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Review article

Challenging *Mycobacterium tuberculosis* dormancy mechanisms and their immunodiagnostic potential

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ABSTRACT

Mycobacterium tuberculosis is the etiologic agent of tuberculosis, one of the world's greatest cause of morbidity and mortality due to infectious disease. Many evolutionary mechanisms have contributed to its high level of adaptation as a host pathogen. Prior to become dormant, a group of about 50 genes related to metabolic changes are transcribed by the DosR regulon, one of the most complex and important systems of host-pathogen interaction. This genetic mechanism allows the mycobacteria to persist during long time periods, establishing the so-called latent infection. Even in the presence of a competent immune response, the host cannot eliminate the pathogen, only managing to keep it surrounded by an unfavorable microenvironment for its growth. However, conditions such as immunosuppression may reestablish optimal conditions for bacterial growth, culminating in the onset of active disease. The interactions between the pathogen and its host are still not completely elucidated. Nonetheless, many studies are being carried out in order to clarify this complex relationship, thus creating new possibilities for patient approach and laboratory screening.

Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), an infectious disease that remains a major global health issue.[1] Each year millions of people are accounted for infection, ranking TB as the second leading cause of death among infectious diseases, after the human immunodeficiency virus (HIV) infection. In 2013, nine million new cases of TB were notified leading to 1.5 million deaths despite the availability of treatment.[1]

One of the hallmarks of *M. tuberculosis* is the ability to establish a latent infection, capable of long persistence in the host, even in the presence of a functional immune system.[2] This persistent subclinical infection is driven by a low number of bacilli, which are kept in check by the host's immune

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system,[3] a situation thought to exist in approximately one-third of the world's population.[1] Of those individuals, 5–10% may develop clinical manifestations of active disease at some point in their lifetime.[4] Most TB cases occur within 2–5 years of the initial infection, especially in young children.[1] Malnutrition, tobacco smoke, indoor air pollution, alcoholism, silicosis, insulin dependent diabetes, renal failure, malignancy, HIV infection, and immune suppressive treatment, are considered risk factors for reactivation.[5]

An important component of a public health response against TB in high-income countries is the investigation of contacts of known TB patients. In contrast, in low- and middle-income countries this policy is still being implemented.[6] In terms of prevalence, 29.1% of the contacts in high-income countries are latently infected, while 1.4% have active disease.[7] In low- and middle-income realities, 51.5% of the contacts have latent TB infection (LTBI), with 3.1% of contacts developing overt disease.[7] Interestingly, in these settings, reactivation rates of up to 40% are observed in children under 2 years old, predominantly within the first year after infection.[8] Healthcare workers are another significant risk group especially in low-and middle-income countries. The prevalence of LTBI among healthcare workers is, on average, 54%, emphasizing the importance of TB control in hospitals.[9] However, there is no agreement on the most common place for LTBI transmission, in addition, there is no gold standard for its diagnosis, which generates inaccuracy and misinterpretations depending on the methodology chosen.[6,10]

Currently in use, but far from gold standard

There are two main immunological approaches for screening *M. tuberculosis* latently infected individuals: the tuberculin skin test (TST), which is an *in vivo* assay, and the interferon-γ release assays (IGRAs), which comprises two *ex vivo* methods.[11] For the execution of the TST, a determined amount of purified protein derivative (PPD) is injected *via* the intradermal route into the forearm of the patient. The PPD is a protein extract from the culture supernatants of *M. tuberculosis*. If the reaction is positive, there is an indication of infection. Regarding the IGRAs, there are two distinct tools, an enzyme-linked immunosorbent assay (ELISA)-based technique, and an enzyme-linked immunospot (ELISpot)-based method.[12] Their most popular commercial versions are QuantiFERON®-TB Gold In-Tube (QFN-GIT; Cellestis Ltd, Carnegie, Victoria, Australia); and T-SPOT®.TB (Oxford Immunotec Ltd, Memphis, Tennessee, USA), respectively.[13] In a general sense, both enzyme-linked assays verify the immunologic memory of the host.[12] While the ELISA-based test determines the concentration of IFN-γ in supernatants of *M. tuberculosis* antigen-stimulated cell cultures, the ELISpot-based assay allows a quantitative assessment of IFN-γ-secreting cells in response to *M. tuberculosis*-specific antigens.[11]

