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

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Abstract

Whereas tuberculous granulomas, comprised of infected macrophages and other immune cells, were considered impermeable structures, recent studies showed 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* BCG similarly home to established granulomas in mice. Furthermore, two prominent mycobacterial virulence determinants, Erp and ESX-1, do not impact this cellular trafficking. These findings suggest that homing of infected macrophages to sites of infection is a general feature of tuberculosis pathogenesis, with important consequences for therapeutic strategies.

Keywords

Mycobacterium; granulomas; caseum; ESX-1; Erp; reinfection

Introduction

Long-term persistence of mycobacteria in the face of an apparently robust immune response thwarts tuberculosis control, and may explain the inefficacy of the 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 re-infection tuberculosis, which is increasingly appreciated to occur in immunocompetent individuals capable of forming granulomas[2]. It has been hypothesized that superinfecting or re-infecting bacteria are able to survive by evading established granulomas where pre-existing 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

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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, super-infecting *M. marinum* similarly penetrated necrotic caseous centers of pre-existing 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* serovar *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 *M. marinum* pathogenesis of the fish and amphibian models used. Genome comparisons of *M. marinum* and *M. 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 establish whether superinfecting *M. tuberculosis* and *M. bovis* BCG also home to established granulomas of 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 (ATCC# BAA-535) was rendered red-fluorescent by transformation with plasmid pMSP12::dsRed2, while *M. marinum* strains RD1-6 (*esx1*::ΔRD1[7]) and KK33 (*erp::aph*[8]) were rendered green fluorescent by transformation with plasmid pGFPHYG2 (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% 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 (CFU) 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 at 37°C.

6–8 week-old female C57B/6 mice (Charles River) were infected by intraperitoneal (IP) injection of red fluorescent *M. bovis* BCG or *M. tuberculosis* Erdman for approximately five weeks to allow granuloma formation, and then superinfected by IP injection with the corresponding green fluorescent strain. Three to seven days later, their organs were removed and fixed overnight in 4% paraformaldehyde in 1X PBS. Tissues were rehydrated and frozen for cryosectioning as described[9]. Wild-caught small male leopard frogs (*Rana pipiens*, Nasco) were infected with red fluorescent *M. marinum* by IP injection for six weeks and superinfected with green fluorescent *M. marinum* or Fluoresbrite Carboxylate YG 1.0 μm microspheres (Polysciences, Inc) for three days at which time their organs were removed and processed as described[9].

CFU from 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. 10-μm thick frozen sections were stained with Slow Fade Gold Anti-Fade reagent with DAPI (Molecular Probes) and imaged using a Cool-Snap 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 defined 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 with *M. bovis* BCG that constitutively express the red fluorescent protein, dsRed via IP injection. This method of inoculation has been used for superinfection studies in frogs[4]. Five weeks after the initial infection, a timepoint at which bacterial counts have stabilized due to 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 three and seven days post-infection, superinfecting bacteria were present in established granulomas (Fig. 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. tuberculosis* Erdman (Fig. 1B). These data demonstrate that mammalian granulomas induced by *M. tuberculosis* complex organisms are permeable to cellular traffic and superinfection. Thus, the propensity of superinfecting mycobacteria to home to granulomas is a general feature of tuberculosis pathogenesis, and not specific to *M. marinum*, fish or frogs.

To probe the mechanism of *Mycobacterium*-directed phagocyte trafficking, two mycobacterial virulence determinants, ESX-1 and Erp, shared by *M. tuberculosis* and *M. marinum*, were analyzed in the frog model. These determinants mediate virulence by distinct mechanisms. Erp acts earlier in infection and is required for intracellular growth and resistance to host defenses [8,13,14]. The ESX-1 locus is not required for bacterial growth within individual macrophages but mediates granuloma formation, macrophage death and intercellular spread of bacteria[7, 15]. Frogs infected with red fluorescent wild-type *M. marinum* for six weeks were superinfected with green fluorescent beads, wild type or Δ*erp M. marinum*. As expected[4], at three days post-infection bacteria were four-fold more likely than beads to be associated with established granulomas (Fig. 2B,C), despite comparable numbers of beads and bacteria infiltrating the surrounding tissue (Fig. 2A). Similar proportions of Δ*erp* and wild-type bacteria were found within granulomas (Fig. 2A, B, and D), indicating that this bacterial determinant is not required for induction of host cell trafficking to granulomas. Furthermore, this result suggests that intracellular bacterial replication is dispensable for homing of infected cells to pre-existing granulomas. In a separate experiment, the ESX-1-deficient ΔRD1 mutant also trafficked to granulomas within three days (Fig. 2E and data not shown) but the small sample size precluded quantitative comparison to wild-type bacteria. Nevertheless, this experiment shows that the ESX-1 locus is not required for rapid delivery of mycobacteria to established granulomas.

