Host Response to Nontuberculous Mycobacterial Infections of Current Clinical Importance

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The nontuberculous mycobacteria are a large group of acid-fast bacteria that are very widely distributed in the environment. While *Mycobacterium avium* was once regarded as innocuous, its high frequency as a cause of disseminated disease in HIV-positive individuals illustrated its potential as a pathogen. Much more recently, there is growing evidence that the incidence of *M. avium* and related nontuberculous species is increasing in immunocompetent individuals. The same has been observed for *M. abscessus* infections, which are very difficult to treat; accordingly, this review focuses primarily on these two important pathogens. Like the host response to *M. tuberculosis* infections, the host response to these infections is of the TH1 type but there are some subtle and as-yet-unexplained differences.

Although nontuberculous mycobacteria have long been recognized, there is far less information regarding their pathogenicity than that of their more famous relatives *Mycobacterium tuberculosis* and *Mycobacterium leprae*. It is becoming increasingly clear, however, that the incidence of certain members of the nontuberculous mycobacterial family may be increasing, an observation not related simply to better recognition, typing, and diagnosis. Of this family, the host response to *M. avium* is perhaps the best understood, but even here there are interesting differences in the expression of immunity that have yet to be explained.

NOMENCLATURE

One cannot begin to address this issue without considering the current nomenclature, a morass in itself. We are using here the term "nontuberculous mycobacteria" (NTM) but could easily use "atypical mycobacteria," "environmental mycobacteria," the subset "rapidly growing mycobacteria" (RGM), "mycobacteria other than *M. tuberculosis*," or even the "quasi-too-complex" term that can be applied to *M. avium* and *M. abscessus*. NTM is perhaps the most widely used, but one has to realize that "tuberculous" refers to "not tuberculosis" not "no tubercles."

ECOLOGY AND EPIDEMIOLOGY

NTM were suspected as potential causes of human infections in the sanatorium era, but it was not until the 1950s that direct evidence became available. Even then, NTM were initially regarded as simple saprophytes of limited, if any, virulence occurring only in people with other predisposing lung conditions (as discussed further below). This opinion, of course, changed dramatically when *M. avium* complex (MAC) species emerged as major opportunistic infections in patients with HIV and more recently with observations of increases in infections with other NTM such as *M. abscessus* in elderly patients.

Because of extensive research, including seminal work by Falkinham, we now know that NTM are widely distributed in the environment and can cause opportunistic infections in multiple mammals, fish, and birds (particularly poultry) (1). In fact, it seems that NTM can grow essentially anywhere (2) and thrive where competing microbes are destroyed, such as in chlorinated water (3). In untreated water, NTM can even parasitize amoebae (4, 5). Even when the water supply is treated, NTM persist, and a very recent study in Holland (6) found NTM in samples of drinking water from eight separate treatment plants.

The first methods for the identification of NTM were developed in the 1950s by Ernest Runyon and were based on pigmentation and growth rates, but the development of 16S rRNA gene sequencing replaced these classical methods, allowing the identification of over 150 species of NTM. Data generated through numerical taxonomy studies that were conducted from the late 1960s through the 1970s, as well as DNA-DNA hybridization analyses, established relationships among NTM strains and provided an important bridge between the purely phenotypic Runyon classification approach and 16S rRNA gene sequencing. Moreover, taxonomic classifications based on DNA-DNA hybridizations were almost always confirmed by 16S rRNA gene sequencing and later by whole-genome sequencing. Accordingly, these applications of molecular techniques allowing genetic analysis of NTM dramatically facilitated classification and subsequent epidemiological studies (7). For example, a recent study described seven examples of identification of NTM in a plumbing system that had a DNA fingerprint identical to that of an isolate from a patient living in the same household (8). Furthermore, whole-genome sequencing of *M. abscessus* in cystic fibrosis patients indicated human-to-human transmission of this infection (9), although it should be stressed that this study has yet to be replicated.

