Thomas et al. Journal for ImmunoTherapy of Cancer (2019) 7:214
https://doi.org/10.1186/s40425-019-0682-1

Journal for ImmunoTherapy of Cancer

RESEARCH ARTICLE Open Access

Development of a new fusion-enhanced oncolytic immunotherapy platform based on herpes simplex virus type 1

Suzanne Thomas[1], Linta Kuncheria[1], Victoria Roulstone[2], Joan N. Kyula[2], David Mansfield[2], Praveen K. Bommareddy[1], Henry Smith[2], Howard L. Kaufman[2,3], Kevin J. Harrington[2] and Robert S. Coffin[1*]

Abstract

Background: Oncolytic viruses preferentially replicate in tumors as compared to normal tissue and promote immunogenic cell death and induction of host systemic anti-tumor immunity. HSV-1 was chosen for further development as an oncolytic immunotherapy in this study as it is highly lytic, infects human tumor cells broadly, kills mainly by necrosis and is a potent activator of both innate and adaptive immunity. HSV-1 also has a large capacity for the insertion of additional, potentially therapeutic, exogenous genes. Finally, HSV-1 has a proven safety and efficacy profile in patients with cancer, talimogene laherparepvec (T-VEC), an oncolytic HSV-1 which expresses GM-CSF, being the only oncolytic immunotherapy approach that has received FDA approval. As the clinical efficacy of oncolytic immunotherapy has been shown to be further enhanced by combination with immune checkpoint inhibitors, developing improved oncolytic platforms which can synergize with other existing immunotherapies is a high priority. In this study we sought to further optimize HSV-1 based oncolytic immunotherapy through multiple approaches to maximize: (i) the extent of tumor cell killing, augmenting the release of tumor antigens and danger -associated molecular pattern (DAMP) factors; (ii) the immunogenicity of tumor cell death; and (iii) the resulting systemic anti-tumor immune response.

(Continued on next page)

* Correspondence: heather.metcalfe@replimune.com
[1]Replimune Inc, 18 Commerce Way, Woburn, MA 01801, USA
Full list of author information is available at the end of the article

(Continued from previous page)

Methods: To sample the wide diversity amongst clinical strains of HSV-1, twenty nine new clinical strains isolated from cold sores from otherwise healthy volunteers were screened across a panel of human tumor cell lines to identify the strain with the most potent tumor cell killing ability, which was then used for further development. Following deletion of the genes encoding ICP34.5 and ICP47 to provide tumor selectivity, the extent of cell killing and the immunogenicity of cell death was enhanced through insertion of a gene encoding a truncated, constitutively highly fusogenic form of the envelope glycoprotein of gibbon ape leukemia virus (GALV-GP-R$^-$). A number of further armed derivatives of this virus were then constructed intended to further enhance the anti-tumor immune response which was generated following fusion-enhanced, oncolytic virus replication-mediated cell death. These viruses expressed GMCSF, an anti-CTLA-4 antibody-like molecule, CD40L, OX40L and/or 4-1BB, each of which is expected to act predominantly at the site and time of immune response initiation. Expression of these proteins was confirmed by ELISA and/or western blotting. Immunogenic cell death was assessed by measuring the levels of HMGB1 and ATP from cell free supernatants from treated cells, and by measuring the surface expression of calreticulin. GALV-GP-R$^-$ mediated cell to cell fusion and killing was tested in a range of tumor cell lines in vitro. Finally, the in vivo therapeutic potential of these viruses was tested using human A549 (lung cancer) and MDA-MB-231(breast cancer) tumor nude mouse xenograft models and systemic anti-tumor effects tested using dual flank syngeneic 4434 (melanoma), A20 (lymphoma) mouse tumor models alone and in combination with a murine anti-PD1 antibody, and 9 L (gliosarcoma) tumors in rats.

