**BRIEF REPORT**

**Trafficking of Superinfecting Mycobacterium Organisms into Established Granulomas Occurs in Mammals and Is Independent of the Erp and ESX-1 Mycobacterial Virulence Loci**

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Although tuberculous granulomas, which are composed of infected macrophages and other immune cells, have long been considered impermeable structures, recent studies have shown that superinfecting *Mycobacterium marinum* traffic rapidly to established fish and frog granulomas by host-mediated and *Mycobacterium*-directed mechanisms. The present study shows that superinfecting *Mycobacterium tuberculosis* and *Mycobacterium bovis* bacille Calmette-Guérin similarly home to established granulomas in mice. Furthermore, 2 prominent mycobacterial virulence determinants, Erp and ESX-1, do not affect this cellular trafficking. These findings suggest that homing of infected macrophages to sites of infection is a general feature of the pathogenesis of tuberculosis and has important consequences for therapeutic strategies.

The long-term persistence of mycobacteria in the face of an apparently robust immune response thwarts tuberculosis control and may explain the inefficacy of the bacille Calmette-Guérin (BCG) vaccine. Tuberculosis begins with the phagocytosis of mycobacteria by macrophages and dendritic cells at the site of infection and the migration of infected cells into deeper tissues, where they aggregate with additional macrophages and other immune cells to form characteristic structures called “granulomas” [1]. The tuberculous granuloma has long been considered an impenetrable barrier structure that contains and restricts mycobacterial infection, and this presumed impenetrability is invoked to explain reinfection tuberculosis, which is increasingly appreciated to occur in immunocompetent individuals capable of forming granulomas [2]. It has been hypothesized that superinfecting or reinfecting bacteria are able to survive by evading established granulomas, where preexisting immune elements are concentrated [3].

This view of the granuloma was called into question by a study that used a *Mycobacterium marinum* infection model to show that superinfection of chronically infected frogs resulted in the rapid trafficking of new bacteria to established granulomas [4]. Newly infecting organisms were transported by host cells to established granulomas and persisted long term therein. Moreover, superinfecting *M. marinum* similarly penetrated necrotic caseous centers of preexisting zebrafish granulomas, demonstrating that infected phagocytes readily access this compartment. These findings highlighted the dynamic nature of tuberculous granulomas and suggested the existence of communication between mycobacteria, infected host cells, and granulomas. This same study showed that trafficking of infected cells is likely induced by a *Mycobacterium*-specific signal; mycobacterial traffic into granulomas is greater than that of latex beads or of *Salmonella enterica* subsp *arizonae*, another macrophage pathogen. Therefore, the unique pattern of host cell trafficking elicited by mycobacterial infection likely involves specific bacterial determinants.

While intriguing, these results left open the possibility that the trafficking of superinfecting mycobacteria is unique to the pathogenesis of *M. marinum* infection in the fish and amphibian models used. Genome comparisons of *M. marinum* and *Mycobacterium tuberculosis* reveal their close genetic relationship [5], explaining the conservation of their essential framework of infection, granuloma formation, and persistence [1]. The present study was undertaken to determine whether superinfecting *M. tuberculosis* and *Mycobacterium bovis* BCG also home to established granulomas in mice and to probe the mechanisms by which mycobacteria influence phagocyte trafficking.

**Methods.** *M. tuberculosis* strain Erdman (gift of W. Bishai) and *M. bovis* BCG substrain Russia [6] were rendered green and red fluorescent by transformation with plasmids pMSP12::gfp and pMSP12::dsRed2 [4], respectively. *M. marinum* strain M

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(ATCC BAA-535) was rendered red fluorescent by transformation with plasmid pMSP12::dsRed2, whereas *M. marinum* strains RD1–6 (exs1::ΔRD1 [7]) and KK33 (erp::aph [8]) were rendered green fluorescent by transformation with plasmid pG-FFHYG2 (a hygromycin-resistant derivative of pMSP12::gfp). Bacteria were grown in static culture in Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.005% oleic acid, 0.2% dextrose, 0.5% bovine serum albumin (BSA), 0.085% NaCl, and 0.05% Tween-80, in the presence of 20 μg/mL kanamycin sulfate or 50 μg/mL hygromycin B, as appropriate. Colony-forming units were enumerated on Middlebrook 7H10 agar supplemented with 0.5% glycerol, 0.005% oleic acid, 0.2% dextrose, 0.5% BSA, and 0.085% NaCl. *M. marinum* was grown at 33°C, and *M. tuberculosis* and *M. bovis* BCG were grown at 37°C.

