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Use of a Leukocyte-Targeted Peptide Probe as a Potential Tracer for Imaging the Tuberculosis Granuloma

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Summary

Granulomas are the histopathologic hallmark of tuberculosis (TB), both in latency and active disease. Diagnostic and therapeutic strategies that specifically target granulomas have not been developed. Our objective is to develop a probe for imaging relevant immune cell populations infiltrating the granuloma. We report the binding specificity of Cyanine 3 (Cy3)-labelled cFLFLFK-PEG₁₂ to human leukocytes and cellular constituents within a human *in vitro* granuloma model. We also report use of the probe in *in vivo* studies using a mouse model of lung granulomatous inflammation. We found that the probe preferentially binds human neutrophils and macrophages in human granuloma structures. Inhibition studies showed that peptide binding to human neutrophils is mediated by the receptor formyl peptide receptor 1 (FPR1). Imaging the distribution of intravenously administered cFLFLFK-PEG₁₂-Cy3 in the mouse model revealed probe accumulation within granulomatous inflammatory responses in the lung. Further characterization revealed that the probe preferentially associated with neutrophils and cells of the monocyte/macrophage lineage. As there is no current clinical diagnostic imaging tool that specifically targets granulomas, the use of this probe in the context of latent and active TB may provide a unique advantage over current clinical imaging probes. We anticipate that utilizing a FPR1-targeted radiopharmaceutical analog of cFLFLFK in preclinical imaging studies may greatly

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contribute to our understanding of granuloma influx patterns and the biological roles and consequences of FPR1-expressing cells in contributing to disease pathogenesis.

Keywords

Mycobacterium Tuberculosis; Granuloma; Phagocytes; Infection Imaging; Peptide Probe; FPR1

1.1 INTRODUCTION

Active Tuberculosis (TB) disease afflicts 10.4 million people and accounts for 1.4 million deaths per year [1]. These numbers represent the tip of the iceberg since the majority of individuals who become infected develop latent TB infection (LTBI) with the potential to reactivate later in life [2]. Alarming, our progress in halting infection and disease is being threatened by drug-resistant cases, which require prolonged antibiotic treatment times with suboptimal therapies and outcomes, potentiating disease transmission. These statistics highlight the need not only for new diagnostic and therapeutic solutions, but also the need to gain a more mechanistic understanding of the immunological events that dictate disease outcome. In this regard, the events leading to LTBI and active disease take place in and around the dense, multi-cellular granuloma: the body's signature response for achieving containment of infection. The bacterium *M. tuberculosis* (*M.tb*) survives in granulomas during LTBI until host immunity and granuloma structure break down, leading to active disease and transmission. Granulomas can vary widely in terms of their containment ability and cellular makeup, even within a single host [3]. They are also not static entities, undergoing dynamic shifts in cell populations and in metabolic profiles presumably through different cell activation states with characteristic surface protein phenotypes [4–6]. Owing to its noninvasive and organ-level 3D assessment capability, imaging approaches are providing new and exciting opportunities to investigate host-pathogen interactions in TB.

Positron emission tomography (PET) imaging of [^{18}F] fluorodeoxyglucose (FDG) has been applied to study granuloma glucose utilization over the course of TB disease in animal models [7,8]. While FDG offers exquisite sensitivity, its main limitations when applied to TB are that it is difficult to associate FDG signal with specific cell types within the granuloma and to interpret the meaning of FDG uptake. For example, activated immune cells and a healing endothelium/epithelium both have elevated glucose demands [9] but are likely associated with different disease trajectories. Additionally, the emergence of FDG-avid lesions in the lung following a microbiologically confirmed cure further highlights the challenges of FDG uptake interpretation in the context of TB [10].

Advancing on this approach, longitudinal imaging of finely tuned probes that have favorable pharmacokinetics and show cell selectivity in their binding would greatly contribute to our understanding of the TB granuloma. The development of imaging probes that directly target pathogenic bacteria, including mycobacteria, is a promising approach. Para-aminobenzoic acid [11] and antimicrobial peptide-derived pharmaceuticals [12,13] are being developed to visualize *M.tb* but it is not certain how effective they will be in targeting *M.tb* within granulomas as well as during periods of bacterial dormancy. Alternatively, probes that target

inflammatory cells involved in TB-associated inflammation could be valuable for tracking *M.tb*-host interactions within the granuloma. The ligand DPA-713 has been shown to target and bind macrophages expressing the translocator protein in settings of neuroinflammation and *M.tb* infection models [14]. Preclinical imaging studies with DPA-713 have shown promise in targeting activated macrophages in pulmonary granulomas. However, the probe does accumulate in normal thyroid and gallbladder tissue and the biological interpretation of DPA-713 uptake in granulomas is yet to be determined.

The peptide cinnamoyl-phenylalanine-(D) leucine-phenylalanine-(D) leucine-phenylalanine (cFLFLF) has high affinity for leukocytes yet does not activate or hinder cell chemotaxis upon binding [15,16]. Data accumulated across several studies have implicated the formyl peptide receptor 1 (FPR1) as the primary receptor recognized by the peptide [16–18]. FPR1 belongs to a family of G protein-coupled pattern recognition receptors, which are mainly expressed by mammalian leukocytes and are important in innate immunity and host defense [19]. Cell surface FPR1 expression can be upregulated by certain stimuli and under inflammatory conditions [20], making it an attractive target for imaging inflammatory-driven diseases. To improve solubility and bioavailability for successful imaging *in vivo*, the peptide is routinely conjugated to a polyethylene glycol (PEG) moiety. Labeled and PEG-conjugated cFLFLF was previously shown to successfully target neutrophils and macrophages in several different inflammatory models including those involving the acute infections in the lung [15]. No studies to date have investigated the use of this probe in the context of imaging TB-associated inflammation. In this study, we evaluated cFLFLFK-PEG₁₂-Cy3 as an imaging biomarker for neutrophils and potentially other inflammatory cells in the TB granuloma environment. Immune cells purified from human blood were used to initially characterize the cFLFLFK-PEG₁₂-Cy3 binding profile. A human cell-based *in vitro* model was used to evaluate peptide binding to multi-cellular granuloma-like structures induced by *M.tb*. Finally, a mouse model was used to evaluate the *in vivo* cellular specificity of cFLFLFK-PEG₁₂-Cy3 in the setting of lung granulomatous inflammation.

1.2 EXPERIMENTAL DESIGN AND METHODS

1.2.1 Synthesis and Labeling of cFLFLFK-PEG₁₂, cFLFLFK-PEG₁₂-Cy5, and cFLFLFK-PEG₁₂-NOTA

All chemicals obtained commercially were of analytic grade and used without further purification. Starting peptides and their conjugates displayed herein were synthesized in two stages. The peptide cinnamoyl-phenylalanine-(D) leucine-phenylalanine-(D) leucine-phenylalanine lysine (cFLFLFK) was synthesized via a solid-phase Fmoc method. Fmoc-N-amido-dPEG₁₂-acid (molecular weight, 839 D) was obtained from Quanta Biodesign, LTD. S-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) was obtained from Macrocyclis, Inc. The peptide synthesis was performed as follows. For each mmol of amine on the Rink amide resin, 4 mmol of protected amino acid was activated with 4 mmol of the coupling agent HBTU and 8 mmol of Diisopropylethylamine (DIEA) for 5 min. The activated acid was then transferred to the amine resin on the solid phase and the reaction vessel was shaken for an hour. The final products and the protection groups were released from the resin using 10 mL of a cocktail containing trifluoroacetic acid (TFA),

phenol, tris-isopropylsilane (TIPS) and water at a ratio of 95:2:2:1. The process was repeated and the mixture was then precipitated into methyl-*tert*-butyl ether. The precipitate was filtered and the crude solid was purified by preparative high-performance liquid chromatography (HPLC, Shimadzu, LC-8A) using a solvent system of A (0.1% TFA in water) and B (0.1% TFA in acetonitrile). The proportion of acetonitrile in solvent B ascended from 10 to 100% during the run on a Sunfire C18 column (50 × 250 cm, i.d., 10 μm particle size, Waters, Milford, MA, USA) with a 60 min runtime at 1 mL/min. Peaks were visualized with a UV-Vis detector (220 nm) and the purity of the product was determined by HPLC analysis. The product mass was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy (Bruker Daltonics, Bremen, Germany). The fractions with the required mass and purity were pooled and freeze dried to yield the product as a colorless fluffy solid. The lyophilized products were reconstituted in DMSO and water, aliquoted, and kept at −20°C until use.