The highly hydrophobic cell wall of NTM may facilitate aerosolization (5), and indeed, such insights began to provide an explanation as to why HIV-positive individuals were exposed to *M. avium* as a primary opportunistic pathogen. NTM can adhere to surfaces, and many NTM are resistant to both antibiotics and disinfectants such as chlorine. Many are oligotrophic (10), requiring low levels of two-carbon sources and limited access to metal ions, permitting significant survival and persistence in the envi-

ronment. (Interestingly, a similar picture may be emerging in the context of *M. tuberculosis* persisting in necrotic tissue [11, 12].)

A key ability of NTM, becoming regarded as very important, is the formation of biofilms in the environment and hypothetically *in vivo* (13). Moreover, biofilm development and formation can consist of several distinct types of structures. Survival in this state may allow these bacilli to persist for very lengthy periods of time in the environment.

CLINICAL ASPECTS

NTM infections in humans fall into three main categories (14). Hypersensitivity pneumonitis is thought to be triggered by inhalation of NTM in water droplets from sources such as shower water (aerosolized by the shower head), baths, and hot tubs (hot-tub lung) (15). This may have been the primary source of MAC infections in HIV patients in the United States (where people tend to shower more often than they bathe).

Cavitary (tuberculosis-like) disease can be caused by multiple NTM species, again predominantly MAC. There is a strong association with underlying lung disease, such as chronic obstructive pulmonary disease (COPD), and with smoking or prior tuberculosis. Like patients with tuberculosis, these patients tend to have upper lobe cavitary disease, as well as standard tuberculosis-like symptoms (2).

Nodular bronchiectasis is associated mostly with MAC and can sometimes occur in mixed infections with *M. abscessus*. It is often seen in older nonsmoking females. These ladies are often thin, hence the name "Lady Windermere syndrome." (from Oscar Wilde's famous play; the title lady is prim and proper, but at that point, the connection to a thin lady coughing seems to completely end).

Disease can also occur in the context of preexisting conditions. NTM infections occur in patients with cystic fibrosis; in such cases, 75% of the infections are caused by MAC (16, 17). NTM (most of which were *M. gordonae*) were cultured from 22% of patients showing signs of acute exacerbation of COPD (18). Nosocomial NTM infections are sometimes associated with water sources such as showers, Jacuzzis, swimming pools, saunas, and hospitals (18).

Drug therapy of NTM infections can be very difficult (for an outstanding review on this specific topic, see reference 19), with therapy long and costly (14, 20). One important factor is antibiotic inactivation, with resistance mechanisms that include beta-lactamases, aminoglycoside phosphotransferases, and aminoglycoside acetyltransferases. In addition, the p55 efflux pump confers resistance to tetracyclines and aminoglycosides, and multiple other efflux pumps likely cause antimicrobial resistance and persistence (20). Lastly, the erythromycin ribosomal methylase (*erm*) gene found in almost all *M. abscessus* subsp. *abscessus* and *M. fortuitum* strains (but not in *M. chelonae*) results in methylation of the 23S rRNA, rendering the bacteria resistant to macrolides, a mainstay of many NTM treatment regimens. Induction of this gene is not detected by conventional susceptibility testing, as this requires extended incubation and observation for up to 2 weeks.

A further issue of concern is the fact that multiple cases of *in vitro* susceptibility testing do not correlate with clinical outcomes (19). One possibility here is that drug susceptibility testing performed with single-cell suspensions in nutrient broth may not actually reflect the resistance of bacteria forming biofilms in necrotic lung tissue. Indeed, our laboratory has suggested this as a

basis for the prolonged period of chemotherapy needed in animal models of tuberculosis, particularly the guinea pig model, where bacilli persist in necrotic tissue by forming biofilm-like communities (necrosis-associated extracellular clusters) (11, 21).

MAC infections are usually treated with rifampin, ethambutol, and a macrolide such as azithromycin (the finding that such macrolides could kill MAC had a substantial impact on the treatment of MAC in AIDS patients). *M. kansasii* tends to be susceptible to standard isoniazid, rifampin, and pyrazinamide therapy, similar to *M. tuberculosis*, and the addition of clarithromycin can also be beneficial. For infections with *M. abscessus*, outcomes are much worse; in fact, this organism seems to be essentially untreatable, with only ~50% sputum conversion seen in chemotherapy studies. If lung resection is not an option, then amikacin, cefoxitin, and imipenem are tried, and in some cases, the isolate may respond to macrolide therapy, as indicated by drug susceptibility testing. As noted, *M. abscessus* has subspecies; as does *M. massiliense*, which lacks a macrolide resistance gene and hence is fully susceptible (22). In many cases, however, given the toxicity of these second-line drugs, it may be hard for the patient to tolerate therapy.