Six- to 8-week-old female C57B/6 mice (Charles River) were infected, via intraperitoneal (ip) injection, with red fluorescent *M. bovis* BCG or *M. tuberculosis* Erdman for 5 weeks to allow granuloma formation and then superinfected, via ip injection, with the corresponding green fluorescent strain. Three to 7 days later, their organs were removed and fixed overnight in 4% paraformaldehyde in 1× PBS. Tissues were rehydrated and frozen for cryosectioning as described elsewhere [9]. Wild-caught small male leopard frogs (*Rana pipiens*; Nasco) were infected, via ip injection, with red fluorescent *M. marinum* for 6 weeks and then superinfected with green fluorescent *M. marinum* or Fluoresbrite carboxylate yellow-green 1.0-μm microspheres (Polysciences) for 3 days, at which time their organs were removed and processed as described elsewhere [9].

Colony-forming units in liver homogenates were enumerated on 7H10 agar. Beads were enumerated by spotting aliquots of tissue homogenate on a slide and visual counting using fluorescence microscopy. Frozen sections (10-μm thick) were stained with Slow-Fade Gold antifade reagent with 4',6-diamidino-phenylindole dihydrochloride (DAPI; Molecular Probes) and imaged using a CoolSNAP HQ camera (Photometrics). Established granulomas were defined by both the presence of red fluorescent bacteria and a dense clustering of DAPI-stained host cell nuclei, which served as a conservative measure of their boundaries [4]. Localization of superinfecting particles was scored by visual inspection of sections; all green fluorescent bacteria or beads were counted and scored as having migrated into granulomas as described here or not.

**Results.** To examine the trafficking of newly infected cells with respect to established granulomas in the mouse model of tuberculosis, C57B/6 mice were first infected, via ip injection, with *M. bovis* BCG that constitutively express a red fluorescent protein, dsRed. This method of inoculation has been used for superinfection experiments in frogs [4]. Five weeks after the initial infection, a time point at which bacterial counts stabilize because of the onset of adaptive immunity [10], livers were found to contain small, compact paucibacillary granulomas typical of this organ [11, 12]. The mice were then superinfected with green fluorescent *M. bovis* BCG. At both 3 and 7 days after infection, superinfecting bacteria were present in established granulomas (figure 1A and data not shown). Similar rapid trafficking of superinfecting green fluorescent *M. tuberculosis* Erdman bacteria was observed into granulomas established using red fluorescent *M. marinum*, and *M. tuberculosis* Erdman bacteria was then superinfected with green fluorescent *M. tuberculosis* Erdman 4.5 weeks later. Liver was harvested 4 days after secondary infection and processed as described in Methods. The granuloma shown is from 7 days after infection and is representative of mixed lesions found in 1 mouse at 3 days after infection and in 3 mice at 7 days after infection. In panel B, a C57B/6 mouse was infected with 1 × 10⁷ red fluorescent *M. tuberculosis* Erdman bacteria and was then superinfected with green fluorescent *M. tuberculosis* Erdman 4.5 weeks later. Liver was harvested 4 days after secondary infection and processed as described in Methods. The scale bar is 50 μm.

![Figure 1](image-url)
mas. Furthermore, this result suggests that intracellular bacterial replication is dispensable for homing of infected cells to preexisting granulomas. In a separate experiment, the ESX-1–deficient ΔRD1 mutant also trafficked to granulomas within 3 days (figure 2E and data not shown), but the small sample size precluded quantitative comparison with WT bacteria. Nevertheless, this experiment shows that the ESX-1 locus is not required for rapid delivery of mycobacteria to established granulomas.