Fluorescent labeling—4-methyl morpholine (5 μL) was added to equimolar amounts of purified peptide (10.3 mg, 0.66 μmols) and Cy3-NHS ester (4.0 mg, Lumiprobe) in dry DMSO (250 μL). The resultant mixture was incubated at 40°C for an hour. After the completion of the reaction, the product was isolated by preparative HPLC on a SunFire C18 column (30×250 mm i.d., 5 μm particle size, Waters) with a 30 mL/min flow rate. The solvent system consisted of solvent A (0.1 % TFA in water) and B (0.1 % TFA in acetonitrile) with gradient of solvent B ascending from 5 to 70 % over 60 min. The HPLC peaks for the dye-conjugated product were visualized with a fluorescence detector (RF-10AXL, Shimadzu) to determine the purity by relative HPLC peak area at 650–720 nm. The product was confirmed by MALDI-TOF mass spectroscopy and the final compound was collected and lyophilized.

NOTA conjugation—A solution of p-SCN-Bn-NOTA (8.08 mg) in DMSO (100 μL) was added to cFLFLFK-PEG₁₂ peptide (22.0 mg, 0.00142 mmols) in dry DMSO (300 μL) at ambient temperature followed by Diisopropylethylamine (70 μL, 5 Eq.). The resultant mixture was incubated at 40°C for 1 h. After the completion of the reaction, the product was isolated by preparative HPLC on a Sunfire C18 column (Waters) as described in this section above. The product was confirmed by MALDI-TOF mass spectroscopy and purified by HPLC chromatography.

1.2.2 Ethics Statement

Human blood samples were collected and processed from otherwise healthy LTBI and uninfected individuals following signed written informed consent using an approved institutional review board protocol. LTBI individuals were identified as having had a positive test result for *M.tb* latent infection by the Mantoux screening test [21] and/or interferon gamma release assay (IGRA) [22] within the previous 12 months. Mouse studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Ohio State University (2015A00000021).

1.2.3 Leukocyte Isolation from Whole Blood

To assess peptide binding to different circulating immune cells relevant to TB granulomas, heparinized blood was drawn from healthy volunteers. Using a technique previously described [23], the blood was gently overlaid with Ficoll-Hypaque (Amersham, Pittsburgh, PA) and centrifuged to achieve peripheral blood mononuclear cell (PBMC) separation from neutrophil-rich pellets. The thin cushion containing PBMCs was gently harvested and the cells were washed, counted, and re-suspended at a concentration of 4×10^6 cells/mL in RPMI.

The pellets were fractionated by dextran sedimentation to separate the neutrophils as previously described [24]. Briefly, the pellets were diluted 1:1 with normal saline and an equal amount of 3% dextran was added to sediment the RBCs. After 30 min incubation on ice, the top layer containing mostly neutrophils was transferred to a new tube and centrifuged at $800 \times g$ for 15 min. The RBCs that remained in the pellet were lysed with sterile water. Following one more wash, the cells were re-suspended in HBSS without calcium or magnesium at a concentration of 4×10^6 cells/mL. Trypan blue exclusion test was used to determine the percentage of viable cells. The PBMCs and neutrophils were kept on ice until ready for experimentation.

1.2.4 Binding of cFLFLFK-PEG₁₂-Cy3 to Human Leukocytes

Peptide binding to human leukocytes was measured by flow cytometry. Briefly, 4×10^5 neutrophils or 1×10^6 PBMCs were seeded into wells of a non-tissue culture treated round bottom 96 well plate. The plate was centrifuged at $800 \times g$ for 5 min and the supernatant removed. The cells were re-suspended in 50 μ L of HBSS with calcium and magnesium (HBSS+) and incubated at 37°C for 15 min to promote surface expression of FPR1 [25]. Next, cell suspensions were incubated for 15 min at 37°C in the presence of increasing concentrations of cFLFLFK-PEG₁₂-Cy3 (1, 10, 20, 30, 40, and 50 nM). At the conclusion of the incubation, the cells were washed three times and re-suspended in 200 μ L of wash buffer (PBS supplemented with 1% BSA) and placed on ice. The cell solutions were transferred to flow tubes and the samples were analyzed using the 560 nm laser on the Aria III instrument. In the neutrophil samples, neutrophils were observed to have distinct FSC and SSC profiles from cellular debris, allowing for their easy identification. In the PBMC samples, lymphocytes and monocytes were differentiated on the basis of forward and side scatter characteristics following a previously published protocol [26]. Mean fluorescence intensity (MFI) of the Cy3 signal for each dose level was measured for each population. In addition to traditional flow studies, samples were analyzed on the ImageStreamX (Amnis Corporation, Seattle, WA) image-based cytometer outfitted with a 560 nm laser to visualize peptide association to the different cell types. Using the IDEAS software (Amnis), the cell populations were defined using the same gating strategy described in this section above. Bright field images of individual cells were captured at 20 $\times$ magnification.

1.2.5 Validation of FPR1 as the cFLFLFK-PEG₁₂-Cy3 Binding Partner on Human Neutrophils

The fungal-derived FPR1 antagonist Cyclosporin H (CsH) was used to validate FPR1 as the binding partner of cFLFLFK on human neutrophils. CsH was prepared for these studies by

dissolving it in dimethyl sulfoxide (DMSO) to 10^{-2} M and stored at -80°C until use. At the time of experiment, aliquots were thawed on ice and serial dilutions were made with HBSS+. CsH was added to the cells at concentrations ranging from 100 to 100,000 nM and incubated with the cells for 30 min on ice prior to the warming up phase. Next, a fixed concentration of cFLFLFK-PEG₁₂-Cy3 (20 nM) was added to the cells and incubated for 15 min at 37°C . Finally, the cells were washed and re-suspended in 200 μL of wash buffer and placed on ice. Cell associated Cy3 fluorescence was measured as described in section 1.2.4 and the average inhibitory concentration of 50% (IC_{50}) value of CsH from 3 different donors was calculated. The IC_{50} values for all experiments were calculated by fitting the data to a one-site binding curve model with nonlinear regression using GraphPad Prism v5 (GraphPad Software, Inc.). All samples were performed in duplicate.

1.2.6 Effect of NOTA Conjugation on Peptide Binding to Human Neutrophils

Briefly, freshly isolated human neutrophils were seeded into wells of a 96 well plate at a density of 4×10^5 cells per well. Increasing concentrations of cFLFLFK-PEG₁₂-NOTA were added to the cells ranging from 100 to 100,000 nM and incubated with the cells for 30 min on ice. Following this incubation phase, cFLFLFK-PEG₁₂-Cy3 was added and incubated with the cells for 15 min at 37°C at a final concentration of 20 nM. Finally, the cells were washed, re-suspended in wash buffer, and kept on ice through the flow analysis. All concentrations of cFLFLFK-PEG₁₂-NOTA were tested in duplicate. IC_{50} values for cFLFLFK-PEG₁₂ and cFLFLFK-PEG₁₂-NOTA were obtained by fitting the data to a one-site binding curve model using non-linear regression analysis in Graphpad Prism. Average IC_{50} values of cFLFLFK-PEG₁₂ and cFLFLFK-PEG₁₂-NOTA from 3 different donors were measured and compared.

