Detection of Graft Versus Host Disease with [^{18}F]F-AraG, a positron emission tomography tracer for activated T cells

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Arm 1) Patients highly suspected to have acute Graft versus Host Disease (blue arrow applies)
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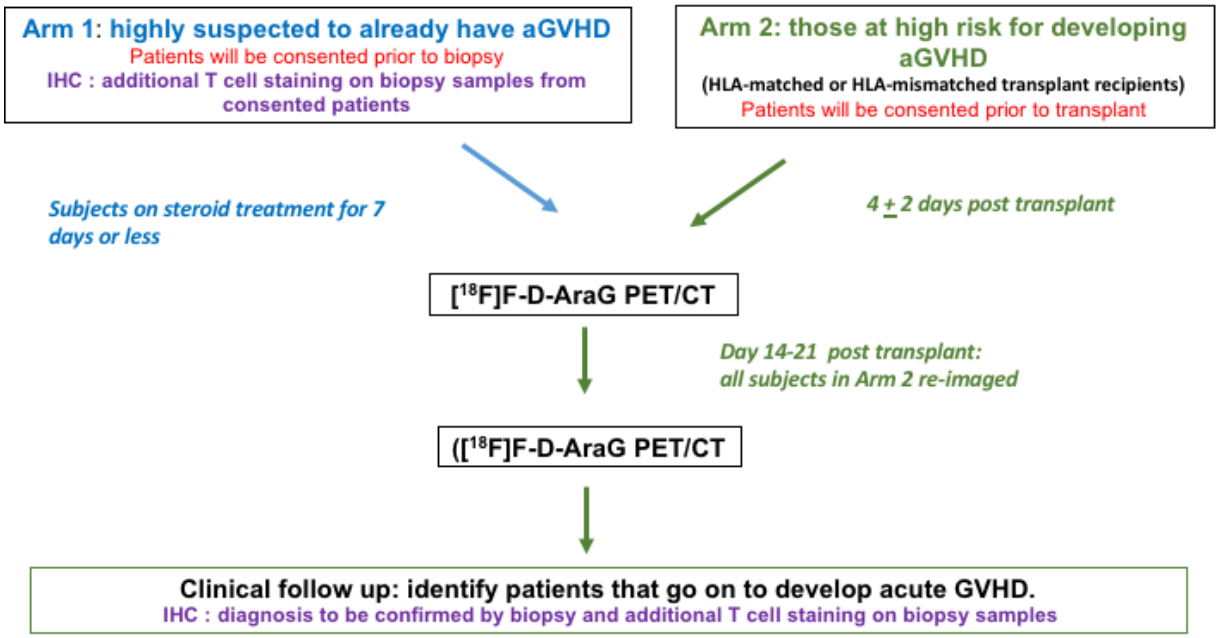
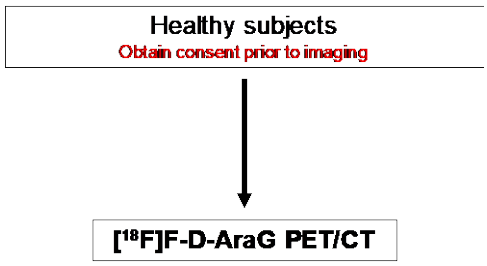


SCHEMA 2: Healthy subjects



List of Abbreviations and definitions of terms

aGVHD	Acute Graft versus host disease
BMT	Bone marrow transplant
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CDA	Cytidine deaminase
[^{18}F]-CFA	[^{18}F]Clofarabine; 2-chloro-2'-deoxy-2'-[(18)F]fluoro-9-β-d-arabinofuranosyl-adenine
CMC	Chemistry, Manufacturing and Controls
dCK	Deoxycytidine kinase
dGK	Deoxyguanosine kinase
[2]-F-D-AraC	1-(2′-deoxy-2′-[18F]fluoro-β-D-arabinofuranosyl)cytosine
[^{18}F]F-AraG	2'-deoxy-2'-[18F]fluoro-9- β -D-arabinofuranosyl guanine
F-18, ^{18}F	Fluorine 18
GI	Gastrointestinal
HCT	Hematopoietic cell transplant
HLA	Human Leukocyte Antigen
F-18, ^{18}F	Fluorine 18
FDA	Food and Drug Administration
FDG	2-deoxy-2-(^{18}F)fluoro-D-glucose
IND	Investigational new drug
IRB	Institutional Review Board
IV	Intravenous
MRI	Magnetic resonance imaging
PET/CT	Positron emission tomography – computed tomography
SUV	Standardized Uptake Value
ROI	Region of interest
IHC	Immunohistochemistry
SUV	Standard uptake value

1. OBJECTIVES

We have developed a Fluorine-18 PET tracer 2'-deoxy-2'-[18F]fluoro-9-β-D-arabinofuranosyl guanine ([^{18}F]F-AraG), based on a drug called AraG that is known to be selective towards T cells. Our initial findings with this tracer have shown similar mechanism(s) of uptake as AraG and preferential accumulation in activated T cells. The selected positron emission tomography (PET) tracer ([^{18}F]F-AraG has shown promise in preclinical studies in a mouse model of acute Graft versus Host Disease (aGVHD). We have shown the ability to use [^{18}F]F-AraG PET imaging to quantitatively visualize locations of T cell accumulation during and early late-stage GVHD in this model. Moreover the tracer has already undergone the first in human studies in healthy human volunteers where it showed a favorable biodistribution, clearance profile and *in vivo* stability. With promising preclinical and clinical data, the tracer warrants further characterization in aGVHD patients where there is a pressing and unmet need for early detection of the disease. We hypothesize that *in vivo* molecular imaging can be safely utilized to identify sites of GVHD and therefore propose the following specific aims:

1.1. Primary objective

Evaluate the feasibility of [^{18}F]F-AraG PET/CT scanning in patients with graft versus host disease

We hope to determine whether [^{18}F]F-AraG, demonstrates good uptake in regions of GVHD as confirmed by GI biopsy. The performance of [^{18}F]F-AraG PET/CT will be assessed on a per-lesion based analysis in a cohort of up to 25 GVHD patients. The tracer's biodistribution, and dosimetry will be studied in all aGVHD patients. All critical data shall be reported to the FDA.

The ultimate goal of this study is to investigate the predictive power of [^{18}F]F-AraG and whether it can be used to diagnose GVHD at its earliest stages to allow for more timely treatments to be applied in the future, to monitor treatment efficacy, and to hopefully improve the outcome of patient's with this devastating and frequent complication of allogeneic hematopoietic cell transplantation.

1.2. Secondary Objectives

The tracer's pharmacokinetics, biodistribution, dosimetry and toxicity will also be studied in GVHD patients having been previously studied in healthy volunteers. Further, extended characterization of tracer biodistribution will be carried out in a small number of healthy subjects.

2. BACKGROUND

2.1. Acute Graft versus Host Disease

Allogeneic hematopoietic cell transplantation (HCT) is an increasingly used curative option for many hematological malignancies, and other life-threatening genetic and hematological diseases [3, 4]. HCT is capable of destroying residual tumor cells that persist after radiation treatment or chemotherapy, an effect called the graft-versus-tumor reaction. However, the main complications of HCT are infections and acute graft-versus-host disease (aGVHD). Whilst infections can be treated successfully, patients with aGVHD by the time of diagnosis are less responsive to treatments. Thus a principle challenge for more successful implementation of HCT has been avoidance or better management of aGVHD. Whilst considerable medical advancements have been made in the last two decades, the diagnosis and treatment of this devastating disease has only marginally improved during that time.

This immunological disorder arises when donor T cells react to “foreign” host human leukocyte antigens (HLA), causing a wide spectrum of host tissue injuries in the gastrointestinal (GI) tract, liver, skin, and lungs [3, 5]. Two phases of the disease have been described; first the migration of CD4+ and CD8+ donor T cells to secondary lymphoid organs where they are activated by innate immune cells that become reactive by the conditioning therapy for HCT[6]. The second phase is characterized by massive proliferation of these alloreactive T cells which then home to various organs (GI tract, liver and skin are tissues that are commonly affected) and start to attack host cells via direct cytolysis or by cytokine production causing a wide range of symptoms [3].

Incidence rates of GVHD are high and depend on the degree of HLA histocompatibility, the prophylactic regimen used, patient age, and the number of T cells in the graft [5]. GVHD can affect 20-50% of patients receiving related donor transplants [7-10] and upwards of 70% of patients receiving HLA-matched unrelated donor transplants [7, 10]. The overall grade of GVHD is known to have a major effect on patient outcome, with grade I or II cases associated with little morbidity and no mortality, and grade IV cases associated with close to 100% mortality [5]. Hence, GVHD is a leading cause of morbidity and mortality of HCT therapy and improved ways of managing this complication could lead to more widespread use of this effective therapeutic paradigm for hematological malignancies, autoimmune diseases, or organ transplant rejection [11, 12]. Since long term survival of grade III-IV aGVHD is a dismal 10-20% [13], it is important to detect patients who will progress to high-grade aGVHD earlier in their course.

Unfortunately, early definitive GVHD diagnosis is often difficult due to its non-specific symptoms and numerous differential diagnoses (e.g., drug toxicity, infections, and side effects from conditioning regimen) [14, 15]. GVHD is typically diagnosed at late stages when overt symptoms such as skin, GI, or liver problems become apparent, and is often one of exclusion, delaying the onset and effectiveness of potentially life-saving prophylactic interventions [16, 17]. Moreover, invasive endoscopic guided biopsy (often from liver or GI tract) and subsequent histology are still the state-of-the-art way to confirm a GVHD diagnosis [7]. These can be challenging to perform due to bleeding and critical illness associated with HCT. Dramatic differences in estimated response rate at a particular disease stage have been reported including 63% to 95% for grade II, 17% to 30% for grade III, and 0% to 6% for grade IV patients [18]. The severity of GVHD is also difficult to quantify, with staging relying on the assessment of the clinical changes seen in the skin, GI tract, liver, and performance status [5, 16, 17]. Therefore, **non-invasive methods that can effectively track alloreactive donor T cell dynamics, stratify the risk of aGVHD prior to overt symptoms and thus diagnose GVHD at earlier stages are urgently needed but entirely lacking**.

Present day clinical management

Several imaging techniques have shown promise for definitive diagnosis of GVHD, particularly GI manifestations of the disease, including magnetic resonance imaging (MRI), computed tomography (CT), high-resolution transabdominal ultrasound and color Doppler ultrasound [19, 20]. These techniques primarily focus on structural manifestations such as detection of edema and thickening of the intestinal wall that become apparent during advanced stages of GVHD, and are therefore likely limited for early disease detection. Molecular imaging of aGVHD, which could yield a more sensitive detection, has been very limited focusing mostly on PET imaging using [^{18}F]FDG [21].

[^{18}F]FDG PET has shown promise for imaging intestinal inflammation associated with GVHD in both mouse models and patients with early clinical symptoms, even allowing prediction and

monitoring of therapy response in these patients [21]. However, the utility of [^{18}F]FDG PET imaging for early detection prior to clinical symptoms is not known and the non-specific nature of [^{18}F]FDG imaging for a variety of activated immune cell types should enable a selective advantage of [^{18}F]F-AraG PET imaging for this purpose.

Numerous candidate blood biomarkers are also under active investigation including soluble CD30 [22], regenerating islet-derived 3-alpha (REG3a) [23], microRNAs [24], amongst others [25, 26]. de Bock et al have also recently identified variations in a panel of proteomic blood biomarkers approximately 15 days before definitive GVHD diagnosis [27]. These candidates however require further validation prior to widespread clinical use and thus to date not a single biomarker has been approved for early aGVHD diagnosis [27-29]. Cellular blood biomarkers of GVHD have also been explored. Bauerlein et al. have shown that, during their migration to effector organs, peripheral blood detection of alloreactive T cells expressing homing receptors (a4b7 integrin and P-selectin ligand) can be used to define a diagnostic window for the improved treatment of GVHD [28]. Notably, if immunosuppressive (i.e., Rapamycin) treatment is administered at the time of earliest alloreactive T cell detection then progression to lethal GVHD was prevented [28]. We believe that whole body [^{18}F]F-AraG PET imaging that can quantify activated T cell accumulation during the initiation phase may provide a highly complimentary technique to these *in vitro* diagnostics to provide more definitive GVHD diagnosis and with spatial information. Hypothetically, it may even enable earlier diagnosis than a blood-based strategy by detection of T cells in priming sites prior to migration of cells or shedding of cellular proteins into the peripheral blood. Overall the continued evaluation of [^{18}F]F-AraG PET imaging for GVHD diagnosis and treatment monitoring could enable this technology to fulfill a critical need in the management of this terrible disease to allow more widespread success of HCT therapy.

2.2 Study Agent/Device/Imaging procedure

Positron emission tomography (PET) imaging has several key strengths including high sensitivity (10^{-9} to 10^{-12} M), quantitative capability, and limitless depth of penetration [30]. To date, few PET radiotracers have been developed for imaging the immune system and even fewer for specific imaging of distinct immune cell types such as activated T cells [2, 31, 32].

