Treatment of melanoma with a serotype 5/3 chimeric oncolytic adenovirus coding for GM-CSF: Results in vitro, in rodents and in humans

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Metastatic melanoma is refractory to irradiation and chemotherapy, but amenable to immunological approaches such as immune-checkpoint-inhibiting antibodies or adoptive cell therapies. Oncolytic virus replication is an immunogenic phenomenon, and viruses can be armed with immunostimulatory molecules. Therefore, oncolytic immuno-virotherapy of malignant melanoma is an appealing approach, which was recently validated by a positive phase 3 trial. We investigated the potency of oncolytic adenovirus Ad5/3-D24-GMCSF on a panel of melanoma cell lines and animal models, and summarized the melanoma-specific human data from the Advanced Therapy Access Program (ATAP). The virus effectively eradicated human melanoma cells in vitro and subcutaneous SK-MEL-28 melanoma xenografts in nude mice when combined with low-dose cyclophosphamide. Furthermore, virally-expressed granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulated the differentiation of human monocytes into macrophages. In contrast to human cells, RPMI 1846 hamster melanoma cells exhibited no response to oncolytic viruses and the chimeric 5/3 fiber failed to increase the efficacy of transduction, suggesting limited utility of the hamster model in the context of viruses with this capsid. In ATAP, treatments appeared safe and well-tolerated. Four out of nine melanoma patients treated were evaluable for possible therapy benefit with modified RECIST criteria: one patient had minor response, two had stable disease, and one had progressive disease. Two patients were alive at 559 and 2,149 days after treatment. Ad5/3-D24-GMCSF showed promising efficacy in preclinical studies and possible antitumor activity in melanoma patients refractory to other forms of therapy. This data supports continuing the clinical development of oncolytic adenoviruses for treatment of malignant melanoma.

Malignant melanoma is an aggressive cancer and the most common cause of mortality from skin cancer worldwide.1 The survival rate is low in patients with unresectable or metastatic disease,2 which characteristically is refractory to current therapies, such as irradiation and chemotherapy.3 Recently, randomized clinical trials with the anti-cytotoxic T-
lymphocyte-associated antigen 4 (CTLA-4) antibody ipilimumab and the BRAF inhibitor vemurafenib have resulted in improved survival in patients with metastatic melanoma compared to conventional therapies, but survival benefits are only seen in a minority of patients. However, ipilimumab provides proof-of-concept that immunotherapy can work in melanoma, albeit at a high cost and with severe adverse events. Ipilimumab is most likely effective in melanoma as this type of cancer is among the most immunogenic of all solid cancers, as demonstrated by the presence of circulating cytotoxic CD8+ T-cell populations specific for melanoma-associated antigens in patients. Moreover, preclinical and clinical immunotherapy studies have shown that a higher density of dendritic cells (DCs) and activated T-lymphocytes within the tumor is positively correlated with better prognosis. In this regard, oncolytic viruses offer an attractive therapeutic platform, where on one hand tumors are destroyed by virus replication, thereby releasing tumor antigens, and on the other hand virus infection evokes robust immune responses. One example is T-Vec, an oncolytic herpes simplex virus expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), which met its primary endpoint of durable response rate (DRR) in a global randomized phase 3 trial. GM-CSF expression can promote antitumor immune responses by recruiting natural killer (NK) cells, stimulating DCs, and promoting macrophage differentiation and activity. However, positive overall survival data has not been published from this trial and thus there may be a need for improvements in efficacy even in the context of melanoma virotherapy.

We have characterized the anti-melanoma activity of Ad5/3-D24-GMCSF, a 5/3-capsid chimeric oncolytic adenovirus coding for GM-CSF, which has been previously described for the treatment of advanced solid tumors. Previous preclinical and clinical data indicates selectivity for cancer cells, good tolerability, and promising antitumor efficacy. In an attempt to identify patient populations for clinical trials focusing on specific cancer types, we now evaluated Ad5/3-D24-GMCSF specifically for the treatment of melanoma in preclinical models, and collected the clinical data from melanoma patients treated in an Advanced Therapy Access Program (ATAP).

