Title

Immune response is an important aspect of the anti-tumor effect produced by a CD40L-encoding oncolytic adenovirus

Running title:

Oncolytic adenovirus coding for CD40L

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Abstract

Oncolytic adenovirus is an attractive platform for immunotherapy because virus replication is highly immunogenic and not subject to tolerance. Although oncolysis releases tumor epitopes and provides costimulatory danger signals, arming the virus with immunostimulatory molecules can further improve efficacy. CD40 ligand (CD40L, CD154) induces apoptosis of tumor cells and triggers several immune mechanisms, including a T-helper type 1 (Th1) response which leads to activation of cytotoxic T-cells and reduction of immune suppression. In this study, we constructed a novel oncolytic adenovirus, Ad5/3-hTERT-E1A-hCD40L, which features a chimeric Ad5/3 capsid for enhanced tumor transduction, an hTERT promoter for tumor selectivity, and human CD40L for increased efficacy. Ad5/3-hTERT-E1A-hCD40L significantly inhibited tumor growth in vivo via oncolytic and apoptotic effects, and Ad5/3-hTERT-E1A-hCD40L-mediated oncolysis resulted in enhanced calreticulin exposure and HMGB1 and ATP release, which were suggestive of immunogenicity. In two syngeneic mouse models, murine CD40L induced recruitment and activation of antigen presenting cells, leading to increased IL-12 production in splenocytes. This effect was associated with induction of the Th1 cytokines interferon-γ, Rantes, and tumor necrosis factor-α. Tumors treated with Ad5/3-CMV-mCD40L also displayed an enhanced presence of macrophages and cytotoxic CD8+ T cells, but not B-cells. Together, our findings demonstrate that adenoviruses coding for CD40L mediate multiple anti-tumor effects, including oncolysis, apoptosis, induction of T-cell responses, and upregulation of Th1 cytokines.

PRÉCIS: Findings detail the development of a new generation of oncolytic adenovirus that is armed with CD40L, which results in the induction of a Th1-type immune response that causes accumulation of cytotoxic T-cells at the tumor site and increased anti-tumor efficacy.
Introduction

Oncolytic adenoviruses have shown safety in clinical trials and some efficacy has also been seen (1-4). Importantly, it has been discovered that immunological factors are critical with regard to efficacy of oncolytic viruses (5) and some investigators consider them a sophisticated form of immunotherapy (6). However, clinical and preclinical results show that treatment with unarmed oncolytic viruses is usually not immunostimulatory enough to result in sustained therapeutic immune response (5). In this regard, oncolytic viruses can be armed with immunostimulatory molecules. Moreover, viral replication and expression of immunomodulatory proteins within the tumor potentiates the immune system by inducing cytokine production and release of tumor antigens (7).

CD40L is a type II transmembrane protein expressed predominately on CD4^+ T-cells and it binds to the CD40 receptor on antigen-presenting cells (APCs) (8, 9). CD40 is expressed on macrophages and dendritic cells (DCs) where its activation by CD40L leads to antigen presentation and cytokine production followed by T-cell priming and a strong innate immune response (10). Interactions between CD40L and its receptor CD40 provide critical costimulatory signals that trigger T-lymphocyte expansion (8), and increase IL-12 production which is required for the engagement of cytotoxic T lymphocytes (CTL) in the anti-tumor immune response (11, 12). Previous reports indicate that recombinant soluble protein CD40L (rsCD40L) has direct effects in suppression of tumor cell proliferation in vitro (13, 14) and in vivo (15, 16). Other direct effects of rsCD40L are stimulation of survival signaling pathways and induction of apoptosis in carcinoma cells (15, 17). Clinical trials performed with rsCD40L have generally been safe, and while there are many examples of patients benefiting from treatment, the overall level of activity has not been high enough to result in successful
phase 3 studies heretofore (18). Side effects at non-target sites limit the concentration achievable at the target which may restrict the efficacy of systemic rsCD40L.

The vector approach is an improvement in this regard as it can yield higher local CD40L concentrations while reducing systemic exposure. In this regard, a non-replicating adenoviral vector coding for CD40L (19) has been safely tested in humans. Nevertheless, the non-replicating platform may not be potent enough for treatment of advanced tumors and it has only been used in local bladder tumors so far.

