

Development of ligand-coated beads to measure macrophage antimicrobial activities

Trinh T. B. Tram^{*} , Vu T. N. Ha^{*}, Do D. A. Thu^{*}, Tran D. Dinh^{*}, Srinivasan Vijay^{*†}, Hoang T. Hai^{*}, Nguyen T. Hanh^{*}, Nguyen H. Phu^{*}, Guy E. Thwaites^{*†} and Nguyen T. T. Thuong^{*1}

^{*}Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam and [†]Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom

Background information. After macrophage recognises and phagocytoses the microorganism, their phagosome undergoes a maturation process, which creates a hostile environment for the bacterium. The lumen is acidified, and proteolysis occurs to kill and degrade pathogen for further antigen presentation. It is important to understand the association between the macrophage intracellular activities and the outcome of infection. Different methods have been developed to measure the phagosome dynamics of macrophages, but there are still limitations.

Results. We used *Mycobacterium tuberculosis* (*Mtb*) antigens, the causative agent of tuberculosis (TB), as a model of infectious disease. Adopting a fluorescent bead-based assay, we developed beads coated with trehalose 6,6'-dimycolate (TDM) from *Mtb* cell wall and β -glucan from yeast cell wall to measure the macrophage phagosomal activities using a microplate reader. We examined the consistency of the assay using J774 cells and validated it using human monocyte-derived macrophages (hMDM) from healthy volunteers and TB patients. There was a decreased pH and increased proteolysis in the lumen of J774 cells after phagocytosing the ligand-coated beads. J774 macrophage showed no difference in the acidification and proteolysis in response to control IgG beads, TDM and β -glucan beads. hMDM from healthy volunteers or TB patients showed heterogeneity in the intracellular activities when treated with ligand-coated beads.

Conclusions and significance. The beads coated with specific ligands from *Mtb* worked well in both macrophage cell line and human primary macrophages, which can be exploited to further study the phagosomal function of macrophage in TB. Our bead model can be applied to different ligands from other pathogens, which could extend the understanding of the associations between macrophage antimicrobial functions and outcomes of infectious diseases and the possible cellular mechanisms involved.



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Phagosome maturation is one of the central processes contributing to important role of macrophages in innate immunity. The process is initiated by recognising and internalising the invading pathogen into phagosome through phagocytic surface receptors,

such as Fc receptors, complement receptors and various scavenger receptors. Following this, the lumen of the phagosome develops into a toxic environment by acidifying, acquiring a range of hydrolytic enzymes and producing toxic radical compounds [Flannagan et al., 2009]. Acidic pH is prerequisite for the optimal functioning of hydrolytic enzymes and degradation of phagosomal content, and is unfavourable for the

¹To whom correspondence should be addressed (email thuongntt@oucru.org)

Key words: acidification, ligand-coated beads, macrophages, proteolysis.

Abbreviations: FBS, foetal bovine serum; hMDM, human monocyte-derived macrophages; *Mtb*, *Mycobacterium tuberculosis*; PBMC, peripheral blood monocyte cells; PTB, Pulmonary tuberculosis; TB, Tuberculosis; TDM, trehalose 6,6'-dimycolate.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

growth of the pathogen. The hydrolytic enzymes can directly kill the bacteria by interrupting their membrane integrity, but also help in degrading the pathogen. Antigen generated from such degradation is presented to T lymphocyte via MHC class II molecule, which further enhances the elimination of the pathogen by adaptive immune response.

Previous studies have showed that an impairment in phagocytic capacity of macrophages could lead to the development of several pulmonary diseases caused by *Haemophilus influenzae*, *Streptococcus pneumoniae* [Taylor et al., 2010] or *Coxiella burnetii* [Ghigo et al., 2004]. Several professional intracellular bacteria such as *Mycobacterium tuberculosis* (*Mtb*), *Listeria monocytogenes* and *Legionella pneumophila* have evolved the mechanisms to avoid phagosome maturation, so that bacteria can replicate within macrophages and establish the infection [Flannagan et al., 2009]. It is likely that the outcome of infection depends on the innate interaction between bacteria and macrophages [Brighenti and Lerm, 2012]; any defects in the phagosomal function of macrophage could have major impact on the establishment of the infection. Therefore, it is important to assess the macrophage phagosomal function, which may help to understand not only the contribution of macrophage in the host defence and susceptibility to the infectious diseases but also the manipulation of macrophages by the pathogen.

Many assays have been developed to examine the phagosome maturation within macrophages. Most approaches use immunofluorescence colocalisation with well-characterised markers [Kelley and Schorey, 2003; Ghigo et al., 2004; Huynh et al., 2007]. However, these methods are subjective unless accompanied with quantification of signal intensities, have low sensitivity and may not reflect the physiological environment within the phagosome [Rohde et al., 2007; Welin et al., 2011]. Russell and colleagues developed multiple assays to measure in real-time, quantitatively functionally relevant parameters of phagosomal luminal biology [Yates and Russell, 2008; Russell et al., 2009; Podinovskaia and Russell, 2015]. These assays exploit the model of beads that were coated with three components including immunoglobulin G (IgG), fluorogenic substrates and calibrators. IgG is recognised by the Fc gamma receptor; hence, it helps facilitating the uptake of the beads. Fluorogenic substrates are specific for different

phagosomal activities, which enable the assessment of the activities on different analytical platforms, including flow cytometry, confocal microscopy and fluorescence microplate reader. The flow cytometry provides detail at the individual cell level within and between cell populations. Visualisation by a microscope provides spatial and temporal details at the level of single phagosome. These methods are time consuming, lacking statistical power and only handle limited experiment conditions at a time; whereas the microplate reader allows monitoring of multiple conditions with many replicates [Podinovskaia and Russell, 2015].

