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PERSPECTIVE

Postprimary Tuberculosis and Macrophage Necrosis: Is There a Big ConNECtion?

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ABSTRACT **Adult or postprimary tuberculosis (TB) accounts for most TB cases. Its hallmark is pulmonary cavitation, which occurs as a result of necrosis in the lung in individuals with tuberculous pneumonia. Postprimary TB has previously been known to be associated with vascular thrombosis and delayed-type hypersensitivity, but their roles in pulmonary cavitation are unclear. A necrosis-associated extracellular cluster (NEC) refers to a cluster of drug-tolerant *Mycobacterium tuberculosis* attached to lysed host materials and is proposed to contribute to granulomatous TB. Here we suggest that NECs, perhaps due to big size, produce a distinct host response leading to postprimary TB. We propose that vascular thrombosis and pneumonia arise from NEC and that these processes are promoted by inflammatory cytokines produced from cell-mediated delayed-type hypersensitivity, such as interleukin-17 and gamma interferon, eventually triggering necrosis in the lung and causing cavitation. According to this view, targeting NEC represents a necessary strategy to control adult TB.**

Tuberculosis (TB) is one of the most successful pathogens in humans. The causative agent of TB, *Mycobacterium tuberculosis*, has coexisted with humans since the earliest history of humankind (1). It continues to cause appalling morbidity and mortality rates (2). Most pulmonary TB cases and almost all transmission of the disease are due to postprimary TB, which is also known as adult or secondary TB (3). Postprimary TB is characterized by pulmonary cavitation in the upper lobes of lungs. Individuals can die from an acute pulmonary cavitation, which presents with profound coagulopathy and coughing up large necrotizing pneumonic materials, or they die from fibrocaseous TB, which is the most common form of TB and has much less coagulopathy (R. Hunter, personal communication). Fibrocaseous TB takes longer to develop and presents with extensive granuloma within pneumonia that cannot be coughed up (4). Survivors become long-term *M. tuberculosis* carriers when lung cavities are connected to airways from which *M. tuberculosis* is coughed out to air.

Postprimary TB develops mostly in immunocompetent adults who gained immunity earlier in their life from their first *M. tuberculosis* exposure and primary TB (3). Individuals who have acquired strong cell-mediated immunity to *M. tuberculosis* proteins, as detected by tuberculin (*M. tuberculosis* extract) skin test, are more likely to develop and die from cavitary disease (5). This is consistent with Koch's phenomenon, in which TB patients became severely ill or died after receiving tuberculin (6). In contrast, in young individuals, *M. tuberculosis* induces granulomas characterized by local accumulation of immune cells surrounded by epithelioid macrophages, Langerhans giant cells, and a rim of fibrous tissue without cavitation. Disseminated tuberculosis in immunosuppressed individuals is not discussed here. As cavitation is believed to be caused by necrosis of granulomas in which *M. tuberculosis* persists or replicates, most TB research has largely been focused on granuloma formation (7). However, in primates, granulomas are associated with *M. tuberculosis* killing, whereas pneumonia is associated with *M. tuberculosis* replication (8). Histology of postprimary TB in humans indicates that lung necrosis and pneumonia, but not granuloma, is associated with pulmonary cavitation (3). Also, pneumonia and lung necrosis are the leading cause of death among untreated adults with acute TB (3, 9, 10).

Hunter et al. (3, 9) and others (10, 11) suggested that vascular thrombosis and delayed-type hypersensitivity (DTH) are associated with tuberculous pneumonia in postprimary TB. Vascular thrombosis occurs when blood clots due to blood vessel injury. DTH is a T cell-mediated inflammatory response. Lando and Edgington identified DTH correlates with induction of macrophage procoagulant activity by activated T cells (12). Recent progress on understanding the mechanism of thrombosis may shed light on the underlying mechanism of procoagulant activity induction by DTH. Here we apply this knowledge to understand how vascular thrombosis is formed and the role of DTH in the context of postprimary TB. Our goal is to understand how *M. tuberculosis* induces tuberculous pneumonia and what host factors contribute to necrosis.

