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Opening Pandora's box: Mechanisms of *Mycobacterium tuberculosis* resuscitation

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Abstract

Mycobacterium tuberculosis (*Mtb*) infection characteristically causes an asymptomatic infection. While this latent tuberculosis infection (LTBI) is not contagious, reactivation to active tuberculosis disease (TB) causes the patient to become infectious. A vaccine has existed for TB for a century while drug treatments have been available for over 70 years; despite this, TB remains a major global health crisis. Understanding factors which allow the bacillus to control response to host stress and mechanisms leading latency are critical for persistence. Similarly, molecular switches which respond to reactivation are important. Recently, research in the field has sought to focus on reactivation, employing system-wide approaches and animal models. Here, we describe the current work that has been done to elucidate the mechanisms of reactivation and stop reactivation in its tracks.

The TB battleground

Mycobacterium tuberculosis (*Mtb*), the causative agent of TB, is believed to latently infect approximately 1/3 of the human population [1]. While a small percentage of infected individuals progress to active, symptomatic disease, most individuals develop latent tuberculosis infection (LTBI) characterized by immune-mediated control and lack of symptoms. Control is however eventually lost in a proportion of these individuals leading to reactivation from LTBI [2, 3]. Many factors can increase the risk of reactivation, including human immunodeficiency virus (HIV) infection, use of some immunosuppressant therapies, diabetes, smoking or even constant re-exposure [3]. Even though the BCG vaccine was developed a century ago and antibiotics have been employed against *Mtb* for 70 years, the disease still profoundly impacts humanity [4]. In resource-poor areas, drug treatment during latency is not generally practical; thus treatment begins only after symptoms develop [1]. At

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this point, the patient may already be infectious. If the risk for reactivation could be accurately predicted then treatment could be targeted to those individuals nearing reactivation, both preventing infection of others and reducing disease in the patient. Developing a more successful vaccine also requires a greater knowledge of *Mtb* pathogenesis and immune control. Antigen-specific adaptive immune responses, particularly those mediated by CD4⁺ T cells are required, although insufficient for, protection from TB [5]. We need to understand if the breadth as well as the magnitude of antigen-recognition is altered during reactivation, relative to active TB or LTBI. Furthermore, we need a better understanding of phenotypic changes in adaptive immune responses to TB during reactivation. Studying successful containment during LTBI and its failure during reactivation may, therefore, lead to better targeted-vaccines.

During LTBI, *Mtb* is thought to survive in a dormant state [6]. Dormancy is defined as a phenotype of the bacilli while latency is a clinical definition of a lack of symptoms. Dormancy has been extensively studied *in vitro* [7–19], *in vivo* [20–25], and *in silico* [26]. *In vitro* modeling has been key to developing our understanding of the transcriptome and proteome of dormancy, and has guided *in vivo* studies of these processes resulting in increased knowledge of the signaling mechanisms. The DosR regulon has been identified as a key regulon to initiate dormancy both *in vitro* [8] and required for persistence *in vivo* [23]. During reactivation of LTBI, the dormant bacilli are believed to resuscitate and resume normal growth and metabolism. Resuscitation is the recovery from dormancy and a phenotype of the bacilli and reactivation is the clinical definition of the recurrence of disease after latency (Figure 1). However, fewer studies have attempted to examine *in vitro* modeling of reactivation using re-aeration [13, 14, 18, 19, 27, 28], many of which are recent. As the field strives to develop new drugs and vaccines, understanding and identifying the signaling networks associated with reactivation will be critical. If we can stop the transition from non-contagious LTBI to infectious TB, the chain of transmission can be broken.

