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Use of a leukocyte-targeted peptide probe as a potential tracer for imaging the tuberculosis granuloma

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ABSTRACT

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)-labeled cFLFLFK-PEG_{12} 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-PEG_{12}-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 contribute to our understanding of granuloma influx patterns and the biological roles and consequences of FPR1-expressing cells in contributing to disease pathogenesis.

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]. Alarmingly, 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

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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-PEG$_{12}$-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-PEG$_{12}$-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-PEG$_{12}$-Cy3 in the setting of lung granulomatous inflammation.

2. Experimental design and methods

2.1. Synthesis and labeling of cFLFLFK-PEG$_{12}$, cFLFLFK-PEG$_{12}$-Cy3, and cFLFLFK-PEG$_{12}$-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-dPEG$_{12}$-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, Breman, 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.

2.1.1. 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.

2.1.2. NOTA conjugation

A solution of p-SCN-Bn-NOTA (8.08 mg) in DMSO (100 μL) was added to cFLFLFK-PEG$_{12}$ 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.

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.