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Review

Multinucleated Giant Cell Formation as a Portal to Chronic Bacterial Infections

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Abstract: This review provides a snapshot of chronic bacterial infections through the lens of *Burkholderia pseudomallei;* detailing its ability to establish multi-nucleated giant cells (MNGC) within the host, leading to the formation of pyogranulomatous lesions. We explore the role of MNGC in melioidosis disease progression and pathology by comparing the similarities and differences of melioidosis to tuberculosis, outlining the concerted events in pathogenesis that lead to MNGC formation, discussing the factors that influence MNGC formation and how they fit into clinical findings reported in chronic cases. Finally, we speculate about future models and techniques that can be used to delineate the mechanisms of MNGC formation and function.

Keywords: *Burkholderia pseudomallei*; *Mycobacterium tuberculosis*; Multi nucleated giant cell; persistence; chronic infection

1. Introduction

Melioidosis is a severe disease caused by the Gram-negative pathogen *Burkholderia pseudomallei* (*Bpm*). *Bpm* is a Category B, Tier 1 select agent, based on the CDC classification. The hotbed of melioidosis is Southeast Asia and northern Australia but increasing surveillance and diagnostic capabilities have revealed that *Bpm* is present as a soil contaminant in the Middle East, sub-Saharan Africa, the Caribbean islands, and the Americas [1]. Recently, a non-travel related case of melioidosis was identified in Texas and together with another prior case in the same area, suggest that *Bpm* might be present in the soil of the continental United States [2]. It has been estimated that there are 165,000 cases of melioidosis a year with 89,000 deaths worldwide [1]. Melioidosis can manifest in a variety of signs and symptoms [3], giving *Bpm* the nickname "the Great Mimicker" because the disease progression is easily misdiagnosed as tuberculosis or other diseases. The majority of human melioidosis cases are classified as acute while around 18% result in chronic or latent infections [3]. These latent infections are generally manifested as symptomatic or asymptomatic, leading to abscesses in the liver, spleen, or lung. The severity of the abscess seems to correlate to the number of bacteria taking refuge within the lesion and have a bias for the spleen and lung; however, liver abscesses have also been observed [4]. These abscesses have been clinically identified as granuloma-like lesions and often are confused with extrapulmonary tuberculosis. Only after a series of diagnostic tests can the *Bpm*-induced lesions be distinguished from *M. tuberculosis*, as the histopathology is nearly identical [5-7]. One hallmark of *Bpm* infection, both during the acute and chronic stages, it is the formation of multinucleated giant cells (MNGCs) [8]. It has been postulated that *Bpm* uses MNGC as a mechanism to spread from cell-to-cell and evade the external immune system. However, their role in pathogenesis and the mechanisms of formation and immunological avoidance in the host remains unknown. The aim of this mini review is to provide an overview of *Bpm* induced MNGC formation, factors that influence their development, the role of these lesions in disease, and highlighting the parallel features to chronic tuberculosis (TB) infections. Finally, we will also provide

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insights for future studies and directions to understand the biological relevance of MNGCs in melioidosis.

2. *Bpm* pathogenesis process leading to MNGC formation

Within a mammalian host, *Bpm* acts as an intracellular pathogen that has been shown to invade and survive within nearly all cell types and tissues; phagocytic and nonphagocytic alike [9, 10, 32]. Whitely et al, demonstrated the ability of *Bpm* and the closely related BSL2 pathogen *B. thailandensis* to establish an infection in a variety of primary cell lines and noted that the bacteria thrived particularly in bronchial epithelial and vein endothelial cells; suggesting those locations as possible unidentified *in vivo* colonization sites [9]. The intracellular life cycle of *Bpm* can be broken down into three distinct stages; invasion/endosomal escape, cytoplasmic replication/motility, and cell-to-cell spread.

When invading cells, *Bpm* is taken up within an endosome and by preventing lysosomal fusion, it escapes into the cytoplasm in a type 3 secretion system (T3SS)-dependent manner. *Bpm* wields three T3SS, named as 1, 2 and 3. The T3SS-1 & 2 mainly appear to be involved in virulence against plants because they share homology with T3SSs found in plant pathogens [11]. The T3SS-3 is heavily involved in mammalian invasion and has been named the *Burkholderia* secretion apparatus (Bsa) [12]. T3SS-3 mutants exhibit decreased invasiveness and partial attenuation *in vivo* [13]. The exact mechanism of endosomal escape has yet to be elucidated but effectors from T3SS-3 have been implicated in later pathogenesis events, such as regulation of both actin-based motility and MNGC formation [14].

