Towards understanding the functional diversity of cell wall mycolic acids of *Mycobacterium tuberculosis*

Jan A. Verschoor*[1], Mark S. Baird[2] and Johan Grooten[3]

[1]Department Biochemistry, University of Pretoria, Pretoria 0002, South Africa.
E-mail: jan.verschoor@up.ac.za
[2]School of Chemistry, University of Wales, Bangor LL57 2UW, United Kingdom.
E-mail: chs028@bangor.ac.uk
[3]Department of Molecular Biomedical Research, Molecular Immunology Unit, University ofGent, Gent 9052, Belgium. E-mail: Johan.Grooten@dmbr.UGent.be
*Corresponding author. Tel.: +27 12 4202477; fax: +27 12 3625302

Abstract: Mycolic acids constitute the waxy layer of the outer cell wall of *Mycobacterium* spp and a few other genera. They are diverse in structure, providing a unique chromatographic foot-print for almost each of the more than seventy *Mycobacterium* species. Although mainly esterified to cell wall arabinogalactan, trehalose or glucose, some free mycolic acid is secreted during *in vitro* growth of *M. tuberculosis*. In *M. tuberculosis*, alpha-, keto- and methoxy-mycolic acids are the main classes, each differing in their ability to attract neutrophils, induce foamy macrophages or adopt an antigenic structure for antibody recognition. Of interest is their particular relationship to cholesterol, discovered by their ability to attract cholesterol, to bind Amphotericin B or to be recognised by monoclonal antibodies that cross-react with cholesterol. The structural elements that determine this diverse functionality include the carboxylic acid in the mycolic motif, as well as the nature and stereochemistry of the two functional groups in the merochain. The functional diversity of mycolic acid classes implies that much information may be contained in the selective expression and secretion of mycolic acids to establish tuberculosis after infection of the host. Their cholesteroid nature may relate to how they utilize host cholesterol for their persistent survival.
Key words: Mycolic acid, cholesterol, tuberculosis, *Mycobacterium*, foamy macrophages, antigenicity

1. Introduction

It is approaching a hundred years since Anderson began to publish a ground-breaking series of papers on the isolation of novel lipid fractions from the extracts of cells of mycobacteria [1]. Within those extracts were the molecules now known as mycolic acids that have been found to show many unique and important biological properties. Mycolic acids (MAs) are long chain β-hydroxy fatty acids that can mostly be represented by the general formula **1** (Fig. 1) in which the 'mycolic motif' contains a non-functionalised long alkyl chain and the 'meromycolate chain' generally contain up to two functional groups X and Y that are described below. Mycolic acids are mainly bound to the cell wall through esters formed between the acid group and an alcohol of a sugar, often as penta-arabinose tetramycolates. They are also present as non-bound solvent extractable esters, such as trehalose mono- and dimycolates (TMM and TDM), glucose monomycolate (GMM) and glycerol monomycolate, and as free mycolic acids.

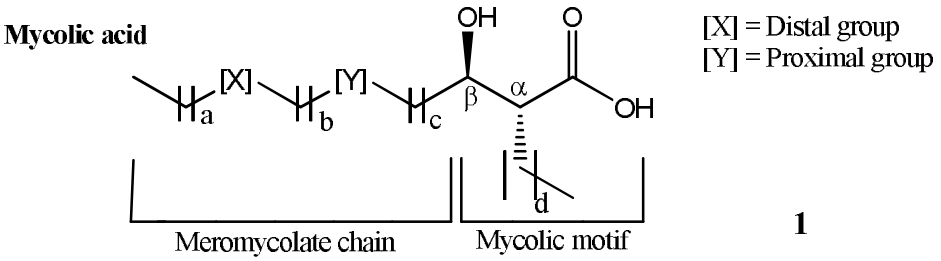

Figure 1. General structure of a mycolic acid. Varying lengths of methylene chains are indicated by a-d.

Mycolic acids occur in a distinct group of Gram-positive bacteria, classified in the suborder of *Corynebacterineae*, including the genera *Mycobacterium*, *Nocardia, Rhodococcus* and *Corynebacterium*, mentioned here in the order in which they contain large-and-complex to small-and-simple mycolic acids. In *Corynebacterium* the mycolic acids are smallest (C_{28}-C_{40}) and seem only to provide a beneficial, but not life-essential permeability barrier for the exchange of nutrients and antibiotics over the cell wall [2]. In contrast, *Mycobacterium* species are fully dependent for growth and survival *in vitro* and *in vivo* on their mycolic acids, which are the largest (C_{60}-C_{90}) in nature [3, 4]. *Rhodococcus* has mycolic acids in the size range C_{30}-C_{54}, and *Nocardia* in the size range C_{42}-C_{66} but in both cases with no functional groups in the mero-chain other than the possibility of one or more double bonds [5, 6, 7, 8] .

In the pathogenic mycobacteria, such as *M. tuberculosis* and *M. leprae*, mycolic acids have distal chiral, oxygenated and proximal and/or distal chiral, non-oxygenated functional groups in the mero-chain defined by X and Y respectively, and different chain lengths a-d (Fig. 1), all of which seem to endow important structural properties on the mycolic acids, which determine virulence and manipulation of the host immune response to establish a host-pathogen relationship that brings about the particular disease syndrome (eg. [9]).

When considering the structure-function relationship of mycolic acids, it is important to put it into the context of the three ways that they are expressed by the mycobacteria, viz. as bound to the arabinogalactan of the cell wall, in cell wall extractable esters such as trehalose dimycolate (TDM), or secreted as the free mycolic acids. When mycobacterial mutants are prepared that produce modified mycolic acids due to genetically impaired or deleted enzymes, then their changed behaviour as live mycobacteria relates overall to the three ways in which they are expressed in the mycobacterium. Examples of these are PcaA and MmaA2 mutants that express mycolic acids without α-mycolate cyclopropanation [10, 11], CmaA2 mutants that are unable to effect *trans*-cyclopropanation in the oxygenated mycolates [12], or MmaA4 and MmaA3 mutants that produce mycolic acids without distal functionality of the oxygenated mycolates [13, 14]. The significance of secreted free mycolic

acids as potential role players in the manifestation of tuberculosis was recently noted when Ojha *et al.* (2008) [15] demonstrated their role in biofilm formation during *in vitro* growth of *M. tuberculosis*.

In this review, the focus is on the structure-function relationship of free mycolic acids, in particular their immunoregulatory and cholesteroid nature, as discovered by their isolation and chemical modification, their stereo-controlled chemical synthesis, their immune steering properties and their recognition by monoclonal and polyclonal antibodies.

2. Structural properties of mycolic acids of *Mycobacterium* species

2.1. Mycolic acid patterns characteristic of mycobacteria

The initial work on the characterization of mycolic acids from *Mycobacterium tuberculosis* by Asselineau and Lederer [16], was followed by the assignment of complete overall structures to a number of classes of mycolic acid through the work of, e.g., Etemadi and Lederer and Minnikin and Polgar. Thus in 1966, the first structures of three major classes of mycolic acid, α-mycolic acid (2) [17], keto-MA (3) and methoxy-MA (4, 5) were proposed (Fig. 2) [18, 19].

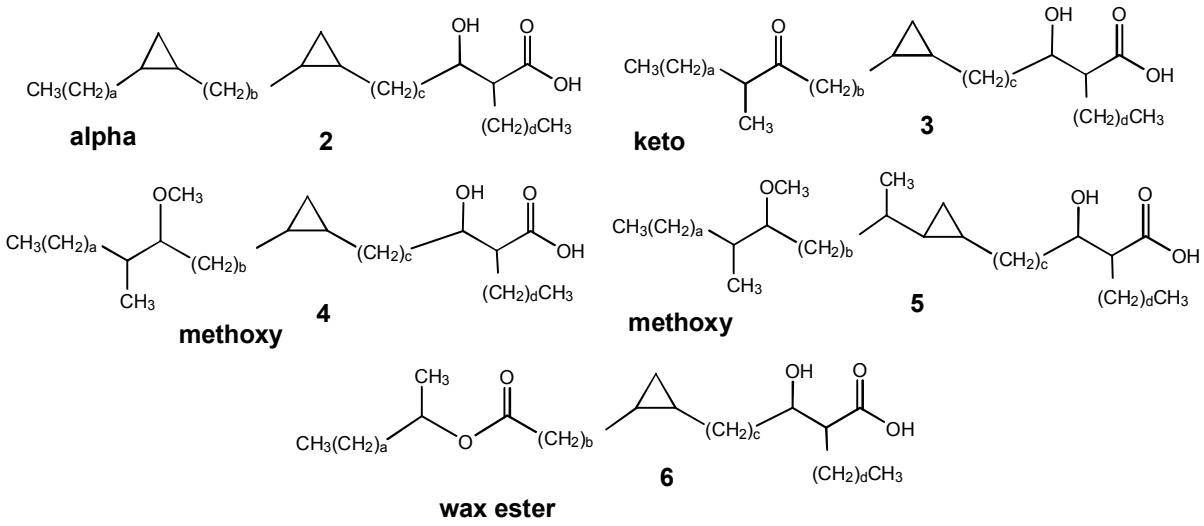

Figure 2. Major types of mycolic acid from *M. tuberculosis* complex (2) - (5) [17-19] and a related wax ester from *Mycobacterium paratuberculosis*

The stereochemistry of the β-hydroxy-acid fragment was assigned as *R,R* [20,21]. In addition, it was shown that the cyclopropane units present in α-MA, methoxy-MA or keto-MA could either have *cis*-stereochemistry as in (4) or α-methyl-*trans*-cyclopropane stereochemistry as in (5), with the methyl substituent on the distal side of the cyclopropane from the hydroxy-acid [22, 23].