Finely Honed Senses

Much of *Mtb*'s success as a pathogen comes from its ability to sense and adapt to its environment [29–31]. *Mtb* encodes 13 sigma factors, the highest ratio of sigma factors to genome size of any obligate human pathogen [32, 33]. Sigma factor A is the only essential sigma factor of *Mtb*, however, many of the alternative sigma factor mutants have been shown to be attenuated for virulence in a variety of systems [32, 33]. SigH directly controls 31 genes [30] and indirectly controls approximately 20% of the genome [34]. SigH is activated in response to heat shock, redox stress, nitrosative stress, and phagocytosis [34, 35]. SigH has been shown to induce the transcription of *sigE*, *sigB*, *sigD*, *sigG* and *sigH* itself directly and indirectly, in addition to a host of other transcription factors [34]. *SigB* and *sigE* are the only transcription factors that have been observed to be induced during hypoxia [32, 36]. Additionally, *Mtb* encodes approximately 200 other DNA-binding proteins, many of which have been documented to govern transcriptional modifications in response to the changing environment [37]. Many transcription factors and kinases are known to act together as part of a signaling cascade in response to specific stressors including redox stress or pH as well as the signals to enter dormancy and have defined pathways. Many specific signaling cascades still elude the field particularly those initiated by the more recently identified

Ser/Thr family of kinases [32]. This preponderance of transcriptional modulators likely reflects the need of *Mtb* to rapidly respond to an array of stress environments.

The transcription factors of *Mtb* have been found to be tightly regulated. Transcription factors can be coupled to kinases as part of two-component systems of which *Mtb* has 12 known system and 4 orphan regulators with no known cognate kinase [32, 38]. One of the most studied two-component systems is the DosR system comprised of DosR as the transcription factor and its cognate kinases DosS and DosT [8, 39]. DosS and DosT are able to sense extracellular changes, such as hypoxia and nitric oxide (NO), and then phosphorylate DosR to activate its 47 gene regulon, and begin the process of dormancy [39]. *Mtb* also encodes 11 Ser/Thr kinases, tyrosine kinase, and three phosphatases. These kinases act as environmental sensors for the complex interwoven signaling mechanisms that allow precise control of bacterial processes such as through activation of transcription factors [38]. SigH is regulated by its anti-sigma factor RshA as well as by the Ser/Thr kinase PknB. RshA binds SigH to block its activity, but this protein-protein interaction can be altered by changes in the redox status of the cell or by phosphorylation of RshA by PknB [40, 41].

Transcriptomic experiments have been extensively employed to define *Mtb* responses to stress *in vitro* [7–19], *in vivo* [20–25], and *in silico* [26]. The findings of these studies were confirmed by detecting the presence of some of the key genes, including members of the DosR regulon, in human samples [42]. The DosR regulon, which was identified *in vitro* [8, 43], is required for persistence in the nonhuman primate (NHP) model [23] defining a key regulon for dormancy *in vivo*. The importance of the DosR regulon has been shown using proteomics as well. The DosR regulon members made up nearly 20% of all proteins expressed after 20 days of *in vitro* hypoxic conditions [14]. Furthermore, some members of the DosR regulon are strong T-cell antigens during latency in humans confirming their expression during human infections [44]. Other *in vitro* work has identified the enduring hypoxic response (EHR) regulon comprised of genes identified during long-term dormancy [16].

Resuscitation has not been studied as extensively as the processes involved in dormancy or its responses to intraphagocytic stressors. Some *in vitro* work has investigated resuscitation, evaluating extensive transcriptomics and some proteomics *in vitro* [13, 14, 18, 19, 27, 28]. This work has used the re-aeration model of resuscitation which presents limitations due to only one variable being studied in this model. While there are several animal models of reactivation, studies have generally only used these models as indicators of sterility in the lung or studying the immune response post-reactivation; rather than focusing on the mechanisms of reactivation [5, 47, 53]. Utilizing these models to specifically study the bacilli is important to further our understanding of the pathogenesis of TB, and to develop better treatments and vaccines.

Modeling resuscitation

In vitro modeling of *in vivo* processes is fundamental to properly target *in vivo* experiments. High throughput screens can be performed to identify genes or proteins of interest for further study in *in vivo* models, reducing the number of animals needed. *In vitro* models

