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Title: Imaging of Activated T Cells

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Abstract

The adaptive immune response plays a critical role in detecting, eliminating, and creating a memory towards foreign pathogens and malignant cells. Demonstration of the specific and effective target killing of T cells in cancer has reignited interest in the study and therapeutic manipulation of the interaction between tumor and immune system. To both improve therapeutic efficacy and reduce adverse events, accurate monitoring of the activation of T cells is required. A number of approaches to monitoring not just the presence, but importantly the activation of T cells have been developed. Herein, we review the recent advances in T cell activation imaging and future directions for potential implementation into clinical utility.

INTRODUCTION

T cells represent the target of many immunotherapies, including checkpoint inhibitors, bi-specific T cell engagers, and immune agonists (*1*). Additionally, T cells themselves have been investigated as therapies either using chimeric antigen receptor addition or autologous adoptive transfer in hematological malignancies (*2*). In solid tumors, although some success has been achieved by checkpoint inhibitors targeting programmed death protein-1 (PD-1), cytotoxic lymphocyte antigen-4 (CTLA-4), and lymphocyte activation gene-3, most approaches have not increased overall survival (*3,4*). Furthermore, approaches to activate T cells to kill cancer can induce severe immune-related adverse events, and in some cases, lead to death (*5*).

The dynamic and spatially heterogeneous nature of immune responses makes predicting and monitoring immune activation difficult *in vivo*. Currently, imaging approaches to monitor response include quantifying therapeutic targets (i.e. PD-L1), the presence of immune cells (i.e. CD8) or T cell activation. Although the presence of therapeutic target and T cells are necessary, they alone are not sufficient, as other immunosuppressive factors may abrogate anti-tumor response. T cell activation ultimately represents the integral of the pro- and anti-tumoral signaling within a tumor, providing a simplified readout of a complex process, including antigen presentation, T cell priming, antigen recognition and appropriate co-stimulatory signal transduction (*6*). Given the whole-body, non-invasive, and quantitative nature of positron emission tomography (PET) imaging, many groups have developed novel imaging agents for interrogating various aspects of the T cell response. In this review, we will cover the recent advances in imaging activated T cells. In general, the approaches to monitoring activated T cells have been focused on several differentiating aspects of activated T cells, including metabolism, secreted molecules, and surface receptors. The strengths and weaknesses of each target, as well as current imaging agents and approaches will be reviewed herein. Finally, future directions and potential next-generation targets will be examined.

METABOLISM

Arabinosylguanosine

Arabinosylguanosine (AraG), a nucleoside analogue, has been shown to exert selective killing against T lymphoblasts (*7*). The nucleoside analogue undergoes phosphorylation by deoxyguanosine kinase, in particular when there is lower concentrations of the substrate in the mitochondria and the cytoplasm (*8*). Namavari et al. first synthesized the tracer 2′-deoxy-2′-^{18}Ffluoro-9-β-D-arabinofuranosylguanine (^{18}F-AraG) and demonstrated uptake in activated T cells (*9*), however Levi et al. were the first to evaluate immune checkpoint blockade (ICB)-related changes in immune response using the tracer. Longitudinal ^{18}F-AraG imaging in a murine sarcoma virus-moloney leukemia virus induced rhabdomyosarcoma model from week 1 to week 3 followed the course of immune activation with stable increase in tumor and draining lymph nodes signal overtime. ^{18}F-fluorodeoxyglucose (^{18}FDG) tumor signal, however, followed the pattern of tumor growth starting from initial increase to decline from week 1 to week 3. Furthermore, ^{18}F-AraG PET imaging in MC38-bearing mice showed a significant difference in tumor uptake between the baseline and only 2 days following initiation of treatment with anti-PD-1 therapy, with a nonsignificant increase in non-responders. Overall, the tracer predicted response to anti-PD-1 therapy early in the course of the disease (*10*). In a separate study, Levi et al. attempted to characterize crucial anticancer immune cell infiltration in the tumor microenvironment by PET imaging with ^{18}F-AraG and changes associated with chemotherapy (*11*). Murine syngeneic tumors with varied immune infiltration profiles had differing uptake of the tracer in both tumors and tumor-draining lymph nodes. A significant positive correlation was reported between tracer uptake and the number of CD8+PD-1+ cells. Furthermore, the PET signal was significantly higher after the introduction of immunogenic cell death inducing chemotherapy in comparison to baseline in both MC38 and A9F1 murine tumor models (*11*). Outside of cancer immunotherapy, Ronald et al. demonstrated the capability of the tracer to detect sites of early T cell activation in a tested mouse model of acute graft versus host disease. The significant pre-clinical evidence for this tracer has led to clinical investigation in a number of on-going trials focused in non-small cell lung cancer, however to date only biodistribution and safety in healthy volunteers (NCT02323893) has been published (*12*).

