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Short Communication

Extracellular forms of *Mycobacterium bovis* BCG in the mucosal lymphatic tissues following oral vaccination

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ABSTRACT

Oral vaccination with BCG provides protective systemic immunity against pathogenic mycobacterial challenge. In this study, the anatomical distribution of *Mycobacterium bovis* BCG following oral vaccination was investigated. Replicating bacteria in the Peyer's patches and mesenteric lymph nodes were present as solitary rods or clusters of two to three bacteria, the majority of which were isolated *ex vivo* as extracellular forms. Only a minority were shown to be associated with typical antigen-presenting cells. Acid-fast staining of mast cell granules in lymphoid tissues revealed a potential pitfall for these analyses and may explain previous reports of acid-fast 'coccoid' forms of mycobacteria in tissues.

Introduction

Tuberculosis (TB) is a worldwide disease of considerable mortality and morbidity with 8.8 million incident cases and 1.4 million deaths in 2010 [1]. BCG is the only available vaccine and is routinely administered intradermally; however, recent studies have highlighted the potential of the mucosal route of delivery of BCG [2–5]. In particular, oral vaccination offers the advantages of ease of application with equivalent, or enhanced T-cell responses, above that achieved with intradermal vaccination [3]. Furthermore, oral vaccination is the only practical solution available for controlling TB in domestic animals and/or wildlife vectors of veterinary mycobacterial disease.

In previous reports, the anatomical distribution of orally administered BCG within the lymphoid and non-lymphoid organs of mice was investigated [2,4]. Following oral administration, BCG seeds the gastrointestinal tract lymphatics, including the cervical, gastric and mesenteric lymph nodes, and Peyer's patches. Although BCG-reactive T-cells are thought to be stimulated within the mucosal lymphatic system, the majority of responding T-cells lack mucosal homing markers and are present in the spleen as a pool of CD4 and CD8 T-cells with IFN-γ secreting potential. Reactive T-cells possess the β1-integrin subunit—potentially involved in homing to the lung [4].

Despite these advances into understanding T-cell responses following oral BCG vaccination, mechanistic data is still lacking on the interaction of BCG with antigen presenting cells (APC). Mycobacteria are thought to exist in a transiently intracellular state in the host, with both intracellular [6–8] and extracellular [7,9,10] phases identified. BCG is effectively internalized into neutrophils, macrophages and dendritic cells [6,7,11]. However, direct infection of antigen presenting cells (APC) is not required for antigen presentation [8,11,12].

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In fact, infection of APC with BCG compromises the induction of co-stimulator molecule and IL-12 expression and increases the susceptibility of infected APC to apoptosis, compared with uninfected APC [6,8]. Instead, soluble products produced from live mycobacteria, or infected cells, are sufficient for the induction of immune responses induced by uninfected APC [4,8,13,14]. In this report, the anatomical distribution of BCG within the mucosal lymphoid tissues has been investigated. The data shows that following oral administration, BCG is present in mucosal lymphatic tissue in a predominantly extracellular form, with evidence for limited intracellular persistence.

Methods

Lipid formulation

BALB/c mice were fed lipid encapsulated BCG Pasteur 1173 (2×10^7 CFU/dose) as previously described [4] and sacrificed at 12 weeks post-vaccination. In other experiments, mice were injected subcutaneously (SC) into the scruff of the neck with 10^6 colony CFU of BCG and sacrificed 2 weeks later. To optimize antibody detection techniques, BCG (10^7 CFU) was administered intravenously (IV) in 100 μl PBS and the mice were sacrificed after 6 h for processing of the spleen for immunohistochemistry (IHC). In some *in vitro* experiments, uptake of GFP-labeled BCG [15] was determined in bone marrow-derived dendritic cell and macrophages as previously described [4].

