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Research paper

Phagocyte interactions with *Mycobacterium tuberculosis* — Simultaneous analysis of phagocytosis, phagosome maturation and intracellular replication by imaging flow cytometry

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ABSTRACT

Utilization of compounds that enhance the innate immune response against *Mycobacterium tuberculosis* is an attractive strategy for combating tuberculosis in the post-antibiotic era. Thus, it is crucial to develop methods that can be used to screen for such compounds and to investigate their mechanisms of action. Here, we used imaging flow cytometry (ImageStreamX Mk II), which enables rapid quantification of microscopic images in flow, to study the interaction between phagocytes and *M. tuberculosis*. Macrophage-differentiated THP-1 cells were infected with GFP-expressing *M. tuberculosis* H37Ra, and methods for rapidly assessing phagocytosis, phagosome maturation, and bacterial replication inside the cells were developed and evaluated. These aspects of innate immunity are essential in determining the outcome of mycobacterial infection of phagocytes. The technique was found effective for monitoring phagocytosis of mycobacteria, phagosomal acidification and phagolysosomal fusion, as well as for measuring mycobacterial replication inside the cells. Several of these aspects could be analyzed simultaneously in the same sample, providing a great deal of information about the phagocyte–mycobacterial interaction at once. Thus, this method has great potential to be useful both for basic research questions and for evaluating compounds that enhance the innate immune response against *M. tuberculosis*.

1. Introduction

Mycobacterium tuberculosis causes tuberculosis (TB), an infectious disease that kills nearly two million people each year worldwide. The bacterium is transmitted by contaminated aerosols released through coughing, and exposure can lead to a spectrum of outcomes; from clearance by the innate or adaptive immune system, to latent infection or active disease (Barry et al., 2009). The importance of innate immunity in protection against TB is illustrated by the fact that up to 50% of exposed individuals clear the infection without the onset of adaptive immunity (Morrison et al., 2008; Verrall et al., 2014). New treatment strategies against TB are sorely needed to combat emerging antibiotic resistance, and an attractive approach is to boost the innate immune response in order to enhance bacterial clearance (Verrall et al., 2014).

Following inhalation, *M. tuberculosis* is phagocytosed by alveolar macrophages in which the bacteria inhibit phagosome maturation to grant intracellular survival and replication. Regular phagosome maturation entails the fusion between the phagosome in which the bacterium resides and lysosomes, as well as the acquisition of vacuolar H^+-ATPases leading to a drop in pH and activation of bactericidal proteases originating from the lysosomes (Sturgill-Koszycki et al., 1994; Vergne et al., 2004; Welin and Lerm, 2012). Lysosomal markers such as CD63 (also known as lysosome-associated membrane glycoprotein 3, LAMP-3), LAMP-1, or functional dyes staining acidic compartments can be used to investigate whether phagosome maturation has occurred (Welin et al., 2008, 2011b). The partial arrest of phagosome maturation by *M. tuberculosis* is an active process dependent on the mycobacterial cell wall component lipoarabinomannan (LAM) (Welin et al., 2008; Welin and Lerm, 2012). The LAM causes inhibition of calcium signaling and of a type III PI 3-kinase necessary for the fusion and fission events involved in phagosome maturation (Vergne et al., 2004). It is known that the degree of antibacterial pressure exerted on *M. tuberculosis* inside the phagosome influences the outcome of macrophage infection (Dhiman et al., 2009; Welin et al., 2011b), and mycobacterial mutants defective in the inhibition of phagosome maturation display reduced survival inside murine macrophages (Pethe et al., 2004). Thus, phagosome maturation is a potential target for new drugs that enhance the innate immune response against *M. tuberculosis*.

In order to find compounds that target the innate immune system, boosting bacterial killing inside the macrophage without causing

Abbreviations: ADC, albumin dextrose catalase; Ch, channel; EDF, extended depth of field; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; LAM, lipoarabinomannan; LAMP, lysosome-associated membrane glycoprotein; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; RMS, root mean square; TB, tuberculosis.

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immunopathology, new methods are needed. Current approaches for quantifying phagosome maturation involve laborious microscopy protocols and manual subjective scoring of phagosomes (Welin et al., 2008; 2011a; O'Leary et al., 2011), or advanced functional assays that probe the phagosomal environment (Rohde et al., 2007). Although both have been very valuable in characterizing the mycobacterial compartment, neither is easily adapted to the screening context even on a small scale. Furthermore, rapid and very useful methods for evaluating bacterial growth or killing inside host cells are available (Eklund et al., 2010; Andreu et al., 2012; Kicka et al., 2014), but not in combination with analysis of phagosome maturation or other intracellular phenomena. Here, we developed a new tool for simultaneous quantification of phagocytosis, phagosome maturation, and bacterial replication inside macrophages interacting with *M. tuberculosis*, based on imaging flow cytometry. Imaging flow cytometry combines fluorescence microscopy and flow cytometry, enabling rapid flow-based quantification of fluorescence microscopic image information, and provides many advantages that can be employed in the search for new treatment strategies against TB as well as for basic research.

