An Update on the Immunology of Tuberculosis

Tanapat Palaga, Ph.D.

Department of Microbiology, Faculty of Science, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330, Thailand.

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Mycobacterium tuberculosis, a causative agent of tuberculosis (TB), is an intracellular pathogen, with infection and transmission in the human population mainly being via an aerosol route. Only a minute dose, perhaps as low as three bacilli, can result in infection, and the bacteria remain viable to drying for several weeks. Thus with such a relative ease of transmission, it is of no surprise that current estimates are that one third of the current world's human population are infected with this pathogen (Kaufmann and Parida, 2007). Interestingly, however, approximately only 10% of the infected human population will develop the primary disease, while the remaining 90% of infected individuals seem to mount a successful protective immune response and will never develop the active disease in their lifetime. This immune response may clear the pathogen or, as is probably true in most cases, may reduce and control it with the infected people remaining as asymptomatic carriers of a latent stage of infection. How much these latently infected people can act as a reservoir for the spread of existing, or of potentially greater concern, the evolution and emergence of new virulent strains, for example via lateral gene transfer or recombination and mutation, remains unknown. Thus while microevolutionary variation is the principal factor affecting the transmission dynamics and relative fitness of antibiotic-resistant strains (Gagneux, 2009), the large genetic diversity with significant phenotypic differences across its biogeographic population structure raises the possibility, for example via recombination following human migration, of new virulent strains emerging. Immunologists have known for a long time that T lymphocyte cells play an important role in controlling this disease, and co-infection of this pathogen with HIV, and the symptomatic appearance of TB infections in HIV patients once CD4 T cell depletion levels are significant, highlights the importance of these immune cells. Indeed, infection with HIV sharply increases the risk of TB reactivation and, *Mycobacterium tuberculosis* is currently the top killer of HIV-infected individuals in Africa, where TB is increasingly becoming a health threat at an alarming rate (Kaufmann and McMichael, 2005).

Although TB presents immunologists with a wide array of intriguing, yet hard, questions, the slow and technically demanding culture nature, combined with the bio-safety containment level required for handling of this pathogen, has led to many immunologists turning their research interests towards other fast turn over and less risky pathogens and diseases. However, with an eerily strong comeback of TB as a health threat of global dimensions, research into the immunology of TB is gaining momentum and many new insights of the host immune responses have emerged in recent years by taking advantage of newly developed tools and the discovery of new players in the immune system. This review will focus on the recent findings on the novel innate mechanism in combating *Mycobacterium tuberculosis* and the roles newly identified T cell subsets play in the protective host immune response (Figure 1).

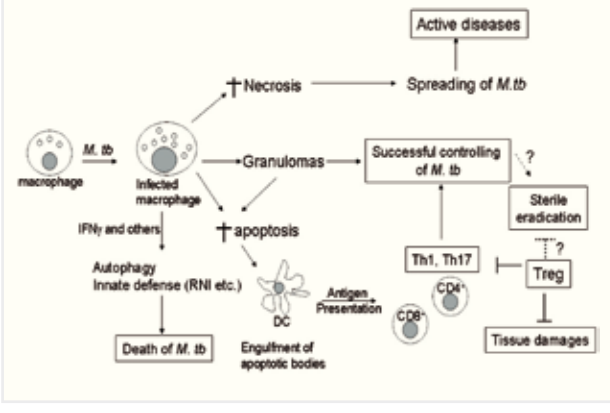

Fig 1. Overview of the immune response to infection with *Mycobacterium tuberculosis*. Infected macrophages play a central role in determining the outcome of the disease. Apoptosis of infected macrophages eliminates pathogens and moreover leads to cross-presentation of antigens via dendritic cells to T lymphocytes. Activated helper T cells (Th1 and Th17) both play a role in the protective immune response. In contrast, necrosis of (heavily) infected macrophages may result in uncontrollable bacterial spread and active disease development.