The detailed patterns of the mycolic acid components of a number of strains of *M. tuberculosis* and some other mycobacteria have been elegantly collected through the work of Watanabe *et al*. [24, 25]. The total mixture of methyl esters was analysed first by ^{1}H NMR spectroscopy, then separated into α-, methoxy and ketomycolates. Each class was further split into those containing no double bonds, one *trans*-alkene and one *cis*-alkene using argentation chromatography. The detailed chain lengths could then be determined using mass spectrometry after pyrolysis to fragment the mycolic acid to produce the meromycolate fragment. For the species examined, which also included *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium kansasii* and *Mycobacterium avium*, the major components of each class contained *cis*- or *trans*-cyclopropanes and lacked alkenes; however, minor proportions of unsaturated compounds were also often seen. A number of strains contained tricyclopropanes with chains extended by 6 to 8 carbons. The methoxy-MA and keto-MA from most *M.tuberculosis* strains had minor components with additional cyclopropane rings; these were major components of the attenuated H37Ra strain which gave a different MA profile to the other Mtb strains. The MAs were categorised according to Fig. 3. In some mycobacteria, such as *Mycobacterium paratuberculosis*, mycolic acids are accompanied by wax esters (6) (Fig. 2) [29].

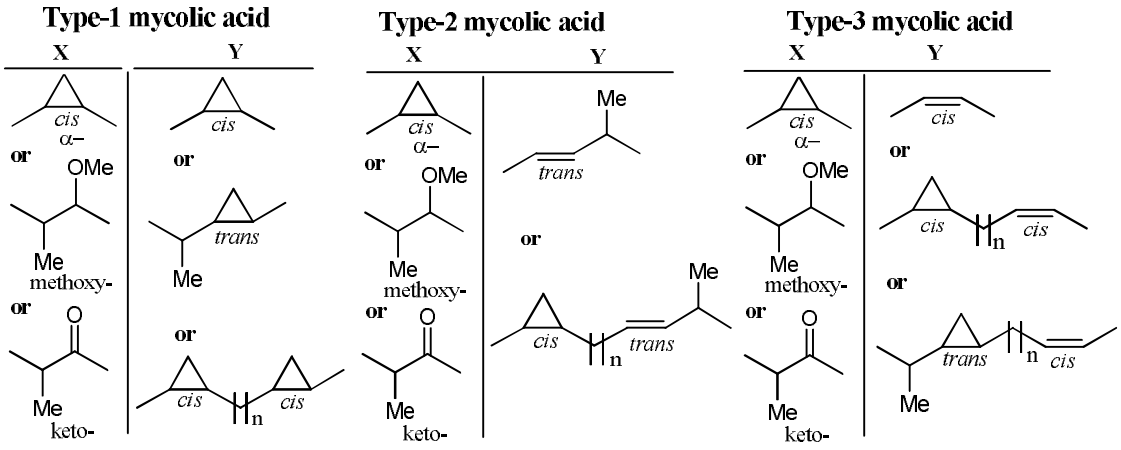

Figure 3. Common categories of mycolic acids [24].

This and earlier work showed very clearly that different mycobacteria contain different complex mixtures of mycolic acids that can provide a fingerprint. Moreover, different strains of *M.tuberculosis* contained significantly different components. Some selected examples are presented in Table 1 [24].

Table 1: The overall balance of mycolic acids in various strains of *M. tuberculosis* [24].

Strain	Alkenes (%)	Stereo-chemistry cis/trans	Cyclopropane (*cis:trans*)	α:methoxy:keto	α:(methoxy+keto)
Aoyama B	6.0	c/t	1:0.18	1:0.74:0.25	1:0.99
MNC1397	4.0	c/t	1:0.11	1:0.78:0.28	1:1.06
O	5.0	c/t	1:0.13	1:0.49:0.10	1:0.59
K	5.1	c/t	1:0.13	1:0.50:0.13	1:0.63
9829/87	9.5	«t	1:0.15	1:0.63:0.34	1:0.97
4610/91	6.4	c/t	1:0.14	1:0.72:0.29	1:1.01
2668/92	9.6	c>t	1:0.14	1:0.57:0.40	1:0.97
Canetti MNC1485	18.0	«t	1:0.19	1:0.64:0.80	1:1.24
H37Rv	10.7	c>t	1:0.14	1:0.54:0.49	1:1.03
H37Ra	22.2	c	1:<0.01	1:0.57:0.45	1:1.02

It is clear from this that there are considerable differences in each of the characteristics reported. However, it must be noted that the strains were obtained from a range of different sources, cultured in somewhat different ways and therefore that the proportions may reflect, or certainly incorporate, factors other than simple strain differences. Thus changes in the proportion of methoxy- and keto-MA had already been reported during cultivation of *Mycobacterium microti* [26]. In a second landmark paper, Watanabe *et al.* identified and collated the positions of the functional groups in the meromycolate fragments of the MA, revealing the full complexity of the mixtures present and again showing considerable difference between H37Ra and other *M.tuberculosis* strains [25]. Table 2 lists the major components of the series of homologues identified for *M.tuberculosis* that contain two functionalities X and Y in the chain. Given that there are also homologous series of compounds containing three functionalities, the overall complexity of the MA mixture is clear.

Table 2: Chain lengths and functional groups of the major mycolic acids of *M. tuberculosis* containing two functionalities X and Y [25].

X	Y	Major a,b,c,d
cis-cyclopropane	*cis*-cyclopropane	19-14-11(13)-23
cis-cyclopropane	α-methyl-*trans*-alkene	19-13-17-23
cis –alkene	*cis*-cyclopropane	19-14-13(11)-23
$CH(CH_3)CH(OCH_3)$	*cis*-cyclopropane	17-16-17-23 (15-14-19-23 in H37Ra)
$CH(CH_3)CH(OCH_3)$	α-methyl-*trans*-cyclopropane	17-16-18-23 (17-16-22(20)-23 in Canetti)
$CH(CH_3)CH(OCH_3)$	α-methyl-*trans*-alkene	17-19-13-23 (17-19-15-23 in Canetti)
$CH(CH_3)CO$	*cis*-cyclopropane	17,18,15,23 (15-16-19-23 in H37Ra)
$CH(CH_3)CO$	α-methyl-*trans*-cyclopropane	17-18-16-23
$CH(CH_3)CO$	α-methyl-*trans*-alkene	17-19-15(13)-23 (17-17-17-23 in Canetti)
$CH(CH_3)CO$	*cis* –alkene	17-18-15-23

The structures in Table 2 represent the major components of a series of homologues in each case. The orientations of the substituents relative to the hydroxy-acid part are as shown in Fig. 3. In addition there are homologous series of mycolic acids containing three functionalities in the chain.

The direct analysis of in-tact mycolic acids by MALDI mass spectrometry was first reported by Laval *et.al.*, who were able to detect less than 10 pmol [27]. For *M.tuberculosis* H37Rv, the typical patterns of groups of α-mycolates at *m/z* peaks of 1146, 1174, 1202, and 1230 are seen to overlap

those of methoxy-MA at 1262, 1290, 1318 and 1346 and keto-MA at 1246, 1274, 1288, 1302, 1316 and 1330. Interestingly, the chain lengths of the MA correlated with the growth rate of the bacterium but also with the strain; thus a comparison of the MA from a wild-type strain of *M.tuberculosis* and an isogenic mutant with an inactive *hma* gene showed that the latter produced almost entirely α-MA and no keto-MA of methoxy-MA. The homologous series of α-MA seen in the wild strain was accompanied by a second series differing by one CH_2-unit and the mass range extended from 1118 to 1258. MALDI-MS has also been used to provide a detailed analysis of the MA components of the cell wall of *M.bovis* BCG Tokyo 172 [28].

The mycolic acids of *Mycobacterium paratuberculosis* are interesting because they contain no methoxy-MA but instead 'wax esters' (**6**) apparently derived by a Baeyer-Villiger type oxidation of keto-mycolates (Fig. 2) [29].

The major MAs of *Mycobacterium smegmatis* are a homologous, alpha´- series containing just a *cis*-alkene in the mero-chain [30-32]. In addition there are two series, one containing two *cis*-alkenes, the other an α-methyl-*trans*-alkene in the proximal position and a *cis*-alkene at the distal position [33]. Similar series of compounds containing four or five *cis*-alkenes have been reported for *Mycobacterium thamnopheos* [34].

Three types of MA have been observed in *Mycobacterium aurum*. The first is di-unsaturated. The second series are keto-MA but the proximal group is either a *cis*- or an α–methyl-*trans*-alkene. The third series are wax ester mycolates (having an ester at the distal position) and again either a *cis*- or an α–methyl-*trans*-alkene at the proximal position [35]. The di-unsaturated MA appears not to be biosynthetically directly related, but the wax ester mycolates appear to be derived from the keto-MA by a biochemical Baeyer-Villiger type process [36].

Epoxy-mycolic acids were isolated from *Mycobacterium fortuitum* [37], *M.smegmatis* [38] and a number of other species, while a novel ω-1 methoxy-MA has been reported in *Mycobacterium porcinum* and a number of other bacteria [39].

