Clinical Cancer Research

Phase I Study of Oncolytic Vaccinia Virus GL-ONC1 in Patients with Peritoneal Carcinomatosis

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Abstract

Purpose: Peritoneal carcinomatosis is common in advanced tumor stages or disease recurrence arising from gastrointestinal cancers, gynecologic malignancies, or primary peritoneal carcinoma. Because current therapies are mostly ineffective, new therapeutic approaches are needed. Here, we report on a phase I study designed to assess safety, MTD, and antitumor activity of intraperitoneal administration of oncolytic vaccinia virus GL-ONC1 in advanced stage peritoneal carcinomatosis patients.

Patients and Methods: GL-ONC1 was administered intraperitoneally every 4 weeks for up to four cycles at three different dose levels (10^7–10^9 pfu) following a standard 3+3 dose escalation design. GL-ONC1 was infused via an indwelling catheter that enabled repetitive analyses of peritoneal fluid biopsies. The primary study objective was safety of GL-ONC1 according to Common Terminology Criteria for Adverse Events, version 4.0 (CTCAEv4.0).

Results: Patients with advanced-stage peritoneal carcinomatosis ($n = 7$) or advanced peritoneal mesothelioma ($n = 2$) received 24 doses of GL-ONC1. Adverse events were limited to grades 1–3, including transient flu-like symptoms and increased abdominal pain, resulting from treatment-induced peritonitis. No DLT was reported, and the MTD was not reached. Furthermore, no signs of viral shedding were observed. Importantly, in 8 of 9 study patients, effective intraperitoneal infections, in-patient replication of GL-ONC1, and subsequent oncolysis were demonstrated in cycle 1. All patients developed neutralizing activities against GL-ONC1.

Conclusions: GL-ONC1 was well tolerated when administered into the peritoneal cavity of patients with advanced stage peritoneal carcinomatosis. Efficient tumor cell infection, in-patient virus replication, and oncolysis were limited to treatment cycle 1 (ClinicalTrials.gov number, NCT01443260).
Clin Cancer Res; 24(18); 4388–98. ©2018 AACR.

Introduction

Many malignancies have the potential to disseminate throughout the lining of the abdominal cavity which is referred to as peritoneal carcinomatosis. Without surgical intervention, patients with peritoneal carcinomatosis generally have an average life expectancy of 6 months with diminished quality of life due to symptoms like malignant ascites and severe bowel obstruction (1–5). Because peritoneal carcinomatosis often is a locoregional disease with no discernable metastasis elsewhere, a multimodal approach combining aggressive cytoreductive surgery with subsequent intraperitoneal hyperthermic chemotherapy (HIPEC) is currently the most promising therapy to significantly increase survival (6). With regard to symptom control the trifunctional antibody catumaxomab is approved for the treatment of malignant ascites only (7–9).

Oncolytic virotherapy is an emerging treatment modality for cancers of various origins and specifications. Oncolytic viruses (OV) are designed to selectively infect and damage cancerous tissues by intracellular replication and subsequent oncolysis without causing harm to normal tissues (10, 11).

On the basis of promising results in preclinical studies, a multitude of different OV species is currently in early stage and advanced clinical development and the herpes simplex virus Imlygic is already approved for melanoma patients (12). Up to now, several strains of vaccinia viruses (e.g., GL-ONC1, Pexa-Vec, vvDD, and MVA-*FCU1*) exhibit an excellent safety profile in multiple phase I studies irrespective of the application route for this OV (13–19).

The oncolytic vaccinia virus GL-ONC1 is a double-stranded DNA virus and a prototype member of the genus Orthopoxvirus (20). Beyond its cytolytic capacity for a broad range of tumor cell types (21), vaccinia virus has many characteristics desirable for clinical applications. These include a short and well-characterized life cycle with efficient cell-to-cell spreading (22), a high genetic stability with replication restricted to the cytoplasm of infected cells, as well as an excellent safety profile

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-18-0244

Translational Relevance

The use of virotherapeutic vectors encoding different marker genes (such as GFP and β-glucuronidase in oncolytic vaccinia virus GL-ONC1) not only makes it possible to directly track infection as well as virotherapeutic destruction of malignant cells, it also allows real-time monitoring of the time course and extent of virotherapeutic oncolysis. Here, we present the results of a phase I study in which the marker gene-expressing oncolytic vaccinia virus GL-ONC1 was administered intraperitoneally via an indwelling catheter, which enabled extensive translational analyses of fluid biopsies in the context of virotherapy. This patient-specific monitoring of "oncolysis thresholds" should help to further optimize virotherapeutic monotherapies or combinatorial therapies with other compounds, such as chemotherapeutics or immune checkpoint inhibitors, in the future.

based on the vast knowledge obtained from its extensive use as smallpox vaccine in millions of people (23). Furthermore, vaccinia virus does not have a natural host and does not cause any known disease in healthy humans. In case of rare uncontrolled systemic infections, vaccinia-specific antidotes are available (e.g., vaccinia immunoglobulin or Cidofovir; ref. 24).

GL-ONC1 comprises three integrated transgenes (encoding Ruc-GFP, β-glucuronidase, and β-galactosidase). Disruption of nonessential vaccinia genes by insertion of these three foreign gene expression cassettes not only attenuates the virus but also enhances its tumor-specific targeting and opens up the possibility of real-time monitoring of tumor cell infections. Thereby, repetitive tumor biopsies (solid or liquid) can be analyzed for key parameters of GL-ONC1 such as efficiency of tumor colonization, in-patient virus replication, and subsequent oncolysis.

The primary purpose of this phase I study was to determine the maximum tolerated dose (MTD) and the spectrum of toxicities associated with first-in-man intraperitoneal administration of a state-of-the-art marker-gene encoding recombinant vaccinia virus (GL-ONC1) to patients with advanced peritoneal carcinomatosis. The ability to track viral replication, the potential clinical efficacy, and the biologic effects of this novel therapeutic strategy in a real-time scenario were assessed by repetitive analyses of liquid biopsies (i.e., peritoneal fluids). This novel approach allows patient–individual monitoring during virotherapeutic treatment and thereby is believed to pave the way to a personalized virotherapy not only in peritoneal carcinomatosis, but also in other malignancies.

Patients and Methods

Study oversight

This study (NCT01443260) was sponsored by Genelux GmbH, which provided the study drug (GL-ONC1) and worked closely with the principal investigator in all study aspects. This study was conducted according to the principles of Good Clinical Practice as described in the International Conference on Harmonization guidelines. The protocol and its amendments were approved by the relevant institutional review boards and ethics committees. All participants provided written informed consent.

