Neoadjuvant Intravenous Oncolytic Vaccinia Virus Therapy Promotes Anti-Cancer Immunity in Patients

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Key words

Clinical trial; Vaccinia virus; Oncolytic virus therapy; T cell immunity; Innate immunity; Immunotherapy; Immune checkpoint modulation

List of Abbreviations

Carcinoembryonic antigen (CEA)
Colorectal cancer liver metastases (CRLM)
Dendritic cells (DCs)
Dimethyl sulphoxide (DMSO)
Enzyme-linked immunosorbent spot (ELISpot)
Granulocyte-macrophage colony-stimulating factor (GM-CSF)
Healthy donor (HD)
Heat-inactivated (HI)
Human serum (HS)
Immunohistochemistry (IHC)
Interferon (IFN
Interferon-stimulated genes (ISGs)
Interleukin (IL)
Intratumoural (*i.t.*)
Intravenous (*i.v.*)
Interferon gamma-induced protein 10 (IP-10)
Lysosome-associated membrane glycoprotein 3 (LAMP3)
Melanoma antigen recognized by T cells-1 (MART-1)
Natural killer (NK)
Neutralising antibodies (Nab)
Peripheral blood mononuclear cells (PBMCs)
Pexa-Vec (*Pexastimogene Devacirepvec*; JX-594, TG6006)
Plaque-forming units (p.f.u)
Spot-forming unit (SFU)
St. James's University Hospital (SJUH)
T cell receptor (TCR

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)

Tumour-associated antigens (TAAs)

Abstract

Improving the chances of cure for cancer patients who have surgery to remove metastatic sites of disease is a priority area for cancer research. *Pexa-Vec* (*Pexastimogene Devacirepvec*; JX-594, TG6006) is a principally immunotherapeutic oncolytic virus that has reached late-phase clinical trials. We report the results of a single-centre non-randomised biological endpoint study, which builds on the recent success of the pre-surgical intravenous (*i.v.*) delivery of oncolytic viruses to tumours. Nine patients with either colorectal cancer liver metastases (CRLM) or metastatic melanoma were treated with a single *i.v.* infusion of *Pexa-Vec* ahead of planned surgical resection of the metastases. Grade 3 and 4 *Pexa-Vec*-associated side-effects were lymphopaenia and neutropaenia. *Pexa-Vec* was peripherally carried in the plasma compartment and not associated with peripheral blood mononuclear cells (PBMCs). Upon surgical resection, *Pexa-Vec* was found in the majority of analysed tumours. *Pexa-Vec* therapy was associated with interferon-α secretion, chemokine induction, and resulted in transient innate and long-lived adaptive anti-cancer immunity. In the two patients with significant and complete tumour necrosis, a reduction in the peripheral T-cell receptor diversity was observed at the time of surgery. These results support the development of pre-surgical oncolytic vaccinia virus-based therapies, to stimulate anti-cancer immunity and increase the chances of cure in patients with cancer.

Trial Registration. EudraCT number 2012-000704-15.

Synopsis: A single intravenous infusion of an engineered immunotherapeutic oncolytic Vaccinia virus (Pexa Vec) in cancer patients, was associated with tumour necrosis, interferon-a secretion, transient innate and long-lived adaptive anti-cancer immunity.

Background

Patients with advanced solid malignancies can be suitable for surgical resection of their metastatic disease, with curative intent. However, only a minority of these patients remain cancer-free long-term, due to either incomplete tumour resection, or the presence of micrometastatic disease at the time of surgery. For patients with colorectal liver metastases (CRLM), survival at five years following liver resection is approximately 50 %, despite the availability of combination peri-operative chemotherapy[1]. Likewise, approximately 60 % of patients with melanoma that has spread to lymph nodes will relapse following surgical resection and adjuvant treatment[2]. For these patients, effective novel systemic therapies administered prior to (neoadjuvant therapy), or shortly after surgery (adjuvant therapy), hold the potential to significantly enhance the chances of cure.

Oncolytic viruses (OVs) are principally immunotherapeutic viruses that preferentially replicate in malignant cells, thereby inducing immunogenic cell death. Several engineered viruses have reached randomised studies, with three agents currently licensed for standard care[3]. One of the most clinically tested oncolytic viruses, *Pexa-Vec* (*Pexastimogene Devacirepvec*; JX-594, TG6006), is an engineered Wyeth-strain vaccinia virus[4], under development by Transgene[5] and SillaJen[6]. *Pexa-Vec* tumour specificity is enhanced by deletion of thymidine kinase, an enzyme of the DNA precursor pathway, which is strictly regulated during the normal cellular cycle, but highly expressed in growing malignant cells[7]. *Pexa-Vec* also expresses granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes anti-tumour immunity by inducing proliferation and differentiation of myeloid precursors, alongside the stimulation, recruitment and maturation of dendritic cells (DCs)[8,9]. Clinical and *in vitro* studies have helped to elucidate the fundamental mechanisms of *Pexa-Vec* therapy, namely tumour-specific virus replication,

153 expression of GM-CSF and the stimulation of cytotoxic T-lymphocyte tumour infiltration[10].
154 Other mechanisms of therapy include antibody-mediated complement-dependent cancer cell
155 cytotoxicity[11] and *Pexa-Vec* replication in tumor-associated endothelial cells, leading to
156 disruption of tumor blood flow, tumour hypoxia and necrosis[12].