In this study we hypothesized that a transcriptionally targeted oncolytic adenovirus, which features a capsid modification and is armed with CD40L, can result in potent oncolytic anti-tumor activity and stimulate an immune response. Our previous studies in vitro (20) showed high cytotoxic effect for Ad5/3-hTERT-E1A, which is an oncolytic adenovirus featuring the hTERT promoter for specific targeting to tumor cells. Also, the 5/3 serotype chimerism approach displays significantly enhanced gene delivery and antitumor effect when compared to adenoviruses with a wild type capsid (21-23).

To this end, we constructed Ad5/3-hTERT-E1A-hCD40L (CGTG-401), a new generation oncolytic virus based on Ad5/3 capsid modification for enhanced tumor transduction, tumor selectivity mediated by the hTERT promoter and armed with CD40L. This virus was tested in vitro and in vivo for specificity, efficacy, induction of immune response and apoptotic effect.
Materials and methods

Cell lines

Low-passage cultures of 293 and A549 from American Type Culture Collection (ATCC-LGS standards, Manassas, VA, USA) were used. EJ cells were provided and authenticated by Dr Aristides Eliopoulos (Crete, Greece). MB49 cells are from Dr K Esavaranatan (Singapore). Their quality and identity has been monitored with regard to growth pattern in vitro and in vivo, in vitro phenotype and y-chromosome positivity. B16-Ova are provided and authenticated by Richard Vile (Rochester, MN).

Adenoviruses

Viruses were generated and amplified using standard adenovirus preparation techniques (20-22, 24-27). More in detail explanation can be found in supplementary material.

The viral particle (VP) to plaque forming units ratios for Ad5/3-Luc1, Ad5/3-hTERT-E1A, Ad5/3-hTERT-E1A-hCD40L, Ad5/3-CMV-hCD40L and Ad5/3-CMV-mCD40L, were 25, 31, 200, 138 and 86, respectively.

Cell viability assay

Cells were infected and 1 hour later were washed and incubated for 4 to 8 days. Cell viability was then analyzed using MTS assay (Cell Titer 96 AQueous One Solution Proliferation Assay, Promega). A competition experiment with anti-CD40L antibody (Table 1) was performed on EJ and A549 cell lines. MTS assay was performed 48 hours upon infection.

Functionality of CD40L

Supernatant collected 48h following infection was filtered with 0.02μm filters (Whatman 6809-1002, Maidstone, England). This was used for two functionality assays which are described more in detail in supplementary material.
EJ cells line was transfected with the plasmid pNiFty-Luc (InvivoGen). Supernatant was added and 1μg/ml recombinant hCD40L protein (Abcam, Cambridge, MA) was used as a positive control. Cells were lysed and luciferase activity was measured (Luciferase Assay System, Promega, Madison, WI). RAMOS-Blue cells were stimulated with the supernatant and their activity was measured using the QUANTI-Blue assay reagent (InvivoGen, San Diego, CA, USA).

**Immunogenicity of cell death**

*Calreticulin exposure* – Cells were infected with 100 VP/cell. Twelve hours later cells were stained with anti-calreticulin antibody and Alexa-Fluor 488IgG was used as secondary antibody for flow-cytometry analysis.

*Extracellular ATP* - Cells were infected for two hours with 100 VP/cell. Supernatant was collected after 18 hours and analyzed with ATP Determination Kit (A22066, Molecular Probes, Invitrogen, San Diego, CA).

*HMGB1* release – Cells were infected with 100 VP/cell. Twenty four hours later supernatant was collected and HMGB1 was measured with ELISA kit (ST51011, IBL International, Hamburg, Germany).

**Apoptosis**

Cells infected with viruses and uninfected cells as mock were analyzed with TACS Annexin-V kit according to manufacturer instructions (4830-250-K, Immunodiagnostics, Finland). Tumors collected from Nude and C57Bl/6 mice were homogenized and stained for Annexin-V with the same kit and results are presented as percentage over the unstained cells.

