expression values were calculated according to the formula: relative expression of gene $X = 2^{-(Cthprt - Ctgene\ x)}$.

Figure 7. CD40L activates endothelial cells and attracts T cells to the tumor. (**a**) Human umbilical vein endothelial cells (HUVECs) were infected with LOAd700, LOAd(−) or left uninfected. After 48 h, the cells were analyzed for cell surface markers using flow cytometry. The fold induction from uninfected cells is shown in the figure. The experiment was repeated twice with similar results. (**b**) HUVECs cells were transduced with murine AdCD40L, AdMock or left untransduced. The cells were collected after 24 and 48 h and analyzed by PCR. The fold induction from untransduced cells is shown in the figure and is calculated using data from three experiments. (**c**) Mice with B16F10 tumors were treated twice with intratumoral AdCD40L (1×10^9 infectious units (IU) per treatment) before intraperitoneal injection of gp100-specific pmel T cells and one additional intratumoral AdCD40L injection. Biopsies ($n = 3$) taken on day 3 post infusion were analyzed by flow cytometry for infiltration of gp100-specific T cells by staining for the congenic marker Thy1.1 and the bulk of activated CD107a+ lymphocytes (**d**). Tumor size (**e**) shown in B16 tumors treated with mAdCD40L or Mock ($n = 5$) at day 5, 8 and 11. When the control group reached maximum growth (day 17), end point statistical difference was calculated. (**f**) Mice with B16 tumors treated as in **e** were followed over time to determine overall survival. Error bars represent s.e.m. and significant differences were calculated using Student's *t*-test (**a**, **d**, **e**) and two-way analysis of variance (**b**, **c**; $P < 0.05$ was judged significant and labeled with *). Survival was evaluated using log-rank test.

Monocyte co-cultures

Monocytes prepared from buffy coats of healthy donors ($n = 3$) were used for co-culture experiments with MiaPaCa2-infected tumor cells. A total of 5×10^5 monocytes per well were cultured in 24-well plates in 0.5 ml Roswell Park Memorial Institute medium described above. MiaPaCa2 cells infected with virus and were diluted to 1×10^6 cells per ml, and 0.5 ml was added to the monocytes in different combinations. Day 3 and 5 post culture initiation, the cells and supernatants were collected. The cells were stained with antibodies specific for CD11b, CD163 and MHC-II (BioLegend) and analyzed by flow cytometry as described above. Supernatants were analyzed by the Meso Scale Discovery platform (Rockville, MD, USA).

Cytokine detection

Supernatants from 293, tumor cell lines and from DCs were analyzed by ELISA (sCD40L, eBioscience, San Diego, CA, USA), and Luminex (Miliplex MAP kit Human Th17 Magnetic Bead Panel HTH17MAG-14 K, Milipore, Billerica, MA, USA).

Animal experiments

Animal experiments were approved by the local animal ethics committee (DNr C54/13, N122_14 and C86/10) and were performed at Uppsala university and by Adlego AB.

Panc01 xenograft model. Female 9–12-week-old-C57BL6 Nu/Nu mice ($n = 6$ per group) were injected subcutaneously with 5 million Panc01 cells. At tumor detection, tumors were either treated with intratumoral virus injections. Tumors were monitored for growth rate and animals were followed for survival. The experiment was repeated several times with different doses and number of treatments as indicated in figure legends. The single-dose-treated animals ($n = 3$) were killed 48 h post treatment. Biopsies were mechanically disrupted and Liberase TL (F Hoffmann-La Roche Ltd, Basel, Switzerland) was added (2 WU ml^{-1}) for 20 min at 37 °C. EDTA was added to a final concentration of 10 mM and the suspensions were filtrated through a 70 μm cell strainer (BD Biosciences) to obtain single-cell suspensions. Cells were kept in medium containing 3 mM EDTA and then stained with antibodies specific for CD11b, F4/80 and CD206 (BioLegend) and analyzed by flow cytometry.

Pmel model. Spleens from transgenic Thy1.1 pmel mice[31] were collected and used to expand gp100-specific T cells by stimulating the splenocytes with human gp100 peptides (1 μg ml^{-1}; $gp100_{25–33}$ KVPRNQDWL) and IL2 (60 infectious units (IU) ml^{-1}) for 7 days in which T cells are expanded and no further enrichment is needed. The cells were collected, counted and 3 million cells per mouse were injected intraperitoneal in Thy1.2 mice with B16F10 tumors established subcutaneously on female 9–12–week-old-C57BL/6 mice (25 000 B16F10 cells per mouse). Mice with B16F10 tumors were untreated or treated with mAdCD40L (1×10^9 f.f.u. per mouse) twice, 3 days apart. On the third day post second injection, the pmel T cells were infused with or without an additional mAdCD40L injection. Three days later, biopsies were collected, mechanically disrupted and filtrated through a MESH membrane to achieve a single-cell suspension. The cells were stained with antibodies specific for Thy.1.1, CD8 and CD107a (BioLegend) and analyzed by flow cytometry. The pmel experiment was repeated twice with similar results. Mice with B16F10 tumors were also treated with mAdCD40L ($3\times$, 1×10^9 f.f.u. per mouse), 3 days apart and compared with mock control for tumor growth and survival.

Splenocyte culture. Spleens were taken from C57BL/6 mice and cultured with murine Panc02 pancreatic cancer cells (10:1 ratio) transduced with LOAd(−), mLOAd700 or left untransduced (MOI1000). Splenocytes were cultured alone for background measurments. Supernatants were collected at day 3 and subjected to cytokine analysis using Meso Scale array (MesoScale Diagnostics) as per the company's protocol.

Statistical analysis

Statistical analyses were performed by using Graphpad Prism (Graphpad Software Inc. La Jolla, CA, USA). Methods used were Student's *t*-test with Welsh correction for comparing two unpaired samples, analysis of variance for comparing several groups and log-rank for survival.

Data availability

LOAd viruses, plasmids and genome description can only be obtained through an MTA with Lokon Pharma AB besides already disclosed genome information in public patent application WO2015155174. Other descriptions and products can be released under MTA with Uppsala University.

CONFLICT OF INTEREST

Dr Loskog, Dr Ullenhag and Dr Alemany disclose conflict of interest connected to the data presented in this paper. Dr Loskog is the CEO, board member, has a royalty agreement and holds a contract research grant from Lokon Pharma AB. She is a scientific advisor at NEXTTOBE AB. Gustav Ullenhag was a medical advisor at Lokon Pharma AB. Dr Alemany had a contract research grant from Lokon Pharma AB. Dr Mangsbo is the founder and CSO of Immuneed AB. The remaining authors declare no conflict of interests.

ACKNOWLEDGEMENTS

We are grateful to Berith Nilsson and Gabriella Paul Wetterberg at Uppsala University for technical support. The study was supported by grants to Dr Loskog from the Swedish Cancer Society, the Swedish State Support for Clinical Research (ALF). Both Dr Loskog and Dr Alemany received grants for contract research from Lokon Pharma AB. The study was supported by grants to Dr Loskog from the Swedish Cancer Society, the Swedish State Support for Clinical Research (ALF). Both Dr Loskog and Dr Alemany received grants for contract research from Lokon Pharma AB.

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Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)