Clinical Cancer Research

Shaping the Tumor Stroma and Sparking Immune Activation by CD40 and 4-1BB Signaling Induced by an Armed Oncolytic Virus

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Abstract

Purpose: Pancreatic cancer is a severe indication with short expected survival despite surgery and/or combination chemotherapeutics. Checkpoint blockade antibodies are approved for several cancer indications, but pancreatic cancer has remained refractory. However, there are clinical data suggesting that stimulation of the CD40 pathway may be of interest for these patients. Oncolytic viruses armed with immunostimulatory genes represent an interesting approach. Herein, we present LOAd703, a designed adenovirus armed with trimerized *CD40L* and *4-1BBL* that activates the CD40 and 4-1BB pathways, respectively. As many cells in the tumor stroma, including stellate cells and the infiltrating immune cells, express CD40 and some 4-1BB, we hypothesize that LOAd703 activates immunity and simultaneously modulates the biology of the tumor stroma.

Experimental Design: Tumor, stellate, endothelial, and immune cells were infected by LOAd703 and investigated by flow cytometry, proteomics, and functional analyses.

Results: LOAd703-infected pancreatic cell lines were killed by oncolysis, and the virus was more effective than standard-of-care gemcitabine. In *in vivo* xenograft models, LOAd703 efficiently reduced established tumors and could be combined with gemcitabine for additional effect. Infected stellate and tumor cells reduced factors that promote tumor growth (Spp-1, Gal-3, HGF, TGFβ and collagen type I), while chemokines were increased. Molecules involved in lymphocyte migration were upregulated on infected endothelial cells. Dendritic cells were robustly stimulated by LOAd703 to produce costimulators, cytokines and chemokines, and such DCs potently expanded both antigen-specific T cells and NK cells.

Conclusions: LOAd703 is a potent immune activator that modulates the stroma to support antitumor responses. *Clin Cancer Res; 23(19); 5846–57. ©2017 AACR.*

Introduction

Pancreatic cancer is a devastating lethal disease increasing in incidence. Yet an orphan indication, pancreatic cancer is one of the most common causes of death due to cancer (1). Gemcitabine treatment has long been the most useful chemotherapy but is of palliative nature without surgery (2). The life expectancy is about 6 months for metastatic disease while patients with locally advanced disease have a median survival of 12 months (3). Combining gemcitabine with nab-paclitaxel increases overall survival from 6.7 months (gemcitabine monotherapy) to 8.5 months (4). The multidrug cocktail FOLFIRINOX has shown even better overall survival (11.1 months), but due to the severe toxicity profile, only patients with a good performance status can be considered (5). Hope was set on checkpoint blockade immunotherapy but these otherwise promising therapeutics failed to show significance in pancreatic cancer (6, 7). Nevertheless, recent data in experimental models demonstrated that resistance to checkpoint blockade therapy could be broken by simultaneous treatment with immunostimulatory agents (8, 9). Hence, besides releasing the breaks of antitumor immunity, the immune system needs to be kickstarted to achieve reliable responses.

Pancreatic tumor lesions are dense and fibrotic with few infiltrating lymphocytes explaining the poor reactivity to checkpoint blockade therapy. Most of the lesions consist of stroma cells including stellate cells, fibroblasts, endothelial cells, and myeloid immune cells such as type II macrophages and myeloid-derived suppressor cells (MDSC). Commonly, less than 20% of the lesion is comprised of malignant tumor cells. The microenvironment plays a critical role in supporting tumor growth, metastatic behavior, as well as resistance to conventional chemo- and radiotherapy (10). Hence, an effective treatment for pancreatic

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Translational Relevance

Pancreatic cancer is a devastating disease refractory to treatment due to a desmoplastic stroma that supports tumor growth by the release of growth- and metastasis-promoting factors, and by acting as a physical barrier. In this article, we present a novel designed oncolytic virus armed with stimulators of the CD40 and 4-1BB pathways. Stimulating these pathways initiates multiple actions that support antitumor activity both in the tumor cells and in the microenvironment. Besides inducing oncolysis, the virus changes the biology of tumor cells as well as the stroma cells by reducing factors promoting tumor growth and metastasis. Simultaneously, the armed virus can robustly activate expression of costimulators, cytokines, and chemokines in dendritic cells. The presented virus is currently ongoing clinical investigation for patients with pancreatic cancer at Baylor College of Medicine (Houston, TX; NCT02705196).

cancer needs to consider both the tumor and its microenvironment. Activation of the CD40 pathway has shown promising responses in early clinical trials in multiple indications including pancreatic cancer (11–17). Most studies have focused on the immunostimulatory role of CD40. However, multiple cell types such as epithelial- and endothelial cells express CD40, indicating a broader role of CD40 stimuli (18) and also risk of toxicity. Indeed, systemic activation of the CD40 pathway leads to dose-limiting liver toxicity (11–14). Instead, local intratumoral *CD40L* gene therapy (AdCD40L) evokes tumor-specific immunity that in the end will affect the treated lesion as well as distant metastases and may be a safer option. Local AdCD40L therapy has shown promise in urinary bladder cancer (15) and malignant melanoma patients (16).

We present herein a novel oncolytic serotype 5/35 adenovirus (LOAd703) armed with a designed trimerized, membrane-bound human CD40L (TMZ-CD40L; ref. 19) and full-length human 4-1BB ligand (4-1BBL). While CD40L has documented antitumor activities including CD40-mediated tumor cell apoptosis (18), and adaptive immune activation (15, 20), 4-1BBL is known to enhance immunologic memory and expand natural killer (NK) cells (21, 22). Because of the actions by the virus *per se* and the CD40 pathway combined with 4-1BB signaling, we hypothesize that the LOAd703 virus has a broad mechanism of action by stimulating several arms of the immune system simultaneously to affecting the biological reality of the tumor and its stroma. Hence, the effects of LOAd703 in tumor cells and key cells defining the stroma were evaluated in this article.

Materials and Methods

Virus construction and production

The gene sequence of trimerized membrane-bound (TMZ)-CD40L alone (LOAd700; ref. 19), or in combination with 4-1BBL (LOAd703) separated with a T2A peptide was synthesized with a cytomegalovirus (CMV) promoter upstream of the 5′ gene and adenovirus-flanking regions for homologous recombination into adenoviral vectors at both ends. The construction of the basic LOAd viruses has been described previously (19), and the transgene cassettes were placed post fiber 5/35 region (Supplementary Fig. S1). Viruses were produced by transfection of 293 cells (ATCC) followed by expansion in A549 cells (ATCC). The virus supernatants were purified by CsCl gradient centrifugation and diluted in 20 mmol/L Tris, 25 mmol/L NaCl, and 2.5% glycerol. Viable virus titer was measured in a fluorescence-forming units (ffu) assay (23). The LOAd(−) is the basic LOAd virus without the CMV transgene cassette.

