Oncolytic viruses infect, replicate in, and kill cancer cells, leaving normal cells unharmed; they also recruit and activate immune cells against tumor cells. While clinical indications for viroimmunotherapy are growing, barriers to widespread treatment remain. Ensuring real-time tracking of viral replication and resulting anti-tumor immune responses will overcome some of these barriers and is thus a top priority. Clinically optimizing trackability of viral replication will promote safe dose increases, guide serial dosing, and enhance treatment effects. However, viral delivery is only half the story. Oncolytic viruses are known to upregulate immune checkpoint expression, thereby priming otherwise immunodeficient tumor immune microenvironments for treatment with checkpoint inhibitors. Novel modalities to track virus-induced changes in tumor microenvironments include non-invasive measurements of immune cell populations and responses to viroimmunotherapy such as (1) in situ use of radiotracers to track checkpoint protein expression or immune cell traffic, and (2) ex vivo labeling of immune cells followed by nuclear medicine imaging. Herein, we review clinical progress toward accurate imaging of oncolytic virus replication, and we further review the current status of functional imaging of immune responses to viroimmunotherapy.

INTRODUCTION

Oncolytic viruses (OVs) are a powerful tool of immunogenicity and are capable of conferring anti-tumor immunity even to disseminated cancers. While one virus is currently US Food and Drug Administration (FDA)-approved for melanoma treatment, barriers remain to the widespread use of viroimmunotherapy in solid tumor treatment algorithms. With an average time to response of approximately 4 months as seen in the OPTIM trial prompting talimogene laherparepvec (T-Vec) approval,1–3 oncolytic virologists and medical oncologists alike are left to guess whether continued OV or other cancer treatment dosing will benefit the patient. This is especially harrowing in the setting of pseudo-progression or progression prior to response, which can occur in up to 49% of responders.4,5 In many cases, we simply continue to treat until a tumor marker rises, or an image demonstrates definitive tumor progression, unaware whether we have benefited the patient with the preceding months of therapy. The inability to non-invasively measure treatment progress in real time is a barrier shared by OVs, immunotherapies, and traditional cytotoxic treatments alike. Non-invasive diagnostics that can provide valid feedback would save money, time, and toxicity for many.

Attempts at optimizing clinical imaging of viral replication in tumors have been ongoing during the last 20 years with limited success.6 Real-time imaging allows OVs to meet their full theranostic potential. Indeed, many OVs currently in clinical testing accommodate transgenes encoding “payloads” that include enhancement of immunogenicity and also reporter genes that allow for real-time tracking of viral replication. Given that many OVs are tumor-tropic, viral imaging may elucidate previously undetected tumors. Ultimately, imaging of OV trafficking and viability could yield truly personalized medicine by guiding variables such as future serial injections for intratumoral models and dose increases for systemic delivery. However, despite years of clinical development spanning disease and vector types, optimal dose timing and the best vector and dosing strategy for each specific tumor remain a challenge.7

Thus far, there are two predominant types of real-time OV imaging: optical and deep tissue functional imaging. Clinically, optical imaging allows direct visualization of fluorescence. In the operating room, special laparoscopes can elucidate fluorescent tissue within body cavities. In the clinic, lamps can reveal fluorescent epidermal or mucosal surfaces.8 Functional viral imaging measures isotope uptake as a surrogate for viral replication with scans such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT). Of the reporter genes in OV clinical trials, human sodium iodide symporter (hNIS) is the most prominent.9 However, only a selected few investigators have published actual human images.10–15

While critical to the success of the field, tracking viral delivery is only half the story. Reliable non-invasive characterization of virally
induced anti-tumor immune responses also remains elusive. In vivo and ex vivo techniques for radiolabeling immune cells, cytokines, and co-stimulators or co-inhibitors are rapidly evolving arenas of clinical imaging. To most comprehensively understand the anti-tumor effects of viroimmunotherapy without invasively sampling tissue, non-invasive imaging should include viral tracking, measurement of immune checkpoint expression, and tracking immune cells into tumors. Herein, we review progress and promise of comprehensive non-invasive imaging of viroimmunotherapy.