Although the MA of mycobacteria have many things in common, as seen above there are also characteristic differences both in the classes of functional group present and in the balance of different chain lengths. The characteristic MA patterns of a large number of mycobacteria were collated using TLC and GC-MS of the meromycolates produced by thermal fragmentation [40, 41]. Although much of the work characterising MA has been carried out by mass spectrometry or GC-MS of meromycolates, HPLC has also been used to provide distinctive profiles of MA. A homologous series of α-mycolates from Mtb H37Ra was purified by HPLC under reverse phase conditions and each component characterised by MS, leading to the identification of 10 components with a range of chain lengths [42]. This has been extended to establish characteristic

HPLC patterns that can be used to distinguish a range of mycobacteria [43, 44]. For example, the MA of *M.bovis* BCG can be distinguished from those of *M.tuberculosis* and *M. bovis* [45]. It can also be used to suggest the phylogenetic position of newly isolated mycobacteria [46]. By coupling HPLC to electrospray ionization quadrupole TOF-MS it is possible to provide both qualitative and semi-quantitative analysis of mycolic acids. In this way the α-MA fraction from *M.bovis* BCG conditions was shown to accumulate during entry to dormancy induced by oxygen deprivation, while the keto-MA fraction was almost entirely eliminated [47].

2.2. Trehalose mycolates

A component of the tubercle bacillus known as 'cord factor' occurs in strains of *M.tuberculosis* which have the ability to multiply *in vivo* and shows a delayed toxicity to mice as well as having a definite role in virulence. Initial analysis of the active component was carried out by Noll and Bloch [48]. Mass spectrometry of these components was described in 1967, though no molecular ions could be observed [49]. The components, trehalose dimycolate (TDM) (**7**), trehalose monomycolate (TMM) (**8**) and related sugar mycolates (Fig. 4) exert a number of very strong immunological effects and are able to stimulate innate, early adaptive and both humoral and cellular adaptive immunity [50].

The distribution of cell surface glycolipids in *M.tuberculosis* strains has been examined by two dimensional TLC coupled to FAB-MS of the intact lipid and GC-MS of the fatty acid part. Both TDM and TMM are present in virulent and avirulent strains, whereas some other lipids are only present in virulent strains [51].

Direct molecular mass determination of TMM from a series of mycobacteria was reported in 2005 using MALDI-TOF mass spectrometry [52]. This showed marked differences between the various species. TMM from *M.tuberculosis* showed a distinctive pattern with abundant odd-carbon-numbered C_{75}-C_{85} α-mycolates containing monocyclopropanoic or monoenoic molecules as well as dicyclopropanes, odd and even carbon methoxy mycolates (C_{83} - C_{94}) and odd and even keto-MA (C_{83} - C_{94}). Some 24 – 38 significant components were identified. The results could be confirmed by hydrolysis and analysis of the derived MA methyl esters. Significant differences were also seen between the TMM components of *M.tuberculosis* H37Rv and Aoyama B, while the TMM from *M.bovis* BCG Connaught lacked methoxy-MA almost completely. The component MAs from *M.tuberculosis* TMM and TDM were very similar, though not identical. In particular, the relative size of MALDI peaks was very significantly different, though the MA pattern from *M. tuberculosis* TDM was very similar to that of membrane bound MA [52].

The analysis of intact TDM is rather more complicated than that of TMM because of the possible combinations of two different MA that are possible. Nonetheless, advised by an analysis of the MALDI patterns for the MA methyl esters derived by hydrolysis of the TDM, it has been possible to analyse these complex mixtures. A marked difference was seen between different mycobacterial species. Thus TDM from *M. tuberculosis* (HR37Rv and Aoyama B) showed over 60 molecular ions while that from *M. bovis* BCG Tokyo 172 showed over 35. In contrast, *M. bovis* BCG Connaught showed less than 35 molecular ions, because of the complete absence of methoxy-MA. Since the physico-chemical properties and antigenic structure of the TDM affect the host immune responses, the detailed profiling of intact TDM represents a significant advance [53, 54]. TDM and TMM prepared from single synthetic alpha- and methoxy-mycolic acids have been reported [55].

2.3. Other mycolate esters

Among the mycobacterial lipids presented on CD1b (see 3.1.1) are free mycolic acids and glucose monomycolate (GMM) [56]. T-cell recognition is highly specific for the precise structure of natural GMM including the glucose, the linkage of the glucose to the mycolate and the *R,R*-stereochemistry of the hydroxyl acid part of the mycolate [57]. Upon infection mycobacteria begin to produce GMM which provides a good indicator of local invasion. The GMM is produced more at 30 ^{o}C than 37 ^{o}C and is recognised by a GMM-specific, CD1-restricted T-cell line isolated from human skin. MALDI-TOF analysis of GMM derived from *M. smegmatis* shows a majority of peaks corresponding to α-mycolates (C_{74}, C_{76}, C_{77}, C_{78}, C_{79}, C_{80}) [58].

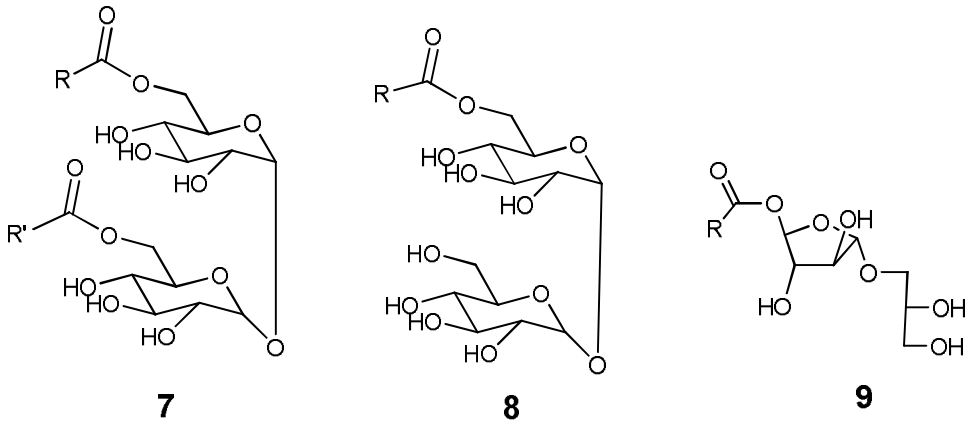

Figure 4. Generic structures of trehalose dimycolate (TDM) (7), monomycolate (TMM) (8) and 5-mycolyl-α-arabinofuranyosyl(1→1')-glycerol (9) (RCOOH, R'COOH = mycolic acid)

A novel 5-mycolyl-α-arabinofuranyosyl(1→1')-glycerol (**9,** Fig. 4) has been isolated from the *Mycobacterium avium-Mycobacterium intracellulare* complex [59]. Even a very simple monomycolylglycerol (MMG) has the ability to stimulate human dendritic cells, while a model based on just a C_{32} chain has similar effects [60].

2.4. The stereochemistry of mycolic acids

In all the cases where it has been established, the β-hydroxy-acid fragment of mycolic acids has been shown to be of *R,R*-absolute stereochemistry. The $CH_2(CHMe)(CHOMe)CH_2$ fragment of methoxy-MA has also been shown to be of *S,S*-absolute stereochemistry. It has been proposed that the biosynthesis of various functionalities in MA proceeds through a common intermediate, a cation produced by alkylation of a *cis*-alkene. This can then either lose a proton from the methyl group to produce a *cis*-cyclopropane, from an adjacent methylene group to produce a methyl-substituted *trans*-alkene or be trapped as an alcohol. The latter can then in turn be converted into methoxy-and keto-MA (Fig. 5 [61].

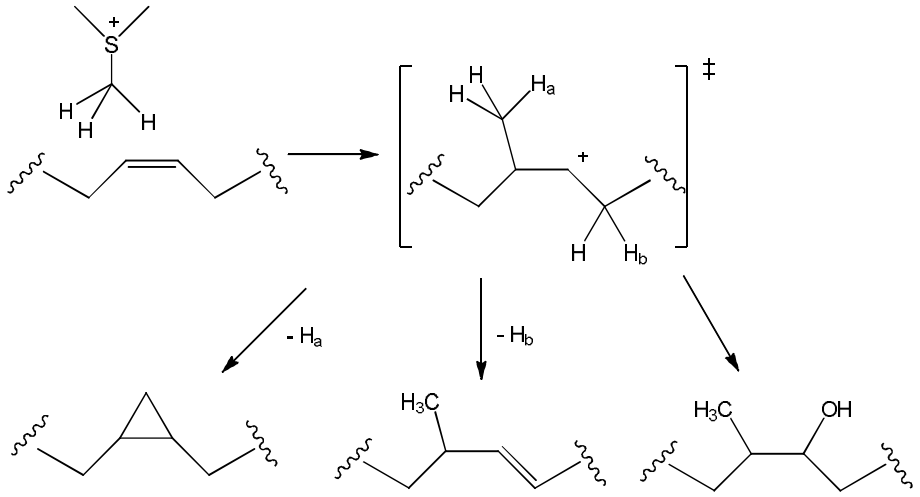

Figure 5. A common pathway to methoxy, keto and cyclopropane mycolic acids [61].

In support of some elements of this mechanism, the fast growing *M.smegmatis* normally produces oxygenated mycolic acids with an epoxy group. However, if a set of four genes from *M.bovis* BCG coding for methyl transferases is transferred into *M.smegmatis*, this produces keto-mycolic acid together with a hydroxy-mycolic acid, both of which are cyclopropanated. There is a perfect match between the structures of the keto- and hydroxy-MA [62]. More detailed analysis showed that both *M.bovis* BCG and *M.tuberculosis* also produce small amounts of hydroxy-MA and again

there appears to be a direct relationship between hydroxy-MA and keto-MA [63]. Although cyclopropanated MAs were previously thought not to abound in *M.smegmatis*, it was recently shown that the genes to effect this are present in the genome and can be induced for expression at low temperature growth conditions [65].