MTS viability assay

Pancreatic cancer cell lines MiaPaCa2, Panc01, PaCa3, and BxPc3 were a kind gift from Dr. Rainer Heuchel (Karolinska Institute, Stockholm, Sweden). Short tandem-repeat analysis of cell lines to confirm identity was performed by the Uppsala Genome Center, Uppsala University (Uppsala, Sweden). MiaPaCa2, Panc01, and PaCa3 were cultured in DMEM GlutaMAX-1 supplemented with 10% FBS and 1% penicillin/streptomycin, whereas BxPc3 was cultured in RPMI1640 medium with the same supplements in addition to 1% HEPES and 0.1% sodium pyruvate. All medium components were purchased from Life Technologies. Upon infection, cells were harvested and washed with serum-free medium and centrifuged. Virus was added to the cell pellet (25–100 MOI) and cells were incubated for 2 hours at 37°C, 5% CO_2. Thereafter, medium with or without 0–1,000 μmol/L gemcitabine was added to dilute the cells to 1×10^5 cells/mL. The cells were plated in 96-well plates in quadruplicates, 100 μL per well. Cell viability was determined at 72 hours using MTS Cell Titer Aqueous One Solution cell proliferation assay (Promega). The experiment was repeated four times. Exocrine pancreatic cells were obtained from four different donors from which the pancreas was obtained for islet cell isolation in a procedure described elsewhere (24). Ethical approval to perform research on the surplus material from such islet cell isolations was obtained from the regional ethics committee in Uppsala, Sweden. Exocrine cells were infected using 100 MOI and analyzed for viability using the MTS assay as described above.

Flow cytometry of tumor cells and HUVEC

Infected (100 MOI) tumor cells were phenotyped using antibodies detecting CD40L and 4-1BBL (clones 24–31 and 5F4, respectively, BioLegend). Briefly, cells were washed in 0.5% BSA in PBS, incubated with antibodies, and washed again. The cell pellet was then resuspended and fixed in 1% paraformaldehyde (PFA) in PBS prior to analysis. HUVEC cells were infected with LOAd viruses (50 MOI) and after 48 hours the cells were harvested and stained with antibodies targeting VCAM-1, ICAM-I, and E-Selectin (clones STA, HA58 and HAE-1f, respectively; BioLegend). The experiments were repeated twice.

Cell preparation for electron microscopy

Panc01 cells, or normal exocrine pancreatic cells, were infected with LOAd703 (100 MOI); 48 hours postinfection, the cells were harvested, centrifuged, and resuspended in fixation solution from Vironova. The electron microscopy was then performed by Vironova's service facility.

Dendritic cell activation and CMV-specific T-cell expansion

$CD14^+$ monocytes were obtained from the peripheral blood mononuclear cell (PBMC) fraction of healthy donor buffy coats (Blood bank Uppsala University Hospital, Uppsala, Sweden) after Ficoll-Paque gradient centrifugation (GE Health Care) and $CD14^+$ magnetic beads MACS sorting (Miltenyi Biotec). The

monocytes were differentiated into immature DCs by culturing in RPMI medium described above with the addition of 0.2% beta-mercaptoethanol, GM-CSF (150 ng/mL) and IL4 (50 ng/mL; Gentaur) for 6 days. Immature DCs were then infected with LOAd viruses (50 MOI). After 48 hours, supernatants were harvested and stored at −80°C, and the cells were analyzed by flow cytometry after staining with antibodies recognizing CD1a, CD40L, 4-1BBL, CD83, CD86, and CD70 (clones HI149, 24–31, 5F4, HB15e, IT2.2 and 113–16, respectively, BioLegend). The $CD1^+$ cells were gated as DCs, and expression of markers determined within this population. The supernatants were thawed and analyzed for the presence of cytokines using luminex methodology (Milliplex MAP kit, Millipore) and by a custom 233-analyte ProSeek array using the company's analysis service (Olink Proteomics). The experiment was repeated using five different donors. Similarly, human immature DCs from CMV-positive donors (HLA-A*02:01) were generated by the 6-day culture as mentioned above. The DCs were infected with LOAd viruses (50 MOI) or stimulated with TNFα (40 ng/mL) and Poly IC (30 μg/mL; Sigma-Aldrich). At day 1, the DCs were pulsed for 4 hours with CMV $pp65_{495\text{-}503}$ NLVPMVATV peptides (Genscript) in the presence of β2 microglobulin (Sigma-Aldrich). The pulsed DCs were then mixed with autologous PBMC (CD14-negative fraction) in a 1:10 DC to PBMC ratio and cultured for 11 days. The cells were then harvested and manually counted, stained with antibodies targeting CD3 and CD8 (clones SK7 and RPA-T8, respectively; BioLegend), Tetramer-PE-HLA-A*02:01 CMVpp65 or negative iTAg Tetramer-PE-HLA-A*02:01 (MBL International), and analyzed by flow cytometry. The experiment was repeated with three donors.

Stellate cell analysis

Stellate cells isolated from the pancreas were purchased from 3H Biomedical (Uppsala, Sweden) and cultured using Stellate Cell Medium provided by the manufacturer. The cells were expanded on poly-L-lysine (PLL)-coated plates prior to experiments (PLL purchased from the vendor). The cells were plated (1×10^5 cells/well) in a flat-bottom 96-well plate (triplicates and two identical plates) and after 1 hour, LOAd viruses were added to the wells (25 MOI). At 48 hours, the plates were centrifuged, the supernatants removed, and cells were harvested for protein lysates. Protein lysates were prepared from one of the plates by adding RIPA lysis buffer (50 mmol/L Tris-HCl pH7.4, 150 nmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mmol/L PMSF, and 10% protease inhibitor cocktail from Sigma). The plates were vortexed and kept on ice for 30 minutes. The lysates were centrifuged at 10,000 × *g* for 15 minutes at +4°C, and the supernatants free from cell debris were stored at −20°C. The protein concentration was determined by Coomassie Plus Protein Assay Reagent (Thermo Fisher Scientific). Protein lysates were stored in −80°C until analysis using a 233-analyte ProSeek array performed by vendor service (Olink Proteomics).

Animal models

Animal experiments were approved by the respective local animal ethics committee and were performed at Uppsala University (Uppsala, Sweden), IDIBELL (Barcelona, Spain), and Adlego AB (Stockholm, Sweden). Human Panc01 cells (5×10^6 cells/mouse) were injected subcutaneously in C57BL/6 Nu/Nu mice. Tumors were monitored for weight and tumor growth. Mice were treated with six peritumoral injections of LOAd viruses ($1–10^{10}$ to 5×10^{11} VP/injection) or PBS-negative control. When gemcitabine was given, mice received 25 mg/kg by intraperitoneal injection, 1× per week. For biodistribution, mice were treated with a single peritumoral virus injection (5×10^{11} VP). At day 2 and 7, mice were euthanized and biopsies taken, snap-frozen, and stored in −80°C until analysis with quantitative real-time PCR.

LOAd detection using quantitative PCR

LOAd DNA was isolated from frozen tissue specimens. First, frozen samples were mashed on dry ice and incubated with buffer ATL (Qiagen) and proteinase K (HoffmannR-La Roche). Serum was incubated with binding buffer (High Pure Viral Nucleic Acid Kit, HoffmannR-La Roche) and proteinase K. DNA was extracted using the High Pure Viral Nucleic Acid Kit for both biopsies and serum. The DNA concentration was then determined using NanoDrop. Dilutions were made and 50 ng/well (biopsies) or 10 ng/well (serum) were mixed with primers specifically recognizing the LOAd genome and reaction buffers. SYBR Green Supermix was used and amplification reactions were done using the Bio-Rad CFX96 and the analysis was performed using the CFX Manager Software (Bio-Rad Laboratories). As a positive control, a spiked liver tissue sample (1×10^7 VP to 15 mg liver specimen) was used while nontemplate samples were used as negative controls. A standard curve was prepared by serial dilution of LOAd703 virus after DNA extraction. All samples, standards, and controls were run in triplicates.