**Quantitative polymerase chain reaction – qPCR**

MB49 cell line was infected for 2h with 100VP/cell. Infection media was removed and cells were collected at different time points. Tumors from Nude mice bearing EJ and A549 tumors
were collected at the end of the experiment and DNA was extracted using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). PCR amplification was based on primers and probe targeting the E4 gene (28).

**Replication assay in vitro**

MB49 cells were infected with Ad5/3-CMV-mCD40L and Ad5/3-Luc1. Cell killing was assessed by MTS assay at different time points after infection.

**Flow-cytometry and FACS-Array**

Cells and tissues were stained according to manufacturer instructions with respective antibodies (Table 1) and analyzed on BDLSR (BD Biosciences, Franklin Lakes, NJ). Results were plotted with FlowJo software (Tree Star, Inc, Ashland, OR, USA). Cytokines were analyzed from supernatant of cultured splenocytes according to the manufacturer’s protocol (BD Cytometric Bead Array Mouse Flex Sets, BD Biosciences, Franklin Lakes, NJ).

Cells infected and fixed with 70% ethanol were stained with propidium iodide (P4864, Sigma Aldrich, Finland) and analyzed by FACS-Array for cell cycle analysis.

**Immunohistochemistry**

Tissue sections were incubated with primary antibody according to manufacturer instructions (Table 1) followed by detection kits either for rabbit using LSAB2+ Dako System (DakoCytomation, Carpinteria, CA, USA (K0673)) or IHC Select kit (DAB150-RT, Millipore, MA, USA) for the antibodies raised in rats. Sections were counterstained with hematoxyline. Pictures were taken with an Axioplan2 microscope (Carl Zeiss) equipped with Axiocam (Zeiss).
Animal experiments

All animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice were obtained from Taconic (Ejby, Denmark) at 4 to 5 weeks of age.

Immune deficient models (nude mice) $10^6$ A549 or EJ cells (n=5 mice/group) and $5 \times 10^5$ MB49 cells (n=6 mice/group) were injected subcutaneously. When tumors reached the size of approximately 5 x 5 mm, virus was injected intratumorally at $10^8$ VP/tumor (A549 and EJ tumors) and $3 \times 10^8$ VP/tumor (MB49 tumors) on days 0, 2 and 4.

Immunocompetent models (C57Bl/6 mice) $5 \times 10^5$ MB49 cells (n=7 mice/group) and $2.5 \times 10^5$ B16-Ova cells (n=8 mice/group) were injected subcutaneously. Viruses were injected intratumorally at $3 \times 10^8$ VP/tumor on days 0, 2 and 4. Spleens from C57Bl/6 mice with MB49 tumors were minced and cultured for cytokine analysis. Tumors and organs from C57Bl/6 mice with B16-Ova tumors were smashed, filtered through a 70 μm filter and cultured for 24 hours. Apoptosis for tumors and flow-cytometry analyses were performed by flow-cytometry according to manufacturer instructions (Table 1).

Enzyme-linked immunosorbent assay (ELISA)

hCD40L and mCD40L concentration in the serum of mice were determined with Human CD40 Ligand ELISA kit (ELH-CD40L-001, RayBiotech Inc, Norcross GA, USA) and Mouse sCD40L elisa kit (BMS6010, Bender Medsystems, Austria) according to the manufacturer’s protocol.

Statistical analysis

Two tailed Student’s t-test was used and a p-value of <0.05 was considered as significant.
Results

In vitro and in vivo characterization of constructed adenoviruses

Replication competent Ad5/3-hTERT-E1A-hCD40L was constructed by inserting the hTERT promoter to control the E1 gene for tumor selectivity while hCD40L was placed in E3 for potentiating the immune response (Fig. 1A). Our arming strategy associates transgene expression with viral replication to ensure high expression of the transgene at the tumor site, starting from 8h after infection (25).

