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Visualizing T cell responses: The T cell PET imaging toolbox

Author list: Chao Li[1,2], Chaozhe Han[1,2], Shao Duan[1,2], Ping Li[3], Israt S. Alam[4,#], Zunyu Xiao[1,4,#]

Affiliation:
1. Harbin Medical University, Harbin, 150086, China
2. Department of Orthopedics, the Second Affiliated Hospital of Harbin Medical University, Harbin, 150086, China
3. Department of Radiology and Nuclear Medicine, the Second Affiliated Hospital of Harbin Medical University, Harbin, 150086, China
4. Molecular Imaging Program at Stanford (MIPS), Department of Radiology, Stanford University School of Medicine, Stanford, CA, 94305, USA

To whom correspondence should be addressed

First author:
Chao Li, M.D., Ph.D.
Harbin Medical University, Harbin, 150086, China
Tel:+(86)13895728281
Email: h05391@hrbmu.edu.cn

Co-corresponding authors:
Zunyu Xiao, M.D., Ph.D.
Harbin Medical University, Harbin, 150086, China
Tel:+(86)18504667955
Email: xiao_zunyu@hrbmu.edu.cn

Israt S. Alam, Ph.D,
Department of Radiology, 1201 Welch Rd, Stanford, CA 94305, USA
Tel:+(1) 650-497-3418
Email: israt@stanford.edu

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ABSTRACT

T lymphocytes are key mediators of the adaptive immune response. Inappropriate or imbalanced T cell responses are underlying factors in cancer progression, allergy and other immune disorders. Monitoring the spatiotemporal dynamics of T cells and their functional status has the potential to provide unique biological insights in health and disease. Non-invasive positron emission tomography (PET) imaging represents an ideal whole-body modality for achieving this goal. With the appropriate PET imaging probes, T cell dynamics can be monitored *in vivo*, with high specificity and sensitivity. Herein, we provide a comprehensive overview of the applications of this state-of-the-art T cell PET imaging toolbox, and the potential it has to improve the clinical management of cancer immunotherapy and T cell- driven diseases. We also discuss future directions and prospects for clinical translation.

INTRODUCTION

T lymphocytes play a central role in the adaptive immune response. The interplay between T cells and extracellular factors maintains a careful balance between activation, proliferation, survival and inhibition. Dysregulation of T cell responses can contribute to cancer progression, allergy and immune disorders (*1,2*). Non-invasive PET imaging represents an ideal approach for visualizing T cell dynamics *in vivo*, which could improve our understanding of their role in disease pathogenesis. This has motivated the development and evaluation of numerous T cell imaging probes in both pre-clinical and clinical settings (Fig 1). The T cell PET imaging toolbox consists of a diverse set of approaches that includes: i) direct labeling of cells *in vitro*; ii) proteins and peptides targeting endogenous T cell surface and secreted biomarkers; iii) small molecule metabolic tracers and iv) engineering cells to express PET reporter genes (*3*). In direct cell labeling, immune cells are incubated with radiolabels *ex vivo* before adoptive transfer into a living subject for subsequent imaging. Although this is a relatively straightforward methodology for T cell tracking, its wider clinical use to date has been limited. Incorporation of radionuclides can cause toxicities, such as radiolysis, and can adversely impact T cell function. The radiolabel itself becomes diluted as cells divide and proliferate *in vivo*, reducing the utility of this approach for longitudinal imaging (*4*). Given the drawbacks of this approach, we will focus our review on the alternative approaches mentioned above, which are summarized in Table 1.

UTILITY OF T CELL PET IMAGING FOR DIAGNOSIS OF ALLOGENIC AND AUTOIMMUNE DISEASES

Inappropriate T cell activation and trafficking are seen in a range of pathologies, including acute graft versus host disease (aGVHD) in the allogenic transplant setting, and autoimmune diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). It is well documented that early diagnosis and timely therapeutic intervention in these scenarios can lead to better outcomes and minimize organ damage (*5,6*), illustrating the urgent need to develop more reliable diagnostic tools. T cell PET imaging represents an ideal approach for achieving non-invasive, early detection of disease, prior to the onset of clinical symptoms.