157 *Pexa-Vec* has shown promising clinical signs of efficacy as a single agent, including in a
158 randomised study between low- ($1x10^8$ plaque-forming units; p.f.u) and high-dose ($1x10^9$ p.f.u)
159 intratumoural (*i.t.*) injection in patients with hepatocellular carcinoma, where overall survival
160 was significantly longer for patients in the high-dose group; 14.1 months versus 6.7 months[13].
161 Furthermore, intravenous (*i.v.*) delivery of *Pexa-Vec* to tumour, a critical feature for the
162 treatment of micrometastatic disease, is achievable using a dose of $1x10^9$ p.f.u.[14].

163 We sought to clinically develop *i.v. Pexa-Vec* delivery as a neoadjuvant multi-mechanistic
164 therapy for patients with metastatic solid malignancies. Herein, we show in nine patients, that the
165 administration of a single *i.v.* infusion of *Pexa-Vec* ahead of planned surgical resection of
166 advanced CRLM or metastatic melanoma, results in acceptable patient safety ahead of surgical
167 tumour resection and pathological evidence for tumour necrosis. *Pexa-Vec* was associated with
168 the plasma compartment of peripheral blood, resulting in delivery of virus to tumour and
169 promotion of innate anti-cancer immunity. Furthermore, a single neoadjuvant infusion of *Pexa-*
170 *Vec* stimulated long-lived T cell anti-cancer immune responses, with repertoire sequencing
171 suggesting that pathological tumour response is associated with a perceived reduction in global T
172 cell diversity.

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Materials and Methods

Experimental Design: EudraCT number 2012-000704-15, open between September 2015 and June 2018 in accordance with Declaration of Helsinki ethical guidelines. This was an open-label, non-randomised study of *Pexa-Vec* given as a one-hour *i.v.* infusion to patients prior to a planned surgical resection of tumour. A total of nine patients each received a single dose of $1x10^9$ p.f.u. Pexa-Vec via *i.v.* infusion 10 to 22 days prior to surgery at St. James's University Hospital (SJUH), Leeds, UK. Three patients had metastatic melanoma and six had CRLM. Eight patients had their planned surgery; one patient (Pt07) had their surgery cancelled when an up-to-date CT scan revealed pulmonary metastases. The primary endpoint of the study was the presence of *Pexa-Vec* in the resected tumour tissue and blood. All patients gave written, informed consent according to good clinical practice guidelines. Protocol, patient information sheet and consent forms were approved by the United Kingdom Medicines and Healthcare products Regulatory Authority, regional ethics review committee, as well as institutional review at SJUH. The trial management committee met on a monthly basis to discuss study progress, including patient safety and adverse events.

Pexa-Vec: *Pexa-Vec* (*Pexastimogene Devacirepvec*; JX-594, TG6006) is a replication-competent, transgene-armed therapeutic vaccinia virus provided by Transgene S.A, France. *Pexa-Vec* is engineered for viral thymidine kinase gene inactivation and expression of GM-CSF and β-galactosidase transgenes under the control of the synthetic early-late and p7.5 promoters, respectively. *Pexa-Vec* was stored at $1x10^9$/mL at -80 °C for use in *in vitro* experiments.

Patient samples: Blood and tissue samples were collected, processed and analysed using the Translational Cancer Immunotherapy Team quality-assured lab manual which included SOPs to standardise all processes.

Peripheral blood was collected into K_3EDTA and serum clot activator vacuette tubes (both Greiner) and processed within 2 hours of venepuncture. Blood samples were taken on day 1 (pre- and 1 hour post-infusion), day 2, day 3 (optional), day 5 (optional), on the day of surgery, one-month post-surgery and 3 months post-surgery. Tumour (and corresponding normal margins, if available) were taken from planned surgical resections.

Isolation of peripheral blood mononuclear cells, plasma and serum from peripheral blood

Plasma: K_3EDTA blood was centrifuged for 10 mins at 2000 g and plasma harvested from the resulting upper layer. Aliquots were stored at –80 °C.

Peripheral blood mononuclear cells: PBMCs were isolated from K_3EDTA blood by density-gradient separation over lymphoprep™ (Axis Shield) as per manufacturer's instructions. Cells were frozen at $1x10^7$/mL in 40 % (*v/v*) Roswell Park Memorial Institute medium-1640 (Sigma) containing 5 mM L-Glutamine & 1 mM sodium pyruvate, 50 % (*v/v*) pooled human serum (HS) and 10 % (*v/v*) dimethyl sulphoxide (DMSO). PBMCs were stored in liquid nitrogen.

Serum: Blood collected in clot activator tubes was left to clot for a minimum of 30 mins post-venepuncture, then centrifuged at 2000 g for 10 mins; serum was harvested from the resulting upper layer. Aliquots were stored at –80 °C.

Full blood counts: Full blood counts were performed where appropriate at SJUH as part of standard clinical care. The Patient Pathway Manager and Results Server systems were used to

acquire total lymphocyte counts (expressed as 10^9/L) throughout treatment. Normal ranges of lymphocytes were defined by St James's University Hospital as 1–4.5 x 10^9/L.

