Immune Response Is an Important Aspect of the Antitumor Effect Produced by a CD40L-Encoding Oncolytic Adenovirus

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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 immunosuppression. 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, a human telomerase reverse transcriptase (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 interleukin-12 production in splenocytes. This effect was associated with induction of the Th1 cytokines IFN-γ, RANTES, and TNF-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 show that adenoviruses coding for CD40L mediate multiple antitumor effects including oncolysis, apoptosis, induction of T-cell responses, and upregulation of Th1 cytokines.

Introduction

Oncolytic adenoviruses have shown safety in clinical trials and some efficacy has also been seen (1–4). Importantly, it has been discovered that immunologic 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 immunostimu-

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heretofore (18). Side effects at nontarget 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 nonreplicating adenoviral vector coding for CD40L (19) has been safely tested in humans. Nevertheless, the nonreplicating 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 antitumor 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 human telomerase reverse transcriptase (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 with 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) were used. EJ cells were provided and authenticated by A.G. Eliopoulos (University of Crete Medical School and Laboratory of Cancer Biology, Heraklion, Crete, Greece). MB49 cells are from Dr. K. Esuvaranathan (National University Hospital, 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).

Adenoviruses

Viruses were generated and amplified with 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 (pfu) 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 by MTS assay (CellTiter 96 AQueous One Solution Proliferation Assay, Promega). A competition experiment with anti-CD40L antibody (Table 1) was carried out on EJ and A549 cell lines. MTS assay was conducted 48 hours upon infection.

Functionality of CD40L

Supernatant collected 48 hours following infection was filtered with 0.02-mm filters (Whatman 6809-1002). This was used for 2 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 1 μg/mL recombinant

Table 1. Antibodies used in the experiments

<table>
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<tr>
<th>Antibody</th>
<th>Product</th>
<th>Method</th>
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<td>BD Biosciences</td>
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Abbreviations: FC, flow cytometry; IHC, immunohistochemistry; NAb, neutralizing antibody experiment.
hCD40L, protein (Abcam) was used as a positive control. Cells were lysed and luciferase activity was measured (Luciferase Assay System, Promega). Ramos-Blue cells were stimulated with the supernatant and their activity was measured with the QUANTI-Blue assay reagent (InvivoGen).

**Immunohistochemistry**

Cells were infected with 100 VP/cell. Twelve hours later cells were stained with anticalretulin antibody and Alexa-Fluor 488 IgG was used as secondary antibody for flow cytometric analysis.

**Extracellular ATP**

Cells were infected for 2 hours with 100 VP/cell. Supernatant was collected after 18 hours and analyzed with ATP Determination Kit (A22066, Molecular Probes; Invitrogen).

**HMGB1 release**

Cells were infected with 100 VP/cell. Twenty-four hours later, supernatant was collected and HMGB1 was measured with ELISA kit (ST5101; IBL International).

**Apoptosis**

Cells infected with viruses and uninfected cells as mock were analyzed with TACS Annexin-V kit according to manufacturer instructions (4830–250-K, Immuno Diagnostic). 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 PCR**

MB49 cell line was infected for 2 hours with 100 VP/cell. Infection removal was achieved 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 by the QIAamp DNA Mini Kit (Qiagen). 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). Results were plotted with FlowJo software (Tree Star, Inc.).

Cytokines were analyzed from supernatant of cultured splenocytes according to the manufacturer’s protocol (BD Cytometric Bead Array Mouse Flex Sets; BD Biosciences).

Cells infected and fixed with 70% ethanol were stained with propidium iodide (P4864; Sigma Aldrich) and analyzed by fluorescence-activated cell-sorting (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 (K0673; DakoCytomation) or IHC Select Kit (DAB150-RT; Millipore) for the antibodies raised in rats. Sections were counterstained with hematoxylin. Photographs 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 (Helsinki, Finland) and the Provincial Government of Southern Finland. Mice were obtained from Taconic at 4 to 5 weeks of age.

Immunodeficient models (nude mice) 10⁶ A549 or EJ cells (n = 5 mice/group) and 5 × 10⁵ MB49 cells (n = 6 mice/group) were injected subcutaneously. When tumors reached the size of approximately 5 × 5 mm², virus was injected intratumorally at 10⁶ VP/tumor (A549 and EJ tumors) and 3 × 10⁵ VP/tumor (MB49 tumors) on days 0, 2, and 4.

Immunocompetent models (C57Bl/6 mice) 5 × 10⁵ MB49 cells (n = 7 mice/group) and 2.5 × 10⁵ B16-Ova cells (n = 8 mice/group) were injected subcutaneously. Viruses were injected intratumorally at 3 × 10⁵ 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 cytometric analyses were conducted by flow cytometry according to manufacturer instructions (Table 1).

**ELISA**

hCD40L and mCD40L concentration in the serum of mice were determined with Human CD40 Ligand ELISA Kit (ELH-CD40L-001; RayBiotech Inc) and Mouse sCD40L ELISA Kit (BMS6610; Bender Medsystems) according to the manufacturer’s protocol.