**Material and Methods**

**Adenoviruses**

Viruses used in this study have been described previously and are summarized in Supporting Information. Ad5wt is the wild-type Ad5 strain Ad300 from the American Type Culture Collection (ATCC, Manassas, VA). Replication-deficient and replication-competent adenoviruses were propagated respectively on 293 and A549 cells and purified on cesium chloride gradients.

**Cell lines**

Human melanoma cell lines SK-MEL-28 (ATCC, Manassas, VA), C8161 (kindly provided by Prof. Danny R. Welch, University of Alabama, Birmingham, AL), A375M (kindly provided by Prof. Isaiah J. Fidler, The University of Texas MD Anderson Cancer Center, Houston, TX) and the human lung carcinoma cell line A549 (ATCC) were cultivated in DMEM (Invitrogen, Karlsruhe, Germany). Human melanoma cell lines Mel888, Mel624 (both kindly provided by Dr. Jeffrey Schlom, National Cancer Institute, Bethesda, MD) and low passage melanoma cells pMeL (purified from skin metastasis) were cultivated in RPMI 1640 (Invitrogen). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, PAA, Göttingen, Germany), 100 IU ml−1 penicillin and 100 µg ml−1 streptomycin (both Invitrogen). The medium for pMeL was additionally supplemented with 10 mM HEPES (Lonza, Basel, Switzerland), 250 ng ml−1 amphotericin B and 100 µg ml−1 gentamycin (both Invitrogen). Cells were grown at 37°C in a humidified atmosphere of 5% CO2. Hamster melanoma cell line RPMI 1846 (ATCC, Manassas, VA) was cultivated in McCoy’s 5a Medium with L-glutamine (Lonza), supplemented with 10% FBS and 1% penicillin/streptomycin. Cell Authentication was done by Multiplexion (GmbH, Immenstaad, Germany).

**In vitro cytotoxicity assays**

Human melanoma cells were seeded in 96-well plates at 5,000 cells/well in 100 µl of growth medium (GM) supplemented with 2% FBS. After 24 hrs, cells were infected with serial dilutions of viruses added in 50 µl of RPMI medium containing 2% FBS or were mock-infected. Tenfold dilutions from 100 viral particles (VP)/cell to 0.01 VP/cell were used. Infections were performed in quadruplicates. After 18 hrs, 100 µl of GM containing 10% FBS was added to each well. Medium was exchanged 6 days post-infection. Upon first signs of lysis of cells (cytopathic effect, CPE) infected with the highest dilution of any of the viruses, cells were stained with 2% crystal violet in 70% ethanol for 10 min. After washing in tap water and drying of the plates, cytotoxicity was quantified by...
measuring the OD395 nm with a LabsystemsMultiscanMS (Thermo Fisher Scientific, Bonn, Germany). Viability of infected cells was calculated in relation to mock-infected cells, whose mean absorbance was defined as 100% viability.

Hamster RPMI 1846 cells were seeded in 96-well plates at 10,000 cells/well in 100 μl of GM supplemented with 5% FBS. After 24 hrs, cells were infected with tenfold dilutions of viruses (from 1,000 VP/cell to 1 VP/cell) added in 100 μl of GM containing 2% FBS or were mock-infected. Infections were performed in triplicate wells. A 100 μl of fresh 10% GM was added to each well 24 hrs post-infection and plates were incubated at 37°C until cytotoxicity was measured. Cytotoxicity assay (MTS-Assay; Promega) was performed 19 days post-infection and quantified by measuring the OD 490 nm using Multiskan Ascent and Ascent Software v2.6 (Thermo Labsystems, Helsinki, Finland). Background absorbance was subtracted and viability of infected cells was calculated relative to mock-infected cells, whose mean absorbance was defined as 100% viability.