Discussion. The previous finding that superinfecting mycobacteria home to established granulomas in *M. marinum*–infected frogs and fish challenged long-held assumptions about the nature of the tuberculous granuloma [4]. That study demonstrated that granulomas, including their caseous centers, are permeable entities and that the concentration of immune elements at these foci fail to protect against naive superinfecting bacteria, which persist long term therein. These findings revealed the limits of antituberculous immunity, drawing into question whether vaccine strategies that use a more persistent or immunogenic strain would be successful. However, although the pathogenesis of *M. marinum* and *M. tuberculosis* infection in their respective ectotherm and mammalian hosts appear to operate via a common functional framework [1], it was unclear whether *M. tuberculosis* complex bacilli would behave similarly. First, *M. marinum*–specific determinants absent from *M. tuberculosis* might direct newly infected macrophages to granulomas. Alternatively, differences in the granulomas of fish and amphibians versus mammals might be responsible for the homing behavior of infected cells. Although *M. marinum* infection is moderated by adaptive immunity, fish and frog granulomas contain many fewer lymphocytes than do mammalian granulomas [16]. A recent study of *M. bovis* BCG granulomas in mouse liver (the tissue used in this study) shows that T lymphocytes are abundant and highly motile therein [11]. Thus, it was possible that the paucity of lymphocytes in fish and amphibian granulomas, although not affecting the ability of the granuloma to restrict preexisting bacteria, might make them more permeable to newly infected phagocytes. Finally, it was possible that *M. marinum*–infected fish and amphibian phagocytes might have intrinsically higher motility than (or other trafficking differences from) mammalian ones, as has been suggested [11]. Therefore, it was important to determine whether this phenomenon occurs in...
mammalian tuberculosis. The present study establishes that the propensity of superinfecting mycobacteria to home to preexisting granulomas is a general feature of the pathogenesis of tuberculosis and raises the possibility that it may occur in humans as well. One aspect of mammalian disease that is not addressed by this study is whether lung granulomas are similarly permeable to superinfecting traffic. Attempts to demonstrate homing of superinfecting organisms by means of aerosol infection of mice were unsuccessful (data not shown), likely because of technical hurdles. Specifically, the number of superinfecting organisms that was achieved with the aerosolization apparatus used was likely too low to allow detection. Alternately, this failure may reflect a biological difference between liver and lung granulomas or between peritoneal and alveolar macrophages. However, this explanation seems less likely given the permeability of frog lung granulomas to superinfecting bacteria delivered ip [4].

Mycobacteria have been shown to be susceptible to host killing by macrophages in vivo [13] but also to utilize these cells to promote their own spread and dissemination [7, 17]. Elucidating the mechanisms by which Mycobacterium infection alters phagocyte traffic may help to define more clearly the seemingly disparate roles played by the mature granuloma as both a host and pathogen-beneficial structure. Thus, a mechanistic understanding of this surprising phenomenon and its consequences for infection will depend on elucidation of the mycobacterial determinants required to induce the host cell trafficking now confirmed for both *M. marinum* and *M. tuberculosis*. In the present study, 2 bacterial virulence determinants shared by both pathogens were examined for their ability to influence host cell trafficking behavior. Erp, a cell-surface protein of unknown function, acts early during pathogenesis in the *M. marinum*-zebrafish embryo model, being required for bacterial growth and/or survival beginning early during infection. Data presented here show that WT and *erp*-deficient bacteria traffic to granulomas with comparable efficiency. This observation demonstrates that Erp is not involved in modulating host cell trafficking and further suggests that the ability to grow and/or survive intracellularly is similarly not required. The second determinant tested, the ESX-1 secretion system, is a virulence determinant in both *M. tuberculosis* and *M. marinum* [7, 15, 18]. Indeed, the primary attenuating deletion in *M. bovis* BCG, RD1 (region of difference 1), removes a substantial portion of the ESX-1 locus [6, 19]. In the present study, both BCG and a *M. marinum* ΔRD1 mutant were shown to traffic into mature lung granulomas. Although it remains possible that a quantitative difference exits, this result demonstrates that an intact ESX-1 locus is not absolutely required for the induction of phagocyte trafficking to granulomas.

That both attenuated strains (Erp and ESX-1 mutants) exhibit some level of persistence in vivo [6, 8, 14] and also home to existing granulomas suggest an intriguing new therapeutic route. If properly modified—for example, for phage [20] or drug delivery—these strains could rapidly deliver antibacterial cargo directly to preexisting granulomas, including the caseum, where the bacilli are thought to be hardest to eradicate [21].

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**References**