T cell imaging with 2′-Deoxy-2′-^{18}F-Fluoro-9-β-D-Arabinofuranosylguanine (^{18}F-AraG) has successfully detected activated T cells in secondary lymphoid organs at both early and late stages of disease in a murine aGVHD model (*7*) (Fig 2A) and is now being evaluated clinically in hematopoietic stem cell transplant (HSCT) recipients (NCT03367962). Imaging of the T cell surface lineage marker CD3 successfully visualized the total T cell compartment *in vivo* in a humanized GVHD model (*8*). However, downregulation of CD3 expression during T cell activation poses a limitation to this approach. A ^{64}Cu-labeled OX40 monoclonal antibody (mAb) demonstrated excellent diagnostic potential in a murine aGVHD model, detecting T cell activation early in disease and prior to presentation of overt clinical symptoms (*9*) (Fig 2B). However, the agonist OX40 mAb clone employed in this model also accelerated aGVHD lethality, even when administered at the

relatively low mass doses used for imaging. These findings highlight the importance of testing for biological perturbations and overall safety prior to clinical translation. Given the utility of OX40 as a biomarker of GVHD, using an OX40 antagonist clone may be a safer imaging approach for GVHD and other indications where T cell activation may be deleterious.

Imaging activated T cells also represents a promising strategy for early RA diagnosis. Although 2-deoxy-2-^{18}F-fluoro-D-glucose (^{18}F-FDG) had been tested in pre-clinical and clinical RA studies, the glucose metabolism pathway is shared by multiple tissues, which results in a high rate of false-positives, especially when the region of interest is adjacent to metabolically active tissues (*10*). Evaluation of ^{18}F-AraG in a preclinical adjuvant-induced arthritis model (*11*) (Fig 2C) showed significantly higher accumulation of ^{18}F-AraG in RA-affected paws in both the acute and chronic phases of disease, demonstrating its potential utility for RA diagnosis. Due to the enormous challenge of developing small molecule binders, antibody-based PET tracers known as immunoPET probes are favored as a reliable tool for immune cell imaging given their robust and facile radiolabeling protocols. Immuno-PET tracers targeting T cell surface or secreted biomarkers should also warrant evaluation for RA detection in future studies (*12*).

For IBD detection, an anti-CD4 mAb labeled with ^{111}In was previously evaluated for SPECT imaging of CD4+ T cells. More recently, an ^{89}Zr labeled CD4-specific GK1.5 cys-diabody was developed and evaluated in a mouse model of dextran sulfate sodium (DSS)-induced colitis (*13*) (Fig 2D). Increased accumulation of ^{89}Zr-maleimide-deferoxamine (malDFO)-GK1.5 cDb was detected in the distal colon of colitis mice and further corroborated by increased CD4+ immunohistochemistry staining, demonstrating the sensitivity of this probe for CD4+ T cells *in vivo*. Whether PET quantification correlated with disease severity was not discussed in this study, so further evaluation is required to explore the feasibility of this approach for determining disease severity. Additionally, naïve CD4+ T cells residing in the gut may raise the background signal in CD4-targeted imaging. Since activated T cells are the true mediators of IBD pathogenesis, PET tracers specific to activated T cells may be preferred.

T CELL PET IMAGING FOR PREDICTING/MONITORING IMMUNE RESPONSE TO CANCER IMMUNOTHERAPIES

Immuno-oncology has rapidly evolved over the last decade (*14*) with numerous clinical trials demonstrating the unprecedented success of cancer immunotherapies in treating late-stage and recurrent malignancies, including relapsed or refractory B-cell malignancies, melanoma, bladder cancer and non-small cell lung carcinoma (*15-18*). Despite the meteoric rise of these innovative therapies, only a small fraction of patients exhibits durable responses, highlighting the urgent need for reliable tools to monitor and predict therapeutic response (*19*). Anatomic imaging often lacks sensitivity and does not collect direct molecular or functional information about the T cell compartment. Biopsies are invasive, risk iatrogenic complications, fail to capture whole body information, and do not assess tumor heterogeneity (*20,21*). Longitudinal PET imaging and

quantitative analysis of T cell distribution in tumors and/or secondary lymphoid organs pre- and post-treatment may correlate with therapeutic response, helping clinicians to identify patients most likely to benefit from treatment (*22*). With this motivation, a wide range of T cell specific PET tracers have been developed and evaluated in preclinical studies and clinical trials.