The sensitivity of the TST, which is the most widespread test, is extremely dependent on the patient's immunological status, being lower in immunocompromised patients.[13] There is growing evidence that the sensitivity of the IGRAs are higher than that of the TST; however, the lack of studies carried out in immunocompromised subjects and the impossibility of a clear differentiation between active disease and LTBI are major setbacks.[14] As another important consideration, the sensitivity of TST is also dependent on the choice of the cut-off value, thus, entangling with the specificity, which is affected by the cross-reactivity between *M. tuberculosis* antigens and environmental mycobacteria.[15] Various groups have performed reviews and meta-analysis of sensitivity and specificity values to help clinicians and agencies to develop guidelines toward the diagnosis of LTBI. According to Pai et al.,[16] the sensitivity of IGRAs scored a value of 70% for QFN-GIT and 90% for T-SPOT.TB, while TST reached a mark of 77%. Diel et al.[17] verified that IGRAs were more reliable for the identification of non-infected individuals, as compared to TST. The specificity for QFN-GIT, T-SPOT.TB, and TST were 99.4%, 98%, and 88.7%, respectively.[17] However, in high-incidence settings, a positive IGRA may not necessarily indicate TB, as much as a negative IGRA or negative TST cannot rule out the possibility of active disease.[16]

In high-income countries, in which low TB rates are found, LTBI screening is recommended in the subjects at increased risk of developing the disease as a matter of public health.[18] However the lack of clear cut-off values for serial testing and unclear interpretation make useful tools like the IGRAS still hard to be implemented in all realities.[10,18] Besides, the exact proportion of positive tests for LTBI and the number of patients that still have not received preventive chemotherapy is still unknown. Public health policies and clinical management for the identification and treatment of latently infected subjects would be improved with a better understanding of the nature of LTBI.[2]

The key mechanism for latent infection

For survival and persistence in extreme conditions, *M. tuberculosis* must be able to sense environmental signals and utilize them to trigger its adaptation machinery, then making its endurance possible in a new ambient.[19] Low oxygen tension, oxidative stress, and NO are factors that are frequently associated with the establishment and maintenance of LTBI.[20] In this context, the transcription factor Rv3133c, named dormancy survival regulator (DosR), directly coordinates the expression of approximately 50 genes, which altogether make up the regulon DosR, preparing the metabolic changes that will allow the mycobacteria to enter dormancy.[21,22]

Under aerobic conditions, the transcription factor PhoP (Rv0757) is responsible for the maintenance of basal levels of Rv3133c.[23] When hypoxia is set, Rv3133c may have more five-fold increase compared to its original values,[24] enabling the entire regulon to be induced.[23] Remarkably, the first cellular changes can occur under low concentrations of Rv3133c. So, the first genes to be transcribed are those related to protein stability and homeostatic regulation, such as *hspx* (*rv2031*) and *rv1738*, respectively, preparing the cell for further metabolic changes.[25]

Though strictly aerobic, *M. tuberculosis* may face low oxygen levels during the course of infection, typically in late granulomas, which are characteristically avascular, inflammatory, and necrotic. Those conditions have been demonstrated as hypoxic.[26] *In vivo* models that mimic hypoxic conditions

have shown that the more oxygen is consumed, the more mycobacterial growth decreases, until it eventually ceases.[27] A decrease in both *in vitro* and *in vivo* oxygen tension leads to a state of non-replication, which causes the latent infection as a mechanism of adaptation.[28] Given the necessary conditions for the dormancy state induction, the DosR regulon is activated through the action of two histidine kinase sensors, DosT (Rv2027c) and DosS (Rv3132c). Both of them are capable of associations with divalent gases such as NO, CO, and O_2, the regulators of their activity.[29]

The absence of aerobic respiration renders few metabolic options to the bacilli. Thus, the induction of the DosR regulon must be triggered prior to complete hypoxia, allowing adaptation and survival mechanisms to be transcribed and translated right before ordinary energy sources are depleted.[30] Despite its importance, the role DosR regulon plays reaches its apex during the preparation for the dormancy state, changing energy sources and reducing energy expenditure.[31] When dormancy is fully set, another group of genes called *enduring hypoxic response* (EHR) takes over until the conditions for optimal growth are once more resumed.[32]