Study design

This is an open label, dose escalating, nonrandomized, single center phase I study on oncolytic vaccinia virus GL-ONC1. The virus was administered in three dose levels ranging from 10^7 to 10^9 plaque forming units (pfu) per cycle via intraperitoneal infusion of a total volume of 520 mL employing an indwelling catheter. In total, 9 patients were treated in the three different dose levels or until the MTD was reached. The primary endpoint of this study was to determine the MTD of GL-ONC1 and the dose-limiting toxicities (DLT) when being administered intraperitoneally. Secondary endpoints included tolerability, detection of GL-ONC1 replication in body fluids and excrements (viral shedding analyses), GL-ONC1 delivery to malignant and normal cells, evaluation of anti-GL-ONC1–neutralizing antibody development, and evidence of antitumor activities. DLT was defined as grade $\geq$4 toxicity or grade $\geq$3 neurotoxicity or cardiotoxicity or any other drug-related nonhematologic grade $\geq$3 toxicity lasting >5 days or grade $\geq$4 toxicity (including flu-like symptoms, nausea and vomiting, which take place despite appropriate prophylactic measures). DLT was further defined as drug-related hematologic toxicities with absolute neutrophil count (ANC) $<0.5\times10^9$/L lasting for >5 days, or ANC $<0.5\times10^9$/L with an axillary temperature >38.5°C lasting >1 hour (febrile neutropenia), or with platelet count $<25\times10^9$/L. All AEs and SAEs were graded using the Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0. During the study, samples of body fluids [urine, sputum, anal swab, blood fractions, and peritoneal fluid (naturally developed peritoneal fluid [PF] and/or peritoneal lavage [PL])] were taken in each cycle (Cx) at day 1 (D1) before treatment (CxD1 pre), 2 hours after treatment (CxD1), and at days CxD2, CxD3, CxD5, and CxD8. In case peritoneal fluid was available, samples at varying additional time points were used for supplemental analyses. Performed analyses included viral plaque assay, β-glucuronidase assay (β-gluc), GL-ONC1 neutralization assay, and immunocytochemical analyses.

Patients

Eligible patients ($\geq$18 years) had histologic or cytologic documented advanced stage of peritoneal carcinomatosis or peritoneal mesothelioma being refractory to standard therapies and exhibiting a likely survival of >4 months. Other inclusion criteria were an Eastern Cooperative Oncology Group performance status score $\leq$2. Furthermore, all acute toxic effects of any prior radiotherapy, chemotherapy, or surgical procedures had to be resolved to grade $\leq$1, according to CTCAE. In addition, any chemotherapy had to be stopped at least 28 days prior to receiving study drug. Required baseline laboratory data included an absolute neutrophil count (ANC) $\geq1.5\times10^9$/L; platelets $\geq75\times10^9$/L; hemoglobin $\geq$9.5 g/dL; serum creatinine $\leq2\times$ upper limit of normal (ULN); total bilirubin $\leq5\times$ ULN; AST/ALT $\leq7.5\times$ ULN, and serum albumin $\geq$2.5 g/dL.

Exclusion criteria were vaccination or immunotherapy for 28 days before study therapy; vaccination with any of the vaccinia strains within the past 10 years; brain metastases; fever and any active immunosuppressive systemic infection or a suppressed immune system; previous organ transplantation; splenectomy; clinically significant dermatologic disorders, skin lesions or ulcers, any history of atopic dermatitis, or any history of Darier disease; a clinically significant history of cardiac disease; known allergy to ovalbumin or other egg products; and pregnancy or breast-feeding.

Oncolytic vaccinia virus GL-ONC1

GL-ONC1 is the proprietary name of GMP-derived material of genetically modified oncolytic vaccinia virus GLV-1h68, which is a derivative of the *Lister* strain of vaccinia (25).

Cell lines

For all analytic assays (viral plaque assay, β-gluc assay, and GL-ONC1 neutralization assay) we used African green monkey kidney fibroblasts (CV-1 cells). CV-1 cells were purchased from ATCC (CCL-70) and Mycoplasma testing was performed regularly every 3 months (MycoTOOL PCR Mycoplasma Detection Kit, Roche). For all analytic assays CV-1 cells were exclusively used between passages 20 and 40.

^{18}F-FDG-PET-CT

Tumor response was assessed by whole-body imaging comparing tumor mass at three different time points: (i) before the study (^{18}F-FDG-PET-CT); (ii) mid-term (prior to cycle 3; "conventional") contrast-enhanced CT; and (iii) after treatment (^{18}F-FDG-PET-CT). PET-CT acquisition protocol was performed as described previously (26). RECIST version 1.1 and Choi criteria were used for the determination of disease status.

Vaccinia virus plaque assay

Viral plaque assays were performed for determination of infectious virus particles in clinical samples from patients treated with study virus GL-ONC1 at indicated sampling days. Dilutions of urine, sputum, anal swab, blood fractions, and peritoneal fluid were titrated on confluent layers of CV-1 cells in 24-well plates as described previously (25). Productive virus infection was determined via staining with a vaccinia virus-specific primary antibody (Quartett) and a secondary horseradish peroxidase-conjugated polyclonal anti-rabbit antibody (Dianova). After an incubation period of 24 hours, cell monolayers were stained with Histogreen Immunostaining Kit (Linaris) according to the manufacturer's instructions. The accuracy of this biological assay encompasses a deviation of the resulting pfu values in the range of ± 0.5 logs.

Immunocytochemistry

Sections of cell blocks with ascitic cells were stained either with hematoxylin and eosin (HE) for morphologic evaluation or underwent analysis by immunocytochemistry. Primary antibody against GL-ONC1 (VACV-A27L; Abcam) was applied to assess vaccinia virus protein A27L expression. For identification of peritoneal tumor cells, a mouse anti-EpCAM antibody (BerEP4; Dako) was used with exception of patients with mesothelioma.

β-Gluc assay

Oncolytic activities of GL-ONC1 were quantified by the detection of β-gluc. Dilutions of PF and EDTA plasma samples were analyzed as described previously (27).

GL-ONC1 neutralization

GL-ONC1–neutralizing titers were measured in serum samples directly before each cycle application (C×D1 pre), in cycle 1 at day 8 (C1D8), and if available also at later time points. Preexisting antibodies were analyzed in samples taken directly before GL-ONC1 was applied for the first time in cycle 1 (C1D1 pre). For this purpose, $\log_2$ dilutions of serum samples were mixed with control virus and transferred to CV-1 cells. After 48 hours, resulting plaques were detected using a vaccinia virus–specific primary antibody (Quartett). Fifty percent neutralization was given when the number of plaques counted for one dilution was equal or less than 50% of the number of plaques that appeared in the negative control.

