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REVIEW

Mycobacterium tuberculosis Biofilms: Immune Responses, Role in TB Pathology, and Potential Treatment

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Abstract: Tuberculosis (TB) is a major public health problem worldwide, and the burden of drug-resistant TB is rapidly increasing. Although there are literatures about the *Mtb* biofilms, their impact on immune responses has not yet been summarized. This review article provides recent knowledge on *Mycobacterium tuberculosis* (*Mtb*) biofilm-immunity interactions, their importance in pulmonary TB pathology, and immune-based therapy targeting *Mtb* biofilms. Pellicle/biofilm formation in *Mtb* contributes to drug resistance, persistence, chronicity, surface attachment, transfer of resistance genes, and modulation of the immune response, including reduced complement activation, changes in the expression of antigenic proteins, enhanced activation of T-lymphocytes, elevated local IFNγ+ T cells, and strong antibody production. The combination of anti-TB drugs and anti-biofilm agents has recently become an effective strategy to improve TB treatment. Additionally, immune-targeted therapy and biofilm-based vaccines are crucial for TB prevention.

Keywords: *Mycobacterium tuberculosis*, immune response, biofilm, treatment

Background

Mycobacterium tuberculosis (*Mtb*), an intracellular pathogen that causes tuberculosis (TB), continues to be a global public health concern.[1] According to the latest report from the World Health Organization (WHO), approximately 10.6 million people received a TB diagnosis in 2021, an increase of 4.5% from 2020, and 1.6 million people died of the disease (including 187,000 HIV-positive individuals).[2] The highest percentage of TB cases was found in the WHO regions of Southeast Asia (44%), Africa (25%), and the Western Pacific (18%).[3] Between 2020 and 2021, the burden of drug-resistant TB increased by 3%, with 450,000 new cases of rifampicin-resistant TB in 2021.[2,4] The mechanisms of *Mtb* survival under antibiotic therapy include the acquisition of gene mutations conferring drug resistance, cell wall structure alteration, production of certain efflux pumps, and biofilm formation.[5]

Costerton et al first described the modern concept of biofilm in 1978.[6] Today, as reviewed by Flores-Valdez et al, research on Mtb biofilms/pellicles is receiving more attention, which is engaged in antibiotic resistance, persistence, chronicity, surface attachment, and transfer of resistance genes.[7,8] However, the immunological effects of *Mtb* biofilms have not yet been thoroughly investigated. Studying about biofilm-immunity interactions is crucial to improve TB treatment, modify host immunity, and applying new vaccine strategies. In this review article, *Mtb* pellicle/biofilm formation, their interaction with host immunity, and their role in TB pathology are described. Moreover, indications for the application of immune-based therapies and biofilm-based vaccines targeting *Mtb* biofilms to enhance host defense mechanisms against TB infection are discussed.

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Literature Search Method

This narrative review of recent literature was performed using Google Scholar and PubMed databases on available articles published without publication year restrictions. The following keywords were used: ("immune response" OR "immunity" OR "innate immunity" OR "adaptive immunity" OR "antibody" OR "vaccine") AND ("tuberculosis" OR "TB" OR "Mycobacterium tuberculosis" OR "M. tuberculosis" OR "Mtb" OR "pulmonary tuberculosis") AND ("biofilm" OR "pellicle"). Although the search and study selection were not conducted systematically, the articles were restricted to the English language and full text, and the references of the included literature were also assessed. Search results were precisely summarized using the following headings: "biofilm formation in Mtb", "how Mtb biofilms involve and affect the lung phenomenon?", "Mtb biofilms and the immune system", "current solutions for Mtb biofilms", and "application of biofilm-based vaccines: future perspectives".