Of the PET tracers that have been developed for the purpose of imaging T cells, to date none have been specific for activated T cells [1, 2, 31, 33]. Of these only one is a small molecule enzyme substrate, the preferred type of molecular imaging probe due to inherent signal amplification from the catalytic trapping of many probes by a single enzyme. This probe is the radiotracer 1-(2'-deoxy-2'-[^{18}F]fluoroarabinofuranosyl cytosine ([^{18}F]F-AraC, also called [^{18}F]FAC). It was identified based on its higher uptake in proliferating versus resting CD8+ T cells and was proven useful for visualizing regions of immune activation *in vivo* through its specificity for deoxycytidine kinase (dCK) [31]. However, [^{18}F]F-AraC was found to rapidly undergo deamination in the blood by cytidine deaminase (CDA), confounding its utility for imaging dCK in species with high CDA levels such as humans [34]. Like drugs with a similar structure such as gemcitibine and cytarabine it solely targets dCK activity and is shown to be taken up into many immune cell types *in vivo*, with higher per cell uptake in myeloid (CD11b^{+}) and B (B220^{+}) cells compared to T cells [31] and also showed high uptake in dCK^{+} tumors. In fact the final applications of significantly improved [^{18}F]FAC derivatives and similar molecules such as [(^{18}F]Clofarabine; 2-chloro-2'-deoxy-2'-[(^{18})F]fluoro-9-β-d-arabinofuranosyl-adenine ([^{18}F]CFA), in the clinic has been the imaging of dCK+ tumors.

At Stanford, we have developed the radiotracer 2'-deoxy-2'-[^{18}F]fluoro-9-β-D-arabinofuranosyl guanine ([^{18}F]F-AraG) [35] (Fig. 1b). [^{18}F]F-AraG is an analog of arabinosyl guanine (AraG), a

compound identified to have specific cytotoxicity towards T-lymphocytes and T-lymphoblastoid cells versus other immune cell types (Fig 1a.) [36-38]. Nelarabine, the water-soluble AraG prodrug, is indicated for the treatment of patients with refractory/relapsed T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL) [39]. AraG enters cells via one of two nucleoside transporters followed by the initial and rate-limiting phosphorylation of AraG to AraG-monophosphate (AraGMP) by either cytosolic deoxycytidine kinase (dCK) or mitochondrial deoxyguanosine kinase (dGK) (Fig. 1c) [39, 40]. Continued phosphorylation to AraG-triphosphate (AraGTP) can inhibit DNA synthesis and induce cytotoxic effects specifically in T lymphocytes and T-lymphoblastoid cells [37, 39]. Thus [^{18}F]F-AraG PET imaging may provide a novel method for imaging of activated T cell dynamics in living subjects without toxicity since the tracer is given in very low (ng-μg) mass levels where no therapeutic effects are observed.

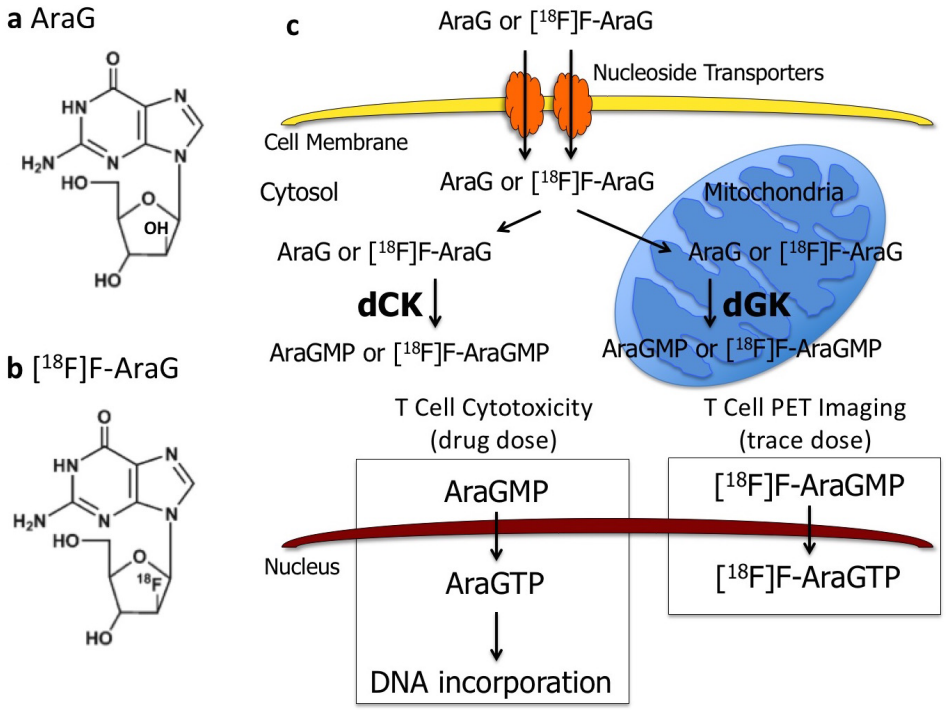
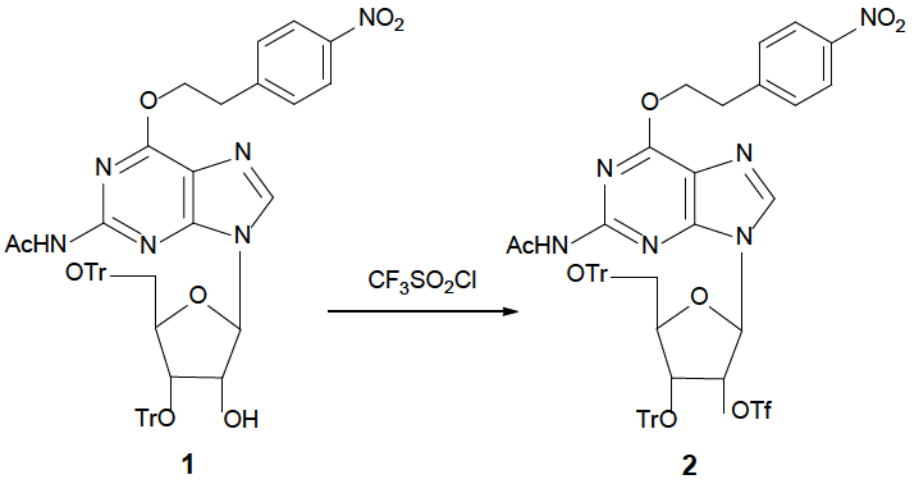


Fig 1. a) Chemical structure of arabinofuranosyl guanine (AraG) and b) 2'-deoxy-2'-[^{18}F]fluoro-9-β-D-arabinofuranosyl guanine ([^{18}F]F-AraG). c) Mechanism for cell entry and trapping. AraG and putatively the radiotracer [^{18}F]F-AraG, are transported into cells via two nucleoside transporters, followed by the rate-limiting conversion to AraGMP or [^{18}F]F-AraGMP via phosphorylation by either cytosolic deoxycytidine kinase (dCK) or mitochondrial deoxyguanosine kinase (dGK). Further phosphorylation leads to AraGTP or [^{18}F]F-AraGTP formation. Bottom left: AraG given at drug doses, once converted to AraGTP, can outcompete deoxyGTP to be incorporated into DNA during cellular mitosis, leading to chain termination and triggering of the apoptotic cascade in T cells. Bottom right: [^{18}F]F-AraG given at trace doses should not cause any pharmacological effects but its accumulation in activated T cells can be utilized to visualize these cells in PET imaging studies.

This imaging strategy could allow clinicians to utilize existing or novel interventions in a timely fashion and to more effectively monitor the outcome of such treatments. The ultimate utility of such efforts would be to prevent progression to higher-grade disease and improve patient outcome.

Tracer Synthesis

We have previously described and published the synthesis of [^{18}F]F-AraG via a direct fluorination method [35] also outlined in Scheme 1 and 2. Scheme 1 shows the synthesis of [^{18}F]F-AraG precursor; 2-N-Acetyl-6-O-((4-nitrophenyl) ethyl)-9-(3,5-di-O-trityl-2-trifyl-β-D-ribiofuranosyl) guanine (2). Treatment of 2′,5′- di-O-trityl guanosine derivative 1 with CF3SO2Cl/DMAP yields the [^{18}F]F-AraG precursor (2) with a 65% yield.



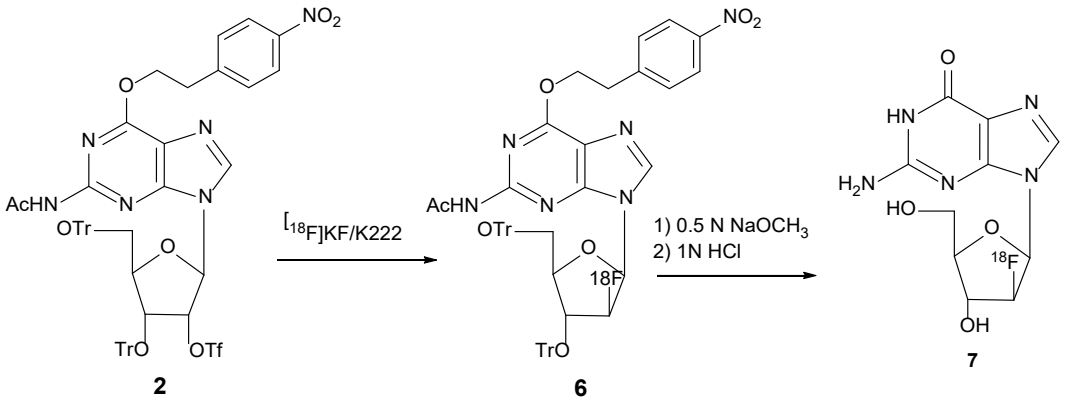
1 2

Tr: trityl, Tf: trifyl, Ac: acetyl

Scheme 1. Synthesis of 2-N-Acetyl-6-O-((4-nitrophenyl)ethyl)-9-(3,5-di-O-trityl-2-trifyl-□-D-ribofuranosyl)guanine(2), the [^{18}F]F-AraG precursor.

[^{18}F]-labeled guanosine derivative (6) (Scheme 2) is then prepared by nucleophilic displacement of triflate in 2 by [^{18}F]fluoride ion in DMSO at 85°C for 45 min. Purification of [^{18}F]6 via HPLC is required to avoid contamination of the final product [^{18}F]F-AraG (7) with the de-protected starting material (2) (AraG). [^{18}F]6 is smoothly hydrolyzed first by base (0.5 M NaOCH3) and then by acid (1 N HCl) to yield [^{18}F]F-AraG 7.

The chemical and radiochemical purity of the final product is confirmed by a reverse-phase analytical HPLC method, by co-injection of a cold F-AraG standard (Fig 2.). Purity levels achieved are often 95% or more.



Scheme 2. Synthesis of 2'-deoxy-2'-[^{18}F]fluoro-9-β-D-arabinofuranosylguanine 7 ([^{18}F]F-AraG).

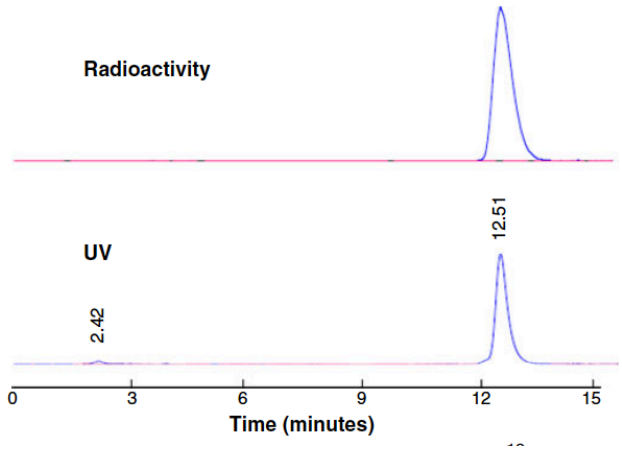


Fig 2. Confirmation of the purity of ([^{18}F]F-AraG synthesis by analytical HPLC profile of co-injection of [^{18}F]F-AraG with cold F-AraG standard (using 5% acetonitrile: 95% water; 1 mL/min, 254 nm, Phenomenex Gemini C18 column, 5µm, 4.6Å~ 250 mm).