SECRETED MOLECULES

Granzyme B

Granzyme B (GZB), a serine protease that induces apoptosis in target cells, is released by activated CD8+ T cells and NK cells, in addition to some immunosuppressive cells (*13*). Granzyme B peptide (GZP) was the first probe used to image the serine protease by Larimer et al. (*14*). Following injection of the radiolabeled

tracer ^{68}Ga-NOTA-GZP into mice, PET imaging was able to predict response to ICBs before divergence into responders and non-responders based on tumor volumes, with the former having higher GZP uptake (*14*). In addition, further studies were performed to evaluate the ability of GZP to predict response to combination therapy of both ICBs and chemotherapy (*15*). In an effort to explore cancer immunotherapy driven adverse events, Ferreira et al. investigated the role of GZP PET imaging in a regulatory T cell depleted model of immunotherapy induced adverse events (*16*). The model mice demonstrated significant increased uptake of the tracer in the colon in comparison to appropriate controls, which was reversed by the addition of steroids, supporting the potential translational capability of GZP to monitor immune-related adverse events.

Granzyme B proteolytic activity has also recently been imaged by Zhao et al. using the multidomain tracer Granzyme targeting Restricted Interaction Peptide specific to family member B (GRIP B) (*17*). Upon cleavage of a granzyme B-specific domain in the center of a peptide, a radiolabeled ubiquitous cell binding domain is unmasked and binds irreversibly to the nearby phospholipid bilayer. The granzyme B specific activation of binding allows measurement of GZB activity over prolonged periods of time upon radiolabeling with copper-64 (^{64}Cu). The tracer accumulated in tumors both faster and with higher uptake in ICB-treated than vehicle-treated mice. Furthermore, spleen uptake of the tracer was higher with ICB treatment. To show utility in non-cancer T cell activation applications, increased GZB activity in the lungs, thymus and spleen of mice was demonstrated following intratracheal instillation of lipopolysaccharides.

Clinically, PET imaging of granzyme B has had an early clinical readout from Zhou et al. using a ^{68}Ga-labeled peptidomimetic (*18*). Two patients, one with lung adenocarcinoma and another with sarcomatoid carcinoma of the lung undergoing checkpoint blockade were imaged after treatment initiation. The lung adenocarcinoma, a partial metabolic responder had a granzyme B PET tumor SUV_{max} of 4.1 and a tumor to blood ratio of 1.2, whereas the sarcomatoid carcinoma was determined to be a partial non-responder with a tumor SUV_{max} of 2.0 and a tumor to blood ratio of 0.8. These data are preliminary, but consistent with pre-clinical studies demonstrating the predictive capabilities of granzyme B PET imaging.

Interferon-gamma

Interferon-gamma (IFN-γ) is a cytokine released by a myriad of cells, including cytotoxic T lymphocytes, T helper 1 cells, natural killer T cells, B cells, and natural killer cells. The downstream effects of the cytokine are pleiotropic, and can either promote or inhibit inflammation (*19*). Gibson et al. first investigated the role of IFN-γ PET imaging to predict response to anti-cancer immunotherapy (*20*). An anti-IFN-γ monoclonal antibody radiolabeled with zirconium-89 (^{89}Zr) (^{89}Zr-anti-IFN-γ) was used in several cancer models. Increased localized tracer uptake in the spleen of mice that received CpG-oligodeoxynucleotide to induce IFN-γ release was observed by PET imaging 72 hours post injection of the tracer. In another experiment, the tracer was injected in mice bearing neu+ TUBO cancer cells to uncover active immune response after administration of two doses of HER2/neu DNA vaccines. Mice that received the vaccine started to respond after the second dose and had tumors with significantly higher uptake of the tracer than control mice. Furthermore, higher uptake of the tracer following Her2/neu DNA vaccination and subsequent Treg depletion was shown in a spontaneous model of cancer using Neu transgenic mice. An inverse relationship between tracer uptake and tumor volume was observed in TUBO tumors implanted in BALB/c mice that received the vaccine. Taken together, the study proposed the role of IFN-γ PET imaging in evaluating active T cell-mediated anti-cancer immunity in situ.

