Published OnlineFirst May 17, 2018; DOI: 10.1158/1078-0432.CCR-18-0244

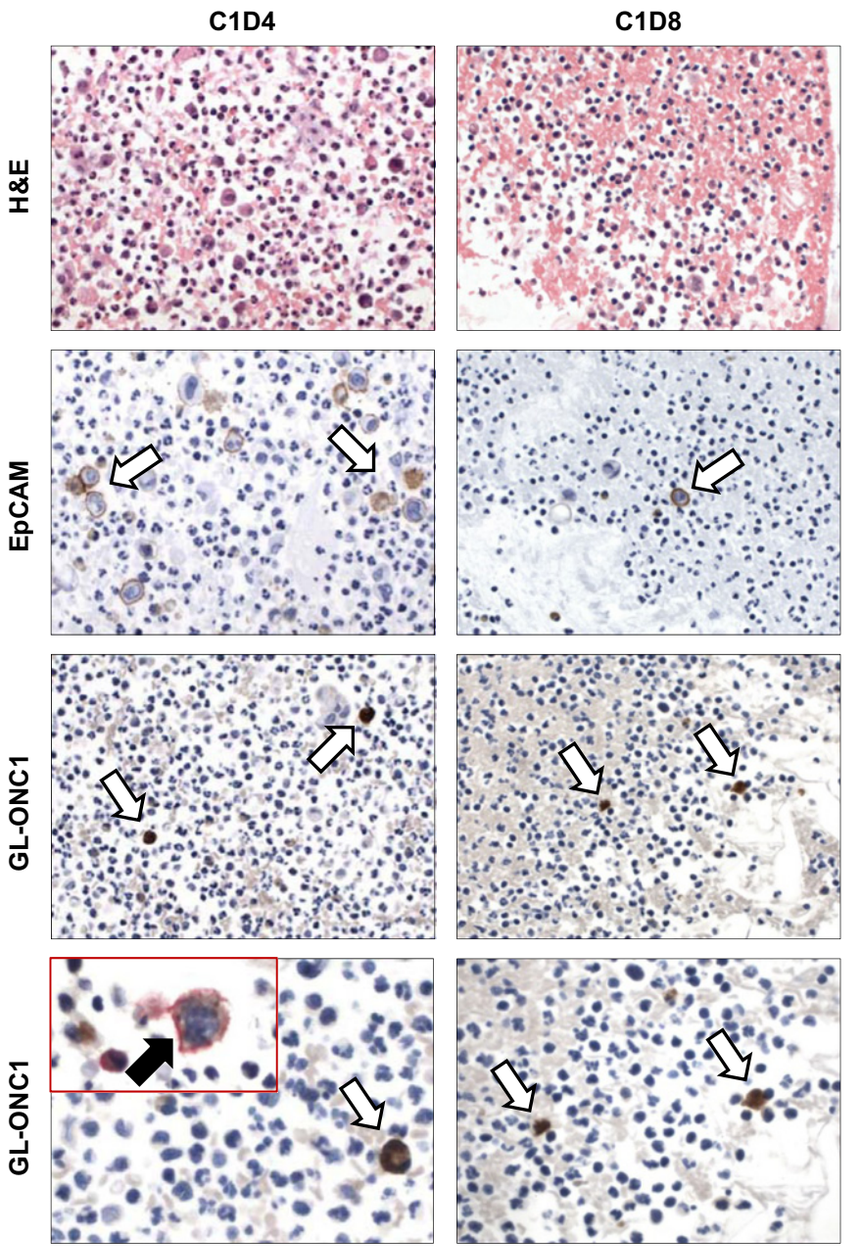

Figure 4.
Cytologic analyses of GL-ONC1–infected tumor cells. Cytologic analyses were performed on ascites samples isolated at C1D4 and C1D8 (patient 01) by staining with hematoxylin and eosin (H&E) as well as with EpCAM- and GL-ONC1–specific antibodies (white arrows indicating positive cells; original 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.

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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

Published OnlineFirst May 17, 2018; DOI: 10.1158/1078-0432.CCR-18-0244

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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Published OnlineFirst May 17, 2018; DOI: 10.1158/1078-0432.CCR-18-0244

Clinical Cancer Research

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Clin Cancer Res 2018;24:4388-4398. Published OnlineFirst May 17, 2018.