Tuberculosis: Immune system, dormancy and resuscitation

Mycobacterium Tuberculosis Infection

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Mycobacterium tuberculosis (M. tuberculosis, Mtb) is a human pathogen causing the chronic infectious disease, tuberculosis (TB). The World Health Organization estimates that there were 10.4 million cases (with clinical disease), of those 6.3 million were new cases (61% of the estimated incidence of 10.4 million), 1.3 million deaths from HIV-negative people, and 53 million deaths between 2000-2016. TB is now ranked above HIV/AIDS as the leading cause of death worldwide from a single infectious agent. From this data, approximately one-third of the global population may have latent TB infection (LTBI), as they do not present outward clinical signs of disease except for a reactive tuberculin skin test. Mycobacterium tuberculosis is a non-sporulating species with a thick and waxy cell surface coating that contains mycolic acid. The bacterial cells do not absorb Gram staining and are stained with "acid-fast" stains. These cells also have a slow growth rate.

A naive host can be infected with Mycobacterium tuberculosis by way of inhalation of bacteria carried in aerosolized particulates. The bacterium can reach the deep respiratory tract entering the alveoli, infecting alveolar epithelial cells, M-cells in the BALT, dendritic cells and macrophages. Chackerian et al. (2002) state that little is understood about how adaptive immunity is initiated within a host after macrophage are infected with Mtb, specifically, how do Mtb antigens leave the lung and enter draining lymph nodes.

After initial infection, Peddireddy et al. (2017) summarize the following possible outcomes. First, the host can successfully clear the bacterial infection by innate immune activation. Second, Mtb multiply and replicate successfully within cells of the immune system, especially macrophages leading to acute clinical primary infection. Third, approximately 5-15% of the incident 1.7 billion infected with M. tuberculosis (WHO, 2017) will develop TB, as the primary infection can be contained without acute clinical disease by entering a state of latency, further discussed within as the interplay between the protective immune functions of the host and dormancy state of Mtb bacteria. Fourth, subclinical infection can be interrupted by loss of dormancy of Mtb leading to acute infection and an active TB state.

The host, in the third state of protective immunity, is usually non-contagious and asymptomatic. The host will, however, react to a tuberculin skin test (Dutta and Karakousis, 2014), even without outward clinical signs and radiographic findings in the lung tissue. In this state of latent TB infection, it has been shown that bacteria from patients can often not be recovered or cultured from respiratory excretions. It is thought that the majority of TB cases in the United States are a reactivation of latent TB infection and this reactivation clinical state of TB is not distinguishable clinically from acute infection after initial infection with Mtb (Dutta and Karakousis, 2014).

Below we focus on the interplay of dormancy of the M. tuberculosis bacteria and the protective immune functions of the host which allow for latent infection and a subclinical state. Specifically, we focus on the host/bacterial niche, functions of the granuloma, and the conditions within the granuloma. We discuss the genetics of dormancy regulation by way of the DosR regulon, which consists of approximately 50 genes (Peddireddy et al., 2017). We also focus on

the loss of this dormant state. There also exists host and bacterial factors leading to reactivation of clinical disease states. Host immune factors leading to the loss or degradation of the environmental niche of the granuloma and the role of resuscitation-promoting factors encoded by the rpf genes, Rpf proteins and their structural elements are discussed. In their 2017 review, Veatch and Kausha state "the rpf genes have been identified as being necessary and generally sufficient for resuscitation from a dormant or nonculturable state."

Other factors affecting the host immune system such as administration of anti-cytokine therapy, such as anti-Tumor Necrosis Factor-alpha (TNF-alpha) therapy for autoimmune disease, are discussed. Veatch and Kaushal also state that it has been shown that TNF-alpha (a T_H1 cytokine) is necessary and sufficient to prevent reactivation in latent mouse models. In line with this, Smith (2003) reviews results that found mice that are unable to produce or respond to TNF-alpha cannot form granulomas. As discussed, granulomas play a role in restricting Mtb dissemination. Finally, we briefly discuss current implications involved with understanding tuberculosis and provide a brief overview of clinical targets and treatments being discussed by those involved in TB research.

Mtb and Dormancy

(Hiral)

Defining dormancy: Dormancy can be defined as a period of time where growth and development is temporarily inactive. Dormancy of the Mtb is caused by its ability to avoid the immune system checkpoints; once dormancy occurs, Mtb can persist in one's body indefinitely. There are many factors that may cause dormancy to occur, environmental factors being one.

