

Antiviral and Antitumor T-cell Immunity in Patients Treated with GM-CSF–Coding Oncolytic Adenovirus

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Abstract

Purpose: Multiple injections of oncolytic adenovirus could enhance immunologic response. In the first part of this article, the focus was on immunologic aspects. Sixty patients previously naïve to oncolytic virus and who had white blood cells available were treated. Thirty-nine of 60 were assessed after a single virus administration, whereas 21 of 60 received a "serial treatment" consisting of three injections within 10 weeks. In the second part, we focused on 115 patients treated with a granulocyte macrophage colony-stimulating factor (GM-CSF)–coding capsid chimeric adenovirus, CGTG-102.

Results: Following serial treatment, both increase and decrease in antitumor T cells in blood were seen more frequently, findings which are compatible with induction of T-cell immunity and trafficking of T cells to tumors, respectively. Safety was good in both groups. In 115 patients treated with CGTG-102 (Ad5/3-D24-GMCSF), median overall survival was 111 days following single and 277 days after serial treatment in nonrandomized comparison. Switching the virus capsid for avoiding neutralizing antibodies in a serial treatment featuring three different viruses did not impact safety or efficacy. A correlation between antiviral and antitumor T cells was seen ($P = 0.001$), suggesting that viral oncolysis can result in epitope spreading and breaking of tumor-associated immunologic tolerance. Alternatively, some patients may be more susceptible to induction of T-cell immunity and/or trafficking.

Conclusions: These results provide the first human data linking antiviral immunity with antitumor immunity, implying that oncolytic viruses could have an important role in cancer immunotherapy. *Clin Cancer Res*; 19(10); 2734–44. ©2013 AACR.

Introduction

Oncolytic viruses replicate selectively in tumor cells, killing them in the process where thousands of new virions are released into the surrounding tumor tissue and vasculature (1). The safety of this approach has been shown in numerous clinical trials but only 2 products have been approved thus far (2). Most clinical and preclinical studies have focused on viral modifications for improved tumor transduction, cancer-specific replication, and arming with

transgenes. Less attention has been given to how treatment should be administered. For example, the effects of multiple dosing, compared with single administration, are not well studied or understood. However, it is well established that immune response can be boosted by multiple dosing making this approach also attractive when adenoviral treatment protocols are designed with immunotherapy goals in mind (3). As many oncolytic viruses are based on common human pathogens, preexisting immunity could impact treatment efficacy (1). Importantly, viruses armed with immunostimulatory genes can be used to induce antitumor immunity (4, 5).

First, our primary goal was to evaluate the safety of multiple versus single administration. Second, we investigated T-cell immunologic effects against tumor and viral epitopes. Patients were also monitored for evidence of antitumor effects by radiology and tumor markers in serum and followed up for overall survival.

Materials and Methods

Patients

Adverse reactions were followed for 28 days and graded according to the Common terminology Criteria for Adverse Events (CTCAE) v3.0. Serious adverse events are defined as

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Translational Relevance

The safety of oncolytic adenoviruses has been good in clinical trials. In an attempt to increase efficacy, armed viruses are increasingly used. An important recent realization is that oncolysis can induce antitumor immunity and in fact the approach embodies a potent form of tumor immunotherapy. We explored the induction of antitumor immunity in patients by comparing a single injection of oncolytic adenovirus to administration of three doses 3 to 4 weeks apart. Immunologic data suggested that oncolytic adenoviruses might be able to break the tumor-associated immunologic tolerance.

resulting in patient hospitalization, prolongation of hospitalization, life-threatening situation, malformation, or death possibly related to therapy. Treatments were given in the context of an Advanced Therapy Access Program (ATAP), which is a personalized therapy approach, not a clinical trial, and the treatments are based on Article 35 of World Medical Association Declaration of Helsinki. All patients signed written informed consent.

The oncolytic viruses administered to patients in ATAP are regulated by Regulation (EC) No. 1394/2007 on advanced therapy medicinal products, amending Directive 2001/83/EC and Regulation (EC) No 726/2004. According to EC/1394/2007, manufacturing of advanced therapy medicinal products shall be authorized by the competent authority of the Member State. The competent authority authorizing the manufacturing of the products administered in ATAP is Finnish Medical Agency (FIMEA). FIMEA also requires reporting of adverse reactions. Patient sample analyses are approved by the local Ethics Committee (HUS 62/13/03/02/2013).

Treatments

All treatments were conducted intratumorally in ultrasound or computed tomography (CT) guidance. The largest safely accessible tumors were selected for injection, and the typical number of needle tracts was up to 10 per patient. Patients were monitored for 24 hours in the hospital and for 4 weeks as outpatients. If there were no contraindications (e.g., gastrointestinal, hematologic, or neurologic), low-dose oral metronomic cyclophosphamide 50 mg/day was administered to reduce regulatory T cells (4, 6, 7). Cyclophosphamide was started 1 day after the first virus injection and continued until disease progression or withdrawal of consent. The definition for serial treatment (including "sero-switch" patients) is 3 rounds of oncolytic adenovirus within 10 weeks; treatments not fulfilling this criteria were evaluated as single treatments.

Analysis of efficacy

RECIST1.1 criteria were applied to overall disease status including injected and noninjected tumors. In addition to the standard criteria, we used minor response (MR = 10%–

29% reduction in the size of lesions) as an indicator of cases where biologic activity might be present. Some patients were not imaged after treatment because the patient elected not to travel for imaging. All imaged patients are reported. In some cases, positron emission tomography (PET)-CT was used instead of conventional CT, using a modification of the PET Response Criteria in Solid Tumors (8). Medical record of each patient was checked to see whether tumor markers (peptides measured from serum) had been used in their treatment evaluations previously. If so, they were measured at baseline and after treatment with oncolytic virus.