REVIEW OF PUBLISHED CLINICAL REAL-TIME VIRAL TRACKING

As demonstrated by our group and others, real-time tracking of viral replication demonstrates tumor tropism whether viruses are administered intratumorally (i.t.), intravenously (i.v.), or intraperitoneally (i.p.) (Figure 1).16,17 These experiments were confirmed in previously published experiments using HCT116 xenografts,17 and also as shown here using HT-29 xenografts infected with a recombinant orthopoxvirus platform (CF33) with tk deletion encoding either hNIS (CF33-hNIS) or firefly luciferase (Fluc) (CF33-Fluc).16,17

Upon comprehensive English literature review from 1995 to present, many abstracts and posters referencing images on replicating OVs were found. However, a surprising paucity of peer-reviewed publications showed images of non-invasive viral replication tracking. We were only able to identify six peer-reviewed publications with images of viral replication: four studies demonstrated successful tracking of NIS-encoding OVs via I-123 SPECT/CT, one study used an 18F-labeled penciclovir analog, and one study used a 124I-labeled substrate for herpes simplex virus 1 (HSV-1)-tk to monitor thymidine kinase gene expression (Table 1). In each of the described studies, the success of imaging appeared to be dose-dependent. In the NIS-based studies, images appeared most consistently 7–8 days after treatment.10–12 In the tk imaging papers, Jacobs et al.15 show [124I]-FIAU retention 68 h after injection, whereas Peñuelas et al.14 examined the [18F]-FHBG signal 1 week after injection.14,15 In the remaining trial referenced in Table 1, investigators of a GFP-encoding vaccinia used fluorescent lamps in the clinic to examine a pox-like rash occurring in treated patients with head and neck carcinomas. While this does not represent imaging of viral replication in tumors, the investigators emphasize that such a rash confirms successful systemic viral replication.8

Clinical OV image optimization has remained a challenge despite numerous creative adjuncts such as oral contrast,18 chemo-tagged radiotracers, and novel highly specific tracers.19 Moreover, in addition to the expected variability of viral replication between tumor types, even similar types of tumors in identical anatomic locations exhibit show differences in viral replication. For instance, Rajecki et al.20 treated a cervical cancer patient with Ad5/3-D24-hNIS, acting based upon the findings of Barton et al.13 using Ad5-yCD/mutTKSR39-hNIS in prostate cancer. Unfortunately, Rajecki et al. saw no evidence of an OV-based signal. This may have been due to their study of both a different vector with hNIS on a different promoter, and also an entirely different disease type. Groups using hNIS-based imaging have seen more consistent results at higher doses and with more uniform disease states as detailed in Table 1. However, published images demonstrate that further optimization is needed to achieve clinical relevance. Perhaps clinical optimization using more potent and rapidly replicating virus platforms such as CF33 or HSVs encoding hNIS will render consistent high-yield imaging to guide future therapies. If properly established, real-time non-invasive deep tissue imaging will enable more rapid incorporation of imageable viroimmunotherapies into solid tumor treatment schema.

VIRAL REPLICATION CO-LOCALIZES WITH TUMOR T CELL INFILTRATION

To further assess whether non-invasive viral imaging can serve as a linear surrogate for both viral replication and T cell infiltration, we
confirmed that immunofluorescent vaccinia staining corresponds to immunohistochemical (IHC) staining showing T cells co-localizing with viral infection (Figures 2A and 2B). Moreover, in subsequent experiments, we evaluated immune cell infiltration and confirmed these IHC findings quantitatively using flow-cytometry on single cells obtained from tumors to find that CD8+ tumor infiltration is higher in viral-treated tumors (Figure 2C). We and others have shown that CD8+ T cells co-localize to actively replicating virus.21 This is aligned with findings by Sampath et al.22 that showed direct synergistic interactions between an enveloped vaccinia virus and immune cell components. While co-localized viral particles and immune cells suggest that non-invasive imaging of viral replication corresponds to immune cell trafficking, only by specifically imaging immune cells or invasively sampling tumors can we confirm this.

IMAGING VIRALLY INDUCED IMMUNE CHECKPOINT EXPRESSION

Our group and others have demonstrated upregulated PD-L1 in tumors following poxvirus infection.31,23 Many think that such upregulation mediates the success of combination therapies pairing OVs with checkpoint inhibitors in advanced solid tumors.24 Others think that viruses pair well with checkpoint inhibitors simply because they release inflammatory damage- and pathogen-associated proteins into the tumor microenvironment, thereby recruiting and activating immune cells in the tumor microenvironment.25 In order to find the most effective place in treatment algorithms for OVs amid the already tumultuous sea of immune checkpoint inhibitors available, we must fully characterize both checkpoint expression and immune cell trafficking in real time. While reliably imaging checkpoint expression after immunotherapy treatment of any sort is a tall order, there is some progress with radiolabeled antibodies to a variety of checkpoint proteins (Figure 3). Indeed, one can image any point along the continuum of activating a T cell as it recognizes tumor, from radio-labeled antibodies to cytokines such as interferon (IFN)γ, cluster of differentiation (CD) cell-surface proteins such as CD8, or markers of activation such as granzyme B. At present, in vivo imaging of this nature is plagued by non-specific background uptake. That said, some progress is being made with highly specific radiotracers and antibodies.26

