PET imaging of TIGIT expression on tumor-infiltrating lymphocytes

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Translational Relevance

Checkpoint blockade therapies have expanded to new inhibitory receptors where stratification for patient selection and therapeutic monitoring strategies are needed. This work develops, validates, and applies PET imaging probes for the T-cell immunoreceptor with Ig and ITIM domains (TIGIT). TIGIT is an inhibitory receptor expressed by tumor infiltration lymphocytes (TILs) including T and NK cells, and is a therapeutic target in a range of solid tumors. Current methods to assess TIGIT expression such as biopsy are invasive and do not provide whole-body quantification of TIGIT expression. To address this need, TIGIT-specific, immunoPET imaging probes are developed and validated. These probes are shown to specifically target TIGIT in both xenograft and allograft murine models. TIGIT expression on TILs in a melanoma allograft model is quantified using PET, confirmed via flow cytometry and ex vivo biodistribution studies. Our data suggest this approach can act as a companion diagnostic for current anti-TIGIT therapies.
Abstract

Purpose: Therapeutic checkpoint inhibitors on tumor-infiltrating lymphocytes (TILs) are being increasingly utilized in the clinic. The T-cell immunoreceptor with Ig and ITIM domains (TIGIT) is an inhibitory receptor expressed on T and natural killer (NK) cells. The TIGIT signaling pathway is an alternative target for checkpoint blockade to current PD-1/CTLA-4 strategies. Elevated TIGIT expression in the tumor microenvironment correlates with better therapeutic responses to anti-TIGIT therapies in pre-clinical models. Therefore, quantifying TIGIT expression in tumors is necessary for determining if a patient may respond to anti-TIGIT therapy. Positron emission tomography (PET) imaging of TIGIT expression on TILs can therefore aid diagnosis and in monitoring therapeutic responses.

Experimental Design: Antibody-based TIGIT imaging radiotracers were developed with the PET radionuclides copper-64 ($^{64}$Cu) and zirconium-89 ($^{89}$Zr). In vitro characterization of the imaging probes was followed by in vivo evaluation in both xenografts and syngeneic tumor models in mouse.

Results: Two anti-TIGIT probes were developed and exhibited immunoreactivity of $>72\%$, serum stability of $>95\%$, and specificity for TIGIT with both mouse TIGIT-expressing HeLa cells and ex vivo activated primary splenocytes. In vivo, the $^{89}$Zr-labeled probe demonstrated superior contrast than the $^{64}$Cu probe due to $^{89}$Zr’s longer half-life matching the TIGIT antibody’s pharmacokinetics. The $^{89}$Zr probe was used to quantify TIGIT expression on TILs in B16 melanoma in immunocompetent mice and confirmed by ex vivo flow cytometry.

Conclusions: This study develops and validates novel TIGIT-specific $^{64}$Cu and $^{89}$Zr PET probes for quantifying TIGIT expression on TILs for diagnosis of patient selection for anti-TIGIT therapies.
Introduction

Immune checkpoint receptors expressed on activated T and NK cells play a key role in maintaining physiologic self-tolerance (1). However, these same regulating pathways are exploited by tumor cells to evade immune surveillance (2). This tumor survival strategy has been targeted with checkpoint blockade therapies such as anti-PD-1 and anti-CTLA-4 agents. While these therapies have shown remarkable responses in a subset of patients, primary and adaptive resistance often occurs (3). This is attributed to a variety of mechanisms including downregulating major histocompatibility complex (MHC) to prevent antigen presentation and upregulation of other inhibitory receptors such as TIM-3, LAG-3, and VISTA (4).