Endogenous T cell biomarkers include those specifically expressed on the cell surface, such as T cell lineage markers (CD4, CD8); those secreted by certain T cell subsets, such as granzyme B; and activation markers, such as CD278 and CD134 (Fig 3A and 3B). A major advantage of using immunoPET probes is their high specificity and affinity towards their homologous target, generating high signal-to-noise ratios and high contrast images. Neutral binders without agonist/antagonistic functions have the potential to be widely applicable without concern of perturbative effects. However, due to their large size, intact antibodies (150KDa) can suffer from poor penetration into target tissues and slow clearance. Additionally, the Fc regions of whole antibodies can bind non-specifically to Fc receptors on other cells, such as macrophages and natural killer cells (*23*). To overcome these challenges, antibody fragments such as the minibody, diabody, and nanobody formats and other engineered protein scaffolds have been evaluated as radiotracers (*24,25*). These smaller vectors are likely to exhibit better tissue penetration and faster clearance and are more suited to radiolabeling with PET isotopes with shorter half-lives, making same-day imaging more feasible (*26*).

Radiolabeled small molecules are usually designed to target specific intracellular metabolic pathways, such as carbohydrate metabolism and DNA synthesis. The most widely used clinical PET tracer for cancer diagnosis and staging, ^{18}F-FDG, has also been explored for monitoring immune responses (*27*). Since the glycolytic pathway is shared by both activated immune cells and cancer cells, its specificity for the interrogation of T cell responses is low. Rapidly proliferating T cells rely heavily on the nucleoside salvage pathway for DNA synthesis. Deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) are key enzymes that regulate this pathway, motivating the development of radiolabeled small molecules targeting each. The first dCK-targeting PET tracer, 1-2'-deoxy-2'-^{18}F-fluoroarabinofuranosyl cytosine (^{18}F-FAC), was able to distinguish proliferating CD8+ T cells from naïve T cells (*28*), but rapid catabolism *in vivo* impeded its clinical translation. Another dCK tracer with improved metabolic stability, 2-chloro-2'-deoxy-2'-^{18}F-fluoro-9-b-D-arabinofuranosyl-adenine (^{18}F-CFA), was subsequently developed and evaluated in glioblastoma patients treated with PD-1 blockade. Increased ^{18}F-CFA accumulation was observed in secondary lymphoid organs and tumor tissue, and the PET signal in the latter strongly correlated with the concentration of tumor-infiltrating lymphocytes (TILs) (*29*) (Fig 3C). ^{18}F-AraG, a guanosine analog with high specificity for dGK, was successfully used to detect activated T cells induced by anti-PD-1 therapy in mouse models, enabling early prediction of therapeutic response (*30*). Given its favorable imaging characteristics, several clinical trials are currently evaluating the feasibility of monitoring T cell responses with ^{18}F-AraG (NCT04186988, NCT04726215).