Innate Immune Responses to Infection with *Mycobacterium tuberculosis*

Upon entering the lung, the bacilli encounter the first line of host defense, such as alveolar macrophages and neutrophils. In addition, professional antigen presenting cells, dendritic cells, also need to have a way to recognize these bacteria. How these innate immune cells recognize the bacteria is one of the current intense areas of TB research. Receptors for the pathogen associated molecular pattern (PAMP) such as the toll-like receptor (TLR) protein family, are reported to play an important role for this initial recognition of the bacilli, but TLRs are certainly not the only players (Jo, 2008). Several TLRs, such as TLR-2, -1 and -6, recognize bacterial cell wall components, including lipoproteins, lipoarabinomannan and lipids (Jones et al., 2001; Underhill et al., 1999), and upon binding of the appropriate ligand(s) the TLRs signal through various intracellular mediators, which results in pro-inflammatory responses. MyD88, one of these intracellular mediators, has been linked to the recognition of *Mycobacterium tuberculosis*, but studies in the mouse model were equivocal in their support for this notion with evidence both for and against its role (Feng et al., 2003; Sugawara et al., 2003). Other PAMP receptors linked to the recognition of *Mycobacterium tuberculosis* are the mannose receptor, CD209 (DC-SIGN), the β-glucan receptor dectin-1 and the nucleotide-binding domain leucine-rich repeat containing like protein receptors or NOD-like receptors. DC-SIGN, a c-type lectin that binds to mannose type carbohydrates and is found on macrophages and dendritic cells, is an important co-receptor for HIV recognition in human dendritic cells, and also plays a role in the recognition of *Mycobacterium tuberculosis* in humans (Tailleux et al., 2003). The emerging picture from all of these studies is that not one particular receptor, but rather a combination of various receptors for PAMP, seem to play a part in recognition of the invading *M. tuberculosis* bacilli. One caveat for all these conflicting results is the fact that most studies that have reported on the innate receptors were performed either *in vitro* or in a mouse model. Validating these results with genetic studies in the human population becomes an important and required verification stage, especially given the known divergence in host-pathogen immunology between mice and humans for other pathogens. However, some of these receptors that have been characterized in the mouse models have subsequently been linked genetically to susceptibility to TB in humans, such as polymorphism in the promoters of DC-SIGN and the TLR signaling molecule TIRAP (Barreiro et al., 2006; Berrington and Hawn, 2007), which provides some confirmational support.

Macrophages: Host and Heaven for *M. tuberculosis*

Alveolar macrophages have long been known to be hosts for *Mycobacterium tuberculosis* infection and replication, where the bacilli are either eliminated by innate immune mechanisms or escape killing by macrophages and rather can by themselves induce the necrotic death of the macrophage host (Figure 1). As with most ingested microbes, the fate of ingested *M. tuberculosis* bacteria is the fusion of the phagosome with lysosome and digestion of the bacteria, along with production of antimicrobials like reactive nitrogen intermediates. However many isolates of *M. tuberculosis* block the bridging molecule early endosomal autoantigen 1 (EEA1), preventing fusion of the endosome with the phagosome, and also preventing phagosome maturation and acidification and by neutralizing reactive nitrogen intermediates (Bell, 2005). Under these conditions, the *M. tuberculosis* infected phagosome can still fuse with nutrient vesicles allowing for proliferation of the pathogen within the host macrophage. Macrophages in the lung play a central and essential role in organizing a temporally immune organ-like structure, the so-called "granulomas". Formation of granulomas with caseating centers is the hallmark feature of TB in humans while, in some contrast, granulomas without caseating centers are formed in the mouse model (Flynn and Chan, 2001). Within the granulomas, the bacteria are contained while other immune effector cells, such as neutrophils and T lymphocytes, are recruited to the granulomas. Recently, an elegant work using the new imaging technique of intravital mutiphoton microscopy, revealed live (real time) images of granuloma formation (Egen et al., 2008), which confirmed that *M. tuberculosis* infected macrophages play a central role in organizing this dynamic structure, where they become static within the mature granulomas. In contrast to the dynamic movement of T lymphocytes, infected macrophages act as an immobile cellular matrix for T lymphocytes to crawl around the structure. The results from this study seem to almost suggest that granulomas may be a make-shift peripheral immune organ which is created temporarily to contain the bacteria and present antigens *in situ*.

