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The ins and outs of the *Mycobacterium tuberculosis*-containing vacuole

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Abstract

The past few years have seen publication of reports from several groups documenting the escape of *Mycobacterium tuberculosis* (Mtb) from its intracellular vacuole to access the cytosol. The major questions addressed in these publications are the mechanism(s) underlying this process, the frequency of its occurrence, and most importantly, the biological significance of this phenomenon to bacterial survival, growth and virulence. I believe that the first two questions are moving towards resolution but questions relating to biological context have yet to be answered fully. In this viewpoint article I will try to convince the readers why escape from the vacuole in no way diminishes the significance of Mtb's intravacuolar survival mechanisms, and why, as a lab, we continue to focus the majority of our efforts on the "bug in the bag".

Historically my lab has focused on the biology of intravacuolar *Mycobacterium tuberculosis* (Mtb) based on the belief that it is these bacteria that represent the subpopulation of organisms that is most biologically significant to the maintenance and progression of the infection. Despite the publication of numerous papers that deal with the escape of Mtb from its intracellular compartment I still retain the conviction that it is the intravacuolar bacilli that represent the most relevant target for resolution of this infection. So, why do I still cling to this viewpoint?

The model system on which we base much of our analysis is detailed extensively in the papers by Rohde and colleagues (Rohde *et al.*, 2007, Abramovitch *et al.*, 2011, Rohde *et al.*, 2012). In one of these papers, bone marrow-derived murine macrophages were infected with Mtb CDC1551 strain and the infection followed for 14 days in tissue culture (Rohde *et al.*, 2012). We could not do this with any other Mtb strain. H37Rv and Erdman would have destroyed the cell monolayer within a few days. The efficiency of exit of Mtb from their vacuoles, and hence the rate of death of the host cell, varies considerably with both the bacterial strain, and with the identity of the host cell. This variability is likely a major contributor to the divergent observations reported by many labs. We use CDC1551 deliberately to enable us to study an extended intracellular infection in tissue culture. Analysis of this stable infection model has revealed how the bacterium responds physiologically to its intracellular environment. The bacteria experience host-derived stresses and undergo a period of adaptation prior to entering growth phase. Electron

microscopical analysis of the infected cells demonstrated that, throughout the course of the infection, the bacteria were almost exclusively intravacuolar, yet they were clearly dividing and the host cells remained healthy, Figure 1. These data underpin one of our basic tenets; that one does not have to invoke escape from the vacuole to explain either intracellular survival or bacterial growth.

The ability of Mtb to escape from its vacuole had been reported in isolated publications over the years and had not been given much credence (Leake *et al.*, 1984, Myrvik *et al.*, 1984, McDonough *et al.*, 1993). However, in 2003 a study on *M. marinum* demonstrated conclusively that this *Mycobacterium* spp. could clearly and efficiently escape from its vacuole and survive in the host cytosol (Stamm *et al.*, 2003). In 2007, extensive and systematic documentation of this phenomenon in *M. tuberculosis* was published and the timbre of these discussions changed. Van der Wel and colleagues reported the detailed immuno-electron microscopical analysis of human monocyte-derived phagocytes, mainly dendritic cells, infected with Mtb and *M. leprae* (van der Wel *et al.*, 2007). The images showed clearly that pathogenic *Mycobacterium* spp. translocated into the cytosol of the host cells, in contrast to the non-pathogenic vaccine strain of *M. bovis* BCG that remained within vacuoles. The authors also noted that translocation into the cytosol led to the induction of cell death.

The next few years saw publication of several manuscripts detailing this phenomenon. Although slightly dated, the review by Welin and Lerm provides comprehensive and balanced coverage of the earlier literature (Welin *et al.*, 2012). Brown's lab followed up their original observation on *M. marinum* to demonstrate that the RD1 region, the major region of the *Mycobacterium bovis* chromosome deleted in the non-pathogenic strain BCG, and secretion of ESAT-6 were required for the bacterium to access the cytosol (Gao *et al.*, 2004). ESAT-6 and CFP from Mtb were quickly proposed to be the agents of lysis and have been shown to have membrane disrupting activity (de Jonge *et al.*, 2007, De Leon *et al.*, 2012, Peng *et al.*, 2016).