Biofilm Formation in Mtb

Recently, research on mycobacterial biofilms revealed that *M. tuberculosis* can form multicellular biofilms composed of bacteria and the extracellular polymeric substances (EPSs) they produce, including proteins, DNA, and polysaccharides.[9,10] Mycobacteria can aggregate on surfaces (biofilms), and *Mtb* typically grows in the liquid–air interface (pellicles), which is related to the distinctive features of the mycobacterial cell wall, including the high lipid content that enables bacteria to live in unfavorable environments.[11] Beginning with bacterial adhesion, *Mtb* biofilms develop in a series of stages including surface attachment, sessile growth, matrix production, and dispersal.[12] It is regulated by molecules including polysaccharides, structural proteins, glycopeptidolipids, GroEL1 chaperones, shorter-chain mycolic acids, genetic material, and environmental conditions (nutrients, ions, and carbon sources).[9,11]

The presence of biofilms lowers the susceptibility of *Mtb* to drugs, which is supported by a study demonstrating that the pellicle-defective *Mtb*1mma A4 mutant strain is more susceptible to rifampicin in vitro.[13] Leukocyte extracts favor *Mtb* biofilm production and drug tolerance in vitro.[14] Intracellular thiol reductive stress induces Mtb biofilm development in vitro, which includes drug-tolerant but metabolically active bacteria.[15] Moreover, mutation analysis revealed that isonitrile lipopeptide (INLP) is essential for the architectural formation of *Mtb* biofilms, providing insight into the resilience of biofilms to antibiotic exposure and identifying INLP as a possible biomarker.[16] Polyketide synthase (Pks1) gene and *Mtb* protein tig (Rv2462c) are involved in *Mtb* biofilm formation.[17,18] The *Mtb* disease reactivation and inhibition of antimicrobial treatment attributed to the smoking-induced enhancement of biofilm formation.[19] Additionally, the Protein O-mannosyltransferase Rv1002c decreases cell permeability and promotes biofilm formation.[20]

How Do Mtb Biofilms Involve and Affect the Lung Phenomenon?

Pulmonary infection with *Mtb* causes granulomatous lesions that develop in lung tissue. Inhalation of *Mtb* droplets leads to the formation of granuloma in the alveolar macrophages at 4 weeks post-infection (termed original granulomas) and then becomes mature. A granuloma is a dense immunological structure primarily composed of macrophages at the center that can differentiate into other cells such as foamy macrophages and multinucleated giant cells with lipid droplets. The periphery of the granuloma is comprised of T and B lymphocytes. The *Mtb* bacilli can form a biofilm structure at the periphery of the TB granuloma near T and B lymphocytes, which is composed of an extracellular matrix. Those *Mtb* biofilms interferes with anti-TB drugs (Isoniazid and Rifampicin), affect host defense mechanisms by hindering the entrance of immune cells, and they are a source of TB persistent cells.[21]

The growth of *Mtb* pellicles/biofilms in lung cavities could be clinically essential in the pathogenesis of TB contributing to caseous necrosis and cavity formation in lung tissue, *Mtb* persistence in the infected host, and expansion of drug tolerance.[22] During the transmission of *Mtb*, expectorated aerosols can harbor single cells, which may be shed from pellicle-like biofilms growing in the *Mtb* cavities.[23,24] These mycobacteria can persist for a long time, possibly within pellicles/biofilms, and hidden from immune cells. It has been hypothesized that the extracellular *Mtb* microcolonies seen in animal models are biofilms that develop in vivo.[25,26] Some sources suggest that pellicles may be present in the lung–air interface when humans develop secondary tuberculosis.[22] Furthermore, a study found that the ability to form biofilms is common among *Mtb* isolates, suggesting that this trait is important for TB propagation or persistence.[17]

Mtb Biofilms and the Immune System

Innate Immune Responses

Innate immunity, the non-specific defense, fights infections from the moment of first contact without previous exposure.[27] The earliest innate immune cells involved in lung infection primarily include macrophages (MΦs), dendritic cells, monocytes, and neutrophils, which readily phagocytose and degrade *Mtb*.[28] Although there is a higher clinical significance of biofilms, studies at large have focused on the immune response to microorganisms in the planktonic state than on pathogenic biofilms.[29] Therefore, it is imperative to comprehend the interplay between *Mtb* biofilms and defense mechanisms to identify novel targets and approaches for immune intervention against biofilm-associated pulmonary complications.