(Table 1) are routinely used in *Mtb* research for drug screening [9, 57] and large-scale -omics experiments [13, 14, 19, 58]. An *in vitro* model of *Mtb* resuscitation has been used since it was published by Sherrid *et al.* in 2010 [18]. This model only replicates one small aspect of resuscitation namely re-aeration. A gradient of hypoxia is established in the granuloma with the greatest degree of hypoxia in the center[59]; during reactivation, the bacilli move through areas of increasing oxygen availability as they escape the granuloma [60]. The *in vitro* model requires that the bacilli are first forced into a hypoxic dormant state. Published papers on re-aeration have relied on variations of the Wayne's model of hypoxia to establish dormancy [10]; subsequently, the cultures are exposed to oxygen by shaking with a defined head space [18, 27] or with constant access to oxygen through filter-capped flasks [13, 14, 19, 28]. While most studies retained the original culture media during this process, two changed the media immediately preceding re-aeration [27, 28]. This approach could be extended to any model of dormancy. If a stressor that forces dormancy is applied and then removed, allowing the culture to grow in optimal conditions, a similar model of resuscitation should be achieved. Unfortunately, *in vitro* models are limited in the number of different conditions that can be manipulated and studied. While this specificity can be useful for directly probing the contribution of one factor, it does not allow for a comprehensive, simultaneous examination of all variables. *In vitro* modeling of the granuloma can allow for many aspects of the disease to be studied. Many host cells types are included that are generally found in human TB granulomas and granuloma-like cell aggregates can form in a test tube [61]. Recently, this model has been extended to study resuscitation of *Mtb* by treating the granuloma with anti-TNF- α to force reactivation. Dormant bacilli were shown to be resuscitated following treatment [45]. Unfortunately, this model has not been well utilized to study reactivation or resuscitation but represents a promising avenue to study host-pathogen interactions in a more efficient model. For a more comprehensive examination of resuscitation or reactivation, animal models are used.

All *in vivo* models of reactivation and thus resuscitation first rely on generating a chronic or latent state with what are thought to be dormant bacilli. While conclusive evidence of a dormant *Mtb* remains elusive, the assumption of the field combined with the presence of non-culturable bacilli suggests that the bacilli can indeed be biologically dormant [6, 62]. Conventional murine models of TB generally do not faithfully recapitulate LTBI or much of the typical pathology as observed in human infections of *Mtb* and are considered resistant to *Mtb* infection [55, 63, 64]. A prior model of reactivation relied on using a low infectious dose to establish a chronic infection and allowing the infection to proceed naturally [48]. The low dose model would not work well with more susceptible murine models nor does it mimic the colony forming units (CFUs) patterns seen during latency, namely low to undetectable CFUs [24, 65]. The more common murine model of TB reactivation is called the Cornell model, which involves infecting mice with a medium to high dose and then once infection is established administering a drug treatment to induce undetectable levels of CFU by plating then removing the drugs and allowing the bacilli to resuscitate [66, 67]. The Cornell model has worked well with several murine models of varying degrees of susceptibility to *Mtb* [46–48] as well as other small rodent models of TB such as the guinea pig [68]. Reactivation rates following the removal of drugs can be in the range of 10–20% of the mice [47], which can be used to study how different conditions affect the rates of the

reactivation but not if the aim is to study the processes of resuscitation. Different immunosuppressive regimens can be used to increase the rates of reactivation [48, 69]. The poorly utilized rabbit model of TB does recapitulate the cavitation of granulomas but is considered more resistant than the mouse model and develops true LTBI with no culturable bacilli [51, 52]. The model of reactivation developed for this model uses a low dose infection and the development of chronic disease which is then reactivated with immunosuppression [50, 51].

Small animal models of disease are more cost effective than larger ones and encompass more aspects of the disease than *in vitro* or *ex vivo* models. While the mouse model has been heavily relied upon, it does not faithfully recapitulate human disease. One of the hallmarks of TB is the well-organized granuloma and the hypoxia that occurs in the center of the granuloma [59]. In the traditional mouse models, there is no formation of an organized granuloma rather granulomatous inflammation is observed [55, 63, 64]. While there are mouse models that do display more hallmarks of TB pathology such as the hypoxic centers observed in the C3HeB/FeJ murine strain [64], these models do not accurately model neither latency nor reactivation [70]. The faithful modeling of both human pathology and latent immune control simultaneously has only been demonstrated in larger animals.