Immunohistochemistry (IHC): IHC was performed on formalin-fixed paraffin-embedded tissue obtained from surgical resection of patient tumours. Tissue for IHC was processed using an automated Bond Max system (Leica Biosystems) as described in[15]. IHC for *Pexa-Vec* was performed by Histalim (France) following company validated protocols using polyclonal rabbit-anti-vaccinia virus antibody (Meridian Life Sciences; RRID: AB_153134). Mouse-anti-human CD8 antibody (Dako; RRID: AB_2075537) was used at 1:100 dilution, followed by anti-mouse secondary (Abcam; RRID: AB_10680417) at 1:500; CD8 positivity was detected using ImmPACT® DAB HRP Peroxidase Substrate Kit (Vector Labs; RRID: AB_2336520) or ImmPACT Vector Red (Vector Labs; RRID: AB_2336524). Antibodies against TAA were used as follows: anti-human carcinoembryonic antigen (CEA; RRID: AB_304463) at 5 µg/mL, anti-human Melan-A (RRID: AB_305836) at 1:50 dilution, both with an anti-mouse secondary antibody at 1:2000 dilution (all Abcam). Enzymatic detection was performed using ImmPACT® VIP for the melanoma (Melan-A) tissue and ImmPACT® DAB HRP Peroxidase Substrate Kit (RRID: AB_ 2336520) for the CRLM (CEA) tissue (both Vector Labs). Finally, rabbit-anti-human-PD-1 (1:200; Abcam; RRID: AB-230_881954) and mouse-anti-human-Ki67 (1:75; Dako; RRID: AB_2142367) were detected with the ImmPACT® VIP HRP or DAB Peroxidase Substrate kits. Control sections were processed as above, without the addition of primary antibody. Digital images were acquired at x20 magnification and quantified using ImageScope software (RRID: SCR_014311).

qPCR: qPCR was performed using DNA extracted from PBMCs and plasma using a DNeasy Blood and Tissue Kit and a Circulating Nucleic Acid kit, respectively (Qiagen). Primers

corresponding to the vaccinia E3L gene (TCCGTCGATGTCTACACAGG and ATGTATCCCGCGAAAAATCA) were used to detect for the presence of *Pexa-Vec,* alongside a standard curve of known *Pexa-Vec* DNA concentration, on an Applied Biosystems QuantStudio™ 5 Real Time PCR System (ThermoFisher). PCR data were analysed using a QuantStudio 3D AnalysisSuite Cloud (ThermoFisher).

Cell culture: The African monkey green kidney cell line, Vero, was maintained in full growth medium (Dulbecco's Modified Eagle's Medium (Sigma), supplemented with 10 % (*v/v*) foetal calf serum (Invitrogen) and 1 % (*v/v*) L-glutamine (Sigma)). Cells were routinely tested and found to be negative for *Mycoplasma* infection.

Neutralising antibodies (NAb): NAb were detected using a modified serial dilution assay of heat-inactivated (HI) patient serum as previously described[16]. Briefly, serial dilutions of HI-serum samples were incubated with *Pexa-Vec*. After incubation for 3 hours at 37 °C, the dilutions were transferred onto monolayers of Veros (cells alone or with *Pexa-Vec* dilution only were also cultured as negative and positive controls, respectively). After a further 72 hours of incubation, MTT (5 mg/ml; Sigma) was added to each well and left for 4 hours, before the removal of all medium and addition of DMSO (Fisher Scientific) to each well. Absorbance of samples was then read at 540 nm. NAb titres were calculated as 1/endpoint, which equates to the last serum dilution at which no antibody neutralisation of *Pexa-Vec*-induced killing was observed (n=9).

Luminex: Bio-Plex Pro™ Cytokine and Chemokine Assays (21-plex; human group I and 27-plex; human group II or 48-plex; human cytokine) were used to determine levels of soluble mediators in plasma samples throughout treatment, as per manufacturer's instructions. Levels of

IFN-β were measured in samples using the VeriKine-HS Human Interferon Beta ELISA Kit for plasma (R&D), as per manufacturer's instructions. Data are expressed as relative fold change in post-treatment samples compared to pre-treatment samples. Statistical significance between time points was determined using paired T tests; * $P<0.05$, ** $P<0.01$ (n=9).

Natural Killer (NK) cell degranulation assay of patient PBMCs: PBMCs from specified time points prior to (D1 pre) and following *Pexa-Vec* infusion were co-cultured at a ratio of 1:1 with tumour-associated cell lines (Mel888 or SW620 for melanoma and CRLM patients, respectively) for 1 hour prior to the addition of 1 µl/mL brefeldin (Sigma). Co-culture continued for a further 4 hours before PBMCs were stained for CD3-PerCP (SK7; BD Biosciences), CD56-PE (AF12-7H3; Miltenyi) and CD107a/b-FITC (H4A3; BD Biosciences). CD107 positivity was assessed using a CytoFLEX S flow cytometer and analysis was performed using CytExpert software (RRID: SCR_017217; both Beckman Coulter). Data are expressed as % positive CD107 staining. Statistical significance is determined by paired T tests (* $P<0.05$; n=9).

NK CD69 expression: Assessment of NK CD69 expression was performed using a CytoFLEX S flow cytometer and analysis was performed using CytExpert software. Data are expressed as % positive CD69 (CD69-APC, Miltenyi, RRID: AB_2784271) staining on NK cells (CD3-negative CD56-positive, as above). Statistical significance is determined by paired T tests (* $P<0.05$; n=9).

mRNA expression analysis of patient PBMCs: Analysis was performed using the HTG EdgeSeq Precision Immuno-Oncology Panel by HTG Molecular, Arizona, USA, which contains probes for 1,410 genes. All sequencing was performed on the Illumina NextSeq. Differential expression analysis was performed using the DESeq2 package (version 1.14.1) (Bioconductor;

RRID: SCR_006442). Data are expressed as $\log_2$(CPM) for the average expression of each probe across all groups after normalisation. Statistical significance between time points is shown by an adjusted P value (adjP); * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 (n=7).