To reduce the occurrence of tumor escape, other inhibitory targets that act through alternative pathways are being extensively explored. One such checkpoint inhibitor is the T cell immunoglobulin and ITIM domain receptor (TIGIT). TIGIT is an inhibitory receptor expressed on CD8+ cytotoxic T cells, CD4+ T helper cells, FOXP3+ regulatory T cells, and NK cells (5). Both TIGIT and PD-1 are expressed in >70% of human melanoma tumor microenvironments, with PD-1 mainly expressed on CD8+ T cells and TIGIT expressed on CD8+ T cells, regulatory T cells, and NK cells (6). The ratio of PD-1 to TIGIT expressed by TILs is variable, ranging from 0.75-4.0 in colorectal cancer (5). TIGIT can inhibit immune responses of effector T and NK cells by acting through inhibitory signals (7-9) and affecting effector functions of T cells and antigen-presenting cells (10,11). TIGIT and PD-1 blockade additively increased proliferation, cytokine production, and degranulation on TILs (12), making TIGIT a potential key addition to current immunotherapies. For this reason, a number of clinical trials in solid tumors are combining TIGIT therapies with current checkpoint blockade inhibitors (13-15).
The expression of TIGIT on tumor-infiltrating lymphocytes (TILs) has been reported via immunohistochemistry, western blotting, and PCR on hepatocellular carcinoma (HCC)(16), follicular lymphoma (FL)(17), squamous cell cancers (SCC)(5), Hodgkin’s lymphoma (HL)(18), renal cell carcinoma (RCC)(19), small cell lung cancer (SCLC)(20), and melanoma(21). TIGIT has been validated as a therapeutic target, and anti-TIGIT therapies are currently being evaluated in multiple clinical trials (Supplementary Table 1). An antibody specific for the TIGIT receptor (AB154) is being evaluated in a variety of solid cancers (NCT03628677). This phase I trial is evaluating the safety, tolerability, pharmacokinetics, pharmacodynamics and clinical activity of AB154 as either a monotherapy or in combination with an anti-PD-1 antibody (AB122). In another phase I trial, the anti-TIGIT agent MK-7684 and pembrolizumab was administered in 34 patients with advanced solid tumors for whom standard treatment options had failed (NCT02720068). The response rate was reported as 19% and the disease control rate was 47% (22). While these results are promising for a subset of patients, patient stratification for these therapies would benefit from a quantitative, whole-body measure of TIGIT expression.

Generally high but variable TIGIT expression is seen in solid tumors, making this therapy likely applicable to many cancer types. However, to determine TIGIT expression, invasive biopsy sampling followed by immunohistochemistry (IHC) is currently required. While this procedure is the current gold standard, it does not allow accurate quantitation in heterogeneous samples or on distant metastases. A noninvasive imaging agent could detect and quantify TIGIT within the tumor microenvironment and aid in determining what patients would benefit from TIGIT immunotherapies(23). This approach has been tested with PET agents for PD-1, PD-L1, and Ox40 all being developed as clinical diagnostics (24-26). These PET tracers allow non-invasive, whole-body, quantitative assessment of receptor expression to aid in patient
stratification. Here we develop TIGIT-specific antibody PET tracers and evaluate their application in quantifying TIGIT expression in vivo (Figure 1).

Experimental procedures

Antibodies, Chelators, and Radiometals

Purified anti-mouse TIGIT monoclonal antibody (TIGITmAb, clone 1G9) raised in mouse was purchased from Bio X cell (catalogue # BE0274) (West Lebanon, NH) or Biolegend (catalogue #142102) (San Diego, CA). NHS ester-DOTA (NHS-DOTA, Catalogue # B-280) and p-SCN-Deferoxamine (DFO-NCS, Catalogue # B705) were purchased from Macrocyclics (Dallas, TX). Copper-64 ($^{64}$Cu) ($t_{1/2}$ = 12.7 h, 17.76-162.8 GBq/μmol) was obtained from the University of Wisconsin. Zirconium-89 ($^{89}$Zr) in 1.0 M oxalic acid ($t_{1/2}$ = 3.3 d, purity >99%; 2–3 GBq/mL) was obtained from the University of Alabama at Birmingham (Birmingham, AL). All other general reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Cell Lines ATCC

B16–F10 (murine melanoma) and HeLa (human cervical) cells were purchased from American Type Culture Collection (ATCC ® CRL-6475™). A HeLa cell line that expressing the TIGIT receptor was generated using a plasmid from VectorBuilder (Chicago, IL) consisting of the mouse TIGIT sequence driven by the EF1α promoter, eGFP driven by the CMV promoter, and a puromycin sequence for selection (Supplementary Figure 1). Transfection was performed using Lipofectamine 3000™ (Thermo Fisher Scientific, Waltham, MA). Cells were sorted 3 times on a
FACSaria II sorter with the top 1% of TIGIT staining collected. The transfected HeLa cells (TIGIT-HeLa) were kept under selection using DMEM supplemented with 10% fetal bovine serum (FBS), 1% Antibiotic-Antimycotic, and 40 μmol/mL of puromycin. All cell lines were screened to exclude infection with mycoplasma and preserved for a period of 20 passages in culture.