Statistical evaluation

Statistical calculations were performed using GraphPad Prism (GraphPad Software). For sample sizes ≥7 Gaussian distributions were tested using D'Agostino-Pearson omnibus normality test. If populations were normally distributed, parametric testing was used such as Student *t* test comparing two groups or one-way ANOVA with Tukey multiple comparison test for several groups. If Gaussian distribution was not assumed, two populations were compared using Mann–Whitney testing for cohorts with low variation while Student *t* test with Welsh correction was used for smaller cohorts with large variation. For evaluation of several populations, ANOVA for nonparametric samples was used (Kruskal–Wallis test with Dunn multiple comparisons). For evaluations of several populations with multiple variables, two-way ANOVA with Dunnett multiple comparisons test was used. All testing used a confidence interval of 95% and a P value <0.05 was considered significant.

Results

Selective replication leads to oncolysis of pancreatic cancer

Three oncolytic serotype 5/35 adenoviruses were constructed. LOAd700 contains a CMV promoter–driven transgene cassette with TMZ-*CD40L*, whereas LOAd703 contains a transgene cassette with both TMZ-*CD40L* and full-length *4-1BB ligand* (*4-1BBL*). LOAd(−) is a control virus lacking a transgene cassette. A panel of pancreatic cancer cell lines was infected with the LOAd viruses to evaluate tumor-restricted oncolysis. Tumor cells were efficiently killed in a 72-hour MTS assay (Fig. 1A) but replication was notable already after 48 hours (Supplementary Fig. S2). The standard-of-care treatment for pancreatic cancer is the nucleoside analogue gemcitabine. Gemcitabine concentration was titrated (1–1,000 μmol/L), and more than 10 μmol/L did not significantly alter the effect (Supplementary Fig. S3). In Fig. 1A, gemcitabine significantly reduced tumor cell viability compared with control

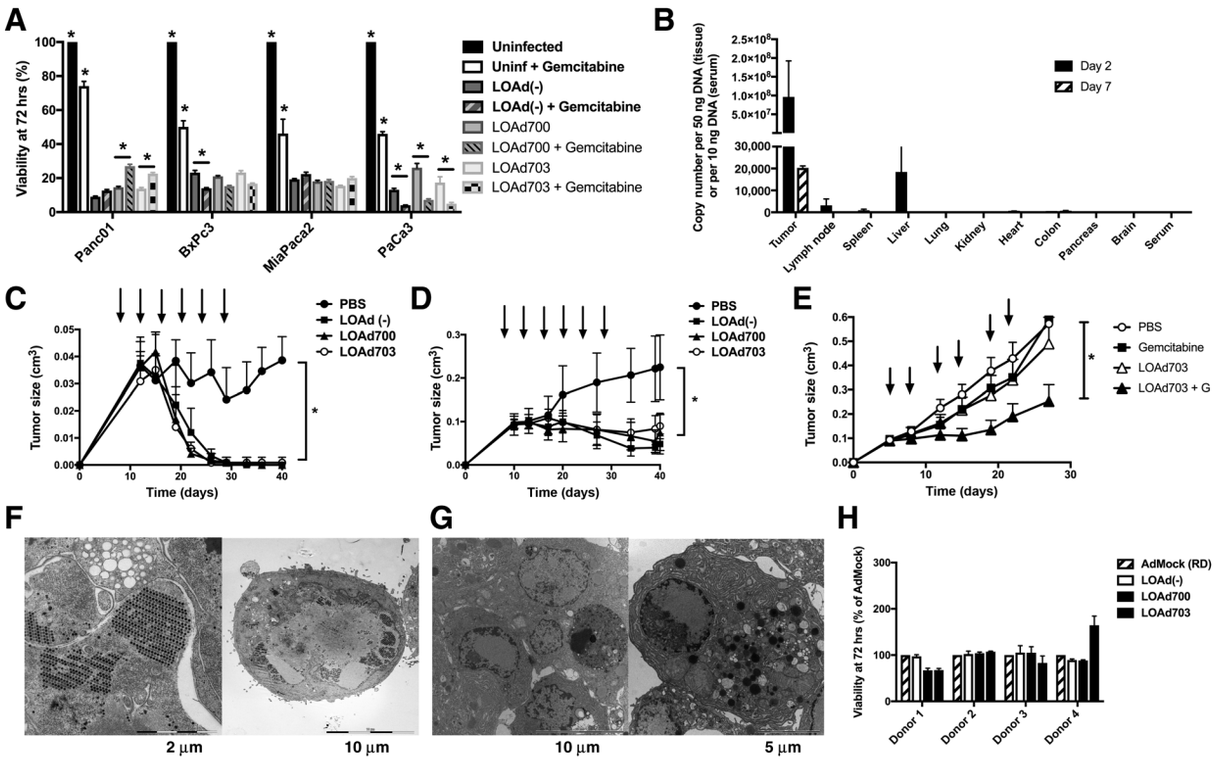

Figure 1.

LOAd703 replication *in vitro* and *in vivo*. **A,** Viability evaluated by MTS assay in LOAd-infected pancreatic cell lines ± 10 μmol/L gemcitabine (quadruplicate samples/line). **B,** The Panc01 xenograft model was utilized to treat established tumors with one peritumoral injection of 5×10^{11} VP LOAd703. Biopsies were taken to evaluate virus biodistribution by quantitative real-time PCR ($n = 2$/group and every sample run in duplicate). The model was then used to treat small (**C**) and large (**D**) tumors with six peritumoral injections of PBS or LOAd viruses ($n = 6$/group). **E,** The Panc01 xenograft model was used to treat established tumors with biweekly intratumoral injections of LOAd703 (1×10^{10} VP) for 3 weeks with or without weekly intraperitoneal gemcitabine (25 mg/kg). **F,** Electron microscopy demonstrating replicating LOAd703 in Panc01 cells. **G,** Electron microscopy demonstrating LOAd703-infected normal exocrine cells isolated from pancreas (donor 4). **H,** Viability of LOAd-infected donor-isolated normal exocrine pancreatic cells ($n = 4$; quadruplicate samples/line) evaluated by MTS assay. A replication-defective (RD) virus was used as a control of overall viability. Statistical calculations were done using a two-way ANOVA with Dunnett multiple comparisons test. Differences in survival were evaluated using the log-rank test.

cultures, but was not as effective as LOAd viruses. When gemcitabine was added to LOAd-infected tumor cells, the cells were still killed at the same rate, or significantly better as noted for Panc01, BxPc3, and PaCa3.