Interleukin-2 Receptor

Interleukin-2 (IL-2) is a cytokine that affects the development and differentiation of several T cell subsets, such as CD8+ T cells, natural T regulatory cells, and CD4+ T helper cells, with resultant either pro- or anti-inflammatory downstream effects depending on the subset of the target cell. The cytokine, however, is particularly essential for the proliferation, effector function, and memory development of CD8+ T cells (*21*). The cytokine binds to its receptor (IL-2R), that is a complex formed by three different subunits in its high-affinity form, namely IL-R2α, IL-2Rβ, and IL-2Rγ (*22*). For PET imaging of IL-2R, IL-2 was radiolabeled with fluorine-18 (^{18}F), by forming the tracer *N*-(4-^{18}F-fluorobenzoyl)interleukin-2 (^{18}F-FB-IL2) (*23*). Later, van der Veen et al. synthesized two IL-2 radiolabeled tracers, ^{68}Ga-gallium- (1,4,7-triazacyclononane-4,7-diacetic acid-1-glutaric acid)-IL2 (^{68}Ga-Ga-NODAGA-IL2) and ^{18}F-fluoride-(restrained complexing agent)-IL2 (^{18}F-AlF-RESCA-IL2), to compare them with the former one and to overcome the hurdles associated with its synthesis (*24*). The authors demonstrated that activated human peripheral blood mononuclear cells (PBMCs) had higher uptake *in vitro* of ^{18}F-AlF-RESCA-IL2 than the other IL-2 labeled tracers. A similar trend was observed in *ex vivo* biodistribution experiments by the bone marrow and spleen. In addition, PET imaging revealed higher uptake of the three tracers by subcutaneously inoculated activated human PBMCs in severe combined immunodeficient mice than

control counterparts. The study proposed ^{18}F-AlF-RESCA-IL2 as a potential candidate for PET imaging of IL-2R expressing cells.

In a non-randomized, open-label clinical trial, a small sample size of patients with stage IV metastatic melanoma were transfused with ^{18}F-FB-IL2 before and through treatment with ICBs in either combination or monotherapy to assess changes in immune response by PET imaging (NCT02922283) (*25*). The maximum standardized uptake value (SUV_{max}) was low for tumors and dropped slightly from 1.8 at baseline to 1.7 at later timepoints while on therapy for patients who had both scans. No correlation between response and changes in uptake were observed. The mean standardized uptake values (SUV_{mean}) in the bone marrow and spleen were reported to be 2.5 and 10.9, respectively. However, the authors did not determine the exact cause behind the inability of their imaging study to detect changes in immune response in tumors following treatment.

SURFACE RECEPTORS

In addition to changes in T cell metabolism, T cells also undergo changes to the surface expression of specific molecules. While some of these cell surface proteins indeed function to attenuate activation, their presence nonetheless indicates activation of immune cells. Cell surface receptors, in comparison to secreted molecules, represent an attractive imaging target as they remain tethered to the cell surface and can be expressed in high concentrations, whereas secreted markers may be diffuse and limit maximal signal concentration .

PD-1

PD-1 is a checkpoint receptor that is upregulated in T cells following T cell receptor stimulation (*26*). Inhibition of PD-1 and its cognate ligand PD-L1 have revolutionized cancer therapy, as re-invigoration of activated T cells by PD-1/PD-L1 blockade can generate durable remission of metastatic disease (*27,28*). PD-1 imaging was first performed in the Gambhir lab using a ^{64}Cu-labeled anti-mouse IgG (*29*). In this seminal work, Natarajan et al. demonstrated persistent accumulation in the lymphoid organs and tumors of transgenic mice bearing B16-F10 melanoma tumors. Subsequent approaches to PET imaging of PD-1 have included using therapeutic antibodies, including ^{89}Zr-df-pembrolizumab and ^{89}Zr-nivolumab, in humanized mouse models, and ^{89}Zr-nivolumab in non-human primates (*30-32*).