2.5. Synthetic mycolic acids

The discussion above emphasises the singular complexity of natural mixtures of mycolic acids and of derived sugar esters. It further highlights the difficulty in isolating a single natural molecule and in assigning a complete structure, including its stereochemistry. Moreover, the important role of such compounds in the immune system and their changing nature during the progression of diseases such as tuberculosis very clearly demonstrate their potential in therapy, prosthetics and in the diagnosis of infection. In natural mixtures, such effects may be a reflection of the overall complex mixture, or may in fact be dominated by individual molecules or groups of molecules. The beneficial effects of the natural mixtures may be outweighed by their toxic effects, as in the case of Freund's complete adjuvant. Moreover, the application of such mixtures produced by cells may be limited considerably by the potential for subtle but nonetheless significant changes in their detailed make up. For all these reasons, it is important to be able to prepare and evaluate single molecules identical to the various components of natural mixtures.

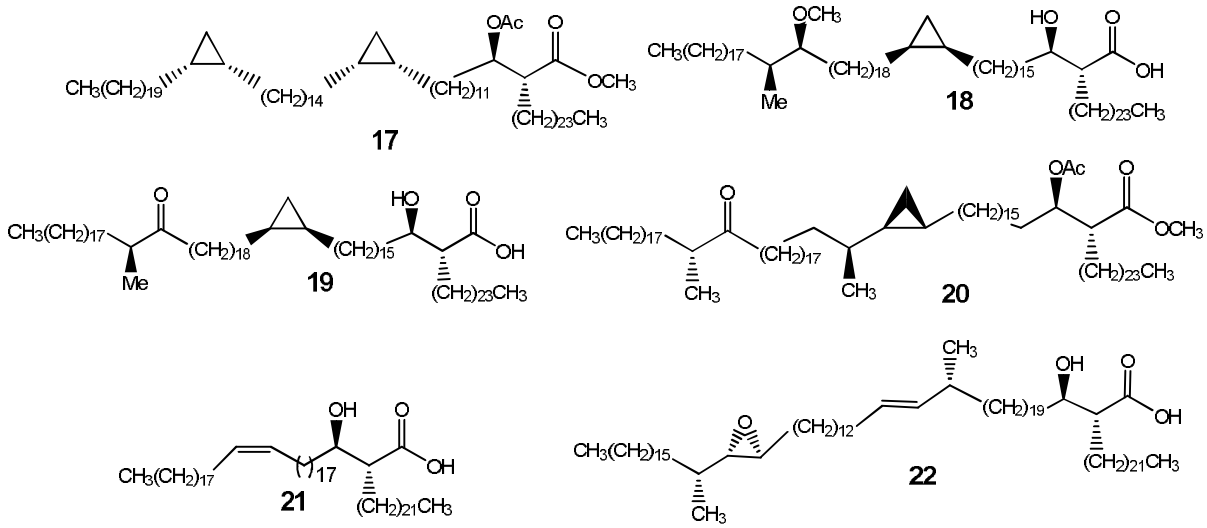

Figure 6. Examples of synthetic mycolic acids.

The first synthetic mycolic acid reported was the protected α-mycolic acid (**17**, Fig. 6) [64]. Since that time, all four possible diastereomers of this di-*cis*-cyclopropane-MA have been synthesised. Based on the model in Fig. 5 and assuming that both proximal and distal cyclopropanes are introduced with the same geometry, isomer **17** has the natural stereochemistry.

In the same way, three stereoisomers of the major methoxy-MA of *M.tuberculosis*, including (**18**) [66] as well as keto-mycolic acids containing both *cis*- and *trans*-cyclopropanes (**19**) and (**20**) have been reported (Fig. 6) [67, 68]. In addition, single synthetic MAs containing just *cis*-alkenes (**21**) [69], *trans*-alkenes [70] and epoxy-MA (**22**) [71] have been reported (Fig. 6).

2.6. Does structure matter?

There are a number of observations suggesting that the detailed structures of natural mycolic acids and their sugar esters are important:

- In at least *Mycobacterium phlei* and *M. smegmatis*, the balance of different classes of MA changes with the temperature at which the organism is grown, possibly to manipulate the membrane properties [65, 72].
- Cells of mycobacteria grown at higher temperature contain increased levels of *trans*-MA and have a higher melting temperature; moreover, they allow a less rapid influx of some drugs [73].
- The proportion of *trans*-substituted cyclopropane at the proximal position of MA is important in determining the fluidity of the cell wall. By over-expressing MMAS-1, an enzyme in the gene cluster responsible for biosynthesis of methoxy-MA, an increase was seen in the production of both *trans*-alkene and *trans*-cyclopropane mycolates, and in the proportion of keto-MA to methoxy-MA. This was accompanied by a substantially decreased growth rate at elevated temperature and an increased permeability of the cell wall [74].
- Cyclopropane modification of *M. tuberculosis* cell envelope TDM is critical for bacterial growth during the first week of infection in mice. TDM isolated from a mutant deficient in the cyclopropane synthase PcaA is hypoinflammatory for macrophages and induces less severe granulatomous inflammation, showing that the fine structure of the mycolates in these lipids directly affects pathogenesis and temporal control of the host immune activation through cyclopropane modification [75].
- TDM isolated from a mutant (Δ*cmaA2*) that lacks the capacity for *trans*-cyclopropanation is five times more potent in stimulating macrophages than TDM isolated from wild-type *M. tuberculosis*. This establishes *cmaA2* dependent *trans*-cyclopropanation of MA as an activity to suppress *M. tuberculosis* induced inflammation and virulence. Moreover, the

stereochemistry of the cyclopropane changes the interaction with the host cell to influence host innate immune responses both positively and negatively [76, 77].

- One of the strategies used by *M. tuberculosis* to avoid elimination by the host is the selective repression of macrophage IL-12p40 production. The mmaA4 gene which encodes a methyl transferase required for the introduction of oxygen substituents at the distal position of MA is a key locus in this suppression. This suggests that *M. tuberculosis* has evolved mmaA4-derived mycolic acids into TDM to manipulate IL-12-mediated immunity and virulence [9].

3. Structure-function relationship of free mycolic acids

3.1. Immunoregulatory properties of natural and synthetic mycolic acids

The different functions exerted by mycolic acids from *M. tuberculosis* and their synthetic counterparts along with their potential consequences for the pathogenesis of tuberculosis are schematically illustrated in Fig. 7.

3.1.1. MA and CD1 restricted T cells

An important aspect of immunoregulation is the presentation of processed antigen on antigen presenting cells (APC) to responder T cells. Protein antigens are processed and peptides presented on major histocompatibility complex (MHC) proteins. Responder T cells are divided broadly into CD4 and CD8 expressing cells, of which CD4 T cells are generally known as helper T cells with main function to facilitate cellular and humoral (antibody, cytokine) immunity, while CD8 T cells are effector cells that kill target cells, but also influence the control of immunity. T cells responding to peptides presented on MHC are 'MHC restricted'. CD4 T cells are MHC-II restricted, with MHC-II membrane proteins, typical of APCs, generally presenting peptides from exogenously captured antigen, while CD8 T cells are MHC-I restricted, with MHC-I membrane proteins, typically present on all nucleated cells, generally presenting peptides from endogenously biosynthetized antigen, such as virus or tumor-associated proteins.

A particular role for mycobacterial mycolic acids in immune regulation was reported in 1994, when Beckman *et al.* [78] showed for the first time that MA presentation on CD1, in particular CD1b of human dendritic cells, stimulated CD4/CD8 double negative T cell lines, representative of a minor population of T cells in the blood circulation believed to be mainly involved in the regulation of auto-immunity. This was the first indication of the function of the CD1 family of membrane proteins as lipid antigen presenting molecules on antigen presenting cells. The role of CD1 as a presenter of various lipids to direct innate and adaptive immune responses was recently reviewed by Cohen *et al.,* 2009 [79]: During the folding of

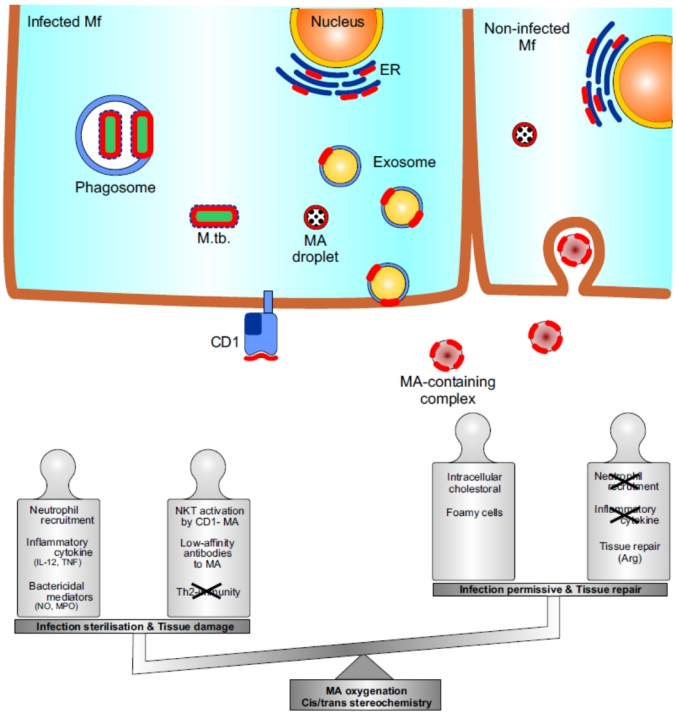

Figure 7. Mycolic acids balance the host immune response to infection.