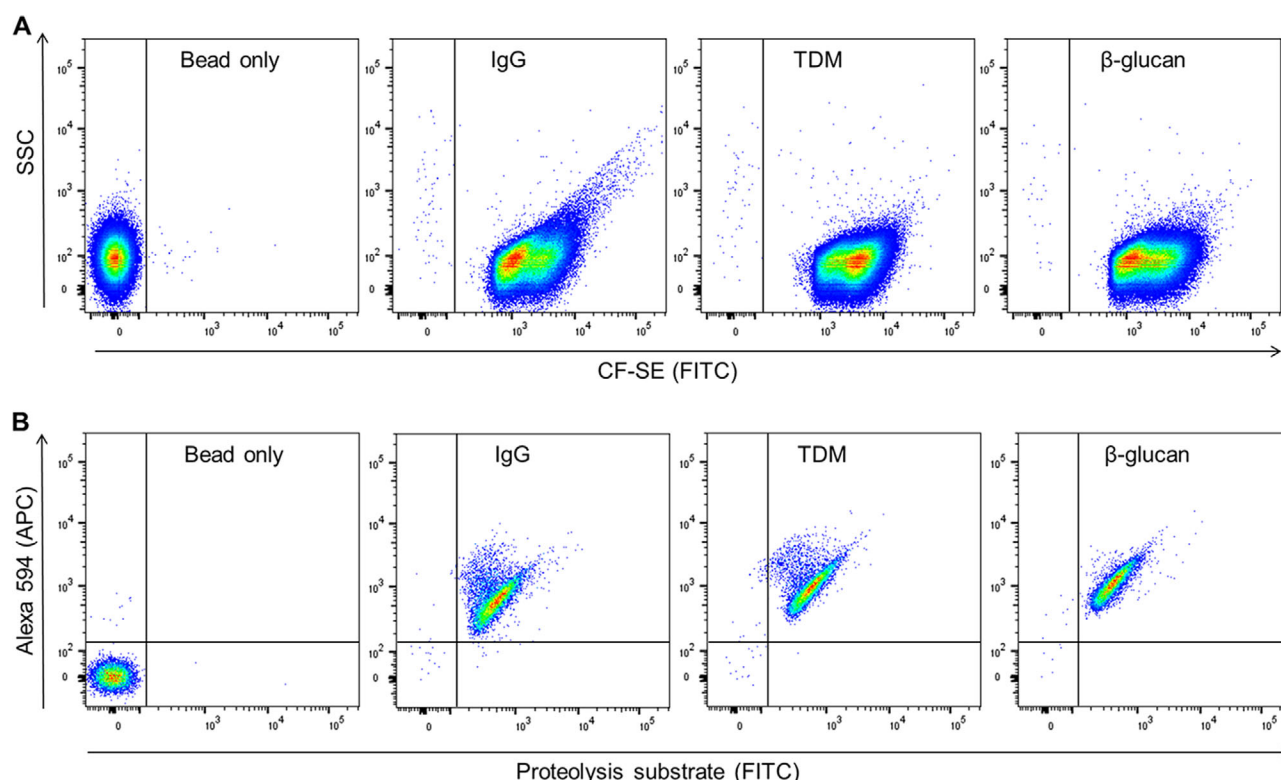
The bead-based assays developed by the Russell group were validated in cell lines such as RAW 264.7 cells or in bone marrow-derived macrophages (BMM) [Yates and Russell, 2008]. The cell lines were used because they are easy to handle, optimal for setting up assays and for analyses requiring large cell numbers, whereas BMM appear to be more accurately in reflecting the properties of the macrophages *in vivo*. However, human primary macrophages most closely reflect the biology of intracellular infections. Therefore, this model could be a practical choice for studying whether the deficiencies in macrophage phagosomal function can predispose a person to the disease.

In addition, different receptors on macrophage surface recognise specific pathogen-associated molecular patterns, which could activate specific signalling pathways and lead to possible distinct anti-pathogen responses. Therefore, coating the beads with different pathogen-derived ligands other than IgG can mimic infection [Podinovskaia and Russell, 2015] and allow us to study the association of macrophage intracellular activities with a wide range of bacterial ligands and dissect the specific pathways involved.

In order to provide a representative model for studying the association between phagosomal function of macrophages and ligands derived from pathogen, we generated reporter beads that are coated with ligands from *Mtb*, which causes tuberculosis (TB), to measure the acidification and bulk proteolysis of macrophages. The assays were examined for consistency using the homogenous J774 macrophage cell line and then were validated in human monocyte-derived macrophages (hMDM), from both healthy volunteers and patients with pulmonary TB (PTB), using a microplate reader.

Figure 1 | Cytometry analysis of acidification and proteolysis beads

(A) Acidification beads are beads coated with a ligand (IgG/TDM/ β -glucan) and acidification indicator CF-SE. The dots represent beads only or acidification beads and were plotted against SSC and FITC channel (for CF-SE). Acidification beads coated with CF-SE exhibited a shift on the FITC channel in comparison with bead only. (B) Proteolysis beads are beads coated with a ligand (IgG/TDM/ β -glucan), proteolysis substrate and calibrator Alexa 594 SE. The dots represent beads only or proteolysis beads and were plotted against FITC channel (for proteolysis substrate) and APC channel (for Alexa 594). Proteolysis beads coated with proteolysis substrate and Alexa 594 exhibited shifts on the FITC channel and APC channel, respectively, in comparison with bead only.