Environmental factors: In ideal situations in which the immune response is compromised with low amounts of nutrients, oxygen active Mtb cells generate a dormant state. This state is achieved by manipulation of toll-like receptors, cytokines, immune cell functions, biochemical resistance to antibiotics and genetic activation of dormancy-associated genes. Due to such a vast array of manipulation activity, approximately 90-95% of new Mtb infections may lie dormant in the host. The manipulation tactics also play a vital role in the establishment of the variety of niche that are created by the Mtb.

Niche: The niche that Mtb are known to create are masking, dormancy, altering innate immune cell fate, enhancing granuloma formation, and developing antibiotic resistance. Another crucial niche is the inhibition of macrophage maturation. Macrophages are a niche needed for Mtb replication and are critical for phagocytosis. Once phagocytosis has occurred by macrophages it allows for Mtb to gain entry to create the niche. Mycobacterial products such as phosphate, SapM, kinase, PknG, and early secretory antigenic target are used to inhibit the maturation of macrophages. Since these listed mechanisms prohibit the macrophages from doing its job, it allows the Mtb to establish its niche and grow and replicate. This will also allow Mtb to remain in the body for a long time. Thus, the most important reason to dormancy is for Mtb to allow itself to create a niche and re-infect the host when conditions are met.

Regulation of dormancy: DosR genes are regulatory entities which consist of various effector proteins that also play a crucial role in maintaining dormancy of Mtb in the host. DosR activates Ca2+ which induces necrosis. Moreover, the activation of Ca2+ leads to ATPase of the plasma membrane to create hypoxic conditions in the mycobacteria. DosR regulon consists of approximately 50 genes which are essential for dormancy regulation. The DosR regulon is broken down to nine blocks in the genome to allow pathogenic, non-pathogenic, and environmental bacteria to thrive in diverse habitats. The function of DosR is to primarily assist Mtb to adapt to anaerobic conditions, this is how it is allowed to live in the hosts granuloma. Expression of DosR proteins are induced under hypoxia, where mycobacterial growth is inhibited by external growth factors such as macrophages. During hypoxic conditions the regulon monitors the conditions of the adaptive immune response to promote the persistence of the infection. Out of the DosR genes expressed for dormancy, 18 of them are T cell responders which trigger an IFN-y response in patients with TB. Although mutations in DosR may occur, these mutations will not establish the death of MTB under hypoxic conditions. This phenomena goes to show that there are other factors which play an important role in dormancy and survival of the Mtb in the host.

Mtb reactivation/resuscitation

(Alisha)

The immune system of the host tries its best to counteract the effects of MTB in many fashions. Macrophages and Th1 cells secrete TNF-alpha and IFN-gamma to recruit cells like neutrophils, dendritic cells, and B cells, to help clear the infection, as well as prevent the bacilli from multiplying. However, some bacilli might escape these innate immune cells, and enter into dormancy which leads to the formation of granulomas. Granulomas are formed in response to infectious and non-infectious stimuli that are associated with various diseases. They consist of a central core of Mtb-infected macrophages surrounded by activated macrophages, giant multinucleated cells, epithelioid cells, lymphocytes, fibroblasts, and dendritic cells. Dying macrophages who are comprised with MTB spread their infestation of MTB to neighboring macrophages. This in turn leads to the expansion and dissemination of granulomas. Mtb reactivates and exits from the granuloma when the immune system is compromised and the bacilli spread to new sites of infection. The occurrence of granulomas are preventable in TB cases globally, due to its enhanced expansion and dissemination of MTB.

Poor nutrition, decrease in the number of T-cells, comprised immune system and HIV coinfection are some of the contributing factors for reactivation. The bacilli undergo multiple strategies in order to form a granuloma and stay latent. Mtb virulence promotes the formation of granulomas even though is it a basic immune response elicited by the host immune system. TNF-alpha plays a significant role in mounting a cytotoxic response to the pathogen all while maintaining the structural integrity of the granuloma. The neutralization of TNF-alpha leads to the reactivation of MTB and an increase of bacilli in the lung tissue. It has been seen that "lipid-rich mycobacterial cell walls composed of trehalose dimycolate induces TNF-alpha induction" and therefore the formation of the granuloma inflammatory response. Mtb virulence also increases the level of TNF-alpha in the macrophages by directly interfering with the cAMP mediated response. Stimulation of immune cells secrete various chemokines, such as CCL-2,

CCL-12, and CCL-13 to recruit macrophages and dendritic cells at the site of granuloma. Extensive calcification of granulomas provides a safe environment for Mtb to remain dormant for extended periods of time, and in turn allows the MTB to be in the host without being detected.