Viruses and quantitative real-time PCR

All viruses used in this study have been published previously; for details, including quantitative PCR (qPCR) conditions, see refs. 5 for CGTG-102, 9 for Ad5-RGD-D24-GMCSF, 4 for Ad5-D24-GMCSF, 10 for ICOVIR-7, 11 for Ad5/3-cox2L-D24, and 12 for Ad3-hTERT-E1. Viruses were produced by Oncos Therapeutics Ltd according to the National and European Union regulations. Virus particles present in the blood were estimated by measuring genomes with qPCR.

Cytokine analysis

Cytokine analysis was conducted with BD Cytometric Bead Array (CBA) Human Soluble Protein Flex Set (Becton Dickinson) according to the manufacturer's instructions. FCAP Array (TM) v1.0.1 software was used for data analysis.

Assessment of tumor- and adenovirus-specific immunity by ELISPOT

Peripheral blood mononuclear cells (PBMC) were used to evaluate the induction of tumor- and adenovirus-specific immunity following treatment as reported (4, 5). PBMCs were isolated by Percoll gradient. Cells were frozen in CTL-CryoABCTM serum-free media (Cellular Technology Ltd.). ELISPOT was conducted according to the manufacturer's (MABtech) instructions (h-INF- γ ELIPOST PRO 10 plate kit). For adenovirus ELISPOT, cells were stimulated with the HAdV-5 Penton peptide pool (ProImmune). For the antitumor response, survivin (BIRC5 PONAB), pool of CEA+Ny-ESO-1, pool of c-Myc +SSX2, MAGE-3, and WT-1 peptides were used (ProImmune). Of note, no pre-stimulation of PBMCs was done to avoid artificial or incorrect signals and to ensure adequate viability of cells that might be compromised during prolonged culture. In data analyses reported in the text, background values were subtracted but they are shown in Fig. 3 and Supplementary Fig. S3. The threshold for increase was set +2 and for decrease –2. All available samples were analyzed.

Statistical analysis

Statistical analyses were done with SPSS v15.0 (SSPS). Two-tailed *t* test was used to assess significance in T-cell phenotype data, whereas nonparametric one-way ANOVA, with Dunn multiple comparisons post test was used for cytokine data. Responses and correlation to ELISPOT data were analyzed with χ^2 and Mann–Whitney *U* tests.

Table 1. Summary of used viral doses in this study

	Viral dose levels ($\times 10^{11}$ VP)				
	<1	1-5	5-10	10-30	30-40
Single treatments ($n = 39$)					
CGTG-102	3	20			
Ad5-d24-GMCSF		7			
Ad5-RGD-d24-GMCSF		3	6		
ICOVIR-7					
Ad5/3-Cox2L-d24					
Ad3-hTERT-E1					
Serial treatments ($n = 21$)					
CGTG-102		26			
Ad5-d24-GMCSF		13			
Ad5-RGD-d24-GMCSF		2	13		
ICOVIR-7		2		2	1
Ad5/3-Cox2L-d24		3			
Ad3-hTERT-E1		1			
All single CGTG-102 treatments ($n = 72$) ^a	7	76			
All serial CGTG-102 treatments ($n = 51$) ^a		111			

^a8 patients received both single and serial treatment. Numbers in table indicate the numbers of treatments for each dose level.

Antitumor and anti-viral T-cell induction was calculated with 2-tailed Fisher exact test. Survival data were processed with Kaplan–Meier analysis and log-rank test.

Results

Patients naïve to oncolytic adenovirus

Thirty-nine patients were treated with a single administration, whereas 21 matched patients received a serial treatment consisting of 3 doses 3 to 4 weeks apart (Fig. 1A, Table 1, and Supplementary Table S1). There were no notable differences in the World Health Organization performance status, age, sex, or previous treatments between groups.

Detection of virus genomes in blood with quantitative PCR

Oncolytic adenovirus DNA was detected in the serum of patients up to 58 days posttreatment (Table 2). All day 0 samples were negative, as expected for patients not treated with viral therapies previously. The frequency of positive samples was usually highest on days 1 to 4 after treatment. The highest virus concentration detected was similar in serial and single patients, neither were clear differences seen in mean and median titers (Table 2). Interestingly, in the serial treatment group, maximum or mean titers did not diminish after the second and third injections. However, the proportion of negative samples increased from 15% to 50% during the course of the serial regimen.

Rate or severity of adverse reactions is not increased with serial administration

The most common adverse reactions were grade 1 to 2 constitutional effects including fever, flu-like symptoms,

fatigue, gastrointestinal complaints, or local pain at tumors. Mild electrolyte disturbances, liver enzyme elevations, and anemia were also common, as well as mild-to-moderate transient thrombo- or leukopenia. Grade 3 adverse reactions were encountered in 23% of single treatments and 5%, 19%, and 14% of serial treatment rounds, respectively (Supplementary Table S2).

There was one grade 4 adverse reaction in each group. In the single treatment group, a patient with carcinoma of the appendix had pulmonary embolism 3 weeks after viral therapy. It was assessed to be unrelated to therapy, but possibly caused by the tumor, as thrombotic events are common in patients with advanced cancer. Grade 4 airway obstruction occurred in a patient with head and neck cancer after the second treatment round in serial treatment group. It was judged that tumor swelling due to virus might have contributed and therefore this was classified as a serious adverse event. No treatment-related deaths occurred in either group (Supplementary Table S2).