CURRENT CLINICAL PROGRESS IN TRACKING IMMUNE RESPONSES TO OV

To date, imaging of immune responses to viral therapy are sparsely explored. In 2013, Weibel et al.27 correlated 19F-magnetic resonance imaging (MRI) with CD68 staining on IHC in xenograft models of human melanoma and breast cancer infected with an oncolytic vaccinia virus GLV-1h68. These macrophage-dense regions within a tumor tended to surround virally infected areas of tumor as fluorescent vaccinia staining corresponds to immune cell infiltration and concomitantly imaging immune cells or invasively sampling tumors can be confirmed this.

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cells during at least several days with serial CT-PET imaging. More- 
over, its relatively lower positron energy fosters enhanced resolution 
of PET images. While other more specific tracers such as copper are 
also being studied, the half-life is comparatively short and the back-
ground signal is also prohibitive in some cases. Zr-labeled T cells 
have been successfully used in clinical settings to image CAR-T cell 
tracking to non-small cell lung cancer (NSCLC), prostate cancer, 
melanoma, and advanced gastrointestinal malignancies as detailed 
in Table 2. While the alternative of MRI uses superparamagnetic 
iron oxide nanoparticles that are ingested by cells intended for 
tracking, this is a much more cumbersome and lengthy image acquisi-
tion process that is also highly dependent on cell function rather 
than precise labeling, as would be required for comprehensive imaging 
of viroimmunotherapy.

The authors propose that an ideal strategy toward comprehensively imaging responses to oncolytic viroimmunotherapy would take into account the “big picture” of a tumor transformation following viral infection, including (1) immediate changes to cancer cells upon viral entry and replication, (2) initial changes to the surrounding tumor immune microenvironment, and (3) alterations in tumor immune cell infiltration (Figure 5A). Each of these three components of virally mediated tumor transformation is imageable by tracking virus to tumor with reporter genes, then flagging upregulation of immune checkpoints, and monitoring effector immune cell traffic in treated tumors (Figure 5B). In so doing, investigators would be able to amend treatment courses in real time to optimize anti-tumor immune responses and prolong patient survival.

CONCLUSIONS
Herein, we have reviewed the published clinical experience with functional viral imaging and proposed additional possible future directions for tracking viral replication in clinical studies. We further reviewed current progress and challenges as well as strategies for future comprehensive imaging of immune responses to oncolytic viral treatment. In conclusion, this paper emphasizes the importance of continued optimization of preclinical and clinical protocols to visualize viral replication in real time. While many trials are currently testing imaging endpoints, we must encourage further investigations to both speed regulatory approvals and incorporate viroimmunotherapy into treatment algorithms. In this era of pay-to-play immunotherapy, patients, clinicians, and payers alike should place a high value
on real-time proof of viral tumor tropism and therapeutic benefit. Strategies to non-invasively and reliably image viral delivery, checkpoint expression, and immune cell trafficking will be critical to advancement of the field.

LITERATURE REVIEW
PubMed and ClinicalTrials.gov were queried for search terms including, but not limited to, oncolytic virus, SPECT, PET, imaging, NIS, GFP, optical imaging, functional imaging, and tracking. All active clinical trials involving oncolytic viral imaging were reviewed. Trial vectors and key words were used in PubMed to search for any publications of results. Many trials are still accruing data.

Identified publications were included in Table 1 only when a clinically generated image was a figure in the manuscript. There were many published abstracts without images available, and we anticipate that images will be forthcoming from several groups in the near future.

VIRUS CHIMERIZATION AND hNIS OR FLUC CLONING
The chimerization, cloning, competitive selection, and sequence of CF33 backbone virus have been described previously. Insertion of the hNIS expression cassette or Fluc under the control of the vaccinia H5 promoter or synthetic early (SE) promoter at the J2R locus has also been described, as has the deletion of the F14.5L gene and insertion of the anti-PD-L1 transgene at the F14.5L locus.