Results

Generation of ligand-coated beads

In addition to IgG, we coated beads separately with TDM from *Mtb* and β -glucan from yeast that are recognised by a scavenger receptor [Bowdish et al., 2009] and C-type lectin [Brown and Gordo, 2001; Kleinnijenhuis et al., 2011], respectively, to study the macrophage antimicrobial activities regarding different signalling pathways. The beads were incubated in an excess of IgG, TDM and β -glucan to ensure the coating of these ligands on the bead surface [Yates and Russell, 2005]. To examine coating efficiency of substrates and calibration fluors on the beads, these components were analysed by flow cytometry (Figure 1). The acidification beads were coated with

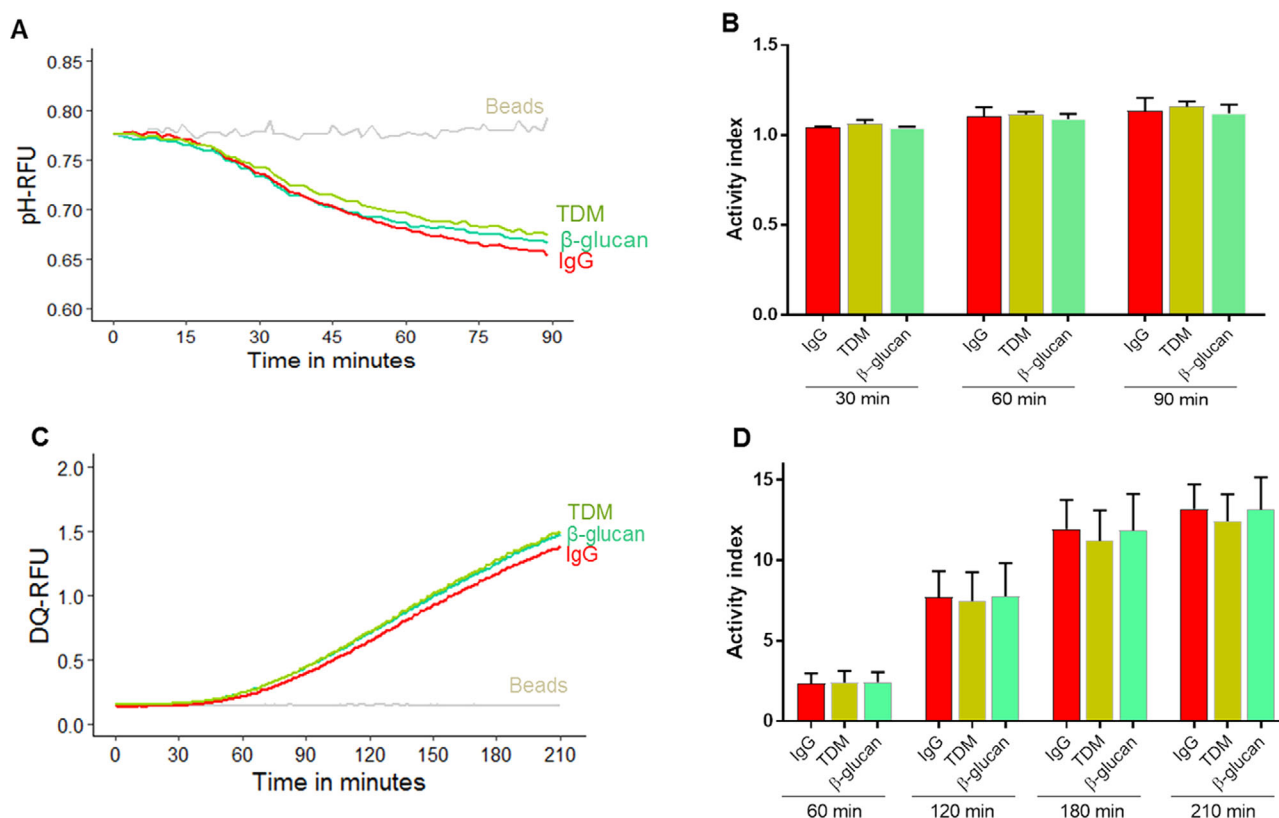
fluorogenic carboxylated-SE indicated by increased FITC signal on the bead surface (Figure 1A). Likewise, the proteolysis beads also exhibited the signal of the proteolysis substrate DQ-BSA (FITC) and signal of the calibrator (Alexa 594) in comparison with the control uncoated beads (Figure 1B).

Consistency of bead-based assays in J774 cell line

We examined whether assays of acidification and proteolysis exploiting ligand-coated beads worked using J774 cell lines. J774 macrophages were fed with the acidification beads to measure the acidification activity. The relative pH in phagosome was expressed by the ratio of fluorescent intensity at pH-sensitive

Figure 2 | Acidification and proteolysis in J774 cell line

Ligand coated beads for acidification (A, B) and proteolysis (C, D) were added to J774 cells. (A) The kinetics of phagosomal pH, plotted as the relative fluorescent unit (RFU) which is ratio of fluorescence emitted at 520 nm upon excitation from pH-sensitive and pH-insensitive wavelengths of 490 and 450 nm, respectively. (B) Acidification activity index of macrophages at time points of 30, 60 and 90 min, calculated by the ratio of RFU at 10 min over RFU at the selected time points. (C) The kinetics of proteolysis, plotted as the RFU which is the ratio of DQ-BSA green fluorescence and calibration fluorescence. (D) Proteolysis activity index at time points of 60, 120, 180 and 210 min, calculated by the ratio of RFU at selected time points over RFU at 10 min. Data were expressed as the mean \pm SD from three independent experiments. *P* value was determined using the Kruskal–Wallis test for comparison across three groups. Beads: beads in assay buffer without macrophages.