(Tran)

Regulation of reactivation/resuscitation

Rpf genes and Rpf protein structure and function

The most crucial part of the survival of Mycobacterium tuberculosis are regulatory proteins that persist the human's immune system under unfavorable conditions. The groups of proteins with genes that upregulate during the process of reactivation of the granuloma are called RPF proteins and DosR gene.

(Michelle)

There are 5 orthologs of the rpf gene in the species M. tuberculosis, resulting in Rpf-like proteins RpfA-RpfE. The most complicated of these proteins is RpfB. It contains 362 amino acids with a catalytic domain (75 amino acids), a G5 domain (with conserved glycine residues) and three DUF348 domains (domain of unknown function). These proteins have hydrolytic enzymatic activity. They are able to hydrolyze peptidoglycan, making them lysozyme-like in function and structurally similar to lysozyme C. Some of the Rpf proteins are secreted and others may be anchored to cell membrane. In a 2016 review, Nikitushkin et al. state that the rpf orthologs have been shown to function similarly; however they may be expressed differently and according to factors in the environment.

(Tran)

Some major functions of Rpf proteins are knocked out of Mtb, which then creates an environment where Mtb is unable to undergo reactivation even after the host immune system is suppressed. There are five Rpf genes (A, B, C, D, E) genes, though all are not required for general viability, but are crucial for reactivation of Mtb and can survive multiple mutations. Rpf genes "Participate in cell wall hydrolysis, an essential early phase step in the reactivation or resuscitation process." these proteins break peptidoglycan of the impermeable cell wall of granulomatous cell. Resuscitation happens when the immune system are suppressed, for instance if there are lower numbers of CD4+ T cells within the system or any other immune cells. Rpf genes will then be secreted by Mtb. Rpf genes are important the breakdown of granulomas, which is demonstrable in cases which granulomas are unable to reactivate when Rpf genes are knocked out and stress is removed from the cell. Therefore, this gene is targeted in use as a potential drug that causes Mtb to exit dormant stage. Use of conventional drugs, or anti Rpf immune responses, can then be elicited afterward.

DosR genes and protein function

Another important gene is the DosR, a regulon gene also called Rv3133c, which contributes to rapid growth of Mtb when they transit from non-respiring conditions back to respiring conditions. It is the regulon responsible for encoding dormancy and reactivation

activities of Mtb. The primary function of the DosR gene is to regulate expression of non-coding short RNAs involved in dormancy and reactivation of the Mtb. Many other gene regulators are also needed to reactivate from Mtb dormancy as well, such as Rv2745c in vitro. Along with those other regulons, the DosR gene needs to be regulated at a different level to contribute to the reactivation process. Therefore, the Rpf proteins and DosR genes are critical in the breakdown of granulomas and reactivating Mtb in favorable conditions.

(Evan)

Effects of anti-cytokine inhibitors on reactivation/resuscitation: The role of cytokines in not only prevention, but distribution of Mtb during infection can not be overstated. Multiple papers studying specifically cytokine interactions with Mtb bacterium outline a strong influence in host outcome by cytokines, chemokines, and their constituents. This close-knit interaction between cytokines and Mtb is illustrated by the World Health Organization's claim that one in three (2.28 billion) humans are latently infection with Mtb. Ten percent of these individuals (around 228 million) are at risk of incurring resuscitated Mtb infection.

The understanding of cytokine interactions and the role cytokines play in preventing re-emergence of Mtb infection stems from studies which correlate HIV incidence with Mtb re-emergence. HIV, known to affect CD4+ T Cells, dramatically increased the likelihood of reactivation of latent Mtb in these studied individuals. Therefore, cytokines became the focus of intense study in determining exactly how much of a role they play in warding off disease. This close interaction was also realized during the 1980's with the rapid emergence of AIDS and associated increase in Mtb infection rates.

CD4+ T cells specifically interact with diseased pulmonary macrophages and limit bacterial growth through IFN-y and TNF-a signalling. Granulomas are formed during this process, but microenvironmental changes lead to an environment which favors latent Mtb infection. Therefore, changes in IFN-y and TNF-a signalling would allow for an environment of rapid Mtb growth and proliferation from a reactivated state. This phenomenon has not only been observed with AIDS/HIV patients, but also in patients taking medications which block CD4+ cell responses.