**Qualitative and quantitative flow cytometry on transfected HeLa cells**

Qualitative flow cytometry was performed on TIGIT-HeLa cells and non-transfected HeLa cells by staining with PE-mTIGIT antibody (2 μL per 10⁶ cells) and DAPI (1 μL of 10mg/mL per 10⁶ cells). Quantitative flow cytometry was performed on an LSR II instrument to determine the average number of TIGIT receptors per cell using a Qifikit (Agilent, Santa Clara, CA).

**TIGIT expression on isolated mouse lymphocytes**

Flow cytometry was completed on isolated, activated C57Bl/6J (Jackson Laboratory) mouse splenocytes to assess antibody affinity for mouse TIGIT. Splenocytes were activated via phorbol 12-myristate 13-acetate (PMA) and calcium ionomycin (50:500 ng/mL for 4 h) at 37°C(27). Cell staining was performed in 96-well microtiter plates (U-bottom). 100 μL of the antibody was mixed with 100 μL cell suspension containing 2 × 10⁵ splenocytes and incubated for 1 h at 4°C. Cells were washed three times with FACS buffer, followed by a secondary stain with 100 μL of anti-mouse IgG-APC (Biolegend, Cat# 409305) pre-diluted to 1:100. The plate was incubated for 30 min on ice in the dark and washed three times. Propidium iodide (PI) was
used as a live/dead stain. Analysis was carried out on a flow cytometer (FACS Aria III, BD Biosciences, San Jose, CA) and data was analyzed by FlowJo FACS analysis software (Tree Star, Ashland, OR).

**DOTA- and DFO- TIGITmAb conjugation**

DOTA was conjugated to mouse TIGITmAb at pH 7.3, 1 mg/mL Ab concentration, and a 20:1 DOTA:TIGITmAb ratio at 4°C for 14 h. The DOTA-TIGITmAb conjugate was purified using 50 kDa spin filters (Millipore) with 0.1 M ammonium acetate (pH 5.4) as the buffer. DOTA-TIGITmAb was washed 3 times and stored at 4°C at a concentration of 1-2 mg/mL.

DFO-TIGITmAb was conjugated to mouse TIGITmAb at pH 8.5 in HEPES buffer, 6 mg/mL Ab concentration, and a 5:1 DFO:TIGITmAb ratio at 37°C for 14 h. The DFO-TIGITmAb conjugate was purified using a SEC 2000 HPLC with 0.1 M ammonium acetate buffer (pH 7) as the mobile phase eluted at 1 mL/min. The purified DFO-TIGITmAb was concentrated to 5 mg/mL using a Vivaspin 30 kDa cut-off centrifugal filter and stored in 100 μl aliquots in 0.1 M ammonium acetate buffer (pH 7.0) at -20°C.

**Radiolabeling DOTA- and DFO- TIGITmAb**

$^{64}$Cu was added to DOTA-TIGITmAb (1 mg/mL) in 0.1M ammonium acetate (pH 5.3) at a ratio of 0.37 MBq/μg Ab and incubated at 37°C for 30-45 min. 10 μL of 50 mM ethylenediaminetetraacetic acid (EDTA) was added for 15 minutes to scavenge free $^{64}$Cu. $^{64}$Cu-TIGITmAb was purified using 50 kDa spin filters with saline as the buffer. $^{89}$Zr in 1.0 M oxalic acid was first brought to a pH of 7.0-7.4 with 1.0 M sodium carbonate. $^{89}$Zr (74–75 MBq; 200
μL) was then added to DFO-TIGITmAb (400 μg; 5 mg/mL) in 1X PBS (pH 7.3) and incubated at 37°C for 1 h. After incubation, 10 μL of 50 mM EDTA was added for 15 min to scavenge free \(^{89}\text{Zr}\). Purification of \(^{89}\text{Zr}\)-TIGITmAb was achieved via SE HPLC in PBS buffer [0.1 mol/L NaCl, 0.05 mol/L sodium phosphate (pH 7.4)] at a flow rate of 1.0 mL/min. The \(^{89}\text{Zr}\)-TIGITmAb peak corresponding to the antibody was collected and concentrated using a Vivaspin 30 kDa centrifugal filter and centrifuged at 3000 g for 15 min. The final product was filtered through a 0.2 μm filter into a sterile vial. Serum stability for \(^{64}\text{Cu}\)-TIGITmAb and \(^{89}\text{Zr}\)-TIGITmAb was conducted by addition of 3.7 MBq radiotracer to 100 μL of mouse or human serum with time points taken every 24 h.