Results

Patient demographics

In total, 9 patients underwent GL-ONC1 treatment (patient demographics summarized in Table 1).

Median patient age was 55 (range, 39–68 years). Patients with ovarian carcinoma, gastric carcinoma, mesothelioma, one primary peritoneal carcinoma, and a single case of CUP (cancer of unknown primary with adenocarcinoma differentiation) were treated. None of the patients had a surgical option because of extended peritoneal carcinomatosis and all had received multiple chemotherapeutic treatments in the past. Preexisting antibodies against GL-ONC1 were analyzed because 6 of 9 patients had received vaccinia vaccines decades before. Patients were enrolled in escalating GL-ONC1 dose cohorts (Table 1). Because of a quite advanced disease status and/or rapid tumor progress under virotherapy, not all patients were able to complete all four treatment cycles as planned in the study protocol. Prior to each study virus application, samples from freshly prepared GL-ONC1 infusions were used to systematically verify *de facto* dosages of GL-ONC1 (Supplementary Fig. S1).

Table 1. Patient demographics and analysis of tumor response

Dosage of GL-ONC1	Pt. ID	Age (sex)	Tumor entity	Vacc.	Dosages ($\Sigma = 24$)	RECIST 1.1 (prim. target lesions)	CHOI (target lesions)
1×10^7 pfu (cohort 1)	01	62 (f)	Gastric	−	1	N.d.	N.d.
	03	54 (m)	Mesothelioma	+	4	SD[a]	SD[a]
	04	47 (f)	Gastric	+	1	N.d.	N.d.
1×10^8 pfu (cohort 2)	05	62 (f)	Ovarian	+	4	PD[a]	PD[a]
	06	40 (f)	Ovarian	−	1	N.d.	N.d.
	07	55 (f)	Ovarian	+	4	SD[a]	PD[a]
1×10^9 pfu (cohort 3)	08	65 (f)	Primary peritoneal carcinoma	+	4	PD[a]	PD[a]
	09	68 (f)	CUP (adeno)	+	2	SD[b]	PD[b]
	10	39 (m)	Mesothelioma	−	3	SD[b]	PR[b]

Abbreviations: pfu, plaque-forming unit; f, female; m, male; SD, stable disease; PD, progressive disease; PR, partial response; Pt. ID, patient identity; Vacc., vaccination status; CUP, cancer of unknown primary; and N.d., not determined.

[a]After final PET-CT.

[b]After midterm CT.

Table 2. GL-ONC1–related adverse events

Drug-related events, number of occurrences (number of patients)	Grade 1	Grade 2	Grade 3	Grade 4
Gastrointestinal disorders				
Diarrhea	2 (2)	—	—	—
Nausea	4 (2)	1 (1)	—	—
Vomiting	4 (4)	—	—	—
Flatulence	3 (1)	—	—	—
Abdominal pain	6 (4)	1 (1)	2 (2)	—
Ascites	—	—	6 (2)	—
Generalized symptoms				
Fatigue	1 (1)	—	2 (1)	—
Pyrexia	16 (8)	3 (3)	—	—
Chills	2 (1)	—	—	—
Abnormal blood parameters				
Alanine aminotransferase increased	—	1 (1)	—	—
Aspartate aminotransferase increased	—	—	1 (1)	—
Alkaline phosphatase increased	1 (1)	—	—	—
Creatinine increased	2 (2)	—	—	—
Gamma-glutamyltransferase increased	—	—	1 (1)	—
Lymphocyte count decreased	—	19 (7)	7 (5)	-
C-reactive protein increased	—	20 (7)	—	—
Hyperhidrosis	1 (1)	—	—	—
Cough	1 (1)	—	—	—
Pain in extremity	1 (1)	—	—	—
Herpes simplex virus infection	2 (2)	—	—	—

Adverse events and safety

Adverse events observed in this study appeared to be dose independent (Table 2); correspondingly, no DLT was reported.

The most common adverse events included decreases in lymphocyte count, increases in C-reactive protein (CRP), pyrexia, and abdominal pain. All alterations were transient in nature and could be managed medically. There were no grade 4 dose-limiting clinical toxicities attributable to GL-ONC1 and no treatment-related deaths occurred. Importantly, no viral shedding was observed in any patient at any dose level (depicted exemplarily for patient 01 in Supplementary Table S1).

Systemic effects

Clinical evaluation and laboratory testing before first-time virotherapy and at several time points thereafter revealed transient inflammatory reactions including (i) elevated body temperature, (ii) increases in CRP, (iii) increases in leukocytes, as well as (iv) significant decreases in lymphocytes in 7 of 9 patients (depicted exemplarily for patient 01 in Supplementary Fig. S2). Of note, peak CRP levels as well as peak leukocyte counts were regularly found in periods of elevated body temperature being observed between day 4 of cycle 1 (C1D4) and day 10 of cycle 1 (C1D10). Also, a maximum decrease of the lymphocyte count could be detected in patient 01 at C1D10.

In the next setting, the neutralizing anti-vaccinia response was determined in serum samples of all 9 patients directly before each cycle application (CxD1 pre), in cycle 1 at day 8 (C1D8), and if available also at later time points (Fig. 1). Preexisting antibodies were analyzed directly before GL-ONC1 was applied for the first time in cycle 1 (C1D1 pre). Interestingly, in only 3 prevaccinated patients (04, 05, and 08) detectable levels of preexisting antibodies could be observed. In all 9 patients, neutralizing capacities against vaccinia virus increased over time with the earliest elevation noticed at 7 days after the first application cycle (C1D8), detected in 4 patients (05, 07, 08, and 10). Prestudy vaccination status as well as different dose cohorts had no impact on this effect.

Detection of GL-ONC1 infection, replication, and virotherapy-mediated oncolysis

Utilizing the patients' indwelling catheter, real-time monitoring of GL-ONC1 functionality could be performed using ascitic fluid (AF) or, in absence of malignant ascites, peritoneal lavages (PL), which were obtained before (pre) and at several time points after intraperitoneal administration of GL-ONC1 to assess (i) GL-ONC1 infection of malignant cells in PF, (ii) GL-ONC1 replication, and (iii) resulting oncolysis, as indicated by the release of GL-ONC1 encoded β-gluc marker protein in the course of tumor cell oncolysis. Remarkably, in 8 of 9 patients, efficient intraperitoneal infection and in-patient replication of GL-ONC1 as well as subsequent oncolysis were demonstrated throughout cycle 1 of GL-ONC1 administration. For patient 01, 8 days after intraperitoneal administration of GL-ONC1 (C1D9), the concentration of viral particles retrieved from AF was more than four times higher (8.8×10^4 pfu/mL) than the original treatment dose (10^7 pfu/520 mL or 1.9×10^4 pfu/mL; Fig. 2A). This finding indicates a significant in-patient viral replication and production of progeny viral particles. Detection of GL-ONC1–encoded GFP marker protein in tumor cells isolated at C1D8 from patient's ascites (Fig. 2B, left) confirmed this assumption. Furthermore, this patient also exhibited a prolonged production and presence of infectious viral particles in PF, being observed for at least 21 days post-treatment (C1D22; Fig. 2A).