Results: The twenty nine clinical strains of HSV-1 isolated and tested demonstrated a broad range of tumor cell killing abilities allowing the most potent strain to be identified which was then used for further development. Oncolytic ability was demonstrated to be further augmented by the expression of GALV-GP-R$^-$ in a range of tumor cell lines in vitro and in mouse xenograft models in nude mice. The expression of GALV-GP-R$^-$ was also demonstrated to lead to enhanced immunogenic cell death in vitro as confirmed by the increased release of HMGB1 and ATP and increased levels of calreticulin on the cell surface. Experiments using the rat 9 L syngeneic tumor model demonstrated that GALV-GP-R$^-$ expression increased abscopal uninjected (anenestic) tumor responses and data using mouse 4434 tumors demonstrated that virus treatment increased CD8+ T cell levels both in the injected and uninjected tumor, and also led to increased expression of PD-L1. A combination study using varying doses of a virus expressing GALV-GP-R$^-$ and mGM-CSF and an anti-murine PD1 antibody showed enhanced anti-tumor effects with the combination which was most evident at low virus doses, and also lead to immunological memory. Finally, treatment of mice with derivatives of this virus which additionally expressed anti-mCTLA-4, mCD40L, m4-1BBL, or mOX40L demonstrated enhanced activity, particularly in uninjected tumors.

Conclusion: The new HSV-1 based platform described provides a potent and versatile approach to developing new oncolytic immunotherapies for clinical use. Each of the modifications employed was demonstrated to aid in optimizing the potential of the virus to both directly kill tumors and to lead to systemic therapeutic benefit. For clinical use, these viruses are expected to be most effective in combination with other anti-cancer agents, in particular PD1/L1-targeted immune checkpoint blockade. The first virus from this program (expressing GALV-GP-R$^-$ and hGM-CSF) has entered clinical development alone and in combination with anti-PD1 therapy in a number of tumor types (NCT03767348).

Keywords: Oncolytic viruses, Herpes simplex-1, Immunotherapy, Anenestic effect

Introduction

Oncolytic immunotherapy has shown single agent clinical activity and synergy with immune checkpoint blockade. However, not all patients respond, and most of the clinical experience has been in melanoma. With the objective of maximally activating a patient's immune system against their own cancer to enhance synergy with anti-PD1/L1 blockade, we have developed a new oncolytic immunotherapy platform based on herpes simplex virus type 1 (HSV-1). This has the dual objectives of robustly killing tumor to provide abundant release of tumor antigens, and potently activating the immune system against these tumor antigens once released. To augment the natural ability of HSV-1 to kill tumors and activate anti-tumor immunity, the viruses developed are armed with therapeutic genes with the expectation that 'arming' will be essential to maximizing clinical activity. Initially, we sampled the genetic variation between strains of HSV-1 by screening twenty nine new clinical strains isolated from volunteers who suffer from cold sores across a panel of human tumor cell lines to identify the strain to be developed. This strain (RH018A) was then engineered for oncolytic use by deletion of the genes encoding ICP34.5 to reduce pathogenicity, deleting the ICP47 encoding gene to enhance viral and tumor antigen presentation by major histocompatibility complex-I (MHC-I), and inserting a gene encoding a potent fusogenic glycoprotein derived from gibbon ape leukemia virus (GALV-GP-R$^-$). Expression of GALV-GP-R$^-$ caused increased immunogenic cell death, assessed by the release of danger-associated molecular pattern factors, activated anti-tumor immunity, and enhanced systemic therapeutic activity against rat and murine tumors in vivo. Additionally, the virus induced expression of PD-L1, and demonstrated enhanced activity in combination with PD-1 blockade. A virus expressing GALV-GP-R$^-$ and hGM-CSF is currently in a Phase 1/2 clinical trial (NCT03767348). Further viruses were constructed based on this virus which additionally express an anti-CTLA-4 antibody or immune co-stimulatory pathway activating ligands, each of which is expected to act at the site and time of immune response initiation in the injected tumor and draining lymph nodes. These viruses demonstrated further increased activity in mice, particularly an enhanced anenestic effect. This data supports the potential for improved therapeutic activity of this new oncolytic immunotherapy platform and demonstrate its use to express immune modulatory proteins which may provide a generalized strategy to improve therapy for patients with cancer. There have been significant advances in the immunotherapy of cancer, most notably through the clinical development of immune checkpoint inhibitors targeting cytotoxic T lymphocyte antigen 4 (CTLA-4) and the pro-pathway [1, 2]. While durable clinical responses have been observed across numerous solid and hematologic malignancies, many tumors do not respond or develop resistance over time [3]. The absence of tumor-specific T cells within the tumor microenvironment appears to be an important feature associated with innate and acquired resistance to checkpoint blockade. New strategies that can induce anti-tumor immune responses with which anti-PD-1/L1 therapy can synergize, reverse the immune-deficient tumor microenvironment, and which can re-establish tumor sensitivity to systemic anti-PD-1/L1 therapy are therefore needed. One promising approach is virus-based oncolytic immunotherapy [4]. Oncolytic viruses preferentially replicate in tumors as compared to normal tissue, and promote immunogenic cell death and induction of host systemic anti-tumor immunity. The oncolytic immunotherapy approach has been clinically validated as demonstrated by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) approval of talimogene laherparepvec (T-VEC), an oncolytic herpes simplex virus type 1 (HSV-1) encoding GM-CSF, for the treatment of advanced melanoma in 2015 [5]. The phase 3 clinical trial which led to the approval of T-VEC demonstrated a 26.4% objective response rate, and a 10.8% complete response rate (rising to 17% at the time of the final analysis [Amgen ODAC presentation May 2015] [6]), in a 436-patient phase 3 study in patients with both previously treated and previously untreated Stage IIIb-IVM1c disease [5].