To date, [^{18}F]F-AraG has been prepared for human imaging at UCSF according to the approved IND Chemistry Manufacturing and Control (CMC) procedures using the Neptis® perform PET synthesizer, a modification our published procedure [35]. The human images are shown further below (Fig 9). At Stanford we plan to use an automated preparation for [^{18}F]F-AraG for human imaging using the GE TRCERLAB FX_{FN} module. Clinical validation runs have been completed and approved by the FDA**.** Briefly, no carrier-added [^{18}F]fluoride ion was prepared on the Stanford University GE PET trace cyclotron, transferred to the GE TRCERLAB FX_{FN} module and captured on a QMA cartridge. The [^{18}F]fluoride ion was eluted with a solution of K_2CO_3 (3.5 mg) and kryptofix 2.2.2 (15 mg) in 1 mL CH_3CN/H_2O (9:1). The fluoride complex was azeotropically dried under vacuum at 65 °C. The residue is taken up in a solution of DMSO(1 mL) containing 5-7 mg of the precursor, 2-N-Acetyl-6-O-((4-nitrophenyl)ethyl)-9-(3,5-di-O-trityl-2-trifyl- β-D-ribofuranosyl)guanine. The solution was heated to 115°C for 45 minutes followed by the first de-protection for 10 minutes at 100 °C with 0.5 mL of 0.5 M NaOMe in methanol. Excess acid (0.5 mL of 1 M HCl) was added and heated to 95°C for 10 minutes to afford the second de-protection. The reaction mixture was cooled to 30 °C and diluted with 0.5 mL of 1N $NaHCO_3$ in 9.5 mL of water. The solution in the reactor was transferred through 4 C-18 plus cartridges. The Cartridges were backwashed to the reactor with 2 mL of methanol.

Methanol was removed under vacuum with the helium flow at 90 ºC for 13 min. After cooling the reactor to 30 ºC, water (2 mL) was added and the solution passed through 0.45 □□filter into a pre HPLC container. The resulting mixture was purified via HPLC (Phenomenex Luna 10µm C18, 10x250mm, 97% 50 mM ammonium acetate/3% CH3CN at 5mL/min, UV was monitored at 254nm and radioactivity detected with a built-in synthesizer detector). The product was collected between 12 and 15 minutes and diluted with 30 mL of water and passed through 4 C-18 plus cartridges. 18F-D-AraG product was removed from the cartridges by 1 mL ethanol followed by 10 mL of saline and passed through a 0.22µm sterilizing filter into a multidose vial. A sample was collected for quality testing. [^{18}F]F-AraG met all quality specifications required for human intravenous injection. The radiochemical yield was 2.5-4% (decay corrected, *n* = 12). The chemical and radiochemical purities of [^{18}F]F-AraG were greater than 95%. The radiosynthesis time was 120 minutes and specific activity was 3-8 Ci/µmol.Prior to injection in human subjects the prepared [^{18}F]F-AraG passed all quality specifications.

Tracer validation *in vitro*

We have carried out extensive *in vitro* and *in vivo* validation of [^{18}F]F-AraG to date. To evaluate the potential utility of [^{18}F]F-AraG, we first assessed uptake and in primary activated versus resting T cells (both murine; Fig. 3 and human; Fig 5c).

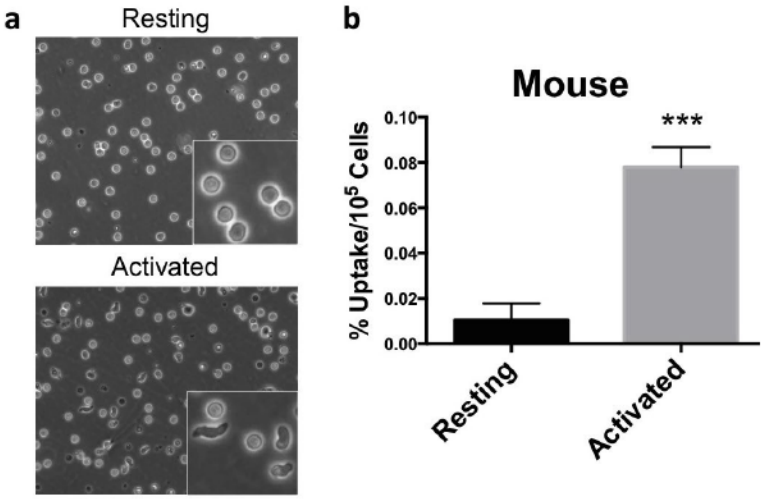


Fig. 3: Uptake of [^{18}F]F-AraG in Activated Versus Resting Murine T Cells.
a) Activated (bottom) murine primary T cells displayed distinct morphological characteristics compared to resting T cells (top).
b) [^{18}F]F-AraG was taken up at significantly higher levels in activated versus resting cells (***p<0.001; n=3 per group).

Activated murine T cells (2 days after activation) appeared morphologically distinct (elongated versus round) compared to resting cells (Fig. 3a) and accumulated significantly higher (~7.8-fold) [^{18}F]F-AraG (p<0.001; Fig. 3b). We have also compared the uptake of the tritiated version of the tracer [^{3}H]AraG, to the tritiated version of the tracer AraC ([^{3}H]AraC) in activated versus resting primary human T cells (Fig. 4).

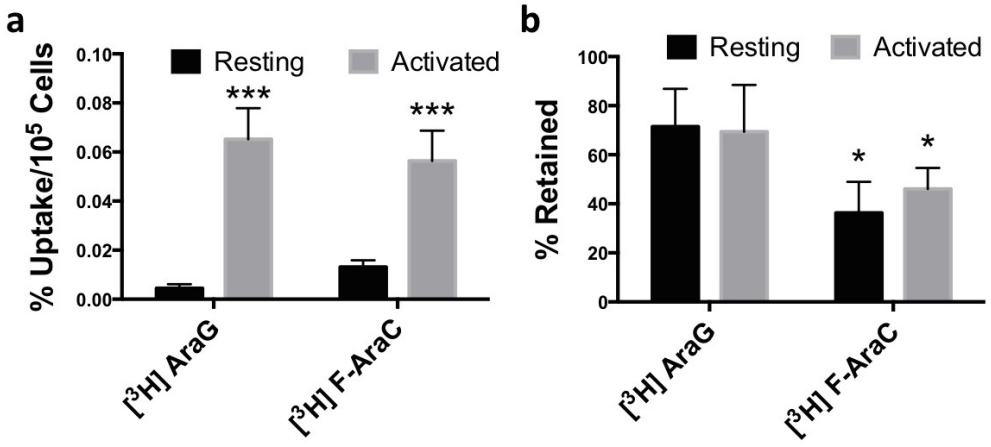


Fig. 4: Uptake and Retention of [^{3}H]AraG and [^{3}H]F-AraC in Resting and Activated Human Primary T Cells. Human primary T cells were isolated from PBMC buffy coats, activated in culture for 2 days, and incubated with either [^{3}H]AraG or [^{3}H]F-AraC to measure uptake **(a)** and retention **(b)**; n=3 per treatment per tracer). Both tracers were taken up at significantly higher levels (~6-7 fold) in activated versus resting T cells (***p<0.001). In contrast, [^{3}H]AraG was retained at significantly higher levels (~30% more) within both resting and activated T cells versus [^{3}H]F-AraC (*p<0.05). Data in all graphs are expressed as mean ± SD.

A 2-day activation protocol of sorted (~94% CD3+ T cells; data not shown) human peripheral blood mononuclear cells (PBMCs) resulted in similar morphological changes as seen in murine T cells. Significantly higher uptake in activated versus resting cells was seen with both [^{3}H]AraG and [^{3}H]F-AraC, but retention in both cell states was significantly higher with [^{3}H]AraG (p<0.05; Fig. 4). Finally, we found significantly higher uptake (~19-fold) of [^{18}F]F-AraG in activated versus resting human T cells (p<0.001; Fig. 5c). Although [^{18}F]F-AraG effluxes significantly out of activated but not resting cells (p<0.05), absolute levels in activated versus resting cells at all time points evaluated were significantly higher (p<0.01). Overall these results support the use of [^{18}F]F-AraG in selective PET imaging of activated versus resting T cells.

We have also evaluated whether [^{18}F]F-AraG accumulates in cells via dCK and/or dGK activity using established cell lines [40]. Loss of dCK in mutant CCRF-CEM T lymphoblast cells (dCK$^-$), as confirmed with Western blot analysis, (significantly impaired [^{18}F]F-AraG uptake (p<0.001; Fig. 5a). Overexpression of dCK in mutant cells (dCK$^+$) showed a trend toward increased tracer uptake compared to dCK$^-$ cells (p=0.17), whereas dGK overexpression (dGK$^+$) in dCK$^-$ cells restored tracer uptake to levels seen in wild-type cells. No effects on tracer retention were seen across any of these cell lines (Fig. 5b).

Our evidence supports that in contrast to [^{18}F]F-AraC that accumulates via dCK, but in line with AraG metabolism [33, 40], [^{18}F]F-AraG accumulates in cells via both dCK and dGK activity. These findings support recently published observations in a study on a novel PET tracer metabolized by dCK, which also noted a role of dGK in the metabolism of [^{18}F]F-AraG[33]; however, we extend upon these previous findings to highlight the additional role of dCK activity in the metabolism of [^{18}F]F-AraG.

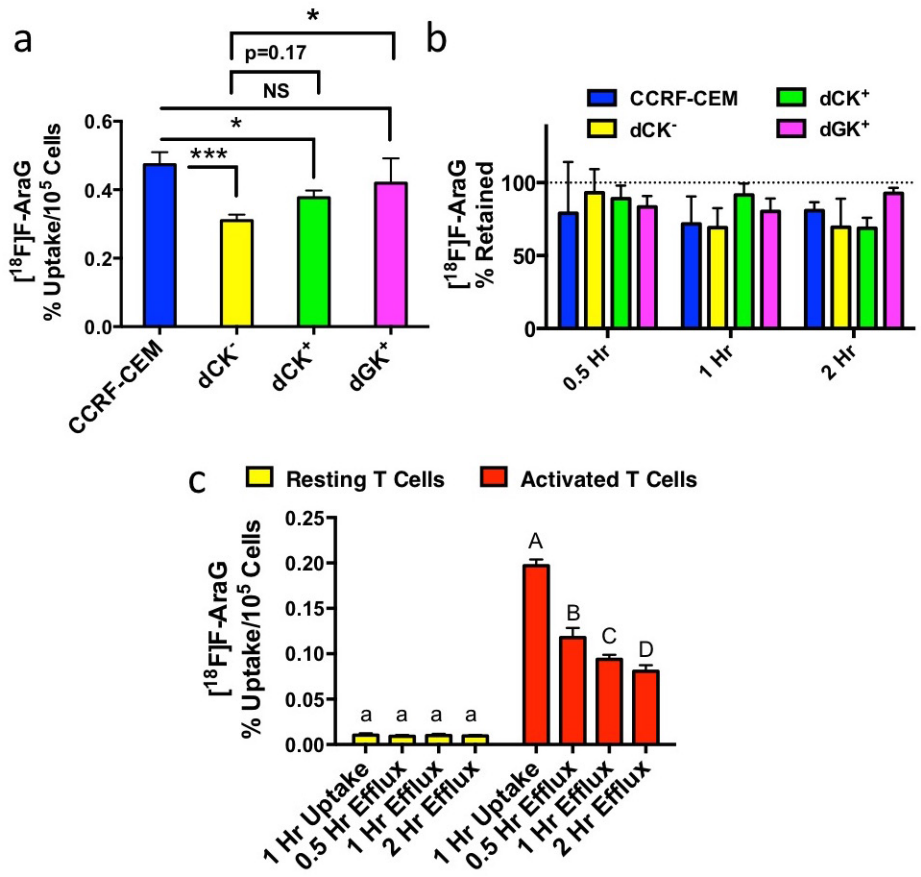


Figure 5: [^{18}F]F-AraG Accumulates in Cells via dCK and dGK Activity and at Increased Levels in Activated Versus Resting Primary Human T Cells.
a) Uptake and **b)** retention of [^{18}F]F-AraG across wild-type CCRF-CEM T lymphoblasts, mutant CCRF-CEM dCK- cells (dCK$^-$), and dCK$^-$ cells overexpressing either dCK (dCK+) or dGK (dGK+) (n=4 per cell type per time point). Significantly less uptake was seen due to the loss of dCK in wild-type cells (CCRF-CEM vs. dCK$^-$), ($p<0.001$). There was a trend towards higher uptake in dCK$^+$ versus dCK$^-$ cells, whereas dGK$^+$ cells had significantly higher uptake compared to dCK$^-$ cells and equivalent uptake compared to wild-type cells. No differences in retention were seen across cell types. **c)** Activated primary human T cells had significantly higher [^{18}F]F-AraG uptake compared to resting T cells at all time points examined (***$p<0.001$; n=4 per cell state per time point). Data in all graphs are expressed as mean ± SD.

Having confirmed the specificity [^{18}F]F-AraG has for activated T cells in vitro, we assessed its suitability for *in vivo* studies. Serum stability studies showed that the tracer was largely stable at 37 °C in phosphate buffer saline (PBS), murine serum and in human serum with more than 85% of the tracer intact after a two hour incubation in the latter. This indicates that the tracer will stay largely intact during *in vivo* preclinical and human imaging.