The second stage of the intracellular life cycle involves *Bpm* replicating within the cytoplasm and the ability to mobilize via hijacking host actin, all while evading and/or subverting the bactericidal pathways being activated within the host cell. A recent study [15] demonstrated that *Bpm* mostly upregulates genes associated with combating oxidative stress once free in the cytoplasm. This is in agreement with another study [16], that showed that *Bpm* induces host factor heme oxygenase 1 (HO-1) which corrals Reactive Oxygen Species (ROS) and promotes cell survival. Addition of a HO-1 inhibitor resulted in decreased bacterial burdens and an increase in survival. The other part during this stage in the lifecycle is the commandeering of host actin by bacteria to facilitate motility. There are several proteins that *Bpm* uses to accomplish this but two that are vital to the system are BimA and BimC [17, 18]. Upon endosomal escape, BimA localizes on one end of the bacterium and oligomerizes to nucleate and polymerize actin. BimC is complexes with BimA but the molecular function of this interaction is unknown. Another protein that is involved in the actin polymerization process is BipC, a Bsa effector. Vander Broek et al., showed that BipC can bind both monomeric actin and filamentous actin but is unable to stabilize it [14]. *Bpm bipC* mutants are attenuated in the Balb/c mouse model of infection and exhibited decreased adherence, phagosome escape and intracellular survival [19].

Upon establishment of actin-based motility, *Bpm* localizes to the plasma membrane and begins to extend the membrane into filopodia-like structures to bring the membrane into proximity to the neighboring cell. This allows for effective engagement of the type 6 secretion system (T6SS); *Bpm* has six T6SSs encoded in the genome, but T6SS-5 is the one shown to be used for bacterial pathogenesis of eukaryotic cells [20]. T6SSs are commonly used for interbacterial competition and delivery of antibacterial effectors; *Bpm* T6SS-1 and T6SS-4 seem to have this functionality [21]. *Bpm* intercellular spread via the formation of MNGCs is T6SS-5 dependent, several studies have demonstrated that individual deletions of essential structural components of T6SS-5 attenuated the infection and abolished cell-to-cell spread [22, 23, 24]. The mechanism for cell fusion and generation of MNGCs is unknown for both the host and the pathogen. The only potential secreted effector molecule that has been identified is VgrG, which is the needle tip protein for all T6SSs [25, 26]. The *Bpm* VgrG-5 contains a specialized C-terminal domain (CTD) with effector functionality. When this VgrG-5 CTD is interrupted, the cell fusion capability is abrogated. The CTD shares no sequence similarity with proteins of known function so its mechanism of action is undetermined [27]. A completely intact T6SS-5 is necessary for cell fusion but the identity and function of any delivered effector molecules,

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beyond VgrG-5, remains to be discovered. On the host side, membrane cholesterol and protein content appear to be important for proper membrane fusion and this is more than likely associated with optimal membrane thickness for T6SS firing and membrane penetration [28]. Another study was able to block MNGC formation by using antibodies that targeted host surface molecules [29]. Taken together, these two studies highlight two key host factors that contribute to MNGC formation but also demonstrate the need for further studies.

3. Similarities and differences between Melioidosis and Tuberculosis

Mycobacterium tuberculosis is unique and a historical human pathogen that exhibits a very distinct infection cycle and manifestations in the infected host. M. tuberculosis is spread via infectious aerosol droplets that are dispelled by the cough of an afflicted individual. Upon inhalation of the infectious droplets, the bacteria travel to the lower airways where it is internalized and begins to replicate within alveolar macrophages. M. tuberculosis accesses the lung parenchyma through an unknown mechanism, but it is hypothesized that the infected macrophages migrate through the epithelium or that M. tuberculosis directly infects the epithelial cells and spreads deeper into the tissue. Once M. tuberculosis has migrated into the tissue, the immune system begins to recruit inflammatory monocytes and leukocytes to contain the infection, this results in the preliminary stages of granuloma development [30]. The granuloma is a very important structure in that it has the duality of containment and haven for the pathogen. The outcome of infection relies on unknown immunological factors, but it is thought that uncontrolled replication and an imbalance between pro and anti-inflammatory mediators drives the bacteria to escape containment and result in active TB [33]. If the balance is right, the bacteria are contained, and the infections remains asymptomatic with mature granuloma structures containing dormant bacteria. The mature granuloma contains cells specialized to seal off the infection, some of these include epithelioid macrophages, foam cells, and MNGCs. The MNGCs that form within the M. tuberculosis granuloma have enhanced antigen presentation but exhibit drastically reduced ability to uptake bacteria [31]. This suggests that these MNGC serve in a mostly surveillance role around the perimeter of the core.