ELISpot: Briefly, $1x10^5$ patient PBMCs were incubated per well in the presence of either 2 µg/mL of CEA or MART-1 over-lapping peptide pools, or with 2 µg/mL CEF peptide pool (positive control; all Cambridge Biosciences), or media alone (negative control) or 10 p.f.u/cell *Pexa-Vec*. IFNγ secretion from activated T cells was detected using a matched paired antibody kit (MabTech) and spot forming units (SFU) were visualised using BCIP/NBT substrate (MabTech). Images were captured using an AID ELISpot reader (AID GmbH). Data are presented as mean SFU per well (in triplicate) ± sem. Statistical significance between time points was determined by fitting a mixed model for one-tailed multiple comparisons; * adjP<0.05 (n=3-9, dependent on sample availability).

Pexa-Vec* peripheral blood carriage*: Peripheral blood from healthy donors was incubated with $2x10^5$ p.f.u/mL *Pexa-Vec in vitro* for one hour at room temperature. Blood was processed into PBMCs and plasma before DNA was extracted, as described previously. qPCR was performed to quantify *Pexa-Vec* DNA, as described above. A standard curve of known *Pexa-Vec* DNA was used to estimate the concentration of *Pexa-Vec* DNA in PBMCs and plasma. Data are shown as ng DNA per 5 mL initial blood volume (n=5 replicates) for 3 donors. Statistical significance is determined by paired T tests; ** P<0.01, *** P<0.001.

TCR B sequencing methodology: TCRB chains were amplified from genomic DNA, extracted from patient samples, using a modified version of the BIOMED-2 sequencing protocol[17]. Adapters attached to the PCR primers allowed sequences to be indexed using the Nextera XT

indexing kit (Illumina) for sample multiplexing. Libraries were sequenced on a single lane of an Illumina MiSeq by the Leeds University sequencing facility using a 2x300bp kit. The quality of demultiplexed reads was assessed by fastqc[18] and reads trimmed to remove adapters and low quality bases using trimmomatic[19] and trim galore[20]. High quality reads (Phred>30) were then overlapped with FLASH[21] and aligned to the IMGT database using MiXCR[22], which discarded erroneous and non-productive rearrangements. Clones were defined by having the same TCRBV/J segment and identical CDR3 sequence and their abundance was adjusted using the absolute T cell count to eliminate amplification errors. For generation of Inverse Simpson Indices, assessment of antigen specificities and generation of Circos (RRID: SCR_011798) plots, the subsequent output was filtered into VDJTools[23] and processed in R[24] using Immunarch[25], circlize[26] and ggplot2[27]. Statistical analysis of the percent change in T-cell diversity at the time of surgery was performed using a one-tailed Mann-Whitney test, * $P<0.05$.

PBMC viability assay: Healthy donor (HD)-PBMC were isolated from leukocyte apheresis cones supplied by the National Health Service Blood and Transplant unit and treated with 0.1 p.f.u./cell *Pexa-Vec* for 72 hours. Viability of PBMC populations were assessed by staining with CD3-PerCP, CD56-APC (REA196, Miltenyi), CD19-FITC (SJ25C1, BD Biosciences), CD14-PE (M5E2, BD Biosciences) and Zombie UV fixable viability kit (BioLegend) following manufacturer's instructions. Flow cytometry analysis was performed using a CytoFLEX S and CytExpert software (both Beckman Coulter).

Immunofluorescence to detect tumour-specific replication of *Pexa-Vec*: Tumour and accompanying normal liver tissue were obtained from freshly resected liver specimens immediately following surgical resection from patients not participating in the trial. Written, informed consent was obtained in accordance with institutional ethics review and approval.

Tissue cores were made using a Tru-Cut® biopsy needle (CareFusion), resulting in cores of 1 mm diameter and 15 mm length; cores were subsequently divided into three 5 mm-length cores. Tissue cores were treated with $1x10^7$ p.f.u. *Pexa-Vec*-GFP or PBS for 96 hours. Prior to image acquisition, 4',6-diamidino-2-phenylindole staining was performed following cell penetration with 0.1% Triton X. High-resolution images were taken using a Nikon A1 Confocal Laser Scanning Microscope (Nikon).

***In vitro* NK cell activation**: HD-PBMC were treated with *Pexa-Vec* for 24 hours with or without (±) type I Interferon α/β blockade or monocyte depletion. For type I IFN neutralisation, PBMC were pre-incubated for 30 mins with polyclonal antibodies to IFNα, IFNβ (both anti-sheep) and IFNα/β receptor chain 2 (anti-mouse) (PBL Interferon Source) or isotype control (anti-sheep; Sigma and anti-mouse; R&D), prior to addition of 1 p.f.u./cell *Pexa-Vec* for 24 hours as previously described[28]. For monocyte depletion, $CD14^+$ cells were depleted from whole PBMC using magnetic cell sorting on MACS® columns (Miltenyi Biotec), according to the manufacturer's instructions. After virus treatment for 24 hours, NK cell CD69 expression and CD107 degranulation were determined using an Attune® NxT Acoustic Focusing Cytometer (Life Technologies) and data were analysed using the Attune® Cytometric Software. (v2.1.0; Life Technologies) as previously described. For NK cell CD107 degranulation, PBMCs (± 1 p.f.u. *Pexa-Vec*) were co-cultured with SW620 cells at 10:1 effector:target ratio, following the same protocol as previously described for the patient CD107 assay.