Discussion

The earlier 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]. This 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 naïve superinfecting bacteria, which persist long term therein. These findings revealed the limits of anti-tuberculous immunity, drawing into question whether vaccine strategies that use a more persistent or immunogenic strain would be successful. However, while *M. marinum* and *M. tuberculosis* pathogenesis 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/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, while not affecting the ability of the granuloma to restrict pre-existing 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 pre-existing granulomas is a general feature of tuberculosis pathogenesis and opens 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 using aerosol infection of mice were unsuccessful (data not shown), likely due to 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 intraperitoneally[4].

Mycobacteria have been shown to be both susceptible to host killing by macrophages in vivo [13] but to also utilize these cells to promote their spread and dissemination[7,17]. Elucidating the mechanisms by which *Mycobacterium* infection alters phagocytes traffic may help to define more clearly the seemingly disparate roles of 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 upon the elucidation of the mycobacterial determinants required to induce the host-cell trafficking now confirmed for both *M. marinum* and *M. tuberculosis.* In this study, two 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 in the pathogenesis program in the *M. marinum*-zebrafish embryo model, being required for bacterial growth and/or survival beginning early in infection. Data presented here show that *erp*-deficient bacteria traffic to granulomas as well as do wild type bacteria. 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, referred to as region-of-difference 1 (RD1), 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 granulomas. While 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.

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

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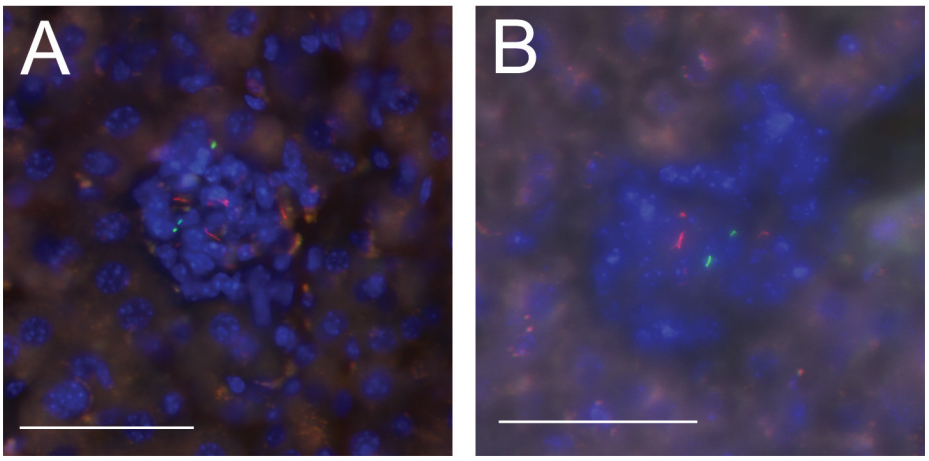

Figure 1. M. tuberculosis

and *M. bovis* BCG traffic to established mouse granulomas. A. C57B/6 mice were infected with 4×10^6 red-fluorescent *M. bovis* BCG bacteria and were then superinfected with 2×10^7 green-fluorescent *M. bovis* BCG five weeks later. Livers were harvested three and seven days post-secondary infection and were processed as described in Methods. Seven day post-infection granuloma shown is representative of mixed lesions found in one mouse at three days post-infection and in three mice at seven days post-infection. B. A C57B/6 mouse was infected with 10^7 red-fluorescent *M. tuberculosis* Erdman, and was then superinfected with green fluorescent *M. tuberculosis* Erdman 4.5 weeks later. Liver was harvested four days post-secondary infection and processed as described in Methods. Scale bar, 50 μm.