In vitro transduction assay

Hamster RPMI 1846 cells were seeded into 24-well plates and infected in triplicates at doses of 1, 10, 100 and 1,000 VP/cell for 2 hrs at 37°C in 200 μl of GM supplemented with 2% FBS. Luciferase assay (E1501 Luciferase Assay System; Promega, Madison, WI) was performed 24 hrs post infection, as described previously.24

Effects of virally expressed GM-CSF on human monocytes

Human melanoma SK-MEL-28 cells were infected with 10 multiplicity of infection (MOI) of either Ad5/3-D24 or Ad5/3-D24-GMCSF, or left uninfected. Cells were collected 48 hrs post-infection, and the supernatants were filtered with 0.22-μm filter (Millex®—GP, Millipore, Ireland) and with 100K spin filter (Amicon® Ultra 0.5 ml, Millipore, Ireland). Human peripheral blood-derived monocytes were isolated from leukocyte-richuffy coats from healthy blood donors (The Finnish Red Cross Blood Transfusion Service) as previously described.25 Cells from three different blood donors were used in each experiment. Monocytes were cultured in Macrophage-SFM media (Gibco) and stimulated 24 hrs post-plating with either supernatant from Ad5/3-D24-GMCSF-infected or Ad5/3-D24-infected SK-MEL-28 melanoma cells. Commercial human recombinant GM-CSF (10 ng ml⁻¹, Immunotomytools), supernatant from uninfected SK-MEL-28 cells, Ad5/3-D24-GMCSF and Ad5/3-D24 viruses were used as controls. The growth medium was changed every other day. RNA was extracted on days 4 and 7 post-stimulation with RNeasy Mini Kit, and QIAcube (Qiagen), including a DNase digestion with RNase-free DNase set (Qiagen).

cDNA synthesis and real-time PCR

cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qPCR analysis of PU.1, FCGR1A, CD163 and GAPDH genes was performed using FAST SYBRgreen master mix and 7500 FAST Real-Time PCR system (Applied Biosystems, SYBRgreen oligonucleotides used are listed in Supporting Information). Primers for analysis of M-CSF, GM-CSF and IFIT2 expression were from Applied Biosystems (assay numbers: M-CSF: Hs00174164_m1; GM-CSF: Hs00929873_m1; IFIT2: Hs01584837_s1). Gene expression of different samples was normalized to GAPDH and comparative threshold-cycles (Ct) method was used to calculate relative expression of target genes.

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. Female nude/NMRI mice were obtained from Harlan (Indianapolis, IN) at 3–4 weeks of age. The animals were quarantined for 10 days and the health status monitored daily. SK-MEL-28 cells (5 × 10⁶ cells/tumor) were injected subcutaneously (s.c.) into both flanks. When tumors reached the size of ~5 mm diameter, mice were randomized into nine groups (four mice/group) and viruses diluted in 0.9% sodium chloride (NaCl) solution (B. Braun, Melsungen, Germany) were injected intratumorally (i.t.) at 7 × 10⁷ VP/tumor (two tumors/mouse) on days 1, 4, 8 and 15. NaCl 0.9% was used as mock treatment. Concomitant low-dose cyclophosphamide (CP: 0.4 mg/mouse) was injected intraperitoneally (i.p.) the day after the first virus injection and every 3 days thereafter for four out of nine groups. Tumor growth was followed by measuring the width and the height of the tumors and approximating the volumes (volume = 0.5 × length × (width)²). Animals were euthanized according to local animal care rules if any sign of pain or distress was evident or if tumors exceeded the maximum allowed size of 15 mm diameter.

Patients

Nine patients with chemotherapy refractory melanoma were treated with Ad5/3-D24-GMCSF in ATAP, which is under regulation of Finnish medicines agency FIMEA as determined by EC/1394/2007. The baseline characteristics of the patients are summarized in Supporting Information Table S1. The treatment was offered only to patients with tumors refractory to conventional therapies, progressive disease, no major organ function deficiencies and no other severe disease or organ malfunction, as described previously.15 The patients signed a written informed consent to accept the experimental approach of oncolytic adenovirus treatment and treatments were administered according to Good Clinical Practice and the Declaration of Helsinki of World Medical Association. Analysis of patient samples has been approved by Helsinki University Central Hospital (HUCH) operative ethics committee (HUS 62/13/03/02/2013). Details on the treatment have been described previously15,16,19 and are summarized in Supporting Information.
Neutralizing antibodies and virus titers in patients
Neutralizing antibody titer measurements using Ad5/3 luc1, DNA extraction and real-time PCR to determine viral titers from patient serum samples were performed as described previously.15,26

Statistical analysis
Statistics were done with SPSS v18.0 (SPSS, Chicago, IL). Two-tailed Student’s t test was used to assess tumor volume for nude mice experiment. p values of <0.05 were considered significant. Kaplan–Meier analysis was used to process survival data.

