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Biofilms in tuberculosis: what have we learnt in the past decade and what is still unexplored?

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Abstract

Elucidating how *Mycobacterium tuberculosis* produces biofilms, and its impact for tuberculosis (TB) pathogenesis is gaining momentum. Here, we discuss recent findings reported over the last decade, which help us gain insights into the association between biofilm formation and TB pathogenesis. A new appreciation of extracellular TB phenotypes found in lung lesions will drive drug and vaccine discovery forward to new possibilities.
Biofilms and tuberculosis (TB)

In 2008, renewed interest in the study of biofilms formed by *Mycobacterium tuberculosis* (*M. tuberculosis*) was launched by the description of these structures covered by a matrix of extracellular polymeric substance (EPS) composed of free mycolic acids, which harbor drug-tolerant bacteria as compared with their planktonic counterparts\(^1\). Mycobacterial genes associated with biofilm formation have roles in virulence and/or drug-tolerance and the study of biofilms was proposed as a means to develop novel TB vaccine candidates (reviewed in\(^2\)). Until a decade ago, there were few reports of *M. tuberculosis* biofilms in the literature, however biofilm-like structures were identified, persisting after drug treatment, in guinea pigs\(^3\). In fact, these extracellular multicellular micro-colonies were located in the acellular rim of granulomas and have been observed in cavities in lung resections from TB patients\(^4\). Despite these findings, the TB field showed reluctance to accept that biofilms formed during TB *in vivo*, this may be for programmatic reasons. TB pathogenesis studies understandably focus on the interactions between bacillus and macrophage, drug discovery programmes on *in vitro* and whole organism efficacy, and vaccine studies on immune responses and host protection. This has left a gap in our understanding of the predominately extracellular “clumps” and “clusters” of bacilli defined over 50 years ago from histopathology studies of human tuberculosis patients. As recognized by Georges Canetti in 1955, who stated that “The fundamental problem, one that requires intensive study, is the fate of the bacilli in the softened caseum”\(^5\).

Are biofilms formed during TB disease?

Part of the reluctance to the notion of biofilms being produced during TB disease comes from the definition of this phenotype as “an assemblage of surface-associated microbial
cells that is enclosed in an extracellular polymeric substance matrix”\textsuperscript{6}, where the attachment to surfaces perhaps is not self-evident in TB. However, the long-term survival of \textit{M. tuberculosis} despite a sustained immune response and antibiotic treatment during active disease resembles chronic infections involving biofilm-forming pathogens. Furthermore, thin-walled cavities could support biofilm-like growth of \textit{M. tuberculosis} with direct access to the airways\textsuperscript{7}, and mycobacterial clusters are also capable of forming without attachment in liquid suspension\textsuperscript{8}. Moreover, it has just been shown that in rabbits, infection with \textit{M. tuberculosis} grown in the absence of detergent, which leads to formation of small clusters (clumps) of cells, promote a more severe lung pathology, differential host inflammatory response, and the induction of matrix metalloproteases when compared to infections with cells grown as planktonic cultures\textsuperscript{9}. To what extent these mycobacterial aggregates share transcriptional or metabolic programmes with biofilms studied by other groups and us, remains to be determined.

Further to these data, the recent demonstration of the presence of cellulose in lung samples obtained from mouse, non-human primates, and humans infected with \textit{M. tuberculosis}\textsuperscript{10}, adds further supporting evidence to the notion of biofilm formation \textit{in vivo} by \textit{M. tuberculosis}. Chakraborty and colleagues\textsuperscript{10} showed that cellulose was present in these lesions by means of staining with calcofluor white (CW), CBD-mCherry staining, and Raman microscopy among other approaches. The authors also reported that increasing the production of a hypothesized mycobacterial cellulase (\textit{celA1}) reduced the \textit{M. tuberculosis} load in lungs of infected mice from weeks 2 to 12 post-infection. Whether cellulose is produced in \textit{M. tuberculosis} biofilms not exposed to the reducing agent dithiothreitol (DTT), or by planktonic \textit{M. tuberculosis}, remains to be determined. It should be noted that the genome of \textit{M. tuberculosis} lacks genes homologous to those involved in cellulose
biosynthesis, raising the question about the origin of cellulose in biofilms reported by Chakraborty et al.\textsuperscript{10}. Could it be that if cellulose is indeed produced by \textit{M. tuberculosis} in biofilms, this could derive from noncanonical hereto unknown pathways? On the other hand, it is worth questioning whether despite the strategies employed by Chakraborty et al., the compound detected is not cellulose but instead another complex carbohydrate, may be glucans? These questions remain to be resolved. There is also a need to ascertain whether another potential EPS (free mycolic acids, abundant in \textit{M. tuberculosis} biofilms formed without DTT\textsuperscript{1}) is present in biofilms formed after exposure to DTT, or whether free mycolic acids and cellulose (or another complex carbohydrate) are produced differentially in response to the changing microenvironment, and also whether other components exist as part of the mycobacterial EPS.