Engineering cells to express reporter genes encoding proteins that can be detected with complementary PET tracers is a promising approach suited to tracking adoptively transferred T cells (*31*). The most extensively evaluated PET reporter gene is the herpes simplex virus type 1 thymidine kinase (*HSV1-tk*) and its mutant version *HSV1-sr39tk*. In a pilot clinical study, $CD8^+$ cytotoxic T lymphocytes were engineered to express both *HSV1-tk* and interleukin-13 (IL-13) zetakine chimeric antigen receptor (CAR), and then adoptively transferred into recurrent high-grade GBM patients. ^{18}F-FHBG was subsequently administered to monitor the trafficking and proliferation of the engineered cytotoxic T lymphocytes in the brain (*32*) (Fig 3D). This landmark study demonstrated the feasibility of using the *HSV1-tk* system to clinically track engineered immune cells. The *HSV1-sr39tk* system can also act as a suicide gene upon treatment with the prodrug ganciclovir, enabling ablation of CAR-T cells for safe control of potential toxicities (*33*). Human PET reporter genes including sodium iodide symporter, norepinephrine transporter, and somatostatin receptor 2 (SSTR2) have also been developed to overcome the potential immunogenicity of *HSV1-tk* and evaluated for CAR-T cell imaging. However, due to their endogenous expression, as well as the internalization of hNET and SSTR2, their application has been limited (*34*). Recently, a highly promising PSMA/^{18}F-DCFPyL reporter system was evaluated for CD19+ CAR-T cell imaging (*35*). To specifically prevent the internalization of PSMA, human CD19-targeted CAR-T cells were transduced with a N-terminally modified variant--tPSMA$^{(N9del)}$. ^{18}F-DCFPyL, a PSMA-specific PET tracer, subsequently allowed detection of these cells with high sensitivity and specificity *in vivo*. Importantly, quantification of the ^{18}F-DCFPyL PET signal from CD19+ CAR-T cells in these tumors led to more accurate assessment of therapeutic response when compared to the measurement of CAR-T cells in peripheral blood.

CONCLUDING REMARKS

Herein, we reviewed state-of-the-art PET imaging approaches for *in vivo* T cell visualization and their applications in T-cell mediated diseases and cancer immunotherapy. As discussed, non-invasive PET imaging has the potential to provide comprehensive information about the distribution and abundance of immune cell subsets in real-time (*36,37*). With these imaging approaches, physicians and research scientists can be better informed about the adaptive immune response and T cell behaviors in different settings, which would greatly facilitate personalized medicine.

For novel T cell PET tracers to translate from bench to bedside, lessons learnt in the clinic should be brought back to the bench to facilitate probe optimization. Although many T cell-targeted PET tracers have yielded promising preclinical results, only a few candidates are currently under clinical investigation and are yet to be approved for routine clinical use. This imbalance may be attributed to redundant preclinical studies. To streamline PET tracer development, unsupervised analysis of prospective biomarkers using both preclinical and clinical 'omic' datasets would help identify the most optimal target for a given application. In a recent study, we utilized RNAseq to compare several candidate T cell activation markers expressed on activated CD19+ human CAR-T cells,

and ICOS was identified as a promising imaging biomarker that was specifically and consistently upregulated; subsequent PET imaging was focused accordingly (*38*). In the future, advanced techniques such as slide seq and CODEX should be incorporated to identify the most relevant biomarker for T cell imaging in a given scenario (*39,40*). Another reason for the imbalance is the slow adoption of novel molecular imaging techniques in the clinic. In conventional clinical workflow, anatomic imaging remains the gold standard for therapeutic response evaluation and monitoring; novel immunotherapy adjuvants are also approved by the FDA under certain criteria. Future clinical studies need to demonstrate that these PET approaches provide actionable insights for improved patient management. In a recent landmark study, PD-L1 imaging in cancer patients indicated that non-invasive immuno-PET outperformed RNA and immunohistochemistry-based biomarker measurements from tissue biopsies in the selection of patients most likely to benefit from PD-L1 blockade (*41*). These are compelling reasons to accelerate the clinical evaluation of new probes and the selection of the most promising candidates for further consideration.

To optimize the T cell PET imaging toolbox, we should carefully compare different biomarkers, pathways, and probe formats. For example, intact antibodies exhibit higher binding affinity compared to small molecules, but lower tissue penetration. While the reporter gene strategy has the advantage of low background, immunogenicity usually limits its wider applications. Thus, among all the potential candidates, we should consider their affinity, specificity, sensitivity, immunogenicity, tissue penetration and clearance, and then select the best candidate for further investigation. Moreover, with current imaging probes, although we can visualize the *in vivo* distribution of distinct T cell populations, we are unable to delineate their antigen-specificity. A recent published study reported a novel engineered PET imaging probe—^{64}Cu labeled synTac (synapse for T cell activation), which was able to distinguish antigen-specific CD8+ T cells from bystander CD8+ T cells (*42*). This powerful approach could allow the detection of cytotoxic CD8^{+} T cells specific for invading pathogens or tumor cells, which would provide more precise assessment of cancer immunotherapy efficacy or disease severity. Finally, the groundbreaking total-body PET will substantially improve sensitivity, enabling enhanced detection of T cells, while also lowering the radioactive dose typically required to acquire high-resolution images, enabling safer repeat imaging (*43*).