Effect of virus administration on white blood cell compartments

The phenotypic panel of circulating white blood cells was analyzed in a cohort ($n = 23$) of patients after a single injection of virus (Supplementary Fig. S1). Fifteen of 23 displayed an increase in the total count of CD8+ cytotoxic lymphocytes (from 0.49×10^9 to $0.64 \times 10^9/L$), whereas only 6 of 23 showed an increase in CD4+ T cells. The total CD3+ T-cell population increased in 10 of 23 patients. The mean ratio of CD8+/CD4+ increased 46% ($P = 0.117$). On the level of individual patients, there was no correlation between an increased CD8+/CD4+ ratio and disease control or survival. Furthermore, the mean CD8+/CD3+ ratio

Table 2. Summary of virus replication and response evaluation data

	Virus in blood (VP/mL) ^a : mean concentration median concentration; range(min-max)			Tumor markers	RECIST	Disease control ^b	Median survival, d
	Pretreatment	After first round	After second round				
Single (<i>n</i> = 39)		7,668		mCR: 0	CR: 0		
		762		mPR: 1	PR: 0	9/22 = 41%	128
	All neg.	500–72,613		mMR: 2	MR: 1		2 alive ^c
		Neg. result		mSD: 5	SD: 8		
		2/38		mPD: 13	PD: 13		
Serial (<i>n</i> = 21)		12,851	30638	95722	mCR: 0	CR: 1	269
		2,450	965	1364	mPR: 2	PR: 1	9/18 = 50%
	All neg.	500–126,738	500–253255	500–611235	mMR: 1	MR: 2	2 alive ^c
		Neg. result	Neg. result	Neg. result	mSD: 0	SD: 5	
		3/20	5/19	9/18	mPD: 5	PD: 9	

Abbreviations: CR, complete response (normalization of markers or disappearance of all tumors in radiologic findings); MR, minor response = 10%–29% reduction in markers or the size of lesions; PD, progressive disease = new metastatic lesions or growth of a previous lesion by 20% or more; PR, partial response = 30% or more reduction in markers or the size of lesions; SD, stable disease = tumor or marker measurements not satisfying the criteria for response or progression; VP, viral particle.

^aLimit of quantification 500 VP/mL.

^bSD or better in imaging.

^cAlive in May 2012.

increased 10% ($P = 0.235$) but increases did not correlate with disease control or survival either.

Adenovirus- and tumor-specific T-cell levels after treatment

On the basis of the trend for increase in CD8+ T cells (Supplementary Fig. S1), and on previous publications (4, 5), we investigated tumor- and adenovirus-specific T-cell responses with ELISPOT (Fig. 1B–D, Supplementary Figs. S2 and S3). As biopsies were not available for assessing the antigens present in each tumor individually, we studied survivin-specific T-cell responses as a measure of antitumor immune activation. Survivin is a classic pan-carcinoma antigen reported to be present in practically all tumors (13, 14), and it has been used to estimate antitumor T-cell responses (4, 5, 9, 6). In the single treated group, 12 of 39 (31%) patients showed an increase in survivin-specific CD8+ lymphocytes after treatment, whereas after serial treatment, these cells were induced at one or more time points in 8 of 21 of patients (38%). The respective frequencies of decrease in survivin-specific CD8+ lymphocytes in blood were 11 of 39 (28%) and 7 of 21 (33%). One explanation for this phenomenon is trafficking of cells from the blood to the tumor as proposed in mouse and human studies (15, 16). However, other explanations could include death of the cells or redistribution to lymphoid organs or other normal tissues. Tumor biopsies before and after treatment, and their comparison to blood data, would be useful for improved understanding of these aspects. Fortunately, a clinical trial featuring tumor biopsies is now ongoing.

Less anergy (lack of T-cell induction or trafficking) was seen in the serial-treated patients (41% vs. 29%; Fig. 1C). In serial treatment, the highest induction of anti-survivin cells was seen after the first injection while trafficking increased over subsequent treatment cycles (Fig. 1D). However, as there was no statistically significant difference between groups, confirmation of hypothesis this requires further work with larger patient cohorts.

Induction of anti-adenoviral T cells was seen in most patients in both groups and the blood frequency of these cells was higher than that of anti-survivin T cells, probably reflecting the relative immunogenicity of viral versus self-epitopes (Supplementary Fig. S2). In the single-treated group, 72% (28/39) of patients showed an increase in the level of Ad5-specific CD8+ lymphocytes whereas the value in serial treatment was 90% (19/21). Possible reasons why antiviral T cells were not induced in all patients include (i) a generally immunosuppressed state and (ii) trafficking of these cells from blood to tissues where the highest concentrations of virus are present.

Inflammation caused by treatment

Signs of inflammation (potentially useful for breaking tumor-associated tolerance) at the tumor were seen in both computed tomographic (CT) imaging and upon inspection (Fig. 2A–B). With regard to potentially dangerous systemic inflammation, elevation of serum cytokines, IL-6 in particular, has been associated with adenovirus-related toxicity (17). Therefore, we investigated the effects of serial treatment on proinflammatory interleukin (IL)-6, IL-8, and TNF- α as well as anti-inflammatory IL-10 (Supplementary