The therapeutic potential of T-VEC can be further enhanced by combination with immune checkpoint inhibitors. In a small phase 1 trial in patients with melanoma, T-VEC in combination with pembrolizumab resulted in a 62% response rate and 33% complete response rate [7]. Similarly promising response rates (> 50%) have also been seen in other small studies with either ipilimumab or pembrolizumab in combination with other oncolytic viruses, such as Cavatak (an oncolytic Coxsackievirus) or HF10 (another oncolytic HSV-1) [4]. Data have also been reported from a 200-patient randomized controlled phase 2 clinical trial with T-VEC combined with ipilimumab compared to ipilimumab alone, where more than a doubling of the response rate was seen in the combination arm [8]. While these studies were all in melanoma, it is important to note that none reported significant additional toxicity compared to that expected with either agent alone. Based on the favorable therapeutic window for T-VEC and other oncolytic viruses, there has been considerable interest in optimizing the oncolytic immunotherapy strategy and using such agents as part of a rational combination regimen in patients with solid cancers.

It is now generally accepted that patients responding to immunotherapy need to have tumors that are im-

although the specific mechanisms that regulate T cell recruitment into established tumors are incompletely understood [9]. Additional factors that favor immune-mediated rejection include high mutation burden, presence of pre-existing immune responses to tumor antigens, particularly tumor neoantigens, and expression of a pro-inflammatory gene signature [10]. While a number of approaches are in development aimed at correcting these deficiencies in non-responsive patients, oncolytic immunotherapies may have particular promise for this purpose as they kill tumors in a highly inflammatory context. This effect is highly immunogenic, including activation of both innate and adaptive immunity, with the potential to create a vaccine "in situ" within the patient against their own cancer. The local production of type 1 interferons induced by oncolytic viruses also results in increased expression of several immune regulatory proteins, including MHC class I and PD-L1 [4].

Thus, oncolytic immunotherapy appears to be particularly well suited for combination strategies with immune checkpoint blockade. We sought to further optimize the approach by maximizing (i) the extent of tumor cell killing, augmenting the release of tumor antigens and danger-associated molecular pattern (DAMP) factors; (ii) the immunogenicity of tumor cell death; and (iii) the resulting systemic anti-tumor immune response. While a range of viral species were considered for development, HSV-1 was selected for several reasons. First, HSV-1 is a very lytic DNA virus; it infects human tumor cells broadly, and when ICP34.5 is deleted exhibits preferential replication in neoplastic tissue. Second, HSV-1 kills mainly by necrosis and activates innate immunity, including through the cGAS/STING pathway. Third, HSV-1 has a large capacity for the insertion of additional, potentially therapeutic, exogenous genes. Finally, HSV-1 has a proven safety and efficacy profile in patients with cancer. While intravenous administration was also considered, an intratumoral approach, i.e. local administration providing systemic immune-based benefit, was selected based on prior clinical validation and the considerable, and potentially insurmountable, biological hurdles to effective intravenous dosing [4, 11]. HSV-1 causes cold sores in humans and is widely prevalent in the population, with up to 90% of individuals testing seropositive by the age of 65 [12]. However, substantial natural variation might be expected amongst clinical strains of HSV-1 (i.e. as sampled from individuals suffering from cold sores) with respect to evolved biological properties such as virulence. This natural variation might also translate into differences in non-evolved properties, such as the ability to infect and kill human tumor cells. Based on the hypothesis that prototypical 'laboratory' strains of HSV-1, such as Strain 17+, extended serial passage or may otherwise not be optimal strains for cancer therapy, T-VEC was initially derived from a clinical strain of HSV-1 after comparing two clinical isolates to Strain 17+. Both of the clinical strains were superior for human tumor cell killing compared to Strain 17+, and the most promising of the two, Strain JS1, was chosen and engineered into T-VEC [13].