Isolation of cells from lymphatic tissue

Isolated mesenteric lymph nodes and Peyer's patches were teased apart in the presence of 20 μg/ml/DNaseI, followed by digestion with 0.5 mg/ml collagenase P (Roche, Auckland, NZ) in Hank's buffered salt solution (HBSS) for 25 min at 37 °C + 5% CO_2 in a tube rotator [16]. After 25 min incubation, 200 μl 0.5 M EDTA (pH 7.2) was added and remaining fragments were pressed through a 70 μm sieve. The preparation was topped up to 50 ml with ice-cold Wuerzburger buffer (Ref. [16]; PBS, 0.3% BSA, 5 mM EDTA and 20 μg/ml DNaseI). Leukocytes and intracellular BCG were pelleted at 515*g*. The remaining supernatant (containing bacteria and less that 1% of total leukocytes; data not shown) was pelleted at 3220*g* for 30 min at room temperature. CFU determinations were performed on 7H11 agar [4].

Fluorescent associated cell sorting (FACS)

Isolated cells were labeled with unconjugated F4/80 (Prof. S. Gordon, Oxford, UK), detected with goat anti-rat-PE (Jackson Immunoresearch, Cat#712–116-153) and CD11c-PE (BD Biosciences, Auckland, NZ) at 5 μg/ml. Cells were sorted according to previously established protocols [16] on a Becton Dickinson FACSaria.

Immunohistochemistry and confocal microscopy

Frozen tissue sections were fixed in 3% paraformaldehyde (PFA; SIGMA, Auckland, NZ) in PBS for 10 min, or 75% acetone/25% ethanol for double labeling immunohistochemistry. PFA treated slides were quenched with 100 mM glycine/PBS and blocked for 10 min with 5% goat serum in PBS. Biotinylated-CD11c (5 μg/ml; clone HL3) or biotinylated-CD11b (5 μg/ml; both BD Biosciences, Auckland, NZ) in 100 μl 1% goat serum was applied and slides incubated for 30 min. Slides were washed with PBS/0.05% Tween-20 and antibodies were detected with 50 μl of 1/200 streptavidin–ALP (Mabtech Cat#3310–10) in TBS and slides incubated for 30 min. After incubation, slides were washed once with TBS and stained cells visualized with Vector blue substrate (Vector #SK-5300) prepared according to manufacturer's instructions and incubated for 15 min. Anti-rabbit–anti-BCG (Biogenesis, Poole, UK) was then applied and detected in the next step with goat anti-rabbit HRP (Sigma Cat. #A0545; 1/100 in PBS) and AEC substrate (Ref. [16]; Sigma, Auckland, NZ). Stained sections were analyzed using an Olympus BX51 microscope with a mounted Olympus DP70 camera using bright field and differential interference contrast. For confocal microscopy, biotinylated-Thy1.2 antibody (clone 30-H12, ATCC, Manassas, VA) was detected with Alexa-488-streptavidin (Invitrogen Cat. #S11223) and the anti-BCG antibody was detected using anti-rabbit Alexa Fluor 594 (Invitrogen Cat. #A11035). Cover slips were mounted using Prolong Gold (Invitrogen Cat. #P36930) according to manufacturer's instructions and sections visualized using a Zeiss 510 confocal microscope. Ice-cold HBSS washes of the peritoneal cavity of BALB/c mice were used to obtain mast cells. Mast cells were detected on cytospin slides using naphthol AS-D chloroacetate (SIGMA, Auckland, NZ) according to Leder [17] and nuclei counterstained with hematoxylin.

Plating and most probable number assay

Lymph nodes or Peyer's patches were aseptically removed from sacrificed mice. Isolated tissue was transferred into a well containing 1 ml of 7H9-SD (7H9 with 0.1% saponin, 20 μg/ml DNAse-I). Tissue was then teased out in single cell suspensions using sterile forceps and further macerated using a plunger of a 10 ml syringe and cells disrupted by vigorous pipetting through a 23-gauge needle and re-suspended in 1 ml of 7H9-SD. Fifty microliters of each dilution (10^{-1}–10^{-9}) was transferred into five wells containing 150 μl 7H9 and incubated for at least 5 days at 37 °C + 5% CO_2. Plates were read every day up to 25 days. Most probable numbers were calculated using the table provided from the US Food and Drug Administration (FDA), source: http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm109656.htm#tab3.