In the mid-1990s in the United States, NTM, especially MAC, caused an alarming number of serious opportunistic infections in HIV-positive individuals, particularly those with low CD4 counts (2, 23). In these patients, these infections tended to occur as a disseminated disease involving multiple organs rather than only the lungs. A major difficulty in treatment at the time arose when it was noticed that rifampin, which often works well against MAC, accelerated the clearance of the antiviral drugs being used to inhibit HIV. In addition, the latter drugs slowed the metabolism of rifampin, letting it reach toxic levels. This problem was finally overcome when the susceptibility of MAC to the new macrolides was discovered.

INCREASING INCIDENCE OF NTM INFECTIONS

There is reason to believe that the incidence of NTM infections is rising, and this may not reflect the possibility that this just represents better detection, which has significantly improved (24). Part of the increase probably reflects better diagnostic methods and increasing recognition of the importance of NTM, but on the basis of tracking records by public health laboratories that have bothered to keep them, this increase is definitely real.

Evidence shows that the incidence of NTM is increasing worldwide, with MAC the most frequent cause (14). Certain geographic areas seem to be foci, such as Taiwan and eastern Canada (25, 26). A recent report from Taiwan (27) noted increases in NTM cases, including a surge in cases of *M. abscessus* infection. Although still rare, mixed infections involving *M. kansasii*, MAC, and *M. abscessus* have been seen (28).

In a huge study (29), Hoefsloot and an army of colleagues recently provided an analysis of the global distribution of NTM infections by using data from the NTM-Network European Trials framework program. These data revealed that 91 species of NTM had been identified in over 20,000 patients from 30 countries. MAC were the most prevalent, followed by *M. gordonae* and *M. xenopi*.

There were obvious geographic differences revealed by this study. MAC is prevalent in North America but much less so in South America. Cases of *M. xenopi* infection tended to focus in Europe and to some degree in eastern Canada. *M. malmoense* tends to be found in northern Europe and the United Kingdom.

Of the RGM subgroup members, *M. abscessus* and *M. fortuitum* were found worldwide. Foci of *M. abscessus* were noted, particularly in Asia. Why these geographic distributions exist is totally unknown.

A further curiosity that has been increasingly observed is a change in human demographics. This reflects evidence indicating a gender shift from mostly males with predisposing conditions to a current 80% incidence in middle-aged to elderly females (30, 31). A similar picture is emerging in Australia (32), where increases in cases of NTM (*M. intracellulare* being the most prevalent) have shifted from middle-aged smoking men to older nonsmoking females. Further examination of this issue seemed to suggest that a greater susceptibility was associated with the body characteristics of these women; they tended to be taller and rather thin, suggesting a genetic trait, and indeed, a third were shown to express the cystic fibrosis-associated transmembrane conductance regulator gene *CFTR* (33). Leptin levels in these females may also contribute (34).

HOST IMMUNE RESPONSE TO NTM

From an immunological point of view, there was little interest in NTM until Stanford and his colleague Rook suggested in 1981 (65) that these bacteria could potentially influence the efficacy of *M. bovis* BCG vaccine, resulting in reduced vaccine-derived protection. Soon after, a study appeared (35) showing that NTM behaves differently in terms of growth in activated murine macrophages; while *M. tuberculosis* was inhibited, *M. kansasii* and *M. avium* were slowed but not killed and *M. intracellulare* seemed unaffected. If mice were infected with these bacilli, the animals were protected against a second infection with *M. tuberculosis* (36).

BCG vaccination was then shown to be protective against infection with *M. kansasii* or *M. avium* but was not effective against *M. simiae* or *M. intracellulare* (37). Studies then showed that protection was mediated by T cells, as shown by passive cell transfer (36).