Top: In infected macrophages, the acidic pH from the phagosome may promote the dissociation of cell wall-associated structures such as LAM, TDM and others, resulting in an increased exposure of cell wall mycolic acids (red stretch). The highly hydrophobic nature of the exposed mycolic acid moieties may promote spontaneous association with the phagosome membrane, thus bringing the mycolic acids in direct contact with the cytosolic cell compartment. In human macrophages, *M. tuberculosis* bacilli also occur outside of the phagosome in the cytosol of the infected cell. Here, enzymatic processing of TDM by serine esterases may promote the formation of free mycolic acids. Again, the highly hydrophobic nature of the free mycolic acids will promote association with intracellular membranes and/or lead to the formation of mycolic acid-containing lipid droplets. ER-associated mycolic acids may in addition be presented on the surface of the cell by the MHC I mimic, CD1. Through as yet poorly understood mechanisms, the intracellular free mycolic acids trigger signaling pathways leading to the activation and differentiation of the infected macrophage. Endocytosis of excreted mycolic acid-containing complexes by non-infected neighboring macrophages may trigger through similar pathways the activation and differentiation of the cell, hereby promoting a bystander effect. Bottom: The functions elicited by free mycolic acids are divergent and may support increased anti-bacterial defenses or increased permissiveness to infection along with tissue repair responses. Increasing evidence supports the notion that the level and type of mycolic acid oxygenation as well as the cis- and trans-cyclopropane stereochemistry are crucial in balancing these opposite macrophage functions.

Arg: arginase; ER: endoplasmic reticulum; M.tb: *Mycobacterium tuberculosis*; MA: mycolic acids; Mf: macrophage; MHC: major histocompatibility complex; MPO: myeloperoxidase; NKT: natural killer T-cell; NO: nitrogen oxide radical.

nascent CD1 in the endoplasmic reticulum, microsomal triglyceride transfer protein (MTP) appears to facilitate the loading of CD1 with self-lipids, that are later replaced by exogenous microbial lipids during endosomal trafficking. Each CD1 molecule may accommodate

different types of lipids and each CD1 molecule has hydrophobic pockets determining preferential binding to different lipids. The various members of CD1(a-e) are loaded early or late in the endosomal/phagosomal maturation pathway. Thus, glucose monomycolate (GMM) with short lipid tail length MAs (32C) is loaded on CD1b early, while GMMs with longer lipid tail length MAs (80C) are loaded late on CD1b in endosomal maturation. CD1e appeared not to be involved directly in antigen presentation as a membrane anchored protein. Rather, it was observed to be involved with the processing of lipid antigens in lysosomes, much like the lipid transfer proteins do [80]. Some of the more complex exogenous lipids require endosomal processing, while others are loaded and presented without processing. There is no published evidence that lipases are involved in lipid antigen processing for CD1 loading. In mice, it was shown for some glycolipid antigens that terminal sugars were removed by specific glycosidases before CD1d loading was permitted [81], analogous to the system for glycopeptide antigen processing before presentation to T cells. Acidification of late stage endosomes is often required to enable loading of CD1, while transfer of the water insoluble larger lipids, including the mycobacterial mycolic acids, from endosomal membranes to CD1 is facilitated by specific lipid transfer proteins of the phagosomal saposin family, selectively active in early or late stage lipid processing aiming towards presentation of the lipids to T cells or NK T cells. Soluble CD1e was first shown to facilitate the removal of sugars from the larger glycolipids before their presentation on CD1b [82]. Later, Facciotti *et al.* [80] demonstrated that this type of facilitation by soluble CD1e happens mainly by its role as a selective lipid transfer protein that can enable both the loading and unloading of CD1 molecules of the CD1b, CD1c and CD1d types.

CD1-restricted T cells appear to respond to CD1 presented antigens that appear on the professional antigen presenting cells, i.e. dendritic cells and B cells. Certain lipid antigens, mainly those with long hydrophobic tails like MA, are presented by the so-called Type I CD1, comprising the human CD1a, CD1b and CD1c. The Type II CD1 presents preferably glycosylated lipids with shorter hydrophobic tails and comprises CD1d in humans. In rodents, only Type II CD1 is found on antigen presenting cells.

Type I CD1 presents lipid antigens to T cells, which respond by polyclonal activation, suggesting a relatively wide range of specificities for antigen binding [83] In contrast, Type II CD1 molecules present lipids to Natural Killer T (NKT) cells with structurally more conserved T cell antigen receptors [84]

In the early days following the discovery that human dendritic cells presented mycobacterial MA to T cell lines, it was believed that only CD4/CD8 double negative and CD8 positive T

cells were responsive to the presented MA [85]. Indeed, it was shown that both these T cell types secreted IFNγ that can protect against *M. tuberculosis* infection [86] and could induce apoptosis of infected macrophages [87]. Later, work on MA stimulation of human T cells by Goodrum *et al*. [88] used whole peripheral blood monocytes from freshly donated normal human blood, with which it was shown that CD4 single positive T cells proliferated strongest, followed by double negative T cells, while very few CD8 single positive T cells responded to MA stimulation. This was corroborated by Montamat-Sicotte *et al*. in 2011 [89], who reported that CD4 single positive cells were the main responders to MA stimulation in the peripheral blood monocytes of human TB patients. In these patients, CD8 single positive cells were also stimulated strongly, while only a small response to MA from double negative T cells were recorded.

The exact role of MA responsive CD1 restricted T cells in tuberculosis remains unresolved. Sieling *et al*. [90] reported that CD4/CD8 double negative T cells from auto-immune systemic lupus erythematosus (SLE) patients induced antibody isotype switching from IgM to IgG in a CD1c restricted manner characterised by Interleukin 4 secretion of the CD1c restricted SLE double negative T cells. CD1c restricted double negative T cells from healthy donors did not secrete IL4 and did not facilitate antibody isotype switching. In the case of tuberculosis, Montamat-Sicotte *et al*. [89] reported that MA appears to be a main target for T cells in tuberculosis. Specific T cells were found to abound at diagnosis, but contracted faster upon treatment of the patients with anti-TB drugs than was the case with TB protein specific T cells, thereby being more sensitive towards pathogen-burden. Although the MA specific T cells were found to exhibit properties of memory T cells, including the property of rapid expansion upon antigen reencounter, the vaccine potential of MA awaits better understanding of the role of CD1-restricted, MA responsive T cells in tuberculosis. For instance, the vaccine potential of the human APC CD1b presented sulfoglycolipid, 2-palmitoyl or 2-stearoyl-3-hydroxyphthioceranoyl-2′-sulfate-α-α′-D-trehalose (Ac2SGL) from *M. tuberculosis* was demonstrated by its ability to stimulate Ac2SGL-specific T cells with concomitant release of interferon γ, recognition of *M. tuberculosis*–infected cells, and killing of intracellular bacteria. The ability to clone such Ac2SGL-responsive T cells from humans strictly depended on the latter's previous contact with *M. tuberculosis,* without necessarily manifesting the disease [91]. Thanyani *et al.* in 2008 [92] associated active TB with biomarker antibodies to MA that may be regarded as auto-immune in that they are associated with a simultaneous increase in anti-cholesterol antibody activity in TB patient sera. How this may relate to the establishment of TB is discussed in more detail below.

3.1.2. MA and macrophages

Specialized immune cells, especially macrophages, mast cells and dendritic cells express a diverse repertoire of germ-line encoded pattern recognition receptors (PRRs) that are pivotal in the early detection of infectious pathogens and the subsequent activation of innate immune defences. This enables the development of adaptive immune defences by steering the differentiation of naïve $CD4^+$ T-lymphocytes towards helper T-cell (Th) subsets supporting cellular (Th_1, Th_{17}) or humoral (Th_2, Th_3) immune defences. In sharp contrast to the highly specific receptors for antigen expressed by B- and T-lymphocytes, PRRs exhibit low specificity and distinguish infectious non-self from non-infectious self on the basis of pathogen-associated molecular patterns (PAMPs). *M. tuberculosis* bacilli express several well characterized PAMPs (reviewed in [93, 94, 95]). Among others, the cell wall skeleton fraction of *M. tuberculosis*, a complex of mycolic acids bound to peptidoglycan through arabinogalactan, mediates inflammatory activation of macrophages and dendritic cells through TLR2 and TLR4 [96, 97]. The peptidoglycan domain rather than the arabinogalactan and mycolate moieties of the cell wall skeleton was later identified as responsible for this TLR2 and TLR4 dependent activation of innate immune cells [98]. Other *M. tuberculosis* cell wall structures representing PAMPs and exerting activation and/or modulation of host immune responses include lipoglycans such as lipoarabinomannan (LAM) [99, 100] and trehalose dimycolate (TDM) [101, 102], the 19 kDa lipoprotein stimulating TLR2 [103, 104], sulfolipids [105], and the cell wall–associated lipid phthiocerol dimycocerosate [106, 107].