MΦ, neutrophils, dendritic cells, natural killer cells, mast cells, and complement are the main players of innate immunity and airway epithelial cells also participate in the defense effort against *Mtb* and could be considered components of innate immunity.[30] Recently, shreds of evidence on biofilm-immunity interactions among different pathogenic bacteria have been developed in vitro and in vivo experimental models. Kaya et al established an in vitro host cell-biofilm interaction model and demonstrated that not only *Pseudomonas aeruginosa* biofilms induced higher activation and response of human peripheral blood mononuclear cell response (PBMC), but also PBMC or their supernatants significantly increased biofilm-associated *P. aeruginosa*, indicating a complex reciprocal relationship between host blood cells and the bacterium.[31] Another study by Gries et al using a novel murine model of *Staphylococcus aureus* implant-associated infection demonstrated that *S. aureus* biofilms inhibit neutrophil chemotaxis, redirecting their migratory patterns to prevent biofilm invasion.[32] In vitro, *b*iofilms aid *Mycobacterium avium* complex in epithelial cell invasion, protect from phagocytosis, and cause premature apoptosis in macrophages.[33]

Similarly, *Mtb* pellicles/biofilms also had an interaction with host immunity.[8] The development of biofilm made *Mtb* more resilient to host immunity and increased the difficulty of its treatment and cure.[34] Small molecules that bacteria release when they change from planktonic to biofilm-associated might exacerbate inflammation, cause cell death, or even result in necrosis.[35] Biofilms minimize the activity of both polymorphonuclear neutrophils and macrophages. Additionally, in the presence of these cells, biofilm formation is actively enhanced, and components of the host immune cells are assimilated into the EPS matrix.[35] The TB bacilli in the lung cavities are also separated from the host's immune defense by the cavity wall that keeps the penetration of viable cells.[36]

The role of the complement cascade in infection and Mtb disease progression is largely unknown, but C5 and C7 components likely play a protective role, and high expression of C1q correlates with worsening clinical status and is associated with latent TB and active TB, but its significance remains uncertain in terms of pathogenesis.[37] A study highlighted the role of carbohydrate alterations during the biofilm growth of *Mtb* and subsequent modulation of the innate immune response through avoiding of phagocytosis due to a reduced complement activation with lower C3b/iC3b deposition.[38]

Adaptive Immune Responses

The adaptive immune response, also known as specific resistance, recognizes identical or similar pathogens through memory cells.[39] In planktonic bacteria, the activation of adaptive immunity often results in the clearance of the infection, due to the combined activity of the innate and adaptive immune reactions. But in the case of biofilm infections, it is very difficult to clear the pathogen.[40] Dendritic cells are essential in linking the innate and adaptive immune systems and have the exclusive capacity to prime naïve T cells into subsequent Th1, Th2, or Th17 cells and responses.[41]

The protective immunity against *Mtb* has been suggested to be associated with polyfunctional T cells. Notably, the quantity of T cells specific to *Mtb* that generate a mix of IFN-gamma, IL-2, and/or TNF-alpha has been observed to be associated with the mycobacterial load; additionally, other research has connected the existence of this particular functional profile as a sign of TB disease activity.[42] It is generally accepted that CD8+ T cells contribute to immunity and protection, even if their exact role in tuberculosis (TB) is less understood than that of CD4+ T cells.[43] The CD4+ T cell helps promote CD8+ T cell effector functions and prevents exhaustion, and the helped CD8+ T cells restrict

intracellular mycobacterial growth. The synergy between CD4+ and CD8+ T cells promotes *Mtb* survival during murine tuberculosis.[44]

A study indicated that nonclassical CD8+ T cells other than the known M3, CD1, and MR1-restricted CD8 +T cells contribute to host immune responses against *Mtb* infection.[45] Chávez-Galán et al reported that TB patients had a high frequency of CD8+ cells in peripheral blood.[46] The relative and absolute number of the effector memory type 1 CD3 +CD8+ cells increased in the peripheral blood of patients with pulmonary tuberculosis compared to the control group.[47] Immunization with a BCG1416c mutant raises IFN-gamma+ in CD4+ and CD8+ lymphocytes.[48] In comparison to the BCG wild type, the BCG1419c and BCG1416c strains grown as surface pellicles, which is the condition used to manufacture the BCG vaccine, both altered the expression of antigenic proteins like DnaK, HbhA, PstS2, 35KDa antigen, GroEL2, as well as AcpM, a protein involved in the synthesis of mycolic acids, molecules relevant to modulating inflammatory responses.[48]

