Chapter 2: The cholesteroid nature of MA as a tool for targeting

2.1 Introduction

Upon inhalation of *M.tb*, the bacteria enter the lungs and are phagocytosed by alveolar macrophages. This causes a proinflammatory response that leads to the recruitment of mononuclear cells. These cells cluster at the site of infection to form granulomas which contain the *M.tb*. Within granulomas a variety of cells are found which include differentiated macrophages, highly vacuolated macrophages and lipid rich foamy macrophages [143]. These *M.tb* infected macrophages induce an accumulation of cholesterol esters that could be a major component of foamy macrophages [144]. Foamy macrophages present in the outer ring of the granuloma were shown to accumulate during the chronic phase of infection in mouse experimental models [85, 145]. Experiments with radio-labelled lipids have shown that the presence of *Mycobacterium leprae* (*M.leprae)* in tissues was associated with increased intracellular levels of cholesterol-esters, which may be responsible for conversion of macrophages into foam cells [84]. In tuberculosis, the foam cells are surrounded by lymphocytes and later fibroblasts [146]. Active disease only comes about when necrosis and cavity formation occur in the lungs, in which massive numbers of bacteria are produced. This occurs concomitantly with changes in the immune status of the host [140].

In order for *M.tb* to survive in a heterogeneous and continually changing macrophage environment, they need to adapt their nutrient sources. Cholesterol is a major sterol in eukaryotic organisms. It is involved in membrane stabilization and plays a role in cell signalling pathways [147]. It can also be used as a carbon and energy source for various microorganisms [80]. Previous papers suggested that pathogenic bacteria use lecithin instead of cholesterol as carbon source [144], but more recent evidence points

to the pathogenic mycobacteria being able to accumulate [80] and consume cholesterol as major nutrient in the persistent stage of the disease [125, 148]. The genomic sequence of *M.tb* revealed the full repertoire of sterol biosynthetic and cholesterol degradation enzymes [149]. Av-Gay and colleagues have suggested that cholesterol could play a role in the prevention of phagosomal maturation of mycobacterially infected macrophages and that soluble cholesterol may accumulate into the cell wall surface of mycobacteria [80].

Brzostek *et al.* 2009 found that *M.tb* can both accumulate and utilize cholesterol. They showed that cholesterol, as primary source of carbon, was degraded via the 4-androstene-3,17-dione/1,4androstadiene-3,17-dione pathway (AD/ADD) together with an intact KstD enzyme. Cholesterol was also shown to accumulate in the free lipid zone of the cell walls of the bacteria and that the accumulation affected cell wall permeability [148]. Whether cholesterol as a carbon source could support long term persistence of tubercle bacilli is not yet known.

It was shown that during the chronic phase of murine infection and in IFN-γ activated macrophages *in vitro* the Mce4 transporter is necessary for cholesterol import into the bacterium and that the bacteria can indeed degrade cholesterol as primary source for carbon and energy [125, 150]. The cholesterol degradation was measured by detecting the no. 4- and no. 26-carbons of the molecule, with C-26 being incorporated into the cell membrane lipids and the C-4 part converted to CO_2 (g) [125]. The study showed that the Mce4 mutant was not able to convert cholesterol. Mce4 was also not required for replication of *M.tb* in resting macrophages. In another study it was shown that the acquisition of cholesterol was dependent on a cholesterol oxidase [151].

Genes that are required for the survival of *M.tb* in the hostile environment of the macrophage were shown with transposon site hybridization (TraSH) using a microarray based technique. Genes that seemed to be critical for survival within macrophages included those involved in lipid transport and degradation as well as those involved in phosphate transport [152]. The TraSH technique was also used to study the *mce4* locus. It suggested that a group of genes were involved in lipid metabolism, including the operon Rv3540-5c [150]. The operon was later called igr (intracellular growth) and was required for growth in THP-1 cells and in a tissue

culture model of infection [153]. It was also shown that mutants thereof were attenuated for growth *in vivo* [154]. In an *M.tb* related actinomycete *Rhodococcus sp.* a 51-gene cluster was identified whose transcription was induced specifically in the presence of cholesterol. The cholesterol catabolic pathway investigated was also shown to be conserved in related species such as *M.tb*, where the matching gene cluster included the igr operon and the *mce4* transport system [155]. Miner *et al.* 2009, showed that an igr deletion mutant could not grow in the presence of cholesterol, whereas the mce4 mutant was only attenuated in mice in late stages, pointing to the notion that cholesterol metabolism was only required in the chronic infection stage [154].