Clinical PD-1 PET imaging was first reported by Niemeijer et al. in 2018 using ^{89}Zr-nivolumab (*33*). Like pre-clinical studies, high uptake was visualized in the spleen, liver, and tumor over time. Tumors with immunohistochemical confirmation of PD-1 presence had statistically significant increases in the SUV_{peak} signal of ^{89}Zr-nivolumab, and subsequent responding tumors also had significantly higher levels of tracer accumulation. In addition to ^{89}Zr-nivolumab, ^{89}Zr-pembrolizumab has also been investigated clinically (*34*). 18 patients, 11 with melanoma and 7 with non-small cell lung cancer were imaged on days 2, 4 and 7 post-injection of 0.37 MBq of 89Zr-pembrolizumab plus 2.5 mg or 7.5 mg unlabeled pembrolizumab. The results were similar to those of ^{89}Zr-nivolumab, with marked uptake in spleen, lymph nodes, tumor and liver. Overall, uptake was significantly correlated with both progression-free and overall survival. To date, PD-1 represents the most advanced PET imaging marker. Although the pharmacokinetics of antibody-based imaging make routine clinical practice challenging, the results of these studies demonstrate significant potential of PD-1 as a predictive biomarker.

OX-40 and Inducible T-Cell Costimulator (ICOS)

OX-40 (CD134) is a member of the tumor necrosis factor receptor superfamily and has been reported to be restricted to antigen-specific activated T cells. The first report of OX-40 PET imaging used a ^{64}Cu-DOTA-conjugated murine anti-mouse OX-40 monoclonal antibody (*35*). In this study, mice bearing dual A20 tumors were administered an in situ CpG oligonucleotide vaccine and imaged 2 and 9 days post-therapy. Significant differences in uptake were quantified between treated and vehicle tumors at 2 days post-injection, and unsupervised hierarchical clustering indicated uptake was predictive of response. A similar approach has been utilized for glioblastoma, however, interestingly the tumor of the vehicle treated mouse demonstrated higher accumulation than the vaccinated mouse (*36*). Lymph node and spleen values were higher in the vaccinated mice, suggesting greater involvement of the peripheral lymphatic system in the efficacy of an intramuscular tumor vaccination approach. The differences demonstrate the varied roles and importance of spatial localization in monitoring activated T cells. Outside of cancer, OX-40 PET imaging has also been used to diagnosis acute graft-versus-host disease, with significant increases in the lungs and liver of mice transplanted with human PBMCs (*37*). Similar to OX-40, an ^{89}Zr-DFO-ICOS antibody has been developed to track T cell activation following introduction of a stimulator of interferon genes (STING) agonist and checkpoint blockade (*38*). In a syngeneic

lung cancer model, PET imaging revealed rapid and persistent upregulation following STING agonist administration that correlated with changes in tumor volume.

CD69

CD69 is an early cell surface marker of activated T cells. It functions as a signal transducer to promote proliferation, but can also be found on monocytes, neutrophils and eosinophils, in addition to constitutive expression on mature thymocytes and platelets. The first targeted imaging agent for CD69 was an affibody, $Z_{CD69:2}$, that bound to both murine and human CD69 (*39*). The affibody was conjugated to DOTA and radiolabeled with ^{111}In for SPECT imaging. The tracer exhibited rapid blood clearance through renal excretion, and accumulation in a lymph node of a healthy rat, as well as accumulation at the site of allograft rejection in a mouse model. More recently, an ^{89}Zr-labeled antibody was used for PET imaging of CD69 (*40*). The authors examined the ability of the tracer to differentiate responding and non-responding syngeneic CT26 tumors during checkpoint blockade. The authors demonstrated significant differences in the blood, spleen, and tumor between treated responders and non-responders. Furthermore, the authors confirmed target specificity using autoradiography and immunohistochemistry, providing robust correlation between PET signal and tissue protein expression.

FUTURE DIRECTIONS

To date, a significant amount of pre-clinical evidence has been generated to support imaging activated T cells to predict response to checkpoint blockade in tumors or monitor the development of autoimmune disease. Early clinical trial results have been mixed, with PD-1 PET imaging correlating with response and IL-2 PET imaging demonstrating no correlation. In addition, some agents, including AraG (NCT04726215, NCT05096234) and NOTA-hGZP (NCT04169321) are currently under investigation in clinical trials. One current limitation of several pre-clinical targets (IFN-γ, OX-40, CD69) is the lack of human-specific probes for translation to clinical trials. The temporal dynamics of the immune system may make antibody imaging challenging due to the extended timelines between injection and image collection. Ideally, high affinity ligands for these targets with rapid pharmacokinetics would allow for robust implementation into a clinical imaging paradigm.

The opportunity for one or more of the current imaging approaches to impact cancer immunotherapy remains high. Approaches that stratify early response in individual patients or provide quantitative assessments of novel single agent or combination immunotherapies in early phase clinical trials would provide the greatest immediate impact to cancer treatment. Continued testing of probes in clinical trials, in addition to translation of promising targets is necessary to advance the field.