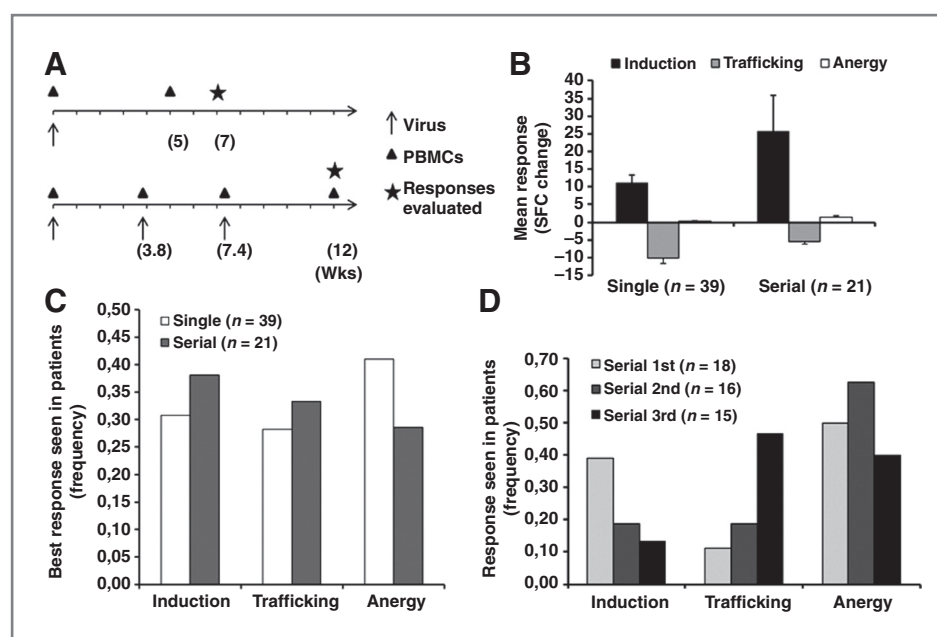


Figure 1. A, schematic presentation of the treatment timeline. The numbers in parentheses indicate the mean time for the PBMC collection, which occurred just before virus injection when conducted. B, PBMCs were isolated from patients before and circa 1 month after treatment and pulsed with a survivin-derived peptide pool and analyzed with IFN- γ ELISPOT. More antitumor T cells were induced in serial treatment but statistical significance was not reached ($P = 0.343$), possibly due to the relatively small number of samples (spot-forming colonies = SFC/million cells). Error bars, SEM. C, both induction and reduction (possibly indicative of trafficking to the tumor) of antitumor T cells was more frequent in serial treatment. Best response for each patient is shown. Anergy (no increase or decrease) was seen less commonly after serial treatment ($P = 0.632$). D, induction of antitumor T cells was most prominent after the first round of treatment while after boosting trafficking to tumor predominated ($P = 0.098$).

Fig. S4). The highest IL-6 elevations seen in our patients were more than 10-fold lower than levels reported in a patient with systemic inflammatory response syndrome (17). In parallel with IL-6, and as reported previously (18), also IL-10 levels increased after treatment. There was significant increase in IL-10 levels in serial treatment, specifically, the second and third serial treatment cycles resulted in higher IL-10 on day 1 compared with serial treatment baseline (both $P < 0.05$).

Serum granulocyte macrophage colony-stimulating factor (GM-CSF) levels were analyzed (Supplementary Fig. S4), as high systemic (as opposed to local) GM-CSF is theoretically counterproductive to immunotherapy because of effects on myeloid-derived suppressor cells (19). Median values were consistently below 20 pg/mL, whereas up to 16,000 pg/mL have been reported for an oncolytic vaccinia virus armed with GM-CSF (20). GM-CSF production by CGTG-102 is restricted to the tumor, which should be favorable for immunotherapy (5).

Disease control, tumor markers, and overall survival in patients without prior virus treatments

Twenty-two of 39 and 18 of 21 patients were evaluable with RECIST1.1 criteria (21) in single and serial groups, respectively (Table 2). Disease control (stable disease or better) was seen in 41% and 50% of treatments, respectively (Table 2). Tumor markers were measured from serum if elevated at baseline (single, $n = 21$; serial, $n = 8$), and the disease control rate was 38% in both groups. Interestingly, minor or partial

marker responses were seen in 38% of patients in the serial group but in only 14% in the single therapy group.

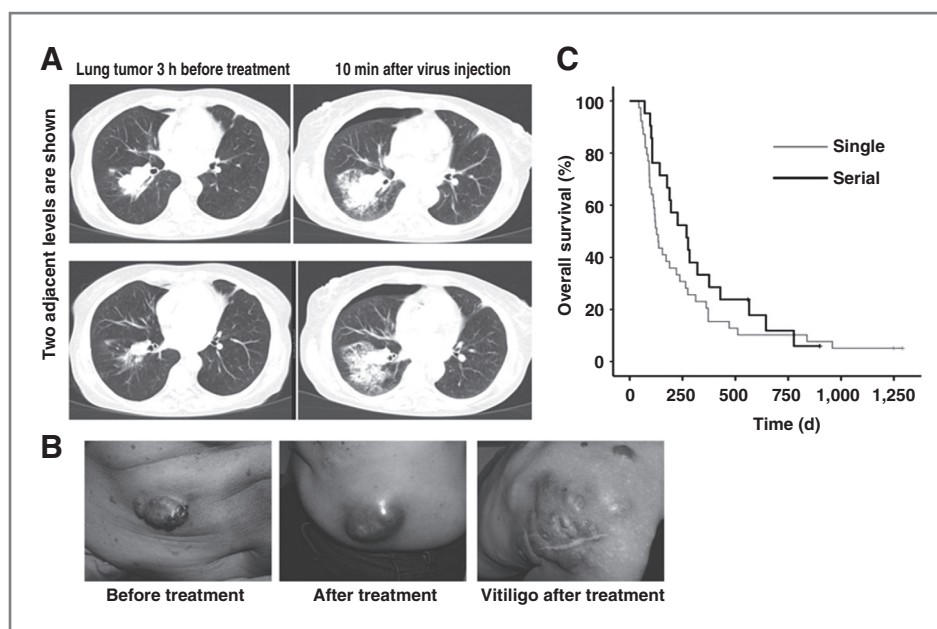
Median overall survival was 128 days for single treated and 269 days for serial treated ($P = 0.183$). Survival at 200 days was 35% and 57%, respectively (Fig. 2C and Table 2).

Adenovirus- and tumor-specific T-cell responses in 115 CGTG-102-treated patients

There were no major differences in patient characteristics between single and serial-treated patients (Table 3). All patients with PBMCs available were analyzed with IFN- γ ELISPOT for T-cell responses (Fig. 3). In the first part of this article, sample availability had limited antitumor T-cell studies to survivin-specific cells. However, in the second part, we were able to study not only anti-survivin but also anti-CEA+Ny-ESO-1 (pooled), anti-c-Myc+SSX2 (pooled), anti-MAGE-3, and anti-WT-1 T cells (Fig. 3).