### In vitro Binding Assays

The immunoreactivity of \(^{64}\text{Cu}\)-TIGITmAb and \(^{89}\text{Zr}\)-TIGITmAb was assessed via Lindmo assay(28). \(^{64}\text{Cu}\)-TIGITmAb and \(^{89}\text{Zr}\)-TIGITmAb were prepared at a concentration of 0.037 MBq in 2 mL of PBS with 1% BSA. 50 μL of the radiolabeled antibody was added to a serial dilution of TIGIT receptor-positive HeLa cells ranging from 5 x 10^4 to 5 x 10^6 cells per vial (500 μL in PBS with 1% BSA added). After an incubation at room temperature for 30 min, the solutions were centrifuged at 300 g, supernatant removed, and pellets counted using a gamma counter. Non-transfected HeLa cells were also tested with the radiolabeled antibodies, as described above. \(^{89}\text{Zr}\)-TIGITmAb binding was also assessed on isolated splenocytes. Two sets of experiments were performed (blocking and non-blocking; n=3). Blocking samples received a 25-fold excess of cold TIGITmAb prior to the addition of \(^{89}\text{Zr}\)-TIGITmAb (2.3 μCi/mL; 200 μL per tube). The solutions were incubated for 1 h at 25 °C, washed, and activity bound to the cell pellets were counted on a gamma counter.
Small Animal PET/CT Imaging

Animal studies were performed in compliance with approval from the Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University. PET/CT imaging was performed on a Siemens Inveon small animal multimodality PET/CT system (Preclinical Solutions; Siemens Healthcare Molecular Imaging, Knoxville, TN). CT imaging was performed at 80 kVp at 500 μA, second bed position, half scan 220° of rotation, and 120 projections per bed position with a cone beam micro-X-ray source (50 μm focal spot size) and a 4064 × 4064-pixel X-ray detector. The data was reconstructed using Shepp-Logan filtering and cone-beam filtered back-projection. The small animal PET scanning [static scans with a coincidence timing window of 3.4 ns and energy window of 350 to 650 keV] was performed at the following time points after the tracer injection: 4, 24 and 48 h for 10 min, and 72 h for 15 min scans. The acquired images were reconstructed with two-dimensional ordered-subset expectation maximization (OSEM 2D) algorithm. PET/CT images and three-dimensional regions of interest (ROI) of organs of interest were computed using Inveon Research Workplace software. The mean pixel values within the ROI volume was converted to radioactivity concentration in counts per milliliter per minute (cpm) by using a predetermined conversion factor. The percentage-injected dose per gram of tissue (%ID/g) was determined by dividing each tissue’s cpm obtained from the region of interest by the injected dose. At each time point before imaging, animals were anesthetized and scanned at the same conditions as detailed above. A Hidex gamma counter was used for ex vivo radio-analysis.
64Cu- and 89Zr-TIGITmAb PET/CT imaging of TIGIT-expressing xenografts

6-10-week-old nu/nu female mice (average weight = 23.0 ± 2.0 g) were injected in the flank with 7.5 x 10^5 cells in 100 μL matrigel. TIGIT-positive HeLa cells were injected into the left flank and TIGIT-negative HeLa cells were injected into the right flank. Xenografts were allowed to develop for 10 days. 64Cu-TIGITmAb (or 64Cu-mouse IgG, n=4 per probe) was injected via tail vein at 2.2 -3.7 MBq (10-15 μg) in 100 μL of saline and imaged at 24 and 48 h followed by a terminal ex vivo biodistribution study. 89Zr-TIGITmAb (or 89Zr-mouse IgG, n=4 per probe) were injected via tail vein at 1.85-3.7 MBq (15-20 μg) in 100 μL of saline and imaged at 24, 48, and 72 h followed by a terminal ex vivo biodistribution study. Gamma counter values were background subtracted and decay corrected to the time of injection and the percent-injected dose per gram (%ID/g) for each tissue sample was calculated by normalization to the total activity injected. Animal experiments were conducted twice.