Both viruses coding hCD40L showed expression of the transgene in vitro on 293 cells (Fig. 1B). Expression of hCD40L and mCD40L was confirmed also in vivo (Fig. 1C). Following intratumoral injection and subsequent secretion of CD40L into blood, infection with Ad5/3-CMV-hCD40L resulted in higher serum levels than Ad5/3-hTERT-E1A-hCD40L. The cells transduced with Ad5/3-CMV-hCD40L continue to produce CD40L ad infinitum, while Ad5/3-hTERT-E1A-hCD40L causes oncolysis which limits the time of CD40L production. Oncolysis of the CD40L producing cell might be advantageous from a safety perspective, as CD40L can cause side effects when present at high concentrations (18). The human maximum tolerated dose of rhCD40L was reported to correspond with a 2900 pg/ml serum concentration which is 100-fold higher than what we saw.

Ad5/3-CMV-mCD40L resulted in lower serum CD40L levels than Ad5/3-CMV-hCD40L, presumably because mCD40L is metabolized by murine tissues and cells, while hCD40L is inactive in mice (29). Alternatively, cells transduced with adenovirus coding for an immunostimulatory molecule could be rapidly cleared in immune competent mice. Accordingly, no mCD40L could be detected on day 8 in the serum of mice with B16-Ova tumors infected with the same virus (not shown).
Two approaches were used to assess the functionality of hCD40L. A549 cells were infected, supernatant was collected and filtered through a 0.02μm filter to obtain the hCD40L protein expressed by the virus. We observed a 2.3 fold increase in NF-κB activation by Ad5/3-hTERT-E1A-hCD40L adenovirus and a 4.5 fold increase in NF-κB/AP-1 in Ramos-Blue cells (Fig. 1D) compared with Ad5/3-hTERT-E1A infected cells. Taken together with ELISA and FACS data (Fig. 1B-C) these results suggest that the constructed viruses express fully functional CD40L both in vitro and in vivo at levels predicted to be safe in humans based on use of recombinant hCD40L (18).

**Presence of CD40 increases virus potency**

Complete cell killing induced by Ad5/3-hTERT-E1A-hCD40L, was seen with 1000 viral particles/cell (VP/cell) in EJ cells (Fig. 2A). In A549 cells oncolysis by Ad5/3-hTERT-E1A-hCD40L was slower compared to the control virus. Ad5/3-hTERT-E1A-hCD40L was more potent in CD40+ EJ cells (Supplementary Fig. S1A) while Ad5/3-hTERT-E1A killed CD40- A549 cells more efficiently (Supplementary Fig. S1B). A competition experiment using an anti-CD40L antibody showed that the cell killing capacity of Ad5/3-hTERT-E1A-hCD40L was abrogated while Ad5/3-hTERT-E1A was not influenced (Supplementary Fig. S1C). As reported by Gomes et al (30), somewhat higher S-phase content was seen in cell cycle analysis following infection with CD40L viruses (Supplementary Fig. S1D).

Moreover, EJ (CD40+) cells showed significant apoptosis when infected with Ad5/3-hTERT-E1A-hCD40L (Supplementary Fig. S2A). In contrast, no apoptosis was seen on A549 cells suggesting that CD40 is needed for CD40L to cause apoptotic cell death while oncolysis is chiefly a non-apoptotic phenomenon at least in this cell line (Supplementary Fig. S2A). These experiments indicated that the innate oncolytic potency of Ad5/3-hTERT-E1A-hCD40L is comparable to a highly potent control virus and that CD40+ cells are killed more efficiently than CD40- cells.
Immunogenicity of CD40L coding viruses in vitro

Calreticulin exposure, ATP and HMGB1 release have been proposed as in vitro measurable indicators of immunogenic cell death (31). We found significant enhancement of each of these features after infection of CD40+ EJ cells with Ad5/3-hTERT-E1A-hCD40L (Fig. 2B-D). In contrast, only HMGB1 release was enhanced in CD40- cell line A549 (Fig. 2D).

In vivo efficacy of adenoviruses expressing hCD40L in immunodeficient animals xenografted with EJ CD40+ and A549 CD40- tumors

Lack of productive replication of human adenovirus in mouse cells and inactivity of hCD40L in mouse tissues complicate preclinical evaluation of Ad5/3-hTERT-E1A-hCD40L. We elected to isolate the antitumor mechanisms into different mouse models.