and -insensitive wavelengths, with a decreased ratio indicating a drop in phagosomal pH (Figure 2A). Conversion from the fluorescent ratio value to pH could be achieved through polynomial regression of a standard curve generated by the calculation of the fluorescent ratio in environments of known pH (Figure S1 in the Supporting Information). In macrophages treated with IgG beads, pH reduced significantly within 90 min from pH 6.9 to approximately pH 5.5 (Figure 2A). Likewise, we observed the reduced phagosomal pH of macrophages treated with TDM- and β -glucan beads, indicated by the decreased relative fluorescent intensities. The activity index was

used to compare the acidification activity of J774 in response to IgG, TDM and β -glucan coated beads. At either 30, 60 or 90 min after bead addition, there was no difference in capacity of J774 to acidify when treated with different ligands coated beads (Kruskal–Wallis test, $P = 0.08, 0.63, 0.63$ for 30, 60 and 90 min, respectively) (Figure 2B).

To measure the proteolytic activity, J774 macrophages were treated with ligand beads coated with fluorogenic substrate DQ green BSA. IgG bead-treated macrophages showed an acquisition of proteolysis initially at 30 min, followed by an increase in the activity during 210 min of measurement

(Figure 2C). The increase in proteolysis activity was also observed in macrophages treated with TDM- or β -glucan-coated beads. The proteolysis activity of macrophages in response to different ligand-coated beads was compared using activity index. At either 60, 120, 180 and late time-point of 210 min, the proteolysis activity of J774 cells was similar when treated with IgG, TDM or β -glucan beads (Kruskal–Wallis test, $P = 0.07, 0.99, 0.83, 0.66$ for 60, 120, 180 and 210 min, respectively) (Figure 2D).

The result for each assay was averaged from three independent experiments with small variation, indicating the consistency of the assay in J774 cell line.

Evaluation of bead-based assays in human hMDM

We next examined whether the assays of acidification and proteolysis worked in hMDM from healthy volunteers ($n = 10$) or PTB patients ($n = 10$). As in J774 macrophages, hMDM treated with acidification beads showed a decreased phagosomal pH by time and the bead-containing phagosomes were acidified completely after 60–90 min of bead treatment (Figure S2 in the Supporting Information). Consistently, by calculating the activity index, we observed an increased acidification activity at 60 and 90 min compared to 30 min in bead-treated macrophages from either healthy volunteer or PTB patients (Figure 3A). In addition, we also observed variation in the acidification activity of macrophages among individuals when treated with either IgG-, TDM- or β -glucan-coated beads.

When hMDM were treated with proteolysis beads, we observed an increased fluorescence in the cells from two groups of subjects (Figure S3 in the Supporting Information), indicating the proteolysis assays worked in this model. Macrophages from either healthy volunteers or TB patients showed a wide range of proteolysis activity in response to ligand-coated beads at early or late time points after bead internalisation (Figure 3B). For example, the coefficient of variation (%CV) (mean and SD) of macrophage activity from healthy volunteers in response to TDM-coated beads at 60, 120, 180 and 210 min were $45.4 (2.9 \pm 1.3)$, $49.7 (9.5 \pm 4.7)$, $44.1 (13.3 \pm 5.9)$, and $41.4 (14.3 \pm 5.9)$, respectively while that from TB patients were $52.8 (4.0 \pm 2.1)$, $41.7 (11.2 \pm 4.7)$, $35.6 (14.8 \pm 5.3)$ and $33.7 (15.8 \pm 5.3)$. These results suggested a diversity in activity among individuals.

Stability of ligand-coated beads

To examine long-term fluorescent intensities of beads, every 6 months during 18 months of storage the acidification and proteolysis beads were added to J774 cells and activity index of J774 at these time points were determined. The acidification activity and proteolysis activity of J774 in response to ligand-coated beads remained stable during 12 months (Figure 4). Fluorescent signals of both activities tended to be decreased after 18 months of storage, and there was a significant reduction in proteolysis activities regardless of ligands (paired t -test, 0 month vs. 18th month, $P = 0.02$) (Figure 4B). These results suggested that the acidification and proteolysis beads were better to use by 12 months after storage.