In this report, we describe the generation and characterization of a new HSV-1-based oncolytic immunotherapy platform which utilizes a strain of HSV-1 selected from twenty nine newly isolated clinical strains on the basis of increased oncolytic activity in vitro. This was then engineered for tumor selectivity and to express a potent fusogenic membrane glycoprotein (GALV-GP-R$^-$) to increase the extent and immunogenicity of tumor cell death. Various fusogenic proteins including from measles virus and various retroviruses have previously been tested in replicative and non-replicative virus-mediated gene therapy approaches to the treatment of cancer in pre-clinical models [14], including when delivered by oncolytic versions of HSV [15]. Fusogenic cell death has also previously been demonstrated to be highly immunogenic [14]. Genes encoding GM-CSF, an anti-CTLA-4 antibody-like molecule and a number of immune co-stimulatory pathway-activating ligands were then inserted, intending to further enhance the systemic, immune-mediated, anti-tumor effects achieved.

Methods

Assessment of GALV-GP-R$^-$ mediated fusion

The cell lines used for the fusion assays were A549 (ECACC 91072201), HT29 (ECACC 91072201), HT1080 (ECACC 85111505), MDA-MB-231 (ECACC 92020424), miaPaCa-2 (ECACC 85062806) and SK-mel-28 (ATCC® HTB-72™). The monolayers were infected using a range of multiplicity of infection (MOI) from 0.01 to 0.0001. The infected cell monolayers were observed for GFP expression at 24 h. and 48 h. post-infection and then fixed and stained with crystal violet.

Western blots and ELISA

For detection of anti-CTLA-4 expressed from Virus 27, supernatant was used from BHK cells infected at MOI =1 in serum-free mediuma for 24 h. The proteins were separated on 10–20% sodium dodecyl polyacrylamide gel (Thermo Fisher CAT No: XP10200BOX) and transferred to polyvinylidene difluoride membrane (Life Technologies Cat No: LC2005). The membrane was probed with goat anti-mouse IgG1 heavy chain (alkaline phosphatase) (Abcam Cat No: ab97237). BCIP®/NBT Liquid Substrate System (Sigma Aldrich Cat No: B1911) was used for the detection.

For detection of CD40L, 4-1BBL and OX40L from

infected at MOI =1 for 24 h. To confirm expression of 4-1BBL from Virus 33, microplates were coated with the capture antibody (0.5μg/ml, R&D Systems Cat No:-AF1246) and incubated overnight at 4 °C. Following blocking, standards (R&D Systems Cat No 1256-4 L, 40 ng/ml- 0.63 ng/ml) and samples were added and incubated at 37 °C. The wells were then probed with anti-mouse 41BBL (Bioxcell Cat No: BE0110) after which HRP Tagged antibody (Sigma Aldrich Cat No: A5795) was added and incubated for 1 h. TMB was added and incubated for 5 mins and sulphuric acid was added to stop the reaction. The plates were read at 450 nm. ELISA for CD40L (Abcam Cat No: ab119517) and OX40L (Thermo Fisher Cat No: EMTNFSF4) was performed using kits as per the manufacturer's instructions.