In immunodeficient mice the replication deficient virus Ad5/3-CMV-hCD40L had no effect on A549 CD40- tumors while in EJ CD40+ tumors induced a significant decrease of tumor growth (Fig. 3A and B; Supplementary Fig. S2B). The oncolytic Ad5/3-hTERT-E1A-hCD40L was found as potent as the positive control virus in both tumor models (Fig. 3C and D), and signs of virus replication was also seen in both sets of mice (Supplementary Fig. S2C). Thus, the oncolytic effect of Ad5/3-hTERT-E1A-hCD40L is not abolished by transgene expression or CD40L/CD40 mediated biological effects (Fig. 3D). Nevertheless, it was interesting that less virus genomes were seen in CD40L+ tumors, perhaps suggesting less viable tumor cells capable of virus production (Supplementary Fig. S2C). Alternatively, apoptosis induction could affect virus titers.

CD40L promotes apoptosis in CD40+ EJ tumors in nude mice

It has been suggested that interaction of CD40L with CD40 can cause apoptosis of tumor cells (15, 32) and thus this was studied by staining for caspase-3. Some apoptosis was induced by control oncolytic adenovirus Ad5/3-hTERT-E1A as reported for oncolytic adenoviruses (33). Also, replication deficient adenovirus Ad5/3-CMV-hCD40L induced
apoptosis in tumors due to hCD40L expression and its apoptotic effect (15). Nevertheless, much more apoptosis was seen in the tumors injected with Ad5/3-hTERT-E1A-hCD40L (Fig. 4). The caspase-3 data was confirmed by analyzing Annexin V (Supplementary Fig. S2A).

**Antitumor activity of CD40L in syngeneic immunocompetent animal models**

In immunocompetent mice with subcutaneous MB49 CD40+ bladder carcinoma tumors (34) there was a significant increase in antitumor activity in the group treated with Ad5/3-CMV-mCD40L (Fig. 5A). Ad5/3-CMV-mCD40L induced apoptosis suggesting that anti-tumor activity was partially due to apoptosis induced by either binding of mCD40L to CD40 or immunological effects triggered by mCD40L (Fig. 5B). As apoptosis was not seen in the same experiment performed *in vitro* (Supplementary Fig. S3A), the latter may be more likely. Interestingly, T-cells seemed necessary for the therapeutic effect, since when the same experiment was performed in T-cell deficient mice, no efficacy was seen (Fig. 5C). However, the presence of a therapeutic effect in the absence of T-cells could depend on the cell line to some degree (Fig. 3 and (30)).

In an immunocompetent but poorly immunogenic model (B16-Ova), Ad5/3-CMV-mCD40L was just as effective as the control virus, the efficacy likely mediated by immune recognition of adenovirus *per se*. Also in this model apoptosis was seen suggesting that it may be involved in immunological clearance of infected tumor cells (Fig. 5D).

**CD40L induces anti-tumor immune responses by recruiting cytotoxic T-cells at the tumor site and modulating the cytokine profile towards Th1 responses**

An important part of the putative antitumor activity of CD40L coding viruses is their effect on antigen presenting cells. Splenocyte analysis showed increased cytokine levels in the group treated with Ad5/3-CMV-mCD40L (Fig. 6A). IL12 induction suggests activation of APC including macrophages and dendritic cells. IFN-γ, TNF-α and RANTES are indicators of Th1 type immunity and suggest induction of a cytotoxic T-cell response.
To correlate this to the cellular level, we analyzed histological sections of tumors. Enhanced recruitment of macrophages (F4/80) and leukocytes (CD45) was seen, but only a small increase in B-lymphocytes (CD19), suggesting that the infiltrate was mostly T-cells (Fig. 6B). Analysis of T-cell subsets showed that most of these cells were CD8+ cytotoxic T-cells, although a smaller increase was seen also in CD4+ helper T-cells (Fig. 6C). These findings indicate that production of mCD40L in syngeneic MB49 tumors prompted a strong anti-tumor immune response mediated through Th1 responsive elements and cytotoxic T-cell infiltration (Fig. 6A-C). The effect was due to mCD40L since it was not seen with the control virus. Also, the effect was not impacted by oncolysis as Ad5/3-CMV-mCD40L is an E1-deleted virus not capable of replication in MB49 cells (Supplementary Fig. S3B and S3C).
Discussion