1.2.7 Binding of cFLFLFK-PEG12-Cy3 to Granuloma-Like Structures

PBMCs from LTBI individuals were infected with *M.tb* H₃₇R_v (MOI 1:1) on glass coverslips in 24-well tissue culture plates as previously described [27]. Robust, granuloma-like structures were visually confirmed at 7 days post-infection and fixed with 4% paraformaldehyde. The structures were washed and incubated overnight at 4°C with a 30 nM solution of cFLFLFK-PEG₁₂-Cy3 diluted in HBSS+. The next day, granulomas were washed three times with PBS, blocked with 10% goat serum and 5% bovine serum albumin in PBS for 1 h, and incubated with a mouse anti-human CD68 antibody (DAKO) for 1 h. Goat anti-mouse AF647 (Invitrogen Life Technologies, Carlsbad, CA) secondary antibody was applied for 1 h after additional washes. Cell nuclei were stained with 400 ng/ml DAPI DNA stain (Molecular Probes, Carlsbad, CA). Finally, the coverslips were washed three times with distilled water and mounted on glass slides using ProLong Gold Antifade (Invitrogen Life Technologies). All incubations were performed at room temperature. Images of peptide binding and CD68 expression on granulomas were captured on an FV3000 confocal laser scanning microscope (Olympus, Tokyo, Japan) using a 60 $\times$ oil DIC objective.

1.2.8 Mouse Model of Lung Granulomatous Inflammation

Female C57BL/6 mice (8 weeks old, 18–20 g body weight) were purchased from Jackson Laboratory (Bar Harbor, ME). An emulsion of purified trehalose dimycolate (TDM, Sigma-

Aldrich, St. Louis, MO) was freshly prepared the same day of injection using a previously published protocol [28]. Briefly, TDM was solubilized in hexane and transferred to a Potter-Elvehjem glass pestle. The solvent was evaporated under a stream of nitrogen gas. Sterile mineral oil was added to the dried TDM in the pestle and ground on ice for 1 minute. Next, 0.2% Tween-PBS was added followed by a second round of grinding until an emulsion was obtained. The final concentration of TDM in the emulsion was 1 mg/mL. A vehicle solution was prepared as described above in the absence of TDM. One hundred microliter of the emulsion (100 µg) or vehicle was loaded into syringes and injected into 6 week old C57BL/6 mice via the tail vein.

Lung histology was performed 5 days after TDM challenge to confirm granulomatous responses. Briefly, mice were euthanized and their thoracic cavity exposed. Ten percent phosphate-buffered formalin was infused into the lung via the trachea to gently distend the alveolar spaces and fix the tissue. The lungs were then harvested *en bloc* for paraffin embedding, sectioning, and hematoxylin and eosin (H&E) staining. The formation of discrete, granuloma-like structures and the presence of recruited immune cells were verified by examining stained lung sections by light microscopy.

1.2.9 In Vivo Evaluation of Peptide Targeting and Cellular Specificity

Five female C57BL/6 mice (8 weeks old, 18–20 g body weight) were intravenously injected with 100 µg of TDM. Five days later, three of the mice were intravenously injected with 50 µg of cFLFLFK-PEG₁₂-Cy3 and two mice were injected with PBS to serve as controls. After a 4 h circulation time, the mice were euthanized by CO₂ asphyxiation for tissue harvest. Quickly after euthanasia, the thoracic cavity was exposed and the pulmonary circulation flushed via the right ventricle with cold PBS containing 50 U/mL heparin. The lung and heart were removed *en bloc* and the heart, trachea, and large vessels trimmed away. The superior right lung lobe was excised and reserved for histology and the remaining lung was processed for single cell preparations as described previously [29]. Briefly, lungs from individual mice were placed in an enzymatic digestion solution containing 0.7 mg/mL collagenase A (type XI, Sigma, St Louis, MO, USA) and 30 mg/mL type IV bovine pancreatic DNase (type IV, Sigma, St Louis, MO, USA) in GentleMACS C-tubes. The lungs were mechanically disrupted using a GentleMACS dissociator (Miltenyi Biotec, Boston, MA) and incubated at 37°C for 30 min to further promote enzymatic digestion. Lung cell suspensions were passed through a 70 µm nylon cell screen and residual erythrocytes were lysed with Gey's solution (NH₄Cl (4.5 g) and KHCO₃ (0.5 g) dissolved in 500 mL tissue culture grade water). Cells from each lung were counted and re-suspended in HBSS+ to a concentration of 2×10⁶ cells/mL. LIVE/DEAD Fixable Blue Dead Cell Stain (Invitrogen) was applied to the cells for 30 min on ice per product instructions. The cells were washed, blocked for 30 min with blocking buffer containing 40% mouse serum, and transferred to a 96 well plate at a density of 1×10⁶ cells per well. Cells were labeled for 30 min at 4°C with different monoclonal antibodies (mAbs). As shown in Table 1, mAbs specific for Ly6G, CD3, CD11b, and CD11c were used as direct conjugates to allophycocyanin (APC), Alexa fluor 488, peridinin chlorophyll protein complex (PerCP)-Cy5.5, and APC-Cy7. Cells were washed three times with ice cold wash buffer, re-suspended in 200 µL of wash buffer supplemented with 0.1% sodium azide, and kept

overnight in the fridge on ice. Samples were analyzed the next morning on the Aria III flow cytometer (BD Biosciences) with 100,000 live events acquired for each sample. Compensation was performed using FACSDiva software prior to data export and analysis using FlowJo software (TreeStar). A gating scheme was devised to measure Cy3 association on the following cell populations: i) neutrophils (CD11b+, Ly6G+), ii) lymphocytes (CD3+), iii) alveolar macrophages (CD11c+, CD11b-), iv) macrophages and dendritic cells (CD11b+, CD11c+), v) monocytes and immature macrophages (CD11b+, CD11c-), and vi) other cells (negative for all mAbs). The relative contribution of Cy3 signal from each cell population was calculated by multiplying the population frequency in the living gate by the Cy3 MFI of that population.

To investigate peptide distribution *in situ*, lung sections were analyzed by confocal microscopy. The superior right lung lobes were harvested from each of the 5 TDM-challenged mice as described in section 1.2.8. Using a tissue preparation technique adopted from Kim *et al.* [30], the lobes were fixed in 4% paraformaldehyde for 3 h followed by submersion in 20% sucrose in PBS for 24 hr. The individual lobes were snap frozen by briefly dipping the tissue in isopentane submerged in a bath of liquid nitrogen. Serial 10 μ m-thick cryostat tissue sections were made and mounted on glass slides. The sections were washed 3 times with PBS, incubated with 400 ng/mL of DAPI for 10 minutes in the dark, and mounted with prolong gold. Images of cell nuclei and cFLFLFK-PEG₁₂-Cy3 distribution in the lung sections were captured with the Olympus FV1000 confocal microscope.

1.2.10 Statistical Analysis

Group data are expressed as the mean $\pm$ standard error of the mean (SEM). Student's *t*-test was used to compare results among groups. Statistical significance was set at $P < 0.05$. Graphpad Prism was used for statistical testing and for calculating inhibitor IC₅₀ values by nonlinear regression with the built-in one-site receptor competitive binding model. R^2 values were used to evaluate goodness-of-fits.