Results

Fractionation of leukocyte-associated and extracellular BCG from mesenteric lymph nodes

In order to determine the cellular localization of BCG within the mesenteric lymph node, differential centrifugation was performed to fraction leukocyte-internalized BCG from extracellular BCG. Cells were then sorted using FACS and antigen-presenting cell subsets lysed prior to CFU determination. As

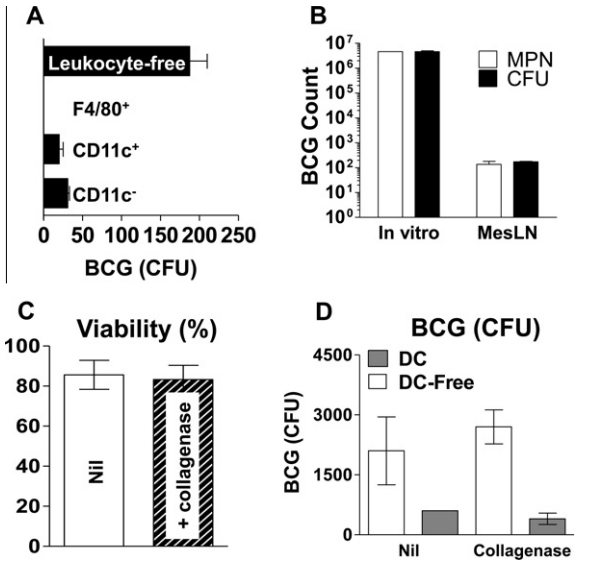

Fig. 1 – Extracellular location of BCG within mucosal lymphoid tissue. (A) Cell suspensions were labeled with CD11c and F4/80 and sorted by FACS into the indicated subsets. Isolated cells were then lysed in saponin and plated onto 7H11 agar for CFU enumeration, together with the leukocyte-depleted (leukocyte-free) fraction for comparison. (B) Equal proportions of mesenteric lymph node cell suspensions, or *in vitro* cultured BCG at mid-log phase were enumerated using solid agar (CFU) or broth culture (MPN). (C) Effect of collagenase on viability of DC. Bone marrow-derived DC were incubated with 0.5 mg/ml collagenase for 25 min. Propidium iodide was then added and viability determined by flow cytometry. (D) Effect of collagenase on intracellular/extracellular fractionation of BCG-infected DC preparations. Bone marrow-derived DC was infected with BCG at a 1:1 MOI for 18 h. Preparations were then fractionated by differential centrifugation into leukocyte (DC) and leukocyte-free (DC-free) suspensions and preparations enumerated for CFU on 7H11.

shown in Fig. 1A, the majority of BCG organisms present in mesenteric lymph nodes fractionated into the leukocyte-free fraction. It has previously been reported that resuscitation of latent mycobacteria from host tissues is enhanced using liquid culture systems [18]. To ensure optimal recovery from both leukocyte-free and leukocyte-bound fractions, the recovery of BCG isolated from mLN (at 12 weeks post-vaccination) was tested by comparing CFU enumeration on solid agar to broth with most probable number (MPN) calculations. The results were compared to BCG grown to mid-log phase *in vitro*. As shown in Fig. 1B, recovery of BCG from mLN was not enhanced by broth culture above that of CFU determination. This suggests that the majority of BCG in mucosal lymphatic tissue is amenable to CFU enumeration on solid agar. It was possible that the tissue disaggregation procedure lysed APC harboring BCG. To rule out possible harmful effects of collagenase on the integrity of plasma membranes of APC, dendritic cells (see phenotype of Supplementary figure) were exposed to collagenase and determined that viability was not affected by collagenase exposure (Fig. 1C). In addition, collagenase treatment of BCG-infected DC cultures did not enhance BCG release from cell bound fractions (Fig. 1D).