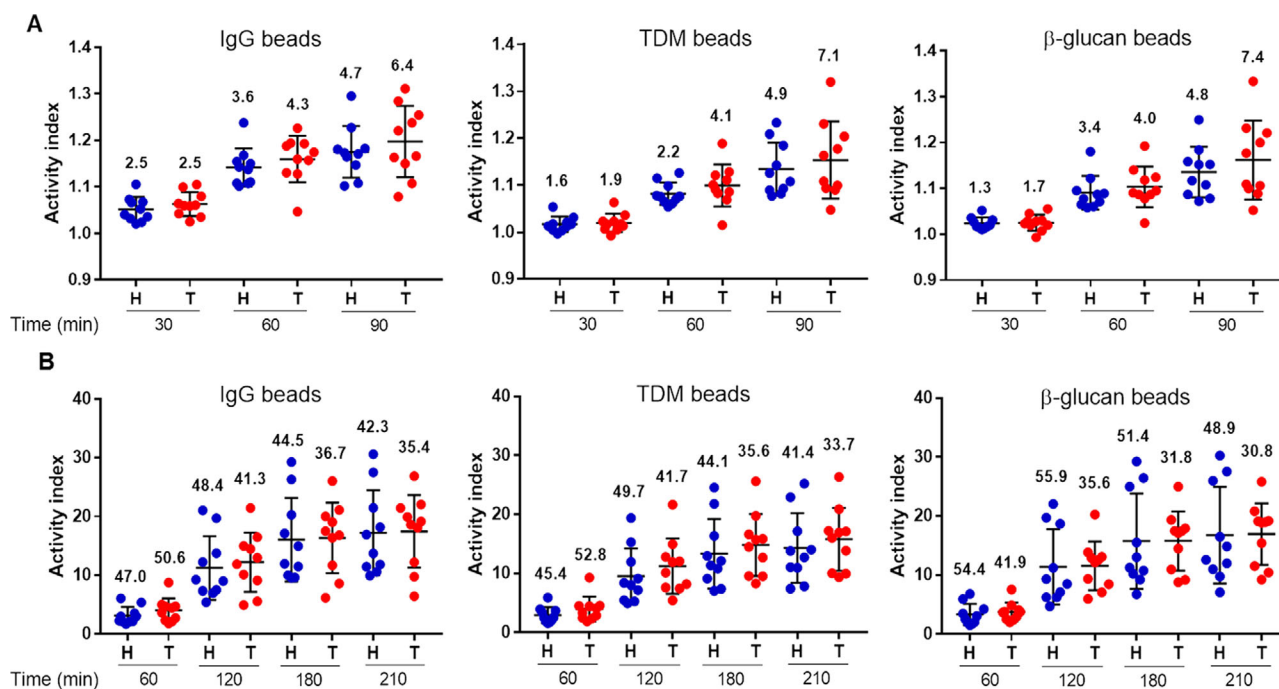
Discussion

The bead-based assays were used to measure the phagosomal activities of macrophages in previous studies; however, the beads were only opsonised with IgG to facilitate the uptake by macrophages. It remains unclear whether different routes of entry into macrophages may lead to differences in subsequent events within the host such as signal transduction, immune activation and survival of the pathogen, as well as outcome of infection [van Crevel et al., 2003]. Taking TB as an example, we generated the beads coated with specific antigens from *Mtb* such as TDM and β -glucan that activate different signalling pathways to measure the macrophage phagosomal activities. The assays exploited these beads showed consistent results in the model of cell line and revealed the heterogeneity in intracellular activities of macrophages among either healthy individuals or TB patients.

We observed the change in acidification and proteolysis in the phagosomal lumen in response to bead coated with TDM or β -glucan in J774. Previous studies showed that activation of BMM or hMDM macrophages with Lipopolysaccharides results in delayed acidification and reduced proteolysis activity compared with IgG-treated macrophages [Yates et al., 2007; Podinovskaia et al., 2013]. These results suggest a shift in macrophage function from its role in homeostasis to the role in immunity. More clearly, the homeostatic function of macrophages is to remove dead cells or cell debris; hence, their hydrolytic activity is programmed to occur with high

Figure 3 | Acidification and proteolysis in hMDM

Ligand-coated beads for acidification (A) and proteolysis (B) were added to hMDM from 10 independent healthy volunteers and 10 PTB patients. (A) Acidification activity index of hMDM, calculated by first determining the RFU which is the ratio of fluorescence emitted at 520 nm upon excitation from wavelengths of 490 and 450 nm at 10-, 30-, 60- min time points, and then dividing the RFU 10 min by RFU at 30, 60 min. (B) Activity index of hMDM, calculated by first determining the RFU which is the ratio of DQ-BSA green fluorescence and calibration fluorescence at 10-, 60-, 120-, 180- and 210-min time points, then dividing the RFU at 60, 120, 180 and 210 by RFU at 10 min. H: Healthy volunteers, T: PTB patients. Data are expressed as the mean \pm SD. The number indicated %CV (coefficient of variation).



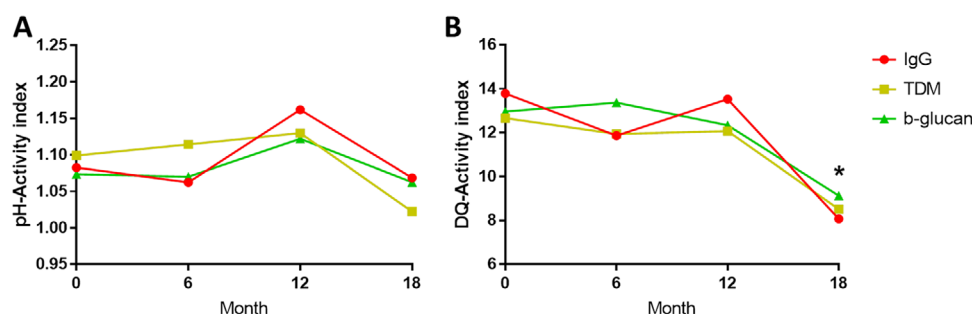
efficiency for complete degradation of those materials. Under activation by cytokines or bacterial components, the hydrolytic activity is reduced to ensure the balance of effectively killing of pathogen and efficient antigen processing for presenting to T cells [Yates et al., 2007]; however, there was no difference in the intracellular activities of J774 macrophages in response to ligands of TDM or β -glucan in comparison to IgG in our study. The discrepancy could result from variation in response among different cell types and ligands in different studies.