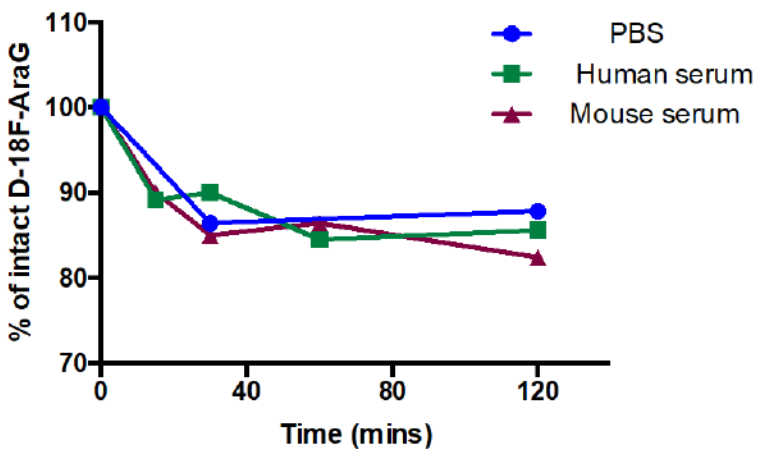


Fig. 6: Stability of [^{18}F]F-AraG in PBS, mouse and human serum at 37 °C as assessed by HPLC.

***In vivo* studies in aGVHD mouse model**

The ability to visualize sites of activated T cell accumulation in aGVHD with [^{18}F]F-AraG PET imaging was assessed in a well-established MHC mismatch aGVHD mouse model [28, 41-43]. In this model donor T cells constitutively express the bioluminescence reporter gene firefly luciferase (Luc) enabling *in vivo* bioluminescence imaging (BLI) to monitor sites of donor T cell accumulation and proliferation over time (Figs. 2a and 3a). BLI enables the visualization of the initiation the effector phase of the disease. This initiation phase occurs prior to overt symptoms and consists of T cell migration to secondary lymphoid organs where they become activated by host antigen presenting cells. This is followed by the effector phase that involves dissemination of homing receptor-expressing T cells out of these initial priming sites to effector sites such as the GI tract, liver and skin.

Static PET imaging procedure:

We performed clinical scoring, *in vivo* and *ex vivo* BLI, [^{18}F]F-AraG PET/CT imaging, tracer biodistribution studies, and *ex vivo* immunofluorescence studies at both at an early initiation phase (day 3) and late effector phase (day 10) after model initiation in both control (irradiated and bone marrow transplanted mice without donor T cells) and aGVHD (irradiated and bone marrow transplanted mice with Luc+ donor T cells) mice. Consistent with previous results and based on a standard scoring system [44], both day 3 and day 10 mice in this model are considered within the T cell activation phase of the disease.

For PET/CT imaging, mice were anesthetized and injected with ~200 □Ci of [^{18}F]F-AraG via the lateral tail-vein. Sixty minutes after injection, static PET images (10 minute acquisition time) were collected using a microPET/CT hybrid Inveon scanner (Siemens). A CT image was acquired just before each PET scan to provide an anatomic reference frame for the respective PET data. All PET images were reconstructed with a 3-dimensional ordered-subsets expectation maximization algorithm and co-registered with CT images using the Inveon Research Workplace (IRW) image analysis software (version 4.0; Siemens). To quantify tracer uptake in PET images, 3-dimensional regions of interest were drawn over the CLN, a 50% threshold was applied to each selected region, and partial volume corrected uptake values were expressed as percentage injected dose per gram of tissue (%ID/g).

Ninety minutes after tracer administration mice were euthanized, various tissues (blood, heart, small intestine, kidney, liver, lung, muscle, spleen, MLN, and CLN) were collected, weighed and radioactivity was measured using an automated □ counter (Cobra II; Packard). Radioactivity was decay-corrected to the time of radiotracer injection using diluted aliquots of the initial administered dose as standards. Data is expressed as percentage injected dose per gram of tissue (%ID/g) values

Day 3 aGVHD mice lacked noticeable symptoms and are at a time point when T cell activation is being initiated, whereas day 10 mice display clinical symptoms and are considered at the peak of T cell activation (data not shown). Disease progression is also reflected via significantly higher total donor T cell numbers (higher total body BLI signal) at day 10 (Fig. 8a) versus day 3 (Fig. 7a) ($p<0.01$; quantitative data not shown). As previously shown [28, 41, 42], *in vivo* (Fig. 7a) and *ex vivo* BLI revealed that donor T cells in aGVHD mice at day 3 had accumulated preferentially in lymphoid organs such as the spleen, mesenteric lymph nodes (MLN) and cervical lymph nodes (CLN). On a separate cohort of mice receiving GFP+ donor T cells we qualitatively confirmed progressively higher donor T cell accumulation within the spleen and CLN from day 3 to day 10. Importantly, [^{18}F]F-AraG PET/CT images taken 1 hour after tracer (~200 □Ci) administration showed qualitatively higher uptake in the CLN in aGVHD versus control mice (Fig. 7b). Quantitative image analysis corroborated our findings revealing significantly higher (~1.4-fold) [^{18}F]F-AraG uptake in the CLNs of aGVHD mice ($p<0.05$, Fig. 7c). At 1 hour, tracer uptake within abdominal organs such as the liver and kidneys obscured the ability to visualize *in vivo* differences in tracer accumulation within the MLN and spleen (Fig. 7b). However, tracer biodistribution studies assessed at 90 minutes after intravenous tracer injection revealed significantly higher [^{18}F]F-AraG accumulation in both the CLN ($p<0.05$; ~2.3-fold) and the MLN ($p<0.05$; ~2.0-fold, (Fig. 7d)), and a trend towards higher uptake in the spleen ($p=0.12$). No significant differences were noted across all other organs between control and aGVHD mice. Thus, at an early stage of disease development prior to clinical symptoms in this model, [^{18}F]F-AraG accumulated in lymphoid organs (MLN, CLN, and spleen) harboring activated T cells and [^{18}F]F-AraG PET imaging enabled visualization of early T cell accumulation within the CLN.

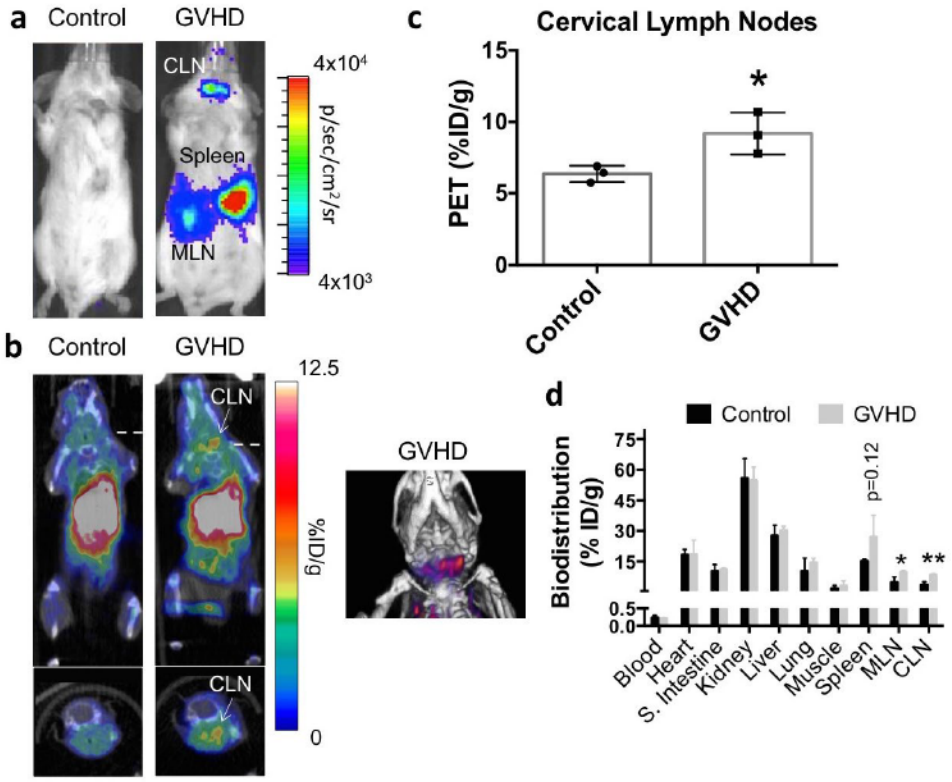


Figure 7: Bioluminescence and [^{18}F]F-AraG PET Imaging of Donor T Cell Dynamics During the Initiation of T Cell Activation in a Mouse Model of acute GVHD.
a) Bioluminescence imaging of control and GVHD mice 3 days after HCT revealed homing of luciferase-positive donor T cells to secondary lymphoid organs such as the spleen, cervical lymph nodes (CLN), and mesenteric lymph nodes (MLN) in GVHD mice. **b)** Representative [^{18}F]F-AraG PET/CT images (10 minute static scan; 60-70 minutes post-tracer administration; ~200 □Ci) at this time point showed visibly higher tracer uptake in the CLN of GVHD mice versus control mice (n=3 per group). **c)** Quantitative region of interest PET image analysis of the CLN corroborated our qualitative assessments, demonstrating significantly (*$p<0.05$) higher tracer uptake in the CLN of GVHD mice versus control mice (%ID/g; percentage injected dose per gram of tissue). **d)** Biodistribution studies (90 minutes after tracer administration) also supported our imaging findings showing significantly (*$p<0.05$) higher tracer uptake in CLNs in GVHD versus control mice. Significantly (*$p<0.05$) higher tracer uptake was also apparent in the T-cells harboring MLN and a trend ($p=0.12$) towards higher uptake was seen in the spleen. Data in all graphs are expressed as mean ± SD.

At day 10, at the peak of T cell activation, [^{18}F]F-AraG PET/CT images showed an even more apparent increase in tracer accumulation in the CLN of aGVHD compared to control mice (Fig. 8b), which was quantitatively confirmed via PET image analysis (Fig. 8c). Tracer biodistribution analysis revealed significantly lower tracer uptake in the spleen at this time point ($p<0.05$, Fig. 8d), but higher uptake in both the CLN and the MLN ($p<0.05$ and $p<0.01$, respectively). Amongst aGVHD mice, we also showed significantly higher BLI and PET signal within the CLNs at day 10 versus day 3 ($p<0.05$; Fig. 8e), indicating promise for monitoring disease burden via this surrogate imaging biomarker. These findings were recently published in Cancer Research (77(11); 2893–902, 2017).

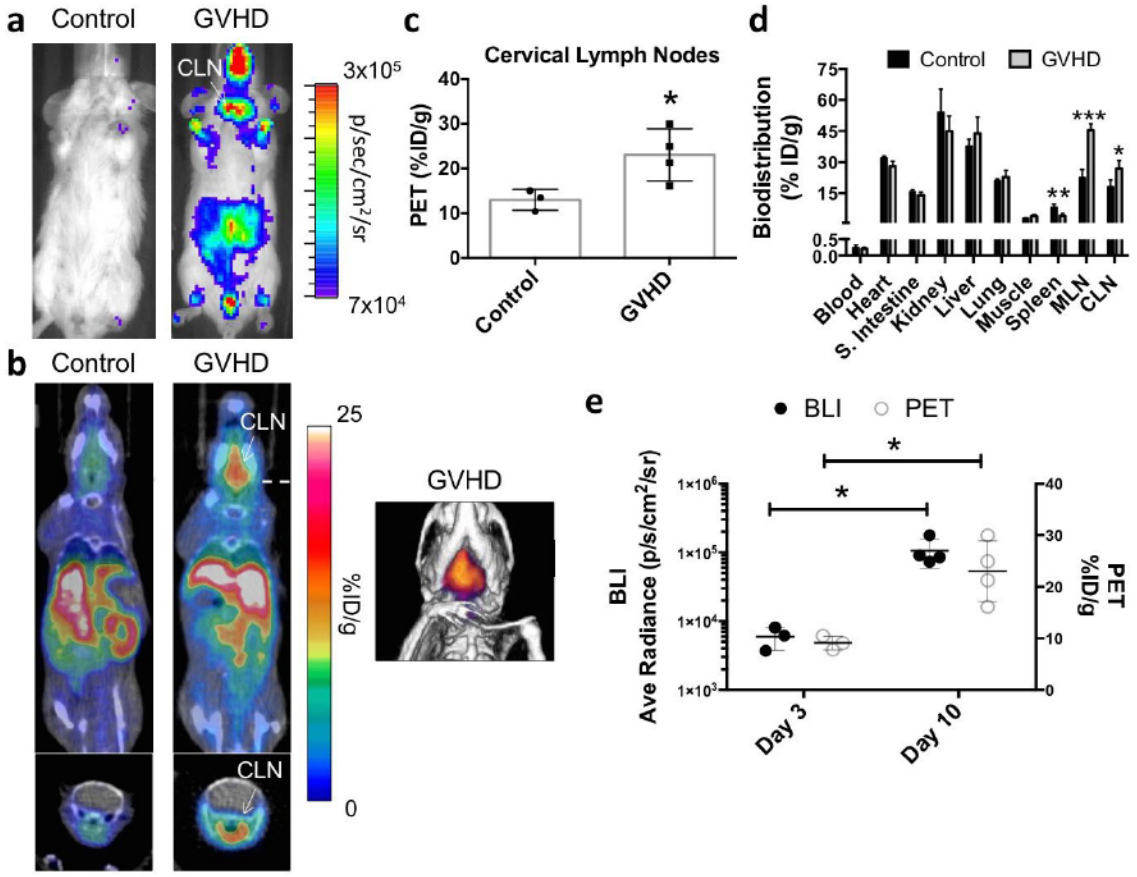


Figure 8: Bioluminescence and [^{18}F]F-AraG PET Imaging of Donor T Cell Dynamics During the Peak of T Cell Activation in a Mouse Model of acute GVHD
a) Bioluminescence imaging of control and GVHD mice 10 days after HCT revealed more widespread distribution of luciferase-positive donor T cells in GVHD mice but still localized accumulation within the cervical lymph nodes (CLN). Note the order of magnitude difference in scale between the images shown here and those in Fig. 2a (Day 3 after cell transplantation). **b)** Representative [^{18}F]F-AraG PET/CT images (10 minute static scans, 60-70 post-tracer administration; ~200 □Ci) at this time point showed visibly higher tracer uptake in the CLN of GVHD (n=4) versus control mice (n=3). **c)** Quantitative region of interest PET image analysis of the CLN corroborated our qualitative assessments, demonstrating significantly higher tracer uptake in the CLN of GVHD versus control mice (*p<0.05) (%ID/g; percentage injected dose per gram of tissue). **d)** Biodistribution studies (90 minutes after tracer administration) also supported our imaging findings showing significantly higher tracer uptake in CLNs in GVHD versus control mice. Significantly higher tracer uptake was also apparent in the MLN (*p<0.05). At this time point significantly lower tracer uptake was noted within the spleen (*p<0.05). **e)** Comparison of Day 3 and Day 10 bioluminescence and PET image analysis of CLNs shows both significantly higher BLI and PET signals on Day 10 compared to Day 3. Data in all graphs are expressed as mean ± SD.