There are many parallels between melioidosis and tuberculosis infections, which has led to occurrences of misdiagnoses in instances where proper diagnostic methods are not properly applied or available. Some of these parallels include the ability to result in an aggressive, difficult to treat pulmonary or extra-pulmonary disease, as well as an asymptomatic infection that can reactivate decades later. Both chronic infections tend to result in granuloma-like structures that are often confused with each other; however, because tuberculosis disease is more prevalent worldwide, that is the obvious diagnosis. Bpm and M. tuberculosis are both intracellular pathogens with a propensity for using macrophages as a replicative niche and a trojan horse to disseminate to other regions of the body. The risk factors for infection are shared between the two; however, HIV infection is the largest risk group for TB, but the relationship between Bpm and HIV co-infection is less understood. These diseases can often be confused but they each display unique characteristics that differentiate them, for example, M. tuberculosis is a strictly human pathogen whereas Bpm is a soil bacterium that evolved to cause mammalian disease. M. tuberculosis has very predictable manifestations with primarily pulmonary involvement and in certain cases dissemination resulting in extrapulmonary tuberculosis. Bpm is an opportunistic pathogen in the sense that it can cause disease in any tissue which creates a diverse profile of signs and symptoms, making diagnosis very difficult.

4. Role of MNGCs in Disease

The formation of MNGCs is a feature characteristic to *Bpm* and the closely related bacteria, *B. mallei* and *B. thailandensis*. The MNGC strategy allows the bacteria to spread cell-to-cell without being exposed to the extracellular milieu or immune components. MNGCs provide the bacteria with a safe environment to replicate with an abundance of nutrients and resources. The role of MNGCs in whole organism virulence is unclear but the attenuation of T6SS-5 mutants indicates this secretion system is critical to *Bpm* infection [23-25]. *Bpm* can form large MNGCs and heterogenous MNGCs composed of macrophages and neutrophils. The inclusion of epithelial and endothelial cells in heterogenous

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MNGCs has not been demonstrated but the possibility of *Bpm* being capable of accomplished this is highly likely based on in vitro studies [9]. MNGCs have been found within the granuloma-like lesions formed during chronic infections, suggesting a larger role in long term colonization [7]. A hypothesis that we have been investigating is that MNGCs are initially a sanctuary for *Bpm* and then act as a nucleation point for the generation of a granuloma-like structure. Factors that influence the switch between acute infection and chronic colonization is largely unknown, but the induction of toxin-antitoxin systems in *Bpm* has been implicated in the generation of metabolically dormant persister cells [34]. *Bpm* infection exhibits cell death to varying degrees, and the structural components of the T3SS have been shown to be potent inducer of caspase-1-dependent IL-1β secretion and, in turn, pyroptosis in murine macrophages [35]. During the pyroptosis process, the intracellular niche would be destroyed and exposed the bacteria to the external immune system; however, *Bpm* upregulates cytoprotective host factors to preserve the integrity of the intracellular environment [16]. The presence of bacterial effectors that manipulate cell death pathways hasn't been established but it is likely that they exist in some capacity, based on the successful intracellular lifestyle of *Bpm* and the wide array of weaponry the pathogen uses within the cell [36]. The transient re-activation of persister bacteria would shut down the cytoprotection and manipulation exhibited by *Bpm* and cause the MNGC to undergo cell death, creating foci of necrosis and inflammation, like the ones seen at the center of the mature granuloma-like lesions of chronic melioidosis patients.