Having exploited the stability of macrophage cell line, we used it as a model to set up and optimised the ligand bead-based assays. All the assays were shown to work well giving consistent results. Our assays also worked in the hMDM model from both healthy volunteers as well as PTB patients, which displayed variations in antimicrobial activities

of macrophages from different subjects. hMDM were differentiated *in vitro* from peripheral blood monocytes (PBMCs) of PTB patients during 7 days, suggesting that these cells are inactivated or in resting stage, and their activities are not affected by other activated cell types in the patients through intercellular communication. A previous study showed no difference in phagocytic function of hMDM between TB patients and healthy volunteers; however, there was a wide range of phagocytosis abilities among individuals, which is associated with host genotype [Thuong et al., 2016]. There has been limited understanding of intracellular killing activities of macrophages and its variations in patients with TB. Therefore, our bead-based assays could be applied to further study the association of defective macrophage functions with different clinical outcomes of *Mtb* infection and signalling pathways driving such

Figure 4 | Long-term fluorescent stability of acidification and proteolysis beads

Every 6 months for up to within 18 months, J774 macrophages were treated with beads coated with IgG, TDM or β -glucan and activity index were determined. (A) Acidification activity index at 60 min after bead treatment. (B) Proteolysis activity index at 180 min after bead treatment. *P* value was determined using the paired *t*-test for comparison of activity at 0 month versus that at other months. *: *P* value < 0.05.



associations. In a broader view, beads could be coated with specific ligands from a target pathogen and an array of acidification, and proteolysis from macrophages of patients infected by the pathogen can be measured. The assays using ligand-coated beads could not only help to identify host phenotypes associated with susceptibility to infectious diseases or any macrophage-associated pathology as using only IgG-coated beads [Russell et al., 2009; Jambo et al., 2014], but also shed light on understanding the possible underlying mechanisms.

Our result showed that the beads should be generated and used by 12 months due to the loss of fluorescence after long-term storage. Therefore, studies on association of intracellular activities of macrophages and susceptibility to infectious disease should take into account the stability of beads. Since the frozen PBMCs can be used in substitution of fresh PBMCs in several functional assays such as phagocytosis [Stevenson et al., 2018], hence PBMCs from patients could be optionally isolated and cryopreserved to further measure all activities of hMDM at the same time to avoid the influence of bead storage.

In summary, we developed the beads coated with ligands from *Mtb* and tested them in macrophage cell line and human primary macrophages, which enabled us to further study the phagosomal activities of macrophages from patients with TB. The ligand bead-based assays provide a useful tool to gain a deeper understanding of specific immunological components or pathways involved in the cellular response to infectious diseases.

Materials and Methods

Preparation of acidification and proteolytic beads

Silica particles (or beads) were coated with ligands (IgG, TDM or β -glucan), fluorogenic substrates and calibration fluor. Twenty-five milligrams (or 500 μ l) carboxylated and 3 μ m silica particles (Kisker Biotech) were washed three times in 1 ml of PBS by vortexing and centrifugation at 2000 $\times g$ for 1 min. Beads were incubated at room temperature in 25 mg/ml cyanamide (Sigma-Aldrich), which works as a cross-linker, in PBS along with 15 min agitation. Beads were washed twice in 1 ml coupling buffer (50 mM borate buffer in PBS, pH 8.0) to remove excess of cyanamide, incubated in 0.5 ml coupling buffer with 1.0 mg defatted bovine serum albumin (Sigma-Aldrich) and 0.1 mg human IgG (Molecular Probes), or 0.25 mg ligands [TDM (Enzo Life Sciences) or β -glucan/whole glucan particles (Invivogen)] and then dispersed for 12 h with agitation. Coated beads were washed three times in 1 ml quench buffer (250 mM glycine in PBS, pH 7.2; Sigma-Aldrich) to quench the unreacted cyanamide. Subsequently, for acidification activity, beads were labelled with the pH-sensitive reporter carboxyfluorescein-SE as described in Yates and Russell [2008] (10 μ l of the 5 mg/ml stock; Molecular Probes) and agitated for 2 h. Beads were washed three times in 1 ml quench buffer, re-suspended in 1 ml PBS with 0.02% sodium azide and stored at 4°C.

For general proteolytic activity, beads were coated with defatted BSA, IgG, TDM or β -glucan/whole glucan together with DQ green BSA [Yates and Russell, 2008] (2 mg/ml, Molecular Probes) and incubated with agitation for 12 h. Next, they were washed three times in 1 ml quenching buffer, re-suspended in 1 ml coupling buffer containing 10 μ l of the 5 mg/ml stock of the calibration fluor Alexa Fluor 594 SE and agitated for 1 h. Beads were then washed with quenching buffer and stored at 4°C similarly to acidification beads.

Human subjects

Healthy volunteers (*N* = 10) were recruited from Vietnamese staff working at Oxford University Clinical Research Unit (OUCRU), Vietnam. PTB patients (*n* = 10) were from a cohort of participants with PTB who were recruited from two district TB

control units in Ho Chi Minh City (HCMC), Vietnam between January 2015 and October 2016 as described previously (Vijay et al., 2017). Patients had clinical symptoms of active PTB, which was confirmed by chest X-rays and sputum positive with acid-fast bacilli by Ziehl–Neelsen stain. All were adults and HIV negative.

Written informed consent was obtained from each participant. Protocols were approved by human subjects review committees at the Hospital for Tropical Diseases and Pham Ngoc Thach Hospital for TB and Lung Disease, HCMC, Vietnam. Ethical approval was also granted by the Oxford Tropical Research Ethics Committee (UK).