1.3 RESULTS

1.3.1 Chemistry Results

Chemical structures of the peptide analogs prepared in this study are shown in Supplementary Fig 1. The theoretical molecular weights of cFLFLFK-PEG₁₂, cFLFLFK-PEG₁₂-Cy3, and cFLFLFK-PEG₁₂-NOTA were 1542, 1981, and 1993, respectively, which were confirmed by mass spectrometry (Supplementary Fig 2). Chromatographic evaluation by HPLC confirmed >90% purity of the peptide conjugates (Supplemental Fig 3). The lyophilized products were reconstituted in DMSO and water, aliquoted, and kept at -20°C until use.

1.3.2 cFLFLFK-PEG₁₂-Cy3 Preferentially Binds Human Neutrophils In Vitro

Neutrophils, monocytes, and lymphocytes were isolated from heparinized blood from healthy volunteers with greater than 90% viability as determined by the Trypan blue exclusion test. Binding of cFLFLFK-PEG₁₂-Cy3 to the three immune cell types was

quantified by flow cytometry. Representative gates used to define the populations for MFI analysis are shown in Fig 1A. Dose-dependent binding was observed on all cell types, however, neutrophils displayed significantly higher cFLFLF-PEG₁₂-Cy3 MFI values compared with monocytes and lymphocytes over the dose range studied ($***P < 0.0001$, $**P < 0.01$, or $*P < 0.05$ corresponding to cFLFLF-PEG₁₂-Cy3 concentrations of 30–50 nM, 20 nM, and 10 nM, respectively, Fig 1B). Increased peptide binding to neutrophils compared with monocytes and lymphocytes is presented in representative panels of bright-field and fluorescence images acquired on the Flowsight system (Fig 1C).

1.3.3 In Vitro Binding of cFLFLFK-PEG₁₂-Cy3 to Neutrophils is Mediated by FPR1

As shown in Fig 2, pretreatment of neutrophils with CsH ranging in concentration from 10 to 100,000 nmol/L led to a concentration-dependent inhibition of cFLFLFK-PEG₁₂-Cy3 binding. Binding data was well fit with a single site binding model with an IC₅₀ of 88.7 ± 6.8 nM ($R^2=0.90$). High concentrations of CsH led to a near abolishment of cFLFLFK-PEG₁₂-Cy3 binding, indicating a prominent role of FPR1 in human neutrophil binding.

1.3.4 NOTA Conjugation Moderately Affects Peptide Binding to Human Neutrophils

In addition to cFLFLFK-PEG₁₂-Cy3, an analog of cFLFLFK-PEG₁₂ was conjugated with a chelator for future radiolabeling and PET imaging application. We sought to determine if the binding of cFLFLFK-PEG₁₂-NOTA to human neutrophils is altered compared with the parent analog. To facilitate radiolabeling for *in vivo* tracking by PET imaging, cFLFLFK-PEG₁₂ was successfully conjugated with the bi-functional chelator NOTA. We studied whether NOTA conjugation has an effect on peptide binding to human neutrophils. Upon incubation with increasing concentration of cFLFLFK-PEG₁₂-NOTA, we observed a decrease in cFLFLFK-PEG₁₂-Cy3 binding indicative of the displacement of the fluorescently labeled analog. The IC₅₀ values calculated for cFLFLFK-PEG₁₂-NOTA and cFLFLFK-PEG₁₂ was 1086 ± 2.5 nM and 597.2 ± 3.4 nM, respectively (Fig 3). Model fitting resulted in R^2 values $>95\%$ for both compounds.

1.3.5 cFLFLFK-PEG₁₂-Cy3 Binds to Macrophages in Human Granuloma Structures

The staining pattern of cFLFLFK-PEG₁₂-Cy3 in the representative granuloma highly correlated with CD68 expression, indicating that macrophages are the primary cellular target of peptide binding in these structures (Fig 4).

1.3.6 TDM Administration Induces Granulomatous Responses in the Mouse Lung

Preliminary analysis by flow cytometry confirmed the expansion of several immune cell populations in the TDM-challenged lung. Specifically, compared to vehicle-injected mice, neutrophils, alveolar macrophages, macrophages and dendritic cells, monocytes and immature macrophages showed frequency increases of $9.1\% \pm 6.4\%$, $1\% \pm 1.1\%$, $25\% \pm 6.4\%$, and $21 \pm 2.2\%$, respectively, 5 days after TDM challenge (data not shown; see below for information on defining these populations). The lung frequency of CD3+ lymphocytes was unchanged by TDM challenge. The percentage of neutrophils, alveolar macrophages, lymphocytes, immature macrophages/monocytes, and macrophages/dendritic

cells relative to all living cells for five TDM-administered mice tested was $9.2\% \pm 3.7\%$, $6.3\% \pm 1.7\%$, $10.7\% \pm 2.5\%$, $20.1\% \pm 3.1\%$, $17.7\% \pm 7.7\%$, respectively.

Histological analysis by H&E staining showed a minimal lung inflammatory response in mice injected with the vehicle emulsion 5 days after injection (Fig 5A). In contrast, patchy regions of granulomatous inflammation were observed at this time in TDM-challenged mice consistent with prior studies (Fig 5B) [28,31]. This granulomatous response appears to be the result of a coordinated, multicellular effort to wall off lipid droplets presumably containing TDM. Representative high magnification images revealed normal lung architecture for the vehicle injected mouse (Fig 5C) and the presence of neutrophils and macrophages with some lymphocytes in the inflammatory infiltrate for the TDM-injected mouse (Fig 5D).

1.3.7 cFLFLFK-PEG₁₂-Cy3 Targets Phagocytes in the TDM-Challenged Lung

The TDM model was used to evaluate the binding specificity of cFLFLFK-PEG₁₂-Cy3 in the setting of lung granulomatous inflammation. Five days after TDM challenge, flow cytometry of digested lung samples was performed 4 h after cFLFLFK-PEG₁₂-Cy3 administration. As shown in Fig 6A, the gating scheme devised for this study identifies 6 cell populations from the 4 color antibody panel: i) neutrophils, ii) alveolar macrophages, iii) macrophages and dendritic cells, iv) monocytes and immature macrophages, v) lymphocytes, and vi) other cells. The average relative frequencies of the 6 cell types in the lung of 3 TDM-challenged mice are depicted as a pie chart in Fig 6B. Histograms depicting Cy3-associated fluorescence for each population show that neutrophils and cells of the monocyte/macrophage lineage stain positively for cFLFLFK-PEG₁₂-Cy3 (Fig 6C), while lymphocytes and other cells were minimally stained. A pie chart was generated to visualize the cell population-specific cFLFLFK-PEG₁₂-Cy3 signal as a fraction of overall Cy3 signal attributable to all live lung cells, accounting for both cell frequency and MFI of each population (Fig 6D). The pie chart shows that cFLFLFK-PEG₁₂-Cy3 associated with the following cell populations to a similar degree: i: neutrophils, ii) alveolar macrophages, iii) macrophages and dendritic cells, and iv) monocytes and immature macrophages. Of note, neutrophils account for 18.2% of the overall Cy3 lung signal, while only making up 9.2% of the live lung cells. Similarly, alveolar macrophages contribute 20.8% of the lung Cy3 signal, while only making up 6.3% of the cellular population. Lymphocytes and other cells negative for the antibodies were not targeted by cFLFLFK-PEG₁₂-Cy3.

1.3.8 Fluorescence Microscopy Reveals cFLFLFK-PEG₁₂-Cy3 Uptake in Inflamed Areas in the TDM-Challenged Lung

As a complementary approach to the flow cytometry studies, confocal images of frozen lung sections were captured to visualize the distribution of cFLFLFK-PEG₁₂-Cy3 in the TDM-challenged lung. As shown in Fig 7A, no signal was detected in the lung from the TDM-challenged mouse injected with PBS. In contrast, the TDM-challenged mouse injected with cFLFLFK-PEG₁₂-Cy3 had punctate Cy3 signal patterns in areas of the lung with prominent cellular inflammation (Fig 7B).