Of the available animal models, the NHP model of TB mostly closely recapitulates human TB disease [55]. Several species of primates have been used successfully to study TB with a range of susceptibilities and similarities to human pathology and disease progression. The NHP model of TB has been extensively reviewed previously [55, 71, 72]. There are two main strategies for inducing reactivation in the NHP models regardless of the species used. TNF- α blocking therapy induces reactivation and has been used in several studies [55, 56] (Table 1). This method of reactivation mimics certain drugs used for human autoimmune disorders and which, of course, come with a risk of reactivating TB if the patient is latently infected with *Mtb*. The second method of reactivation is co-infection with simian immunodeficiency virus (SIV) [5, 53, 55, 73]. SIV is closely related to HIV and is used to model HIV infection in primates. HIV and *Mtb* co-infection is a major human health concern and increases the risk of reactivation of *Mtb* from 10% over a patient's lifetime to 10% a year [4, 74]. These factors make the SIV-*Mtb* co-infection method of reactivation particularly relevant to human disease progression. When primates are infected with a low dose of *Mtb* and develop LTBI, a small percentage reactivate spontaneously [5, 23, 75]. The proportion of spontaneous reactivation is too low for practical use as a model of reactivation in an experimental setting even though it closely mimics human disease progression. The *Mtb*/SIV coinfection model increases the percentage of reactivation to ~65% and has been used extensively as an indicator for sterilization of *Mtb* from the lung and to study immune responses but very little has been done to study resuscitation of the bacilli [5, 23, 53, 56, 65, 75, 76] (Table 1).

Resuscitation as the End of Dormancy

Several studies have utilized the *in vitro* re-aeration method to study transcriptomics but with some differences in protocol. Namely, three studies examined re-aeration after seven days of hypoxia [13, 18, 19] while three more studies examined re-aeration after 20 or 25

days of hypoxia [14, 27, 28]. In addition to changes in the length of the study, two strains of *Mtb* have been studied; CDC1551 [13, 19] or H37Rv [14, 18, 27, 28]. Given the limited resuscitation work, researchers have targeted much of their investigation on examining signaling methods that are known to be involved in dormancy. Nearly all the *in vitro* work currently published has included at least a part of the DosR regulon in their analysis of re-aeration. These differences in study design did not change the overall expression patterns of the DosR regulon observed. Expression of the genes of the DosR regulon was near baseline following resuscitation though there was limited differential expression between the papers when resuscitation expression was compared to logarithmically growing cultures. For all but one paper [27], hypoxic data was included and expression values of the DosR regulon genes are uniformly lower following re-aeration than during hypoxia. When longitudinal data was included, nearly every gene of the DosR regulon examined had returned to very near baseline levels by 72 hours post re-aeration [13, 28]. Thus, once re-aeration is established, the DosR regulon may no longer be required and expression levels return to baseline levels, hypothetically due to the presence of oxygen changing the confirmation of DosT and no longer activating DosR [39].

In vivo research of reactivation has focused on reactivation as an ending of clinical latency and resuscitation of *Mtb* from dormancy. The mouse model highlights this approach as it has primarily been used to study drugs ability to sterilize the bacilli within the mouse [47]. The Cornell model of reactivation is entirely built upon this concept [66, 67]. While bacterial transcriptomics has not been studied in the mouse model of reactivation to the best of the writers' knowledge, studies with *Mtb* mutants have been done [77, 78]. But again, researchers have used this to examine what can affect dormancy and cause or hinder reactivation, rather than to identify signals or pathways involved in the transition. The NHP model has rarely been used to study reactivation because even using methods to force reactivation only a subset of animals will reactivate increasing the number of primates needed to reach statistical significance [55, 73].

Elucidating Drivers of Reactivation

In vitro studies have identified pathways differentially expressed during resuscitation that are not seen during hypoxia and thus are specific to returning to normal growth and metabolism [13, 19, 28]. Understanding the resuscitation-specific processes that *Mtb* uses will allow researchers to not only better understand resuscitation but dormancy as well. The *in vitro* granuloma model identified differential staining indicative of changes in the lipid cell wall between dormant and resuscitating. This phenotype was contributed to the actions of *lipY* [45]. *lipY* and *lipX* both PE/PPE proteins were identified as upregulated by one of the *in vitro* re-aeration studies [19]. In addition to *lipY*, genes involved in ATP synthesis and RNA polymerase subunits were identified as up-regulated during resuscitation. Interesting, all genes identified as up-regulated in dormant cells remained up-regulated during resuscitation. This study only examined resuscitation over 12 hours which implies that the dormant transcripts of those genes have half-lives longer than the study time [45]. One can also hypothesize that lipids accumulated during hypoxia which serve as a carbon source are rapidly metabolized during resuscitation as a quick access food supply and a cleaning house measure [45].