Although the exact role of humoral adaptive immunity in tuberculosis remains unknown, new research indicates that humoral immunity and B cells may be able to control the immune response to a variety of intracellular infections, including *Mtb*.[49] According to a study, mice exposed intradermally to an exoproteome extract of an exopolysaccharide-dependent *S. aureus* biofilm developed a humoral immune response and produced IL-10 and IL-17.[50] Moreover, vaccination of BALB/c mice with immune structural proteins extracted from *Mtb* biofilm elicited a strong humoral immune response with high IgG1 and IgG2a titers and showed a higher Th1 response relative to a control group that had more Th2/Treg responses[51] (Table 1).

Current Solutions for Mtb Biofilms

The microbial biofilm serves as the best model for analyzing the effectiveness of antibacterial therapies. Scientists have shown the effects of different agents against many *Mycobacterium species,* but in this review, we have focused on recent targets against *Mtb* biofilms (Table 2). As reviewed by Oluyori et al, the administration of anti-TB drugs along with anti-biofilm agents such as bioactive natural products and synthetic analogs has been a recent effective strategy to improve TB treatment.[55] In a study of the nanobiotechnology approach, the biofilm formation of *Mtb* is inhibited by titanium dioxide (TiO2) nanoparticles, and an increase in TiO2 nanoparticle concentration was found to cause a three- to four-fold

Table 1 The Relationship Between Mtb Pellicle/Biofilm and Immune Response

Author, Year	Experimental Model	Finding	Ref
Mishra et al, 2023	C3HeB/FeJ mice	Biofilm-like intracellular *Mtb* cords compress host cell nuclei, suppress immune signaling, and reduce tissue inflammation.	[52]
Keating et al, 2021	In vitro assay	Alterations in *Mtb* biofilm cell wall carbohydrates reduced complement activation with lower C3b/iC3b deposition.	[38]
Segura- Cerda et al, 2018	BALB/c mice	In comparison to the BCG Pasteur vaccinated group, the CD4+ T and CD8+ T lymphocytes recovered from the BCGDBCG1416c vaccinated group displayed a larger proportion of IFN-c+ T cells in response to the purified protein derivative.	[48]
Pedroza-Roldán et al, 2016	BALB/c mice	Mice vaccinated with the BCGΔBCG1419c strain, which generates more pellicles in vitro, showed reduced bacterial burden in the lungs of the mice, improved activation of some T lymphocytes, and higher local IFNγ+ T cells.	[53]
Kerns et al, 2014a	Guinea pig	Guinea pigs infected with *Mtb* produce host humoral response against specific proteins present in vitro-grown biofilms.	[54]
Kerns, 2014b	BALB/c mice	Immunogenic proteins extracted from a biofilm for the formulation of a sub-unit vaccine elicited a strong IFN-γ response and humoral immune response with high IgG1 and IgG2a titers against *Mtb.*	[51]