Results
Ad5/3-D24-GMCSF causes oncolysis of human melanoma cells
To study the susceptibility of melanoma cells to oncolysis by Ad5/3-D24-GMCSF, cytotoxicity was quantified on a panel of cell lines and compared to wild type adenovirus serotype 5, an isogenic control virus Ad5/3-D24 without GM-CSF, and a replication-defective Ad5/3luc1 vector. While there was some cell line-dependent variation in the kinetics of oncolysis between the different viruses, all cell lines were permissive to Ad5/3-D24-GMCSF, which killed >90% of cells by 13 days at 100 VP/cell (Fig. 1). As expected, in pMelL cells which do not express the coxsackie-adenovirus receptor (CAR), the primary receptor for Ad5, both fiber-chimeric recombinant viruses induced greater cell killing than Ad5 wt (p < 0.001) (Fig. 1g).

Ad5/3-D24-GMCSF in combination with low-dose cyclophosphamide led to complete tumor regression in a melanoma xenograft mouse model
We evaluated the efficacy of Ad5/3-D24-GMCSF in a SK-MEL-28 melanoma xenograft model in nude mice (Fig. 2). Concomitant low-dose cyclophosphamide (CP) was included to enhance the antitumor effect of Ad5/3-D24-GMCSF.27 All of the viruses tested inhibited tumor growth (Fig. 2, Supporting Information Fig. S1a), whereas CP treatment on its own showed no effect (Fig. 2, Supporting Information Fig. S1b). In Ad5/3-D24-GMCSF treatments, with or without CP, tumor growth was significantly reduced compared to mock or mock + CP groups, respectively, on Day 35 (p < 0.01) and on day 41 post-infection (p < 0.001) (Fig. 2a). With regards to additional antitumor effect, low-dose CP showed a statistically significantly enhancement of tumor growth inhibition when combined with the Ad5/3-D24-GMCSF virus (p < 0.05, Fig. 2a). Mice treated with Ad5/3-D24-GMCSF alone had near-to-complete tumor regression (4/8 tumor regressed), and the group treated with Ad5/3-D24-GMCSF in combination with low-dose CP exhibited complete tumor regression (8/8 tumors regressed) (Fig. 2a). Ad5/3-D24-GMCSF and the isogenic control virus Ad5/3-D24, lacking a transgene, displayed a similar degree of efficacy throughout the experiment in this model where human GM-CSF is not bioactive. In the groups treated with Ad5/3-D24 + CP and Ad5wt + CP, 6/8 and 1/8 tumors regressed respectively (Figs. 2b and 2c). Overall, it can be concluded that Ad5/3-D24-GMCSF displayed antitumor activity in the human melanoma xenograft model.

RPMI 1846 hamster melanoma cells were resistant to (Ad5/3D24) and wild-type adenoviruses
We performed a cytotoxicity assay to investigate the oncolytic potency of Ad5/3-D24-GMCSF in a hamster melanoma cell line. None of the viruses (Ad5/3-D24-GMCSF, Ad5/3-D24, Ad5wt and Ad5luc1) were able to kill this cell line in vitro (Supporting Information Fig. S2a). Therefore, we assessed transduction by adenoviruses with different capsid
modifications. We observed a trend for higher transgene expression with Ad5luc1, compared to the capsid-modified viruses. Ad3CMV-luciferase failed to transduce the cell line at any VP number/cell. Transduction with Ad5lucRGD virus was comparable to Ad5luc1 and Ad5/3luc1 (Supporting Information Fig. S2b).