Both DNA repair genes and *clp* protease genes have been identified as specific to resuscitation [13, 18, 27]. The *clp* genes are controlled by the transcription factor ClgR. An isogenic *clgR* deletion mutant in the *Mtb* strain CDC1551 was demonstrated to not only reduce the expression of the *clp* genes but also the expression of the transcription factors genes *sigH*, *dosR*, and *sigE* during resuscitation [13]. *SigH* and *sigE* are both observed to be upregulated during resuscitation by multiple studies [19, 28]. DNA and proteins are likely damaged during hypoxia and must be corrected once metabolism has resumed with active growth. The signaling mechanisms may involve feedforward loops. SigH was observed to induce *clgR* during re-aeration [13], a lack of ClgR decreased *sigH* expression implying that ClgR directly or indirectly induces the expression of *sigH* [58] (Figure 2). Surprisingly, the ribosomal subunit genes have not been shown to be induced during early re-aeration; rather their expression is near or lower than during logarithmic growth [13, 18]. The expression of these ribosomal genes have been shown as induced compared to levels during hypoxia and may take longer to return to expression levels observed during logarithmic growth than has been studied [18]. These findings are supported by recent transcriptomic work with sputum samples from active TB patients who can be assumed to have progressed from LTBI. This study found a decrease in expression of ribosomal machinery and aerobic metabolism genes in the sputum compared to logarithmically growing *Mtb* [79]. While in wildtype *Mtb* the ribosomal subunit genes are similar to baseline during resuscitation, a *clgR* isogenic deletion mutant was shown to have induced expression of ribosomal genes after 4 hours of re-aeration. These genes are thought to be targets of the Clp protease system [13]. As the transcripts are being studied, we cannot assume that the protease system is involved in the reduction of ribosomal gene expression [13]. We can hypothesize that ClgR may have additional regulatory roles which include suppressing ribosomal gene expression. It is likely that resuscitation is a state separate from a return to logarithmic growth, but specialized for proper transmission and recovery from dormancy. Combined with the increased expression of DNA repair genes and the Clp protease system and a lack of expression of ribosomal and aerobic metabolism genes implies the bacilli must first expend energy repairing damage accumulated during dormancy before the rebuilding process can begin. Hypothetically, the bacilli may also be maintaining aspects of the dormant state, specifically the low aerobic metabolism, to repair damaged macromolecules and begin replication while avoiding increased susceptibility to the immune system and antibiotics. Another logical hypothesis is that a lack of new damage due to decreased immune activity may signal *Mtb* to begin robustly repairing damage before logarithmic growth begins.

Identifying signals for the transition from dormancy represents a unique challenge for which *in vitro* studies are particularly suited. Signals to transition occur prior to resuscitation during LTBI which is very difficult to capture in animal models. To study events just prior to reactivation and thus resuscitation in an animal model, we would first need to be able to definitively state which animal is about to reactivate. Our current models all show a range in the timing of reactivation, including some animals that maintain latency [5, 46–48, 53]. *In vitro* models allow researchers to know the exact timing of resuscitation and to sample many time points from the early stages to the late stages of resuscitation. This aspect is both a blessing and a curse as we cannot be certain that the sudden return of oxygen is a natural signal for rapid replication. However, bacilli are present in hypoxic granulomas and the

breakdown of those granulomas, and thus so is the return of oxygen, is an aspect of reactivation leading to resuscitation [4]. It is also likely that restoration of normoxia is an oversimplification in modeling resuscitation. Other signals could prompt the end of dormancy, including greater access to essential elements and preferred carbon sources.