To better comprehend the host-pathogen relationship during dormancy various research groups have been carrying out experiments to unravel the mechanisms by which *M. tuberculosis* survives the environmental changes elicited by the host's immune system. Bold et al.[33] demonstrated that the first changes in gene expression during the chronic phase would be sufficient to decrease the frequencies of protective $CD4^+$ IFN-γ^+ T cells in the lungs of C57BL/6 mice. As a result of the down regulation of genes expressed during the active growth phase followed by the up regulation of those expressed during dormancy, the antigen presenting cells are not able to perform a proper activation of $CD4^+$ T cells. Thus, there is not sufficient production of IFN-γ to control the infection.[34] Besides, as stated by Mehra et al.,[35] the activation of the DosR prevents the immune system to establish a proper T helper 1-type of response against the pathogen.

However, one cannot associate the establishment of dormancy with a state in which *M. tuberculosis* would be fully protected from the immune system in all situations. Actually, as demonstrated by Mariotti et al.,[36] dormant mycobacteria lack the capacity to subvert the host immune system due to its inability to block the phagosome maturation. Such findings could be associated with persistent antigen presentation to $CD4^+$ T cells by dendritic cells. However, it may be considered unexpected that dormant mycobacteria is recognized by T lymphocytes with higher efficiency than active growing mycobacteria. It is unlikely that dormant mycobacteria could infect tissue macrophages during latency, as they do not replicate and cannot invade host cells unless they reactivate to sense the environment.[36] Another mechanism for host protection against *M. tuberculosis* infection was recently described by Venkatasubramanian et al.[37] A particular subset of regulatory T cells, named $CD4^+$ $CD25^+$ $FoxP3^+$ $D4GDI^+$ T cells, was found to contribute in the contention of *M. tuberculosis* in both human and murine models. These cells do not produce immunomodulatory cytokines, such as TGF-β or IL-10, secreting Rho GDP dissociation inhibitor (D4GDI) instead. This factor acts on macrophages, enhancing the production of IL-1β, TNF-α, and reactive oxygen species, indirectly contributing, to DosR regulon's activation, as a mechanism of mycobacterial growth arrest.[37]

Recent works like the one carried out by Ryndak et al.,[38] have shed some light toward the niches where the mycobacteria actually actively grows. Remarkably, it was verified that alveolar epithelial cells are interesting sites for mycobacterial active growth, prior to macrophage infection.[38] However, the physical location during LTBI is poorly understood. It is conceivable that dormant bacteria reside in cells which have a scarce ability to act as antigen presentation cells, and subverted macrophages in mature granulomas.[39] These structures physically separate T lymphocytes from infected cells by a fibrous wall of caseum,[40] limiting their interactions, creating a reservoir for *M. tuberculosis*.[41]

DosR antigens and diagnosis

Diagnosing TB continues to be a challenge, especially in low-income countries with high prevalence of the disease.[1] Classic methods such as sputum smears and cultures are relatively cheap and widespread; however, their major drawbacks are low sensitivity for direct sputum analysis and the long time required for the culture.[42] Molecular biology is also an alternative. Despite the rapid diagnosis afforded by this technique, its relatively high cost and moderate sensitivity are factors that make it unsuitable for all realities.[43] Thus, the search for alternative approaches implementing new markers and antigens capable of a better differentiation between LTBI and overt disease have become the subjects of many research groups.[1,5,42]

Among the most important and well-studied DosR regulon-encoded genes, the gene that codifies for the protein Rv2031c, also known as the 16 kDa antigen, whose levels elevate as dormancy sets in, and is kept at low amounts during mycobacterial exponential growth.[39,44] This antigen, as demonstrated by Demissie et al.,[45] elicited a higher IFN-γ response by T cells from asymptomatic subjects with history of previous infection by *M. tuberculosis* in both Ethiopian and Gambian populations. The opposite happened toward the recognition of the active phase antigen ESAT-6, which was better suited for people with active disease or people who had been in recent contact with tuberculous patients.[45] In agreement, Goletti et al.[46] observed a correlation between LTBI and higher IFN-γ responses elicited by DosR-encoded antigens. The following antigens were tested *via* ELISA: Rv2626c, Rv2627c, Rv2628, Rv2031c and Rv2032. However, only Rv2628 elicited a high and significant response for both latently infected and recently cured TB groups. The ability of Rv2628 for predicting LTBI had a specificity of 87.5%, and sensitivity of 76%. As internal control, QFN-GIT did not detect a response capable of discriminating different stages of the disease. In another study, IFN-γ response against mycobacterial dormancy antigens was higher in those subjects who tested positive for TST, while the response against the active phase antigen CFP-10 was significantly greater in people with active disease. The dormancy antigens Rv1733c, Rv2029c, Rv2627c, and Rv2628 were the ones that elicited the highest IFN-γ responses in latently infected subjects.[47]