Anatomical distribution of BCG within lymphoid tissues

In order to determine the cellular localization of BCG in mucosal lymphatic tissue, an antibody-based technique was employed, with reported sensitivities exceeding that of acid-fast stain by several log [19]. The technique was first tested on spleens from mice sacrificed 6 h after receiving 10^7 BCG IV. As shown in Fig. 1A, BCG were readily visualized in the spleen as rods—their appearance slightly 'fattened' by the deposition of AEC substrate. Applying this technique to Peyer's patches and mesenteric lymph nodes, the extreme paucity of bacteria in individual Peyer's patches (Fig. 2D and E) or mesenteric lymph nodes (Fig. 2B and F) was first noted—with a maximum of only one to three bacteria visible on a selection of sections prepared from each organ. Using double labeling immunohistochemistry, it was possible to label both BCG and host APC. The results highlight the variety of BCG interactions with cells of the mucosal tissues. BCG typically formed small groups of 1–3 rods present in both intracellular and extracellular locations. Interestingly, BCG was present as extracellular forms in T-cell-dependent areas, as shown in Thy1.2 labeling in the confocal image (Fig. 2F).

Identification of mycobacterial 'coccoid forms' as mast cell granules

When an attempt was made to confirm the results using acid-fast staining, typical BCG organisms could not be reliably identified in lymphoid tissues of orally vaccinated mice. Instead, aggregates of intracellular acid-fast 'coccoid structures' were visible in the mesenteric lymph nodes of orally vaccinated mice. Acid-fast positive 'coccoid structures' were also present in peripheral lymph nodes (brachial/axillary) of mice primed 2 weeks previously with BCG. In peripheral lymph nodes, cells harboring these coccoid structures were in proximity to the sub-capsular sinus (Fig. 3), yet failed to label with CD169, ruling out their identity as sub-capsular macrophages (data not shown). When lymphoid tissue was examined by electron microscopy, it became apparent that the coccoid structures were actually mast cell granules, as shown by the evidence presented in Fig. 3D. To further confirm that the Ziehl–Neelsen stain was labeling mast cell granules, mast cells were isolated from the peritoneal cavities of mice. As shown in Fig. 3E, acid-fast carbol fuchsin labeled mast cells in peritoneal cell washes with a similar intensity to those stained with the classical mast cell esterase stain (Fig. 3F).

Discussion

Although progress has been made in recent years with regard to understanding the role of T-cell-mediated responses in immunity to mycobacteria, determining how mycobacterial antigens are sampled and presented by the immune system remains a challenging field. Extracellular mycobacteria have been highlighted as the predominant surviving form that