Adenoviruses have many appealing characteristics as replicating oncolytic agents, including their unparalleled capacity for infection of a wide range of tumors, stability \textit{in vivo} and a good efficacy/safety profile in humans (2, 4, 35, 36). Importantly, they can be armed with transgenes to improve their efficacy. One perceived limitation of adenoviruses is their immunogenicity. However, as the immune system of cancer patients has failed to eliminate the tumor because of the immunosuppressive nature of the tumor environment, immunogenicity becomes an advantage. This effect can be potentiated by retaining replication competence and arming with immunostimulatory molecules such as CD40L.

We constructed Ad5/3-hTERT-E1A-hCD40L which features six important aspects. a) Tumor transduction is improved by Ad3 serotype chimerism. b) Tumor selectivity is achieved by inserting the hTERT promoter in front of E1A. c) Recruitment and stimulation of APCs for induction of a Th1-type and cytotoxic T-cell response by CD40L. d) Apoptosis of CD40+ tumors through CD40-CD40L interaction. e) The gp19k/6.7K deletion in E3A to increase tumor selectivity (25) and f) anti-tumor immune response (25). Adenoviruses were found effective in inducing high level CD40L expression in CD40+ and CD40- cells. The levels of CD40 and CD40L expression could be crucial in regulating two important processes with opposite consequences: proliferation or retardation of tumor cell growth. For example, in lymphomas low levels constitutive engagement of CD40 can result in neoplastic cell growth (32) whereas high concentrations of CD40L induce inhibition of tumor growth (13, 14). Thus, in the worst case, using recombinant CD40L or a non-replicating virus as a gene transfer vector might enhance growth of some tumors. Therefore, it is more attractive to use an oncolytic platform, which ensures that transduced tumor cells are ultimately killed by oncolysis. In this approach, CD40L secretion or release from lysing cells can nevertheless
cause an apoptotic bystander effect on tumor cells near-by. However, the main utility may be the immunostimulatory effect which was the focus of this study.

Our previous data showed that Ad5/3-hTERT-E1A has significantly higher oncolytic potency compared with wild type Ad5 (24). Moreover, an oncolytic adenovirus driven by hTERT promoter has shown good safety data in humans (37). In fact, Ad5/3-hTERT-E1A is the fastest oncolytic adenovirus we have developed and thus it is an ambitious control virus (38). We compared Ad5/3-hTERT-E1A-hCD40L with Ad5/3-hTERT-E1A and found that both viruses were equally effective with regard to oncolytic potency \textit{in vivo} (Fig. 3). This was an important finding as expression of transgenes can sometimes inhibit the potency of viruses (39) and Ad5/3-hTERT-E1A-hCD40L was slower than Ad5/3-hTERT-E1A on A549 cells \textit{in vitro}, which might have been due to a 6-fold difference (200 versus 31 VP/PFU) in total to functional particles and viruses were dosed according to the former. It is therefore striking that Ad5/3-hTERT-E1A-hCD40L was as potent as Ad5/3-hTERT-E1A on CD40+ cells (Fig. 2-3). \textit{In vitro}, Ad5/3-hTERT-E1A-hCD40L had more antitumor activity on CD40+ cells than on CD40- cells, while opposite was true for Ad5/3-hTERT-E1A (Supplementary Fig. S1A and S1B). Since tumor size measurements may not be the optimal approach for studying therapeutics with immunological modes of action, further studies – including survival experiments – would be useful. Ultimately, human data is needed to evaluate the actual benefit of arming with CD40L.

Although the biggest utility of Ad5/3-hTERT-E1A-hCD40L might be in the context of CD40+ tumors, where all three anti-tumor activities (oncolysis, apoptosis, immune stimulation) would contribute, there are reports demonstrating that CD40L activates APCs even when the tumor is CD40- (40, 41). Thus, the potential utility of the virus is not restricted to CD40+ tumors. In particular, the relative contribution of apoptosis versus immune response to the overall efficacy needs to be studied further. Also, it is not yet fully
clear which immune cells are most relevant for antitumor effects and our data suggest a putative role for many classes of cells. As even unarmed oncolytic adenoviruses have shown utility in humans (2, 4, 42), Ad5/3-hTERT-E1A-hCD40L might represent an improvement regardless of CD40 status of the tumor.