Next, the *in vivo* capacity of LOAd oncolysis was investigated. Established human Panc01 tumors in immunodeficient mice were treated with a single peritumoral injection of LOAd703 virus. After 2 and 7 days, respectively, mice were sacrificed and biopsies were analyzed for the presence of virus particles to determine biodistribution. Highest level of LOAd703 DNA was seen in the tumor biopsies and to lower extent in the liver and lymph nodes at day 2. At day 7, virus particles were mainly found in the tumor biopsies (Fig. 1B). Repeated (6×) intratumoral injections of LOAd viruses could abolish *in vivo* growth of human Panc01 tumors when treatment started upon tumor detection (Fig. 1C). To challenge the virus capacity, larger tumors were treated. However, the LOAd viruses could still significantly control tumor growth (Fig. 1D). Large tumors were then treated with six injections of a reduced virus dose with or without weekly administration of gemcitabine. Gemcitabine, or LOAd703, modestly decreased progression. However, the combination of gemcitabine and LOAd703 resulted in significant reduced overall growth rate (Fig. 1E).

As adenoviruses with serotype 35 knobs poorly infect, or replicate, in murine cells, potentially harmful replication of the virus in normal cells cannot be evaluated in mice. Hence, to demonstrate the restricted replication in tumor cells, Panc01 cells and donor-isolated normal exocrine pancreatic cells were infected with LOAd viruses. In Fig. 1F, replication in Panc01 is demonstrated by electron microscopy. The nucleus was disrupted and the morphology severely altered. Newly synthesized virus particles were present in large paracrystalline arrays or scattered in the cell. However, virus replication was not detected in normal exocrine cells, although an occasional particle could be seen demonstrating virus infection (Fig. 1G). Cell viability was evaluated in four normal donors using a MTS assay, and it was confirmed that normal exocrine cells were not affected by the virus (Fig. 1H).

Virus infection regulates tumor-promoting proteins

LOAd700-infected tumor cells expressed the TMZ-CD40L transgene while LOAd703-infected cells expressed both TMZ-CD40L and 4-1BBL (Fig. 2A). To investigate the role of virus

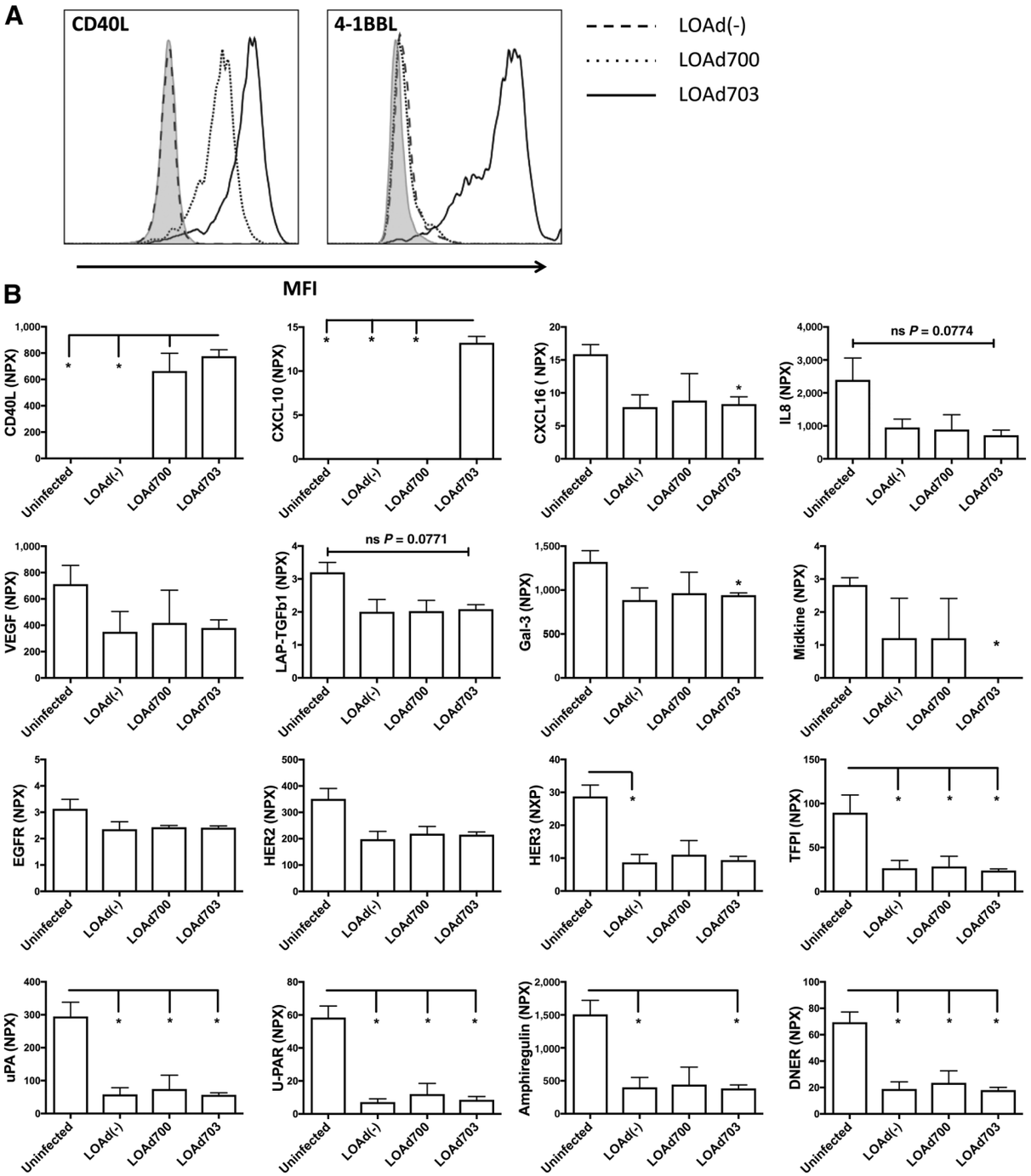

Figure 2.

LOAd-infected tumor cell proteomics. **A,** CD40L and 4-1BBL transgene expression in MiaPaCa2 cells infected with LOAd(−), LOAd700 or LOAd703 shown by flow cytometry. Isotype control is demonstrated by a filled curve. **B,** Proteomic profile of MiaPaCa2 cells (uninfected) or infected with LOAd(−), LOAd700 or LOAd703 as evaluated by ProSeek (duplicates) and relative values are plotted as NPX. Statistical calculations were done using one-way ANOVA with Tukey multiple comparisons test. A single asterisk (*) represents significance either using *t* test if Gaussian distribution was confirmed by D'Agostino–Pearson omnibus normality test, or using *t* test with Welch correction. Both methods compared differences with uninfected.

infection and the transgenes in the tumor cells before oncolysis occurred, cell lysates made from infected cells (25 MOI) at 48 hours postinfection were analyzed by a proteomic array. Most analytes were not present or were unchanged compared with uninfected tumor cells. Markers of interest are shown in Fig. 2B. CD40L expression could be confirmed in LOAd700- and