In our preclinical model of aGVHD, [^{18}F]F-AraG therefore enabled the visualization of activated donor T cells, prior to overt clinical symptoms. before the While in mouse studies we were unable to visualize *in vivo* differences of tracer uptake in both the MLN and spleen, two organs

we know harbor activated donor T cells. Tracer *ex vivo* biodistribution results supported higher uptake in the MLN of aGVHD versus control mice at both early- and late-stages of disease we were unable to identify the MLN in our PET (or CT) images. This was mostly due to high tracer uptake in adjacent organs such as the kidneys and liver at 1 hour after tracer administration. While this is problematic in mice due to their small size and closely positioned organs, this will be less of a concern in humans as supported by the low tracer uptake in the GI tract in human PET images of normal volunteers.

Toxicology Study on F-AraG (the non-radioactive version of the tracer)

A toxicity study of 9-(2-deoxy-2-fluoroarabinofuranosyl) guanine (F-AraG) in Sprague Dawley rats was conducted by Sobran, Inc, Bioscience Division, Sobran Rangos Animal Facility (Baltimore, MD). A complete report by Sobran entitled "14-Day Single Intravenous Dose Toxicity Study of 9-(2-deoxy-2-fluoroarabinofuranosyl) guanine (F-AraG) in Sprague Dawley Rats" dated April 10, 2014, is summarized below.

Twenty rats of each sex (10/sex/group, 5/sex/sacrifice time point) were assigned to a dose group and a vehicle control group and administered 9-(2-deoxy-2-fluoroarabinofuranosyl) guanine (F-AraG) intravenously via the tail vein at 5ml/Kg as a single dose on Study Day 1 with either vehicle (3%ethanol and 50mM Ammonium acetate solution in sterile water) or F-AraG at 44.6ug/kg. Groups of 5 animals/sex and from each sex were sacrificed on Study Days 3 (48 hours) and 15 for evaluation of clinical pathology and organ toxicity.

The animals were monitored prior to the administration of the test article, 9-(2-deoxy-2-fluoroarabinofuranosyl) guanine, and up to 14 days following the administration of the test article. Parameters evaluated for test article effect included survival, clinical observations, body weight, body weight gain, clinical pathology, gross pathology, organ weights, and microscopic pathology. No morbidity or mortality was observed in the study. All rats survived to the scheduled termination date and remained bright, alert and responsive during the study. No abnormal findings were indicated during mortality checks (cagesides) or hands-on observations. There were no treatment-related findings from clinical chemistry, hematology, and coagulation samples collected on Study Days 3 and 15.

Animals remained bright, alert, and responsive at all times and did not exhibit signs of toxicity during the conduct of the study. No treatment-related differences were noted in mean body weights and body weight changes, clinical chemistry, hematology, or coagulation parameters. In addition, no treatment-related effects were observed organ weights or in gross and microscopic pathology. Under the conditions of this study, there were no treatment related findings in Sprague Dawley rats three or fifteen days after a single intravenous dose of F-AraGat 44.6µg⁄mg/kg.

Clinical characterization and dosimetry studies in healthy volunteers

The suitability of [^{18}F]F-AraG for human studies has been validated in humans at the University of California San Francisco (UCSF). [^{18}F]F-AraG PET/CT scans were performed on six healthy human volunteers and dosimetry was also calculated. [^{18}F]F-AraG exhibited hepatobiliary and renal clearance with highest uptake in associated organs with SUV mean values (normalized to body weight) of 13.47 ±1.46, 20.72±5.26 and 17.27±4.19 in liver, right and left kidneys respectively at 47-77 minutes post tracer injection (Table 1). High uptake was also observed in the myocardium as seen in mice, while relatively low background was observed in the thorax and lower abdomen. No significant adverse events or blood lab changes were observed due to

tracer administration in any of the volunteers other than slightly increased urine esterase levels. Radiation dosimetry results are shown in Table 2. These findings were recently published in Cancer Research (77(11); 2893–902, 2017).

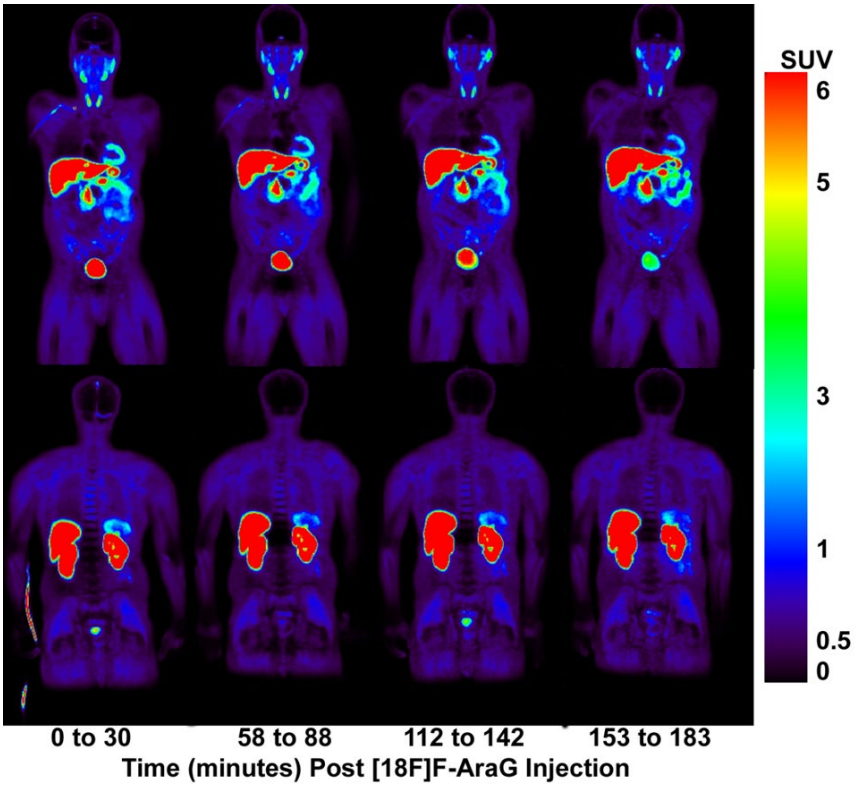


Fig. 9: Whole body PET images of [^{18}F]F-AraG in a healthy human volunteer. Frontal (top panel) and dorsal (bottom panel) coronal PET images taken immediately after intra-venous injection of the tracer and at serial time points in one of the 6 humans imaged. Highest activity is observed in the liver, kidneys and bladder, associated with [^{18}F]F-AraG clearance from the body. Uptake is also observed in the heart and spleen while relatively low background activity is observed in all other tissues. Similar images were obtained from the other 5 subjects.

Site	SUV-BW $_{mean}$	SUV-BW $_{max}$
Brain Tissue	0.02 ±0.01	0.08±0.02
Cervical Lymph Node	5.2 ±1.15	7.13±1.76
Heart	2.12 ±0.72	4.72 ±1.37
Myocardium	3.38 ±1.19	4.72±1.37
Liver	13.47 ±1.46	16.18±2.00
Kidney (right)	20.72±5.26	44.47±12.44
Kidney (left)	17.27±4.19	41.35±10.65
Bladder	8.28 ±6.15	10.28± 7.66
Muscle	0.98 ±0.44	1.42±0.66

Table 1: [^{18}F]F-AraG standard uptake values for key tissues- normalized to body weight (SUV-BW) at 47-77 minutes post tracer injection in six healthy human volunteers. SUV-BW mean and max values shown. Data shown represent averages ± standard deviation; n=6 for each site. Average of both right and left sides of subject shown for brain tissue and cervical lymph node.

These preliminary studies in healthy human volunteers have been promising with low background in the thorax and GI tract, making it favorable for thoracic and pelvic imaging. Favorable kinetics of [^{18}F]F-AraG in humans along with the preclinical data support its potential as a promising tracer for early detection of GVHD and warrants its further clinical evaluation in aGVHD patients. Given the paucity of PET tracers for activated T cells in the clinic this work lays the important foundation for [^{18}F]F-AraG PET imaging in aGVHD patients and the potential to provide crucial information to aid in the detection of activated T cell dynamics during the activation phase of aGVHD.

Organs	Male	Female
Adrenals	0.046567	0.067133
Brain	0.006380	0.007407
Breasts	0.005757	0.007547
Gallbladder Wall	0.051400	0.063033
LLI Wall	0.085107	0.012150
Small Intestine	0.047033	0.073000
Stomach Wall	0.018667	0.025367
ULI Wall	0.022600	0.033067
Heart Wall	0.052533	0.096933
Kidneys	0.877000	1.133333
Liver	0.211000	0.266667
Lungs	0.027167	0.033133
Muscle	0.009190	0.012167
Ovaries		0.016967
Pancreas	0.037300	0.050200
Red Marrow	0.013600	0.016833
Osteogenic Cells	0.007390	0.010593
Skin	0.004543	0.005790
Spleen	0.093600	0.163667
Testes	0.002780	
Thymus	0.005710	0.007800
Thyroid	0.001357	0.001633
Urinary Bladder Wall	0.085800	0.088167
Uterus	0.014133	0.017500
Total Body	0.018767	0.025200
Effective Dose	0.0138 mSv/MBq	

Table 2: [^{18}F]F-AraG human radiation dosimetry data. Mean radiation dosimetry data in 3 males and 3 females in REM/mCi. The kidneys receive the highest radiation dose followed by the liver.

For our studies at Stanford, the tracer will be administered as a single bolus injection of approximately 5 mCi into a hand or arm vein. The rationale for the starting dose is based on dosimetry studies already performed in healthy human volunteers where the dose limiting organ was found to be the kidney and the annual dose characterized as 5mCi. Please refer to the imaging protocol for recommended limits for male and female subjects. The major route of clearance based on initial clinical studies is the liver and kidney.

2.3 Clinicaltrials.gov

We are currently in communication with the company CellSight Technologies, Inc. (Dr. Gambhir is a founder) that licensed the [^{18}F]F-AraG patent (US Patent #US20110059014 A1) from Stanford. This company has been clinically evaluating this tracer in parallel to our proposed studies. Toxicity testing and dosimetry has already been performed by the company under FDA IND #123591, and the company has evaluated this tracer in normal subjects prior to testing in cancer patients receiving immunotherapy.

CellSight Technologies has given permission for this study to be submitted to their existing IND 123591.

2.4 Rationale

Benefits: The results may allow for improved, early and sensitive diagnosis of acute GVHD in patients that have undergone a bone marrow transplant. Please refer to pages 6-9 for additional rationale.

2.5 Preliminary results

Please refer to pages 15-22.

2.6 Study Design

Study participants will consist of healthy volunteers for extended studies on radiotracer behavior and patients belonging to a GVHD group. Two patient categories exist within the GVHD group- those highly suspected to have acute GVHD and those that are at high risk for developing acute GVHD. We will recruit healthy volunteers through local advertising. We will recruit high risk and confirmed GvHD patients from the Bone Marrow and Transplantation Clinic at the Stanford Cancer Institute.

Normal subjects will be recruited by publicly disseminating the information about the clinical trial through printed media such as a flyer, or by sending out an email to the Stanford community. We will ask for volunteers. A consent form for healthy volunteers is also included. Patients will undergo preliminary evaluation to ensure eligibility, receive and sign an informed consent, be enrolled in the trial, and then have [^{18}F]F-AraG PET/CT.