Dynamic interactions between host and mycobacterial factors may play an important role in mycobacterial survival [140]. Mycobacterial cell wall components include phosphoinositol mannosides (PIMs) interacting with cholesterol-enriched microdomains of the host [156], ManLAM induces reorganisation of lipid membranes, preventing vesicle fusion, thereby inhibiting phagosome maturation [157]. Phthiocerol dimycocerosates (DIM) have been shown to be involved in the prevention of phagosomal acidification, which precedes phagolysosomal fusion and eventual destruction of internalized mycobacteria. DIM is also involved in receptor dependent phagocytosis (rather than macropinocytosis) into macrophages that are dependent on an intact actin filament network. This is done by a possible mechanism of targeting of lipids and disturbing their organisation in the host membrane [83]. Interestingly it has been shown that *M.tb* forms biofilms that is distinct from planktonic growth and that free mycolic acids were present in the extracellular matrix of these films [141].

Host membrane cholesterol seems to play a role in at least one route of entry and survival of mycobacteria in macrophages, also facilitating the phagosomal association of TACO on mycobacteria-containing phagosomes. TACO inhibits lysosomal delivery of the mycobacteria by activating a Ca^{2+} dependent phosphatase calcineurin [90]. The TACO protein is associated with the prevention of phagosome-lysosome fusion [81, 96, 154]. In *M. avium* infected mouse bone marrow macrophages, phagosome maturation and lysosome fusion occurred when the macrophages were

depleted of cholesterol. Cholesterol replenishment showed that mycobacterium could rescue itself from phagolysosomes [96]. It was shown that in *M.tb* a compound with epitopes similar to the human cholesterol specific receptor – C_k like molecule was found that has an affinity for cholesterol, and that it was responsible for entry into macrophages as well as the mediation of phagosomal association with Coronin-1A, which inhibits endosomal/lysosomal fusion [92, 93].

The rationale for using free mycolic acids as targeting agent in nanodrug delivery is due to their cholestroid nature [121] and their attraction to cholesterol. It may therefore target cholesterol enriched mycobacterially infected areas. Further investigation into the characteristics of mycolic acids was done in order to establish how structural changes affect the cholesteroid nature of the MA.

To measure the interaction between Amphotericin B and MA as indicative of the cholesteroid function of MA, an ESPRIT SPR biosensor was used. The principle of the SPR biosensor is based on the change in refractive index on a modified thin gold film surface when mass is accumulated onto an immobilized ligand [158]. This interaction is measured in real time. An important advantage that this technique has above the conventional methods is that no labeling is required and that low affinity specific binding can be detected. The instrument was also used to determine the effect of labelling of MA on its manifestation of the cholesteroid nature, the binding interaction between amphotericin B and either cholesterol-, MA-, or fluorescein-labeled MA-containing immobilized liposomes. In addition to the amphotericin B affinity experiments on the SPR biosensor, the antibody recognition of modified MA was tested in an ELISA based experiment. Thereafter the relationship between the structure and the cholesteroid nature of MA was investigated by testing different stereo-controlled synthetic MA to antibodies in TB patient serum.

2.2 Hypothesis

The cholesteroid nature of MA derives from a specific folding of the mero-chain that is stabilized by hydrogen bonding to the carboxylic acid.

2.3 Aims of study

- Comparing the ability of cholesterol, fluorescein-MA and MA to associate with AmB
- Investigating the tolerance of structural changes of natural mixed mycolic acids for human antibody recognition.
- How structure of synthetic mycolic acids relates to antigenicity in tuberculosis

2.4 Materials

2.4.1 Consumables

Amber vials	Separations Pty Ltd, Randburg, RSA
Amphotericin B	Sigma-Aldrich, Steinheim, Germany (isolated from *Streptomyces* to 80% purity, identified by HPLC)
5-Bromomethyl fluorescein	Molecular Probes, Leiden, The Netherlands
Chloroform	Merck, Darmstadt, Germany
18-crown-6 ether	Sigma-Aldrich, Steinheim, Germany

Dimethyl formamide	Merck, Darmstadt, Germany
Ethanol	BDH, Gauteng, RSA, Analytical grade
Hydrochloric acid	Saarchem, Gauteng, RSA
Methanol	Merck, Darmstadt, Germany
Molybdatophosphoric acid	Merck, Darmstadt, Germany
Mycolic acids	Sigma Chemical Co., St Louis, USA
Octadecanethiol	Sigma Chemical Co., St Louis, USA
Phosphatidyl choline	Sigma Chemical Co., St Louis, USA, (99 % pure)
Potassium chloride	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Potassium hydrogen carbonate	Merck, Darmstadt, Germany
Potassium hydroxide	Saarchem, Gauteng, RSA
Silica plate	ALUGRAM SIL G/UV, layer: 0.2mm silica gel 60 with fluorescent indicator UV254, Düren, Germany
Sodium azide	Sigma Chemical Co., St Louis, USA
Sodium chloride	Saarchem, Gauteng, RSA
di-Sodium hydrogen phosphate	Merck, Darmstadt, Germany
Ethylenediaminotetraacetic acid salt	Merck, Darmstadt, Germany