LOAd703-infected tumor cells using the proteomic array. Interestingly, CXCL10, known to attract T cells, was upregulated by LOAd703 while CXCL16, known to promote invasiveness, was significantly downregulated. Virus infection tended to reduce the angiogenesis promoter IL8, while the VEGF was not significantly altered. TGFβ is released as a latent form complexed with latency-associated peptide (LAP) and latent TGFβ binding protein (LTBP). In the tumor milieu with increased proteases (e.g., plasmin and matrix metalloproteinases) and reactive oxygen species (ROS), the LAP–TGFβ complex is resolved which releases the active form of TGFβ (25). The pancreatic cancer cells tended to reduce LAP-TGFβ expression after virus infection at the same time significantly downregulating the enzyme uPA and its receptor (uPAR) that converts plasminogen to plasmin in the vicinity of the tumor cell (26). Galectin-3 (Gal-3) is a beta-galactoside–binding protein that promotes cell proliferation via activation of the Ras pathway in pancreatic cancer cells with mutated Ras (27). In our analysis, LOAd703 significantly downregulated Gal-3. Midkine was also significantly reduced by LOAd703 infection. Midkine promotes cell proliferation and migration in pancreatic cancer (28, 29) and is upregulated upon EGF signaling (28). However, the EGF receptor (EGFR) itself was not altered by virus infection. HER-2 and HER-3 were also present in the tumor cells and HER-3 was significantly downregulated by LOAd(-) infection. Interestingly, delta/notch-like EGF-related receptor (DNER) was expressed by the tumor cells and was significantly reduced by all LOAd viruses upon infection. Tissue factor pathway inhibitor (TFPI) and amphiregulin, both associated to enhanced progression and invasiveness in pancreatic cancer (30, 31), were also significantly downregulated by the LOAd viruses. Hence, prior to oncolysis, the infected tumor cells may reduce growth rate, metastatic behavior, and affect the surrounding tumor microenvironment.

LOAd700 and LOAd703 modulate key regulators of the tumor microenvironment

As most of the pancreatic tumor lesions consist of nonmalignant cells, the LOAd703 virus will likely infect more stroma cells than actual tumor cells. Because of the CMV promoter, all infected cells will express the transgenes independently of virus replication that only occurs in malignant cells. The role of LOAd viruses and their transgenes were therefore evaluated in pancreatic stellate cells and in human umbilical vein endothelial cells (HUVEC). CD40L was detected in both LOAd700- and LOAd703-infected stellate cells as expected (Fig. 3). Stellate cells also expressed CD40, but in cells expressing CD40L, the detection was significantly decreased which can be due to CD40/CD40L binding. Similarly, 4-1BB was increased upon LOAd700 infection but in the LOAd703 group that expresses the 4-1BBL, the presence of 4-1BB was decreased. Stellate cells express several factors that stimulate cell division such as FGF5, amphiregulin, Gal-3, and TWEAK (10, 27, 30). All these were significantly decreased using either LOAd700 or LOAd703 suggesting CD40L was the promoting component. Placental growth factor (PlGF) was recently shown to drive obesity-induced tumor progression via induction of tumor-associated macrophages and angiogenesis (32). Herein, LOAd700 and LOAd703 infection of stellate cells reduced PlGF significantly. Other angiogenic factors such as IL8 and VEGF-A were not altered. TGFβ and IL6 are involved in collagen production and suppression of immune activation via STAT-3 phosphorylation (33). Both TGFβ and collagen type I were significantly reduced in the pancreatic stellate cells by the LOAd viruses, while IL6 was similarly produced independently of virus infection.

Factors that promote metastasis and mobility in pancreatic cancer such as Spp-1 (34), hepatocyte growth factor (HGF), uPA and tPA were also produced by the stellate cells but downregulated after virus infection (10, 28). However, epithelial cell adhesion molecule (EpCAM) was upregulated. Another adhesion molecule, junctional adhesion molecule-A (JAM-A), was also increased and low level of JAM-A has been connected to poor prognosis in pancreatic cancer (35). Furthermore, the tumor suppressor SIRT-2 (36) tended to increase after virus infection. The transgenes induced CCL20 and CXCL10, both involved in the recruitment of lymphocytes and DCs. CXCL6, recruiting neutrophilic granulocytes, was insignificantly decreased.

Virus-infected HUVEC cells were investigated for their expression of molecules involved in lymphocyte attachment and transmigration. Postinfection, all LOAd viruses enhanced VCAM-I, ICAM-I, and E-selectin. Both LOAd700 and LOAd703 were more potent than LOAd(−) to upregulate these markers (Fig. 4).

LOAd703 shows superior capacity to activate immune responses

To evaluate the immunostimulatory function of LOAd viruses, human monocyte–derived immature DCs from five healthy blood donors were infected and analyzed for activation markers (Fig. 5A). First, phenotypes were confirmed by flow cytometry. LOAd703-infected DCs were viable and expressed both CD40L and 4-1BBL while LOAd700-infected DCs expressed CD40L only. LOAd(−)-infected cells did not differ from uninfected DCs in regard to transgene expression. The virus could by itself increase CD86 expression on DCs, but only LOAd700 and LOAd703 induced expression of the maturation markers CD83 and CD70. Second, the supernatants were analyzed for release of cytokines. LOAd703 induced a robust T helper (Th) 1-type cytokine profile (Fig. 5B). DCs stimulated by any virus could drive proliferation of antigen-specific T cells as good as positive control–stimulated DCs as demonstrated in an *in vitro* CMV-specific T-cell model in which the DCs were differentiated from CMV^+ blood donors and then cocultured with autologous unsorted peripheral blood mononuclear cells (PBMC). While the total number of viable cells in our cocultures was higher in the group using LOAd703-stimulated DCs (Fig. 5C), the total number of T cells or CMV-specific T cells, tended to be lower than in the positive control group (Fig. 5D and E). However, in-depth flow analyses showed that the LOAd703 virus had simultaneously expanded NK cells present in the PBMCs (Fig. 5F). To obtain a broader view of the different DC signatures activated by LOAd viruses, the supernatants were evaluated by a proteomic array and analyzed with a focus on immune markers. The LOAd(−) virus induced expression of chemokines and midkine while reducing expression of the TNF-like weak inducer of apoptosis (TWEAK) compared with uninfected cells (Fig. 6A and B). LOAd700-infected DCs showed a further increase of chemokines but also expression of a battery of cytokines and costimulatory molecules (Fig. 6C). TWEAK was even further reduced. When both TMZ-CD40L and 4-1BBL were expressed in LOAd703-infected DCs, the cytokine expression was further increased together with costimulatory molecules (Fig. 6D). MICA, a MHC-like molecule that can activate NK cells (37), was also upregulated by LOAd703. TWEAK was still further reduced, and the level was significantly lower than in

