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Thioaptamer targeted discoidal microparticles increase self immunity and reduce *Mycobacterium tuberculosis* burden in mice

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ABSTRACT

Worldwide, tuberculosis (TB) remains one of the most prevalent infectious diseases causing morbidity and death in > 1.5 million patients annually. *Mycobacterium tuberculosis* (*Mtb*), the etiologic agent of TB, usually resides in the alveolar macrophages. Current tuberculosis treatment methods require more than six months, and low compliance often leads to therapeutic failure and multidrug resistant strain development. Critical to improving TB-therapy is shortening treatment duration and increasing therapeutic efficacy. In this study, we sought to determine if lung hemodynamics and pathological changes in *Mtb* infected cells can be used for the selective targeting of microparticles to infected tissue(s). Thioaptamers (TA) with CD44 (CD44TA) targeting moiety were conjugated to discoidal silicon mesoporous microparticles (SMP) to enhance accumulation of these agents/carriers in the infected macrophages in the lungs. *In vitro*, CD44TA-SMP accumulated in macrophages infected with mycobacteria efficiently killing the infected cells and decreasing survival of mycobacteria. *In vivo*, increased accumulations of CD44TA-SMP were recorded in the lung of *M. tuberculosis* infected mice as compared to controls. TA-targeted carriers significantly diminished bacterial load in the lungs and caused recruitment of T lymphocytes. Proposed mechanism of action of the designed vector accounts for a combination of increased uptake of particles that leads to infected macrophage death, as well as, activation of cellular immunity by the TA, causing increased T-cell accumulation in the treated lungs. Based on our data with CD44TA-SMP, we anticipate that this drug carrier can open new avenues in TB management.

1. Introduction

Tuberculosis (TB) is a major public health problem that affects more than one-third of world's population, accounting for 1.5 million deaths worldwide in 2015 [1]. TB is caused by the intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*), primarily harbored in the lungs within alveolar macrophages [2] where *Mtb* may remain dormant for decades [3]. Standard TB therapeutic regimens combine multiple antibiotics (rifampin, isoniazid, pyrazinamide and ethambutol) for prolonged periods of time from six to nine months, resulting in decreased adherence [4]. Premature withdrawal of treatment can lead to treatment failure, disease relapse, and increased risk of multi-drug resistance (MDR) development, especially for isoniazid [5] and rifampicin [6], requiring more toxic, less effective second-line or third-line drugs to be utilized [7]. With conventional first-line TB therapies, a number of bio-barriers prevent anti-TB drugs from being effective at the infected site. The difficulties of transporting the anti-TB drugs to the infected organ and across the cellular layers present, which in TB lesions include accumulated immune cells pathologically described as granulomas, causing low concentrations of TB drugs at the affected site and within pathogen proximity [8].

The use of nano- and micro-particles in medicine has been intensely studied providing new and improved options for diagnosis and therapy for diseases, such as cancer, cardiovascular disorders and infections [9–13]. Use of particles for TB treatment has been tested both *in vitro* and *in vivo* as summarized in several excellent reviews [14–17]. As an

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example, oral administration of drug-loaded poly(lactide-*co*-glycolide) (PLG) nanoparticles to TB-infected mice showed their ability to overcome first-pass metabolism, increasing therapeutic concentration in the tissue for 9 to 11 days and reducing the dosage needed for bacterial clearance, as compared to unbound drugs [18]. Similar results were observed with pulmonary delivery of particles in guinea pigs [19].

Macrophages are the major host-cell for *Mtb* and nano- and microparticles can potentially improve drug delivery due to their preferential uptake by macrophages. Alveolar macrophages are typically located in close proximity to the epithelial surface of the respiratory system and the capillaries, and are capable of sampling and examining air-borne and blood-borne materials [20,21]. Priming of intravenously (i.v) injected materials by alveolar macrophages [22] has been utilized in previous studies for TB treatment [23,24]. Recent studies demonstrated accumulation of particles carrying ciprofloxacin [25], rifampin [26], or isoniazid and streptomycin [27] in human blood monocytes, enhancing their antimicrobial activity against intracellular *Mtb*.

The approaches proposed to date aim to target only one bio-barrier to enhance TB therapy, thus, a combinatorial approach is likely to be advantageous. In the current study, we proposed to overcome several bio-barriers by combining the targeting to the lung based on the particle shape [28,29] and targeting to the specific ligands on the infected macrophages (Fig. 1). To attain this goal, we use discoidal microparticles that have been previously shown to concentrate in the lungs due to the hemodynamics of the organ [28]. Mesoporous silicon microparticles (SMP) are microfabricated by photolithography and electrochemical etching processes commonly used in the microelectronics industry [30–32]. These SMP have been shown to be biodegradable and biocompatible in a number of *in vitro* and *in vivo* studies [28,30,31,33–35] and can be easily loaded with chemotherapeutics and smaller therapeutic particles in their pores [36,37]. We further conjugated thioaptamers targeting CD44 (CD44TA) to the SMP surface (Fig. 2d) to enhance the accumulation of particles in the infected lung loci and on the infected cells. Thioaptamers are oligonucleotide molecules selected from large combinatorial libraries that exhibit specific structures and have a high binding affinity to proteins or other biological macromolecules. Aptamers and thioaptamers have been emerging as possible alternatives to conventional ligands, such as antibodies or peptides, due to their advantageous properties: easy production, robustness and their ability to be chemically modified [38]. Thioaptamers differ from conventional aptamers in their thiophosphate ester, which enhances their affinity and specificity for proteins and stability [39].