**Ad5/3-D24-GMCSF-infected human melanoma cells produce GM-CSF that activates monocyte-macrophage differentiation**

The transgene expression of Ad5/3-D24-GMCSF is associated with virus replication and the bioactivity of virus-produced GM-CSF has been confirmed on human lymphocytes. Furthermore, it has been previously shown that human recombinant GM-CSF stimulates peripheral blood monocytes in vitro to become activated macrophages important for immune response to tumors. To assess if virus-produced GM-CSF mediates monocyte-macrophage differentiation, particularly in the context of infected melanoma cells which may secrete immunosuppressive cytokines that potentially could interfere with GM-CSF activity, we stimulated human monocytes isolated from healthy blood donors with either supernatant from virus-infected SK-MEL-28 melanoma cells or commercial human recombinant GM-CSF. Monocytes cultured with the recombinant GM-CSF exhibited a macrophage-like morphology both four (Fig. 3a, Supporting Information Fig. S3a) and seven (Fig. 3b, Supporting Information Fig. S3b) days after stimulation. Similarly, monocytes cultured with supernatant from Ad5/3-D24-GMCSF-infected cells developed macrophage-like morphology after stimulation, whereas supernatant from Ad5/3-D24 infected or uninfected cells failed to induce monocyte-to-macrophage differentiation (Fig. 3, Supporting Information Fig. S3).

To confirm monocyte-macrophage differentiation, we quantified the expression of GM-CSF-inducible genes (PU.1 and M-CSF) and macrophage-activation marker genes (CD163 and FCGR1A) (Supporting Information Fig. S4). Four days after stimulation, monocytes cultured with supernatant from Ad5/3-D24-GMCSF-infected cells expressed significantly more GM-CSF and M-CSF mRNA, compared to control groups (p < 0.01 and p < 0.05 respectively). PU.1 mRNA was significantly more expressed by monocytes cultured with recombinant GM-CSF (p < 0.05), but other significant changes were not seen between the groups. There was no statistically significant difference in the expression of the macrophage-activation marker genes CD163 and FCGR1A. We also quantified the expression of the interferon (IFN)-inducible gene IFIT2, which plays an important role in prevention of tumor progression. Interestingly, there was a trend for more expression of IFIT2 by monocytes cultured with Ad5/3-D24-GMCSF virus alone, compared to control groups including supernatant from Ad5/3-D24-GMCSF-infected cells and the recombinant GM-CSF (Supporting Information Fig. S4).

**Safety of ad5/3-D24-GMCSF in melanoma patients**

In the context of an advanced therapy access program (ATAP), a total of 15 Ad5/3-D24-GMCSF treatments were given to nine patients whose advanced melanomas were...
progressing after previous therapies (Supporting Information Table S1). The most common adverse reactions were grade 1–2 constitutional symptoms (fever, fatigue, edema, dizziness and rigors), nausea and pain (Supporting Information Table S2). Grade 3 adverse reactions were reported for three patients, but none were classified as a SAE (serious adverse events possibly related to the treatment and leading to patient hospitalization, malformation or death). Specifically, patient I266 had grade 3 fatigue, and grade 4 dyspnea and pericardium/pleural fluid, both caused by disease progression. Five out of nine patients showed a transient decrease in lymphocyte numbers in the peripheral blood, a phenomenon frequently observed in association with oncolytic adenovirus treatments and possibly related to redistribution of lymphocytes.15,16,18,19,31 No treatment related deaths occurred.

Neutralizing antibody titer and viral genomes in patient serum after treatment

Three out of six evaluable patients had low neutralizing antibody titer against the Ad5/3 capsid before treatment with Ad5/3-D24-GMCSF, but no patient had medium or high titers at baseline. After treatment, the antibody titer increased in all six patients (Table 1). There was no viral DNA present in serum at baseline while four out of six patients had measurable oncolytic adenoviral DNA levels on day 1, and three out of six beyond day 2. In two patients, the viral DNA load increased between day 1 and 4, strongly suggesting virus replication32 (Table 1). There was no clear correlation between neutralizing antibody titer and virus levels in the blood, which is in accord with previous observations.15