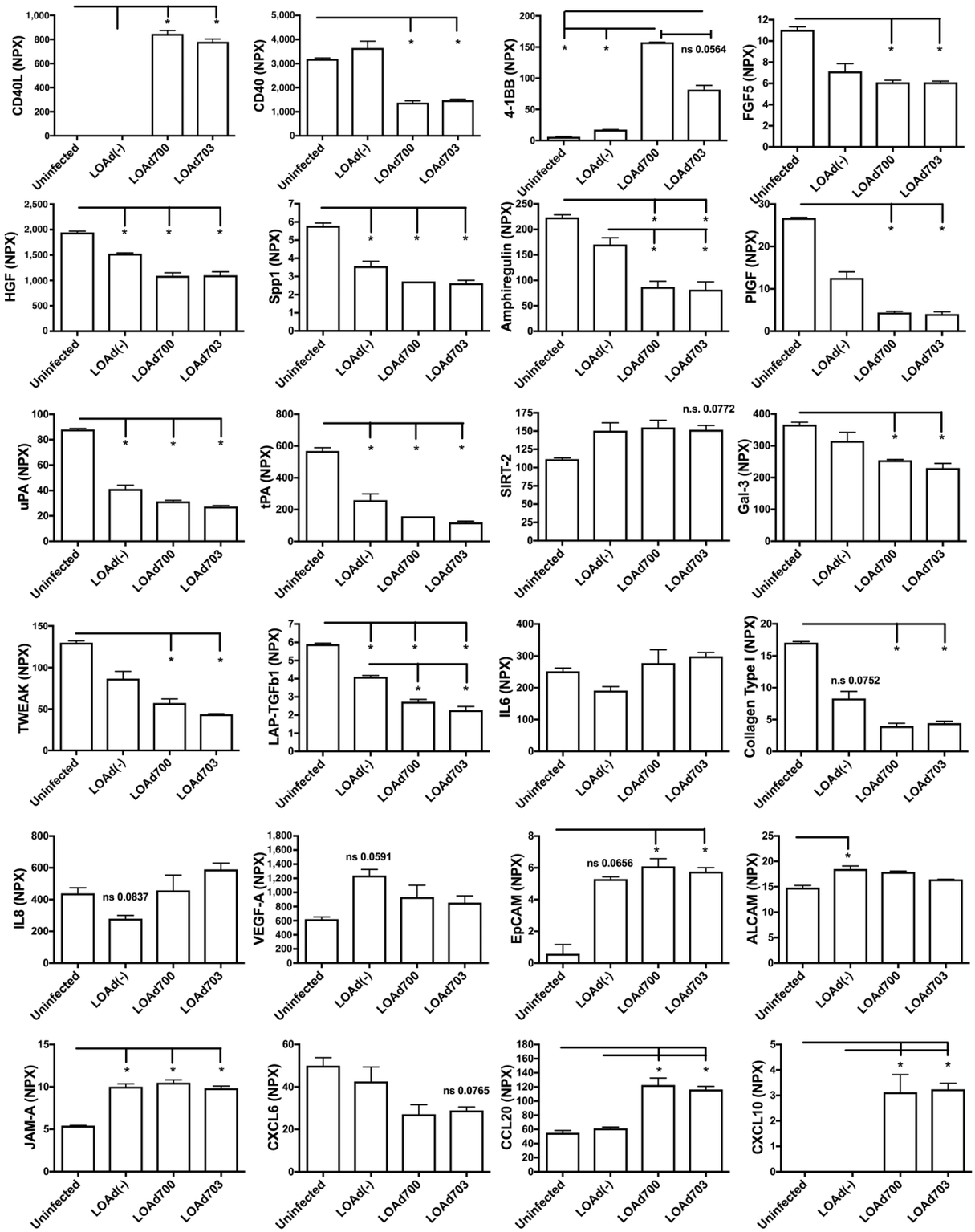

Figure 3.

The LOAd effect on stellate cells. Human pancreatic stellate cells were infected with LOAd viruses (triplicates) and harvested at 48 hours for lysate preparations. The lysates were analyzed by ProSeek proteomics (duplicates) and relative values are plotted as NPX. Statistical calculations were performed using *t* test with Welch correction.

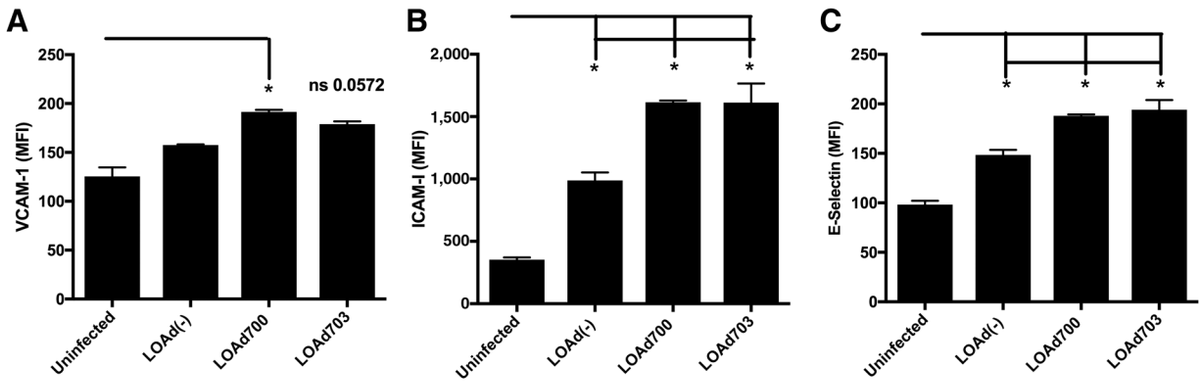

Figure 4.

Endothelial cell activation after LOAd infection. HUVEC cells were infected with LOAd viruses and at day 2, the cells were harvested and analyzed by flow cytometry for the markers VCAM-1 (**A**), ICAM-I (**B**), and E-Selectin (**C**). The experiment was repeated twice. Statistical calculations were performed using *t* test with Welch correction.

the LOAd700 group. Noteworthy, PDL1 was expressed by both LOAd700- and LOAd703-infected DCs.

Discussion

Pancreatic cancer shows a remarkable resistance to various chemotherapeutic strategies as well as to irradiation. The resistance is due to both the biological feature of the tumor cells and the supporting stroma but also to physical barriers such as the dense nature of the tumor stroma, dysregular blood vessels and high interstitial fluid pressure (10). Both *in vitro* and *in vivo*, the LOAd viruses presented herein were able to replicate and kill pancreatic cancer cells via oncolysis. As chemotherapy may