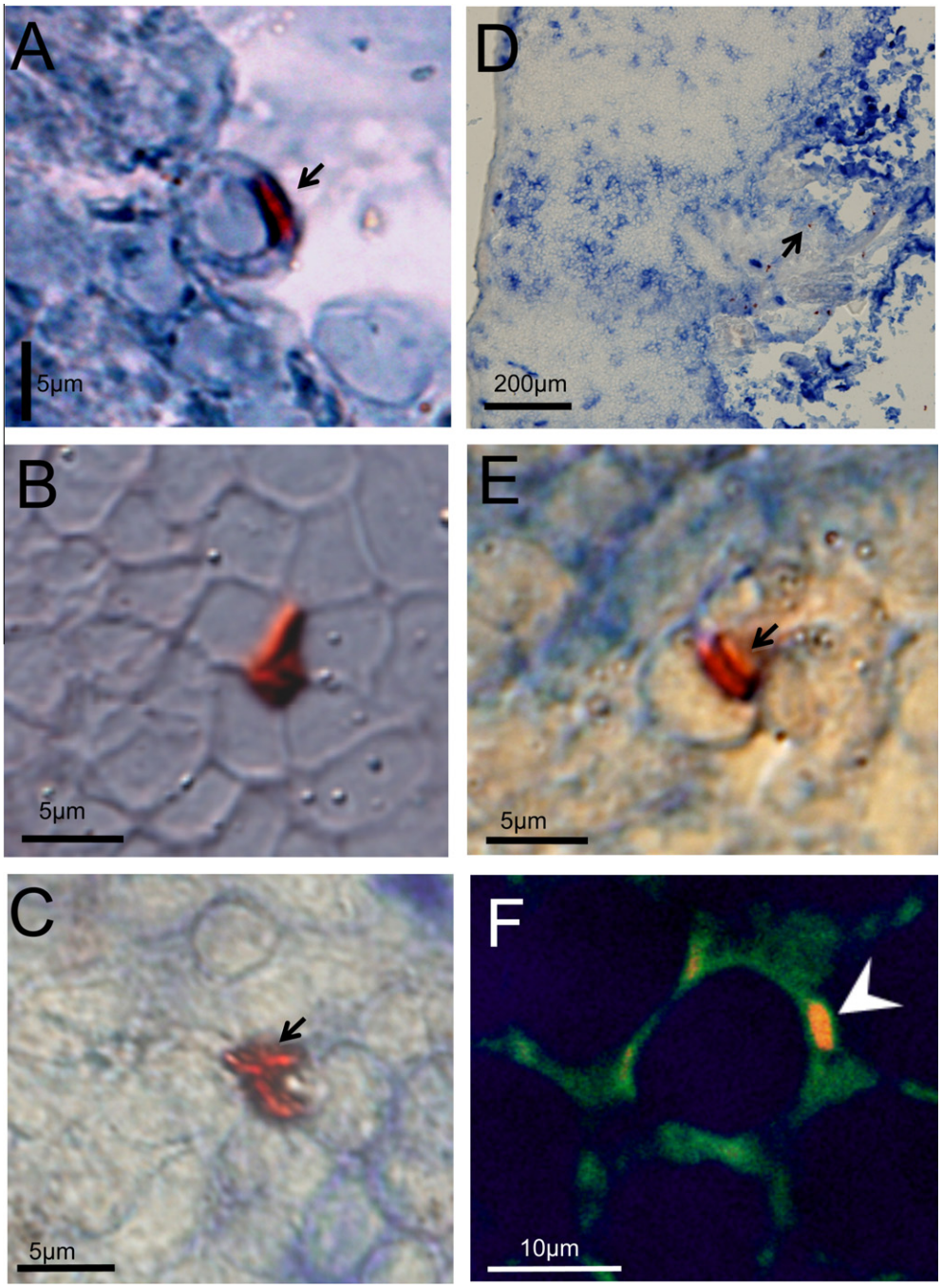

Fig. 2 – Location of BCG within mesenteric lymph nodes and Peyer's patches. (A) BCG rod within a leukocyte, orientated proximal to the luminal aspect of a Peyer's patch from a BCG-orally vaccinated mouse. (B) BCG rods within mesenteric lymph node observed using differential interference contrast to enhance the contrast of plasma membranes of adjacent leukocytes. (C) Double-labeling IHC technique performed on splenic tissue: frozen spleen sections of mice inoculated IV 6 h previously with 10^6 BCG were labeled with F4/80 followed by anti-BCG anti-serum. (D and E) Double labeling of Peyer's patch with CD11c and anti-BCG. (D) Low power view with the location of BCG rods shown in (E) arrowed. (E) Note the location of rods within a cell staining weakly for CD11c. (F) Merged confocal image of BCG rod between the membranes of Thy1.2$^+$ labeled T-cells in a mesenteric lymph node from an orally vaccinated mouse.

persists beyond drug treatment and, due to metabolic changes, possess an inherently drug-resistant state [9,10]. In this study, it has been shown that BCG colonization of mesenteric lymphatic tissue occurs in a predominantly extracellular fashion, with a small number (1–3) of bacilli present at each site. This is in contrast to *in vitro* observations of infection of antigen-presenting cells, e.g. DC or macrophages (Supplementary figure) with BCG. Although it has been previously observed that BCG colonization of the spleen results in an influx of inflammatory CD11b$^+$ cells or granuloma formation,