The most well-researched possible bacterial signals to exit dormancy are the resuscitation promoting factor (*rpf*) genes. The first *rpf* gene was identified in *Mycobacterium luteus* [80, 81]. *Mtb* contains five *rpf* genes, *rpfA-E* [81, 82]. The *rpf* genes have been identified as being necessary and generally sufficient for resuscitation from a dormant or non-culturable state [62, 82]. The Rpf proteins appear to function as a dual lysozyme and lytic transglycosylase and act intercellularly, [81, 83] the proteins have been reviewed recently [84]. The *rpfD* deletion mutant in mice has been shown to have delayed reactivation, measured by CFU, using the Cornell model. In the same study, the *rpf* genes had decreased expression during reactivation compared to the chronic phase of infection [85]. In human patients, the proteins elicit an increased T-cell response during latency compared to active pulmonary disease and T-cell responses to RPF antigens may be indicative of a protective response during latency [22, 86, 87]. Animal models have detected the presence of *rpf* transcripts during latency and after reactivation using immune suppression in rabbits [50] as well as being detected by T-cells during murine infection [88]. *In vitro* transcriptomics reveals a similar pattern of expression to the *in vivo* studies. *In vitro* studies that have shown hypoxia transcriptomic data sets observed slight induction of at least one of the *rpf* genes during hypoxia [13, 28]. The *in vitro* granuloma model observed *rpfA-C* expressed during dormancy and at somewhat elevated levels during resuscitation [45]. Only one study has shown their expression increases dramatically during re-aeration [28] but this study changed the media between hypoxic condition and re-aeration. It is possible that the increased expression during hypoxia establishes necessary signaling cascades for re-aeration, if the media is not changed, the signaling takes place with no additional Rpf proteins needed. Robust T-cells responses may be a signal for *Mtb* to remain dormant due to not only proper immune responses but also the possible destruction of the Rpf antigens. If the immune system weakens and the Rpf proteins can build up extracellularly this may signal the bacilli to resuscitate. While the bulk of the research has studied transcript and protein, post-translational and epigenetic mechanisms may play a role in signaling the exit from dormancy. These mechanisms are poised to respond quickly to changing environments and the post-translation modification phosphorylation has been recognized lately has having a much larger role [38].

Host Response and Reactivation

Some work has attempted to elucidate the immune system's contribution to reactivation. As we have previously discussed, the standard model of reactivation of *Mtb* in the NHP model is to perturb the immune system either through SIV co-infection or anti-TNF- α therapy (Table 1), clearly implicated a proper immune response to maintain latency and prevent reactivation. Most of the immune studies in the mouse model of latency and reactivation have focused on the role of TNF- α preventing reactivation [89–91]. This work has demonstrated that TNF- α is necessary [89, 90] and sufficient [91] to prevent reactivation in the latent mouse model of *Mtb*. Two of the *rpf* genes have been studied in the murine model

and their effect on the immune response has been examined. The double deletion mutant of both *rpfA* and *rpfB* showed increased survival in both the normal course of infection and the Cornell model of reactivation. This increased survival of mice infected with the double mutant was independent of antibodies or CD4⁺ T-cells but implicated to be due to innate immunity [92].

NHP studies have been conducted to attempt to identify the host factors that may be risks of reactivation. These studies leverage the fact that not every animal reactivates, even when specific attempts to induce reactivation are made. One study attempted to investigate both gross pathology and bacterial factors to determine the risk of reactivation following anti-TNF- α therapy. This study demonstrated that both larger granulomas and increased inflammation during latency as measured by PET-CT scans were associated with increased risk of reactivation after anti-TNF- α therapy. After reactivation treatment, animals that did not reactivate unsurprisingly had higher bacterial burdens [73]. Another study induced reactivation in the primates with LTBI using SIV co-infection to determine the role of the immune system in maintaining latency and preventing reactivation. This study also found a greater bacterial burden in the primates that reactivated compared to primates that did not. Despite a greater bacterial burden, the differences in the pathology between primates which reactivated and those that did not was attributed to SIV-related pathology rather than TB-related pathology. The greatest immunological difference between the groups was the increased number of CD8⁺ T-cells that expressed granzyme B and the number of proliferating CD8⁺ memory T-cells positivity in the group of animals that did not reactivate [5]. These studies together can give use clues on how to identify patients who are at greater risk of reactivation and what type of immune response will be necessary to help those patients remain latent.