At the time, resistance to mycobacterial infections was thought to be associated with a gene, *Bcg* (now *NRAMP1*). NTM did, indeed, grow differently in *Bcg*^{s} and *Bcg*^{r} strains, but backcross and mouse chimera studies showed this event to be multigenic (38).

The stomach was traditionally thought to be a barrier to mycobacteria, but it was shown that virulent *M. avium* strains could infect mice orally and be found in gut lymphoid tissues. If the beige mutant in the C57 mouse background was used, this was amplified (39, 40). Subsequent studies showed that *M. avium* crossed the gut epithelium via interactions with enterocytes (41). Once *M. avium* was in, a CD4 response was important for host immunity whereas a local CD8 or NK cell response was not.

In addition, as shown earlier, these strains of *M. avium* were slowed but not killed when cultured in activated macrophages. At this time, the role of TH1 cytokines began to be investigated, and an early study suggested that tumor necrosis factor alpha (TNF-α) was more effective than gamma interferon (IFN-γ) in growth inhibition (42). Soon after, it was demonstrated that a virulent colony morphotype smooth transparent (SmT) isolate induced a delayed, relatively small TNF-α response, whereas the smooth domed (SmD) equivalent induced a rapid, large TNF-α response (43). It was then found that while macrophage activation generated reactive oxygen species in response to *M. avium*, isolates expressing the virulent SmT form were unaffected (44). Further

studies showed that, in general, SmT isolates were virulent and SmD isolates were much less so, with rough variants showing an intermediate phenotype (45). As predicted, there were large differences between the inflammatory and protective cytokine/chemokine profiles of SmT and Smd/SmO infections (46, 47).

Nitric oxide (NO) was also produced by activated macrophages, but blocking of this event did not influence growth inhibition, and subsequent studies raised the possibility that acidification of the bacterial phagosome was, in fact, more important in the control of the infection (46). *In vivo*, CD4 involvement was crucial for both protection and granuloma formation, and it was suggested at the time that the TH1 cytokines IFN-γ and TNF-α produced by these cells act in synergy (47). Consistent with the TH1 pathway hypothesis, depletion of interleukin-12 (IL-12) also reduced resistance to *M. avium* infection (48). In addition to CD4 cells, IL-12 was also implicated in TNF-α production by NK cells in *M. avium* infections (49). An early role for IL-6 was suggested, as was a late response involving IL-10 (50). More recently, a role for TNF-α has been illustrated by infections occurring in patients treated with anti-TNF-α biologic reagents for rheumatoid arthritis (51, 52). *M. haemophilum* or *M. avium* was seen in 52 cases, with a high rate of extrapulmonary disease.

Consistent with earlier studies, T cells harvested from mice infected with *M. avium* transferred protection but only if live bacteria were used (53). This was, of course, consistent with observations at the time that only live *M. tuberculosis* was capable of generating protective T cells (54). While CD4 T cells were the primary source of protection, some studies suggested a contribution by CD8 cells (55), although the latter were eventually shown not to be critical.

Further investigation of the beige-mouse model indicated a neutrophil defect, and depletion studies with wild-type C57 mice produced a similar effect (56). This was then shown to reflect poor cellular accumulation due to a diminished chemokine response that was subsequently shown to be directed via CXCR2 expression (57).

A 1999 study (58) provided the unexpected result that mice that lack the *NOS2* gene and cannot make NO within their macrophages were more resistant than wild-type controls to *M. avium* (the opposite of the result obtained with *M. tuberculosis*), at least partially explaining why virulent *M. avium* strains grow in mice despite very strong TH1 responses (59), and subsequent studies also demonstrated a superior fibrotic response in the lungs of gene knockout mice and showed that the increased resistance was consistent when multiple virulent *M. avium* strains were used (60). A possible explanation was the observation that along with TNF-α, NO controlled granuloma integrity rather than being directly antimicrobial (61). TNF-α seemed critical, since in TNF receptor-deficient mice, *M. avium* generated severe, fatal necrosis (62), although a later paper suggested otherwise (63).