The pronounced impact on overall mycobacterial fitness of genetic approaches to describe the biological roles of mycolic acid lipids has as a consequence that it is not possible to segregate a role for free mycolic acids in manipulating host immune responses to infection. For that reason, Korf *et al.* addressed the relation to host immunity of *M. tuberculosis* mycolates by examining the interaction with macrophages of isolated mycolic acids. To overcome the poor solubility in water of the highly hydrophobic, wax-like lipids, liposomes were used as vehicles for subsequent administration into mice [108]. Following phagocytosis by murine macrophages, mycolic acids were found to colocalize with acidic phagosomes. This absence of interference with phagosome maturation and acidification is in contrast with the intact mycobacterium that generally inhibits maturation and acidification of phagocytic compartments as a strategy to promote its own survival [109, 110]. However, administration of mycolic acids to the airways mimicked other features of *M. tuberculosis* infection. Thus similar to the recruitment of neutrophils, monocytes and lymphocytes observed in tuberculosis patients, mycolic acids elicited an acute neutrophilic airway inflammation which was accompanied by a moderate and chronic IL-12 production [108]. Macrophages showed only a mild inflammatory activation but exhibited a strongly increased reactivity when

challenged with suboptimal concentrations of heat-killed *M. tuberculosis*. Among others, the production of the antimicrobial enzyme, myeloperoxidase, and the inflammatory cytokine, IFN-γ were strongly induced under these conditions. Myeloperoxidase is a heme enzyme that generates reactive oxidants and has been implicated in mycobacterial killing [111]. IFN-γ, along with IL-12, has been shown to be a pivotal cytokine in the control of *M. tuberculosis* infection in mice and humans. These observations identify mycolic acids as a (mildly) pro-inflammatory PAMP from *M. tuberculosis* that in addition primes macrophages for a future infection by increasing the capacity of the cells to raise antimicrobial defenses. Strikingly, also Fenhals and colleagues [112] observed expression of IFN-γ in macrophages in human tuberculous granulomas. Isolated mycolic acids therefore appear to elicit innate immune responses characteristically generated during *M. tuberculosis* infection.

T-lymphocyte responses strongly biased towards IFN- γ -producing Th_1 lymphocytes are a hallmark of tuberculosis in patients and mouse models. Chronically increased levels of the instructive cytokines IL-12 and IFN- γ in the infected lung are crucial in promoting this Th_1 bias of the pulmonary immune response. In contrast, immune responses to nonmicrobial airborne environmental antigens in allergic subjects are strongly biased towards Th_2 lymphocytes, instructed by a predominance of the cytokine IL-4. Asthma, a chronic inflammatory disorder of the airways, is driven by a Th_2 cell response to inhaled allergen and presents, as distinctive pathology, a bronchoalveolar inflammatory infiltrate consisting of eosinophils, basophils, and mast cells [113, 114] rather than neutrophils and monocytes/macrophages observed in Th_1-driven inflammatory responses. Interestingly, population studies showed an inverse correlation between the clinical prevalence of asthma and the incidence of microbial infections such as tuberculosis [115]. The failure of PAMPs or bacterial infections to trigger Th_2 cell responses combined with a mutual antagonism between Th_1 and Th_2 responses is believed to be at the centre of the inverse relationship between asthma and tuberculosis incidence. Strikingly, a similar antagonism was reported by Korf and colleagues [109+7] when applying mycolic acids, administered by intratracheal instillation, in a mouse model of asthma. A single treatment with *M. tuberculosis* mycolic acids not only prevented the onset of inhaled allergen-induced eosinophilic airway inflammation but also promoted unresponsiveness to a secondary set of allergen exposures, indicating the occurrence of a Th_2 tolerizing condition in mycolic acid-treated lungs. The authors further showed that the development of this tolerant condition depended on IFN- γ and could be mimicked by adoptive transfer of mycolic acid treated macrophages [116]. Extrapolated to the human condition, these observations indicate that mycolic acids may actively contribute towards preventing the development of a deleterious, non-protective Th_2-lymphocyte host response and support the development of protective Th_1-driven cellular immunity. The next

question is then how the structure of mycolic acids relates to their immunoprotective properties and whether such knowledge can be exploited for the design of vaccine adjuvants to steer immunity towards a beneficial response protective against a variety of infectious diseases.

Vander Beken and colleagues [117] recently addressed this issue by studying the inflammatory properties of chemically homogenous mycolic acids differing in oxygenation class and proximal cyclopropane stereochemical configuration. Using single synthetic mycolic acid isomers, the authors confirmed that the presence and type of an oxygenated distal group in the meromycolate moiety is the main determinant for at least pulmonary inflammatory potency. Thus, whereas alpha-mycolic acid was found not to cause inflammation, oxygenated methoxy- and keto-mycolic acids with *cis*-cyclopropane stereochemistry elicited strong to mild inflammatory responses respectively. Strikingly, keto-mycolic acid with a *trans*-cyclopropane in the proximal position, exerted anti-inflammatory activation of alveolar macrophages and counterbalanced *cis*-methoxy-mycolic acid induced airway inflammation. This fine-tuned relation between (anti-) inflammatory potency, mycolic acid oxygenation and cyclopropane stereochemistry observed with individual mycolic acids may explain some of the apparently contradictory observations made with *M. tuberculosis* mutant strains lacking in specific mycolate classes. Furthermore, a differential innate immune activity of individual mycolic acid isomers may provide a means for *M. tuberculosis* to fine-tune host innate immunity in response to different growth conditions, such as reduced levels of aerogenation or entry into dormancy versus reactivation; conditions that have been shown before to be accompanied by changes in mycolic acid composition [47, 118, 119].

3.1.3. Free mycolic acids in TB

The bioavailability of free mycolic acids during natural infection has been a matter of debate for a long time. Recent new insights indicate that free mycolic acids may become available during infection to exert innate immune activation. Thus Ojha and colleagues [15] reported the formation, under specific growth conditions, of biofilms in cultures of *M. tuberculosis*. In the biofilms, the *M. tuberculosis* bacilli were found to be embedded in an extracellular matrix rich in free mycolic acids. Biofilm formation depended on the expression of groEL1 chaperone and its physical association with KasA, a key component of the type II Fatty Acid Synthase involved in mycolic acid synthesis [120]. *M. tuberculosis* biofilms containing free mycolic acid are not just an artifact from *in vitro* growth but have also been described in the infected organism. Within primary granulomas in guinea pig lungs microcolonies with features reminiscent of biofilms and containing extracellular mycolic acids have been

described [121]. These microcolonies were detected within an acellular rim adjacent to the edge of the mineralizing central necrotic core. Free mycolic acids in these biofilms reportedly are derived from cell envelope glycosylated mycolates and are released by a trehalose dimycolate-specific serine esterase [122]. These new findings support the notion that free mycolic acids in tubercle granulomas may actively contribute to the delicate immune balance maintained in granuloma structures by exerting the divergent pro- and anti-inflammatory activities previously reported with isolated mycolic acids [108, 116, 117].

This leaves open the important issue of the contribution or not of mycolic acids in their natural, cell wall-bound form to the innate immune response of the infected macrophage. Most *in vivo* and *in vitro* studies of free mycolic acids used liposomes or other carriers as vehicle for the water insoluble mycolic acids. Besides efficiently targeting phagocytic cells such as macrophages, liposomes in addition are rapidly engulfed by the phagocyte where they end up in the endosomal to lysosomal compartment [108]. This predominantly intracellular location of administered free mycolic acids may explain why transmembrane PRRs such as TLR2 and TLR4 reportedly do not contribute to the elicited inflammatory response [108] and suggests the involvement of other PRRs with either an endolysosomal location or present in the cytosol of the cell. In particular, the family of nucleotide-binding domain and leucine rich repeat-containing receptors (NLRs) may be implicated in the macrophage activation observed upon administration of mycolic acids. NLRs have been shown to detect a broad range of intracellular bacteria but also to sense stress signals resulting from infection [123, 124] or, in the case of mycolic acids, possibly from an intracellular abundance of non-self mycolate lipids. Considering the variety of additional *M. tuberculosis* PAMPs such as TDM, LAM and others and their pronounced inflammatory potency, mycolic acid immunoregulatory activity may in fact be predominant during dormancy. Here cell wall mycolic acids interacting with cytosolic PRRs such as NLRs may enable an enduring inflammatory conditioning of macrophages harbouring (silent) *M. tuberculosis* bacilli in spite of the absence of extracellular *M. tuberculosis* PAMPs that are mostly associated with active disease.

4. **The human antibody response to mycolic acids**

The first report of human TB patient antibodies to mycolic acids appeared by Pan *et al.* in 1999 [125]. By separating the natural mixture of MA into the three main classes by thin layer chromatography, the authors showed that methoxy- and keto-MA were more antigenic than the non-oxygenated alpha-MA. This was confirmed by Beukes *et al*. in 2010 [126], using various isomers of stereo-controlled chemically synthetic MAs and showing methoxy-MA to be the most antigenic of the three classes in TB patients. Further analysis showed MA antigenicity to be critically dependent on the fine structure