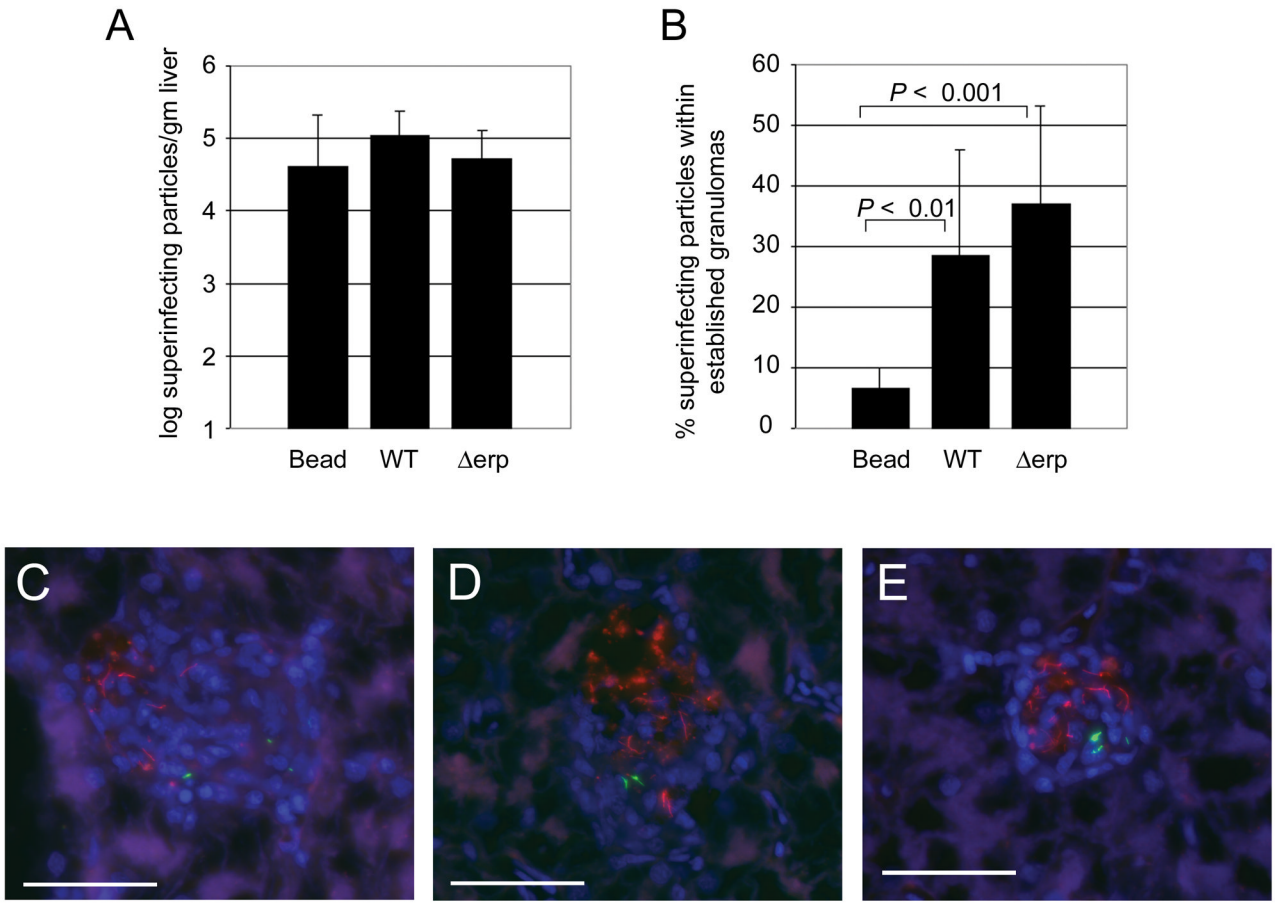

Figure 2.

Wild type, *erp::aph* and ΔRD1 *M. marinum* traffic to established granulomas. A–D) Frogs were infected with 3×10^6 CFU red fluorescent wild-type *M. marinum*. After six weeks frogs were superinfected with 4.5×10^6 green fluorescent beads (11 frogs), 1.1×10^6 green fluorescent wild type bacteria (6 frogs), or 1.6×10^6 green fluorescent Δ*erp::aph* bacteria (8 frogs). Livers were harvested three days post-secondary infection and were processed for CFU and microscopy as described in Methods. (A) Green fluorescent particles (latex beads or bacteria) per gram liver tissue. Differences were not statistically significant (ANOVA with Tukey's post test). (B) Proportion of green fluorescent particles found within established granulomas. Error bars represent standard deviations. Means compared using ANOVA with Tukey's Post test. Wild-type vs. *erp::aph*, not significant. C) Wild-type and D) *erp::aph M. marinum* found inside of established granulomas. E) Frogs were infected with 1.9×10^5 CFU red fluorescent wild-type *M. marinum*. After 10.5 weeks frogs were superinfected with 2.0×10^6 green fluorescent ΔRD1 *M. marinum*. Livers were harvested 3 days post-secondary infection and were processed for CFU and microscopy as described in the Methods. Scale bar, 50 μm.