Results

Clinical outcomes

We recruited nine patients (three patients with metastatic melanoma and six patients with CRLM) to a phase 1b window of opportunity trial. Each patient received a single, 1-hour *i.v.* infusion of 1×10^9 p.f.u. *Pexa-Vec*, 16 (+/- 6) days ahead of planned surgical resection of metastatic melanoma or CRLM (Fig. 1A). *Pexa-Vec*-related grade 3 and 4 adverse events included lymphopenia and neutropenia (Table S1). Patient 09 experienced grade 4 intraoperative hypotension that was regarded to be unrelated to *Pexa-Vec.*

Surgery was undertaken in all but one patient (patient 07), where plans for surgery were abandoned after finding pulmonary metastases on an up-to-date pre-operative CT scan. At the time of writing, after more than three years of follow-up post-*Pexa-Vec* infusion for each patient, five of the nine patients are alive. The three patients that remain cancer-free all had CRLM (patients 05, 06 and 08). Pathological examination of their resected tumour specimens post-*Pexa-Vec* infusion revealed extensive (patient 05) or complete (patient 08) necrosis of the tumour in two of the three patients (Fig. 1B and Table S1). None of the other resected patient tumours showed any signs of necrosis.

Intravenous *Pexa-Vec* associates with plasma, but not PBMCs

In contrast to our previous clinical trial findings using *i.v.* reovirus infusions[29,30], qPCR analysis of peripheral blood compartments revealed that *Pexa-Vec* associates with plasma, but not PBMCs (Fig. 1C), being detectable at 1 hour post-infusion in four of the nine patients. This was confirmed in *Pexa-Vec*-pulsed blood, donated from healthy volunteers, where *Pexa-Vec* could only be detected in the separated plasma fraction (Fig. 1D). However, in patient 05, in whom extensive tumour necrosis was noted following *Pexa-Vec* infusion (Table S1), qPCR of plasma only revealed the presence of virus immediately pre-surgery, 22 days post-infusion (Fig. 1C). This potentially signifies ongoing virus production from the tumour at this time point. In

common with reovirus infusion, *Pexa-Vec* neutralising antibodies peaked either at the time of surgery or one-month post-surgery (Fig. 1E).

Intravenous *Pexa-Vec* infects CRLM

Immunohistochemistry (IHC) analysis of four from the five resected CRLM revealed the presence of *Pexa-Vec* protein in the tumours from patients 02 and 09, on the periphery of the tumour from patient 08, and its absence from the tumour of patient 06 (Fig. 2A). The available tissue from patient 05's tumour could not be assessed due to tumour necrosis. To confirm the specificity of *Pexa-Vec* to CRLM in comparison to the surrounding background liver, we treated *ex vivo* tissue core biopsy samples, from patients undergoing standard CRLM surgical resections outside the trial, with *Pexa-Vec*. At 96 hours post-treatment, large areas of CRLM core biopsies were infected with *Pexa-Vec*, as determined by immunofluorescence for virus-expressed green fluorescent protein (GFP, Fig. 2B). In contrast, separate core biopsies taken from background livers, were resistant to *Pexa-Vec* infection, with GFP being seen only in scattered cells, indicating non-productive infection (Fig. 2B).

***Pexa-Vec* stimulates anti-cancer NK cell activity**

Pexa-Vec infusion was followed, after 24 hours, by a significant increase in plasma interferon (IFN)-α concentrations (Fig. 3A, Table S2), in addition to other pro-inflammatory cytokines e.g. interleukin (IL)-12 (Fig. S1A, Table S2); pro-apoptotic cytokines e.g. tumour necrosis factor-related apoptosis-inducing ligand (TRAIL; Fig. S1A, Table S2) and DC-maturation-associated proteins e.g. Lysosome-associated membrane glycoprotein 3 (LAMP3; Fig. S1B, Table S2)[31]. Gene expression of a panel of interferon-stimulated genes (ISGs) significantly increased in PBMCs 24 hours post-*Pexa-Vec* (Fig. 3B). Inflammatory cytokine protein and RNA levels gradually reduced to baseline levels by the time of surgery (Fig. 3A, 3B, S1A, S1B, Table S2).

Given that NK cells are activated by ISGs, including IL-12[32], we measured patient-derived peripheral blood NK cell activation and cytolytic activity against tumour-relevant cell line targets. We found significantly increased NK cell activation, as measured by CD69 expression, on days 2 and 3, with a reduction to baseline levels by day 5 (Fig. 3C (i)). NK cell cytolytic activity was significantly increased 24 hours following *Pexa-Vec* infusion, before falling to baseline at day 5 (Fig. 3C (ii) and Fig. S2). In common with other oncolytic viruses[29,33] the critical role of IFN-α/β in NK cell activation following *Pexa-Vec* stimulation was confirmed by simultaneous blockade of the type I IFN receptor and of soluble IFN-α/β within *in vitro Pexa-Vec*-treated PBMCs. This resulted in a significant reduction in NK cell activation, as assessed by cell surface CD69 expression and cytolytic activity (Fig. 3D). Previous work had indicated monocytes to be the source of both oncolytic reovirus and herpes simplex virus-induced type I interferons[34,35]. We confirmed a critical role for monocytes in mediating NK activity following *Pexa-Vec* stimulation, whereby depletion of monocytes from PBMCs significantly reduced NK cell activation and function (Fig. 3E).