Although the data should be regarded with caution due to small sample sizes, induction of antitumor T cells against one or several epitopes was seen in 7 of 15 (47%) single-treated patients, whereas the frequency was 2 of 4 (50%) in the serial group. Putative evidence of trafficking of T cells to the tumor (i.e., decrease in frequency in blood) was seen in 8 of 15 (53%) and 2 of 4 (50%) of single and serial-treated patients, respectively (Fig. 3). Mouse studies suggested that a decrease in antitumor T cells in blood can be accompanied by concomitant accumulation in tumors, which is compatible with virus injection-mediated trafficking of T cells from blood to tumors as published for another immunotherapeutic (10).

Figure 2. A, inflammation at the tumor after virus injection. A CT scan of a patient with lung cancer before and 10 minutes after the virus treatment. The intratumorally injected volume was only 3 mL, but the volume of the inflammatory infiltrate (seen due to an increase in water content) encompassing the tumor is more than half a liter. Therefore, the therapy-induced inflammatory response seems to be rapid and capable of enveloping the entire tumor. B, inflammation in skin lesions of a melanoma patient before and after single treatment displaying redness and swelling indicative of inflammation. Another lesion from the same patient shows vitiligo and redness after treatment. C, Kaplan-Meier survival analysis. Patients who were alive at the time of submission of the article were censored (tick marks).



Antiadenoviral T cells presented a trend similar to anti-survivin cells in that there was less anergy in serially treated patients (Supplementary Fig. S2). However, even single injection resulted in clear induction of antiviral cells, and the numbers of cells were higher than with anti-survivin cells, possibly reflecting the stronger immunogenicity of viral versus self-derived tumor epitopes.

In a first-in-human correlation of antiviral to antitumor immunity, it was interesting that 8 of 9 (89%) patients who had induction of antiviral T cells had also induction of one or more types of antitumor T cells. In contrast, patients without induction of antiviral cells were less likely (1/10, 10%) to display induction of antitumor T cells ($P = 0.001$). Thus, there was 17/19 (89%) concordance in behavior of antitumor and antiviral T cells in blood. It should be noted, however, that these patient numbers are quite low and any conclusions should await confirmation from larger cohorts.

Rate or severity of adverse reactions is not increased with serial administration of CGTG-102

Almost all patients encountered grade 1 and 2 adverse reactions as seen in the first part of the study (Supplementary Table S3). Grade 3 reactions were encountered in 21% of single treatments and 2%, 10%, and 4% of serial treatment rounds, respectively. There were 6 grade 4 adverse reactions in the single treatment group and 4 in the serial group. Two were classified as serious adverse events: one airway obstruction (this patient was included also in the first part of the study) and another was fever 1 day after treatment. Overall, there was no statistically significant difference in occurrence of adverse reactions between single and serial-treated patients. Of note, the standard automated blood count does not include lymphocytes (only leukocytes) and thus lymphocyte data are not included in Supplementary Tables S2 and S3. However, the automated

blood count does include a rough estimate of the percentage of lymphocytes out of all leukocytes, and after discovering the ELISPOT data, we calculated lymphocyte values manually for CGTG-102 patients. Transient lymphopenia was seen 1 day after viral treatment in almost all patients (87% and 80%; single and serial-treated, respectively) and 1 of 3 of patients displayed grade 3 or 4 lymphopenia. Future studies should incorporate lymphocyte counts in the routine panel of tests.

Disease control and overall survival in patients treated with CGTG-102

All patients had progressing disease before treatment with oncolytic adenovirus. In radiologic evaluation, disease control rates were 74% ($n = 23$, single) and 48% ($n = 22$, serial; Fig. 4A). "Pseudoprogression" caused by more pronounced inflammatory swelling in serially treated tumors may have contributed to the lower rate of disease control (22, 23). In addition to formal responses, other signs of antitumor activity included disappearance of pleural effusion and ascites (lung cancer patient K75, mesothelioma patient M137) and a complete response in a noninjected liver metastasis (urinary bladder cancer patient V136; ref. 5). With regard to tumor markers, disease control rates were 58% and 48%, respectively (Fig. 4A).

Median overall survival was 111 days for single treatments ($n = 83$) and 277 days for serial treatments ($n = 53$, $P = 0.068$; Fig. 4B). To put these numbers into perspective, we identified patients treated with Ad5/3-Cox2L-D24 at the same institution under similar inclusion criteria ($n = 30$, all single treatment, median survival, 124 days) and found that serial treatment with CGTG-102 resulted in significantly improved survival ($P < 0.0001$). Ad5/3-Cox2L-D24 has 2 levels of selectivity for tumors (a Cox2L promoter and D24 deletion in E1A), a capsid identical to CGTG-102, but lacks