2.4.2 Buffers

PBS-Azide EDTA buffer (PBS/AE): 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per 1 L double distilled, deionized water with 1 mM EDTA and 0.025% (m/v) sodium azide, adjusted to pH 7.4

2.4.3 Instrumentation

For the measurement of the binding properties of mycolic acids and derivatives thereof to other low molecular weight ligands an ESPRIT biosensor from Metrohm Autolab B.V., Utrecht, the Netherlands was used.For preparation of liposomes a Virsonic sonifier, (Virtis, Gardiner, N.Y.) was used. Well Wash4 ELISA washer (Labsystems, Helsinki, Finland) was used for ELISA plates wash steps. Absorbancies were measured with a SLT 340 ATC photometer (SLT Labinstruments, Austria) at 450 nm.

2.5 Methods

2.5.1 Fluorescent labelling of mycolic acids

MA was fluorescently labelled by derivatization with 5-bromomethyl fluorescein (5-BMF) as described by Korf *et al.*, according to a general protocol first described by [159]. The first step in the methodology involves re-saponification of the MA. Briefly, reagent A (1 ml of potassium hydroxide, KOH 25%, i.e. 5 g in ddd H_2O, 10 ml, and methanol 10 ml) was added to MA (1 mg, 8.3 x 10^{-7} mol, 1 eq), and the capped solution was heated for an hour. Following cooling, 1 ml of reagent B (50% aqueous dilution of concentrated Hydrochloric acid (32%)) was added and the solution vortexed. Chloroform (1 ml) was added, the organic layer removed and the aqueous phase washed three times with chloroform. After evaporation of the organic solvent on a heat block with N_2 (g), reagent C, 800 µl (Potassium hydrogen carbonate

$KHCO_3$, 0.01 mol in ddd H_2O, 25 ml and methanol, 25 ml) was added and heated for 1.5 hours at 86 ºC. The saponified MA product was dried and the solvent evaporated with heat under an N_2 (g) atmosphere. Chloroform (500 µl) was added to the dried, saponified MA and mixed, before 18-crown-6 ether (1.1 mg, 4.16 x 10^{-6} mol, 5 eq), dissolved in chloroform, was added and vortexed. The reaction was incubated for 30 min and 5-BMF (712.5 µg, 1.67 x 10^{-6} mol, 2 eq dissolved in 95 µl DMF) was added to the solution, vortexed and heated at 86 ºC for one hour. The solution was left overnight at room temperature. The unbound fluorescence was removed by washing with 1ml chloroform saturated reagent E (reagent B mixed 1:1 with methanol). The washing with concomitant vortexing and phase separation was repeated 17 times. Each chloroform phase wash was monitored by measuring absorbance at 450 nm with 690 nm as reference filter. The organic layer was collected and dried under N_2 (g) after five final washes with $CHCl_3$ saturated HCl:H_2O.

Quality control was performed using TLC on a silica gel thin layer plate. Chromatography was performed in two dimensions, with chloroform:methanol:water as the mobile phase in the first dimension, and 100% methanol as the mobile phase in the second dimension. For visualisation, the TLC was stained with 5% molybdatophosphoric acid solution. Fluorescently labelled MA was incorporated into liposomes for assessment by the biosensor.

2.5.2 Biosensor experiments

2.5.2.1 Preparation of mycolic acids, labelled mycolic acids or cholesterol-containing liposomes

For the preparation of the different liposomes, phosphatidyl choline stock solution (90 µl, 100 mg/ml chloroform) was added to an amber glass vial containing either mycolic acid (1 mg) or an equimolar amount of 5-BMF labeled mycolic acid (1.35 mg). For the preparation of cholesterol containing liposomes, phosphatidyl choline stock solution (60 µl, 100 mg/ml chloroform) was added to a cholesterol solution (30 µl, 100 mg/ml chloroform). The samples were mixed well until dissolved, then dried under a stream of N_2 (g) at 85 ºC. Saline (2 ml) was then added and the sample was heated on a heat block for 20 min. at 85 ºC. The sample was then vortexed for 1 min, sonicated with a Virsonic probe sonicator until a clear solution formed to indicate vesicle formation, aliquoted at 0.2 ml per vial, lyophilized and stored at -70 ºC until use. Before use, lyophilized liposomes were reconstituted with PBS buffer (2 ml). The liposomes were placed on a heat block for 30 min. at 85 ºC. Following incubation, the solution was vortexed for 2 min, then sonified briefly and a final liposome stock concentration of 500 µg lipid/ml was used for experiments.