**Is biofilm production more prominent in some \textit{M. tuberculosis} strains compared with others, and is this clinically significant?**

Despite these exciting findings, one can easily question how extended or conserved among different \textit{M. tuberculosis} lineages is the correlation of “biofilm production = TB pathogenesis”. Chakraborty and colleagues\textsuperscript{10} employed in their animal models the lineage 4 (L4, Euro-American) strain H37Rv, which along with two other strains that also belonged to L4, were all good biofilm producers\textsuperscript{11}. Conversely, three strains from lineage 2 (L2, East Asia) did not produce biofilms at all\textsuperscript{11}. Chakraborty and colleagues did not define the lineages of the \textit{M. tuberculosis} in the human tissue they studied. Given the source of their samples (Chandigarh, India) one could speculate these might be from lineage 1 (L1, prevalent in the Rim of Indian Ocean and Philippines), lineage 3 (L3, India and East Africa)\textsuperscript{12} or the increasingly prevalent Beijing lineage (L2). If from L1 or L3, it raises the
following question: Do different clinical isolates within the same lineage share an *in vivo* biofilm phenotype and differences in their virulence may correlate (or not) with biofilm production? This query arises from the observation that four of six *M. tuberculosis* strains from L1 and L3 tested by Pang and colleagues did not form biofilms *in vitro*, whereas the other two L1/L3 strains evaluated formed thin biofilms compared with L4 H37Rv. If *M. tuberculosis*-producing cellulose (or as questioned above, another complex carbohydrate) was isolated from infected human lungs, would they produce biofilms *in vitro* and did this occur by induction of reductive stress by addition of DTT? Of note, Pang and colleagues did not utilize DTT-treatment to stimulate biofilm production, as opposed to the studies where cellulose was found in *M. tuberculosis* biofilms. DTT could constitute the additional stimulus that Pang, and colleagues hypothesized might be needed for some *M. tuberculosis* strains to produce biofilms, in addition to other yet uncharacterized signals or environmental cues. An alternative explanation might simply be that for unknown reasons; some *M. tuberculosis* isolates do not readily produce biofilms.

**What is required to better understand the role of biofilms in TB disease?**

Thinking of TB as a biofilm infection has implications for the way we conduct *in vitro* laboratory research, and for vaccine and drug discovery programmes. We need to understand more about this mycobacterial phenotype and the ramifications for drug tolerance and the development of antibiotic resistance, to successfully translate into novel and better therapeutics to the bedside. In this regard, it was recently shown that some genes that were necessary for the fitness of *M. tuberculosis* cells within biofilms, but not in planktonic cultures, were implicated in mycobacterial their tolerance to a diverse set of
stressors and antibiotics\textsuperscript{13}, therefore linking biofilm growth phenotypes to the arise of drug tolerance.

To determine the role of \textit{M. tuberculosis} biofilms in TB disease and to assess the impact this under-researched extracellular growth state has on TB pathogenesis, we suggest several research priorities that: (1) define the physiological state of \textit{M. tuberculosis} in these biofilm-like “clumps” and “clusters”; (2) map the dynamic interplay between the biofilm-pathogen and host; and (3) contrast the existing \textit{in vitro} models of \textit{M. tuberculosis} biofilm formation to develop more predictive models of the \textit{in vivo} biofilm states. This will result in an improved understanding of the key molecular and immunological consequences of \textit{M. tuberculosis} biofilm-formation on each side of the host-pathogen dynamic\textsuperscript{14} \textit{in-vivo}, to drive novel strategies towards eliminating patient suffering from tuberculosis across the globe.

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\textbf{Contributions}

The study was conceived by M.A.F.V; J.B., S.J.W, and M.A.F.V. wrote the manuscript.

\textbf{Competing interests}

The authors declare no competing interests.
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