To better describe the effects of these cytokines, CD4+ T cells, and other cell types, a study was performed on mice which were infected with a low-dose aerosol of Mtb, allowing for development of latent infection. This was cross-checked with studies of known Mtb infectious symptoms and physiological changes. Mice were then placed into two groups with a control, one being HIV (SIV) infected mice, and one being non-infected mice (but both groups infected with latent TB). As expected, mice in the SIV group lost CD4+ and effector CD8+ cell responses. Additionally, CRP tests, thoracic X-rays, TB skin tests, and BAL tests were utilized to document progression of disease.

Study results showed reactivation of Mtb correlated with CD4+ cell depletion by virtue of SIV presence. Nonreactive animals had an enhanced CD8+ and CD4+ response, conceivably leading to suppression of viral replication. Having SIV present eliminated the ability of CD4+ cells to secrete cytokines at the site of infection (in the presence of macrophages), thereby affecting the prevention mechanisms of the host organism. Drawing from this, we can understand the profound influence of CD4+ and their native cytokines on Mtb dormancy and

reactivation processes. In this study, researchers comparatively looked at mice which had intact immune systems (CD4+,CD8+,no SIV) and those which did not have intact immune systems (lower levels of CD4+, impaired CD8+ effector function, SIV). The results clearly showed those mice with impaired immune function were much more likely to show reactivation. BALT presence close to granuloma formation in pulmonary sites also correlated with control of TB reactivation. This factor shows B cells are included in the host's defense methods of controlling latent TB. Cross priming of CD8+ cells with B cells and affinity maturation are also thought to be occurring in BALT-macrophage infested areas.

Therefore, we can conclude any factor affecting cytokine responses, as shown in this study, have an impact on whether latent Mtb has a greater incidence of activation. This is not foolproof, although, as some SIV-infected and control mice still showed control of infection, thereby opening up the possibility of other methods of controlling Mtb from reactivation besides CD4+ cytokine functions.

Conclusion

(Alisha)

<u>Current implications and clinical targets:</u>

Due to the secure endowment Mtb has created within the host, it is not susceptible to certain antibiotics and develops resistance to strong antibiotics. Chromosomal mutations during dormancy can also result in drug resistance. Mtb has developed a multidrug resistance, and clinically eight to ten drugs are being used to target Mtb.

Patients with immunodeficiency conditions such as HIV, and AIDS, as well as patients who are treated with anti-TNF have been seen to have a reemergence of TB.

The protein and genes related to Mtb dormancy and reactivation have been studied extensively, however. Targeting granuloma formation is key in causing a decrease in the spread of MTb. "Granuloma-targeted therapy is advantageous because it allows for the repurpose of existing drugs used to treat other communicable and noncommunicable diseases as adjunctive therapies combined with existing and future anti-TB drugs. Thus, the development of adjunctive, granuloma-targeted therapy, like other host-directed therapies, may benefit from the availability of approved drugs to aid in treatment and prevention of TB." (Peddireddy, Doddam & Ahmed, 2017)

(Michelle)

Much work has shown that Rpf proteins play a role in resuscitation of dormant bacteria and stimulating growth. Nikitushkin et al. (2017) discuss that both the enzymatic activity and a possible role in signaling through muropeptides are at least some part of these as yet undefined mechanisms for these outcomes. Hence, many in the field have worked on methods to target Rpf proteins directly in anti-tuberculosis drugs, develop as components of vaccines and aid in diagnosing latent TB in culture. Drugs that inhibit the enzymatic capabilities of Rpf protein. Nitrophenyl thiocyanates (inhibitors of hydrolases) can prevent Rpf from binding peptidoglycan in its active site. These drugs exist now and could be further developed. Some have proposed using Rpf protein to enliven dormant M. tuberculosis within the human host in order to increase

sensitivity to current antibiotic regimens. Because Rpf protein orthologs can be secreted and recognizable by our immune system, they have been tested as vaccine components. As an aid to diagnosing latent TB, Rpf proteins have been added to nonculturable samples from patients with TB and achieved resuscitation of dormant cells. As a diagnostic, use Rpf proteins may decrease false negative rates in the diagnosis of latent tuberculosis.

(Evan)

Finally, utilizing methods where latent TB, reactivated TB and initial TB infection can be studied together greatly increases our understanding of which aspects of the host's immune system has the most profound impact on reactivation and suppression of Mtb. Initial studies have shown Mtb is suppressed by many different parts of the immune system including CD4+, CD8+ effector cells, B cells, macrophages, cytokines, chemokines, and others. Additionally, by understanding the ways in which these systems become nonfunctional (such as in the case of HIV/AIDS), researchers can have a better understanding of which methods treat latent Mtb or at least decrease the likelihood of reactivation.

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