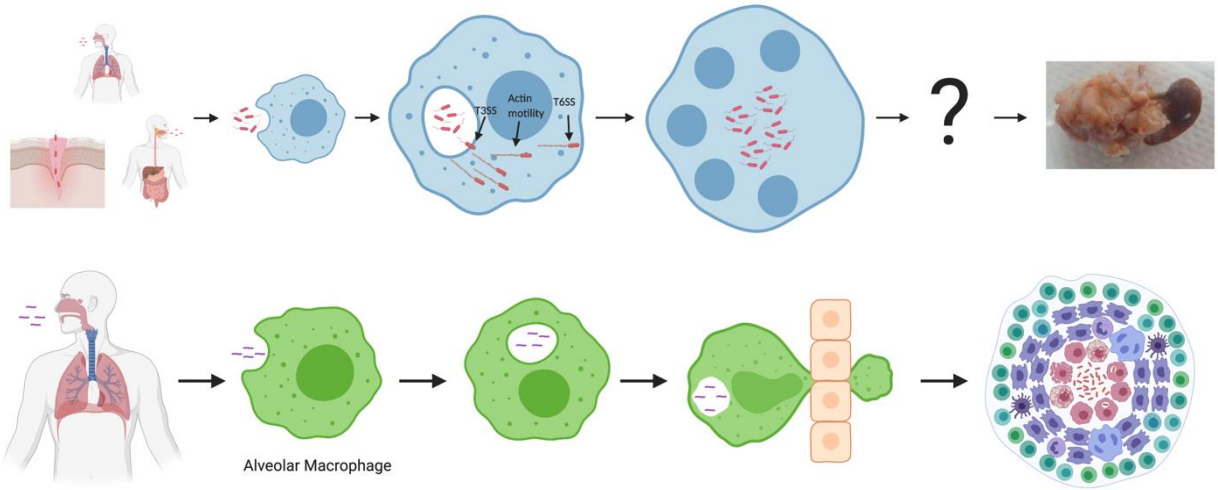

Figure 1. Comparison of pathogenesis events in *Burkholderia pseudomallei* (*Bpm*) and *Mycobacterium tuberculosis*. *Bpm* (top) enters the host through inhalation, ingestion, or percutaneous routes. Once in the host, it enters cells and escapes the endosome/phagosome via T3SS activity and becomes free in the cytoplasm. Hijacking host actin, *Bpm* moves to the perimeter of the cell to engage the T6SS. Using T6SS activity, Bpm fuses neighboring cells and forms MNGC and eventually, they can establish granuloma-like abscesses through an unknown mechanism. *M. tuberculosis* (bottom) is inhaled, where it travels into the airways and encounters alveolar macrophages. Once internalized, *M. tuberculosis* prevents phagolysosomal fusion and proliferates in the phagosome. Alveolar macrophages laden with bacteria penetrate the epithelium and travel deeper into the tissue, where it triggers the response that results in the formation of the granuloma.

As previously stated, MNGCs have been found in the granuloma-like structures of chronic infections but mostly on the perimeter of the central necrotic core that houses most of the bacteria [4, 37]. This is consistent with granuloma structure in *M. tuberculosis* infection where MNGCs are scattered throughout the periphery of the epithelioid cells, foamy macrophages, and lymphocytes. It is unknown whether these MNGCs house bacteria or, like *M. tuberculosis*, they have fused due to an unknown immunological stimulus (Figure 1). Studies have shown that peripheral blood mononuclear cell (PBMCs) exposed to beads coated in *M. tuberculosis* extract begin to form granulomas, including MNGCs, without live bacteria [39]. It is reasonable to speculate that the formation of MNGCs during *M. tuberculosis* infection is a passive process from the bacterial side. This

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contrasts with *Bpm* infection where live bacteria and an intact T6SS-5 are required for MNGC formation. It is possible that the MNGCs observed within the granuloma-like structures are sterile environments and have undergone the same fusion events as the ones within *M. tuberculosis* granulomas. The MNGCs that were actively formed by *Bpm* likely were at the center and resulted in the necrotic core that is full of extracellular bacteria. This idea is supported by post-mortem or post-surgical histopathology of infected spleens where the patients that died of acute melioidosis exhibited higher amounts of MNGCs when compared to the chronically infected patients who had organs removed surgically to find granuloma-like lesions. The later were positive for *Bpm* and negative for *M. tuberculosis* and/or fungal diseases, that could cause the formation of the granuloma [7]. Based on comparisons between laboratory and clinical studies, it is evident that MNGCs are the keystone event of *Bpm* pathogenesis, but many more studies are needed to delineate the mechanisms of their formation and define the function of the factors associated with their function.