On the basis that interferons stimulate upregulation of programmed death protein 1 (PD-1)[29], we analysed expression of this immunosuppressive immune checkpoint in patient 1, comparing the pre-*Pexa-Vec* tumour biopsy to the resected tumour sample. In keeping with the observed induction of ISGs, we found more concentrated PD-1 staining post-*Pexa Vec* infusion (Fig. S3A). PD-1 expressing cells lacked Ki67 staining, confirming that they are non-replicating and likely to be immunologically exhausted. Examination of post-*Pexa Vec* tumour samples from other patients on study similarly revealed areas of PD-1 expressing non-replicating cells (Fig. S3B).

Chemokine Induction

Pexa-Vec infusion was followed by a reduction in the peripheral lymphocyte concentration in all patients at day 2, returning to baseline levels by the time of surgery (Fig. 4A). We confirmed that this was unlikely to be due to lymphocyte cell death in an *in vitro* cell viability assay (Fig. 4B). *Pexa-Vec* was also associated with *CXCL10* gene expression in PBMCs and a corresponding peak in plasma CXCL10 (also known as interferon gamma-induced protein 10; IP-10) protein levels, 24 hours following virus infusion (Fig. 4C, Table S3). IP-10 is a chemokine known to play an important role in recruiting activated T cells into sites of tissue inflammation and is associated with the presence of CD8 T cells in tumour[36,37]. Concordantly, the observed kinetics of peripheral blood lymphopenia closely followed the rise and fall in IP-10 concentrations and *CXCL10* gene expression. On examination, all resected tumours harbored infiltrating CD8 T cells (Fig. 4D). CD8 T cells were frequently associated with malignant cells, as exemplified by the resected lymph node from patient 04, where CD8 T cells were found in higher concentrations in association with melanoma cell clusters (Fig. S4A) than in areas of the same lymph node with few infiltrating melanoma cells. Furthermore, in patient 01, where a pre-*Pexa-Vec* tumour biopsy was available, comparison to the resected tumour sample post-*Pexa-Vec* revealed a shift in the CD8 T cell population from a peri-vascular localisation at baseline, to a wider infiltrative distribution across the tumour post-virus infusion (Fig. S4B). As well as increased *CXCL10*, gene expression of *CCL2*, a chemotactic cytokine that induces directional migration of DCs into infected tissue, also peaked at day 2 and returned to baseline by the time of surgery (Fig. S5)[38].

Pexa-Vec stimulates functional anti-cancer T cell activity

In addition to the observed innate anti-cancer immune effects, successful anti-cancer therapy requires a longer-lived T cell immune response. We therefore measured the effects of *Pexa-Vec* infusion on functional T cell responses, by way of PBMC IFN-γ secretion, using an enzyme-

linked immunosorbent spot (ELISpot) assay. Patient-derived PBMCs were stimulated *ex-vivo* using overlapping peptide pools of TAAs; either melanoma antigen recognized by T cells-1 (MART-1) for PBMCs derived from melanoma patients or CEA for PBMCs isolated from CRLM patients, which were confirmed to be expressed in the corresponding tumour samples (Fig. 5A). Whilst very few MART-1 or CEA-specific IFN-γ-producing cells were observed at baseline pre-*Pexa-Vec*, this greatly increased following *Pexa-Vec* infusion (Fig. 5B). A similar increase was seen in IFN-γ producing cells specific to CEF (Cytomegalovirus, Epstein Barr Virus, Influenza virus) overlapping peptide pools, which were used as a positive control for the assay (Fig. S6A). T cell responses to either CEA and MART-1 for all patients peaked at one month post-surgery, and remained elevated over pre-*Pexa-Vec* baseline levels (Fig. 5C and S6B). T cell responses remained elevated at least 3 months following *Pexa-Vec* infusion, indicating the induction of long-lived T cell anti-cancer immune responses (Fig. 5C). We confirmed these findings for MART-1 and other TAAs by T cell receptor (TCR) sequencing, using DNA derived from trial patient PBMCs at baseline, surgery and at the end of study. Examination of the relative numbers of individual T cell clones was performed against published databases of TCR epitope specificity (VDJdb[39], PIRD[40] and McPAS-TCR[41]), where these data were available. Analysis revealed clonal proliferation of MHC class I-restricted (CD8) T cells following *Pexa-Vec* infusion, targeting MART-1 (Fig. 5D and Table S4) and other TAAs (Table S4).

Reduced T cell clonal diversity is associated with pathological tumour response

Despite the observed NK cell cytolytic activity and the functional T cell anti-cancer immune responses across all the trial patients, tumour necrosis was only observed in the CRLM of patients 05 and 08. We, therefore, characterised changes in peripheral blood T cell clonal

evolution in the nine trial patients, to elucidate whether this could be a determining factor for the observed differences in tumour necrosis.

Longitudinal analysis of these data showed changes in the V-J TCR usage, which are displayed as circos plots (Fig. 6A) where the width of each band is proportional to the frequency of usage of that gene segment. Individual gene regions are labelled and a transient increase in the use of TRBV20-1 can be seen in the two patients who displayed tumour necrosis (patient 05 and 08), along with a similar increase in TCRBV18 in the patient displaying complete necrosis (patient 08). This was in contrast to a more stable gene family usage profile exhibited by other patients with no visible necrosis, as exemplified by patient 06. The small patient numbers involved limited statistical inference however, increased usage of TRBV18 and TRBV20-1 draws parallels with a prior study examining tumour-infiltrating lymphocytes within colorectal cancer biopsies and may indicate a TAA-driven T cell response[42].