A remarkable fact concerning the dormancy related antigens is that they do not show any boost effect related to

M. bovis BCG vaccination. It was well demonstrated by Lin et al.[48] in both animal and human models for the following antigens: Rv1733c, Rv1738, Rv2029c, Rv2031c, Rv2032c, Rv2627c, and Rv2628. The main responsive cellular type to the dormancy antigens in terms of IFN-γ production, is the $CD4^+$ $CD45RO^+$ T cells, which belongs to the T cell memory subtype.[49] The same is also valid for the TST, characterizing a phenomenon found in individuals infected by mycobacteria, but not in healthy subjects. Among the best DosR encoded antigens recognized by T cells, the Rv1733c, Rv2029c, and Rv2031c are the most expressive concerning $CD4^+$, and $CD8^+$ polyfunctional T cells in subjects exposed to *M. tuberculosis*.[50] Both cellular types are phenotypically different from those generated by *M. bovis* BCG vaccination, which are $CD27^+$, while those found in latently infected individuals do not express this marker.[51]

Caccamo et al.[52] verified that the frequencies of polyfunctional $CD4^+$ IFN-γ^+ IL-2^+ TNF-α^+ T cells were significantly higher in patients with active disease, decreasing as chemotherapy progresses. The post-chemotherapy phenotypic pattern consisted mainly of $CD4^+$ IFN-γ^+ IL-2^+ and in minor proportion $CD4^+$ IFN-γ^+ T cells, which was the pattern found in latently infected patients.[53] According to Seder et al.,[53] the initial antigenic load dictates the phenotypic pattern of the lymphocytes, so it is expected that the new pattern will be closely related to the formation of memory cells. This is also in accordance with the pattern of cytokines found after chemotherapy.[54]

An interesting approach toward dormancy antigens is their association with humoral immunity. Thus, the serological detection of antibodies against Rv2031, for example, would provide potential new markers. Raja et al.[55] observed that IgA, IgG, and IgM antibodies against Rv2031c were detected in serum of culture-positive and both smear-positive and smear-negative TB patients, indicating that it could be used as a screening strategy. Interestingly, the combination of IgA, IgG and IgM, generated sensitivities and specificities of 83% and 93%, respectively, allowing the differentiation between active pulmonary disease and healthy subjects. In another study, Brazilian healthcare workers recently exposed to *M. tuberculosis*, showed higher anti-Rv2031c IgM titers than those with previous infection, or uninfected subjects, suggesting that Rv2031c would be a suitable marker for recent infection.[56] Moreover, studies from our laboratory[57] have shown a relation between successful treatment of pulmonary TB and serum IgG1 levels against Rv2031c, ESAT-6 and CFP-10 antigens. During the first three months of treatment, a significant increase in IgG1 antibody titers against Rv2031c was noticed. However, by the sixth month of treatment, IgG1 levels dropped to titers comparable to the ones elicited by the healthy controls, as indicative of successful chemotherapy. Concerning the levels of IgG1 against ESAT-6 and CFP-10, they were already high prior to chemotherapy, but as it progressed, they also dropped to control levels. Apparently, this increase in antibody levels is a consequence of the release of antigens from cytosol during the lysis of the mycobacteria, thus increasing the titers of specific antibodies after the first term of treatment.[58] Such findings are also in agreement with a recent study published by Belay et al.,[59] in which the levels of IFN-γ, TNF-α, and IL-10 in response to Rv2031c stimulation were significantly higher in patients with LTBI after the beginning of the prophylactic chemotherapy.

What should be taken into account?