Table 2 Key Studies Reported Anti-Biofilm Inhibitors of Mtb

Author, Year	Finding	Ref
Kalera et al, 2024	Azidodeoxy and aminodeoxy -D- trehalose analogs inhibit the trehalose catalytic shift, which is required for *Mtb* persister formation in the biofilm model.	[57]
Kumar et al, 2022	D-cycloserine and its metabolite hydroxylamine have a potent anti-*Mtb* biofilm activity.	[59]
Mashele et al, 2022	All anti-TB medication combinations containing Clofazimine demonstrated synergistic inhibitory and bactericidal effects in biofilm-forming cultures, especially when combined with Rifampicin and Isoniazid.	[60]
Bekier et al, 2021	Derivatives of imidazole-thiosemicarbazide can enter human macrophages infected with Mtb, profoundly inhibiting the intracellular proliferation of tubercle bacilli and suppressing the production of *Mtb* biofilms.	[34]
Jiang et al, 2019	Compound 1, Arisaema sinii's active ingredient, can destroy mature biofilms, disperse preformed biofilms, and inhibit the production of new biofilms in a dose-dependent manner.	[61]
Kumar et al, 2019	Cyclosporine-A, acarbose, or GaNP suppressed *Mtb* H_{37}Rv biofilm formation.	[62]
Wang et al, 2019	Recombinant CwlM treatment decreased the development of M. smegmatis and *Mtb*'s biofilms while increasing their autolytic potential.	[63]
Ackart et al, 2014b	2-aminoimidazole derivatives inhibit in vitro-grown *Mtb* biofilms.	[64]
Dalton et al, 2014	Ascorbic acid (vitamin C) can suppress and kill *Mtb* biofilms.	[65]
Wang et al, 2013	The bacillary load in the lungs of infected BALB/c mice is decreased by TCA1, a tiny chemical that suppresses Mtb biofilms in vitro.	[66]

decrease in mycobacteria metabolic activity.[56] The inhibition of the trehalose catalytic shift, which is required for *Mtb* persister formation in the biofilm model, is a viable strategy to target persisters using trehalose analogs.[57] A gene expression profile analysis showed inhibition of the Rv1217c and Rv1218c gene expression repressed *Mtb* biofilm formation.[58]

Application of Biofilm-Based Vaccines: Future Perspectives

Nowadays, several vaccine candidates for the prevention of TB have been identified. However, no effective vaccine is available to prevent infection with drug-resistant *Mtb* strain.[67] Despite the significance of biofilms in disease, vaccines are typically prepared from bacteria grown as planktonic cells in the laboratory. Vaccines based on planktonic bacteria may not be enough to protect biofilm-associated infections.[68] Several studies demonstrated new strategies against *Mycobacteria* biofilms to enhance patient outcomes and treatment effectiveness. This narrative review gives insight into preparing vaccines from biofilm sources in addition to planktonic cells. Finding viable vaccines and immunological therapies to end TB requires examining the immune response to *Mtb* biofilms.[69]

By applying immunology to compare the cellular and humoral immune responses mounted by immunized subjects/models in vivo and/or ex vivo toward particular components relevant to biofilm production, novel vaccine candidates may arise from specific analyses around the biofilm mode of growth.[70] A five-sub-unit biofilm vaccine formulation elicited a strong immune humoral response and reduced lung burden in animals vaccinated with the purified antigen subunits and DDA/MPL adjuvant.[51] Further confirmation of those vaccines is needed for their application in vivo. Vaccines prepared from *Mtb* biofilms will be more efficacious for protecting in vivo biofilm growth during the pathogenesis of TB and more effective against drug-resistant strains, and different stages of the clinical disease such as the chronic state.

Conclusion

Mycobacteria can aggregate on surfaces as biofilms and they tend to develop as pellicles at the liquid–air interface. According to the reports, biofilms can stimulate a unique immune response than planktonic cells. The *Mtb* pellicles/

biofilms alter both innate and adaptive immune responses such as reduced complement activation with lower C3b/iC3b deposition, change in the expression of antigenic proteins, a better activation of specific T lymphocytes, increased local IFN-gamma^{+} T cells, and strong antibody production. A combination of anti-TB drugs with anti-biofilm agents has been a recent effective strategy to improve TB treatment. Additionally, immune-targeted therapy and biofilm-based vaccines should be considered as a new candidate to prevent TB.

Abbreviations

EPSs, Extracellular polymeric substances; INLP, Isonitrile Lipopeptide; *Mtb, Mycobacterium tuberculosis*; MΦs, Macrophages; PBMC, Peripheral blood mononuclear cell; TB, Tuberculosis, WHO, World Health Organization.

Data Sharing Statement

All the necessary data are freely assessed online in the referenced literature and provided upon a reasonable request to the corresponding author.

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Biofilm-immunity interaction is a recent field of interest, which requires further research and the authors thank any scientific authors whose research was not cited in this review.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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