MACROPHAGE NECROSIS AND THE CONCEPT OF NECROSIS-ASSOCIATED EXTRACELLULAR CLUSTER

Induction of macrophage necrosis is a key *M. tuberculosis* virulence mechanism. Inhaled *M. tuberculosis* is first taken up by alveolar macrophages within which it persists or replicates. *M. tuberculosis* grows when more than 10 of these bacteria infect one macrophage (13). If the infected macrophage contains more than 25 *M. tuberculosis* bacteria, the macrophage undergoes necrosis and bursts to release *M. tuberculosis* (14). This process requires the *M. tuberculosis* ESX-1 protein secretion system (15). The killing of macrophages by *M. tuberculosis* can also occur without ESX-1 when the bacterial burden is high (16). However, such a scenario is unlikely to occur if the initial infection dose is low, since ESX-1 is required for *M. tuberculosis* to grow intracellularly (17).

Material from necrotic macrophages may be beneficial to *M.*

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tuberculosis. When cultured with lysed leukocytes, *M. tuberculosis* attaches to extracellular matrix materials and enters into a drug-tolerant persistent state (18). Orme suggested that *M. tuberculosis* in this state forms a biofilm-like structure and referred to these structures as necrosis-associated extracellular clusters (NECs) (19). A single NEC likely contains enough *M. tuberculosis* to kill macrophages upon contact, potentially because big particles make phagocytosis difficult to complete and trigger deaths in macrophages and neutrophils (20). Depending on the local environment, *M. tuberculosis* may remain as a pellicle for years or spread toward oxygen-rich areas such as blood vessels or bronchial airways. Along the way, *M. tuberculosis* can trigger necrotic lesions over time within a larger area of caseous pneumonia (4). The lesions may harden or be healed by fibrosis and calcification. Others can become soft. When this happens across a bronchus, the softened materials are coughed out through the bronchus, and a cavity is formed (4). *M. tuberculosis* can then grow a massive amount by forming a pellicle on the surface of the cavity wall, which can be coughed out for transmission (3, 11).

NEC was initially proposed in an attempt to understand granulomatous TB (19). Here we seek to determine whether the NEC model can be extended to understand postprimary TB. We are particularly interested in applying new findings in the field of thrombosis in the context of postprimary TB.

EXTRACELLULAR TRAP: CONNECTING *M. TUBERCULOSIS* INFECTION TO PNEUMONIA?

Necrotic cells release inflammatory intracellular molecules after the plasma membrane collapses. ETosis describes a necrosis in which a chromatin structure called an extracellular trap (ET) is decondensed and extruded (21). An ET is a stretch of chromosomal DNA and globular protein domains. It traps pathogens and prevents their spreading. *M. tuberculosis* induces ETosis in neutrophils and macrophages and associates with ETs (22, 23). Accordingly, ETosis could generate NECs.

The discovery of ETs provides a molecular basis of vascular thrombosis (24). An ET induces blood clotting by activating platelets and inducing fibrin and thrombus formation (25). A thrombus contains platelets aggregated within a network of fibrin and chromatin DNA. Infected macrophages in tissues normally do not encounter blood components, unless blood vessels are damaged. In a rabbit model of postprimary TB, tissue-damaging MMP-1 activity is responsible for lung damage (26). Such tissue damage may allow mixing of blood contents with *M. tuberculosis*-induced ETs and trigger thrombosis. At this point, extracellular *M. tuberculosis* can encounter neutrophils and induce necrosis of the infected neutrophils (27). Since *M. tuberculosis*-infected neutrophils undergo ETosis, this *M. tuberculosis*-neutrophil interaction may further promote thrombosis and NEC formation (23).

Thrombosis is associated with TB. Cudkowicz identified thrombosis within pulmonary artery branches near tuberculous foci in histological autopsy samples of pulmonary TB (28). Vascular thrombosis has also been observed in patients with postprimary TB by Hunter and colleagues (9). Cases of pulmonary TB patients with pulmonary thromboembolism or venous thromboembolism are noted (29–31). Patients with community-based pneumonia also have an elevated risk of thrombosis-related vascular diseases (32). Various animal models of TB also show evidence of vascular thrombosis (33).

Thrombosis can cause airway obstruction, a feature of postprimary TB also seen in a humanized mouse TB model (34–36). Hunter suggested that bronchial obstruction triggers and exacerbates tuberculous pneumonia, another feature of postprimary TB (35). Thus, vascular thrombosis arising from ET may lead to tuberculous pneumonia. Excessive ETs correlate with inflammation and severe pneumonia (37). ET has been detected in sputum samples from patients experiencing community-based pneumonia who were infected with bacterial or viral pathogens (38, 39). Thus, ETs may represent a link between TB and thrombotic conditions. Studies of active TB patients are needed to test the idea.