To evaluate the extent of GL-ONC1–mediated oncolysis, we first measured the cellular release of LDH, a nonspecific marker indicating gross cellular destruction, into both PF and blood plasma. Because LDH release does not directly prove that GL-ONC1 is responsible for cell destruction, we also determined the cellular release of GL-ONC1–encoded transgenic β-gluc, which is only released after successful virus-induced oncolysis. Therefore, β-gluc constitutes an explicit marker for GL-ONC1–mediated oncolysis (27). As exemplified for patient 01 (Fig. 3A and B), both markers (LDH and β-gluc) increased massively during the first 2 weeks following intraperitoneal

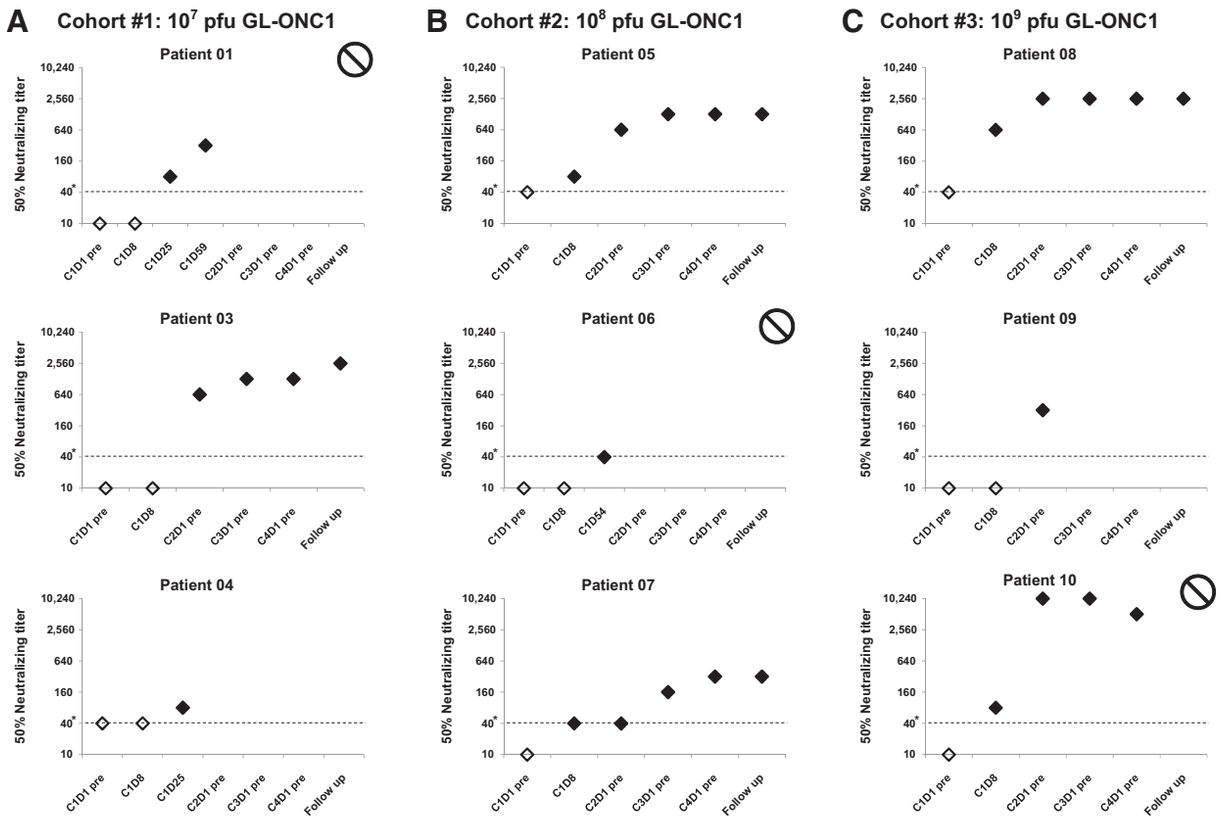

Figure 1.

Neutralizing activities against GL-ONC1. Vaccinia virus–specific antibodies were determined in all 9 patients of study cohorts #1 (**A**), #2 (**B**), and #3 (**C**) at different time points throughout the study (CxD1 pre, pretreatment in cycle x on day 1; further samples were taken as indicated); ⊘ not prevaccinated; open data points, baseline titers before occurrence of GL-ONC1–induced neutralizing antibodies; closed data points, neutralizing titers being elevated significantly; no data points, no samples available; *, detection minimum at 1:40, indicated by dotted lines.

administration of GL-ONC1 and exhibited maximum concentrations in PF at C1D11, which coincided with the period of maximum inflammation (Supplementary Fig. S2). When comparing patients found to be positive for oncolysis concerning their peak levels of β-gluc in both PF and plasma (Fig. 3C), a great variation was found with no correlation to the respective GL-ONC1 dosage levels. However, there was a direct dependency between levels of β-gluc in PF and plasma of the same patient. Notably, only 1 patient (Fig. 3C, patient 07) showed no signs of oncolysis at all and therefore had to be classified as a single nonresponder throughout this study.

Response to virotherapy

To assess the virotherapeutic treatment response, immunocytochemistry was performed in PF samples being available before and at different time points after intraperitoneal application of GL-ONC1. In 4 of 7 patients, virus-infected tumor cells could be detected in PF several days after first intraperitoneal application of GL-ONC1. Because availability of PF differed in each patient, the time points at which we were able to analyze PF samples were not consistent between patients, a fact that has to be considered when interpreting obtained data. In patient 01, PF samples could be analyzed at C1D4 and C1D8. Interestingly, H&E staining of isolated ascitic cells at both time points revealed numerous tumor cells, identified by staining with a tumor cell–specific EpCAM antibody. The virus-specific infection study of those tumor cells was shown by staining with a GL-ONC1–specific antibody as well as by EpCAM/GL-ONC1 double staining (Fig. 4, inserted red box in bottom left panel; Supplementary Fig. S3 represents a further example; patient 08). Semiquantification of those cells (Supplementary Table S2A) at C1D4 revealed that 5% of all ascitic cells were classified as EpCAM-positive tumor cells, while only 10% of these EpCAM-positive tumor cells were already positive for GL-ONC1 infection at this early time point. Four days later (at C1D8), only 1% of all ascitic cells remained EpCAM-positive while now more than 50% of these EpCAM-positive tumor cells were found to be GL-ONC1 positive. Interestingly, on subsequent time points, employing peritoneal lavages at C1D22 and C1D59, no EpCAM-positive cells were found at all (Supplementary Table S2A), indicating a significant reduction of the number of detached/floating tumor cells (i.e., clearance).