1.4 DISCUSSION

Imaging is being increasingly utilized in TB for diagnosing and managing patients as well as clinical trials for therapy evaluation. However, current nuclear medicine imaging tracers are not specific for TB pathologies and anatomical imaging modalities such as computed tomography are not able to distinguish active vs. latent granulomas. Imaging probes that specifically target granulomas are currently unrealized, but would enhance the value of molecular imaging in the context of TB. For example, a probe that targets specific immune cell populations infiltrating the granuloma may offer several advantages. First, it could specifically identify granulomas that are highly infiltrated by active immune cells and thus potentially at risk for losing containment and allowing unrestrained growth of *M.tb*. Second, it could rapidly and quantitatively track therapeutic efficacy and durability once the treatment phase ends. Third, it could provide new information in individuals with LTBI that can be combined with traditional TB tests (Mantoux screening test and/or IGRA) for improved management of TB patients in the clinical setting. Traditional TB tests evaluate only activity of the adaptive immune response and it is unknown how this correlates with the number and types of granulomas present.

Neutrophils have garnered recent interest in TB, although their exact roles have remained elusive. Data from animal studies have shown that in early stages of disease neutrophils facilitate the initiation of the adaptive immune response [32] and regulate the initial construction of the granuloma [33]. In late stage disease, however, neutrophils appear to take on more host-detrimental roles. Clinical examples of this come from late stage tissue biopsy samples in TB patients [34], sputum analysis [35], and signatures of blood transcript levels in circulating neutrophils during active disease [36]. Preclinical studies in mice have corroborated this association by showing a link between infiltrating neutrophils, increased bacterial load [37,38], and exacerbated lung pathology in late stage disease [38–43]. It is clear that neutrophils are infiltrating the lung in late phases of disease and mediating tissue destruction. However, the extent to which neutrophils are fundamentally involved in the early pathogenesis process or simply a late stage consequence of a threshold amount of inflammation is currently not known. Studies aimed at addressing this question have not been performed in part due to a lack of appropriate imaging methodologies. The development of an imaging tool that enables the non-invasive tracking of neutrophils will enhance what we can ultimately learn about the behavior of these cells in TB and help to reconcile seemingly conflicting studies on their roles in TB.

The hexapeptide cFLFLFK has been studied as a leukocyte tracking probe in various disease models. Through different imaging modalities, the peptide has shown promise in imaging acute, neutrophilic inflammation in the lung [15] and elsewhere [16,17,44,45]. Imaging of inflammatory macrophage responses has also been demonstrated with this peptide [17]. Considering the collective evidence, this peptide may be uniquely suited for application to TB for two reasons. First, macrophages are a major component of granulomas and neutrophils are implicated in TB granulomatous inflammation and their degree of infiltration and/or activation may be an indicator of disease severity as discussed above. Second, the peptide has the ability to image inflammatory responses in the lung and has demonstrated low lung retention in the absence of inflammation. The goal of this work was to evaluate the

potential of cFLFLFK for application to TB. To this end, we examined the binding and cellular specificity of Cy3-labelled cFLFLFK-PEG₁₂ in a human cell-based TB granuloma model and a mouse model of lung granulomatous inflammation.

In vitro binding studies showed greater cFLFLFK-PEG₁₂-Cy3 binding to neutrophils compared to monocytes and lymphocytes. To our knowledge, this study is the first to examine the differential binding of cFLFLFK to human blood cells. Previous studies showed that cFLFLFK displaces the binding of a radiolabeled cFLFLF analog on human embryonic kidney (HEK) cells transfected with human FPR1 in a dose-dependent manner [16]. Other work showed a dramatic reduction in cFLFLF binding to macrophages isolated from FPR1 knockout mice [18]. Our data indicate that cFLFLF-PEG₁₂-Cy3 preferentially interacts with FPR1, as the FPR1 antagonist CsH [46] inhibited its binding to neutrophils in a dose-dependent manner. Overall, the collective human and mouse data indicate that FPR1 is the major GPCR targeted by cFLFLFK. Thus, we anticipate that FPR1 expression levels on cells in the granuloma will likely be an important factor in determining cFLFLFK accumulation in this environment.

To test whether cFLFLFK-PEG₁₂ might successfully bind cells present in human TB granulomas, we utilized a human cell-based *in vitro* granuloma model developed in our laboratory that is showing promise as a useful model of the *in vivo* TB granulomatous [27]. This model is based on primary human PBMCs and captures the complex host-pathogen interactions that take place inside human TB granulomas [27]. Although not previously used for this purpose, the model offers a unique platform for studying and screening imaging probes targeted to granulomas before committing to more costly *in vivo* preclinical studies. In this context, we examined the binding of cFLFLFK-PEG₁₂-Cy3 to granuloma structures 7 days after the cells were infected with *M.tb*. Using this model we found high co-localization between cFLFLFK-PEG₁₂-Cy3 and CD68 expression. While we did not specifically stain for macrophage FPR1 expression, these cells have been reported as having high baseline FPR1 transcript levels [47]. Future work will seek to study the kinetics of cFLFLFK-PEG₁₂-Cy3 binding to the developing granuloma over time and correlate probe binding to FPR1 expression and *M.tb* growth kinetics. A current limitation of the model is the absence of neutrophils. Neutrophils are a challenging cell type to utilize for long-term studies and thus are not featured in the model at this time. Short-term studies involving the addition of neutrophils to early forming or established granulomas are possible and are being considered for future advancement of the model.

We validated the cellular specificity of cFLFLFK-PEG₁₂-Cy3 *in vivo* in a mouse model of lung granulomatous inflammation induced by the *M.tb* cell wall component TDM. Flow cytometry data revealed that cFLFLFK-PEG₁₂-Cy3 associated strongly and equally with neutrophils and cells belonging to the monocyte/macrophage lineage. Lymphocytes, as was the case in human cell binding studies, were not targeted by the probe *in vivo*. A limitation to our flow cytometry study was that structural cells of the lung such as fibroblasts and epithelial cells were not specifically examined for cFLFLFK-PEG₁₂-Cy3 association. Although these cells would be included in the live cell gate, we are not able to resolve individual cell populations belonging to these non-myeloid cell lineages as multiple cell

types are being lumped together as “other cells” (Fig 6B). It is of note that a similar cFLFLFK analog was previously shown to not bind mouse fibroblasts [48].

We acknowledge that use of the TDM model over the *M.tb* mouse model is a limitation of this work. However, we believe the TDM model was an appropriate choice for the purposes of this study as it provides two key advantages. First, the TDM model rapidly induces a pulmonary granulomatous response, requiring only five days for the induction of significant lung pathology compared to the slowly developing, multi-stage *M.tb* model. Second, the granuloma-like structures that form in the lung of TDM-administered mice are comprised of cell types known to reside within human TB granulomas, ensuring that the cellular specificity of cFLFLF-PEG₁₂-Cy3 could be assessed in a TB relevant context. Our collective results would predict that neutrophils and macrophages will be the primary cell types targeted in the TB granuloma environment.