In summary, the T cell PET imaging toolbox has great potential for improving clinical management of cancer immunotherapy and diagnosis of T cell-driven immunopathology. Further optimization of these approaches is still needed to overcome limitations in their specificity, sensitivity and safety. We hope that our discussion will highlight the utility of the T cell PET imaging toolbox to both researchers and physicians and encourage the translation and wider adoption of these tools in clinical practice.

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KEY POINTS

QUESTION: How could these state-of-the-art T cell PET imaging approaches improve clinical management of T cell immunopathology and cancer immunotherapy?

PERTINENT FINDINGS: As described in the manuscript, non-invasive T cell imaging technology is able to visualize the distribution and functional status of T cell subsets *in vivo* in real-time, which could help us better understand T cell behavior in disease or in response to therapy.

IMPLICATIONS FOR PATIENT CARE: The T cell PET imaging toolbox has the potential to aid physicians in the accurate diagnosis of T cell-driven diseases and predict treatment response to cancer immunotherapy at early stages. This will aid in clinical decision-making and help to optimize and streamline drug development.

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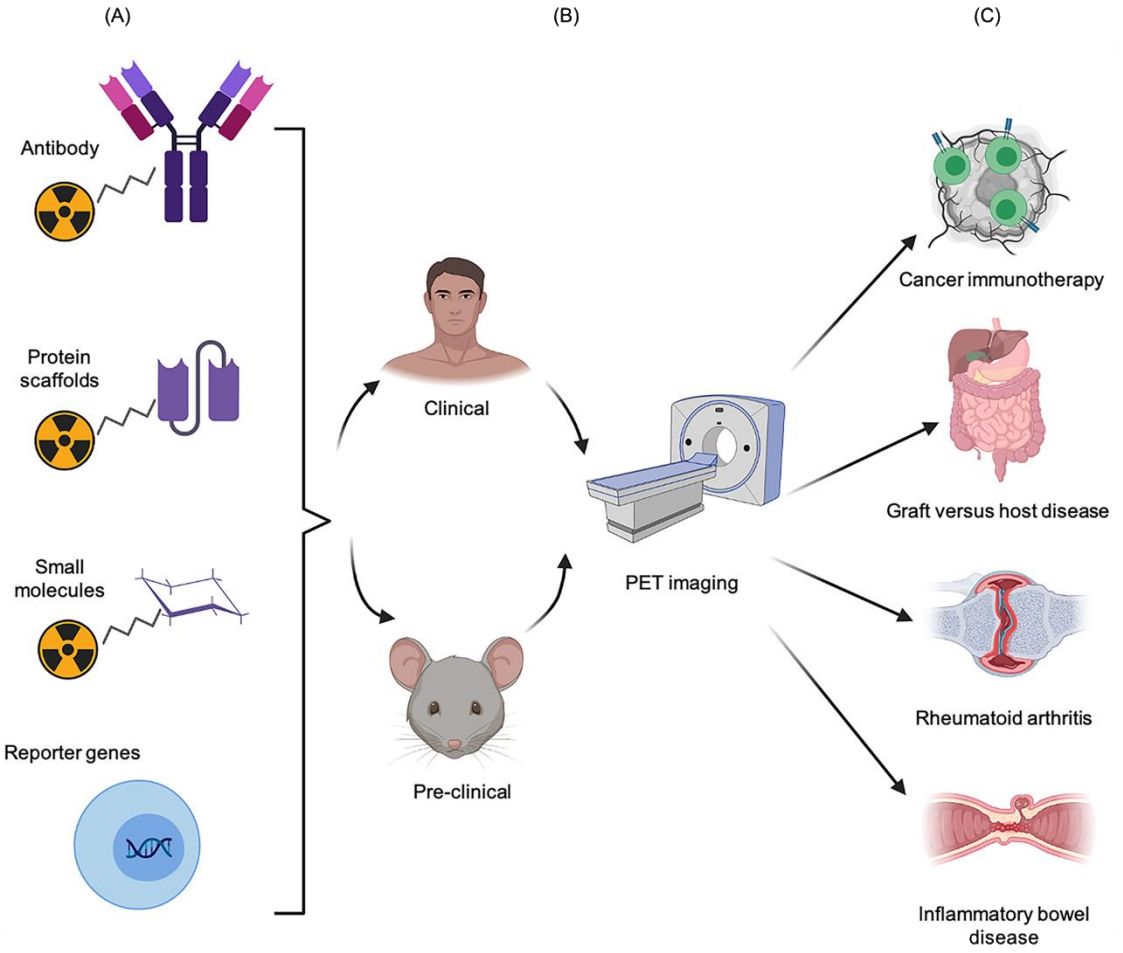