Highly suspected acute GVHD patients will be scanned following biopsy (taken to confirm acute GVHD). The staging and grading of the disease using the Glucksberg grade and International bone marrow Transplant Registry Severity Index (IBMTR) at time of enrollment will be noted. Their consent for a PET-CT scan will be obtained prior to biopsy. Biopsy tissues of consented patients will be analyzed further for T cell involvement.

High-risk patients (recipients of myeloablative or reduced intensity allogeneic transplants using either bone marrow or peripheral blood stem cells from HLA-matched or HLA-mismatched related or unrelated donors-protocols 9917, 9142, 9022, 9924) will have to sign consent forms

before they undergo a transplant. All those that consent will undergo a PET-CT scan day 4 ± 2 days post transplant. Additionally these patients will be scanned again between day 14-21 post transplant. Follow up on these patients will note those that go on to develop aGVHD and the clinical end point will be correlated to the scans to verify any predictive potential of the radiotracer.

For both GVHD groups-results of the scan will be compared to biopsy results and immunohistochemical staining of the biopsy samples for T cells.

Investigational agent: [^{18}F]F-AraG will be formulated in a sterile and pyrogen-free isotonic solution will be administered in a single slow IV injection.

This clinical study will be funded by the Parker Institute of Cancer Immunotherapy at Stanford Medicine Bench to Bedside Program 2017.

Additional resources and costs will be supplemented with the NIH RO1 grant 'A Novel Positron Emission Tomography Strategy for Early Detection and Treatment Monitoring of Graft-Versus-Host Disease' (Grant number 5RO1CA201719-02).

This is a single-arm prospective study of imaging of aGVHD where no randomization will be done (please see section 10 for more information on statistical analysis).

3 PARTICIPANT SELECTION AND ENROLLMENT PROCEDURES

3.1 Inclusion Criteria

This study will enroll three cohorts of participants: healthy volunteers, patients highly suspected to have acute GVHD and requiring systemic therapy, and patients at high risk for developing acute GVHD. For patients highly suspected to have acute GVHD and requiring systemic therapy, biopsy of involved tissue is encouraged to support the clinical diagnosis- this will normally be taken from the gastrointestinal tract. T cell staining will be incorporated in the immunohistochemical analysis of the tissue.

Participants must meet all of the following relevant criteria to participate:

1. Must be 21 years of age or older.
2. Must understand and voluntarily have signed an Informed Consent after its contents have been fully explained.
    a. For patients highly suspected to have acute GVHD and requiring systemic therapy, informed consent should be signed prior to biopsy taken to support clinical diagnosis.
    b. For patients at high risk for developing acute GVHD, informed consent should be signed prior to transplant.
3. For healthy volunteers only: Must have no known medical problems that would make undergoing the scan hazardous to the health of the patient or interfere with the results. In particular subjects should not have any cardiac or immunological disorders as these would likely affect the scan results. Subjects should have had a full physical exam within 6 months of the study. If healthy volunteers have not had a full medical exam within 6 months of the study, one of the nuclear medicine physicians will conduct the medical exam prior to any study procedures.
4. For patients highly suspected to have acute GVHD and requiring systemic therapy only:

Taking steroid treatment for suspected acute GVHD for 7 days or less.

5. For patients at high risk for developing acute GVHD only: Recipients of myeloablative or reduced intensity allogeneic transplants using either bone marrow or peripheral blood stem cells from HLA-matched or HLA-mismatched related or unrelated donors (protocols 9917, 9142, 9022, 9924) who have not yet been placed on any therapy for acute GVHD.

3.2 **Exclusion Criteria**

To participate in this study, subjects must have none of the relevant criteria:

1. Pregnant or nursing
2. Individuals with known or suspected substance abuse, obtained by self-reporting.
3. Uncontrolled infection
4. Relapsed/persistent malignancy
5. Currently receiving immunotherapy

3.3 **Informed Consent Process**

All participants will be provided a consent form describing the study with sufficient information for participants to make an informed decision regarding their participation. Participants will sign the IRB approved informed consent prior to participation in any study specific procedure. The participant will receive, as is mandatory, a copy of the signed and dated consent document. The original signed copy of the consent document will be retained in the medical record or research file.

3.4 **Randomization Procedures**

Patients will not be randomized.

3.5 **Study Timeline**

3.5.1 **Primary Completion:**

The study will reach primary completion 24 months from the time the study opens to accrual.

3.6 **Study Completion:**

The study will reach study completion 36 months from the time the study opens to accrual.

4 IMAGING AGENT/DEVICE/PROCEDURE INFORMATION

4.1 **[18^F]F-AraG: 2'-deoxy-2'-[18F]fluoro-9-β-D-arabinofuranosyl guanine**

The imaging agent to be used in this study is 2'-deoxy-2'-[18F]fluoro-9-β-D-arabinofuranosyl guanine. The radionuclide on this imaging agent being Fluorine-18 (^{18}F). The imaging agent will be administered intravenously.

Clinical grade imaging agent will be synthesized under GMP/GLP conditions at the Lucas Center Cyclotron and Radiochemistry facility, part of the Molecular Imaging Program at Stanford. The imaging agent will be requested as and when subjects become available. The radiotracer will be received at the Nuclear Medicine department at Stanford Hospital in a lead shielded container by an authorized person and with the appropriate transfer sheet.

Dosimetry of this imaging agent has previously been characterized in male and female healthy subjects and the information is included on page 21. Dosimetric calculations were performed

using the OLINDA/EXM 1.1 software using methods previously described using other tracers [Yaghoubi, 2001].

PET/CT is a well established, FDA approved clinical imaging procedure. Each subject will be scanned a maximum of 2 times. For each imaging session the amount of radioactivity that will be administered will be less than 5.7 and 4.4 mCi for males and females respectively.

5 STUDY PROCEDURES

5.1 Imaging Protocol for Patient Groups

Each subject may have up to two imaging sessions. The procedures for each imaging session is detailed below:

Day 1 (Imaging Day)
Overview of procedures prior and post injection of [^{18}F] F-AraG:

1. Subject will participate in an informed consent session (approximately 30 minutes) and if she/he would like to continue after being fully informed she/he will sign consent form.
2. Subject will be asked to drink several glasses of water before arriving at the nuclear medicine clinic and during the study.
3. Subject will be interviewed to obtain medical history.
4. Volunteer height and weight will be measured.
5. Baseline Heart Rate (HR), Blood Pressure (BP), Blood Oxygen %,Temperature, and Respiratory Rate will be collected at baseline (5-120 minutes prior to the injection of the tracer).
6. IV line may be placed in both arms of healthy subjects for PET tracer injection and another may be used for blood collection. The GVHD patients will have double lumen catheters which can these be used for these purposes.
7. For baseline CBC analysis- 3 ml of blood will be collected and 5 ml of blood will be collected for baseline metabolic panel analysis.
8. Subject may either be positioned on the PET/CT scanner bed or another bed in the clinic (where possible the same PET-CT system should be used especially for the healthy subjects).
9. Injection of [^{18}F] F-AraG and post-injection procedures will follow.
10. If possible, subjects should be asked to urinate prior to the start of the PET/CT scans.
11. A bolus < 5.7 mCi of clinical grade [^{18}F] F-AraG for male volunteers or < 4.4 mCi of clinical grade [^{18}F]F-AraG for female volunteers will be injected through IV.
12. A CT image will be acquired with the PET images.
13. The PET scanning procedure may start at the time of tracer injection or at a later time point after tracer injection. The imaging field includes the vertex to thigh for healthy subjects and vertex to feet for GVHD patients. Up to two whole-body PET scans may be performed at various time points after injection of the tracer, not to exceed 2 hours after tracer injection.
14. Blood pressure, Temperature, Blood Oxygen saturation, Heart Rate, Respiratory Rate will be measured immediately after [^{18}F]F-AraG PET-CT procedure.
15. A safety follow up will happen at one time point within the time interval (day 2 – day 7) post-scan.
16. All observed adverse events and subject complaints will be recorded.

Healthy subject protocol

1. Volunteer will be asked to drink 1-2 glasses of water before his/her arrival at the clinic.
2. Volunteer will be consented by a member of the study team
3. Female subjects will have a urine or serum pregnancy test to rule out pregnancy.
4. Subject will be weighed.
5. Subject will have an IV line placed in a hand or arm vein for tracer administration.
6. Subject will have an IV line placed in a hand or arm vein for blood sampling.
    - Subject will have a second IV line placed in the vein of the other hand/arm for venous/hand warmed arterialized-venous blood or in a radial artery for arterial blood sampling for kinetic and radio-metabolite analysis, over the first 90 minutes post tracer injection. The arterial/venous or arterialized-venous blood will be sampled in heparinized coated tubes pre-tracer injection and at 1, 3, 5, 10, 15, 30, 45, 60 and 90 minutes post tracer injection (10 sampling points).

    - During this time, care should be taken to prevent movement in the subject from his or her original position in the scanner. The blood sampling volume will be 3 mL, (2mL for whole blood and plasma radioactivity analysis + 1mL for radiometabolite analysis). The exact time point of the collection of each blood sample will be recorded using the same clock device.If an automated blood-sampling device is available (e.g., Twilite system from Swisstrace GmbH) it should be used for the blood sampling procedure, but if this is not available, then the sampling will be done manually.

    - **Radioactivity analysis of whole blood and plasma**: The blood tubes should be weighed before and after collecting the blood. Whole blood radioactivity will be measured. To measure plasma radioactivity, the whole blood samples will be centrifuged. The radiolabeled tracer concentration in whole blood, plasma and pellet will be measured by a gamma well counter (1-min per sample). The counting rates measured by the well counter will be converted to the PET equivalent counts using a conversion factor from a weekly phantom cylinder study. The counts should be decay corrected back to the injection time.
7. Blood pressure, temperature, respiratory rate, heart rate and pulse oximetry measurements will be taken at baseline.
8. A blood sample will be taken prior to the injection of the tracer, to be used for baseline chemistry and hematology laboratory testing (hematocrit should be an included measure).
9. Participant will have EKG leads placed for cardiac gating and then be placed in the scanner.
10. A low dose attenuation correction CT scan (ACCT) of the heart will be performed for attenuation correction.
11. A small low activity calibration source of known activity, volume, and concentration of F-18 activity will be taped to the furthest corner of the scanner field of view prior to radiopharmaceutical injection. The 30 minute dynamic PET acquisition of the heart will then begin, making sure that the entire heart region is covered in the field of view. All PET/CT scans should be done on the Discovery MI PET/CT scanner or the PET/CT 690 scanner.
12. Right after the start of the PET acquisition, [^{18}F]F-AraG formulated in a sterile and pyrogen-free isotonic solution in less than 10 ml will be administered in a single slow IV injection. The line will be slowly flushed at a constant rate with at least 10 ml normal saline after injection.
13. The initial 5 minutes of the dynamic PET scan will be finely acquired at 5 seconds/frame (total number of frames in initial 5 minutes =60 frames) so as to capture the peak of the cardiac input function and then 1 minute/frame for the next 25 min over the same field of view (FOV). The dynamic PET data should be collected in list-mode and saved

appropriately.
14. Participants will have skull base-to-thigh static PET-CT scans (which includes the brain) obtained again at 60 and 90 minutes (scans #2 and #3). CT will use 120 kV and dose modulation based on body habitus, ranging 10-105 mA for both scans.
15. Blood pressure, temperature, respiratory rate, heart rate and pulse oximetry measurements will be performed post scan 3.
16. All recordings of important time measurements (e.g. time of injection and acquisitions) will be done using the same clock e..g a phone.
17. Participants will be asked to void frequently to reduce radiation exposure.
18. Adverse drug events that have developed during the scans will be recorded in source documents. Any that develop after ^{18}F]F-AraG injection will also be recorded in the source documents when patients seen at the safety follow-up visit at one time point within the time interval (day 2 – day 7) post-scan.
19. After [^{18}F]F-AraG injection, participants will be asked to return to the clinic for a safety follow-up visit for blood sample collection for blood chemistry and hematology and also for measurements of vital signs. Results will be recorded in the case report form.