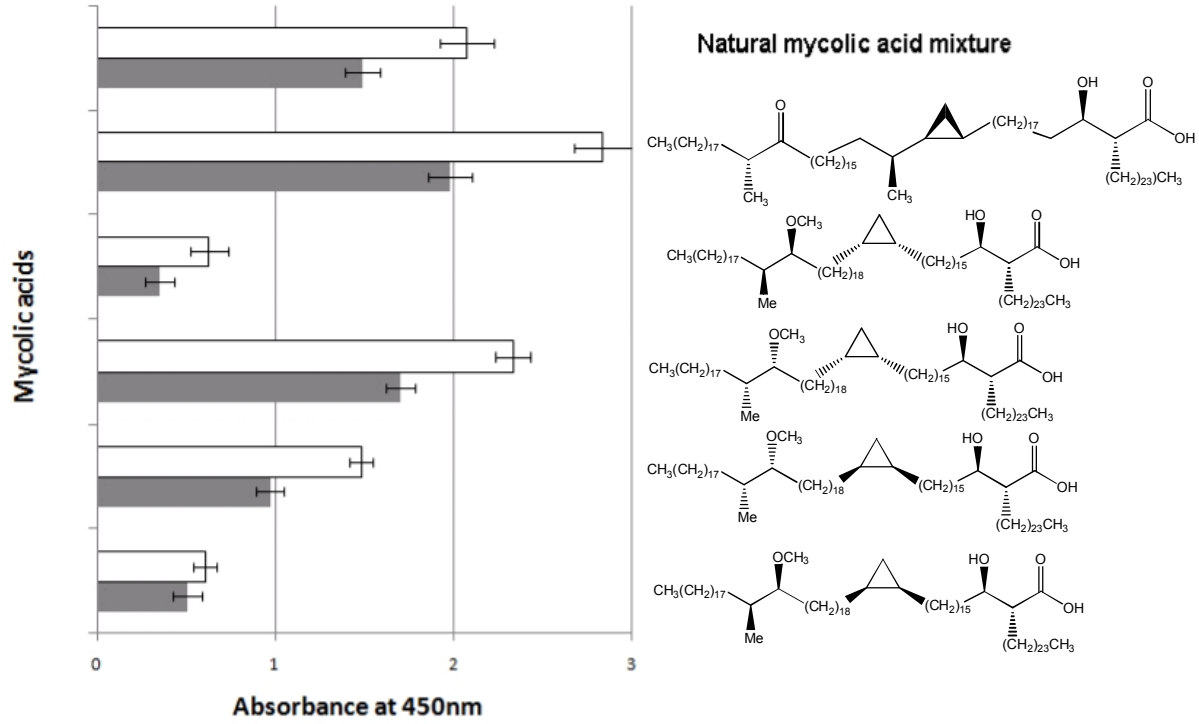

Figure 8. ELISA antibody binding signals of pooled TB positive (blank boxes) and TB negative sera (filled boxes) to natural MA and to five synthetic methoxy MA. The error bars indicate the standard deviation. Natural MA was isolated from *M. tuberculosis* H37Rv. Adapted from [126].

of the functional groups in the MA merochain. Even the stereochemical configuration of the functional groups was important, as demonstrated in Fig. 8 with various isomers of methoxy-MA. The antibody binding signal to isomer 24 was almost double that of isomer 23, although the only difference between them was the stereochemistry of the proximal *cis*-cyclopropane. Likewise, the antibody binding signal to isomer 24 was almost three times that of isomer 25, with the only difference between them being the stereochemistry of the distal methoxy-methyl functional group. Isomer 21, believed to be the closest structural representative of the naturally occurring methoxy-MA of this chain length, was comparatively poorly recognized by TB patient sera. Converting its proximal *cis*-cyclopropane to a methyl-trans cyclopropane (isomer 26) made it the strongest antigenic isomer of the five that were compared in the figure. Antigenicity was also found to depend critically on the structure of the mycolic motif. In particular, methyl esterification of the carboxylic acid in the motif destroyed antigenicity altogether [126], possibly indicating a critical role of the carboxylic acid in stabilizing an antigenic conformational fold of the MA by hydrogen bonding with the oxygenated distal functional group.

Currently, TB diagnosis in HIV burdened populations poses one of the most challenging hurdles towards regaining control of the TB epidemic [127,128]. The potential for detection of antibodies to MA as biomarker for diagnosis of active TB was emphasized when Schleicher *et al*. [129] demonstrated in 2002 that the antibody binding signal to MA was not affected by the degree to which the patient was affected by HIV-co-infection. There remained, however, an overlap between the anti-MA antibody binding signals between TB patient and control sera that made the ELISA outcome not adequately accurate as a diagnostic indicator. One way to get around this problem was to employ modern antibody detection technology where antibody binding is detected in real time, thereby increasing the sensitivity of the reaction [92, 130]. These authors also showed that human TB patient anti-MA antibodies included IgG, by demonstrating that a significant part of the anti-MA antibody activity could be purified with Protein A affinity chromatography, known to be insensitive for IgM. The antibody isotypeswitch from IgM to IgG usually involves antigen specific T cells, hereby distinguishing the anti-MA antibody response as probably a T cell dependent response, as opposed to T cell independent responses that are typical of a number of other lipid antigens, in particular glycolipids, and are mainly constrained to IgM responses. Even with the use of biosensor technology to detect antibodies to MA in TB patients, there remained quite a number of false positive outcomes, leaving the question as to what causes some TB negative patients to express antibodies that are reactive to MA. One possibility to determine this would have been to identify individual MA class structures to which antibodies from TB patient sera bound preferentially, as opposed to other more cross-reactive MA structures. The reality that is clear from the data in Fig. 8 is that none of the MA isomers examined showed any significant difference in distinguishing between sera from TB-positive and TB-negative patients. The same applied to isomers of the keto- and alpha-MA classes [126]. Although the antibody binding activity to all the MA-isomers was higher in (pooled) TB positive patient sera than in (pooled) TB negative patient sera, the ratio of binding activity to MA between TB positive and TB negative patient sera remained similar for each MA antigen tested. Whereas this diminishes the prospects of increasing the accuracy of mycolic acid antigen based immunodiagnostic tests for TB by using particular isomers of chemically synthetic mycolic acids, it eases the necessity for defining exactly the class composition of MA antigens that is used to detect the MA-binding antibodies. These MAs may resemble early stage *M. tuberculosis* when the keto-MA dominates or the late stage when the methoxy-MA dominates [131], with the only difference being slight changes in the magnitude of the signals for both MA antigen sets, with no difference in the ratio of the antibody binding signal from TB positive and TB negative patients.

5. **Cholesteroid nature of mycolic acids**

One lead towards the understanding of the cross-reactivity of MA recognition by TB patient and control sera was the observation that immobilized cholesterol in an ELISA plate could substitute for MA to produce similar antibody binding activity between TB patient and control sera [132]. The authors also showed that cholesterol was attracted to immobilized mycolic acids in an evanescent field biosensor. This could mean that a structural and/or functional relationship exists between MA and cholesterol. The demonstration that Amphotericin B, a cholesterol binding anti-fungal antibiotic, bound equally well to MA as to cholesterol gave credibility to such a notion. Later, Beukes *et al.* [126] provided more direct proof by providing a recombinant scFv antibody fragment expressed from a chicken antibody gene library that recognized both MA and cholesterol, indicating that the antibody binding activity to cholesterol could be due to a true functional cross-reactivity with mycolic acids. It has been firmly established that humans generally have variable levels of IgM and IgG anti-cholesterol antibody activity in the circulation ([133, 134], thereby providing a possible explanation for the high binding activity to MA observed in TB negative patients. Alving and Wassef [135] proposed in 1999 that endotoxin, found ubiquitously in the environment, could act as a potent adjuvant for the induction of antibodies to cholesterol. The cholesterol source of antibody induction could come from localised sites of infection and inflammation where cellular debris is gathered. In particular, it was shown that HIV-infected individuals express higher amounts of anti-cholesterol antibodies, probably due to the role of cholesterol in the cholesterol-rich lipid raft where HIV is budding from the infected cells, as well as the subsequent insertion of cholesterol into the envelope of the HIV virion [136]. When patients were put on anti-retroviral treatment, the anti-cholesterol antibody titre rapidly declined.

Even if the prevalence of truly cross-reactive anti-MA and anti-cholesterol antibodies could be demonstrated in human sera, cross-reactivity could also be due to a mixture of monospecific anti-cholesterol and monospecific anti-MA antibodies. Such monospecific scFv specificities were also selected from the chicken antibody gene library by Beukes *et al*. [126], showing that the recognition of MA by antibody does not necessarily manifest as cross-reactivity to cholesterol. Likewise, scFv antibody fragments to cholesterol did not necessarily cross-react with MA. Whether the antibody cross-reactivity observed in human TB and control patient sera is due to a mixture of monospecific antibodies, a true single antibody cross-reactivity, or both remains to be determined. What seems clear at this stage is that MA and cholesterol may not be recognized as individual haptens, but rather as molecular clusters forming multi-faceted surfaces, of which one or some facets can cross-react, while other facets may be restricted to mono-specific recognition by antibodies to either cholesterol or MA. For this reason the structure of MA recognized by a truly cross-reactive anti-MA/cholesterol antibody could better be referred to as the cholester*oid* face (with *–oid* indicating a particular shape, form or resemblance) of a cluster of MAs constituting an antigenic determinant. Studies of MA monolayers

indeed confirmed that they were not uniform in terms of surface topography, showing significant variation of film thickness and conformational depencency on the presence of water [137]. In the body, MAs can be expected to be present in the context of a phospholipid complex, such as in a membrane or in a lipoprotein, which further complicates the interpretation of ELISA results where MAs are recognised as precipitates on polystyrene.

Cholesterol is not normally present in prokaryotes, including the mycobacteria. The manipulation of the micro-environmental host cholesterol concentration by *M. tuberculosis* may be of strategic benefit for pathogenesis. One possible role for the cholesteroid nature of MA in TB pathogenesis may be found in the role that cholesterol plays in phagosomal maturation during mycobacterial infection of the host macrophage. Cholesterol mediates the binding of TACO, an actin binding protein [138], on live mycobacteria-containing phagosomes in order to prevent lysosomal degradation of the mycobacteria [139]. Upon cholesterol depletion of pre-existing phagosomes, the close apposition between the phagosome membrane and the mycobacterial surface is loosened and fusion with lysosomes occurs [140, 141], leading to destruction of the pathogen. TACO is retained on the phagosome by cholesterol from the host as well as by the mycobacterial protein, coronin interacting protein (CIP)50, later identified as lipoamide dehydrogenase C (LpdC) [142]. LpdC is the E3 component of the pyruvate dehydrogenase complex. Cholesterol depletion of the phagosome membrane inhibited the TACO – LpdC interaction showing the cholesterol dependence of the association. TACO is responsible for activating the Ca^{2+} dependent phosphatase calcineurin. This leads to the inhibition of lysosomal delivery [143, 144]. From the perspective of the property of mycolic acids to attract cholesterol and their demonstrated release from trehalose-mycolates at the mycobacterial cell surface, one can predict that MA in the phagosomal membrane would be able to facilitate cholesterol accumulation to ensure TACO retention at the interface between the phagosomal membrane and the mycobacterial cell surface [145].