Tracking changes in the abundance of T cell clones following *Pexa-Vec* infusion revealed that by the time of surgery, there was an observed reduction in the diversity of the most highly abundant T cell clones derived from patients 05 and 08, when compared to other patients, as estimated by the Inverse Simpson index (Fig. 6B). Statistical analysis revealed that this drop in T-cell diversity for patients 05 and 08 was significant (P=0.036), in comparison to the other patients that had no tumour necrosis at the time of surgery (Fig. 6C). We investigated whether this could be due to differences in T cell responses to *Pexa-Vec*; comparison of longitudinal ELISpot PBMC IFN-γ between patients 05 (extensive tumour necrosis) and 06 (no tumour necrosis) revealed an expected increase in responses at the time of surgery, which reduced at the end of study, but no discernible difference between the two patients (Fig. S7). Therefore, CRLM necrosis in patients 05 and 08 following *Pexa-Vec* infusion appears to be associated with a

reduction in the diversity of the most highly proliferated T cell clones. This observation could in part be accounted for by proliferation of select TRBV18 and TRBV20-1 T cell clonotypes targeting TAAs, in contrast to a more diverse naïve CD8+ TCR repertoire in patients without tumor necrosis. This is reminiscent of the same observation in patients with melanoma who experienced durable progression-free survival following treatment with a neoantigen vaccine and PD-1 inhibition[43]. In these patients, the presence of increased effector memory CD8+ T cells was reflected by a more restricted and less diverse circulating TCR repertoire than in patients who experienced progressive disease.

Discussion / Conclusions

Our results show that neoadjuvant *i.v. Pexa-Vec* therapy is well tolerated in patients with metastatic melanoma and CRLM, has tumour-specific replication and shows evidence of clinical efficacy, including pathological tumour response. *Pexa-Vec* was detected in three of the four available CRLM specimens, confirming previous findings of *i.v.* delivery of *Pexa-Vec* to tumour when using the same or higher doses[14]. In contrast to our previous findings of the cellular carriage of oncolytic reovirus to tumour following *i.v.* infusion[29,30], *i.v. Pexa-Vec* administration resulted in plasma-based carriage, with no association of virus with PBMC. The development of neutralising antibodies to *Pexa-Vec* at the time of surgery is in keeping with the timing of seroconversion previously reported for *Pexa-Vec,* as well as for other *i.v.*-administered oncolytic viruses[44,45]. In contrast to oncolytic reovirus, which is delivered to tumour by peripheral blood cell carriage[46], and results in the shielding of virus from antibody neutralization[47], the development of *Pexa-Vec* antibodies will likely hinder *i.v.* delivery from that time point onwards[48]. Strategies to reduce antibody neutralisation are being tested clinically for other

oncolytic poxviruses, including TG6002, an engineered Copenhagen strain vaccinia virus under clinical development by Transgene, which is being delivered by hepatic artery infusion in patients with CRLM (ClinicalTrials.gov Identifier: NCT04194034).

Our study extensively characterises the innate and adaptive immune response to intravenous *Pexa-Vec* infusion in cancer patients. In accordance with results from other oncolytic virus clinical trials, *Pexa-Vec* administration stimulated IFN expression, which peaked 24 hours post infusion[4950]. This resulted in a broad inflammatory cytokine and chemokine response, despite the potential for immunosuppressive vaccinia virus gene expression of encoding proteins that act as decoy receptors to block the activity of type I IFNs[51]. The expressed cytokines were associated with NK cell activation and the proliferation of CD8 T cell clones specific for TAAs, despite the presence of exhausted cell populations. The correlation seen in our study, of pathological tumour response with a reduction in the perceived peripheral T cell receptor diversity, is worthy of note and requires confirmation in larger trials. Whilst we have not, within the confines of the present study, specified the nature of these T cell populations, we suggest that pathological tumour response is driven by a highly proliferative, low diversity clonal T cell population targeting TAAs. If confirmed in a larger study, our results therefore support a strategy to encode selected HLA-matched tumour-specific epitopes within oncolytic viruses, or non-HLA-specific TAAs, instead of the pursuit of broad-spectrum, low-level T cell stimulation.

Disappointing phase 3 results using *Pexa Vec* in combination with Sorafenib in advanced hepatocellular carcinoma (NCT02562755) indicate the need to better rationalize combinations and schedules of therapy. Despite the size of the present study and heterogeneity of the tumour cohort, our findings, in conjunction with the excellent safety data from our previous neoadjuvant oncolytic virus studies[29,30], supports the development of this class of immunotherapies towards

standard clinical practice in the neoadjuvant setting. Rationalised combinations with non-overlapping toxicities are needed to increase efficacy. The most promising OV combination is with immune checkpoint proteins, which are frequently upregulated in the presence of both pathogenic and therapeutic viral infections[29,52–54], and act to dampen CD8 T cell responses. The sequential combination of an oncolytic virus, followed by PD-1 or PD-L1 blockade has produced remarkable results in pre-clinical models[29,54], and is currently being tested in numerous early-[55] and late-phase (ClinicalTrials.gov Identifier: NCT02263508) clinical trials, within a wide variety of malignancies. Likewise, tumour-conditioning using intratumoural *Pexa-Vec*, followed by PD-1 blockade is currently being tested in patients with advanced hepatocellular carcinoma (ClinicalTrials.gov Identifier: NCT03071094). To aid in the clinical progression of oncolytic vaccinia therapy, further research should seek reliable baseline biomarkers that predict tumour response and clinical prognosis, following virus treatment, in conjunction with the associated clinical disease parameters.