Recognition of infected tissue and macrophages is achieved by utilizing an inflammation targeting thioaptamer: anti-CD44 (CD44TA) [38] for both *in vitro* and *in vivo* models of TB infection. The objective of this study was to evaluate the targeting efficacy of the proposed CD44TA-SMP vectors as well as their effects on the infected cells and Mtb burden *in vitro* and *in vivo*. Interestingly, we serendipitously observed an anti-microbial activity with the CD44TA-SMP targeted drug carrier system, which can be ascribed to the activation of immune system, including the recruitment of T-cells to the infected lung.

2. Materials and methods

2.1. TA synthesis

Initial development and amino acid sequences of the CD44TA have been previously described [40]. For this study, TAs were synthesized on Expedite 8909 oligonucleotide synthesizers, using standard phosphoramidite chemistry [41,42] on standard CPG beads, with reagents purchased from Glen Research Inc. (Sterling, VA). For the selectively monothioated bases, Sulfurization Reagent II was used in place of the normal oxidation reagents. Cyanine 5 dye (Cy5) was added to the 5′-end of the TAs while still on the Expedite synthesizers, the chemical linkers Amino C6 or Carboxy C10 were then subsequently added to the 5′-end of the TAs when needed. Base-protecting groups and the beads were separated from the TAs by strong basic conditions for 24 h at room temperature. When the carboxy-linker was present, the deprotection step utilized 400 mM NaOH in 75% MeOH, otherwise concentrated ammonium hydroxide (in water) was used. After lyophilization (when using NH_4OH) or buffer exchange (when using NaOH) in Amicon centrifugal concentrators, TAs were loaded onto Hamilton PRP-1 semi-prep column using 100 mM triethylamine acetate buffer (pH = 8.4), and eluted off with acetonitrile gradient. Following lyophilization the TAs were solvated in water and subjected to repeated buffer exchanges with water to achieve about 100,000:1 dilution of any small impurities that might remain.

2.2. TA-SMP design

Discoidal SMP with a 1 × 0.4 μm (dxh) dimension were fabricated in a microelectronics facility *via* photolithography and electrochemical etching and modified with 3-aminopropyl-triethoxysilane (APTES) as previously described [43]. The particles were lyophilized using Freezone Freeze Dry System (Labconco, Kansas City, MO). SMP were conjugated with TAs using a Polylink Protein Coupling Kit according to the manufacturer's protocol (Bangs Laboratories Inc., Fishers, IN). The conjugation efficiency was ~9.96×10^6 CD44TA/particle. After every step in the conjugation process, the particles were characterized for their zeta potential using Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, UK), and counted with Multisizer 3 (Beckman Coulter, Brea, CA). Additionally, CD44TA-SMP morphology was characterized using scanning electron microscopy (SEM) and the CD44TA conjugation was confirmed by co-localization of TA-Cy5 with reflectance signals from the SMP using fluorescence microscopy.

2.3. Cell culture

Human primary monocytes were isolated from buffy coat (IRB (2) 1111-0206, Houston Methodist Blood Donation Service). Buffy coat was diluted 1:1 with PBS, overlaid on Ficoll-Paque (GE Healthcare, Pittsburgh, PA), and centrifuged at 400 × *g* for 30 min at 4 °C. Mononuclear cells were collected from the cloudy layer at the interphase and washed with PBS 2 times by centrifugation at 300 × *g* for

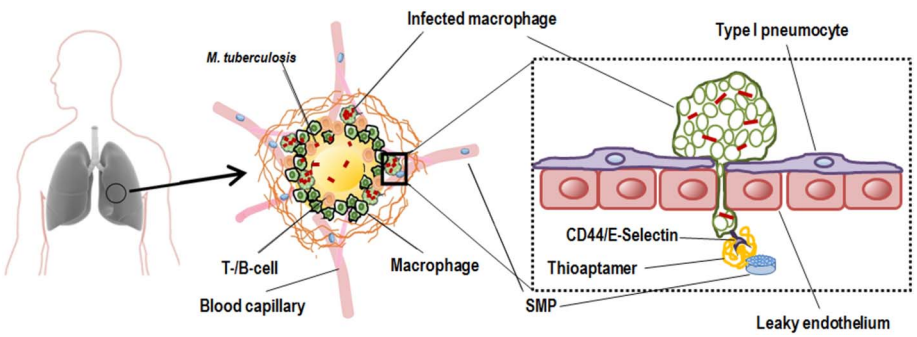

Fig. 1. Components of the studied systems and proposed method of action. Lung hemodynamics and pathological changes in *Mycobacterium tuberculosis*-infected cells enabled targeting of microparticles to the infected tissue. CD44 thioaptamers-conjugated discoidal microparticles enhanced accumulation in the infected macrophages *in vitro* and *in vivo*.