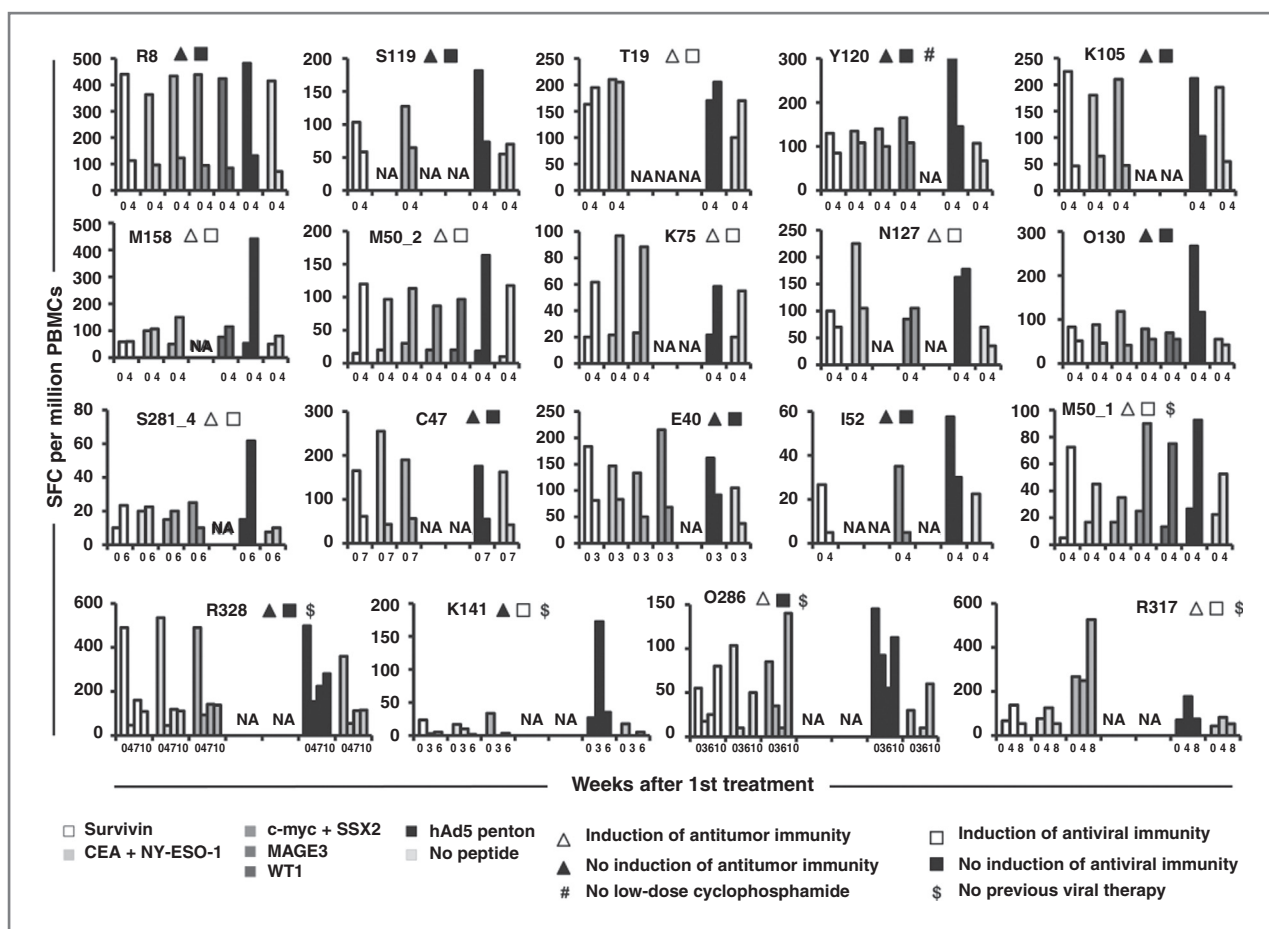


Figure 3. IFN- γ ELISPOT results in CGTG-102-treated patients, peptide-specific spot-forming colonies (SFC)/million cells shown. For evaluation of antitumor response, survivin, a pool of CEA + Ny-ESO-1, a pool of c-Myc + SSX2, MAGE-3, or WT-1 peptides were selected for each patient individually. All peptides could not be analyzed for each patient due to limitations in the amount of PBMC available (NA, not analyzed). Black bars indicate anti-adenoviral response. Triangles and squares indicate a summary of the data for each patient: white symbols indicate induction whereas black symbols indicate no induction of antitumor (triangles) or antiviral immunity (squares). #, patient did not receive low-dose cyclophosphamide (used for reduction of T-Reg). There was a significant concordance (white with white, black with black) between induction of anti-viral and antitumoral T cells (2-tailed Fisher exact test, $P = 0.001$). In particular, 89% of patients with induction of antiviral T cells had induction of one or more classes of antitumor T cells.

an arming device (11, 24). Survival at 200 days was 37% and 27% for CGTG-102 and Ad5/3-Cox2L-D24 single treatments, respectively, and 58% for serial treatment with CGTG-102. It should be noted, however, that none of these comparisons conducted in the second part of this article are neither randomized nor do they comprise matched case-control series as in the first part of this article. Instead, in the second part, we selected to report every patient treated with CGTG-102 in our experimental treatment program.

Figure 4D features an example of PET-CT imaging of patient R247 who received a serial treatment with CGTG-102. Her skin metastases also provide an example of inflammation swelling the tumors and show that size-based measurements may not be optimal for evaluation of oncolytic virus therapy (Supplementary Fig. S5), whereas measurement of metabolic activity of the tumors might be more useful (Fig. 4D; ref. 22).

As the viruses used in this article were not planned to be effective only in certain tumor types, but instead in

advanced solid tumors in general, tumor type was not an inclusion criteria. Thus, the patients had several different tumor types and analysis of each subgroup would be risky because of the small number of cases. Whether or not some tumor types respond better is an interesting question and is best answered in studies focusing on defined tumor types.