Furthermore, accessory cells in PF samples were typified before and at different time points after GL-ONC1 application (Supplementary Table S2B representing results from patient 01). Semiquantification of granulocytes in PF revealed a strong initial increase in the days subsequent to GL-ONC1 application, regardless of the treatment cycle. Subsequently, granulocytes decreased,

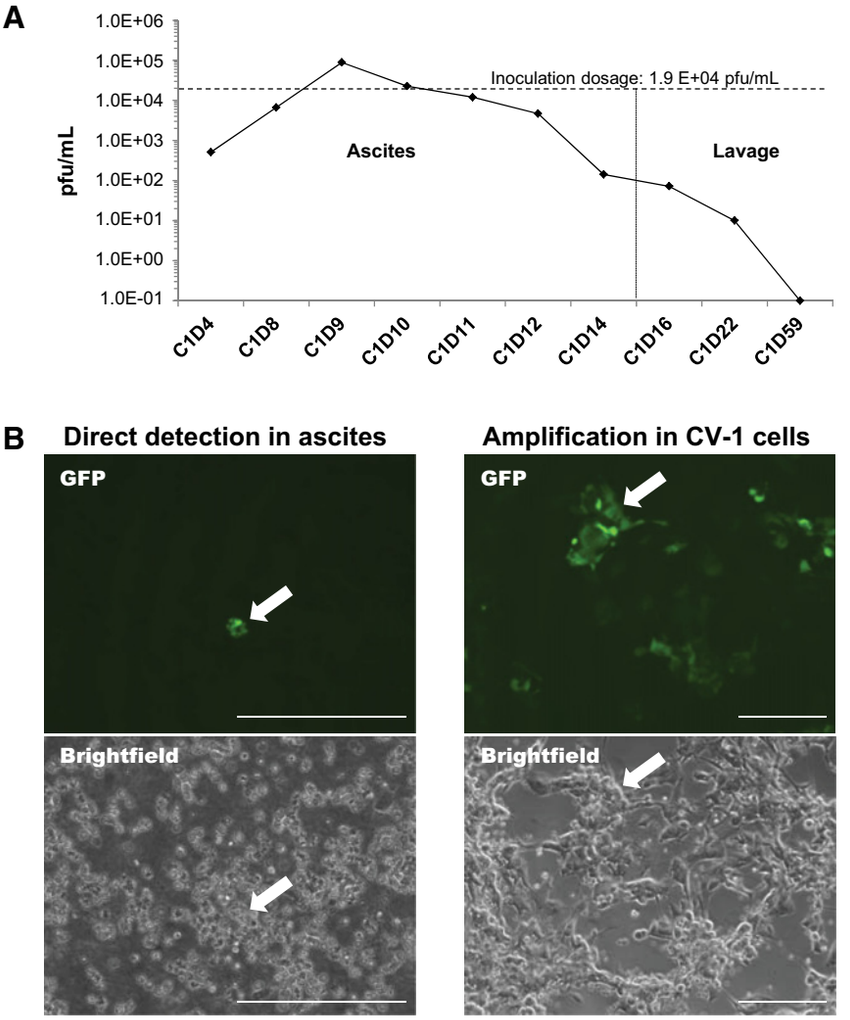

Figure 2.
GL-ONC1 replication in patients with peritoneal carcinomatosis. **A,** Time course of in-patient production of new infectious viral particles (here: patient 01). **B,** Direct detection of GL-ONC1–infected cells in ascites by fluorescence microscopy analysis (here: patient 01, C1D8; left; white arrow pointing out a representative GFP-positive cell expressing GL-ONC1–encoded marker gene GFP); when incubating CV-1 monolayers for 24 hours with the same sample, a significant amplification of GL-ONC1 infection was obtained (right; white arrows) now exhibiting numerous GFP-positive cells; scale bars, 200 μm.

with a minimum level at day 7 after virus application, which usually was the last time point on which samples were taken. Interestingly, semiquantification of lymphocytes in PF revealed opposite results: in the days after virus application, lymphocytes first decreased before they increased, being indicative for an intense antiviral combat taking place within the peritoneal cavity.

When looking on response patterns by tumor imaging, the read-out was limited in this phase I study: (i) 3 of 9 patients were able to receive only a single dose of GL-ONC1 (due to unexpected rapid tumor progress); therefore, no regular postvirotherapy imaging could be performed in these patients; (ii) another 2 patients received two or three dosages of GL-ONC1; accordingly, only a "mid-term" CT (per protocol after two cycles) was performed; as a result, both patients exhibited a stable disease by RECIST 1.1 criteria and one partial response and one progressive disease by Choi criteria, when not only size, but also CT density criteria were included in the evaluation (Table 1); (iii) among the 4 patients who had completed all four cycles of intraperitoneal GL-ONC1 virotherapy, postvirotherapy PET-CT exhibited two stable diseases and two progressive diseases by RECIST 1.1 criteria and one stable disease and three progressive diseases by Choi criteria (Table 1).

Taken together, these data obtained in a cohort of heavily pretreated patients, exhibiting advanced peritoneal carcinomatosis with rather large tumor masses, warrants further investigation in phase II trials, preferably in patients with cancer exhibiting less advanced peritoneal carcinomatosis with less tumor masses.