In vitro luciferase activity was confirmed by infecting HCT-116 cells with CF33-Fluc at varying multiplicities of infection (MOIs). Rapid luciferase activity was observed after 24 h by adding 100× luciferin solution (prepared as below) directly to wells and imaging after 10 min with a Lago X optical imaging system (Spectral Instruments Imaging, Tucson, AZ, USA).

CELL LINES
HT-29 (RRID:CVCL_0320), HCT116 (RRID:CVCL_0291), and African green monkey kidney fibroblasts-CV-1 (RRID:CVCL_0229) cell lines were purchased from ATCC (Manassas, VA, USA). All human colorectal cell lines were maintained in McCoy’s 5A medium (Gibco, Gaithersburg, MD, USA) and CV-1 cells were maintained in Dulbecco’s modified Eagle’s medium (Corning Life Sciences, Corning, NY, USA). MC38 and MC38-Luc cells were a kind gift from Dr. Laleh Melstrom’s laboratory (City of Hope, Duarte, CA, USA). MC38 and MC38-Luc cells were maintained in DMEM. All cells were supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution, both purchased from Corning Life Sciences.

Table 2. Zirconium-89 based imaging to track therapeutics

| Year, Journal, Author | Image modality | What labeled | No. of patients imaged | Disease process | Trial ID |
|-----------------------|----------------|--------------|------------------------|----------------|---------|
| 2016, Journal of Nuclear Medicine, Pandit-Taskar et al. | PET and SPECT | #Zr-Df-IAR2M | 18 | prostate Cancer | ClinicalTrials.gov: NCT02760199 |
| 2017, Nature Communications, Niemeijer et al. | PET-CT | #Zr-nivolumab | 13 | NSCLC | ClinicalTrials.gov: NCT03107663 |
| 2018, J Clinical Oncology, Postow et al. | PET-CT | #Zr-AMG222M2C (anti-CD8) | 3 | melanoma, HCC, NSCLC | ClinicalTrials.gov: NCT02291614 |
| 2019, Clinical Cancer Research, Moek et al. | PET-CT | #Zr-Df-IAR22M2C (anti-CD8) | 9 | advanced GI cancer | ClinicalTrials.gov: NCT03802123 |

HCC, hepatocellular carcinoma; GI, gastrointestinal; RCC, renal cell carcinoma; SCC, squamous cell carcinoma.
The cells were maintained in a humidified incubator at 37°C and 5% CO₂. Efforts were made not to perform experiments past 15 passages of cells. All cell lines were tested for mycoplasma before each experiment initiation.

**PET IMAGING**

*In vivo* I-124 uptake measured by PET/CT

Mice bearing HT-29 flank xenografts were divided into imaging and control groups (n = 4 mice). To analyze tumor imageability after intratumoral delivery, mice received an intratumoral injection of 10⁴ plaque-forming units (PFU) per tumor of either CF33-hNIS, CF33-Fluc, or PBS when tumors reached 100 mm³. At 7, 14, and 21 days after viral injection, mice in each group received 200 μCi of I-124 injected per tail vein. The radioisotope was obtained from the City of Hope Small Animal Imaging Core Radiopharmacy. PET imaging was then obtained 2 h following injection using the small animal PET scanner (microPET R4, Siemens), which provides fully three-dimensional PET imaging with a spatial resolution of better than 2.0 mm and quantitative accuracy for measurement of tissue activity concentration on the order of 10%. Quantitative accuracy is supported by scatter, dead time, and measured attenuation corrections. The system includes a fully developed image analysis package that supports volumetric regions of interest and the fusion of PET with co-registered anatomic CT. To protect mouse thyroids from radioiodine ablation, all mice received T4 supplementation with 5 mg of levothyroxine/L of water beginning 1 week before radioiodine administration.

**LUCIFERASE IMAGING**

Firefly luciferin solution was prepared as per the manufacturer’s instruction (PerkinElmer, Waltham, MA, USA). Imaging was obtained after intraperitoneal delivery of luciferin in a control mouse and all mice treated with CF33-Fluc using a Lago X optical imaging system (Spectral Instruments Imaging, Tucson, AZ, USA) after 15 minutes of incubation.