Cell preparation

J774 murine macrophages (ATCC) were cultured in RPMI (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Sigma), 2 mM L-glutamine (Sigma-Aldrich) and 100 units of penicillin (Sigma-Aldrich). Cells were plated in a 96-well plate at 4×10^4 cells per well. Cells were prepared and incubated at 5% CO₂, 37°C for 14–16 h before experiments.

To prepare hMDM, PBMCs were separated from 20 ml heparinised whole blood by Lymphoprep (Axis-Shield) gradient centrifugation in accordance with the manufacturer's protocol. PBMCs were plated in cell-culture treated 60 mm \times 15 mm petri dishes (Corning) with $(6-8) \times 10^6$ cells per dish in serum-free media containing RPMI-1640 (Sigma), 2 mM L-glutamine (Sigma-Aldrich) and 100 units of penicillin and streptomycin (Sigma-Aldrich) to screen adhered monocytes. After 2 h incubating at 5% CO₂, 37°C, the non-adhered cells were washed off gently three times by warm phosphate buffered saline (PBS) with 3% FBS. On the following day, cells were seeded in the 96-well plate (8×10^4 cells per well) in complete media containing 10 ng/ml human macrophage colony-stimulating factor (m-CSF, R&D system) and incubated at 5% CO₂, 37°C. Complete media was changed at day 4, and experiments were conducted at day 7.

Acidification and proteolysis assays

To measure the ability of macrophages in acidifying and hydrolysing a particle after phagocytosis, the acidification and proteolysis assays, respectively, were performed following previous study [Podinovskaia et al., 2013]. Resting macrophages in the 96-well plate were verified for a monolayer with 80–90% confluence by a microscope, and old media was replaced by 100 μ l pre-warmed assay buffer (1 mM CaCl₂, 2.7 mM KCl, 0.5 mM MgCl₂, 5 mM dextrose, 10 mM HEPES, 10% FBS in PBS). Stored acidification or proteolysis beads were washed three times in PBS by vortexing, and were re-suspended in PBS. Ten microlitres of the beads were added into each well at a concentration to achieve an average of four to five beads internalised per macrophage. Within 2–3 min of bead addition, cells were transferred to 37°C in a fluorescent microplate reader (SynergyH4, BioTek) to initiate the fluorescence data acquisition in real time with top read. This acquisition lasted 90 min for acidification assay and 210 min for proteolytic assay. During this time period, data were collected with an interval of 1.30 min. The relative pH within phagosome was reflected in the relative fluorescent unit (RFU), which is the ratio between fluorescent intensities emitted at 520 nm when excited at a pH-sensitive wavelength of 490 nm and at a pH-insensitive wavelength of 450 nm. Hydrolysed DQ-BSA substrate emits at 520 nm when excited at 490 nm.

Proteolysis activity occurring within phagosome was indicated by the degree of substrate hydrolysis, which was determined by the RFU which is the ratio between fluorescent intensities of substrate and calibration fluor. To facilitate the comparisons of the macrophage acidification or proteolysis activity between different ligands, we used activity index across different kinetic time points as reported previously [Russell et al., 2009; Jambo et al., 2014]. The acidification activity index of macrophages at 30, 60, 70 and 90 min was calculated by ratios between RFU at 10 min over that of 30, 60, 70 and 90 min, respectively. Likewise, the proteolysis activity index at 60, 120, 180 and 210 min was calculated by dividing RFU at these time points, respectively, by that at 10 min.

Consistency of proteolysis and acidification assays was tested using J774 across three independent experiments with triplicate for each experimental condition.

Statistical analysis

Phagosomal activities of J774 in response to different ligands such as IgG, TDM and β -glucan were compared using the Kruskal–Wallis test. To examine the stability of beads, the phagosomal activities of J774 in response to ligand beads at 0 month were compared with that at 6 or 12 or 18 months using the paired *t*-test. The variation of phagosomal activities among either healthy donors or TB patients was expressed by the percentage of %CV calculated by the SD divided by the mean then multiplied with 100. A *P* value ≤ 0.05 was considered statistically significant. Graphs were generated using GraphPad Prism v7.03 (GraphPad Software Inc.) or R program v3.3.1 (R Core Team, 2016).

Author contribution

TT, NT, NP and GT conceived and designed the experiments. TT, VH, DT, TD, SJ, HH and NH did the experiments and collected the data. TT, GT and NT analysed and interpreted the data. TT, VH, DT, TD, SJ, HH, NH, NP, GT and NT drafted, revised the manuscript and approved the final version.

Funding

This work was supported by Wellcome Trust Fellowship in Public Health and Tropical Medicine to NTTTT (097124/Z/11/Z and 206724/Z/17/Z), The World Academy of Sciences to NTTTT (13-051 RG/BIO/AS.I) and Wellcome Trust Major Overseas Program Funding to GT (106680/B/14/Z).

Acknowledgements

We would like to thank all healthy volunteers and patients participating in this study.

Conflict of interest statement

The authors have declared no conflict of interest.