Targeted imaging of FPR1-specific inflammation in the context of TB may offer advantages over ⁶⁸Ga-citrate, a PET tracer that lacks specificity for bacterial infection [49], and other peptides such as VAP [50] or RGD [51], which have yet to be thoroughly investigated in TB. Another probe under investigation known as TBIA101 [52] has shown the ability to delineate sites of mycobacterial infection by PET imaging, but cell types targeted by this probe and its ability to reflect infection severity remain unknown. It is of note that the Cy3 fluorophore used in this study serves as a proof of concept label and can be replaced with a clinically relevant label such as Indocyanine green in future work. Additionally, in preparation for future preclinical studies with PET imaging, cFLFLFK was successfully conjugated to the bi-functional chelator NOTA. NOTA is capable of chelating ⁶⁸Ga (68 min half-life), ¹⁸F (110 min half-life), and ⁶⁴Cu (12.7 h half-life) making it flexible for labeling different radionuclides to match clearance kinetics. To our knowledge, this is the first time cFLFLFK has been successfully conjugated to this chelator. Peptide conjugation to NOTA did result in an approximately 2-fold larger IC₅₀ value relative to the parent peptide. The *in vivo* significance of this will be assessed in future studies. Previous work showed that the conjugation of a tetraglycine chelate upon the lysine residue of the cFLFLFK peptide did not significantly disrupt cellular binding [16]. Other studies on small peptide probes have reported similar shifts in binding following conjugation to radiometal chelators [53,54]. In these cases, acceptable *in vivo* results were obtained despite the observed changes in *in vitro* binding.

The cellular binding profile of cFLFLFK appears to be complementary to another recently developed peptide probe LLP2A targeting the very late Ag-4 (VLA-4) [8]. PET imaging of LLP2A in a non-human primate model of pulmonary TB revealed a targeting bias towards macrophages and lymphocytes with moderate to low binding to neutrophils. Intriguingly, imaging studies using both probes in tandem might provide a comprehensive picture of the dynamic influxes of important cell populations and inform on how shifts or changes in the respective granuloma populations affect susceptibility to *M.tb* and ultimately host survival. Such studies could also reveal new therapeutic directions and biomarkers in TB.

In conclusion, diagnostic and therapeutic strategies that specifically target granulomas have not been developed. The studies outlined above demonstrate that the FPR1-targeting peptide

probe cFLFLFK-PEG₁₂ preferentially binds human neutrophils via FPR1 and macrophages in an *in vitro* human granuloma model. *In vivo* the probe preferentially associated with neutrophils and cells of the monocyte/macrophage lineage in a mouse model of granulomatous inflammation. Given these findings, further investigation into the use of this probe is warranted as it may prove to be a specific imaging biomarker to monitor TB granulomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Organization. WH. [Accessed 10 August, 2017] Tuberculosis Facts Sheet. <http://who.int/mediacentre/factsheets/who104/en/print.html>
2. Salgame P, Geadas C, Collins L, Jones-Lopez E, Ellner JJ. Latent tuberculosis infection--Revisiting and revising concepts. *Tuberculosis (Edinb)*. 2015; 95:373–84. [PubMed: 26038289]
3. Mattila JT, Ojo OO, Kepka-Lenhart D, Marino S, Kim JH, Eum SY, et al. Microenvironments in tuberculous granulomas are delineated by distinct populations of macrophage subsets and expression of nitric oxide synthase and arginase isoforms. *J Immunol*. 2013; 191:773–84. [PubMed: 23749634]
4. Lin PL, Coleman T, Carney JP, Lopresti BJ, Tomko J, Fillmore D, et al. Radiologic responses in cynomolgous macaques for assessing tuberculosis chemotherapy regimens. *Antimicrob Agents Chemother*. 2013
5. Marino S, Cilfone NA, Mattila JT, Linderman JJ, Flynn JL, Kirschner DE. Macrophage polarization drives granuloma outcome during *Mycobacterium tuberculosis* infection. *Infect Immun*. 2015; 83:324–38. [PubMed: 25368116]
6. Brighenti S, Andersson J. Local immune responses in human tuberculosis: learning from the site of infection. *J Infect Dis*. 2012; 205(Suppl 2):S316–24. [PubMed: 22448014]
7. Coleman MT, Maiello P, Tomko J, Frye LJ, Fillmore D, Janssen C, et al. Early Changes by (18)Fluorodeoxyglucose positron emission tomography coregistered with computed tomography predict outcome after *Mycobacterium tuberculosis* infection in cynomolgus macaques. *Infect Immun*. 2014; 82:2400–4. [PubMed: 24664509]
8. Mattila JT, Beaino W, Maiello P, Coleman MT, White AG, Scanga CA, et al. Positron Emission Tomography Imaging of Macaques with Tuberculosis Identifies Temporal Changes in Granuloma Glucose Metabolism and Integrin alpha4beta1-Expressing Immune Cells. *J Immunol*. 2017; 199:806–15. [PubMed: 28592427]
9. Abouzied MM, Crawford ES, Nabi HA. 18F-FDG imaging: pitfalls and artifacts. *J Nucl Med Technol*. 2005; 33:145–55. quiz 62-3. [PubMed: 16145222]

10. Malherbe ST, Shenai S, Ronacher K, Loxton AG, Dolganov G, Kriel M, et al. Corrigendum: Persisting positron emission tomography lesion activity and Mycobacterium tuberculosis mRNA after tuberculosis cure. *Nat Med.* 2017; 23:526.
11. Ordonez AA, Weinstein EA, Bambarger LE, Saini V, Chang YS, DeMarco VP, et al. A Systematic Approach for Developing Bacteria-Specific Imaging Tracers. *J Nucl Med.* 2017; 58:144–50. [PubMed: 27635025]
12. Ebenhan T, Chadwick N, Satheke MM, Govender P, Govender T, Kruger HG, et al. Peptide synthesis, characterization and (6)(8)Ga-radiolabeling of NOTA-conjugated ubiquicidin fragments for prospective infection imaging with PET/CT. *Nucl Med Biol.* 2014; 41:390–400. [PubMed: 24630816]
13. Dutta J, Baijnath S, Somboro AM, Nagiah S, Albericio F, de la Torre BG, et al. Synthesis, in vitro evaluation, and (68) Ga-radiolabeling of CDP1 toward PET/CT imaging of bacterial infection. *Chem Biol Drug Des.* 2017; 90:572–9. [PubMed: 28328161]
14. Ordonez AA, Pokkali S, DeMarco VP, Klunk M, Mease RC, Foss CA, et al. Radioiodinated DPA-713 Imaging Correlates with Bactericidal Activity of Tuberculosis Treatments in Mice. *Antimicrob Agents Chemother.* 2015; 59:642–9. [PubMed: 25403669]
15. Locke LW, Chordia MD, Zhang Y, Kundu B, Kennedy D, Landseadel J, et al. A novel neutrophil-specific PET imaging agent: cFLFLFK-PEG-64Cu. *J Nucl Med.* 2009; 50:790–7. [PubMed: 19372473]
16. Stasiuk GJ, Holloway PM, Rivas C, Trigg W, Luthra SK, Morisson Iveson V, et al. (99m)Tc SPECT imaging agent based on cFLFLFK for the detection of FPR1 in inflammation. *Dalton Trans.* 2015; 44:4986–93. [PubMed: 25603955]
17. Zhang Y, Kundu B, Zhong M, Huang T, Li J, Chordia MD, et al. PET imaging detection of macrophages with a formyl peptide receptor antagonist. *Nucl Med Biol.* 2014
18. Xiao L, Ding M, Zhang Y, Chordia M, Pan D, Shimer A, et al. A Novel Modality for Functional Imaging in Acute Intervertebral Disk Herniation via Tracking Leukocyte Infiltration. *Mol Imaging Biol.* 2017
19. Migeotte I, Communi D, Parmentier M. Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. *Cytokine & growth factor reviews.* 2006; 17:501–19. [PubMed: 17084101]
20. Dorward DA, Lucas CD, Chapman GB, Haslett C, Dhaliwal K, Rossi AG. The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. *Am J Pathol.* 2015; 185:1172–84. [PubMed: 25791526]
21. Huebner RE, Schein MF, Bass JB Jr. The tuberculin skin test. *Clin Infect Dis.* 1993; 17:968–75. [PubMed: 8110954]
22. Gerald H, Mazurek JJ, Andrew Vernon, Phillip LoBue, Stefan Goldberg, Kenneth Castro. Updated Guidelines for Using Interferon Gamma Release Assays to Detect Mycobacterium tuberculosis Infection --- United States, 2010. Division of Tuberculosis Elimination NCFH, STD, and TB Prevention. 2010
23. Rajaram MV, Brooks MN, Morris JD, Torrelles JB, Azad AK, Schlesinger LS. Mycobacterium tuberculosis activates human macrophage peroxisome proliferator-activated receptor gamma linking mannose receptor recognition to regulation of immune responses. *J Immunol.* 2010; 185:929–42. [PubMed: 20554962]
24. Mishra M, Byrd MS, Sergeant S, Azad AK, Parsek MR, McPhail L, et al. Pseudomonas aeruginosa Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. *Cell Microbiol.* 2012; 14:95–106. [PubMed: 21951860]
25. Sengelov H, Boulay F, Kjeldsen L, Borregaard N. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. *Biochem J.* 1994; 299(Pt 2):473–9. [PubMed: 8172608]
26. Azad AK, Rajaram MVS, Metz WL, Cope FO, Blue MS, Vera DR, et al. γ -Tilmanocept, a New Radiopharmaceutical Tracer for Cancer Sentinel Lymph Nodes, Binds to the Mannose Receptor (CD206). *The Journal of Immunology.* 2015:1402005.