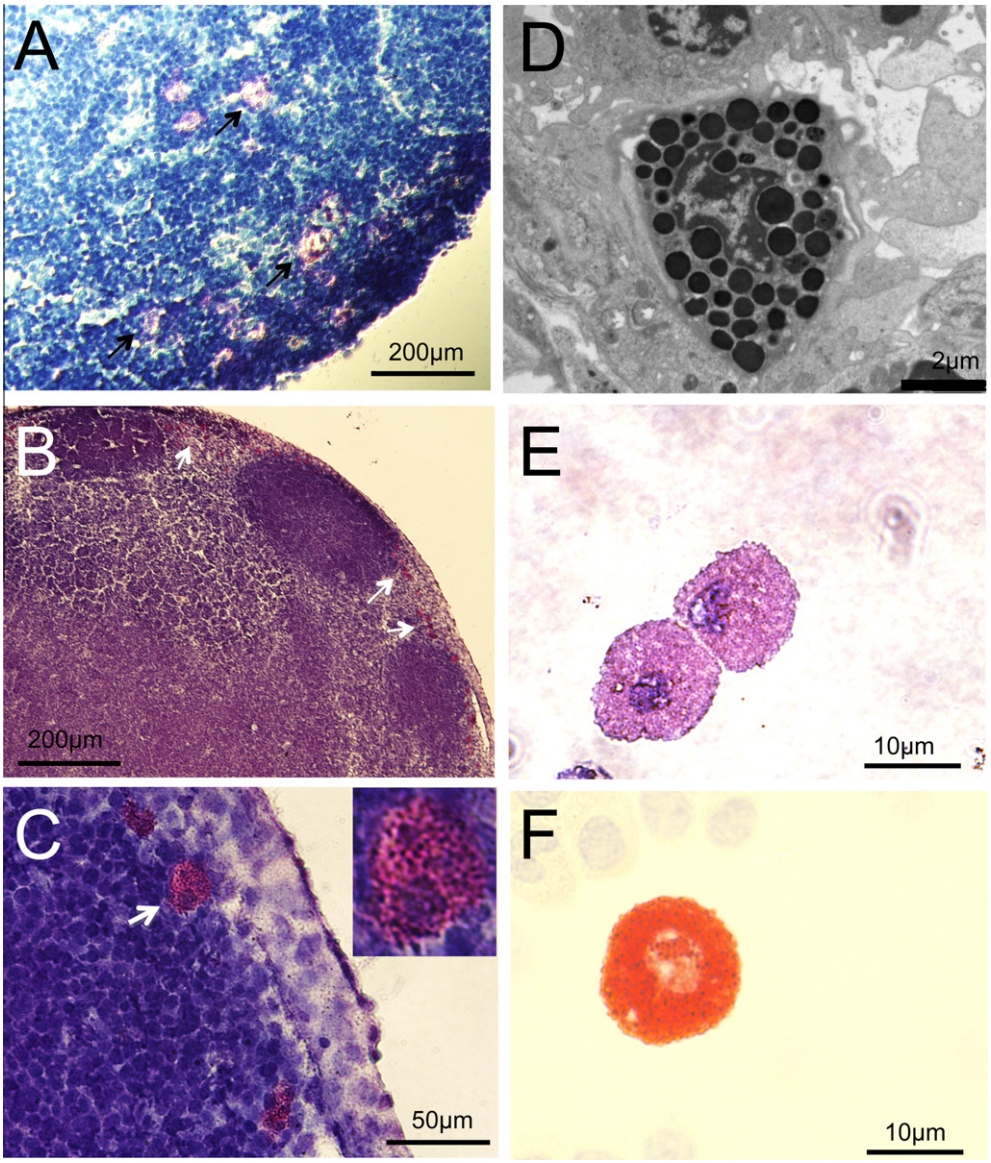

Fig. 3 – Coccoid structures within Ziehl–Neelsen labeled mesenteric and peripheral lymph nodes are mast cell granules. (A) Acid fast (AF) structures with the mesenteric lymph node of an orally vaccinated mouse (arrowed) or within a cervical lymph node of a SC vaccinated mouse (B and C). Note the proximity of the acid fast cells to the sub-capsular sinus. (D) Transmission electron microscopic image of coccoid structures within the cervical lymph node of a SC vaccinated mouse. (E and F) Acid fast (E) or chloroacetate (F) labeling of mast cells within peritoneal wash cell suspensions confirms acid fast labeling of mast cell granules.

this study did not observe either granuloma formation or the influx of $CD11b^+$ cells into areas of the Peyer's patch or mesenteric lymph nodes containing BCG [8].