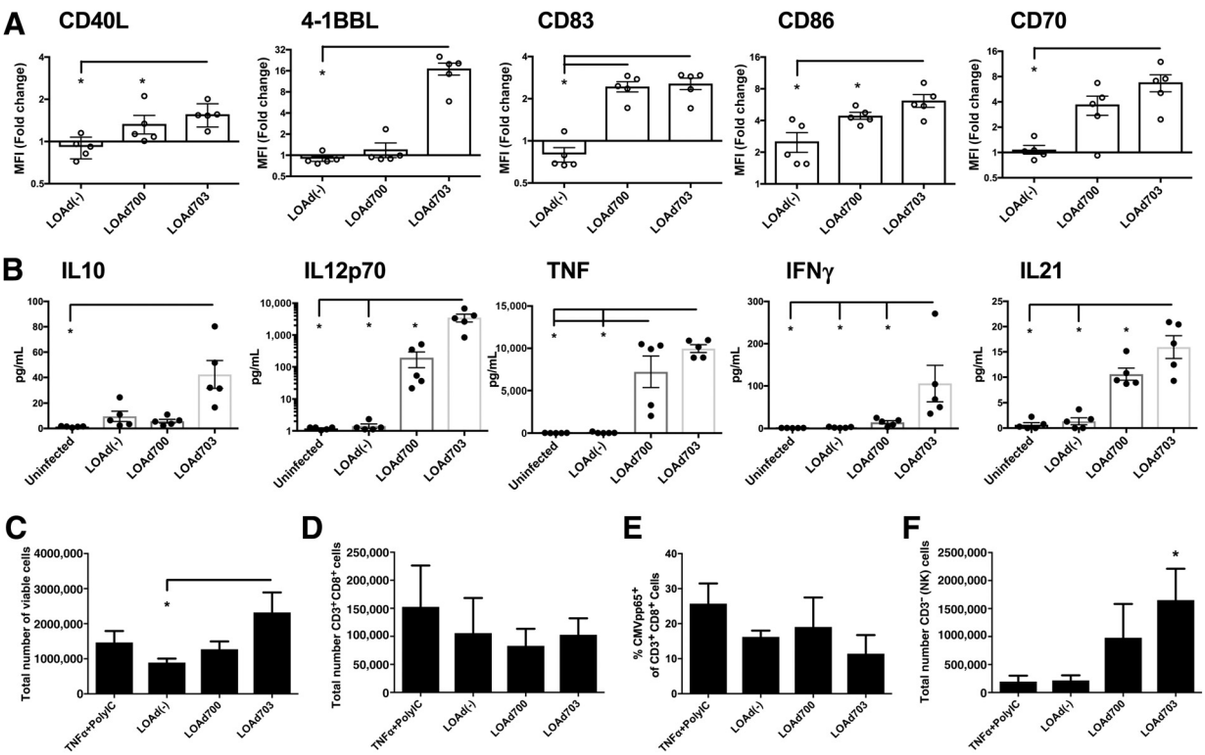

Figure 5.

LOAd-activated DCs promote T and NK-cell expansion. **A,** Human DCs ($n = 5$) were infected with LOAd viruses and phenotyped by flow cytometry. Fold change from uninfected cells is shown. **B,** The supernatants were analyzed for cytokine release using Milliplex MAP. LOAd-activated DCs pulsed with CMVpp65 peptides were cocultured with PBMCs ($n = 3$) to expand CMV-reactive T cells. Total expanded cell number (**C**), total number of $CD3^+CD8^+$ cells (**D**), percentage of CMVpp65 of $CD3^+CD8^+$ cells (**E**), as well as total number of $CD3^-CD8^+$ (NK) cells are shown (**F**). Statistical calculations were done using Kruskal–Wallis test with Dunn multiple comparisons test. A single asterisk (*) represent significance by Mann Whitney test (**A-B**) or t-test with Welsh correction (**C–F**) compared with control group.

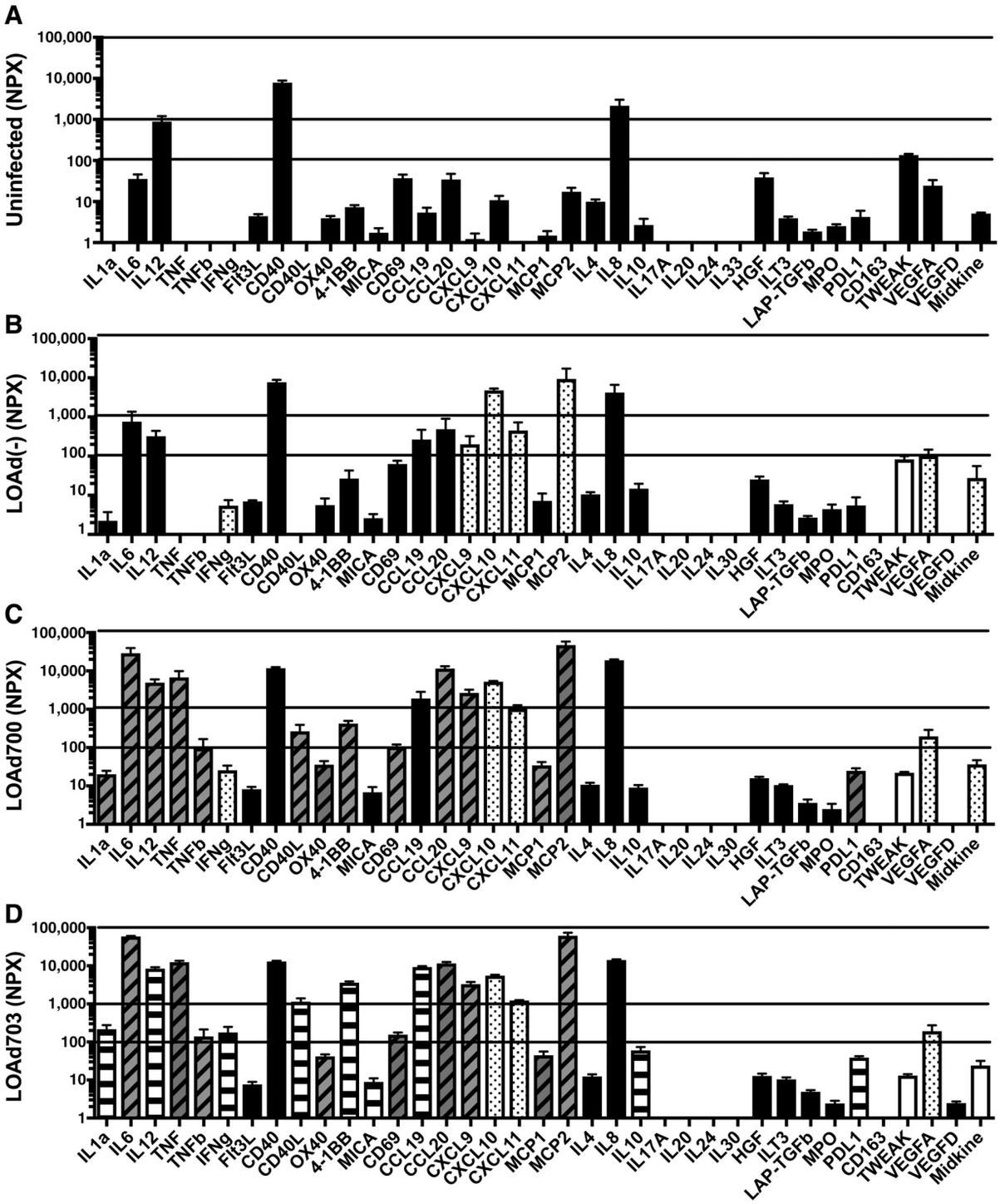

Figure 6.

DC proteomics after LOAd infection. Supernatants from human DCs ($n = 5$) infected with LOAd viruses were analyzed by ProSeek proteomics, and relative values are plotted as NPX. The frequencies of immune modulators were shown in uninfected DCs (**A**), DCs infected with empty control LOAd(−) virus (**B**), LOAd700 (**C**), or LOAd703 (**D**). Statistical calculations were done using Mann–Whitney test (**A** vs. **B**, **B** vs. **C**, and **C** vs. **D**). Black bars indicate no difference from uninfected control, dotted (light gray) bars indicate a significantly higher value than uninfected control, angled lines (dark gray) indicate a significantly higher value compared with LOAd(−) group, horizontal lines (white) indicate a significantly higher value compared with LOAd700, while a white bar indicates a decreased value from the previous graph.

interfere with cell functions, it may as well block the function of a virus therapeutic. We therefore investigated whether the standard of care for pancreatic cancer, gemcitabine, would enhance or possibly block oncolysis by LOAd703. Both *in vitro* and *in vivo*, gemcitabine could be used in combination with LOAd viruses to further reduce tumor viability. Oncolytic viruses may not be restricted by the common resistance mechanisms as they are regarded as live biological therapeutics rather than small-molecule drugs. Indeed, many clinical trials using oncolytic viruses are ongoing and demonstrate promising results in cancer patients (17, 38–43). Talimogene laherparepvec has been subjected to phase III trials and is already approved for the treatment of malignant melanoma (41). This medicinal product is a live herpes simplex virus unlike the adenoviruses presented herein. However, just like the LOAd viruses, talimogene laherparepvec is armed with an immune stimulator (granulocyte macrophage-colony stimulating factor; GM-CSF) to enhance antitumor immunity.