27. Guirado E, Mbawuike U, Keiser TL, Arcos J, Azad AK, Wang SH, et al. Characterization of host and microbial determinants in individuals with latent tuberculosis infection using a human granuloma model. *MBio*. 2015; 6:e02537–14. [PubMed: 25691598]
28. Lee WB, Kang JS, Yan JJ, Lee MS, Jeon BY, Cho SN, et al. Neutrophils Promote Mycobacterial Trehalose Dimycolate-Induced Lung Inflammation via the Mincle Pathway. *PLoS Pathog*. 2012; 8:e1002614. [PubMed: 22496642]
29. Cyktor JC, Carruthers B, Kominsky RA, Beamer GL, Stromberg P, Turner J. IL-10 inhibits mature fibrotic granuloma formation during Mycobacterium tuberculosis infection. *J Immunol*. 2013; 190:2778–90. [PubMed: 23396944]
30. Kim JS, Yoon TJ, Yu KN, Kim BG, Park SJ, Kim HW, et al. Toxicity and tissue distribution of magnetic nanoparticles in mice. *Toxicol Sci*. 2006; 89:338–47. [PubMed: 16237191]
31. Hamasaki N, Isowa K, Kamada K, Terano Y, Matsumoto T, Arakawa T, et al. In vivo administration of mycobacterial cord factor (Trehalose 6, 6'-dimycolate) can induce lung and liver granulomas and thymic atrophy in rabbits. *Infect Immun*. 2000; 68:3704–9. [PubMed: 10816531]
32. Blomgran R, Ernst JD. Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection. *J Immunol*. 2011; 186:7110–9. [PubMed: 21555529]
33. Seiler P, Aichele P, Bandermann S, Hauser AE, Lu B, Gerard NP, et al. Early granuloma formation after aerosol Mycobacterium tuberculosis infection is regulated by neutrophils via CXCR3-signaling chemokines. *Eur J Immunol*. 2003; 33:2676–86. [PubMed: 14515251]
34. Eum SY, Kong JH, Hong MS, Lee YJ, Kim JH, Hwang SH, et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest*. 2010; 137:122–8. [PubMed: 19749004]
35. Ong CW, Elkington PT, Brilha S, Ugarte-Gil C, Tome-Esteban MT, Tezera LB, et al. Neutrophil-Derived MMP-8 Drives AMPK-Dependent Matrix Destruction in Human Pulmonary Tuberculosis. *PLoS Pathog*. 2015; 11:e1004917. [PubMed: 25996154]
36. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature*. 2010; 466:973–7. [PubMed: 20725040]
37. Repasy T, Martinez N, Lee J, West K, Li W, Kornfeld H. Bacillary replication and macrophage necrosis are determinants of neutrophil recruitment in tuberculosis. *Microbes and Infection*. 2015
38. Niazi MK, Dhulekar N, Schmidt D, Major S, Cooper R, Abeijon C, et al. Lung necrosis and neutrophils reflect common pathways of susceptibility to Mycobacterium tuberculosis in genetically diverse, immune-competent mice. *Dis Model Mech*. 2015; 8:1141–53. [PubMed: 26204894]
39. Gopal R, Monin L, Torres D, Slight S, Mehra S, McKenna KC, et al. S100A8/A9 proteins mediate neutrophilic inflammation and lung pathology during tuberculosis. *Am J Respir Crit Care Med*. 2013; 188:1137–46. [PubMed: 24047412]
40. Elkington P, Shiomi T, Breen R, Nuttall RK, Ugarte-Gil CA, Walker NF, et al. MMP-1 drives immunopathology in human tuberculosis and transgenic mice. *The Journal of clinical investigation*. 2011; 121:1827. [PubMed: 21519144]
41. Nandi B, Behar SM. Regulation of neutrophils by interferon- gamma limits lung inflammation during tuberculosis infection. *J Exp Med*. 2011; 208:2251–62. [PubMed: 21967766]
42. Kramnik I, Beamer G. Mouse models of human TB pathology: roles in the analysis of necrosis and the development of host-directed therapies. *Semin Immunopathol*. 2016; 38:221–37. [PubMed: 26542392]
43. Almeida FM, Ventura TL, Amaral EP, Ribeiro SC, Calixto SD, Manhaes MR, et al. Hypervirulent Mycobacterium tuberculosis strain triggers necrotic lung pathology associated with enhanced recruitment of neutrophils in resistant C57BL/6 mice. *PLoS One*. 2017; 12:e0173715. [PubMed: 28306733]
44. Chen J, Cheng H, Dong Q, Chen F, Huang Z, Zhang Y, et al. [(99m)Tc]cFLFLF for Early Diagnosis and Therapeutic Evaluation in a Rat Model of Acute Osteomyelitis. *Mol Imaging Biol*. 2015; 17:337–44. [PubMed: 25323104]

45. Xiao L, Zhang Y, Liu Z, Yang M, Pu L, Pan D. Synthesis of the Cyanine 7 labeled neutrophil-specific agents for noninvasive near infrared fluorescence imaging. *Bioorg Med Chem Lett*. 2010; 20:3515–7. [PubMed: 20488705]
46. Loor F, Tiberghien F, Wenandy T, Didier A, Traber R. Cyclosporins: structure-activity relationships for the inhibition of the human FPR1 formylpeptide receptor. *J Med Chem*. 2002; 45:4613–28. [PubMed: 12361388]
47. Gemperle C, Schmid M, Herova M, Marti-Jaun J, Wuest SJ, Loretz C, et al. Regulation of the formyl peptide receptor 1 (FPR1) gene in primary human macrophages. *PLoS One*. 2012; 7:e50195. [PubMed: 23185575]
48. Yang X, Chordia MD, Du X, Graves JL, Zhang Y, Park YS, et al. Targeting formyl peptide receptor 1 of activated macrophages to monitor inflammation of experimental osteoarthritis in rat. *J Orthop Res*. 2016; 34:1529–38. [PubMed: 26717557]
49. Vorster M, Maes A, Van de Wiele C, Sathekge MM. 68Ga-citrate PET/ CT in Tuberculosis: A pilot study. *Q J Nucl Med Mol Imaging*. 2014
50. Lankinen P, Makinen TJ, Poyhonen TA, Virsu P, Salomaki S, Hakanen AJ, et al. (68)Ga-DOTAVAP-P1 PET imaging capable of demonstrating the phase of inflammation in healing bones and the progress of infection in osteomyelitic bones. *Eur J Nucl Med Mol Imaging*. 2008; 35:352–64. [PubMed: 18038133]
51. Gaertner FC, Kessler H, Wester HJ, Schwaiger M, Beer AJ. Radiolabelled RGD peptides for imaging and therapy. *Eur J Nucl Med Mol Imaging*. 2012; 39(Suppl 1):S126–38. [PubMed: 22388629]
52. Ebenhan T, Mokaleng BB, Venter JD, Kruger HG, Zeevaart JR, Sathekge M. Preclinical Assessment of a Ga-68-DOTAFunctionalized Depsipeptide as a Radiodiagnostic Infection Imaging Agent. *Molecules*. 2017:22.
53. Hernandez R, Czerwinski A, Chakravarty R, Graves SA, Yang Y, England CG, et al. Evaluation of two novel (6)(4)Cu-labeled RGD peptide radiotracers for enhanced PET imaging of tumor integrin $\alpha v \beta 3$. *Eur J Nucl Med Mol Imaging*. 2015; 42:1859–68. [PubMed: 26016906]
54. Perreault A, Richter S, Bergman C, Wuest M, Wuest F. Targeting Phosphatidylserine with a 64Cu-Labeled Peptide for Molecular Imaging of Apoptosis. *Mol Pharm*. 2016; 13:3564–77. [PubMed: 27608290]