COMMON DETERMINANTS FOR LIPID PNEUMONIA FORMATION AND MACROPHAGE NECROSIS

Postprimary TB is characterized by the presence of tuberculous pneumonia surrounding alveolar airways, as observed in primate models of TB (8, 40). Cavitation occurs when lung cells in a patient with pneumonia undergo necrosis. The lipid-rich nature of cells in tuberculous pneumonia was first reported by the pathologist R. Virchow in 1860 (R. Hunter, personal communication). The lipid accumulation correlates with increased expression of host genes for lipid metabolism (41). Cholesterol crystals in lesions are observed in TB patients and in a humanized mouse model of TB (34).

Tuberculous pneumonia is characterized by the presence of lipid-rich foamy macrophages with few neutrophils (35). Foamy macrophages are induced by at least two *M. tuberculosis* mechanisms. One involves the interaction between *M. tuberculosis* keto-mycolic acid and human nuclear receptor 4 (42, 43). Another involves interrupting autophagy (44). Autophagy is a recycling pathway critical for lipolysis (45). *M. tuberculosis* ESX-1 inhibits host lipolysis by disrupting autophagy (46). ESAT-6 perturbs lipid homeostasis and induces foamy macrophages (47). The host factor gamma interferon (IFN-γ) can also promote foamy macrophage formation (48). Elevated intracellular lipid levels then cause macrophage necrosis (49). Since IFN-γ can promote ESX-1-mediated macrophage necrosis (22), it may act in concert with keto-mycolic acid and ESX-1 to induce macrophage necrosis by promoting lipid accumulation.

Few *M. tuberculosis* bacteria or a lack of acid-fast mycobacteria are found in lipid-rich tuberculous pneumonia (4). Acid fastness is a physical property of *M. tuberculosis*'s cell wall. *M. tuberculosis* living in a lipid-rich environment loses its acid fastness (50). The mechanism is unclear but may involve dephosphorylation of a key enzyme for mycolic acid synthesis (51). Acid-fast-negative *M. tuberculosis* is present in sputum of TB patients (19, 52). Acid-fast-negative cases account for nearly a third of the ongoing TB transmissions in China (53). Half of these TB-transmitting patients do not have cavities (53). We propose that sputum samples from these patients contain NECs that originated from tuberculous pneumonia lesions that are undergoing necrosis and have not yet matured into cavitary lesions.

TISSUE DAMAGE MEDIATED BY DELAYED-TYPE HYPERSENSITIVITY

Patients with cavitary TB tend to have a stronger reaction to tuberculin skin test. Canetti indicated that tuberculous lipid pneumonia is more frequent and more severe in hypersensitive hosts (10). This is a classic example of DTH. Sensitization with repeated high doses of heat-killed *Mycobacterium bovis* generates DTH that triggers cavitation in a postprimary rabbit model of TB (26). *M.*

tuberculosis trehalose dimycolate and ESAT-6 can induce DTH (54, 55). DTH is mediated by IFN-γ-producing $CD4^+$ T helper cell (Th1) cells. IFN-γ promotes survival of lightly infected macrophages but induces ETosis in heavily infected macrophages (14, 16, 22). This suggests that infection of lightly infected macrophages is controlled by IFN-γ-mediated DTH responses, without which the infection becomes disseminated, as seen in AIDS patients. However, the same DTH response in immunocompetent individuals may help heavily infected macrophages undergo ETosis and help *M. tuberculosis* persist in a NEC.

The classical view considers IFN-γ as the sole cytokine mediating DTH. The discovery of interleukin-17 (IL-17)-producing T (Th17) cells revises this view. Th17 cells develop after initiation of DTH mediated by Th1 cells. Excessive and prolonged Th17 responses cause tissue damage (56). Th17 cells are implicated in human chronic inflammatory lung diseases (57). All these conditions are linked to airway obstruction, which can facilitate pneumonia and cavitation. In mice, Th17 cells play a protective role during early *M. tuberculosis* infection, whereas excessive Th17 responses lead to severe immunopathology and increased *M. tuberculosis* burden (58–60). This discrepancy may be because, similar to IFN-γ, IL-17's effect depends on how heavily the macrophages are infected.

IL-17 is a cytokine that recruits neutrophils. TB patients with pulmonary cavities have higher neutrophil levels in bronchoalveolar lavage fluid samples than those without pulmonary cavities (61). Inside the pulmonary cavities, there are more neutrophils than macrophages. Also, more than half of the *M. tuberculosis* bacteria are found associated with neutrophils, compared to less than a quarter of *M. tuberculosis* bacteria associated with macrophages (62). If neutrophils encounter an *M. tuberculosis* NEC, they undergo NETosis (NET stands for neutrophil extracellular trap) and release more ETs and other extracellular material to produce a bigger NEC. NETs have been detected in pulmonary cavities and sputum samples from patients with active TB (63). This NET contains tissue-degrading MMP-8 that mediates cavitation (63).