Efficacy of ad5/3-D24-GMCSF in melanoma patients

Four patients were evaluable for treatment response according to modified RECIST 1.1. criteria.33 One patient showed a minor response (MR), constituting a 15.1% reduction in diameter of an injected liver metastasis 6 weeks after a single treatment with Ad5/3-D24-GMCSF, with 50% of the dose given intratumorally and 50% intravenously.15 Two patients had stable disease (SD), 6 and 3 weeks after the last Ad5/3-D24-GMCSF injection, respectively; one patient had a progressive disease (PD), 2.5 weeks after the last virus injection (Table 1). Median survival of all treated patients was 51 days after the first Ad5/3-D24-GMCSF treatment (Supporting Information Fig. S5) and two patients were still alive at the end of follow up (September 2014). One of these long surviving patients had a minor response in the initial radiological evaluation (patient censored 2,149 days post-treatment) and the other patient had stable disease (patient censored at 559 days post-treatment) (Table 1).

Discussion

The oncolytic potency of Ad5/3-D24-GMCSF on melanoma cells was on par with or superior to wild type Ad5. In vitro, GM-CSF production did not lead to additional cell killing, due to the lack of a tumor microenvironment or immune effector cells in the culture. Inserting a transgene in the adenovirus genome can hinder the activity of the virus or make it slower in replication and oncolysis.34,35 As seen before,18 in our study, the direct cytolytic activity of the armed virus
Table 1. Clinical responses, viral genomes and neutralizing antibody titers in patient serum

<table>
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<th>Treatment number</th>
<th>Patient code</th>
<th>Imaging result</th>
<th>Virus in serum (VP/ml), days post-treatment</th>
<th>Neutralizing antibody titer</th>
<th>Survival (days)1</th>
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<td></td>
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<td>1–2</td>
<td>3–7</td>
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<td>I266</td>
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</table>

1Numbers in parenthesis indicate the survival “from the first viral treatment.” Otherwise “from the first Ad5/3-D24-GMCSF treatment.”
2Patient still alive.
3The patient received a “serial treatment” consisting of three injections within 10 weeks.
4Serial treatment, response evaluation done after the third treatment.
5Three weeks after the last treatment. New evaluations 3–5 months after the last treatment showed a PD (+19.8% + new metastases).
6The post-treatment titer was measured at day 1 after the third round of serial treatment (>5 weeks from the first treatment; 3 weeks from the second treatment).

Abbreviations: PD = progressive disease, SD = stable disease, MR = minor response, empty boxes indicate data not available.

versus its unarmed counterpart seemed to depend on the cell line, for unknown reasons. Importantly, the chimeric capsid allowed effective killing of primary melanoma cells such as pMelL, which lack the primary receptor for serotype 5-based viruses (CAR), as proposed also for other tumor types.17,21,36

In the xenograft model we saw complete tumor regression in nude mice treated with Ad5/3-D24-GMCSF in combination with low-dose CP, whereas CP treatment alone did not result in reduction of tumor growth. Also the non-replicating control virus Ad5/3luc1 reduced tumor growth, perhaps due to NK cell activity against infected tumor cells in nude mice (which retain this cell type37), although this remains speculation. A similar result was obtained by a previous study, in which the injection of the control vectors reduced tumor burden ~1.5- and threefold in immunodeficient animals.38 In humans or immune competent animals, the rationale for combining low-dose CP with oncolytic adenovirus treatments could involve three distinct issues: (i) reduction of regulatory T-cells15,27; (ii) the anti-angiogenic properties of CP39,40; (iii) a more poorly defined form of combination effects between alkylating agents and oncolytic adenovirus.19,41 In preclinical work with human xenografts, immune deficient animals (lacking T-cells) are required, precluding assessment of the first aspect. Thus, our result shows that low-dose metronomic CP may have a useful combination effect with the virus despite the lack of T-cells, suggesting the second or the third aspects as possible mechanisms of action.

An independent experiment at multiple CP and multiple virus doses would be required for reliable assessment of synergy versus additive effects. Naturally, as human GM-CSF is not active in mice, the xenograft model cannot be used to demonstrate superiority of the armed virus over its unarmed counterpart.