GVHD patient protocol

1. Subject will be asked to drink 1-2 glasses of water before his/her arrival at the clinic.
2. Subject will be consented by a member of the study team
3. Subject will be weighed.
4. The subject will likely have an existing port (double lumen catheters) for administration of the radiotracer.
5. A blood sample will be taken 5-120 minutes prior to the injection of the tracer, to be used for baseline chemistry and hematology laboratory testing. Blood pressure, temperature, heart rate and pulse oximetry measurements will also be taken up between 5-120 minutes prior to injection (baseline).
6. [^{18}F]F-AraG formulated in a sterile and pyrogen-free isotonic solution will be administered in a single slow IV injection. The line will be flushed with at least 10 ml normal saline after injection. The bed strap should be open until scanning starts.
7. A small low activity calibration source of known activity of F-18 will be placed in the field of view in each image collected
8. Participants will have a static PET scan at 60 minutes after injection of [^{18}F]F-AraG covering all positions from vertex to toe. Attenuation correction will be done with a CT scan (120 kv, 10 mA) usually acquired before the PET scan. There will be 8 bed positions covering head to toe (each position will taking approximately 3 minutes). The data should be collected in list mode and saved. Patients that are able to tolerate longer times in the scanner will also under go an additional scans following the same format described above at 30 minutes post tracer injection.
9. All recordings of important time measurements (e.g. time of injection and acquisitions) will be done using the same clock e.g. a phone.
10. Blood pressure, temperature, respiratory rate, heart rate and pulse oximetry measurements will be taken immediately after the [^{18}F]F-AraG PET-CTscan.
11. Participants will be asked to void as frequently as possible to reduce radiation exposure.
12. Participants will be given a copy of the consent form they signed and will be dismissed.
13. Participants will undergo a safety follow-up at one time point within the time interval (day 2 – day 7) post-scan for blood sample collection for blood chemistry and hematology and also for observation for vital signs. For in-patients we will use the blood samples and vital signs

obtained as part of the standard of care by the Bone Marrow and Transplant group. Out-patients will have to return to the clinic for the safety follow up.
14. Adverse drug events will be recorded at one time oint with the time interval of 2-7 days after [^{18}F]F-AraG injection/scan in the case report form.
15. Patients in the high risk of developing GVHD arm will undergo the above protocol once more 10-17 days after the first scan.
16. A post safety follow up will be conducted for up for 6 months via chart review to assess progression or development of aGVHD.

Safety Follow-up [range: One time point within the interval (Day 2 to Day 7) post-scan
1. BP, HR, Temperature, Blood Oxygen %, and Respiratory Rate will be recorded.
2. Blood will be collected for CBC, and Metabolite Analysis.
3. Adverse events will be collected at one time point within the time interval (day 2 – day 7) post scan.

Healthy subjects and out-patients will need to come in to the clinic for the safety follow up. For in-patients (GVHD group)-we will obtain the blood samples and vital signs obtained as part of the standard of care by the Bone Marrow and Transplant group.

Subjects with suspected acute GVHD OR at high-risk of aGVHD will be followed for 6 months post their last scan to evaluate disease (progression/development) by chart-review.

5.2 Criteria for Removal from Study

Withdrawal of Participants

The Protocol Director may withdraw participants from the study for one or more of the following reasons: failure to follow the instructions of the Protocol Director and/or the study staff; failure to provide informed consent; determination that continuing the participation could be harmful to the subject; the study is cancelled or other administrative reasons.

Discontinuation: Patients can discontinue in case of unacceptable adverse events or patient decision to withdrawn from study.

6 STUDY CALENDAR

6.1 Healthy Volunteers

Table 6-1: Study Calendar for Healthy Volunteers

	Screening	Day 1			Safety Follow-Up
	Day -30 to Day 1 pre-injection	Pre-injection of [^{18}F]F-AraG (Baseline)	Injection of [^{18}F]F-AraG[a]	Post-injection of [^{18}F]F-AraG	Day 2-7 post-scan
Inclusion/Exclusion Criteria	X				
Informed Consent	X[d]	X[d]			
hCG[c]	X[d]	X[d]			
Height		X			
Weight		X			
Vital signs		X		X	X
EKG		X (leads placed prior to scan)	X	X	
CBC w/diff, plts		X			X
Serum chemistry[b]		X			X
IV administration of [^{18}F]F-AraG			X		
PET/CT scan with investigational Agent [^{18}F]F-AraG				X[e]	
Arterial/venous blood sampling for input function				X (0, 1, 3, 5 10, 15, 30, 45, 60 and 90 min after tracer injection)	
Radio-Metabolite Analysis				X (0, 1, 3, 5 10, 15, 30, 45, 60 and 90 min after tracer injection)	
Adverse event evaluation				X	X[f]

a: Investigational Agent: Dose as assigned (5 mCi); route/schedule.
b: Albumin, alkaline phosphatase, total bilirubin, bicarbonate, BUN, calcium, chloride, creatinine, glucose, LDH, phosphorus, potassium, total protein, SGOT[21], SGPT[45], sodium.
c: Urine or serum pregnancy test (women of childbearing potential).
d: Procedure may be done during screening period or on Day 1 prior to injection of study drug.
e: Scan 1: 30-minute dynamic PET of heart (60x5s+25x1min; Scan 2: vertex to mid-thigh static PET 60-minute post injection (includes brain); Scan 3: vertex to thigh static PET 90 minutes post-injection
f: Collected at one time point within the time interval (day 2 – day 7) post scan.

6.2 **Patients highly suspected to have acute Graft versus Host Disease**

Table 6-2: Study Calendar for Patients highly suspected to have acute GHVD

	Screening	**Day 1**			**Safety Follow-Up**	**Disease assessment**
	Day -30 to Day 1 pre-injection	Pre-injection of [^{18}F]F-AraG (Baseline)	Injection of [^{18}F]F-AraG[a]	Post-injection of [^{18}F]F-AraG	Day 2-7 post-scan	Upto 6 month post-scan
Inclusion/Exclusion Criteria	X					
Informed Consent	X[d]	X[d]				
Height		X				
Weight		X			X[c]	
Vital signs		X		X	X[c]	
CBC w/diff, plts		X			X[c]	
Serum chemistry[b]		X			X[c]	
IV administration of [^{18}F]F-AraG			X			
PET/CT scan with investigational Agent [^{18}F]F-AraG				X[e]		
Adverse event evaluation				X	X[g]	
Follow-up for development of aGVHD symptoms/ disease						X[f]

a: Investigational Agent: Dose as assigned (5 mCi); route/schedule.
b: Albumin, alkaline phosphatase, total bilirubin, bicarbonate, BUN, calcium, chloride, creatinine, glucose, LDH, phosphorus, potassium, total protein, SGOT[21], SGPT[45], sodium.
c: Obtained as part of the standard of care by the Bone Marrow and Transplant group for in-patients (for in-patients)
d: Procedure may be done during screening period or on Day 1 prior to injection of study drug.
e: Scan 1: head to toe PET/CT scan 60 minutes post injection); OPTIONAL head to toe PET/CT scan 30-minutes post-injection
f: Chart review for up to 6 months post scan to monitor disease progression.
g: Collected at one time point within the time interval (day 2 – day 7) post-scan

	Screening	**Day 1**			**Safety Follow-Up 1**	**Day 11-18: 2nd scan**			**Safety Follow-Up 2**	**Disease assessment**
	Day -30 to Day 1 pre-injection	Pre-injection of [18F]F-AraG (Baseline)	Injection of [18F]F-AraG[a]	Post-injection of [18F]F-AraG	Day 2-7 post 1st scan	Pre-injection of [18F]F-AraG (Baseline)	Injection of [18F]F-AraG[a]	Post-injection of [18F]F-AraG	2-7 days post 2nd scan	Upto 6 month post-scan
Inclusion/Exclusion Criteria	X									
Informed Consent	X[d]	X[d]								
Height		X								
Weight		X			X[c]	X			X[c]	
Vital signs		X		X	X[c]	X		X	X[c]	
CBC w/diff, plts		X			X[c]	X			X[c]	
Serum chemistry[b]		X			X[c]	X			X[c]	
IV administration of [18F]F-AraG			X				X			
PET/CT scan with investigational Agent [18F]F-AraG				X[e]				X[e]		
Adverse event evaluation				X	X[g]	X		X	X[g]	
Follow-up for development of aGVHD symptoms/ disease										X[f]

6. 3 **Patients at high risk of developing acute Graft versus Host Disease**

Table 6-3: Study Calendar for Patients at high risk of developing acute GHVD

a: Investigational Agent: Dose as assigned (5 mCi); route/schedule.
b: Albumin, alkaline phosphatase, total bilirubin, bicarbonate, BUN, calcium, chloride, creatinine, glucose, LDH, phosphorus, potassium, total protein, SGOT[21], SGPT[45], sodium.
c: Obtained as part of the standard of care by the Bone Marrow and Transplant group for in-patients (for in-patients)
d: Procedure may be done during screening period or on Day 1 prior to injection of study drug.

e: On Day 1:Scan 1: head to toe PET/CT scan 60 minutes post injection); OPTIONAL head to toe PET/CT scan 30-minutes post-injection;
On Day 11-18: Scan 2: head to toe PET/CT scan 60 minutes post injection); OPTIONAL head to toe PET/CT scan 30-minutes post-injection
f: Chart review for up to 6 months post scan to monitor disease progression
g: Collected at one time point within the time interval (day 2 – day 7) post-scan #1 and at one time point within the time interval (day 2 – day 7) post-scan #2

6 ADVERSE EVENTS AND REPORTING PROCEDURES

7.1 Potential Adverse Events

Risks associated with this Investigational Agent

[^{18}F]F-AraG has been tested in 6 healthy human volunteers, 3 advanced bladder cancer patients (2 of whom received 2 doses each), 2 breast cancer patients, and 3 squamous cell carcinoma of the head and neck cancer patients (2 of whom received 2 doses each). Any observed side effects were mild. The only side effect that occurred in more than one volunteer/patient was positive WBC esterase in the urinalysis without accompanying urinary symptoms.

Risks associated with Investigational PET/CT Imaging

The administration of the radioactive substance will feel like a slight pinprick if given by intravenous injection. Patients who are claustrophobic may feel some anxiety while positioned in the scanner. Also, some patients find it uncomfortable to hold one position for more than a few minutes. The subjects will not feel anything related to the radioactivity of the substance in their body. The substance amount is so small that it does not affect the normal processes of the body. Procedures such as isotope injection are standard procedures currently used in the clinical PET and Nuclear Medicine clinics and the usual sterile methods will be used.

Risks from PET imaging include exposure to ionizing radiation and the placement of an intravenous line. Total time required for each PET study will be approximately 90-120 minutes. The radiation the participants will receive as a result from participating in this study includes radiation from administration of [^{18}F]F-AraG and the accompanying CT scans for attenuation correction. This radiation exposure is for research purposes only.

- The amount of radiation dose the healthy group will receive from one of [^{18}F]F-AraG PET/CT is 11.7-12.3 mSv (2.7mSv from 163MBq for females and 3.3 mSv from 211MBq of [^{18}F]F-AraG for males plus a further 9mSv from the three CTs (one cardiac and two whole body CT scans) for attenuation correction acquired usually before the PET acquisitions.

- The amount of radiation dose the GVHD group will receive from one [^{18}F]F-AraG PET/CT session is 5.7-6.3 mSv (2.7mSv from 163MBq for females and 3.3 mSv from 211MBq of [^{18}F]F-AraG for males plus a further 3mSv from the whole body CT scans for attenuation correction. If a patient is able to withstand longer times in the scanner they will undergo a further PET acquisition at 30 minutes for which 1 more CT scans will be performed usually before the PET acquisition (and an additional 3mSv dose will be received).

This amount of radiation involves minimal risk and is necessary to obtain the research information desired. Procedures will be in place for verification of correct radiopharmaceutical dose and route of administration (i.e., each dose will be double checked for dosimetry and quality by a researcher and technologist).

Risks associated with aGVHD

Patients highly suspected to have aGVHD or who are at high risk of developing aGVHD may experience side effects from underlying disease while participating in this study. Acute GVHD is characterized by a wide spectrum of host tissue injuries in the gastrointestinal (GI) tract, liver, skin, and lungs; causing a wide range of symptoms [3, 5]. In this setting, a very large number of

Grade 1 and 2 adverse events (AEs) are expected to occur, regardless of whether or not a patient is participating in a research study. Expected AEs include a number of specific AEs that are associated with acute GVHD.

The Protocol Director (PD) or designee will assess each AE to determine whether it is unexpected according to the Informed Consent, Protocol Document, or Investigator's Brochure, and related to the investigation. Unless specifically determined otherwise by the investigator, the following events are considered expected in the early post-transplant setting.

- Alopecia
- Anemia
- Anorexia
- Bleeding, including requiring transfusions
- Cardiac arrhythmias
- Central venous catheter infections
- Constipation
- Diarrhea
- Edema
- Fatigue
- Febrile episodes
- Gastritis
- Graft failure
- Graft versus host disease
- Hematuria
- Hypertension
- Hypotension
- Hypoxia
- Incontinence
- Infections, including sepsis
- Insomnia
- Laboratory abnormalities
- Mental status changes and mood alterations
- Mucositis
- Nausea
- Neutropenia
- Pain
- Pleural effusion
- Pneumonitis
- Rash
- Seizures
- Sinusoidal obstructive syndrome
- Tachycardia
- Thrombocytopenia
- Thrombotic microangiopathy
- Tremor
- Vomiting

7.2 **Adverse Event Collection**

Adverse event collection will be focused on adverse events useful for assessment of the safety of [^{18}F]F-AraG. The following AE monitoring schema is intended to capture all AEs that are clinically significant and/or impactful, but minimize "not informative" AE collection. Adverse events will be graded according to CTCAE v4.0.