It is not possible to evaluate the immunologic capacity of LOAd703 *in vivo* as the LOAd viruses have poor murine cell infectivity and human CD40L do not cross-react with murine CD40 (19). The immunologic mechanisms of action were, hence, further evaluated in human *in vitro* models to understand the potential of the transgenes. We have previously shown that CD40L-based gene therapy induces typical Th1 cytokines such as IL12 and IFNγ as well as T-cell infiltration into tumors in mouse models (44) and in clinical trials (15, 16). Similarly, Diaconu and colleagues evaluated another oncolytic adenovirus carrying wild-type CD40L and showed that antigen-presenting cells also were increased along with T-cell infiltration and a Th1-type of cytokine pattern (42). In the current study, human DCs were infected with the LOAd viruses to evaluate the effect of the virus backbone and the addition of TMZ-CD40L or the addition of both TMZ-CD40L and 4-1BBL. The virus *per se* increased CD86 and chemokines but did not significantly increase cytokine production but IFNγ and IL6 that tended to increase. These data indicate that even if adenoviruses can activate immune reactions, there is clearly a need to potentiate the responses with immunostimulatory transgenes to optimize the chance of evoking antitumor activity. Of note, virus infection increased VEGF-A, and the level of this angiogenic stimulator remained in the presence of the transgenes indicating that LOAd virus therapy may be of interest to combine with anti-VEGF therapy. In comparison with the nonarmed virus control, LOAd700 generated a broader response in the DCs with increased expression of costimulatory molecules and cytokines as well as upregulation of chemokines. LOAd703 showed a similar broad activation but even further increased the expression level of important Th1 factors such as IL12, IFNγ, IL21, and CD70. Interestingly, chemokines including CCL19 that is known to attract DCs and central memory T cells (45) were also increased. Any LOAd-infected DCs could stimulate proliferation of antigen-specific T cells but LOAd703-infected DCs expanded NK cells as well, which is likely due to 4-1BBL as it is a known potent stimulator of NK-cell expansion (21, 22). Similarly, an article by Galivo and colleagues demonstrated that adenoviruses with CD40L can stimulate T-cell responses against tumor antigens, and not only to adenoviral antigens which unfortunately was the case when using a vesicular stomatitis virus (46). PDL1 was also significantly upregulated on LOAd700- or LOAd703-infected DCs, which is a common event during activation to restrain responses. If this is limiting the efficacy, LOAd therapy can be combined with anti-PD1 antibody therapy.

An obstacle for effective immunotherapy of solid malignancies is the barrier preventing T-cell infiltration into the tumor parenchyma. The LOAd viruses tackle this by several mechanisms of action, likely due to the immunogenicity of the virus itself, and others due to the transgenes. First, adenoviruses are immunogenic viruses as they can stimulate Toll-like receptors (TLRs) and yet undefined cytosolic receptors in antigen-presenting cells leading to IFN type I responses (47). This activation is not dependent whether LOAd703 is infecting a tumor cell or a normal cell including immune cells infiltrating the tumor lesions. Second, the two transgenes, TMZ-CD40L and 4-1BBL will be expressed independently of replication as they are driven by a promiscuous CMV promoter. CD40 stimulation activates NFκB, JNK, and the p38/MAPK pathways that regulate multiple genes involved in cell biological processes and immune activation (cytokines, chemokines, costimulatory proteins; ref. 48) These pathways are shared with 4-1BB activation (49). Chemokines are needed to attract the right set of immune cells to the tumor area. Our results demonstrate that both infected tumor cells, DCs, and stellate cells upregulated chemokines important to attract T cells, NK cells, and DCs. LOAd703 also induced CCL19 that attracts central memory T cells, which may be crucial for long-term responses (45). In previous work using CD40L-based gene therapy we, and others, have shown that CD40L can revert the suppressive environment toward immune activation and lymphocyte infiltration (19, 42, 44–46, 50). The studies presented in this article suggest that the combination of TMZ-CD40L and 4-1BBL is even further promoting Th1 immunity by enhanced cytokine production and costimulatory molecules. Third, the tumor endothelium is commonly dysregulated and has poor expression of receptors that trigger T-cell attachment, rolling, and transmigration (51). Both LOAd700 and LOAd703 robustly induced an increase of such receptors in infected endothelial cells and this effect was likely CD40-mediated considering that LOAd700 and LOAd703 were equally effective.

The dense stroma in pancreatic cancer is characterized by an abnormally high level of collagen type I as well as the presence of immunosuppressive cells and inhibitory cytokines (10). Stellate cells are defining the desmoplastic microenvironment in pancreatic cancer and are fibroblast-like cells (10). Interestingly, virus infection *per se* downregulated the presence of many factors that otherwise promote stellate cell survival, division, and capacity to support the malignant cells. This may be due to the E2F-binding sites in the LOAd genome but also to TLR stimulation due to the virus backbone. Nevertheless, the stellate cells were CD40-positive and upon CD40 activation they also expressed 4-1BB. Both TMZ-CD40L and 4-1BBL could further potentiate the effect of virus infection by downregulating FGF5, Gal-3, amphiregulin, PlGF, and HGF. Furthermore, collagen type I was significantly decreased by both LOAd700 and LOAd703, which may be connected to the simultaneous decrease of TGFβ that is known to promote collagen production by stellate cells (52). On the other hand, the inflammatory trigger upon CD40 activation upregulated chemokines on the stellate cells. The stellate cell data suggests that these viruses have the potential to modulate the tumor microenvironment, which may even potentiate treatment with conventional therapeutics. However, *in vitro* data cannot fully mimic a viable tumor and it remains to be investigated how the LOAd703 virus can modify a growing tumor in patients.

Taken together, the triple combination of an adenovirus, TMZ-CD40L and 4-1BBL initiates and regulates many signaling

pathways that can affect both the tumor and shape its microenvironment. LOAd703 showed superior efficacy compared with LOAd700 considering the plethora of investigated parameters. A clinical trial is ongoing to investigate the safety and efficacy of repeated LOAd703 intratumoral injections combined with standard of care in patients diagnosed with pancreatic cancer not eligible for surgery (NCT02705196).

Disclosure of Potential Conflicts of Interest

J. Leja-Jarblad and G. Ullenhag are consultant/advisory board members for Lokon Pharma AB. R. Alemany reports receiving other commercial research support from Lokon Pharma AB. A. Loskog reports receiving commercial research grants from Lokon Pharma, and is a consultant/advisory board member for NEXTTOBE and Olink Proteomics. No potential conflicts of interest were disclosed by the other authors.

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Other (executed laboratory work): J. Leja-Jarblad

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