To study the immunostimulatory functions of GM-CSF, an immunocompetent animal model would be appealing. We have previously used Syrian hamster models successfully, since this animal is semi-permissive for both human adenoviruses serotype 5 and human GM-CSF.15,18,26,42,43 Our previous study showed that the transduction with Ad5/3 was low in semi-permissive hamster cancer cells, as compared to human cells18 which results in lower oncolytic potency than seen in human tumors.15 Even though it is still less well understood if Syrian hamster is permissive also for Ad5/3 chimeric viruses which enter cells differently,15,18 it has been considered the “best available model” for the study of oncolytic adenovirus vectors43 and thus we utilized the model in the context of melanoma. Although melanoma is rare in Syrian hamsters we were able to obtain one Syrian hamster melanoma cell line, RPMI 1846. These cells were not resistant to virus entry, as evident in the transduction assay. However, they could not be lysed with the studied oncolytic viruses, and the cell line was also resistant to development of cytotoxic effect by wild-type Ad5, as reported also by others.44 Thus, RPMI 1846 hamster cells probably have additional mechanisms that limit the adenoviral replication-cycle and therefore further work is required for development of hamster melanoma model.

The differentiation of human monocytes into macrophages and the induction of DC generation are important
steps for antigen recognition, T-cell activation and antitumor immune response.\textsuperscript{45} A previous study reported that purified recombinant human GM-CSF was capable of stimulating human peripheral blood monocytes to become tumoricidal against the human malignant melanoma cell line A375.\textsuperscript{14} In our experiment, GM-CSF production, associated with virus replication in human SK-MEL-28 melanoma cells, led to differentiation of human primary monocytes into macrophages. This confirms that virally encoded GM-CSF, produced from tumor cells, can achieve this important biological effect. The induction of DC generation by the virus requires further studies.

Long-term GM-CSF production and accumulation may induce the generation of myeloid-derived suppressor cells (MDSC). A previous study showed that these unwanted effects of GM-CSF are mediated by the systemic concentration of this cytokine above a certain “safety level.”\textsuperscript{26,46} Local production levels of GM-CSF might be beneficial on antigen presenting cells, without increasing systemic concentrations. We have previously shown that intratumorally injected Ad5/3-D24-GMCSF produces GM-CSF in immunocompetent Syrian hamsters and cancer patients, without significant changes in systemic levels.\textsuperscript{15}

Ad5/3-D24-GMCSF was well-tolerated in nine melanoma patients and the treatments resulted in disease stabilization or tumor shrinkage in three out of four evaluable patients, two of which have an unusually long and ongoing survival given their baseline situation of metastatic melanoma progressing after routine therapies. However, the median survival of all treated patients was rather unimpressive, underlining the aggressive nature of metastatic melanoma progressing after routine therapies. Immunotherapies may require time to start working and thus patients with very advanced and aggressive disease may not be able to receive optimal treatment benefits. Individualized treatments, such as ATAP, are not always optimally informative for designing clinical trials. A major issue (with regard to scientific interpretation) in the personalized setting is the heterogeneity of the treatment on an individual level, which aims at patient benefit instead of scientific rigor. Personalized treatments were not designed, nor can they never be a replacement for clinical trials.\textsuperscript{47,48} On the other hand, a positive aspect of ATAP is that the treated patients are not part of a highly selected trial population but they are real-life patients.

In conclusion, Ad5/3-D24-GMCSF appears suitable for melanoma therapy and continued clinical investigation is supported. Because of their excellent safety profile, it is easy to envision oncolytic viruses combined with other forms of therapy. In the context of melanoma, attractive combinations could include checkpoint-inhibiting antibodies such as ipilimumab and nivolumab, which target respectively the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the programmed cell death 1 (PD-1) receptor on T cells.\textsuperscript{49} The oncolytic virus can provide a strong proimmunogenic signal at the tumor site while the antibody would reduce immunosuppression. Another attractive approach could be first using vemurafenib (which targets the BRAF V600E-mutated protein)\textsuperscript{50} for debulking and reducing associated immunosuppression, and then continuing with virus treatments, prior to progression due to the disease acquiring resistance to vemurafenib.

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\textbf{References}