Disclosure of Conflicts of Interest: BL is a consultant and shareholder in Cytosite Biopharma Inc. No other potential conflicts of interest relevant to this article exist.

FIGURE LEGEND

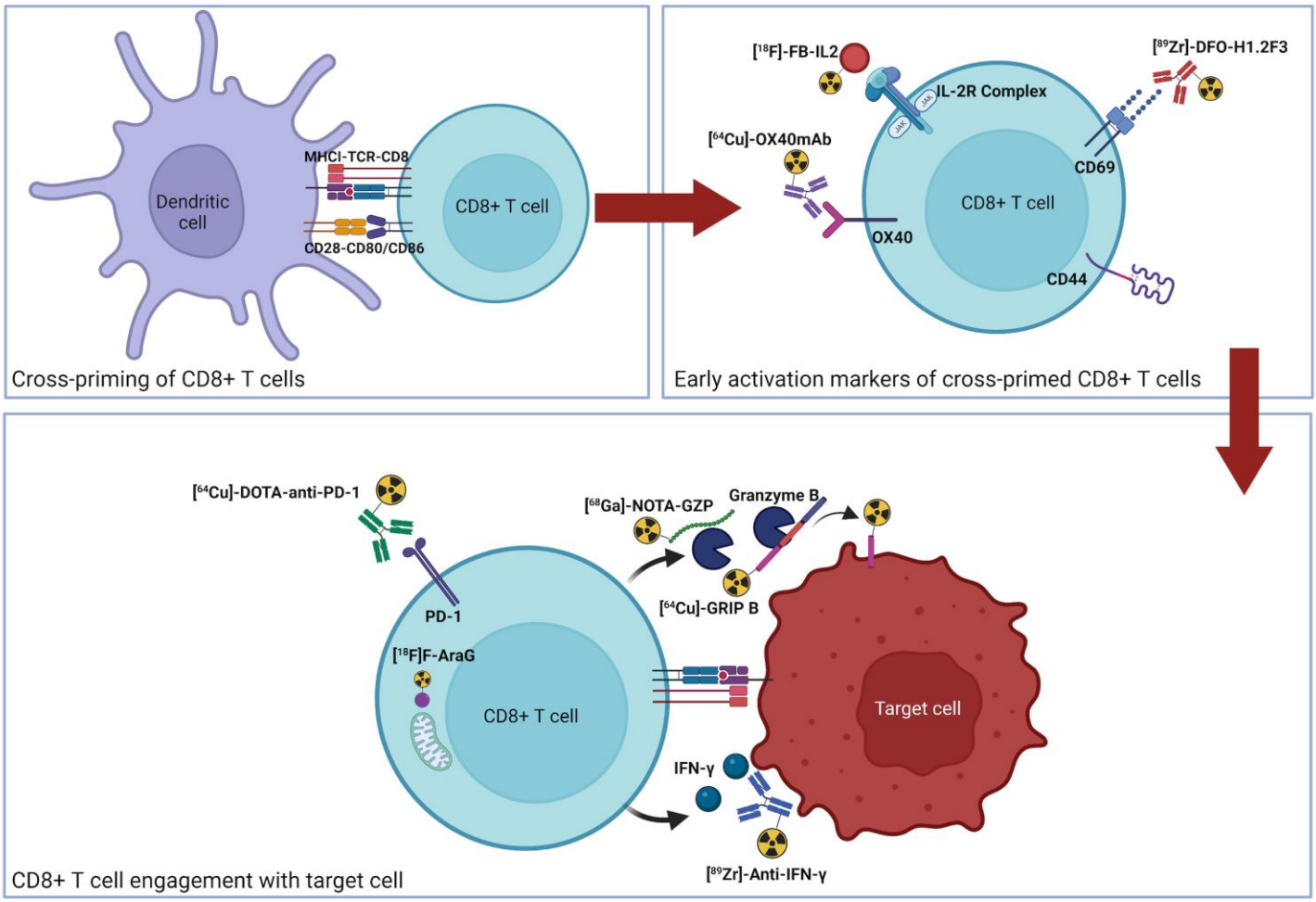

Figure 1 – Graphical representation of T cell activation and corresponding PET imaging agents. Upper left – Cross-priming of naïve T cells leads to activation (Upper right). Upon recognition of cognate antigen, functional release of granzyme B and interferon-gamma is induced.

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