The absence of an association of BCG with typical antigen-presenting cells within mucosal lymphoid tissue is not surprising given recent findings that the presentation of BCG antigens occurs independently of intracellular infection of antigen-presenting cells [8]. Rothfuchs et al. [8] found that IL-12 p40 was predominantly released from splenic DC that did not contain intracellular BCG. Furthermore, their *in vitro* studies showed that infected DC products could stimulate non-infected DC *in trans*. Another study has demonstrated a role for neutrophils in containing cutaneously administered BCG [12], with *in vitro* evidence for the transfer of BCG from neutrophils to DC [11].

It was previously shown that oral vaccination with lipid-encapsulated BCG leads to the formation of reservoirs of BCG within the alimentary tract lymphatics, with the highest numbers of replicating bacteria associated with the mesenteric lymph nodes and Peyer's patches 12 weeks post-vaccination. This time point coincides with the induction of maximal protective immunity [2]. In the present study, BCG persisted in groups of 1–3 extracellular rods that were excluded from the follicular area, but rather distributed in the T-cell-dependent areas of mesenteric lymph nodes. In Peyer's patches, BCG was similarly present in inter-follicular areas, proximal to the

luminal aspect. Given that BCG was visualized close to the lumen, it is possible that the lymphoid tissue analyzed is not the site of replication of BCG, but rather that mesenteric lymph nodes and Peyer's patches act to capture BCG from non-lymphoid sites within the gastrointestinal tract. However, the complete absence of cultivable BCG from the ilea that have been resected free of lymphoid tissue argue against a non-lymphoid origin for the replicating BCG within sites (unpublished data). An interesting feature of BCG distribution within these lymphoid sites is the predilection of BCG for selected lymph nodes; for example, although BCG initially seeds the spleen and gastric lymph nodes, it fails to replicate in these sites and is absent at later time points [4]. In contrast, replicating pools of BCG are found within cervical lymph nodes, mesenteric lymph nodes and the Peyer's patches [4].

This study confirmed a report published in the early 1960s warning of possible confusion of mast cell granules with acid-fast bacteria [20]. Mast cells are rich in metachromatic granules into which several common laboratory stains have been shown to partition [20]. The source of mast cells in the lymph nodes is likely via afferent lymph from mucosal [21] or peripheral tissues [22] and are likely senescent or apoptotic mast cells translocated from sites of inflammation [21]. Thus, reports of coccoid forms of mycobacteria within solid tissues [23] or peritoneal exudates [24] may need to be reinterpreted in the light of this study.

In conclusion, this study demonstrates the complexity of interactions between BCG and phagocytes within the gastrointestinal tract lymphatics. Further studies will be necessary to determine the mechanism of BCG antigen presentation *in vivo*; in particular, the relative roles of intracellular vs. extracellular forms in the induction of immunity to mycobacteria.

Conflict of interest

F.E.A. is an Inventor on a patent related to the Liporale technology and is a Director on a company (Immune Solutions Ltd.) which has a vested interest in commercializing Liporale as an oral delivery platform. Liporale is covered by the following pending patents and applications: "Antigenic Compositions" WIPO WO/2003/009868 (PCT/NZ2002/00132, NZ 546141, AU 2002/326233, US 2004/0234533, EP 02760915.5, CA 2454920, ZA 2004/1211, CN 02817408.9, IN 00302/DELNP/2004, JP 2003-515260, HK 04109263.7). The authors declare no other financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmyco.2012.11.004.

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