Besides the classical innate immune response of macrophages, such as production of pro-inflammatory cytokines and reactive oxygen and nitrogen intermediates, recent evidence suggests that cellular "autophagy" is crucial for eliminating intracellular *Mycobacterium tuberculosis* (Vergne et al., 2006). Autophagy, also known as macroautophagy, is an evolutionally well conserved cellular homeostatic event, and has been best studied genetically in yeast. Under nutrient starvation or stress, cells make an attempt to try to survive by forming cytoplasmic structures called autophagosomes and accelerate the degradation of cellular proteins or cytoplasmic organelles by fusion of the autophagosome with late endosomes or lysosomes (Levine and Deretic, 2007). Autophagy plays various other physiological roles, such as removing damaged organelles, turning over long-lived or damaged proteins and supplying nutrients for nutrient starving cells. It turns out that the immune system also adopts this autophagy tactic to combat intracellular pathogens such as bacteria, parasites and viruses. During infection with *Mycobacterium tuberculosis*, triggering autophagy in macrophages promotes maturation of *Mycobacterium* phagosomes and, as a result, a decreased survival of bacteria inside the infected cells was observed (Gutierrez et al., 2004). Furthermore, the pathway leading to degradation of the contents inside autophagosomes is also linked to antigen presentation in such a way that cytosolic antigens are transferred to late endosomal or lysosomal compartments for presentation, in contrast to exogenous antigens which enter cells via endocytosis and are processed for presentation (Levine and Deretic, 2007). Research into the role of autophagy in immune cells is a rapidly growing field with high expectations to shed

new light on cellular immunity and to develop new tools to successfully combat intracellular pathogens.

In addition to macrophages, dendritic cells also play an important part in the initial recognition of the bacilli. For example, a recent study revealed that *Mycobacterium tuberculosis* can escape into the cytosol in dendritic cells by translocating from the phagolysosome (van der Wel et al., 2007). The fate of the translocated bacilli, however, is not known, but it was suggested that this may be another way to present antigens via Major Histocompatibility Complex (MHC) to T lymphocytes (see below).

T Lymphocytes: New Players Get Involved

T lymphocytes, both helper (Th) and cytotoxic (Tc) cells have been recognized to be important for the acquired immune response against TB. Classically, helper T cells were divided into two principal types or classes, i.e. Th1 and Th2, based on the cytokine patterns and immune effector functions they mediate (Rengarajan et al., 2000). A Th1 based immune response is highlighted by production of the pro-inflammatory cytokine interferon gamma (IFNγ) and is mainly effec tive against intracellular pathogens including viruses and *Mycoplasma*, but over activation of the Th1/Th2 balance can lead to autoimmunity or even tolerance. Various lines of evidence have confirmed the central and essential roles of Th1 responses in a successful immune response against TB. Recent advances in understanding the biology of helper T cells, however, has identified two new major subsets of helper T cells, i.e. regulatory T cells (Treg), and IL-17-producing helper T cells (Th17) (Stockinger and Veldhoen, 2007). The Treg subset is mainly subdivided into naturally occurring Treg (typically defined as $CD4^+$ $foxp3^+$ $CD127^{low}$; but other equivocal subsets exist) and adaptive (or induced) Treg. Although the forkhead family transcription factor 3 (Foxp3) is by far the most well-known intracellular marker for Treg, definitive molecular markers to distinguish between the two subsets of natural and induced Treg cells are not known. Treg, as its name suggests, plays an essential role in negatively regulating an immune response, by suppressing the effector functions of various immune cells, including T lymphocytes (Sakaguchi et al., 2008). The biology concerning the development of Treg cells and the molecular mechanism of immune suppression has been extensively reviewed elsewhere (Wing et al., 2006). For infectious diseases in the murine models, Treg, on one hand, seems to play a role in minimizing collateral tissue damages during an immune response, while, on the other hand, they help pathogens establish persistent infections (Rouse and Suvas, 2007). Studies in human subjects with TB are consistent with this notion, where it was reported that the population of Treg, defined here as $CD4^+CD25^+$ $Foxp3^+$ $CD127^{low}$, were significantly expanded in asymptomatic carriers, that is *M. tuberculosis* skin test positive (PPD^+) healthy individuals, and in TB patients (Garg et al., 2008). In a mouse model, Treg proliferates and accumulates at the site of infection and prevents effective clearance of *Mycobacterium tuberculosis* (Kursar et al., 2007; Scott-Browne et al., 2007).