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FIGURES AND LEGENDS

Figure 1: *Pexa-Vec* peripheral blood carriage

A) Trial schema showing the timing of *Pexa-Vec* infusion and collection of translational blood samples. B) H&E staining of tumour sections from patients 05 and 08 showing areas of necrosis. 'L' indicates lymphocytic infiltrate. Bars indicate 400 μm. C) qPCR analysis for the presence of *Pexa-Vec* in patient PBMCs or plasma at all time points; '+' and '-' indicate positive and negative detection, respectively. D) qPCR quantification of *Pexa-Vec* in three healthy donor plasma or PBMC fractions, following *ex-vivo* addition of virus to whole blood. Data are shown as ng DNA in PBMCs or plasma extracted from an initial 5 mL peripheral blood. **P<0.01, ***P<0.001 by unpaired T tests; n=9. E) Neutralising antibodies to *Pexa-Vec* in patient serum following *i.v.* infusion; plot shows pre-virus, peak at surgery/1M post and end of study titres in n=9 patients.

Figure 2: *Pexa-Vec* detection in resected CRLM specimens

A) The presence of *Pexa-Vec* in surgical tissue was assessed by IHC following *i.v.* infusion. Representative slides from four patients shows vaccinia protein (brown), secondary antibody-alone controls and H&E stains in consecutive sections. B) Tissue core biopsies, taken from resected CRLM and background liver samples from patients undergoing standard surgery outside the clinical trial, were treated with *Pexa-Vec-GFP* or PBS prior to confocal fluorescent imaging. Images shown are for one of ten representative patients. All bars indicate 200 μm.

Figure 3: Innate immune response to *Pexa-Vec*

A) Peripheral blood plasma IFN-α concentration following *Pexa-Vec* infusion was determined by multi-plex analysis. Data are shown as fold-change from baseline (D1 pre) samples. *P<0.05 by paired T tests; n=4. B) Differential ISG expression analysis of mRNA isolated from CRLM trial patient PBMCs. Data are expressed as log_2(CPM). AdjP value was determined after adjustment using the Benjamini and Hochberg (1995) method for controlling the false discovery rate; *P<0.05, **P<0.01; n=6. C) Patient NK cell activation shown by (i) CD69 expression and (ii) NK degranulation against tumour-specific targets (Mel888 for melanoma patients; SW620 for CRLM patients) shown as % positive CD107 expression. *P<0.05 by paired T tests; n=9 for both. D) NK cell activation (represented by CD69 expression) (i) and NK degranulation (ii) of healthy donor PBMCs following stimulation with *Pexa-Vec* in the presence of IFN-α/β blockade or isotype control. *P<0.05 by unpaired T tests; n=4. E) NK cell activation (i) and degranulation (ii) of healthy donor PBMC ± monocyte depletion prior to stimulation with *Pexa-Vec*. *P<0.05 by unpaired T tests; n=4.

Figure 4: Chemokine expression and CD8 T-cell tumour infiltration

A) Trial patient peripheral blood lymphocyte concentrations prior to (D1 pre) and post-*Pexa-Vec* infusion. *P<0.05 by paired T test; n=9. B) Cell death of healthy-donor PBMC cell populations treated with *Pexa-Vec* or PBS (n=3). C) *Pexa-Vec*-treated trial patient PBMC samples were assessed for (i) mRNA expression quantification of the *CXCL10* gene (AdjP value was determined after adjustment using the Benjamini and Hochberg (1995) method for controlling the false discovery rate ****P<0.0001; n=7) and (ii) multiplex quantification of CXCL10 protein (IP-10) in plasma (*P<0.05 by paired T tests; n=9). D) IHC staining (brown) of CD8-expressing cells within representative trial patient tumours following *Pexa-Vec* infusion. Bars represent 100 μm.

Figure 5: T-cell functional anti-cancer responses

A) Representative IHC of patient tumours showing MART-1 (patient 01) and CEA (patient 05) expression (purple and brown, respectively), with corresponding secondary antibody controls. Bars represent 200 µm. B) Representative ELISpot images from patients 01 and 05, where PBMCs were stimulated using MART-1 and CEA overlapping peptide pools, respectively. Duplicate wells are shown for each time point. Data is shown as SFU per well; each spot represents an IFNγ-secreting T cell. C) A summary graph of CEA and MART-1 IFN-γ ELISpot responses from all 9 patients. Data is shown as mean ± sem SFU/well. *P<0.05 by paired T tests; n=7-9, dependent on sample availability. D) Estimated numbers of CD8 T-cells that belong to specific MART-1 TAA clones. Data shown for melanoma patients 01, 03 and 04.

Figure 6: TCR sequencing of trial patient PBMC

A) Circos plots showing the association of V-gene (lower half of plot) and J-gene (upper half of plot) segments at different time points for patients 05, 06 and 8. Width of the ribbon is indicative of the relative usage of each segment at each time point. B) Percent change in T-cell receptor diversity as calculated by Inverse Simpson Index. C) Percent change in the diversity of T-cell clones at surgery as a comparison between patients with defined necrosis and patients whose tumour showed no necrosis. * P<0.05 by one-tailed Mann-Whitney test (n=