89ZrTIGITmAb PET/CT imaging of TIGIT expressing TILs in melanoma allografts

6-8 weeks old female B6 mice were implanted with 1x10^6 B16F10 melanoma cells in the right shoulder and developed allografts over 10-12 days. Two groups of mice (blocking and non-blocking; n=5) received 89Zr-TIGITmAb (200 μL, 1.7-2.1MBq, 15-16 μg of DFO-TIGITmAb) via tail vein injection. Blocking was completed by injection of cold TIGIT (200 μg in 100 μL) 2 h prior to 89Zr-TIGITmAb administration. The mice were imaged at 4, 24, 48 and 72 h post-injection and immediately followed by a terminal biodistribution study.
Ex vivo flow cytometry of mTIGIT on melanoma allografts

B6 mice (n=5) were implanted with B16F10 melanoma allografts as described above. After 10 days of tumor growth, mice were euthanized, and tumors removed. Liberase TL (300 µg/mL) and DNase I (100 µg/mL) in 5 mL HBSS were added to dissociated tumors for 1 h at 37°C with gentle mixing every 15-20 minutes followed by washing in HBSS. Subsequently ACK buffer was added to samples for 10 minutes to lyse red blood cells followed by two washes in cell stain buffer. Single cell suspensions were prepared and stained for mTIGIT (2 µL Ab per 10^6 cells) and DAPI (10 µg/ml final concentration) as a viability stain as described above. Samples were run in triplicate, and at least 2×10^5 live cells per sample were collected. Analysis was completed on an LSR II flow cytometer. Ex vivo flow cytometry was completed twice.

Statistical Analyses

Unpaired Student’s t-test (two-tailed, unequal variance) was used for data comparisons. P values of less than 0.05 were considered statistically significant.

Results

Mouse TIGIT expression on engineered cell lines and activated splenocytes

Following 3 rounds of cell sorting under puromycin selection, >98% of HeLa cells expressed the mouse TIGIT receptor (Fig. 2A, Supplementary fig. 2) with 95200±15900 TIGIT receptors/cell (Fig. 2B). Splenocytes activated ex vivo were positive for TIGIT and DFO-
conjugated TIGITmAb showed similar affinity as the unconjugated TIGITmAb for activated splenocytes, demonstrating that DFO conjugation did not adversely affect binding (Fig. 2C). These results validate the expression of TIGIT in transfected HeLa cells, as well as the specifically of TIGITmAb for the TIGIT receptor on primary splenocytes.

**Synthesis and radiolabeling of Immunoconjugates**

DOTA and DFO were successfully conjugated to the TIGITmAb with 1.59±0.30 DOTA molecules and 0.90±0.17 DFO molecules per TIGITmAb (Supplementary Table 2), as determined by MALDI-MS (Supplementary fig 3). The radiochemical yield of $^{64}$Cu-TIGITmAb was 60-75% and the specific activity of 0.222-0.370 MBq/µg, while $^{89}$Zr-TIGITmAb had a radiochemical yield of 60-80% and a specific activity of 0.074-0.185 MBq/µg. The final purity of both radiotracers was >98% (Supplementary fig. 4). The serum stability of $^{64}$Cu-TIGITmAb was > 95% out to 48 h (Figure 3A) and that of $^{89}$Zr-TIGITmAb was >95% out to 96 h (Figure 3D). Lastly, $^{64}$Cu-TIGITmAb was further evaluated using SE HPLC and showed a radiochemical purity of 98.3±1.5% (mean ± STD) (Supplementary fig. 4C).

$^{64}$Cu- and $^{89}$Zr-TIGITmAb binding assays demonstrate high specificity and immunoreactivity

$^{64}$Cu-TIGITmAb and $^{89}$Zr-TIGITmAb showed at least 40-fold higher binding to TIGIT-HeLa cells, compared to the radiolabeled isotype control and low non-specific binding to TIGIT-negative HeLa cells ($p < 0.05$, Figure 3B, 3E). $^{64}$Cu-TIGITmAb and $^{89}$Zr-TIGITmAb had immunoreactivities of 72.7±1.7% and 80.5±2.2 %, respectively (Figure 3C, 3F). $^{89}$Zr-
TIGITmAb demonstrated high specificity on activated splenocytes when uptake study was performed with in the presence of excess cold TIGITmAb (Figure 3G).

$^{64}$Cu-TIGITmAb and $^{89}$Zr-TIGITmAb PET Imaging of TIGIT-expressing xenografts in nu/nu mice demonstrate the specificity of probes uptake

Both PET probes showed specific uptake in HeLa-TIGIT expressing xenografts ($n = 4$), compared to TIGIT-negative xenografts or isotype controls ($p < 0.05$). $^{64}$Cu-TIGITmAb showed specific uptake in HeLa-TIGIT at 24 h, with increased uptake at 48 h (Fig. 4A-B). The 48 h ex vivo biodistribution (Fig 4C) of $^{64}$Cu-TIGITmAb and $^{64}$Cu-mIgG showed $^{64}$Cu-TIGITmAb uptake in HeLa-TIGIT xenografts of 21.9±3.5 %ID/g, compared to $^{64}$Cu-mIgG uptake of 9.8±2.2 %ID/g ($p < 0.05$). However, blood activity levels at 48 h were 13.4±0.8 %ID/g due to the pharmacokinetics of the antibody.