One of the most important features of *M. tuberculosis* for its success as a pathogen is its high level of adaptation to the host.[60] The consensus is that the bacteria, when in a state of latent infection, stops growing and enters a stationary phase due to the granuloma walling off.[39] Eventually, when favorable conditions are re-established in the surrounding environment, the mycobacteria resumes its normal growth.[39] Despite the important role played by the granulomas during this process, the exact localization of the dormant mycobacteria is still not well known.[61] Garton et al.[61] showed that high numbers of mycobacteria sharing typical dormancy patterns can be found in the sputum of patients with active disease. Thus, what conventionally is known as latent infection LTBI or dormant state is in fact, part of a greater spectrum in which different levels of replications are found.[61,62]

As verified by Arlehamn et al.[63] in a study carried out with latently infected subjects, the same T cell memory phenotype described by Schuck et al.,[49] was also accounted for the majority of responsive T cells for both CFP-10 and Rv2031c antigens. The phenotype in question is composed by $CD4^+$ $CD45RA^-$ $CCR7^-$ effector memory T cells.[64] Moreover, it is unknown whether or not there is a certain degree of replication in latently infected patients in a way that the memory effector lymphocyte subtype is kept. However, this is the major subtype partaking in the immune response against *M. tuberculosis*.[50] Still, further studies are necessary in order to determine if this pattern of response is due to persistence of certain antigens, an undetected bacterial replication, or to longevity of the immune response even in the absence an antigenic stimulation.[63]

The different responses of peripheral blood cells and the ones at the site of infection is a matter of great importance. As stated by Chiacchio et al.,[65] there is a higher frequency of monofunctional IFN-γ-secreting effector memory T cells specific to RD1 (active phase genic locus) antigens and to the DosR-encoded antigen Rv2628 in the bronchoalveolar lavage of patients with active TB than in their peripheral blood. Taking this into consideration, the approach toward latently infected patients possesses an interesting feature. While there was a predominant Rv2628 response in the peripheral blood, there were not any differences between both RD1 and DosR antigens in the bronchoalveolar lavage. So, one can assume that the response to a DosR antigen in patients with active TB, and antigens related to active disease in latently infected patients could be an indicator of the continuum spanned by the life cycle of the mycobacteria.[61,62]

Another important point to be explored is the possibility of cross-reaction between dormancy antigens and those originated from environmental non-tuberculous mycobacteria, which are facultative intracellular bacteria that live in specific niches in the environment.[66] Apparently, 41 of the 50 antigens codified by the DosR regulon have homologues in different species of environmental mycobacteria, and in some common environmental bacteria.[67] Lin et al.[67] carried out an

experiment testing subjects infected with *Mycobacterium marinum* or *Mycobacterium kansasii* on the responsiveness of their peripheral blood cells against stimulus with DosR antigens. The results indicated that the antigens Rv1733c and Rv2627 were highly cross-reactive antigens, but despite the great level of homology of the antigens Rv2029c, Rv2626c, and Rv2031 in environmental mycobacteria, virtually no cross-reactions were observed.

Apart from the points previously discussed, two key aspects should be taken into account toward the use of these antigens as diagnostic tools: the genotype of the *M. tuberculosis* strain and the ethnicity of the population to be tested. To exemplify this topic, Riaño et al.,[68] observed that latently infected subjects from Colombia produced higher IFN-γ levels in response to the following DosR antigens: Rv1737c, Rv2029c, and Rv2628. However, the antigens Rv1733c, Rv1735c, and Rv2006 have not elicited a significant IFN-γ response. In contrast, Rv1733c, Rv1735c, and Rv2006 were ranked among the best recognized antigens in terms of IFN-γ response by latently infected populations from South Africa, Ghana, and Uganda.[69]

Concluding remarks

In this review article we briefly discussed some mechanisms by which *M. tuberculosis* is able to keep itself at a low metabolic profile, allowing it to survive in an aggressive environment as is frequently found in its host. Such mechanisms work under fine genetic control, and its products may affect bacterial structure and metabolism leading to escape the host's immune system. As previously observed, DosR regulon-encoded antigens may be used as a powerful factor to improve the current understanding about the relationship between the host immune response and the establishment of LTBI. However, the main point to be taken into consideration is that latent infection is actually part of a greater spectrum in which the life cycle of *M. tuberculosis* is inserted. Thus, this must be taken into consideration when analyzing the overall immune response against the pathogen as well as in research for new diagnostic tools for tuberculosis control.

Conflicts of interest

The authors declare no conflicts of interest.

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