Human studies have observed LTBI patients with increased risk of reactivation or examined humans with LTBI or active pulmonary TB which can be assumed is mostly reactivated disease. Similar to NHP studies, many human studies of reactivation use HIV⁺ cohorts with an increased reactivation risk. Two studies of HIV⁺ LTBI patients observed CD4⁺ T-cells with a central memory phenotype directed at DosR regulon and *rpf* protein antigens are indicative of protection from reactivation [86, 87]. While CD8⁺ T-cells were more important in protection from reactivation due to SIV infection in NHPs [5], the human HIV⁺ cohorts are treated and maintain higher CD4⁺ T-cell counts which may explain the different findings in the relative importance of CD4⁺ or CD8⁺ T-cells but both humans and NHPs demonstrated the involvement of memory T-cells to maintain latency. Another study identified a Th2/M2 shift (less inflammatory) from Th1/M1 (more inflammatory) in an HIV⁺ cohort as well as decreased expression of the DosR regulon which could be correlated to increased risk of reactivation. This study postulated that the shift to Th2/M2 lead to decreased NO and hypoxic shift and reduced the induction of the DosR regulon. The initial shift may be due to HIV-specific pathology that then leads to changes in the *Mtb* pathology and an increased risk of reactivation [42]. In the recent NHP using SIV infection as the method of reactivation, increased total lung pathology in reactivating NHPs compared to non-reactivating NHPs was observed including increased pathology due to SIV infection in the reactivating NHPs [5] which is consistent with the hypothesis that a change in the lung environment due to SIV or HIV infection signals reactivation. So far results have varied

between studies depending on the cohort observed, but a consistent increase in CD4⁺ T cells with responses to DosR or RPF antigens have been observed lately as protective from reactivation [42, 86, 87].

Concluding Remarks

Reactivation from LTBI is a major battleground for preventing the spread of *Mtb* disease. Despite the importance of studying reactivation, limited progress has been made in our understanding the host responses or the signaling pathways leveraged by the bacilli during the transition. More information is available about the signaling pathways of bacterial dormancy which are associated with LTBI and the persistence of the bacilli in the host during this stage. Much of this information revolves around the DosR regulon. The activation of DosR and the processes involved in establishing dormancy are well characterized. It is important that we better understand the bacterial mediators of reactivation, e.g. the RPFs. While it is understood that these proteins are necessary and sufficient for reactivation in culture models, the signals that trigger their induction and the mechanisms by which they promote reactivation is not understood (Figure 3, Key Figure). Finally, it is possible that post-transcriptional mechanisms amplify and control key signals leading to the onset of reactivation. These could potentially include small RNA molecules, some of which are known to be induced by low oxygen tension and in a DosR-dependent manner [93, 94]. Because *Mtb* has a redundant genome, there may be post-translational signaling or other pathways involved such as the clp proteolysis pathway that has been shown *in vitro* to be active during re-aeration. It has been postulated that Clp-mediated proteolysis is a rapidly effective way to silence the expression of a particular pathway [95]. It is conceivable that it could also be utilized to degrade specific mediators of dormancy. Hence, the ClgR regulon could serve as a true correlate of resuscitation, and a polar opposite of the DosR regulon. Proteins are likely to be damaged during the jumpstart from a stagnant position coupled with a rapid increase in access to oxygen and nutrients. The end goal of TB research is to develop a more successful vaccine and/or better drug therapies. If *Mtb* infections were stopped in the latent stage either through drugs or a vaccine, there would be no new infections and the progress of the infection would be halted. Preventing reactivation will prevent the spread of the disease.

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Outstanding Questions

- While we know that the Rpf proteins are important for resuscitation, what signals their expression and what are the specific mechanisms that lead to resuscitation?
- ClgR, SigH, and other transcription factors are induced during resuscitation but which of these regulons are essential to resuscitation and reactivation of disease?
- Other than an increase in oxygen, what are the *in vivo* signals for resuscitation from dormancy?
- What signals can be identified to discern in animal models which animals will reactivate and what is the relative contribution of host genetics?
- In the background of a normal immune system, which aspects of the immune system are interacting with *Mtb* and allowing or preventing resuscitation?
- Are there differences in the cell division or changes in epigenetic or post-translation regulation that cause resuscitation in addition to transcriptional changes?