At 72 h post-injection, $^{89}$Zr-TIGITmAb demonstrated higher tumor-to-background signal ratios than $^{64}$Cu-TIGITmAb due to higher tumor uptake and lower blood activity (Fig. 5A). This was confirmed by ex vivo biodistribution results at 72 h (Fig. 5B), where $^{89}$Zr-TIGIT uptake in HeLa-TIGIT xenografts was 29.3±4.5 %ID/g, compared to $^{89}$Zr-mIgG uptake of 8.8±0.8 %ID/g ($p < 0.05$). Blood activity levels at 72 h were 9.7±1.0 %ID/g, significantly lower than $^{64}$Cu-TIGITmAb at 48 h ($p<0.05$). $^{89}$Zr-TIGITmAb was chosen for further studies due to the combination of higher tumor uptake and lower blood activity levels compared to $^{64}$Cu-TIGITmAb at later time points.
\(^{89}\)Zr-TIGITmAb PET/CT imaging of TIGIT expressing TILs in melanoma allografts demonstrates quantification of TIGIT in the tumor microenvironment

\(^{89}\)Zr-TIGITmAb PET signals at the tumor site indicated the presence of TIGIT on TILs. Representative axis and coronal immunoPET images of mice bearing melanoma at various time points (4, 24, and 72 h post injection of tracer) are shown in Figure 6A. PET ROI quantification (Fig. 6B) correlated with biodistribution results at 72 h post injection (Fig. 6C). \(^{89}\)Zr-TIGITmAb uptake by TIGIT bound TILs in non-blocking and blocking mice at 72 h was (mean % ID/g ± SD) 7.4 ± 0.9 and 3.81 ± 0.75, respectively (p < 0.05). Tumor-to-muscle uptake ratios for \(^{89}\)Zr-TIGITmAb in non-blocking mice were 4.1 ± 0.2 and 7.4 ± 0.7 at 4 h and 72 h (black bar), respectively (p < 0.05) (Fig. 6D).

*Ex vivo* flow cytometry on melanoma allografts and immunofluorescence staining

Flow cytometry on single cell suspensions of B16 tumors from five mice showed TIGIT expression on infiltrating TILs. 10.91+/−2.22% of live cells in the tumor microenvironment were positive for TIGIT (Fig. 6E), showing TIGIT expression in this tumor microenvironment as previously shown (29). Further, from a separate group (n=4) melanoma allograft tissue was harvested and prepared several sections from inter and intra melanoma. These FFPE-tissue slides were stained to determine the expression pattern of both TILs and TIGIT by using CD45 and TIGITmAb (Supplementary Figure 5). Low TIGIT expression correlated with low CD45 staining (Supplementary Figure 5D) with the majority of TIGIT expression associated with the CD45⁺ TILs (Supplementary Figure 5C).
**Discussion**

This work develops and validates TIGIT-specific PET agents for quantification of TIGIT expression in the tumor microenvironment. We demonstrate that $^{64}$Cu-TIGITmAb and $^{89}$Zr-TIGITmAb are highly specific molecular imaging probes for assessing TIGIT expression *in vivo*. While both probes detect the TIGIT receptor in TIGIT-HeLa xenografts, $^{89}$Zr-TIGITmAb showed superior contrast due to better matching of full-length antibody pharmacokinetics, which are slow to clear from circulation. $^{89}$Zr-TIGITmAb was therefore evaluated in immunocompetent B6 mice with B16 melanoma allografts and demonstrated the ability to quantitatively image TIGIT expression from 4 to 72 hours post-injection. Blocking studies with excess cold TIGITmAb decreased the PET signals in the tumor, demonstrating specificity of $^{89}$Zr-TIGITmAb. Flow cytometry confirmed TIGIT expression on T and NK cells of TILs in the tumor microenvironment. These results indicate $^{89}$Zr-TIGITmAb as a potential companion diagnostic for current and upcoming TIGIT therapies. The ability to quantify TIGIT expression in solid tumors could aid in treatment strategies prior to starting a particular immunotherapy regimen.