Clinical and preclinical work in the field of tumor immunology and vaccine development has demonstrated that induction of an antitumor immune response can be achieved with several approaches (43). However, this has only rarely correlated with tumor control in patients. Instead, the first successful immunotherapeutics feature either trained/stimulated T-cells to overcome tumor mediated immune suppression or antibodies capable of down-regulation of immune suppression (44-46). Many investigators also use preconditioning to “make room” for activated T-cells and reduce immune suppressive cells (47). Thus, a critical lesson is that breaking the immunological tolerance acquired by tumors may be required for successful immunotherapy.

With regard to oncolytic adenoviruses coding for immunologically active transgenes, there are no suitable animal models in which adenoviruses can exert their replication effect together with evaluation of the immunological effect. Syrian hamsters are semipermissive for human adenovirus (5, 28) but no rodent models are known to be sensitive to human CD40L. Therefore, we used different models to isolate the effect of oncolysis, apoptosis and induction of anti-tumor immunity. Of particular interest are the immunological aspects of the approach, which were studied with Ad5/3-CMV-mCD40L. Even in the absence of oncolysis, Ad5/3-CMV-mCD40L significantly inhibited tumor growth in the syngeneic MB49 model and some animals were eventually cured, which is well in accordance with previous data obtained with a non-capsid modified virus Ad5-mCD40L (26).
Initial reports suggested that CD40-CD40L interactions play a role in potentiating B-cells accompanied by B-cell proliferation and differentiation for consequent induction of humoral responses (8, 48). The effect of CD40L on B-cells (Supplementary Fig. S4) and T-cells (Supplementary Fig. S5 and S6) might depend on context; the expression of CD40L in tumors might skew the response in the direction of a cytotoxic response while expression in lymph nodes could have a different effect. In our case, when MB49 tumors were analyzed for CD19, we did not notice a significant increase of these cells in the tumor. The same was seen in organs from syngeneic B16-Ova bearing mice (Supplementary Fig. S6). Intriguingly, in T-cell deficient mice with MB49 tumors we noticed a significant increase of B-cells in the spleens but not at the tumor site (Supplementary Fig. S4). However, even looking at tumors may not accurately reflect the entire story as T-cell may spend themselves by their anti-tumor actions.

This is supported by our findings in the syngeneic MB49 model where macrophages and T-cells, instead of B-cells, were recruited to tumors (Fig. 6). Macrophages are potent APCs and known for their capacity to induce IL-12. In turn, IL-12 production stimulates release of Th1-type cytokines including Rantes, IFN-γ and TNF-α for induction of a cytotoxic T-cell response. All of these effects were seen in our studies (Fig. 6).

In conclusion, we report significant anti-tumor effects for CD40L expressing adenoviruses including Ad5/3-hTERT-E1A-hCD40L. An important part of the effect is induction of a Th1-type immune response which results in accumulation of cytotoxic T-cells at the tumor site. Taken together, these data set the stage for clinical studies with Ad5/3-hTERT-E1A-hCD40L, which are currently ongoing.
Acknowledgements

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References


Table 1
Antibodies used in the experiments

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BD Biosciences, Franklin Lakes, NJ; Proimmune, Oxford, UK; eBioscience, Hatfield, UK; Abcam, Cambridge, UK; Invitrogen, San Diego, CA; Santa Cruz Biotechnology,

IHC=immunohistochemistry; FC=flow cytometry; Nab=neutralizing antibody experiment
Figure legends

Figure 1

Functionality and expression of constructed adenoviruses: *in vitro* and *in vivo*

(A) Schematic representation of virus construction. (B) Flow cytometry analysis for hCD40L expression in 293 cells at 24 hours post infection with 10VP/cell. IC=isotype control (C) *In vivo* expression of CD40L in serum of mice. Results are presented as mean from all mice/group + SEM. pg/ml=picogram/milliliter. (D) Functionality of virus produced hCD40L in *vitro*. Filtered supernatant was added on EJ transfected cells and Nf-κB activity is expressed in fold increase of luciferase expression (relative light units, RLU). Mock values were subtracted. Functionality of virus produced CD40L was also confirmed by studying NF-κB/AP-1 activation in Ramos-Blue cells (right panel). The assay was performed three times and each time was assessed in triplicates. Data are presented as mean ± SEM. ; ***,P<0.001.