The relatively newly discovered Th17 helper T cell subset is characterized by the predominant production of the cytokines IL-17 (A and F), IL-21 and IL-22. The differentiation of Th17 cells, which are considered to be distinct from Th1 and Th2 T cells, is apparently mediated in mice by extrinsic signals from IL-6 in combination with TGF-β and expanded by IL-23 (Bet-telli et al., 2006; Stockinger and Veldhoen, 2007), although IL-21 may induce Th17 differentiation via a different route (Korn et al., 2007). Both IFN-γ and IL-4, the two key antagonistic Th1 and Th2 cytokines, respectively, are inhibitory to Th17 development. From the cytokine pattern of Th17, which secretes the effector cytokines IL-17 (A and F), IL-21 and IL-22 (Ouyang et al., 2008), which have been speculated to be involved in inflammatory responses, such as neutro-phil recruitment to the site of immune response and granulopoiesis. Furthermore, many studies have revealed a role in autoimmune diseases, such as multiple sclero-sis and arthritis (Ouyang et al., 2008). During infection with *Mycobacterium tuberculosis*, both Th1 and Th17 are induced, but a protective immune response cannot be mounted without Th1, suggesting that Th17 may play a minor role during primary infection (Khader and Cooper, 2008). In the vaccinated situation, however, the absence of Th17 cells decreased the production level of the chemokines, CXCL-9, -10 and -11, and as a result led to a decreased accumulation of $CD4^+$ T cells producing IFNγ in the lung (Khader et al., 2007). Therefore, Th17 seems to be essential for recall of protective responses during a later challenge in TB. In addition, gamma delta T cells, rather than Th17, are reported to be a dominant source of IL-17 during infection with *Mycobacterium tuberculosis* (Lockhart et al., 2006).

Death of Host Cells: The Good, the Bad and the Ugly

Cellular death of pathogen infected host cells during infection is considered to be one of the host innate immune responses. Intracellular pathogens, trapped inside the host cells, are eliminated together with the dying host cells. During infection of macrophages with *Mycobacterium tuberculosis*, apoptosis of macrophages has been observed with decreasing survival of the bacilli inside (Keane et al., 1997; Riendeau and Kornfeld, 2003). How infection with the bacilli leads to apoptosis of macrophages is not entirely known, but has been reported to be partly due to TNF-α (Clay et al., 2008). The importance of apoptosis of infected macrophages as a protective immune response was confirmed by the finding that the host tuberculosis resistance associated gene, *lpr1*, regulates apoptosis in macrophages (Pan et al., 2005). Some isolates of the bacilli have found a way to escape this host response as it was reported that virulent, but not avirulent, strains of *M. tuberculosis* can modulate host cell apoptosis either by inhibiting apoptosis preventing host cell death, or by switching it from apoptotic to necrotic cell death (Keane et al., 2000; Pan et al., 2005). This escape mechanism is mediated, in part at least, by the induced production of TNF-R2 and also by inducing the expression of the host anti-apoptotic gene, *Mcl1* (Balcewicz-Sablinska et al., 1998; Sly et al., 2003).

One major contribution of the apoptotic cell death of *M. tuberculosis* infected macrophages to the immune response is that it can further lead to a protective immune response via activation of MHC class I-restricted cytotoxic T cells. During apoptosis, apoptotic vesicles are formed that carry mycobacterial antigens to bystander antigen presenting cells. The uptake of these vesicles by dendritic cells leads to cross-presentation of

the antigen via MHC class I and CD1b (Schaible et al., 2003). A further elegant study has shown that apoptotic vesicles, when used as a vaccine, could protect mice from TB (Winau et al., 2006).

However, cell death of the *M. tuberculosis* infected host cells can also proceed through necrotic cell death of the host cells. In contrast to apoptotic cell death, necrosis is another form of cell death where the cellular contents may leak out of the cells during cellular disintegration because of the rupture of the cells' plasma membrane. Necrotic cell death is mainly observed in macrophages with a high burden of *Mycobacterium tuberculosis* (Lee et al., 2006). Such a balance between the host-desired apoptosis and that of pathogen avoidance by inhibition of apoptosis and or switching to host necrotic cell death is predicted by host-pathogen coevolutionary theory, with the evolution of apoptosis avoidance systems by virulent pathogen strains. Consistent with this notion then are the two observations that, firstly, for the host, as described previously, the presence of the product of the host *lpr1* gene is able to switch cell death of infected host cells from necrosis to apoptosis, resulting in a protective immune response (Pan et al., 2005). Secondly, a virulent strain of *Mycobacterium tuberculosis* has been shown to prevent host cell apoptosis but rather to induce necrosis, by inhibiting the formation of an apoptotic envelope via suppression of annexin-1 crosslinking (Gan et al., 2008). Therefore, the cell death of *M. tuberculosis* infected host cells can be a either useful (apoptosis) or harmful (necrosis) to the host, depending on the form of cell death which is induced.