2. Materials and methods

2.1. Culture of M. tuberculosis

The avirulent *M. tuberculosis* strain H37Ra (ATCC #25177, Biosafety Level 2) was used for all experiments. Early passages were prepared and stored at −70 °C. The bacteria were cultured in Middlebrook 7H9 broth with 0.05% Tween-80, 0.5% glycerol and albumin dextrose catalase (ADC) enrichment (BD) at 37 °C for 2–4 weeks, and then passaged and incubated for 1 week before use in experiments. For constitutive expression of green fluorescent protein (GFP), H37Ra bacteria were transformed with the pFPV2 plasmid carrying the gene for GFP, and plasmid-carrying mycobacteria were selected using 20 μg/ml kanamycin (Welin et al., 2011a). Heat-killed fluorescein isothiocyanate (FITC)-labeled *M. tuberculosis* H37Ra was prepared by incubation of H37Ra (without the pFPV2 plasmid) in a water bath at 80 °C for 1 h, multiple passages through a syringe equipped with a 27-gauge needle, followed by labeling with 2 μg/ml FITC (Sigma) and several washes, and stored at -20 °C until use.

2.2. Cell culture

The monocytic cell line THP-1 (ATCC #TIB-202) was cultured by standard procedures in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Fisher Scientific). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and passaged every 3–4 days. For differentiation to macrophage-like cells and use in experiments, THP-1 cells were seeded (1×10^6/well) in 6-well plates in fresh medium supplemented with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma), and incubated for 18 h (Theus et al., 2004). Efficient differentiation was defined as adherence to the plastic. The cells were then allowed to rest in RPMI without PMA for at least 2 h.

2.3. Preparation of phagocytic prey

The H37Ra suspension was washed in phosphate-buffered saline (PBS) supplemented with 0.05% Tween-80. Single bacilli were obtained by passing the suspension 10 times through a syringe equipped with a 27-gauge needle, a further wash, resuspension in plain RPMI (*i.e.* without additives), and finally another 10 passages through the needle (Welin et al., 2011a). In some experiments, FITC-labeled zymosan particles (Life Technologies) were used as phagocytic prey. The concentration of bacteria and zymosan was determined using an Accuri C6 flow cytometer (BD). Opsonization of bacteria was carried out in Protein LoBind tubes (Eppendorf). For serum opsonization, the phagocytic prey was incubated with normal human serum (Sahlgrenska University Hospital Blood Bank, 25% or 50% as stated), at 37 °C for 30 min. For IgG opsonization of mycobacteria, the bacilli were incubated with 20 μg/ml polyclonal rabbit anti-*M. tuberculosis* antibody (Abcam ab905), after removal of sodium azide from the buffer using protein G-coated Dynabeads® (Life Technologies), under the same conditions. The prey was not washed after opsonization.

2.4. Infection of cells

Adherent THP-1 cells were washed in plain RPMI to remove serum and antibiotics, and the phagocytic prey was added at the indicated ratio (prey:cell), diluted in plain RPMI. Synchronization of phagocytosis was achieved by centrifugation of the plate (3000 ×*g*, 5 min), after which the plate was incubated at 37 °C for 2 h or the indicated time. For long-term incubations over several days, the medium was exchanged after 2 h for RPMI supplemented with 10% fetal calf serum and 2 mM L-glutamine, with or without the addition of antimycobacterial antibiotics (100 μg/ml streptomycin) as stated.

2.5. LysoTracker and CD63 staining

For staining of acidified compartments in THP-1 cells, LysoTracker® Deep Red (Life Technologies) at 75 nM was added to the medium during the final 20 min of infection (Welin et al., 2011a). LysoTracker stains compartments with a pH of about 6 and below (von Bargen et al., 2009), and the *M. tuberculosis*-containing phagosome has an average pH of about 6.4, as opposed to a mature phagosome with a pH of less than 5 (Rohde et al., 2007), making LysoTracker an appropriate probe. The cells were removed from the surface using a cell scraper following incubation with lidocaine hydrochloride monohydrate (Sigma, 4 mg/ml) at 37 °C for 15 min, and washed with PBS by centrifugation (500 ×*g*, 5 min). LysoTracker-stained cells were then resuspended in 25 μl PBS and placed on ice for immediate imaging flow cytometry analysis. CD63 was used as a late endosomal/lysosomal marker (Welin et al., 2011a). Indirect intracellular immunofluorescence staining of detached, fixed, and permeabilized cells was performed as previously described (Welin et al., 2013), using a monoclonal mouse-anti human CD63 antibody (Sanquin, 4 μg/ml) followed by an Alexa Fluor 647-conjugated goat anti-mouse ($F(ab')_2$ fragment) secondary antibody (Life Technologies, 2.5 μg/ml). Cells were washed and resuspended in 25 μl PBS before analysis by imaging flow cytometry.

2.6. Imaging flow cytometry

All samples were analyzed using an ImageStreamX Mk II imaging flow cytometer (Amnis), followed by IDEAS analysis software (v. 6.1, Amnis) as described in Section 3 below. The ImageStreamX simultaneously collects six multi-mode images of each event in flow, including brightfield, darkfield (corresponding to side scatter) and up to four fluorescence colors. Images are then analyzed using IDEAS software, enabling quantification of different aspects of the obtained image data. A "mask" defines the region of interest in the cell, and quantification of different parameters inside the mask is then performed using a "feature", which can be custom-made. Here, the 488 nm laser was used for excitation of GFP or FITC and the 642 nm laser for excitation of Alexa Fluor 647 or LysoTracker Deep Red. The 60× magnification objective was used, providing a pixel size of 0.33 μm^2 and a depth of field of 2.5 μm. 20,000 events were collected per sample (at a speed of about 50–150 cells per second), ensuring a sufficient number of events remaining for statistically robust analysis after all the gating steps (generally above 500 cells). Single stain samples were routinely collected using the same settings and used as compensation controls to generate compensation matrices in IDEAS. The extended depth of field (EDF) option on the ImageStreamX can be used to project structures in a wide focal