Figure 2

(A) Oncolytic potency of Ad5/3-hTERT-E1A-hCD40L on EJ (CD40+) and A549 (CD40-). Infected cell lines were analyzed by MTS assay. Cell viability was assessed relative to mock uninfected cells. (B) Calreticulin exposure on EJ (CD40+, left panel) and A549 (CD40-, right panel). (C) Extracellular ATP release on EJ (CD40+, left panel) and A549 (CD40-, right panel). The experiments (B and C) were performed three times. (D) HMGB1 release on EJ (CD40+ left panel) and A549 (CD40- right panel). Data are presented as mean from triplicates plus SEM for panels B,C and D. *P<0.05, ***,P<0.001.

Figure 3

Anti-tumor efficacy in immune deficient mice
(A, B) Efficacy of replication deficient adenovirus Ad5/3-CMV-hCD40L in A549 (CD40-)
(A) or EJ (CD40+) (B) tumors. (C, D) Efficacy of replication competent adenoviruses in
CD40- (C) and CD40+ (D) tumors. Data are presented as mean ± SEM. Arrows indicate virus
injection. Tumor growth is expressed as percentage increase from first day of virus injection.
*, P<0.05; **, P<0.01; ***, P<0.001.

Figure 4

Caspase-3 expression in EJ (CD40+) tumors
Nude mice bearing tumors EJ tumors were injected thrice with viruses. Tumors were
collected 26 days after first virus injection and analyzed by immunohistochemistry for
induction of apoptosis (active-caspase). Staining was performed on all tumors and
representative pictures are shown. Positive staining is shown in brown. Pictures were taken at
20x magnification.

Figure 5

Ad5/3-CMV-mCD40L inhibits MB49 (CD40+) tumor growth in an immunocompetent
animal model but has no effect in the absence of T-cells
(A) Efficacy of Ad5/3-CMV-mCD40L in C57Bl/6 mice bearing subcutaneous MB49 tumors
Tumor growth is expressed as percentage increase from first day of virus injection. Data are
presented as mean ± SEM. Arrows indicate virus injection. ***, P<0.001. (B) immunohistochemistry analysis of apoptosis (active caspase-3) in MB49 tumors. Active
caspase-3 expression is shown in brown. Pictures were taken at 10x magnification. (C) Left
panel: efficacy of Ad5/3-CMV-mCD40L nude mice bearing MB49 tumors. Tumor size was
followed and plotted relative to the size on first day of injection. Data are presented as mean
+ SEM. Right panel: flow cytometry for Annexin-V in MB49 tumors from nude mice.
Arrows indicate virus injection. ***, P<0.001 (D) – (left panel) Efficacy of Ad5/3-CMV-
mCD40L in C57Bl/6 mice bearing B16-Ova tumors and (right panel) apoptosis (Annexin-V)
in the same tumors. Arrows indicate virus injection. Tumor growth is expressed as percentage increase from first day of virus injection. **, P<0.01.

**Figure 6**

Host immune responses in C57Bl/6 syngeneic murine models

(A) Cytokine analysis in supernatant from cultured splenocytes of C57Bl/6 mice bearing MB49 tumors. **, P<0.01.

(B, C) immunohistochemistry analysis of MB49 tumors from C57Bl/6 mice: (B) macrophage-F4/80, leukocytes-CD45 and B-lymphocytes-CD19 and (C) helper-CD4 and cytotoxic-CD8 T-cells. Positive staining for all these markers is shown in brown. Pictures were taken at 10x magnification.
Fig. 2 Diaconu et al
Fig. 3 Diaconu et al
Fig. 6 Diaconu et al
Immune response is an important aspect of the anti-tumor effect produced by a CD40L-encoding oncolytic adenovirus

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