Dormant for more than a decade, the idea that NTM could interfere with BCG vaccination itself reactivated. Studies by Brandt et al. showed that if mice were immunized with various NTM prior to BCG vaccination, the vaccine could not proliferate to any extent and efficacy was subsequently reduced (64). An initial explanation (65), put forward by Rook et al., was that the mixture of infections unbalanced immunity and did not allow activity by regulatory T cells. This is a rather elegant idea, and recent unpublished studies in our laboratory indicate that exposure in the gut to *M. avium* induces regulatory T cells that then
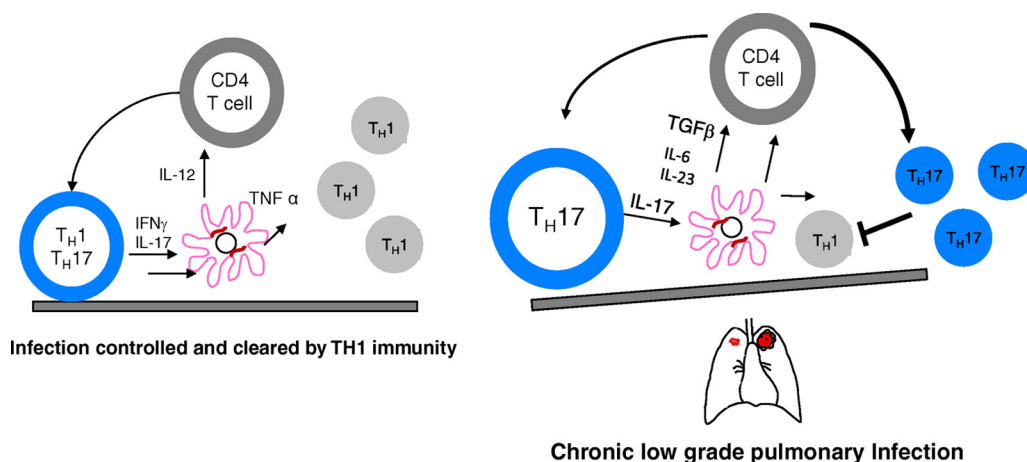
FIG 1 Hypothetical model explaining the persistence of *M. abscessus* and establishment of chronic disease. Mouse models clearly indicate that TH1 immunity predominates in situations where this pathogen can be cleared, but there is little evidence as yet explaining its survival and chronic disease. One possibility involves T cell plasticity in which a TH17 response becomes dominant, resulting in loss of a protective TH1 response and its replacement by cells that can continually drive low-grade inflammation.

counterbalance BCG-induced effector cells in the lungs after a challenge. A subsequent review by Andersen and Doherty nicely put all of this in perspective (66) by explaining that NTM can, in some cases, block effector immunity, diminishing protection by BCG, or can add to it. It is plausible that live NTM generating T cell immunity cross-reactive to *M. tuberculosis* antigens can mask the effects of BCG by itself. This idea was more recently confirmed by studies showing that people exposed to NTM have IFN-γ⁺ T cells that recognize multiple proteins from the DosR gene complex (67).

In 1999, a paper appeared that indicated that when *M. avium* grew in macrophages, some bacilli grew well but a second static population was also present (68). One explanation that appeared 4 years later was the observation that *M. avium* can form biofilms (69). It was then found that the capacity to form these biofilms seemed to involve glycopeptidolipid (GPL) expression by the bacillus and that mutants unable to do so were far less invasive. Our recent unpublished studies showed that *M. tuberculosis* mutants that cannot form biofilms also cannot form pellicles *in vitro* and fail to persist in animals *in vivo*. A very recent paper (70) associated disruption of the *pks12* gene in *M. avium* with loss of biofilm formation (preliminary [unpublished] data suggest the same for *M. tuberculosis*).

TH1 immunity can be triggered through Toll-like receptors (TLRs), and it was found that mice lacking TLR2 were more susceptible to *M. avium* infection (71, 72). Signaling via TLR2 operates via the mitogen-activated protein kinase pathway in a TNF- and TRAIL-independent manner (72). TLR9 signaling may also play a small role. Recent evidence (73) suggests that NTM initially trigger immunity via the AIM2-inflammasome whereas *M. tuberculosis* does so via NLRP3.

Chronic infection of mice not only can result in severe necrosis but also (unlike tuberculosis) can result in a gradual loss of T cells (lymphopenia) (74). Such observations illustrate that the pathogenesis of these diseases is still less well understood (75), with elements of the disease process in response to certain NTM infections showing clear differences (76), lymphopenia being one such example.