IL-17 stimulates MMP-1 expression from fibroblasts (64, 65). Sputum MMP-1 activity from patients with TB correlates with lung pathology (66, 67). In the rabbit postprimary TB model, MMP-1 activity correlates spatially with tissue necrosis and pulmonary cavitation (26). Transgenic mice expressing human MMP-1 exhibit necrosis of lung tissue and lipid pneumonia upon *M. tuberculosis* challenge (68). Collectively, Th17 cells may promote cavitation through IL-17-induced MMP-1.

Th17 differentiation can be promoted by cholesterol crystals through NETosis and priming of macrophages for IL-1β production (69). Interestingly, diabetes is associated with elevated intracellular cholesterol and primes neutrophils to undergo NETosis (70, 71). Diabetes is a risk factor of TB and pulmonary cavitation (70, 72, 73). ETs are detected in the bronchoalveolar lavage fluid samples from diabetic mice with TB (14). Pulmonary TB patients with higher frequencies of Th1 and Th17 cells are more likely to have diabetes (74). It may be possible that *M. tuberculosis* exploits cholesterol-rich environments to promote NETosis, Th17 differentiation, and ultimately, cavitation.

More Th17 cells were observed in peripheral blood samples from TB patients than in healthy controls after *M. tuberculosis* antigen stimulation (75). However, similar results were not found in another study using peripheral blood and bronchoalveolar lavage fluid samples (76). *M. tuberculosis*-specific Th17 cells might localize in tuberculous lesions and escape detection. Alternatively, heterogeneity of *M. tuberculosis* strains from different geographic regions might generate different Th17 responses. Clinical *M. tuberculosis* isolates secrete different levels of ESAT-6, which induces Th17 differentiation (77). Finally, patients might develop TB through a mechanism independent of a DTH response, such as involving Th2 cells that contribute to TB pathogenesis by antagonizing Th1 responses.

What is the source of *M. tuberculosis* that triggers tuberculous lipid pneumonia in postprimary TB? Increasing evidence from high-burden settings indicates that exogenous reinfection contributes considerably to postprimary TB in adults (78, 79). The lymphatic system is a proposed reservoir of latent *M. tuberculosis* (80, 81) and may provide another source. Either way, deposition of *M. tuberculosis* into the upper lobes of lungs can trigger DTH pathology toward *M. tuberculosis*. Skin graft rejection due to DTH is associated with neutrophil recruitment and widespread microvascular injury and is mediated by IL-17 and IFN-γ (82, 83). Immunomodulation by mesenchymal stem cells suppresses DTH and extends skin graft survival (84). This approach shows promising results when used as an adjunct therapy to treat drug-resistant TB (85). It may be possible to treat or prevent postprimary TB by a host-directed approach that targets DTH.

CONCLUDING REMARKS

Here we present a model of tuberculous pneumonia based on the concept of NEC. Induction of macrophage ETosis by *M. tuberculosis* causes release of intracellular material such as ETs that can initiate thrombosis. The intracellular material can also interact with *M. tuberculosis* to form a biofilm-like NEC that is tough to clear by host immunity or antibiotics. The persistent nature of NEC might sustain tuberculous pneumonia. DTH responses mediated by IFN-γ and IL-17 then inflict tissue damage on the lung of an individual with pneumonia. Long-term interaction with the human immune system may have selected for *M. tuberculosis* bacteria that are efficient at forming NECs for survival and exploiting the human DTH for transmission. Accordingly, inhibition of pneumonia and macrophage ETosis caused by *M. tuberculosis* should help TB control, as has been shown recently in a mouse model of pneumonia and TB (86).

Our NEC model of TB has several implications for developing novel therapies for controlling TB. (i) Efforts need to be made to develop vaccines that prevent the formation of the biofilm caused by ET. This might be achieved by developing antibody responses that prevent the biofilm formation or target their dissolution. (ii) We need new chemotherapeutic strategies to kill *M. tuberculosis* in a biofilm with drugs that either dissolve the biofilm or kill *M. tuberculosis* bacteria that have entered into a persistent state. (iii) Mycobacteriophages may provide attractive therapeutic reagents for killing extracellular *M. tuberculosis*.

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