Discussion

This is a report on a phase I study of GL-ONC1, a Lister strain–derived oncolytic vaccinia virus, employed as a locoregional treatment in patients with advanced peritoneal carcinomatosis. When assessing the study's primary endpoint of safety, GL-ONC1 was well tolerated and no dose-limiting toxicities were observed, that is, no MTD was reached when being administered intraperitoneally. All adverse events were limited to flu-like symptoms and were transient in nature. Interestingly, unvaccinated patients ($n = 3$) did not show any evidence of increased side effects compared with prevaccinated patients ($n = 6$). Furthermore, we did not find any evidence of GL-ONC1 shedding in body fluids and, even more important, did not obtain any report on horizontal

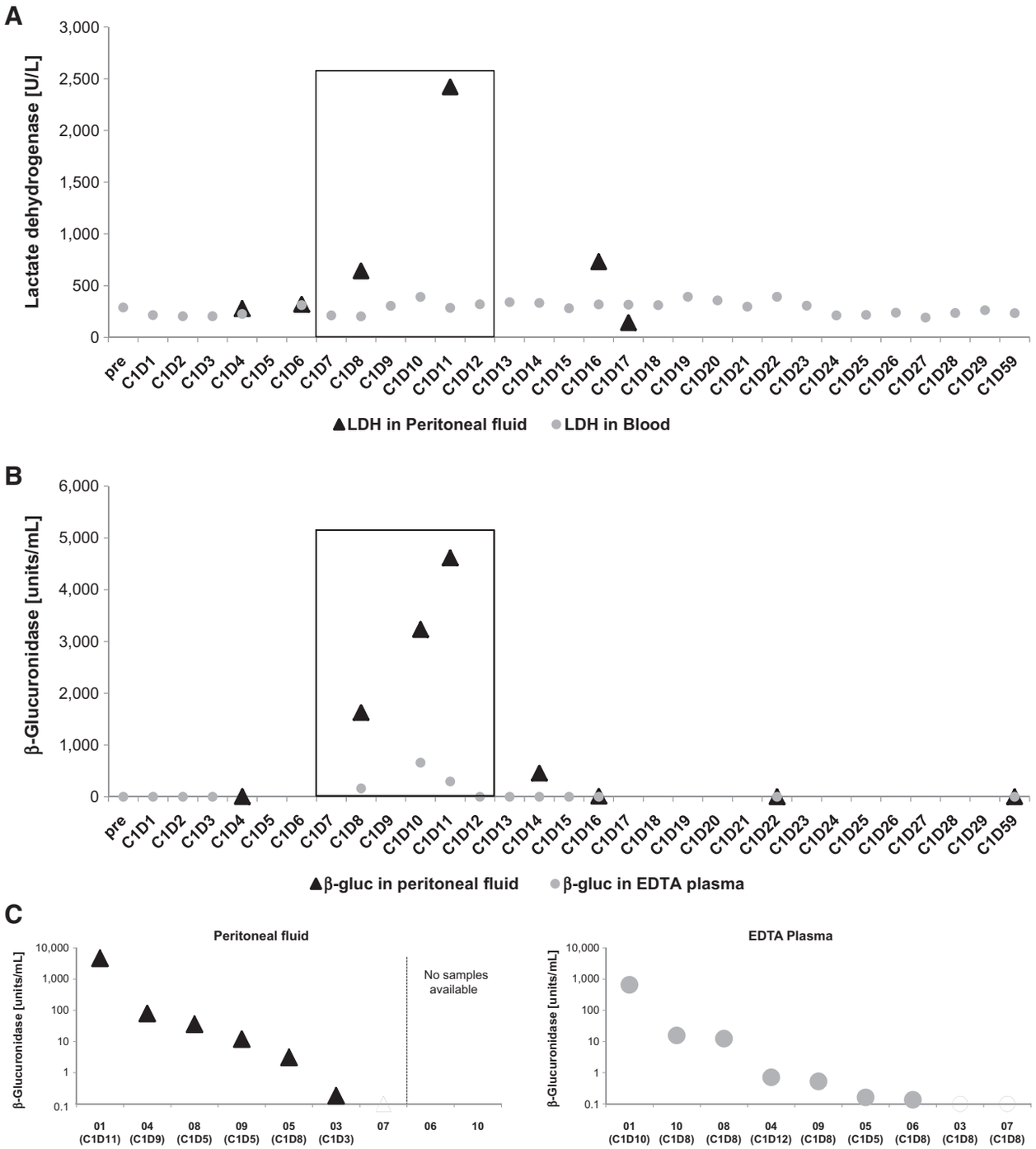

Figure 3.

GL-ONC1 oncolysis in patients with peritoneal carcinomatosis. Oncolytic activity of GL-ONC1 determined in samples of PF and EDTA plasma (here: patient 01) by measuring cellular release of lactate dehydrogenase (LDH; **A**) and virus-encoded β-gluc (**B**). High levels of LDH and β-gluc were detected in PF in the period of elevated body temperature (large boxes in black) with a peak value for patient 01 at C1D11; β-gluc levels in EDTA plasma increased in the same time period. pre: pretreatment. **C,** Comparison of β-gluc peak levels in PF and EDTA plasma for all 9 study patients (sorted in descending order, respectively); open data points, samples negative for β-gluc.

transmissions. When looking on study's secondary endpoints (i) the recommended dose (RD) for subsequent phase II studies was 10^9 pfu per intraperitoneal application; furthermore, (ii) immunocytochemical analyses on ascitic cells provided clear evidence for tumor delivery, that is, specific colonization of intraperitoneal tumor cells by GL-ONC1;