Trends

- New strategies are needed to limit the increasing burden of drug resistant TB.
- While extensive work has been done to understand dormancy, elucidating the signaling mechanisms during resuscitation is only now being attempted.
- Strong immune responses to DosR regulon members and Rpf proteins may be indicative of protective immunity and maintenance of latency.
- Resuscitation is characterized by different metabolic pathways and processes than in logarithmically growing cultures.
- The signaling for resuscitation may be laid out during dormancy such as the extracellular accumulation of Rpf proteins. Hence dormancy could be considered as a precursor for and a preparation for resuscitation/reactivation. Mtb may be programmed to undergo dormancy.
- Variation may exist in how different strains of Mtb enter and exit the dormancy program, and this may be key to their differential virulence.

Figure 1.

Definition of clinical and bacterial terms. Latency and reactivation refer to clinical states of disease during TB. Latency is a period during which there are no symptoms of the disease and the patient is not infectious. Reactivation is the return of symptoms and the patient is infectious. During latency, the bacilli are hypothesized to be dormant. Dormancy is defined as low levels of replication, minimal metabolism, and a thick cell wall. In favorable conditions, the bacilli is thought to resuscitate and begin replication and metabolism again and this is believed to lead to reactivation of disease.

Figure 2.

Expression of probable key regulons during the transition from dormancy to resuscitation. *In vitro* expression of *dosR* (*devR*), *sigH*, *sigE*, and *clgR* regulons after 6 hours of re-aeration. *clgR*, *sigH*, and *sigE* regulons are both generally expressed while the *dosR* regulon is being down-regulated including those genes shared with the other regulons. These regulons may be interacting through the shared members. Red indicates induction of that gene while blue indicates repression of that gene. Genes with a gray background do not have expression data. The members of the regulons were identified using the cutting-edge technology, ChIPseq [96]. The expression data was generated by our lab and previously published [19] and the program Cytoscape [97] show interconnected regulons.

Figure 3.

Bacterial changes from dormancy to resuscitation. Dormancy is characterized by low metabolism and growth and increased lipids and DosR regulon expression. Accumulation of Rpf proteins may be part of the groundwork for future resuscitation. Resuscitation is characterized by decreased DosR and increased ClgR regulon expression and increased metabolism using lipids as an energy source which are different pathways than aerobic respiration metabolism. The bacilli will increase in number and escape the granuloma.

Table 1**Models of Resuscitation and Reactivation**

	Basic Method	Advantages	Disadvantages	Refs
<i>In vitro</i>				
Reaeration	Shaking cultures after hypoxia w/or w/o new media	Faster, high throughput	One small aspect of resuscitation	[13, 18, 19, 27, 28]
<i>In vitro</i> Granuloma	Many host cells cultured together to form granuloma-like aggregate. Treated with TNF- α to induce resuscitation	More variables of bacteria and immune system can be studied	Natural influx and efflux of immune cells are absent.	[45]
Small Mammal				
Cornell Model	Infect with medium to high dose then treat to establish latency. Treatment is removed and a proportion of the mice will reactivate	Allows for sacrifice groups to study process over time, high throughput, drug treatment assures a period of 'latency'	Most small mammal models do not recapitulate human pathology; Latency is established by exogenous killing rather than endogenous control	[46, 47]
Low Dose Model	Infect with ~5 to 10 CFU and allow infection to proceed naturally or induce with immunosuppression (rabbit)	Allows for sacrifice groups to study process over time, high throughput, closer to true disease progression	Most small mammal models develop disease; timing is more uncertain so larger groups would be necessary	[48–52]
Primate				
SIV Co-Infection	Low dose TB infection followed by SIV co-infection	Monkey model closest to human pathology. TB- HIV is the greatest co- morbidity for both	Does not show natural reactivation in the absence of comorbidity.	[5, 53, 54]
TNF- α therapy	Low dose TB infection followed by TNF- α Treatment	Monkey model closest to human pathology. TNF- α treatment reactivation can occur in humans.	Does not show natural reactivation in the absence of comorbidity.	[55, 56]