Figure 1: Approaches for PET imaging of T cells. The T cell PET imaging toolbox had expanded rapidly over the last decade. (A) Numerous T cell specific PET tracers have been developed, including radiolabeled antibodies and antibody fragments, protein scaffolds, small molecules and those complimentary to reporter genes that can be used to track engineered T cells. (B) These approaches have been evaluated in both preclinical and clinical studies. (C) Noninvasive PET imaging of T cells, has the potential to be highly useful, allowing preclinical researchers and clinicians to predict or monitor therapeutic response to cancer immunotherapy, and enable early diagnosis of inflammatory diseases, like graft versus host disease (GVHD), rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) for timely and effective intervention.

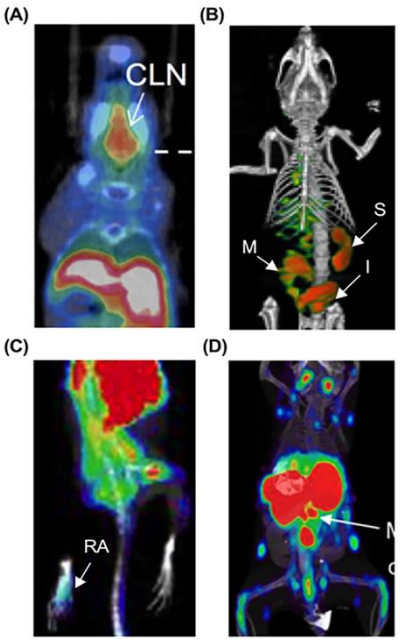

Figure 2: PET imaging of T cells for early detection of inflammatory diseases. (A) ^{18}F-AraG enables detection of T cell activation in the cervical lymph nodes (CLN) during acute graft versus host disease (aGVHD)(*7*); (B) OX40 immunoPET allows early diagnosis of aGVHD, prior to overt clinical symptoms (M; mesenteric lymph node, S; spleen, I; intestine) (*9*); (C) ^{18}F-AraG imaging detects rheumatoid arthritis (RA) in a mouse model of adjuvant-induced arthritis (*11*); (D) ^{89}Zr labeled CD4 targeting cys-diabody allows detection of inflammatory bowel disease (IBD), (white arrow indicating the mesenteric lymph nodes) (*13*).