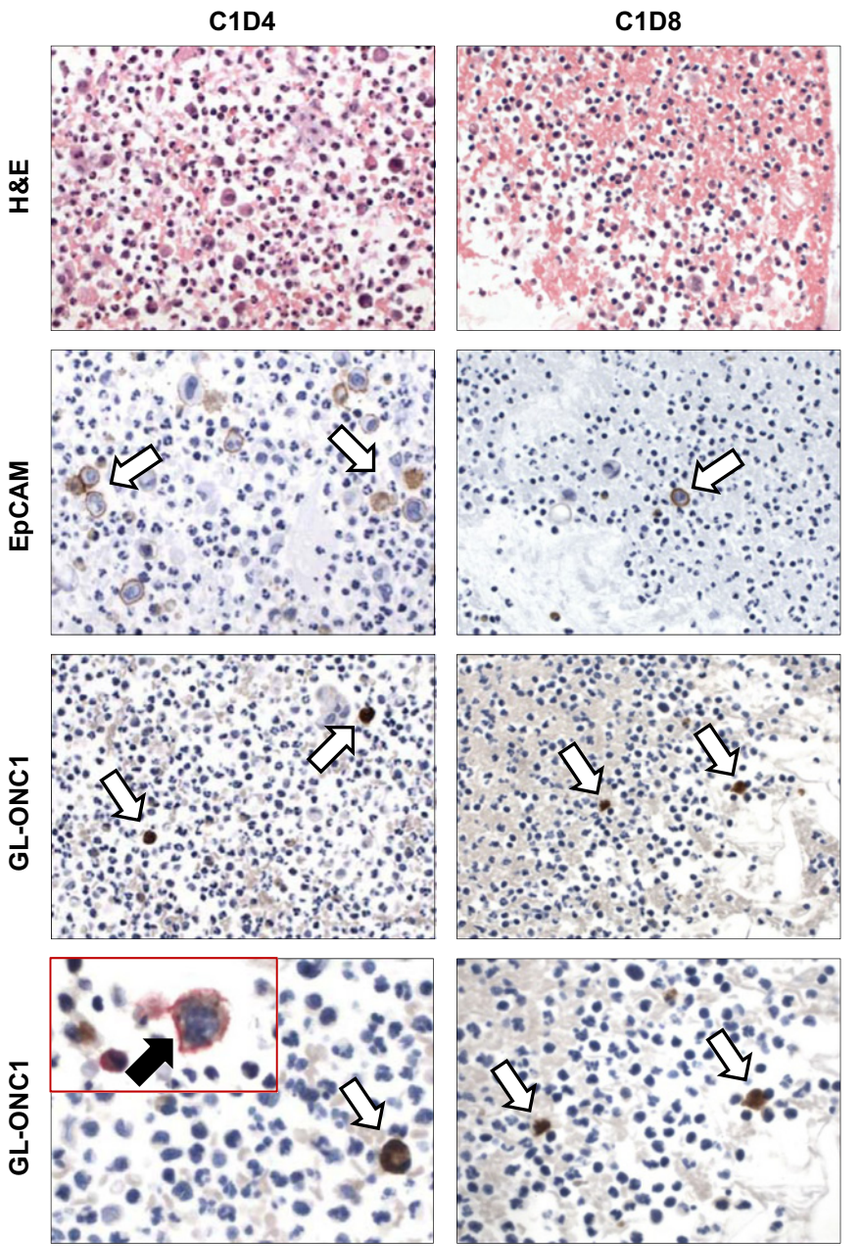

Figure 4.
Cytologic analyses of GL-ONC1–infected tumor cells. Cytologic analyses were performed on ascites samples isolated at C1D4 and C1D8 (patient 01) by staining with hematoxylin and eosin (H&E) as well as with EpCAM- and GL-ONC1–specific antibodies (white arrows indicating positive cells; original magnification 400×; lowermost images: 630×). Inserted red box shows double staining of a representative EpCAM-positive tumor cell (red membranous staining pattern) infected with GL-ONC1 (brown granular cytoplasmic staining pattern) at C1D4.

(iii) when titering GL-ONC1 from ascitic fluid, intraperitoneal replication of GL-ONC1 could be demonstrated at significant levels for up to 22 days after virotherapy; (iv) significant, direct antitumoral activity could be demonstrated by cellular release of GL-ONC1–encoded transgenic β-glucuronidase; (v) neutralizing activities against GL-ONC1 could be detected as early as 1 week after its first application in both prevaccinated and unvaccinated patients with cancer. On the basis of these comprehensive phase I data, it was concluded that intraperitoneal application of GL-ONC1 is both safe and efficient, warranting its clinical development in further phase II studies. In this regard, a phase Ib/II study was initiated recently, where GL-ONC1 is administered intraperitoneally now in the form of multiple dosages specifically in patients with peritoneal carcinomatosis originating from ovarian cancer (NCT02759588).

We further reported the evidence of GL-ONC1 infection, inpatient replication, and subsequent oncolysis of tumor cells in 8 of 9 study patients. These results are not only based on the recovery of infectious virus particles, but also on detection of virus-encoded marker protein β-glucuronidase (β-gluc) in PF samples obtained via repetitive paracentesis through the indwelling peritoneal catheter. Taking into account (i) that the extent of ascites production differed markedly between ascites-positive patients and (ii) that peritoneal lavages had to be conducted in ascites-negative patients (resulting in dilution phenomena), the best conclusions could be drawn by comparing a variety of different parameters in identical samples.

Exemplarily, in patient 01, treated with the lowest GL-ONC1 study dose (10^7 pfu), a massive increase of infectious virus particles was detected in PF samples between C1D4 and C1D9 (Fig. 2A). Furthermore, cytologic analysis of ascites samples obtained at C1D4 and C1D8 from this patient also revealed increasing rates of tumor cell infection over time as well as a profound destruction of malignant cells (Supplementary Table S2A). Oncolysis could be proven by the detection of GL-ONC1–encoded β-gluc in cell-free PF samples (Fig. 3B). Because β-gluc strictly remains intracellular until (onco)lysis of infected cells occurs, detection of this marker protein in cell-free PF constitutes a direct marker for in-patient virus replication and subsequent virus-induced "direct" oncolysis (27). In patient 01, the increase in β-gluc perfectly went along with the amount of infectious progeny virus particles being detected in ascitic fluid (compare Figs. 2A and 3B). Interestingly, there was a slight delay of 2 days between the peak of GL-ONC1 replication (Fig. 2A, C1D9) and the peak of β-gluc release (Fig. 3B, C1D11), which simply can be explained by the "time consuming" process required from the onset of virus-induced breakdown of the tumor cell metabolism until the final execution of oncolysis and concomitant release of β-gluc marker proteins.

Interestingly, the occurrence of viral replication between C1D4 and C1D8 matches with data from previous clinical studies with Pexa-Vec, an oncolytic vaccinia virus of the Copenhagen strain expressing the GM-CSF, in subjects with advanced hepatocellular carcinoma. After intratumoral injection, patients were shown to have subsequent intravenous exposure of Pexa-Vec genomes due to replication in the tumor approximately 1 week after dosing (16, 28). Furthermore, in a phase Ib trial of biweekly intravenous Pexa-Vec administration to patients with colorectal cancer, significant increases from baseline in plasma GM-CSF concentrations could be detected at day 5 after virus application, which constitutes a specific but insensitive marker for transgene expression and therefore viral replication at this timepoint (15).

When comparing all 9 study patients for this unique set of parameters (GL-ONC1 infection, in-patient replication, and subsequent oncolysis), a large variety of reaction patterns was observed, including one single nonresponder patient showing no signs of oncolysis at all (Fig. 3C). These differences in outcome might be explained in part by differences in PF composition of the respective study patients that are known to contain different immune relevant factors like cytokines, chemoattractants, and especially components of the complement system (29, 30). In this context, it is of major interest that the complement system is known to trigger several effector functions that contribute to an instant, highly effective virus inactivation and elimination (31), taking place not only in the blood stream, but most probably also in the peritoneal cavity. Interestingly, malignant cells isolated from the ascitic fluid of patient 04 showed a much higher rate of infection with GL-ONC1 under *ex vivo* conditions when cultivation took place in cell culture medium (definitely being free of any biologically active complement factors), and not in whole ascitic fluid of patient 04 (data not shown). Furthermore, *ex vivo* inactivation of GL-ONC1 by whole ascitic fluid of patient 04 could be partially reversed by standard heat inactivation procedures known to destroy all complement factors (data not shown). These findings support our assumption of a significant, at least partial, complement-mediated inactivation of GL-ONC1 directly after application into the peritoneal cavity. Preclinical studies of Evgin and colleagues demonstrated that short-term complement inhibition not only stabilized vaccinia virus in the blood after intravenous application and led to improved delivery to tumors, but also enhanced tumor infection when virus was directly injected into tumors of a rat mammary adenocarcinoma model (32). On the basis of these findings, a phase 1b study was initiated that evaluates the safety and effect of GL-ONC1 administered intravenously in combination with eculizumab (terminal complement inhibitor) to patients with solid organ cancers (NCT02714374).