Effect of capsid switching on adverse reactions and survival

As switching the virus capsid has been proposed to allow partial avoidance of neutralizing antibodies (25–28), 22 patients received 3 different viruses in a serial treatment in a first-in-human application of "sero-switching" with oncolytic viruses (Table 3). There was no difference in the adverse reaction profile (Supplementary Table S3). Regarding treatment efficacy, disease control was seen in 50% and 46% in "including CGTG-102" and "CGTG-102 only" groups, respectively (Fig. 4A). With regard to tumor markers present in serum, disease control rates were 46% and 50%,

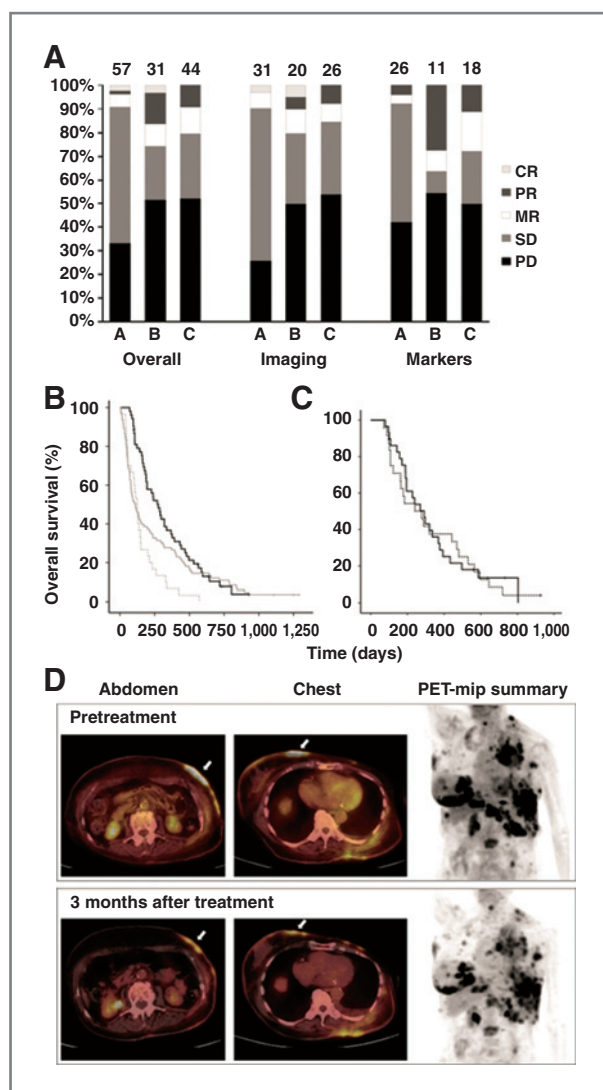


Figure 4. A, tumor responses in single versus serial treated patients. Single treatment (A): serial treatments are further divided to treatment with 3 different viruses; (B) CGTG-102 being one of them; or (C) treatment with CGTG-102 only. Values above columns indicate the number of patients analyzed. B, survival of patients treated with CGTG-102 in serial treatment (bold), a single round (gray), or with unarmed control virus Ad5/3-Cox2L-D24, which has an identical capsid (dotted). Serial treatment with CGTG-102 resulted in longer median overall survival than single treatment with CGTG-102 ($P = 0.068$, i.e., not statistically significant) or Ad5/3-Cox2L-D24 ($P < 0.0001$, significant). Please note that these are nonrandomized comparisons. Patients who were alive at the time of submission of the article were censored (tick marks). C, serial treatment with three rounds of CGTG-102 (bold) or 3 rounds of virus including CGTG-102 (dotted) resulted in similar survival ($P = 0.843$). D, breast cancer patient imaged with PET-CT imaging before and 3 months after serial treatment with CGTG-102 only. Arrows (left 2 columns) and black areas (right column) indicate tumor before (top row) and after treatment (bottom row).

respectively. Median overall survival rates were 241 days (200 days survival, 54%) and 291 days (200 days survival, 61%), respectively ($P = 0.843$, Fig. 4C) and therefore, the theoretical advantage of avoiding neutralizing antibodies

(25–28) did not convert into a survival advantage. These are the first human data on the relative importance of effective transduction (achieved with 3 different capsids) versus enhanced immunity (no change of virus capsid). Interestingly, these data seem to be in accord with the correlation of antiviral and antitumoral T-cell immunity (Fig. 3), suggesting that the former may be able to influence the latter.

Discussion

Oncolytic viruses are entering the clinical arena in a rapid manner (29–33). Although few products have been approved heretofore (29), interest in the field has increased together with maturation of trials from early phase to randomized settings (30). It seems likely that many different viral backbones, with or without transgenes, have the potential for efficacy (1, 31, 32). However, one aspect that has so far received little or no study is whether multiple dosing is better than a single dose. Given the emerging realization that immune response may determine the efficacy, or lack thereof, for many oncolytic viruses, such considerations should take immunologic issues into account.

An ongoing discussion topic is the relationship between antiviral and antitumor immunity (33). At first glance, the former might seem disadvantageous, whereas the latter is obviously appealing. However, in practice, the situation might be less straightforward as reaction against the virus could result in antitumor response through at least 2 mechanisms: breaking of tumor-associated immunologic tolerance and epitope spreading. Regarding the former, most, if not all, advanced solid tumors have developed immunosuppressive mechanisms to sustain numerous potentially immunogenic mutated epitopes. If a strong danger signal can be provided at the tumor, it may be able to break such tolerance. Given the evolutionary antagonistic codevelopment of many viruses and the human immune system, immunogenic viruses such as adenoviruses are appealing candidate for breaking tolerance. Viral epitopes are among the strongest pathogen-associated molecular patterns and therefore logically immunodominant over self-derived tumor epitopes. However, it is an appealing notion that this might in fact help with induction of antitumor immunity.

In this article, we first studied safety and immunologic activity in matched oncolytic virus-naïve patients, who were treated with a single injection or multiple rounds of therapy. Interestingly, multiple injections did not seem to reduce virus replication, as estimated by virus genomes present in blood. However, the frequency of virus negative samples did increase. With regard to T-cell reactions, the data suggested enhanced induction of antitumor T cells, a higher number of patients with a decrease in antitumor T cells in blood compatible with trafficking to the tumor (15), subsequently less anergy while safety was not affected.