Animal Models: Still Searching

Studying pathogenesis and immune responses of infectious diseases can be greatly facilitated by using animal models. For example, aside from the ethical issues of infection with pathogens in human subjects, they offer inbred (essentially genetically homogenous and controlled) and transgenic opportunities. In fact, various mouse models have been used as model systems for many infectious diseases, mainly because of their ease to handle and culture, relative fast generation times, the availability of many genetically controlled inbred lines, the ability to further make knock out or transgenic lines, the availability of an almost complete mouse genome sequence and expanding proteome data bases, and the existing extensive immunological knowledge and variety of immunological tools and databases. Similar to other diseases, mice have thus been used as a model system to study the immunology of TB for a long time, and a great deal of invaluable information has been generated. However, many aspects of the murine immune system are different to humans limiting the application of data derived from the murine model systems, which appears to include TB-host immunity. Thus, TB in mice have several major differences from human TB, aside from isolate-specific differences, such as: 1) latent tuberculosis is not observed unless manipulated by drug treatment: and 2) granulomas with a caseating center are not observed (Flynn and Chan, 2001). However, even amongst all these short comings, mice are still the best characterized model of TB. Therefore, developing new animal models is urgently needed and other animal models such as guinea pigs, rabbits and non-human primates have been used sporadically for the research of TB immunology.

Vaccine Development: an Update

Research into the host immune response against infectious diseases has one main goal, which is to develop an effective vaccine for prevention and therapy. A widely used vaccine for TB, *Mycobacterium bovis* BCG, is effective in protecting newborns and young children from severe disseminated TB, but the efficacy in preventing adult pulmonary TB is questionable (Kaufmann and Parida, 2008). With increasing antibiotic resistance and the emergence of new virulent strains added to the existing ~1.5 million human deaths annually due to TB, the development of new vaccines for TB is, therefore, increasingly urgently needed and more so than ever before. Some of the candidate vaccines which are under development are summarized in Table 1 (Kaufmann, 2006). Due to the extraordinary long period to follow the efficacy of a TB vaccine, and the relatively short lifespan of traditional host models like mice compared to humans, there is additionally and importantly the need to develop reliable diagnostic markers for monitoring and evaluating protective efficacy. To this end research into novel biomarkers that may be an indicative of a protective efficacy are under development, together with vaccine research, using relatively new techniques such as metabolomics and transcriptomics (Jacobsen et al., 2008). In addition, advances in bioinformatics including protein modeling systems may allow modeling of pathogen-host interactions and future prediction of the evolution of both pathogen escape mechanisms and drug design, which has shown some recent success with, for example, Influenza virus-human interactions.

Concluding Remarks

TB is currently re-emerging as a global health threat and the evaluation of the immunology of TB in recent years has revealed many new insights into the host response to this pathogen. The old players, such as macrophages and dendritic cells, are still the focus of immunologists, whilst the new players, such as Treg and Th17 cells, are entering the picture of the host immune response to TB in this pathogen-host coevolutionary arms race. Cellular immunology has revealed

TABLE 1. Some vaccine candidates for TB.

Type of Vaccine	Composition	Ref
Subunit Vaccine Mtb72F	Fusion protein of Rv0125 and Rv1196 in adjuvant AS02A	(Skeiky et al., 2004)
Subunit Vaccine Ag85B-ESAT6 fusion protein	Fusion protein of Ag85B and ESAT6 in adjuvant IC 31	(Olsen et al., 2004)
Recombinant viral vaccine MVA85A	Replication deficient recombinant vaccinia virus expressing Ag85A	(Hawkridge et al., 2008)
Recombinant BCG ΔureC/Hly$^+$rBCG	Recombinant BCG engineered to expressed listeriolysis O and is urease deficient	(Grode et al., 2005)

new host defense mechanisms, such as apoptosis and autophagy, in combating the attack of the bacilli, along with TB counter measures, such as the repression of apoptosis or conversion to necrotic cell death via, for example, inhibition of annexin-1 crosslinking. With the rapid advances in molecular biology and bioinformatics, as well as new tools and techniques to work with, immunologists working in the field of TB are in for an exciting and entertaining time.

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