References

- Bowdish, D.M.E., Sakamoto, K., Kim, M.J., Kroos, M., Mukhopadhyay, S., Leifer, C.A., Tryggvason, K., Gordon, S. and Russell, D.G. (2009) MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. *PLoS Pathog.* **5**, e1000474
- Brightenti, S. and Lerm, M. (2012) How *Mycobacterium tuberculosis* manipulates innate and adaptive immunity – new views of an old topic. In *Understanding Tuberculosis—Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity* (P.-J. Cardona, ed.) pp. 208–234. IntechOpen, London. doi: urn:nbn:se:liu:diva-75444LA-English
- Brown, G.D. and Gordo, S. (2001) A new receptor for beta-glucans. *Nature* **413**, 36
- Flannagan, R.S., Cosío, G. and Grinstein, S. (2009) Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat. Rev. Microbiol.* **7**, 355–366
- Ghigo, E., Honstetter, A., Capo, C., Gorvel, J., Raoult, D. and Mege, J. (2004) Link between impaired maturation of phagosomes and defective *Coxiella burnetii* killing in patients with chronic Q fever. *J. Infect. Dis.* **190**, 1767–1772
- Huynh, K.K., Eskelinen, E.-L., Scott, C.C., Malevanets, A., Saftig, P. and Grinstein, S. (2007) LAMP proteins are required for fusion of lysosomes with phagosomes. *EMBO J.* **26**, 313–324
- Jambo, K.C., Banda, D.H., Kankwatira, A.M., Sukumar, N., Allain, T.J., Heyderman, R.S., Russell, D.G. and Mwandumba, H.C. (2014) Small alveolar macrophages are infected preferentially by HIV and exhibit impaired phagocytic function. *Mucosal Immunol.* **7**, 1116–1126
- Kelley, V.A. and Schorey, J.S. (2003) *Mycobacterium*'s arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. *Mol. Biol. Cell* **14**, 3366–3377
- Kleinnijenhuis, J., Oosting, M., Joosten, L.A.B., Netea, M.G. and Van Crevel, R. (2011) Innate immune recognition of *Mycobacterium tuberculosis*. *Clin. Dev. Immunol.* **2011**, 1–12
- Podinovskaia, M., Lee, W., Caldwell, S. and Russell, D.G. (2013) Infection of macrophages with *Mycobacterium tuberculosis* induces global modifications to phagosomal function. *Cell. Microbiol.* **15**, 843–859
- Podinovskaia M. and Russell, D. (2015) Detection and quantification of microbial manipulation of phagosomal function, *Methods in Cell Biology. Methods Cell Biol.* **126**, 305–329
- R Core Team (2016) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at <https://www.r-project.org/>, accessed: May 28, 2018
- Rohde, K., Yates, R.M., Purdy, G.E. and Russell, D.G. (2007) *Mycobacterium tuberculosis* and the environment within the phagosome. *Immunol. Rev.* **219**, 37–54
- Russell, D.G., Vandervan, B.C., Glennie, S., Mwandumba, H. and Heyderman, R.S. (2009) The macrophage marches on its phagosome: dynamic assays of phagosome function. *Nat. Rev. Immunol.* **9**, 594–600
- Stevenson, H.C., Bonvini, E., Favilla, T., Miller, P., Akiyama, Y., Hoffman, T., Oldham, R. and Kanapa, D. (2018) Characterization of purified cryopreserved human monocyte function in assays of superoxide production, accessory cell function, chemotaxis, and in fluorescent cell sorter analysis. *J. Leukoc. Biol.* **36**, 521–531
- Taylor, A.E., Finney-Hayward, T.K., Quint, J.K., Thomas, C.M.R., Tudhope, S.J., Wedzicha, J.A., Barnes, P.J. and Donnelly, L.E. (2010) Defective macrophage phagocytosis of bacteria in COPD. *Eur. Respir. J.* **35**, 1039–1047
- Thuong, N.T.T., Tram, T.T.B., Dinh, T.D., Thai, P.V.K., Heemskerk, D., Bang, N.D., Chau, T.T.H., Russell, D.G., Thwaites, G.E., Hawn, T.R., Caws, M. and Dunstan, S.J. (2016) MARCO variants are associated with phagocytosis, pulmonary tuberculosis susceptibility and Beijing lineage. *Genes Immun.* **17**, 1–7
- van Crevel, R., Ottenhoff, T. H. M. and van der Meer, J. W. M. (2003) Innate immunity to *Mycobacterium tuberculosis*. *Adv. Exp. Med. Biol.* **531**, 241–247
- Vijay, S., Vinh, D.N., Hai, H.T., Ha, V.T.N., Dung, V.T.M., Dinh, T.D., Nhung, H.N., Tram, T.T.B., Aldridge, B.B., Hanh, N.T., Thu, D.D.A., Phu, N.H., Thwaites, G.E. and Thuong, N.T.T. (2017) Influence of stress and antibiotic resistance on cell-length distribution in *Mycobacterium tuberculosis* clinical isolates. *Front. Microbiol.* **8**, 1–12
- Welin, A., Raffetseder, J., Eklund, D., Stendahl, O. and Lerm, M. (2011) Importance of phagosomal functionality for growth restriction of *Mycobacterium tuberculosis* in primary human macrophages. *J. Innate Immun.* **3**, 508–518
- Yates, R.M., Hermetter, A., Taylor, G.A. and Russell, D.G. (2007) Macrophage activation downregulates the degradative capacity of the phagosome. *Traffic* **8**, 241–250
- Yates, R. M. and Russell, D. G. (2005) 'Phagosome maturation proceeds independently of stimulation of toll-like receptors 2 and 4. *Immunity* **23**, 409–417
- Yates, R.M. and Russell, D.G. (2008) Real-time spectrofluorometric assays for the luminal environment of the maturing phagosome. *Methods Mol. Biol. (Clifton, N.J.)* **445**, 311–325

Received: 31 May 2019; Revised: 17 July 2019; Accepted: 6 August 2019; Accepted article online: 10 August 2019