As observed in several virotherapy clinical trials before, infusion of GL-ONC1 in all treated patients in each cycle led to induction of transient signs of inflammation of different intensities, including elevated body temperature, massive increase of acute phase proteins such as CRP, leukocytosis, as well as lymphopenia (Supplementary Fig. S2). Furthermore, cytologic analysis of ascitic cells of patient 01 revealed a massive GL-ONC1–induced peritonitis (Supplementary Table S2B).

Because of an unscheduled (not per protocol) ^{18}F-FDG-PET-CT performed in patient 01 at C1D24, we had the unique chance to gain insight into GL-ONC1–induced intraperitoneal reactions. This scan, taken more than 3 weeks after the first virotherapy application, showed a very strong uptake of FDG in all regions of the peritoneal surface (Supplementary Fig. S4, top right). Of note, the corresponding scan performed before virotherapy (Supplementary Fig. S4, top left) merely exhibited a localized pattern of FDG uptake, typical for multifocal peritoneal carcinomatosis. Unfortunately, it is currently unknown whether this largely increased intraperitoneal metabolic activity is either due to (i) a massive virus-induced peritonitis going along with high glucose consumption, (ii) a massive GL-ONC1 replication in close to 100% of the peritoneal tumor nodules (now including also tiny ones), requiring a highly increased glucose uptake for widespread mass production of progeny viral particles, or (iii) an extremely rapid (highly unlikely) tumor progression. To address these thrilling hypotheses, a laparoscopy would have been required for macroscopic inspection as well as sampling of solid probes from the peritoneal lining. However, due to ethical reasons, our study protocol did not allow such an invasive procedure.

GL-ONC1 treatment induced a humoral antiviral immune reaction in all 9 patients, which increased over time with the earliest detected elevation at C1D8 (Fig. 1). Vaccination status as well as different dosages of GL-ONC1 (low, medium, and high) had no impact on the extent of this effect. It is important to point out that all effects of tumor colonization, in-patient replication, and oncolysis observed in this study were found to be limited to the first cycle of treatment (data not shown). According to the fact that neutralizing activities could be detected as early as one week after the first application of GL-ONC1 (C1D8), it seems likely that this profound humoral immune reaction constitutes a major factor for the restricted efficacy of virotherapy in subsequent cycles, at least when looking on the direct mechanism of our study virus (i.e., oncolysis resulting from mass production of progeny virus particles), and not on the putative induction of a profound, long-lasting antitumoral immune response.

This finding of intraperitoneal neutralization of the study virus [due to the effects of seroconversion (in unvaccinated patients) and boostering (in vaccinated patients)] went along

with our observation that periods with fever and inflammation started much earlier in cycles 2–4, that is, already between 1 hour and 1 day after reapplication of GL-ONC1 (data not shown). When comparing data from other clinical trials investigating different strains of oncolytic vaccinia viruses (e.g., Pexa-Vec, vvDD), an induction of anti-vaccinia antibodies until day 29 after dosing could also be observed, irrespective of the application route used (15–18).

Originally, oncolytic viruses were designed to function solely as tumor-lysing therapeutics but now have been clinically shown to initiate powerful multicellular immune surveillance mechanisms being instrumental in the elimination of solid tumors. On the basis of current knowledge, at least three requirements are assumed for an effective "immuno-virotherapy" response: (i) targeted replication of oncolytic viruses in the tumor bed; (ii) initiation of an immune-stimulating or immune-recruiting inflammatory response; and (iii) exposure of tumor-associated antigens (TAA) that can be targeted by the immune system to establish an effective and long-lasting antitumoral immune response (11). Therefore, further clinical trials with locoregional application of GL-ONC1 should focus mainly on multiple dosing regimens in a "first week/1 week time frame" before neutralizing antibodies do arise. The most important goal will be to achieve a profound and evenly distributed intratumoral virus replication, followed by a systemic immune cell–recruiting inflammation to initiate a strong adaptive antitumoral immune response before neutralizing antibodies may eliminate viral particles.

However, in this phase I trial, nearly all study patients had advanced tumors that were refractory to all available conventional therapies prior to study entry. Therefore, we could not expect to observe elimination of tumors throughout the body and long-term disease-free survival in these patients. One major lesson learned from this as well as other studies is that virotherapy should intervene early enough, preferably already in first- or second-line settings and going along with low tumor burden, to provide enough time for a full execution of its profound antitumoral potencies, that is, not only profound direct oncolytic tumor mass destruction (as evidenced here exemplarily for patient 01), but also oncolytic virus–induced long-lasting immune responses. On the basis of preclinical findings from Acuna and colleagues (33), adjuvant vaccinia-based virotherapeutic interventions are also conceivable for patients with peritoneal carcinomatosis, subsequent to aggressive tumor mass reductions achieved by cytoreductive surgery plus HIPEC chemotherapy, meeting the aforementioned requirement of low to very low tumor masses at the time point of virotherapy.

Taken together, key findings of this study warrant the further development of the intraperitoneal route of virotherapy, especially in patients exhibiting peritoneal carcinomatosis. Beyond that, employment of virotherapeutic vectors encoding different marker proteins (such as GFP and β-gluc in GL-ONC1) does not only make it possible to directly track infection as well as destruction of malignant cells in PF, but also enables a real-time monitoring of the time course and extent of oncolysis. Such a patient-specific monitoring of "oncolysis thresholds" could help to stratify future virotherapeutic applications. Furthermore, combined treatment with other compounds, such as chemotherapeutics or checkpoint inhibitors, also could be optimized in dependence on the results of a patient-specific monitoring of "oncolysis thresholds" as being performed in our study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank Irina Smirnow, Andrea Schenk, and Christine Geisler for excellent technical assistance and acquisition of data. They also thank Markus Burkard for his excellent support during preparation of the figures. This study was sponsored by Genelux GmbH, which provided the study drug (GL-ONC1) and designed the study protocol together with the principal investigator (U.M. Lauer). The authors thank all staff from Genelux for their support. Collection, analysis, and interpretation of data were performed exclusively by the Tübingen study team.

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Received January 22, 2018; revised April 5, 2018; accepted May 14, 2018; published first May 17, 2018.

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