ATP release

Cells were plated at 2×10^5 cells per well in 1 mL, in 12-well plates, and incubated overnight. Cells were then infected with Virus 23 or Virus 17 the following day. Twenty-four and 48 h after treatment, cell supernatants were collected and centrifuged at 2000 rpm for 4 mins. Cell-free supernatants were then measured for ATP by CellTiter-Glo Luminescent Cell Viability Assay (CTG, Promega, UK). Fifty microliter of CTG was added per 200 uL sample and incubated for 10 min. Luminescence was measured on a Victor 2 V plate reader (Perkin Elmer).

High mobility group box 1 protein (HMGB1) release

Cells were plated at 2×10^5 cells per well in 1 mL, in 12-well plates, and incubated overnight. Cells were infected with Virus 23 or Virus 17 the following day. Forty-eight h after treatment, cell supernatants were collected and centrifuged at 2000 rpm for 4 mins. Cell-free supernatants were then measured for HMGB1 by an ELISA Assay (IBL International GmbH Cat No: ST51011) as per the manufacturer's instructions.

Cell surface calreticulin expression

Cells were plated at 2×10^5 cells per well in 1 mL, in 12-well plates, and incubated overnight. Cells were infected with Virus 23 or Virus 17 the following day at various MOI. Forty-eight h after treatment, un-permeabilized samples were stained with viability dye (Thermo Fisher Cat No: 65–0865-14), with anti-calreticulin antibody (Abcam Cat No: ab92516), or isotype control antibody (Abcam Cat No: ab172730), and flow cytometry was performed. Surface calreticulin expression was shown as median fluorescence intensity (MFI). Data was analyzed

In vivo efficacy testing

Bilateral mouse A20 lymphoma tumors were grown in of Balb/c mice or human A549 or MDA-MB-231 tumors grown in the right flanks of Balb/c nude mice until average tumor diameters were > 5 mm. Right flank tumors were then injected 3 times (every other day) with the indicated virus and dose in 50 μl or with vehicle (PBS) and tumor diameters were then followed. For experiments in rats, rat 9 L glioma tumors were grown in the left and right flanks of Fischer 344 rats until tumors were 0.75-1 cm in diameter and right flank tumors then dosed 5x (approximately every other day) with the indicated virus at a dose of 5×10^6 pfu in 50 μl or with vehicle and tumor diameters then followed. For experiments in combination with anti-murine PD1, clone RMP1–14 (BioXCell) was given by the intraperitoneal route at 10 mg/kg every 3 days for a total of 9 doses.

Vectra staining

Vectra staining was performed on tumors to identify tumor infiltrating immune cells as previoulsy described [16]. Bi-flank 4434 murine melanoma tumours grown in C57BL/6 mice were treated with Virus 16 on days 1, 3 and 5, then collected at day 10 after the first injection, fixed overnight in 10% neutral buffered formalin and then transferred to PBS prior to processing and embedding. Tissue sections were labeled with immunofluorescent stains as follows; CD8 (Cat No: 14–0808-82), CD4 (Cat No:14–9766-82), and foxp3 (Cat No: 14–5773-82), all from eBioscience. Images were then quantified by an automated cell segmentation and phenotyping algorithm, using inForm analysis software (Perkin Elmer). Four thousand four hundred thirty-four cells are a murine melanoma tumor cell line generated in house at The Institute of Cancer Research, London.

FACS analysis of tumours

C57BL/6 mice were subcutaneously implanted with 4×10^6 4434 murine melanoma cells suspended in 0.1 mL PBS per flank in a bi-flank model. Tumours were allowed to grow to 6–8 mm and randomized into study groups. The right flank was injected with 5×10^6 plaque forming units (pfu) of Virus 16 in 50 μl or a mock group received formulation buffer (vehicle), given on days 1, 3 and 5. Mice were euthanized when a tumour reached 15 mm in any direction. Tumours were harvested and minced with scissors in digestion mix (0.01% trypsin, 2.5 mg/mL collagenase, 2 mg/mL dispase and 1 mg/mL DNAse in RPMI), and incubated at 37 °C for 30 min. Thereafter, samples were kept on ice. Suspensions were passed through a 70 μm strainer using a 2.5 mL syringe plunger and washed through with RPMI + 5 mM EDTA until only connective tissue remained. Samples were

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.