**TUMOR MODELS AND VIRUS DOSING**

For the HCT116 xenograft model, 2–3 × 10⁶ of HTCT116 cells were injected into 6- to 8-week-old female nude mouse flank using a total of 100 μL of PBS containing 50% Matrigel for each tumor. When the average tumor size approached 150 mm³, mice were divided into experimental groups and treated with 10⁵ PFU of CF33-Fluc in 50 μL of PBS by intravenous or intraperitoneal injection.
Flank tumors of MC38 and MC38-Luc were established using 3–5 × 10^5 cells in Matrigel. Tumor measurements and mouse weight were monitored twice weekly using calipers to calculate tumor volume, V (mm^3) = (0.5) × A^2 × B, where A is the shortest measurement and B is the longest measurement. Treatment typically occurred when tumors reached 100–200 mm^3 (approximately 10 days after cell injection), following which mice were randomized into treatment groups (n = 4) such that average tumor volume in each group was similar. C57BL/6J mice 8–12 weeks of age were used for most experiments (Jackson Laboratory, Bar Harbor, ME, USA and Charles River Laboratories, Wilmington, MA, USA, RRI-D:IMSR_JAX:000664, RRID:IMSR_CRL:027). Six-week-old Hsd athymic nude-Foxn1nu female mice (Envigo, Indianapolis, IN, USA) were purchased and acclimatized for 7 days.

Mice were maintained in a biosafety containment level 2 facility within our vivarium where the environment was temperature and light controlled with 12-h light/12-h dark cycles, and food and water were ingested ad libitum. All animal experiments were performed with approval of the City of Hope Institutional Animal Care and Use Committee (IACUC).

IHC

Tumors were harvested and fixed with 10% formalin. Paraffin-embedded 5-μm-thick tumor sections were obtained. The slides were deparaffinized followed by heat-mediated antigen retrieval per the manufacturer’s protocol (IHC World, Ellict City, MD, USA). Tumor slides were then permeabilized with cold methanol and blocked for 30 min with TNB blocking buffer (PerkinElmer, Waltham, MA, USA). Tumor slides were incubated with a rabbit anti-vaccinia virus antibody (Abcam, Cambridge, MA, USA, RRID:AB_778768) 1:100 in TNB blocking buffer in a humidified chamber at 4°C overnight. The next day, tumor slides were stained with Alexa Fluor 488-conjugated goat anti-rabbit (Abcam, Cambridge, MA, USA, RRID:AB_2630356) 1:200 in TNB blocking buffer for 1 h at room temperature. Finally, the slides were counterstained with 4’,6-diamidino-2-phenylindole (DAPI). IHC for CD8 was performed by the Pathology Core at City of Hope. Images were obtained using the NanoZoomer 2.0HT digital slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) or Ventana iScan HT (Roche, Basel, Switzerland).

FLOW CYTOMETRY

Single cells from tumors were generated using a mouse tumor dissociation kit utilizing a gentleMACS dissociator (Miltenyi Biotec, Cologne, Germany). Cells were stained with Live/Dead Fixable dye (Invitrogen, Carlsbad, CA, USA) in PBS for 30 min at 4°C in the dark. Next, Fc receptors on the cells were blocked using an anti-CD16/32 antibody (BD Biosciences, Franklin Lakes, NJ, USA, RRID:AB_394657 in FACS buffer (PBS containing 2% FBS) for 10 min and then stained for 30 min at 4°C in the dark using the following antibodies: mouse CD45-peridinin chlorophyll protein complex (PerCP) (BioLegend, San Diego, CA, USA, RRID:AB_893340), mouse CD3-fluorescein isothiocyanate (FITC) (eBiosciences, San Diego, CA, USA RRID:AB_2572431), mouse CD4-allophycocyanin (APC) (BioLegend, San Diego, CA, USA, RRID:AB_389325), and mouse CD8- VioGreen (Miltenyi Biotec, Cologne, Germany, RRID:AB_2659495). The data were acquired using the MACSQuant analyzer 10 (Miltenyi Biotec, Cologne, Germany). Data were analyzed using FlowJo software (v10, Tree Star, Ashland, OR, USA).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism (version 7.01, GraphPad, La Jolla, CA, USA). A Student’s t test was used to evaluate statistical significance. p < 0.05 was considered significant. Where present in figures, error bars indicate SD or SEM as defined in legends.

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AUTHOR CONTRIBUTIONS

Study concept and design, S.C., S.-I.K., M.O., Y.F., and A.K.P.; data collection, analysis, and interpretation, S.C., S.-I.K., M.O., J.L., S.K., Z.Z., A.Y., Y.F., and S.G.W.; manuscript preparation and critical revision, all authors; and final approval of manuscript, all authors.

DECLARATION OF INTERESTS

Y.F. receives royalties from Merck and from Imugene. CF3 platform is licensed to Imugene by the City of Hope. The remaining authors are City of Hope employees.

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