**Statistical analysis**

Two-tailed Student t test was used and a P value of less than 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 whereas 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 8 hours 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, whereas 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 levels.
concentrations (18). The human maximum-tolerated dose of rhCD40L was reported to correspond with a 2.900 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, whereas hCD40L is inactive in mice (29). Alternatively, cells transduced with adenovirus coding for an immunostimulatory molecule could be rapidly cleared in immunocompetent 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 0.02-μm 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 1,000 viral particles per cell (VP/cell) in EJ cells (Fig. 2A). In A549 cells oncolysis by Ad5/3-hTERT-E1A-hCD40L was slower than the control virus. Ad5/3-hTERT-E1A-hCD40L was more potent in CD40+ EJ cells (Supplementary Fig. S1A) whereas 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 whereas Ad5/3-hTERT-E1A was not influenced (Supplementary Fig. S1C). As reported by Gomes and colleagues (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 whereas oncolysis is chiefly a nonapoptotic 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 with 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 and ATP and HMGBl 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 whereas 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 biologic effects (Fig. 3D). Nevertheless, it was interesting that less virus genomes were seen in CD40- 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 were 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 antitumor activity was partially due to apoptosis induced by either binding of mCD40L to CD40 or immunologic effects triggered by mCD40L (Fig. 5B). As apoptosis was not seen in the same experiment carried out in vitro (Supplementary Fig. S3A), the latter may be more likely. Interestingly, T cells seemed necessary for the therapeutic effect, as when the same experiment was carried out 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; ref. 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 immunologic clearance of infected tumor cells (Fig. 5D).

**CD40L induces antitumor immune responses by recruiting cytotoxic T cells at the tumor site and modulating the cytokine profile toward T-helper cell responses**

An important part of the putative antitumor activity of CD40L coding viruses is their effect on APCs. Splenocyte...
analysis showed increased cytokine levels in the group treated with Ad5/3-CMV-mCD40L (Fig. 6A). IL-12 induction suggests activation of APC including macrophages and DCs. IFN-γ, TNF-α, and RANTES are indicators of T-helper cell (TH1) type immunity and suggest induction of a cytotoxic T-cell response.

To correlate this to the cellular level, we analyzed histologic 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+ T cells (Fig. 6C). These findings indicate that production of mCD40L in syngeneic MB49 tumors prompted a strong antitumor immune response mediated through T<sub>H1</sub> responsive elements and cytotoxic T-cell infiltration (Fig. 6A–C). The effect was due to mCD40L as 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 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 patients with cancer 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 6 important aspects. (i) Tumor transduction is improved by Ad5 serotype chimerism. (ii) Tumor selectivity is achieved by inserting the hTERT promoter in front of E1A. (iii) Recruitment and stimulation of APCs for induction of a T<sub>H1</sub>-type and cytotoxic T-cell response by CD40L. (iv) Apoptosis of CD40<sup>+</sup> tumors through CD40–CD40L interaction. (v) The gp19k/6.7K
deletion in E3A to increase tumor selectivity (25) and (vi) antitumor 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 2 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 nonreplicating 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 nearby. However, the main use 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 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 in vitro, which might have been due to a 6-fold difference (200 vs. 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 (Figs. 2 and 3).

In vitro, Ad5/3-hTERT-E1A-hCD40L had more antitumor activity on CD40+ cells than on CD40−/− cells, whereas opposite was true for Ad5/3-hTERT-E1A (Supplementary Fig. S1A and S1B). Because tumor size measurements may not be the optimal approach for studying therapeutics with immunologic modes of action, further studies—including survival experiments—would be useful. Ultimately, human data are needed to evaluate the actual benefit of arming with CD40L.

Although the biggest use of Ad5/3-hTERT-E1A-hCD40L might be in the context of CD40+ tumors, where all 3 antitumor activities (oncolysis, apoptosis, and immune stimulation) would contribute, there are reports showing that CD40L activates APCs even when the tumor is CD40−/− (40, 41). Thus, the potential use 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 use 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 shown 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 immunotherapeutic feature either trained/stimulated T cells to overcome tumor-mediated immunosuppression or antibodies...
capable of downregulation of immunosuppression (44–46). Many investigators also use preconditioning to "make room" for activated T cells and reduce immunosuppressive cells (47). Thus, a critical lesson is that breaking the immunologic 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 immunologic 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 antitumor immunity. Of particular interest are the immunologic 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

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. Photographs were taken at ×10 magnification. C, left, efficacy of Ad5/3-CMV-mCD40L in 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, flow cytometry for Annexin-V in MB49 tumors from nude mice. Arrows indicate virus injection. ***, P < 0.001. D, left, efficacy of Ad5/3-CMV-mCD40L in C57Bl/6 mice bearing B16-Ova tumors. Right, 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.
data obtained with a noncapsid-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 Figs. 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 antitumor 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 TNF-α-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 antitumor 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.

**Disclosure of Potential Conflicts of Interest**

A. Hemminki is shareholder and consultant/advisory board member for Oncos Therapeutics, Ltd. I. Diaconu has ownership interest (including patents) for Oncos Therapeutics. A. Kanerva has ownership interest (including patents) and is a consultant/advisory board member for Oncos Therapeutics. A.S.I. Loskog has ownership interest (including patents) as an inventor of patent owned by Alligator Bioscience AB, and is the consultant/advisory board member for NEXTTOBE AB. S. Pesonen has ownership interest (including patents) in stock options from Oncos Therapeutics Inc. No potential conflicts of interest were disclosed by the other authors.
Oncolytic Adenovirus Coding for CD40L

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