Although multiple virulence factors associated with the NTM have been proposed, this picture is still not fully clear. At one point, possession of a virulence plasmid was suggested but never verified. The biological activity of various cell wall GPLs (77) has also been suggested as a factor, but this also has not been verified.

IMMUNITY TO *M. ABSCESSUS,* AN EMERGING PATHOGEN

Although identified as a member of the 150-plus NTM organisms, *M. abscessus* was barely noticed until it started to become evident over the past decade or to be clinically important. Even now, however, we know little about the host response to it (34, 78).

Compared to *M. avium* (*M. abscessus* has the reverse phenotype), rough strains of *M. abscessus* tend to be virulent while smooth strains are less so (79). Like *M. avium*, the organism expresses GPL, but rough mutants have been found that do not (80). Although much less virulent than *M. avium*, *M. abscessus* can persist *in vivo* in recently developed animal models such as SCID, nude, and granulocyte-macrophage colony-stimulating factor gene disruption mice and cystic fibrosis models causing a progressive pulmonary infection (unpublished results). Whereas wild-type isolates generate strong TH1 responses and are easily cleared in animal models (81), GPL-defective mutants can cause rapid death (82). Given the very rapid death, within a week, this cannot be explained in terms of bacterial growth and instead suggests some sort of shock reaction (no autopsy data were provided in that report). In addition, death resulting from infection with a rough variant has been reported (83).

Induction of host immunity did not initially appear to be associated with TLR2 signaling, since both rough and smooth variants triggered a TLR2-mediated response equally (72). However, a further study described a GPL⁺ strain that could form a biofilm but could not trigger a TLR2-mediated response, whereas a rough variant of this was GPL⁻ and could not form a biofilm but could trigger a TLR2-mediated response (84). This led to the hypothesis that GPL on the outer cell wall of *M. abscessus* allows the bacillus to initially avoid inducing immunity by avoiding TLR2 triggering, but when it is not present, this unmasks other materials, possibly phosphatidylinositol mannosides or lipoarabinomannans, that

are known to be TLR2 agonists. More recently, disruption of the *mmpL4b* gene, which is involved in GPL biosynthesis, resulted in bacilli that could trigger TLR2 in macrophage cultures (85, 86); these mutants lack GPL but also seem to upregulate the production of cell wall lipoproteins (87). What has yet to be explained, however, is why mechanisms other than TLR2 signaling do not compensate and prevent GPL⁻ *M. abscessus* from so quickly killing mice.

Other recent studies using the aerosol route of infection provided similar results, with evidence of strong TH1 responses and granuloma formation in both the mouse and guinea pig models (78); however, it was noted that relatively high doses were needed to establish any degree of infection by aerosol. Under these experimental conditions, this seems to drive a TH1 response (predominantly IFN-γ-secreting CD4 T cells) that seems capable of controlling this infection in immunocompetent mice and clearing it. How this can progress to a chronic, uncontained infection is far from clear, but it might involve mechanisms similar to those seen in *M. tuberculosis* infections in which T cell plasticity shifts to a less favorable TH17 type of response (as illustrated in Fig. 1). There is little evidence to go on as yet.

KNOWLEDGE GAPS AND FUTURE DIRECTIONS

The current epidemiological data seem to indicate that the incidence of the pathogens *M. avium* and *M. abscessus* is increasing, and this information needs to be regarded with concern. The host response to these pathogens involves the TH1 response, and while this is unsurprising, there are still large gaps in our knowledge. First, there are facets of the host response, particularly to MAC, that remain enigmatic. These include the loss of reactive T cells and the apparent resistance of MAC to reactive nitrogen. Second, with regard to *M. abscessus*, animal models are currently very limited and host immunity and pathogenesis are difficult to measure unless very large doses of bacilli are given intravenously. If small doses are given, there is little evidence that a productive infection is even fully established. As a result, better models are needed, particularly in terms of elucidation of pathogenesis, enabling better new drug regimens for *M. abscessus* infections, where current therapeutic outcomes are very poor.

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