5. Future Directions and Models

It is becoming clear across all disciplines that physiology happens in three dimensions (3D) and many responses are dependent on cells interacting with other cells and/or the extracellular matrix (ECM) proteins in a spatiotemporal manner. Many of the common cell culture techniques fail to faithfully recapitulate the responses that occur in vivo because they lack the 3D interactions with the environment [40]. That is not to say that these methods are invalid but the translational power behind them is less than some of the novel 3D cell culture systems being developed. The implications of 3D technologies on a wide array of cellular responses has been recently reviewed [41] and indicates that interactions which occur in 3D have a drastic effect on the nature of the responses. For example, in 2D cultures, necrotic cells detach and float into the media while *in vivo* and 3D cultures, necrotic cells are trapped, and the surrounding cells are forced to interact with the remnants of the dying cell. With the complex nature of *Bpm* pathogenesis culminating in cell fusion events and granuloma-like lesion formation in chronic cases, it would be very useful to coopt some of the advanced cell culture techniques to study MNGC and granuloma formation in systems that more closely resemble the organs where the events take place. The tuberculosis field has made great progress on this area and many novel *in vitro* techniques have been used to define the role of these cell structures in the pathogenic mechanisms [42]. *Bpm* and *M. tuberculosis* are similar in certain aspects which makes the immediate coopting of these methods convenient and useful but limited in scope due to being primarily focused on the lung. Organoids are simplified, miniature versions of tissue that are generated *in vitro* and still have characteristics of the *in vivo* anatomy. Organoids represent an attractive situational alternative to *in vivo* and *in vitro* modeling due to the levels of customization, complexity and control it offers the laboratorian. *Bpm* can colonize and cause disease in most tissues of the body, so expansion of the repertoire of organoid models would be needed to characterize the behavior of *Bpm* across the different organs and systems. Fortunately, many of these organ models are already in development or in use in other areas of biomedical research [43-45]. Depending on the nature of the study, the existing organoid models could be used as is or altered to accommodate the ability to simulate the chemotaxis/infiltration of leukocytes into the system.

Another alternative modeling system that shows potential for studying MNGC and granuloma formation is the zebrafish. The zebrafish is commonly used with *Mycobacterium marinum* as a model to study granuloma formation in a surrogate organism of *M. tuberculosis*. The granuloma structures formed in the zebrafish model of *M. marinum* are nearly identical to those formed by *M. tuberculosis* [46]. This model is attractive for many reasons, one being the ability to examine the contributions of innate and adaptive immune components separately due to the fact that the embryonic stage of the zebrafish only possesses the innate system. Another attractive characteristic is the growing number of genetic knockout strains of zebrafish. This model has been used as a bridge between in vitro models and rodents to test the efficacy of therapeutics with a high degree of success. The zebrafish is already in use in the *Burkholderia* field to study *Burkholderia cepacia* complex (Bcc) therapeutics and pathogenesis [47-48]. Establishing a zebrafish model for *Bpm* infection has two immediate obstacles; biosafety concerns that come with infecting an aquatic animal with a tier 1 select agent and the

possibility that *Bpm* might be too aggressive of a pathogen. Both obstacles can be easily overcome by using *B. thailandensis* which is used as a BSL2 surrogate for *Bpm* and exhibits many of the same hallmarks and characteristics of infection, including MNGC formation.

6. Final Remarks

The goal of this mini review was to shed light on MNGC formation and function during *Bpm* infection. MNGCs are linked to the function of *Bpm* T6SS-5 and interruption of the formation of these lesions results in attenuation in murine models of melioidosis, suggesting a critical role of this secretion system in disease. Although critical, very little is known about these structures associated with the secretion of effector proteins; *Bpm* can fuse multiple cell types into large, heterogenous MNGCs but the mechanism of fusion is unknown. We believe that MNGCs play a critical role in chronic infection and granulomatous lesion formation, so it is appropriate to use the parallels with latent *M. tuberculosis* infection. More work is needed to understand the MNGCs formed by *Bpm* and how they compare to the MNGCs that form within granulomas. To gain further insight, we strongly believe that co-opting 3D cell culture methods to closely mimic the microenvironment within the host will provide useful information. As indicated, these types of organoid methods, as well as the zebrafish, have been widely used within the *M. tuberculosis* field, which makes commandeering them an attractive option for *Bpm* studies. Overall, the MNGC is an understudied area of *Bpm* but has proved to be difficult and therefore, innovation is a necessity to begin unraveling the mysteries within.

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