Further analysis of the biology of vacuolar escape by Mtb was aided tremendously through an elegant method developed initially in *Shigella* by Enninga and co-workers (Ray *et al.*, 2010). In brief, the technique utilized surface-expressed β -lactamase on the bacterium and the loading of a fluorogenic substrate into the cytosol of the host cell. When the bacterium lysed its vacuole the substrate was hydrolyzed and became fluorescent. This method was used on Mtb by Simeone *et al.* to visualize the escape process in real-time and at single cell level (Simeone *et al.*, 2012). This enabled the correlation between escape from the vacuole and progression to cell death to be established as a biological continuum.

In assessing the impact of escape from the vacuole on the virulence of this infection the majority of studies have been conducted on organisms deficient in the ESX-1 secretion system. The complication in the analysis of these studies is that ESX-1 is responsible for the export of numerous substrates that are still being identified (Champion *et al.*, 2014, Kennedy *et al.*, 2014). Therefore to assign the phenotypes and impaired virulence of ESX-1 mutants solely to their inability to escape from their confining vacuole is highly likely to be an over-interpretation of the data. For example, ESX-1-dependent substrates also affect phagosome

maturation (MacGurn *et al.*, 2007, Stoop *et al.*, 2012), which is known to impact bacterial growth and viability (Pethe *et al.*, 2004). Furthermore, disruption of ESX-1 impairs cell wall integrity, which could have broad reaching consequences for bacterial fitness (Garces *et al.*, 2010).

To find data that addresses the impact of ESAT-6 and CFP10 and escape from the vacuole on the virulence of Mtb specifically one has to combine information from separate publications. Houben and colleagues presented data demonstrating that a mutant of Mtb expressing a form of ESAT-6 with a truncated C-terminus was impaired in its ability to escape from its vacuole (Houben *et al.*, 2012). They argued that ESX-1-mediated translocation regulated the virulence of Mtb, however despite this conclusion the paper actually contained no bacterial growth or survival data. The correlation between ESAT-6 expression and bacterial growth during infection had been published previously by Bordin *et al.*, where a panel of mutations in ESAT-6 were probed for their phenotypes in mouse infections, and multiple mutants in ESAT-6 were shown to be impaired in growth (Bordin *et al.*, 2005, Bordin *et al.*, 2006). So whilst I accept that ESAT-6 is linked to escape from the vacuole and that Mtb expressing modified ESAT-6 can be impaired in growth in murine infections, there is no single published study that ties these critical pieces of data together.

The escape of Mtb from its vacuole leads to the induction of cell death in the host cell and I believe that this link is highly significant in placing the phenomenon into the context of infection. Simeone and colleagues established the connection between vacuole escape and cell death at the cellular level by fluorescent readout and went on to generate data in support of this occurring *in vivo* in murine infections (Simeone *et al.*, 2012, Simeone *et al.*, 2015). Kornfeld's lab has published several papers looking at the kinetics of induction of necrosis and programmed cell death in different phagocyte populations *in vitro* and *in vivo* (Repasy *et al.*, 2013, Repasy *et al.*, 2015). They demonstrated that host cell survival correlated inversely with bacterial burden indicating that cell death was a dose-dependent phenomenon. Their data argue that faster replicating Mtb strains induce greater levels of necrosis, which lead to enhanced neutrophil recruitment and increased pathology. And I think that it is this linkage that is most important. In contrast to murine Mtb infections, human tuberculosis is paucibacillary with very few bacteria present in the granulomas. In humans, the only time when large numbers of bacteria are present is at the surface of active cavities (Kaplan *et al.*, 2003), which are characterized by extensive neutrophil recruitment and necrosis. In active disease bacteria are frequently recovered from sputum in neutrophils (Eum *et al.*, 2010). Moreover, in Mtb-infected phagocytes recovered by bronchoalveolar lavage of patients with active TB, the bacteria were observed in vacuoles that were non-acidified and did not fuse with lysosomes (Mwandumba *et al.*, 2004). While I appreciate that this is a snapshot of a single time point nonetheless the bacilli observed in this study were almost exclusively intravacuolar. The analysis of TB granulomas in humans and in experimental infections in macaques, where the bacterial number is also low, indicates that the granuloma contains different regions and that inflammatory signaling is spatially organized (Mattila *et al.*, 2013, Marakalala *et al.*, 2016). Those regions rich in neutrophils correspond to those regions with the highest bacterial burden (Mattila *et al.*, 2015). It is reasonable to propose that the bacteria encounter different host environments in this tissue, that their growth status differs with

environment, and that those regions that support greatest bacterial growth correspond to the regions of tissue damage and are indicative of progressive disease.