- All AEs, both non-serious and serious, attributed to investigational agent or study procedure will be collected and documented with causality attribution (see Section 7.2 Adverse Event Attribution) on an adverse event log (AE log).
    - An adverse event is considered serious if it fulfills one of the following criteria per 21CFR§312.32(a):
        - Results in death
        - Life-threatening (patient at risk of death at the time of the event)
        - Requires inpatient hospitalization or prolongation of existing hospitalization
        - Results in persistent or significant disability
        - Other medical events that may not be immediately life-threatening or result in death or hospitalization by may jeopardize the patient or require intervention to prevent one of the outcomes listed above

- In addition, all AEs meeting the criteria of "serious" as defined at 21CFR§312.32(a), ***and*** are Grade 3 or above, will be collected regardless of attribution; and documented on an AE log, including causality attribution.
- AEs not meeting these criteria will not be collected, except as follows. AEs resulting in subject withdrawal from the study will be collected.

All Serious Adverse Events (SAEs) will be tracked until resolution or at least 30 days after the last dose of the study treatment.

The development of acute GVHD, its date of onset and overall grade, is a key component of the primary outcome measure for patients on this trial and will be monitored by chart review. Acute GVHD will be recorded on an AE log.

7.3 **Adverse Event Reporting**

Both serious and non-serious AEs will be clearly noted in source documentation and listed on study-specific Case Report Forms (CRFs). Non-serious adverse events will be reported annually to FDA via an Annual Report (IND: 123591) and to IRB via Continuing Review. SAEs Grade 3 and above, and all subsequent follow-up reports will be reported to the Stanford Cancer Institute Data and Safety Monitoring Committee (DSMC) using the study specific CRF regardless of the event's relatedness to the investigation. Following review by the DSMC, events meeting the IRB definition of 'Unanticipated Problem' will be reported to the IRB using eProtocol within 10 working days of DSMC review, or within 5 working days for deaths or life-threatening experiences.

Adverse events deemed serious, unexpected (i.e. not described in the protocol, Investigator's Brochure or informed consent documents) and related to study drug must be reported to the IND Holder (CellSight Technologies) using the FDA MedWatch form 3500a within 24 hours of knowledge of event. The IND Holder is responsible for deciding whether the event meets IND Safety Reporting criteria.

If the Principal Investigator determines an unanticipated adverse effect presents an unreasonable risk to subjects, the study will be terminated as soon as possible, but no later than 5 working days after the PI makes the determination and no later than 15 working days after first receiving notification of the effect.

8 REGULATORY CONSIDERATIONS

8.1 **Institutional Review of Protocol**

The protocol, the proposed informed consent and all forms of participant information related to the study (e.g. advertisements used to recruit participants) will be reviewed and approved by the Stanford IRB and Scientific Review Committee (SRC). Any changes made to the protocol will be submitted as a modification to FDA and will be approved by the IRB and SRC prior to implementation. The Protocol Director will disseminate the protocol amendment information to all participating investigators.

8.2 **Data Management Plan**

The study will be registered on Oncore, a centralized database used by CCTO to manage clinical trials. All associated approvals and subject information will also be stored on this platform. Any case report forms (CRFs) will be stored in a locked office in the Nuclear Medicine clinic. Records will be kept using OnCore.

Data will be managed using Excel software where the following important parameters will be documented; subject ID (number assigned based on order of scan), the type of subject (healthy/highly suspected GVHD or high risk GVHD group), sex of the subject and their weight, height and age. The date and time of radiotracer injection, the dose injected, time and duration of PET scan will also be noted.

For data analysis the following parameters will be documented; region of interest (Ie.g. liver, heart, cervical lymph nodes), volume of the region of interest and the associated mean, maximum and minimum values for the Standard Uptake Values (SUV).

8.3 **Data and Safety Monitoring Plan**

During the clinical investigation, the Protocol Director will evaluate the progress of the trial, including periodic assessments of data quality and timeliness, participant recruitment, accrual and retention, participant risk versus benefit, performance of trial sites, and other factors that can affect study outcome. Monitoring of the trial will occur every 8 weeks and a record of monitoring activities will be maintained by the study team.

The Stanford Cancer Institute Data and Safety Monitoring Committee (DSMC) will audit study related activities at least annually in accordance with the DSMC SOP to determine whether the study has been conducted in accordance with the protocol, local standard operating procedures, FDA regulations, and Good Clinical Practice (GCP). This may include review of regulatory binders, case report forms, eligibility checklists, and source documents. In addition, the DSMC will regularly review serious adverse events and protocol deviations associated with the research to ensure the protection of human subjects. Results of DSMC audits will be communicated to the IRB and the appropriate regulatory authorities at the time of continuing review, or in an expedited fashion, as needed.

9 MEASUREMENTS

9.1 **Primary and Secondary Outcome measures**

Primary outcomes will be the severity/score of aGVHD or the development of aGVHD in the highly suspected and high risk groups respectively. Diagnosis and staging of the disease are ultimately based on skin, liver and gut involvement and will be further supported by the secondary outcome of IHC analysis of the gastrointestinal tract. The latter mostly focuses on the detection of dead regions as determined by haematoxylin and eosin staining. For this study, an additional secondary outcome will be to further stain tissues for T cell markers to determine T cell infiltration (CD3, CD4 amd CD8) and T cell activation markers (e.g. OX40) to determine T cell status since activated T cells underlie the development of aGVHD in the early stages.

9.1.1 Primary Outcome Measurement Methods

Static images acquired will be used to determine the bio-distribution of the imaging agent in the GVHD groups. Past images as well as newly acquired data from healthy subjects will be used as comparisons. Regions of interest will be drawn on various organs such as the liver, cervical lymph nodes and gut and quantified using imaging software (e.g. Inveon Research Workplace or Amide). In addition any areas that show up differently in the GVHD groups, to the normal bio-distribution seen in healthy subjects will be identified and ROIs will also be drawn over these regions.

Bio-distribution data will be expressed as % injected dose/gram (%ID/g) and/or as standard uptake values (SUV) with respect to the organ being studied. In the high suspected aGVHD group, [^{18}F]F-AraG PET results will be compared to any grades and scores of aGVHD severity attributed to subjects. In the case of the high-risk group, after the second scan, patients will be monitored for development of aGVHD and the clinical endpoint (whether or not the high risk patient develop aGVHD) will represent the primary outcome variable. In addition, IHC analysis of biopsies taken in either GVHD group will help in the interpretation of the primary results and thus potentially support interpretation of the PET data. The IHC analysis will involve determining the amount of cell death or T cell infiltration and activation.

Vital signs and blood laboratory data collected before and after the i.v. injection of [^{18}F]F-AraG will allow evaluation of any unexpected side effects.

To measure plasma radioactivity, whole blood sample will be centrifuged. The radiolabeled tracer concentration in whole blood, pellet post centrifugation and plasma will be measured by a gamma well counter (1-min per sample). The counting rates measured by the well counter will be converted to the PET equivalent counts using a conversion factor from a weekly phantom cylinder study. The counts should be decay corrected back to the injection time.

Plasma parent tracer and metabolite concentrations will be determined using solid phase extraction combined with HPLC technology.

Clinical assessment of disease severity will be made by the aGVHD grade. Staging and grading of the disease will be performed by Clinicians at the Bone Marrow and Transplant Clinic using the Glucksberg grade and International bone marrow Transplant Registry Severity Index (IBMTR) at time of enrollment and imaging.

9.1.2 Primary Outcome Measurement Time Points

Duration of the follow up will extend to 6 months post final scan for all aGVHD subjects by chart review.

9.1.3 Response Review

The SUVmax values will be analyzed by Nuclear Medicine and Radiology physicians blinded to the diagnosis and results of the other scan, in a randomized order to avoid bias.
IHC values will be assessed by trained Pathologists.

9.2 **Secondary Outcome**

Healthy subjects group: Biodistribution information and kinetic behavior of the radiotracer.

The secondary outcome is the IHC confirmation of abnormal T cell status in those patients who have had a biopsy.

10 STATISTICAL CONSIDERATIONS

10.1 **Statistical Design**

Multi-arm prospective study of imaging of aGVHD (one group of highly suspected aGVHD and one group at high risk of developing aGVHD) and healthy volunteers.

10.2 Randomization
No randomization will be done.

10.3 Interim analyses
No interim analyses are planned.

10.4 Descriptive Statistics and Exploratory Data Analysis
(see below)

10.5 Analysis
10.5.1 Analysis Population
Analysis population: all ROIs drawn on various organs, as well as areas with elevated SUV relative to healthy volunteers.

10.5.2 Analysis Plan
Although the study will have low power due to the constrained sample size, in addition to descriptive statistics we will perform the following statistical analyses:

- In the highly suspected group:
  Spearman rank correlation between median or maximum SUV values across ROIs and disease severity
- In the high-risk group:
  Spearman rank correlation between SUV values in the first and second scans prediction of disease status at end of follow-up (aGVHD or no aGVHD) from SUV values of the first and second scans (using logistic regression)

10.6 Secondary Analysis
10.6.1 Analysis Population
Analysis population: biopsy samples from the high suspected aGVHD group, as well as any later performed on the high-risk aGVHD group.

10.6.2 Analysis Plan
Although the study will have low power due to the constrained sample size, in addition to descriptive statistics we will perform Spearman rank correlation between the SUV values and IHC measurements in biopsy locations.

10.7 Sample Size
This is a pilot study, for the first time in GVHD patients including up to 25 GVHD patients (roughly half each in the highly suspected and high-risk groups). An additional 5 healthy volunteers are also being included to add to existing bio-distribution data crucial to understand tracer characteristics. While the small sample size provides insufficient power for formal statistical tests, we will examine the data and expect a high level of agreement between uptake of [^{18}F]F-AraG with severity and progression of disease, and IHC results from biopsies

10.8 Accrual estimates
We expect the accrual of 5 healthy volunteers and 25 patients over 24-36 months. Approximately 1-2 high risk patients for GVHD are seen monthly in the Bone Marrow and

Transplant clinic at Stanford Cancer Institute. Established GVHD patients are frequently seen in the clinic (approximately 5 high intensity and 10 low intensity patients at a given time). We consider this planned accrual achievable given our experience with other protocols and the expressed support from the referring physicians, Professor Negrin and Dr Sally Arai.

We plan to prioritize patients from Arm 1; imaging up to 10 patients highly suspected to have aGVHD and analyze scans to ascertain if the tracer is able to image the disease, before recruiting patients from Arm 2 (those that are at high risk of developing the disease post transplant).

10.9 **Criteria for future studies**

If the results of this pilot study are encouraging, a larger phase II study will be designed.

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Appendix A: Inclusion/Exclusion Criteria Checklist

*All subject files must include supporting documentation to confirm subject eligibility. The method of confirmation can include, but is not limited to, laboratory test results, radiology test results, subject self-report, and medical record review.

Protocol Title:	Detection of Graft Versus Host Disease with [^{18}F]F-AraG, a positron emission tomography tracer for activated T cells
Protocol Number:	IRB-38850 / BMT323
Principal Investigator:	Sanjiv Gambhir, MD, PhD

II. Subject Information:

Subject Name/ID:
Gender: ☐ Male ☐ Female

III. Inclusion/Exclusion Criteria

Inclusion Criteria (From IRB approved protocol)	**Yes**	**No**	**Supporting Documentation***
1. Must be 21 years of age or older.	☐	☐	
2. Must understand and voluntarily have signed an Informed Consent after its contents have been fully explained. a) <u>For patients highly suspected to have acute GVHD and requiring systemic therapy</u>, informed consent should be signed after biopsy supporting clinical diagnosis. b) <u>For patients at high risk for developing acute GVHD,</u> informed consent should be signed prior to transplant.	☐	☐	
3. <u>For healthy volunteers only:</u> Must have no known medical problems that would make undergoing the scan hazardous to the health of the patient or interfere with the results. In particular subjects should not have any cardiac or immunological disorders as these would likely affect the scan results. Subjects should have had a full physical	☐	☐	

exam within 6 months of the study. If healthy volunteers have not had a full medical exam within 6 months of the study, one of the nuclear medicine physicians will conduct the medical exam prior to any study procedures.			
4. <u>For patients highly suspected to have acute GVHD and requiring systemic therapy only:</u> Taking steroid treatment for suspected acute GVHD for 7 days or less.	☐	☐	
5. <u>For patients at high risk for developing acute GVHD only:</u> Recipients of myeloablative or reduced intensity allogeneic transplants using either bone marrow or peripheral blood stem cells from HLA-matched or HLA-mismatched related or unrelated donors (protocols 9917, 9142, 9022, 9924) who have not yet been placed on any therapy for acute GVHD.	☐	☐	
Exclusion Criteria (From IRB approved protocol)			
1. Pregnant or nursing	☐	☐	
2. Individuals with known or suspected substance abuse, obtained by self-reporting.	☐	☐	
3. Uncontrolled infection	☐	☐	
4. Relapsed/persistent malignancy	☐	☐	
5. Currently receiving immunotherapy	☐	☐	

IV. Statement of Eligibility

This subject is [☐ **eligible** / ☐ **ineligible**] for participation in the study.

Signature:	Date:
Printed Name:	