We then focused on 115 patients treated with a single virus. Again, serial treatment did not increase adverse reactions, whereas antitumor immunologic reactivity seemed

Table 3. Characteristics of CGTG-102-treated patients

	All CGTG-102 patients (n = 115)	Single (n = 72)	Serial all (n = 51)	Serial incl. CGTG-102 (n = 22)	Serial only CGTG-102 (n = 29)
Sex	No. of patients	No. of patients	No. of patients	No. of patients	No. of patients
Male	43 (37%)	29 (40%)	15 (29%)	8 (36%)	7 (24%)
Female	72 (63%)	43 (60%)	36 (71%)	14 (64%)	22 (76%)
Age, y					
Median	58	59	57	59	56
Range	7–78	7–78	9–78	9–71	35–78
WHO performance status					
0	14	9	8	3	5
1	56	35	26	15	11
2	39	23	16	3	13
3	6	5	1	1	0
Tumor type					
Ovarian cancer ^a	23	14	10	3	7
Breast cancer ^a	16	4	13 ^b	5 ^b	8
Sarcoma ^a	15	11	7	5	2
Lung cancer ^a	10	4	7	2	5
Melanoma	9	7	2	—	2
Colon or rectal cancer	7	6	1	1	—
Mesothelioma ^a	6	4	3	2	1
Gastric or esophageal	5	5	—	—	—
Pancreatic cancer ^a	5	4	2	1	1
Renal cancer	4	4 ^c	—	—	—
Cholangiocarcinoma	4	3 ^c	1	—	1
Head & neck cancer	3	2	1	1	—
Prostate cancer	3	1	2 ^b	2 ^b	—
Thyroid cancer	2	1	1	1	—
Endometrial cancer	1	1	—	—	—
Neuroblastoma	1	1	—	—	—
Neuroendocrine (adrenal) cancer	1	—	1	—	1
Parotid cancer	1	—	1	—	1
Wilms tumor	1	1	—	—	—
Previous Treatments					
Surgery	75	44	35	14	21
Chemotherapy	114	72	50	21	29
Median of regimens (range)	4 (0–14)	3 (1–10)	4 (0–14)	4 (0–11)	5 (1–14)
Radiotherapy	55	30	28	12	16
Stem Cell Transplantation	1	1	—	—	—

^aEight patients are reported in both groups.

^bOne patient having both breast and prostate cancer.

^cOne patient having both cholangio and renal cancer.

more pronounced in the serial cohort and survival was also better, especially at earlier time points. Eventually, most patients progressed and died of their tumor, but it should be noted that in many cases, progression occurred while off therapy. Studying the effect of CGTG-102 on survival and optimal dosing would benefit from rigorous clinical trials. The data in this article are well in accord with the previous articles reporting similar viruses (5, 6, 18, 11, 10). However, reliable comparison of viruses would require a randomized setting and a strictly defined patient population. In essence,

our data suggest that a single treatment with CGTG-102 provides a strong stimulus for induction of antitumor T cells. However, further danger signals, provided by additional injections of virus, are useful for trafficking of those cells to their target.

Comparison of the "CGTG-102 only" to the "including CGTG-102" cohort provides an interesting opportunity to study the merits of avoiding neutralizing antibodies versus enhancing immune response (Fig. 4C). The former group received one virus thrice, whereas the latter were treated

with viruses with different capsids in an extension to previous work (25–28). This is the first time 3 different oncolytic viruses have been used for the treatment of human patients. The fact that there was no difference in overall survival suggests that the advantage with regard to transduction, transgene expression, and/or oncolysis in the latter group could be counteracted by the benefits of increased antiviral immunity (leading to antitumor immunity) in the "CGTG-102 only" group. An alternative explanation could be that the preclinical advantage in transduction did not convert to clinical advances. Unfortunately, these issues are difficult to assess in immunocompetent model systems due to species incompatibility issues with regard to permissivity to adenovirus and human transgenes.

With regard to epitope spreading, preclinical data and clinical data from cell therapy trials have suggested that a strong response against one epitope might have the potential of resulting in "bystander effects" against the other epitopes nearby (34). If more immune response is beneficial for the goals of oncolytic virotherapy, boosting with repeated administration could be appealing. Our study provides first-in-human immunologic data suggesting that the generation of antiviral immunity might correlate with generation of antitumor T-cell immunity. Thus, epitope spreading might be possible not only between tumor epitopes but also between viral and tumor epitopes. An alternative explanation for the correlation is that some patients are more likely to mount a T-cell response in general. Patient-by-patient variation could be explained by, for example, different levels of immunocompetence.

In oncology, antitumor agents are traditionally given multiple times and therefore many virotherapy trials have used this approach. However, as oncolytic viruses should be able to replicate and spread throughout the tumor, it is less obvious if repeated administration is required. Preliminary experiments in severe combined immunodeficient mice suggested that this might not be the case (35), but the lack of an immune system renders such data useless with regard to immunologic boosting. The necessity and/or use of repeated administration relates to important practical, humanistic, and socioeconomic aspects. The data reported here indicate that multiple injections are safe and could be useful.

In summary, our results suggest that serial treatment does not impact safety while it could increase potentially beneficial immunologic reactions, whereas the signs of danger-

ous inflammatory overreaction were not detected. Putative correlation between antiviral and antitumor T-cell responses (requiring confirmation in future studies) provided tantalizing preliminary evidence compatible with epitope spreading in the context of oncolytic virus treatment of humans. These results have led us to use multiple rounds of injection in our ongoing clinical trials with CGTG-102. As GM-CSF and other immunostimulatory transgenes are popular in the field of oncolytic viruses, our results could be relevant for the entire field.

Disclosure of Potential Conflicts of Interest

A. Hemminki is employed (other than primary affiliation; e.g., consulting) as a shareholder and consultant in Oncos Therapeutics Ltd; has a commercial research grant and ownership interest (including patents) in Oncos; and is a consultant/advisory board member of Oncos. No potential conflicts of interest were disclosed by the other authors.

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