Our interpretation of these observations is that survival in the vacuole is critical for Mtb, and that, in humans, the infecting bacilli will spend the greatest proportion of their time inside vacuoles, presented diagrammatically in Figure 1D. We hypothesize that escape from the vacuole represents a transient state that could be critical to the rapid expansion of the bacterial population. We know that Mtb survive and grow, albeit slowly, in vacuoles in tissue culture infections of primary human and murine macrophages. Escape from the vacuole is therefore not a requirement for either survival or growth. It is certainly possible that the cytosol is a more permissive environment that could support rapid bacterial growth but occupation of that environment is transient because access to it is a prelude to the death of the host cell.

I don't deny the capacity of Mtb to escape the vacuole and I don't deny that it likely has biological relevance to progression of the infection *in vivo*. I just believe that it is the bacterium within the vacuole that represents the more significant target from a therapeutic standpoint. Recently developed reporter strains of Mtb show how physical attributes within the vacuole impact bacterial transcriptional responses both in tissue culture and in murine infections (Abramovitch *et al.*, 2011, Tan *et al.*, 2013, Sukumar *et al.*, 2014). We know Mtb modulates its metabolism when inside the vacuoles of its host cell and utilizes cholesterol and fatty acids as a preferred carbon source (McKinney *et al.*, 2000, Lee *et al.*, 2013). We know that the host cell's metabolism is also modified and retention of cholesterol and fatty acids is enhanced (Lee *et al.*, 2013, Podinovskaia *et al.*, 2013), and that this is also observed in human TB granulomas that have progressed to caseation (Kim *et al.*, 2010, Subbian *et al.*, 2015). A large empirical chemical screen for compounds active against CDC1551 in the macrophage-like cell line J774 identified inhibitors of cholesterol metabolism under conditions where we know the bacterium remains intravacuolar (VanderVen *et al.*, 2015), and these compounds were active against Mtb in primary human macrophages. Furthermore, in an immune environment, the level of sensitivity of Mtb to frontline TB drugs is impacted negatively by the activation status of the host macrophage both *in vitro* and *in vivo* (Liu *et al.*, 2016), a phenotype that is effectively recreated in the tissue culture infection model with intravacuolar bacilli. These data all emphasize the pivotal importance of successful adaptation to and survival within the intravacuolar environment of the host cell.

So in conclusion, I think that escape from the vacuole is likely to be an important step in the pathology that accompanies progression of tuberculosis infection to active disease, and there is undoubtedly value in studying this process both *in vitro* and *in vivo*. However, I don't believe that escape from the vacuole in any way diminishes the significance of the intravacuolar survival mechanisms or reduces my belief that it is this physiological state that represents the more significant target for new therapeutics. But in truth what we strive for in the lab is the development of rich biological platforms that facilitate the screening of compound libraries, whilst making as few assumptions as is possible. The environment within the host cell plays a critical role in shaping bacterial metabolism, and in shaping Mtb's response to drug pressure. It makes sense to incorporate this environment in ongoing

drug screens, and to achieve this with an over-arching gestalt, rather than emphasizing or prioritizing individual virulence mechanisms.

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Figure 1.

Electron micrographs of murine bone marrow-derived macrophages infected with *Mtb* CDC1551 and maintained in culture for 2, 4, and 14 days respectively. In panels A & B the cells were incubated with 15 nm colloidal gold for 2 hrs followed by a 45 min chase prior to processing. The gold identifies the lysosomal compartments that fail to fuse with the *Mtb*-containing vacuoles. At 14 days post-infection, panel C, even though the cell is carrying an impressive bacterial load, in this infection model the bacteria appear almost exclusively intra-vacuolar. Panel D illustrates the hypothesis that escape from the vacuole and the induction of necrotic cell death could play a critical role in late stage disease that in the mouse leads to death, and in humans leads to transmission. In this model the percentage of cell-associated *Mtb* that are intravacuolar represents the majority of organisms and is relatively constant until individual granulomas progress to active disease. Interestingly, in experimental infections with fluorescent reporter *Mtb* strains in non-human primates we do observed extracellular bacteria in the caseum. These bacilli are fluorescent but occur as

singletons suggesting they are viable but non-replicating (unpublished data, Russell and Flynn).

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