Figure 1.

cFLFLFK-PEG₁₂-Cy3 preferentially binds to human neutrophils in vitro. **A)** Gates for neutrophils (i), monocytes (ii), and lymphocytes (iii) were defined based on forward and side scattering properties. **B)** Average dose-response curves from three different donors showing Cy3 MFI for the different cell populations incubated with increasing concentrations (1, 10, 20, 30, 40, and 50 nM) of cFLFLFK-PEG₁₂-Cy3. Each peptide concentration level was run in duplicate for a given donor. Errors bars represent the standard error of mean. Neutrophils showed significantly higher cFLFLFK-PEG₁₂-Cy3 MFI values compared to monocytes and lymphocytes (***p < 0.0001, **p < 0.01, and *p < 0.05). **C)** Bright field (BF) and Cy3 fluorescent images of individual cells representing the three cell populations following incubation with 20 nM of cFLFLFK-PEG₁₂-Cy3. These images, which are from one representative donor, were acquired on the Flowsight imaging system. Scale bar = 20 μm.

Figure 2.

Cyclosporin H inhibits binding of cFLFLFK-PEG₁₂-Cy3 to human neutrophils. The FPR1 antagonist Cyclosporin H (CsH) inhibits binding of cFLFLFK-PEG₁₂-Cy3 to human neutrophils in a dose dependent manner ($IC_{50} = 88.7 \pm 6.8$ nM). The y-axis indicates the percentage of binding of 20 nM of cFLFLFK-PEG₁₂-Cy3 relative to the control situation (absence of CsH). Data points shown represent mean $\pm$ SEM from 3 independently performed experiments (donors). Each CsH concentration level was tested in duplicate for each donor. Fitting the data to a one-site binding model in Graphpad Prism resulted in an R² of 0.90.

Figure 3.

Conjugation of cFLFLFK-PEG₁₂ to the NOTA chelator modestly alters peptide binding to human neutrophils compared to the parent peptide. Both cFLFLFK-PEG₁₂ and cFLFLFK-PEG₁₂-NOTA inhibited binding of cFLFLFK-PEG₁₂-Cy3 to human neutrophils in a dose-dependent manner. Y-axis values show percentage binding of 20 nM of cFLFLFK-PEG₁₂-Cy3 co-incubated with non-fluorescent cFLFLFK-PEG₁₂ or cFLFLFK-PEG₁₂-NOTA over the indicated concentration range compared to the control situation (absence of unlabeled peptide). The calculated IC₅₀ values for cFLFLFK-PEG₁₂ and cFLFLFK-PEG₁₂-NOTA were 597.2 ± 3.4 nM and 1086 ± 2.5 nM, respectively. Data points shown represent mean $\pm$ SEM from 3 independently performed experiments (donors). Each peptide concentration level was run in duplicate for each donor. Fitting the data to a one-site binding model in Graphpad Prism resulted in R² values >0.95 for both compounds.

Figure 4. cFLFLFK-PEG₁₂-Cy3 selectively binds to CD68+ macrophages in an in vitro human cell model of TB granulomas. Seven days after *M.tb* infection of PBMCs, granulomas were fixed and incubated with mouse anti-human CD68 antibody (green) and cFLFLFK-PEG₁₂-Cy3 (red). Binding was analyzed by confocal microscopy. (A) Confocal image of a representative granuloma showing no staining of the isotype control Ab (no cFLFLFK-PEG₁₂-Cy3 incubation). (B) Confocal image showing a strong and highly co-localized CD68 and cFLFLFK-PEG₁₂-Cy3 staining pattern in the granuloma. DIC, differential interference contrast. Scale bar = 10 μm.

Figure 5.

TDM-challenged mice develop robust, granulomatous lesions in the lung. TDM was administered as oil-in-water emulsion containing 100 µg of TDM via the tail vein. Representative light microscopy images of hematoxylin and eosin (H&E) stained lung sections harvested from vehicle (A) and TDM-challenged (B) mice at day 5. Compared to the vehicle-injected mouse, the TDM-challenged mouse contains areas of dense, patchy immune cell infiltration. A representative high magnification view of a lung section from a vehicle-challenged mouse shows normal lung architecture and a minimal inflammatory cell infiltrate (C). In contrast, a robust inflammatory infiltrate is observed in the TDM-challenged mouse lung, appearing to surround lipid droplets presumably containing TDM (D). In this image, examples of macrophages, neutrophils, and lymphocytes are indicated by black arrows, black arrowheads, and gray arrows, respectively.

Figure 6. cFLFLFK-PEG₁₂-Cy3 selectively binds neutrophils and macrophages in vivo. Five days after TDM challenge mice were injected with 50 µg of cFLFLFK-PEG₁₂-Cy3 (n=3) or PBS (n=2). A mixture of mAbs was incubated with lung single-cell suspensions and Cy3 levels were measured on different cell populations. **(A)** The gating scheme applied to living cells for identifying: i) neutrophils, ii) alveolar macrophages, iii) macrophages and dendritic cells, iv) monocytes and immature macrophages, v) lymphocytes, and vi) other cells (not shown). **(B)** Pie chart showing the average relative frequencies of the 6 cell types in the TDM-challenged lung pooled together from 3 mice. **(C)** Cy3 signal levels in each cell population (solid gray histograms) as shown through histograms. Unfilled histograms represent TDM-challenged animals injected with PBS for controls. **(D)** Pie chart showing the relative Cy3 signal contribution per cell type to overall signal pooled together from 3 TDM-challenged mice.

666

Figure 7. cFLFLFK-PEG₁₂-Cy3 accumulates in areas of granulomatous inflammation in the TDM-challenged mouse lung. Representative confocal micrographs of frozen lung sections (10 µm) from TDM-challenged mice receiving no peptide (**A**) or 50 µg of cFLFLFK-PEG₁₂-Cy3 (**B**) administered via the tail vein. The images revealed punctate uptake of cFLFLFK-PEG₁₂-Cy3 in areas of robust inflammation in the TDM-challenged lung. DIC, differential interference contrast. Scale bar = 20 µm.

Table 1

Antibody	Clone	Source	Final Concentration
Ly6G-APC	1A8	BD Biosciences	2.4 µg/mL
CD3-Alexa488	17A2	Biolegend	12 µg/mL
CD11b- PerCP-Cy5.5	M1/70	BD Biosciences	2.4 µg/mL
CD11c-APC-Cy7	HL3	BD Biosciences	5 µg/mL