While this work has demonstrated the ability of quantitative TIGIT imaging in solid tumors, it must be noted that the murine TIGIT receptor was targeted. This was necessary to test the probe in TIGIT-expressing immune cells in immunocompetent mice; however, developing a human TIGIT imaging probe is necessary for clinical translation. A companion diagnostic for
TIGIT therapy, much like $^{64}$Cu-pembrolizumab for human PD-1 could aid in therapeutic decisions prior and throughout the regimen. These results warrant the future development of a human TIGIT PET imaging agent for quantifying TIGIT expression in the tumor microenvironment for stratification of patients for anti-TIGIT therapy.

Conclusions

In conclusion, we developed and radiolabeled novel anti-TIGIT probes for immunoPET imaging of TIGIT expression in xenograft and melanoma allograft murine models. Furthermore, specific in vivo binding of anti-TIGIT probes were confirmed via quantitative flow cytometry and ex vivo biodistribution studies. This study demonstrates the potential for clinical translation of these radiotracers.
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Conception and design: T.M. Shaffer, A. Natarajan, S.S. Gambhir

Development of methodology: T.M. Shaffer, A. Natarajan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.M. Shaffer, A. Natarajan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.M. Shaffer, A. Natarajan

Writing, review, and/or revision of the manuscript: T.M. Shaffer, A. Natarajan, S.S. Gambhir

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.M. Shaffer, A. Natarajan

Study supervision: S.S. Gambhir
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Legends for Figures

**Figure 1.** Quantifying TIGIT expression on TILs can aid in therapeutic decisions. A TIGIT PET agent can act as a companion diagnostic selection of patients for anti-TIGIT therapies. TCR=T cell receptor, MHCI= major histocompatibility complex I, CD155=ligand for TIGIT receptor.

**Figure 2.** Flow cytometry of the mTIGIT receptor. A) HeLa cells transfected with a plasmid expressing the mTIGIT receptor were stained with PE-TIGIT (light blue) or PE-IgG (red). B) Quantitative flow on transfected TIGIT-expressing cells shows an average of $9.5 \times 10^4$ receptors/cell. C) Live cell binding assay for the determination of binding specificity of TIGITmAb and DFO-TIGITmAb on activated mouse splenocytes. Splenocytes were activated with PMA and calcium ionomycin (50:500 ng/mL for 4 h). Cells ($1 \times 10^6$) were stained with isotype control mouse-IgG, TIGITmAb or DFO-TIGITmAb, followed by anti-mouse-IgG-APC staining. Histogram traces correspond to induced splenocytes stained with the following mAbs (1 µg/0.1mL): mouse IgG isotype control (open histogram), DFO-TIGITmAb (dotted histogram), and unmodified TIGITmAb (filled histogram) for TIGIT expression.

**Figure 3.** Radiochemical and *in vitro* evaluation of the TIGIT PET probe. A) $^{64}$Cu-TIGITmAb serum stability is >95% over 48 h. B) $^{64}$Cu-TIGITmAb demonstrated high specificity for TIGIT-HeLa cells compared to IgG controls and TIGIT-negative cells. C) $^{64}$Cu-TIGITmAb had an immunoreactivity of 72.7%. D) $^{89}$Zr-TIGITmAb serum stability is >95% over 96 h. E) $^{89}$Zr-
TIGITmAb demonstrated high specificity for TIGIT-HeLa cells compared to IgG controls and TIGIT-negative cells. **F)** $^{89}$Zr-TIGITmAb had an immunoreactivity of 80.5%. **G)** $^{89}$Zr-TIGITmAb showed high specificity for activated splenocytes expressing the TIGIT receptor. Mean and standard deviation shown in A, B, D, E, G. 95% confidence intervals shown in C and F (red dotted line).

**Figure 4.** $^{64}$Cu-TIGITmAb PET/CT imaging and *ex vivo* biodistribution in xenografts (n=4 per group). $^{64}$Cu-TIGITmAb showed higher uptake in the TIGIT-positive Hela tumor (left flank, solid circle) compared to TIGIT-negative HeLa WT tumor (right flank, dotted circle) at 24 h (**A**) and 48 h (**B**) time points. **C)** *Ex vivo* biodistribution confirmed the $^{64}$Cu-TIGITmAb specificity, although blood activity was still high at 48 h (13.4±0.8 %ID/g), n=7, conducted independently two times.