The mycolic acids that are bound to the cell wall arabinogalactan form the inner part of an outer membrane that allows adhesion at an oil-water interface to facilitate feeding from oils and fats. This property gave *Rhodococcus* species potential for industrial application to clean up sites of oil pollution [137]. In *Mycobacterium tuberculosis*, this property is manifested as an ability to attract cholesterol into the lipid-free zone that surrounds the cell wall mycolic acid layer [146] and is at the basis of the requirement for host cell membrane cholesterol to enable entry of the bacillus into its host macrophage [139, 147]. Besides being essential for the phagocytosis of the bacterium by the macrophage, cholesterol is also used by the bacilli as an energy and carbon source that is critically linked with mycobacterial persistence [148, 149] and depends on a sufficient availability of the sterol within the host cell. Mycolic acids may in fact constitute a direct means by which intracellular *M. tuberculosis* bacilli ensure a sufficient lipid import by the host macrophage. The property of MA to

attract cholesterol may mean that free MA, known to be released from the bacilli for the purpose of biofilming [15, 122], could fulfil the role of attracting cholesterol into the biofilm as a carbon source for the slow growing, persistent mycobacteria. Cholesterol is known to be able to diffuse across biological membranes along a concentration gradient without the need for specific transport mechanisms. It is therefore the ideal carbon source for a pathogen that survives by isolating itself from the host vasculature that normally provides for the pathogen's nutrient requirements. Should the mycolic acids be able also to attract cholesterol *in vivo*, then their function in the biofilm may well include the creation of the cholesterol concentration gradient necessary to facilitate cholesterol transfer from host to pathogen. Brzostek *et al*. [146] demonstrated that the ability of *M. tuberculosis* to attract cholesterol close to its cell wall depended on the presence of the extractable lipid component of the cell wall, which includes the free mycolic acids and the trehalose mycolates from which they are released [122].

In their pioneering paper, Korf and colleagues [108] were the first to demonstrate that mycolic acids strongly interfered with the lipid metabolism of mouse macrophages, triggering intracellular accumulation of cholesterol, increased cell size, and multiple vacuole formation, all typical features of macrophage foam cells. Foamy macrophages containing multiple cholesterol loaded lipid bodies constitute a distinct trait of tuberculous granulomas and a safe haven for mycobacteria, since they have been found to harbor multiple bacilli [150, 151]. Induction by mycolic acids of macrophages rich in lipid bodies was further confirmed on human monocyte-derived macrophages [152]. Here, the authors further showed that oxygenated mycolic acids, mostly of the keto class, play a leading role in the observed foamy macrophage formation.

In non-pathological conditions, macrophage lipid import and export are tightly regulated processes. Excessive cholesterol accumulation therefore is indicative of a disturbed lipid homeostasis in the cells. Liver X receptors (LXRs), LXRα and LXRβ, are master regulators of macrophage transcriptional programs involved in cholesterol homeostasis [153, 154, 155]. LXR activation by oxidized forms of cholesterol (oxysterols) induces expression of several genes involved in cholesterol trafficking and efflux, such as *ABCA1*, *ABCG1/ABCG4*, and *apoE* [156, 157]. An increased cholesterol influx and accumulation in infected macrophages may as a consequence trigger the activation of LXR nuclear receptors and LXR target genes. This was recently confirmed using a mouse model of *M. tuberculosis* pulmonary infection. Here, alveolar macrophages isolated from the bronchoalveolar lavage and lung tissue showed besides an increased expression of the inducible *LXRα* isoform also increased expression levels of the LXRα target genes, *ABCA1*, *apoE* and *SREBP-1c* [158]. This infection-induced activation of LXRα transcriptional activity may be of direct relevance for the course of the infection. Thus mice deficient in *LXRα* showed an increased susceptibility to infection, developing higher bacterial burdens and an increase in size and number of granulomatous lesions. This increased susceptibility was accompanied by a failure of the *LXRα*$^{-/-}$ mice to mount an

effective early neutrophilic airway response and, at a later stage, by a near-complete abrogation of the infection-induced Th_1 and Th_{17} response in the lungs [158]. Whereas these immune consequences of a deficient LXRα function obviously may directly contribute to the increased susceptibility of the mice, it is noteworthy that mice deficient in *LXR*α also accumulate intracellular cholesterol within tissue macrophages, resulting in a foamy macrophage-like morphotype (Unpublished results). It can therefore not be excluded that the increased intracellular cholesterol content in *LXR*$\alpha^{-/-}$ macrophages, comparable to the cholesterol-loaded foamy macrophages induced by mycolic acids, generates an energy-rich environment for the bacilli, promoting *M. tuberculosis* persistence and indirectly contributing in *LXR*$\alpha^{-/-}$ mice to increased susceptibility.

The cholesteroid antigenic nature of MA may add to the propensity of forming energy rich foamy macrophages at the site of mycobacterial infection to benefit the parasitic bacilli [159]. Thus it is possible that the antibodies to MA that are cross-reactive with cholesterol may behave as anti-cholesterol antibodies do: at high concentration the latter are known to bind to VLDL/IDL and LDL, but not to HDL [160]. This was found to be due to the higher concentration of non-esterified cholesterol on the surface of VLDL/IDL and LDL in comparison to that on HDL. In the body, this function relates to the role that HDL plays in sequestering cholesterol from the surfaces of cells for transport to the liver, while VLDL and LDL have the function to distribute cholesterol from the intestines and liver to the tissues. Antibody opsonized lipoproteins of the VLDL or LDL type will then be selectively phagocytosed by macrophages through the Fc receptors, thereby creating or adding to the lipid abundance of such cells to support the parasitic growth of the mycobacteria on a mainly cholesterol diet.

6. Conclusion

From simple, short chained MAs with one or a few unsaturated bonds in *Corynebacterium* to long chain MAs of *Mycobacterium* endowed with chiral functional groups, nature displays how a bacterial wax is phylogenetically developed from just being a cell wall barrier against the environment (*Corynebacterium, Rhodococcus, Nocardia)* to a sophisticated, conformationally sensitive, pleiotropic cell wall system (*Mycobacterium*) designed to also direct cellular host-pathogen relationships and steer host immunity to benefit the survival of the prokaryotic parasite. Besides the proven role of various aspects of function of MA in defining virulence of mycobacterial pathogens, mainly demonstrated by recombinant mutants of mycobacteria that are defective in some aspect of decoration of MA with functional groups during biosynthesis, free mycolic acids that are released from sites of infection elicit immunity in a structure dependent way. Mycobacterial MAs elicit both innate immune steering mechanisms and adaptive humoral and cellular immunity. Innate immunity to MA exposure is characterized by granulocyte, macrophage and dendritocyte activity with specific cytokine responses to steer immunity. Adaptive immunity to MA is characterized by B cells producing IgG

antibodies to MA and by T cells of both the effector and memory type coming about and responding to MA in rapid expansion upon reencounter of MA. We have reviewed the evidence that the innate immune response to MA is differentiated into MA class specific activities, such as neutrophil attraction by methoxy-MA and macrophage foam cell induction by keto-MA. Likewise, the adaptive antibody response to MA is differentiated into high antigenicity (methoxy-MA) and low antigenicity (alpha-MA), of which all are responsive to the stereochemical configuration around the functional groups of MA.

How this knowledge can be exploited for intervention of tuberculosis in the management of the global TB epidemic depends on a better understanding of the role of MA elicited immune responses in protection of the host, prevention of auto-immunity or parasite beneficiation. Brigl and Brenner in 2004 [161] reviewed evidence of several examples of where CD1 restricted immune responses are involved in various auto-immune diseases, both in infectious and non-infectious disease conditions. Although much more is known about Group II CD1d restricted responses in auto-immunity, Group I restricted T cell involvement has also been reported for auto-immune related diseases, particularly in inflammatory neuropathies and rheumatoid arthritis. Thus, the potential for using MAs as a treatment for asthma, demonstrated in mice by Korf *et al.* in 2006 [116], awaits an understanding of how administered MAs affect auto-immunity, disease susceptibility or other essential pathways of metabolism. Diagnosis of TB by means of biomarker anti-MA antibody detection in patients [92] awaits a better understanding of the cross-reactivity of such antibodies with cholesterol and the binding properties of the universally prevalent interfering anti-cholesterol antibodies in humans. The inclusion of MA in anti-TB vaccines [89] awaits the understanding of whether the MA reactive memory and effector T cells actually protect against TB.

The discovery of the cholesteroid nature of MA reviewed here provides a strong clue to the better understanding of how mycolic acids contribute to establish tuberculosis, in particular how free mycolic acids may influence the availability of cholesterol as a prime nutrient for mycobacteria in persistent TB and how the induction of cholesterol cross-reactive anti-MA antibodies may aid towards the delivery of cholesterol to sites of infection.

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