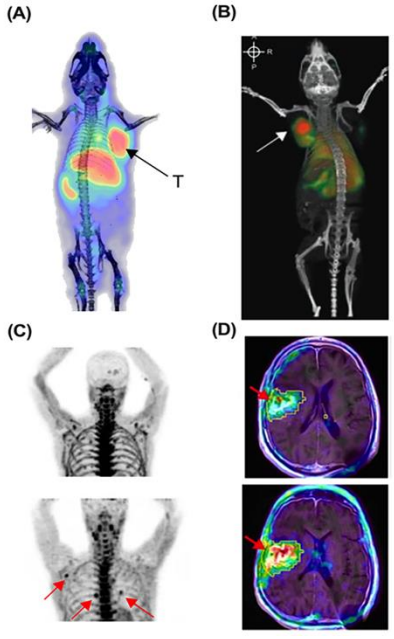

Figure 3: PET imaging of T cells enables monitoring of treatment response in cancer immunotherapy. (A) ICOS immunoPET with ^{89}Zr-DFO-ICOS mAb visualizes and predicts therapeutic response in a mouse model of Lewis lung cancer treated intratumorally with STING agonist and PD-1 blockade (T; treated tumor) (*37*). (B) OX40 immunoPET imaging enables visualization of activated T cells in A20 tumor bearing mouse treated intratumorally with CpG (white arrow represents CpG treated tumor) (*44*). (C) Elevated uptake of ^{18}F-CFA was observed in several lymph nodes (red arrow) of a recurrent glioblastoma (GBM) patient post-treatment with tumor lysate-pulsed DCVax and PD-1 blockade (lower panel), compared with pre-treatment (upper panel) (*29*); (D) ^{18}F-FHBG enables visualization of HSV-TK1 reporter gene modified IL-13 CAR-T cells in recurrent GBM patients (upper, pre-CAR-T infusion; lower, post-CAR-T infusion) (*32*).

Table 1: Candidate biomarkers for PET imaging of T cell responses (PET tracers targeting the biomarkers listed here are also discussed in references 3, 22 and 35).

Target	Type	Stage	Application
OX40	Surface biomarker	Preclinical	TILs, GVHD
CTLA-4	Surface biomarker	Preclinical	TILs, GVHD
CD3	Surface biomarker	Preclinical	TILs
CD4	Surface biomarker	Preclinical	TILs, IBD, HSCT, Lymphoid organs
CD8	Surface biomarker	Preclinical/Clinical	TILs, Lymphoid organs
ICOS	Surface biomarker	Preclinical	TILs, CAR-T
PD-1	Surface biomarker	Preclinical/Clinical	TILs
IL-2R	Surface biomarker	Preclinical/Clinical	TILs, ONNV, Lymphoid organs
TCRmu	Surface biomarker	Preclinical	CAR-T
VLA4	Surface biomarker	Preclinical	Tuberculosis
Granzyme B	Secreted biomarker	Preclinical	TILs
dCK	Intracellular enzyme	Preclinical/Clinical	TILs, Autoimmune hepatitis
dGK	Intracellular enzyme	Preclinical/Clinical	GVHD, TILs, RA
HSV1-tk	Reporter gene/encoded protein size (46KDa)	Preclinical/Clinical	CAR-T
HSV1-sr39tk	Reporter gene/encoded protein size (42KDa)	Preclinical	RA
PSMA	Reporter gene/encoded protein size (100KDa)	Preclinical	CAR-T
NET	Reporter gene/encoded protein size (69KDa)	Preclinical	TILs
SSTR2	Reporter gene/encoded protein size (41KDa)	Preclinical	CAR-T
DHFR	Reporter gene/encoded protein size (18KDa)	Preclinical	CAR-T
NIS	Reporter gene/encoded protein size (69KDa)	Preclinical	CAR-T
2D12.5/G54C	Reporter gene/encoded protein size (52KDa)	Preclinical	CAR-T

Abbreviations: tumor infiltrating lymphocytes (TILs); graft versus host disease (GVHD); cytotoxic T-lymphocyte antigen-4 (CTLA-4); inflammatory bowel disease (IBD); hematopoietic stem cell transplantation (HSCT); interleukin-2 receptors (IL-2R); O'nyong-nyong virus (ONNV); murine T cell receptor beta domain (TCRmu); very late antigen-4 (VLA4); deoxycytidine kinase (dCK); deoxyguanosine kinase (dGK); rheumatoid arthritis (RA); herpes simplex virus type 1 thymidine kinase (HSV1-tk); prostate-specific membrane antigen (PSMA); norepinephrine transporter (NET); somatostatin receptor 2 (SSTR2); dihydrofolate reductase enzyme (DHFR); sodium iodide symporter (NIS); Anti-lanthanoid-DOTA antibody (2D12.5/G54C)

Graphical abstract