**Figure 5.** $^{89}$Zr-TIGITmAb PET/CT imaging and *ex vivo* biodistribution in xenografts (n=4 per group). **A)** $^{89}$Zr-TIGITmAb showed uptake in the TIGIT-positive Hela tumor (left flank, solid circle) compared to TIGIT-negative HeLa WT tumor (right flank, dotted circle) at 72 h post-injection. **B)** *Ex vivo* biodistribution confirmed the $^{89}$Zr-TIGITmAb specificity along with lower blood activity levels (9.7±1.0 %ID/g) at 72h compared to $^{64}$Cu-TIGITmAb at 48 h (p<0.05), n=7, conducted independently two times.

**Figure 6.** *In vivo* imaging and quantification of PET signals corresponds to $^{89}$Zr-TIGITmAb in B6 mice with B16 allografts. **A)** PET/CT images of in B6 mice with B16 allografts injected with $^{89}$Zr-TIGITmAb were imaged at various time points (n=3). Blocking mice received a cold TIGITmAb pre-dose (200 µg) 2 h prior to tracer injection. L denotes liver, H denotes heart and T
denotes tumor. B) PET quantification shows specificity of $^{89}$Zr-TIGITmAb for Nblk tumor is 7.4 ± 0.9 %ID/g (p<0.05). C) Ex vivo biodistribution shows specificity of $^{89}$Zr-TIGITmAb for TIGIT expression on TILs. D) Tumor-to-muscle ratios of 7.4 ± 0.7 demonstrate the specific uptake of $^{89}$Zr-TIGITmAb in the tumor, with a reduced ratio in mice that were pre-administered with the cold TIGIT. White bars represent mice that received the blocking antibodies, and black bars present mice that did not receive the blocking antibodies. E) Flow cytometry on excised B16 tumors (n= 5) shows TIGIT expression on cells in the tumor microenvironment. TIGIT-positive cells ranged from 8.88 -12.39% of the live cells within the tumor microenvironment (n=5 subjects). Grey histogram represents the isotype control, red histogram represents the TIGIT staining. * denotes p<0.05.
Figure 1

Anti-TIGIT therapy

= Anti-TIGITmAb blocks the TIGIT receptor in TME to activate NK and T cells

Alternate therapy
Figure 2

A. Flow cytometry on TIGIT-expressing cells

B. TIGIT receptor expression on HeLa cells

C. Flow cytometry activated splenocytes

Legend:
- PE-Ab
  - PE-TIGITmAb
  - PE-IgG

- mlG-isotype
- Unmodified TIGITmAb
- DFO-TIGITmAb
**Figure 3**

**A** 64Cu-TIGITmAb serum stability

![Graph showing serum stability of 64Cu-TIGITmAb](image)

**B** 64Cu-TIGITmAb specificity

![Graph showing specificity of 64Cu-TIGITmAb](image)

**C** 64Cu-TIGITmAb immunoreactivity

**D** 89Zr-TIGITmAb serum stability

![Graph showing serum stability of 89Zr-TIGITmAb](image)

**E** 89Zr-TIGITmAb specificity

![Graph showing specificity of 89Zr-TIGITmAb](image)

**F** 89Zr-TIGITmAb immunoreactivity

![Graph showing immunoreactivity of 89Zr-TIGITmAb](image)

**G** 89Zr-TIGITmAb affinity for activated splenocytes

![Graph showing affinity for activated splenocytes](image)
Figure 4

A 24 h  15%ID/g  B 48 h

$^{64}$Cu-TIGITmAb  $^{64}$Cu-IgG  $^{64}$Cu-TIGITmAb  $^{64}$Cu-IgG

$^{64}$Cu-TIGITmAb biodistribution

$P < 0.05$

$\%$ID/g

- $^{64}$Cu-TIGITAb
- $^{64}$Cu-mlgG

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**Figure 5**

**A**

72 h

**B**

$^{89}$Zr-TIGITmAb biodistribution

$P < 0.05$

- $^{89}$Zr-TIGITmAb
- $^{89}$Zr-IgG

%ID/g

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**Figure 6**

**A** PET/CT images of $^{89}$Zr-TIGITmAb

**B** $^{89}$Zr-TIGITmAb PET quantification

**C** $^{89}$Zr-TIGITmAb tracer BioD in melanoma mice at 72h p.i.

**D** $^{89}$Zr-TIGITmAb Tumor-to-muscle ratios

**E** DAPI and PE-TIGITmAb fluorescence.
PET imaging of TIGIT expression on tumor-infiltrating lymphocytes

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