2.5.2.2 Measuring the affinity between Amphotericin B and either mycolic acids, labelled mycolic acids or cholesterol

The binding interaction between Amphotericin B and either cholesterol, MA or fluorescein labelled MA immobilized liposomes were tested on an ESPRIT biosensor. The gold coated sensor disk used for immobilizing the lipids on, was first incubated with 10 mM octadecanethiol (ODT), dissolved in absolute ethanol for 16 hours. An automated software program sequence was created to control the addition of all the samples and liquids into the cuvette [117]. Filtered PBS/AE was used as buffer, and

He (g) was used to degas the buffer when needed. The sequence included flushing the cuvette with 500 µl ethanol (96%) using the automatic dispenser with simultaneous draining, followed by brief washing with PBS/AE. SPR 'dips' were continuously measured to ensure the quality of the surface for surface plasmon resonance. Cleaning of the ODT coated surface was done with 96% ethanol and a mixture of isopropanol and 50 mM sodium hydroxide (2:3, v/v) before the start of the experiment. The samples were transferred from a 384 multi-well plate (Bibby Sterilin Ltd, Stone, UK) to the cuvette surface by an auto pipettor. The baseline of the ESPRIT biosensor was formed with 10 µl PBS/AE, followed by addition of 50 µl test liposomes on the disc for 20 min. Washing was done 5 times with 100 µl non-degassed PBS/AE with mixing to obtain a baseline. Amphotericin B (1 x 10^{-4} M) was added to the liposome layer and the direct binding interaction was recorded for 10 min., after which the disk was washed 5 times with non-degassed PBS/AE, and left for 5 min.

2.5.3 Enzyme-linked immunosorbent assay (ELISA) to test the antigenicity of MA derivatives

For coatings done in PBS, methylester MA (ME-MA) or free MA (250 ug) was dissolved in 1 x PBS (4 ml, pH= 7.4) by heating at 90º C for 20 min on a heat block. PBS (4 ml) served as control. The solutions were vortexed for 30 s before sonifying for 2 min. using the Virsonic sonifier at output of 2. The warm solutions were subsequently loaded onto the ELISA plates (50 µl per well) and the presence of oily drops viewed under a light microscope. The plates were kept at 4 ºC overnight in plastic bags. For the coatings done using hexane as coating solution, the lipid samples (250 µg) were dissolved in hexane (4 ml, distilled) and vortexed for 30 s. Hexane (4 ml) served as control. Solutions were coated using a Hamilton syringe (50 µl per well) and the liquid was loaded in the centre of the wells. Lipid was visible as a circular waxy layer after 2 hours of evaporation of the hexane at room temperature. The plates were then stored in plastic bags at 4 ºC overnight [160].

The rest of the ELISA experiment and the experiment done with the fluorescein-MA were carried out as described below.

2.5.4 Enzyme-linked immunosorbent assay (ELISA) of synthetic MA

In the ELISA experiments, pooled TB positive and TB negative sera for active disease were used. Both groups were HIV negative. The sera dated from the year 2000 and was initially collected for another study [118]. To coat the ELISA plates with the different synthetic mycolic acid subclasses, natural mycolic acids or modified mycolic acids, the lipids were dissolved in hexane (3 µg/ 50 µl) and vortexed one minute, heated (at ~85 ºC) for a minute and allowed to stand at room temperature for 15 minutes. Hexane coating as such served as a control. The ELISA plates were coated with the different mycolic acids at 50 µl per well using Hamilton syringes. The lipids were visible as a waxy coating after 2 hours of evaporation at RT. Plates were stored in a plastic bag at 4 ºC overnight.

The following day the plates were blocked with 400 µl of 0.5% Casein / PBS pH 7.4 for 2 hours. The blocking buffer was removed and the wells were aspirated to dryness under vacuum and then coated with 50 µl per well of serum (1:20 dilution in 0.5% Casein / PBS pH7.4) for an hour. The plate was washed 3 times (Well Wash 4 ELISA washer) with 0.5% Casein / PBS pH 7.4. The plates were then coated with the goat anti-human Immunoglobulin G (H + L) peroxidase conjugate (50 µl / well) for 30 min. at RT. After the incubation the wells were washed three times with 0.5% Casein / PBS pH 7.4, then coated with 50 µl per well of the substrate solution consisting of 10 mg of OPD plus 8 mg of Urea- H_2O_2 in 10 ml of citrate buffer to measure the peroxidase activity. After 30 min. incubation at RT the plate was read with a SLT 340 ATC photometer at 450 nm with a reference filter at 690 nm. Background binding of the serum to the plate was corrected for by subtracting the average binding signal of serum to MA from that registered for the hexane coated wells. The results obtained were analysed by the making use of the Student's t test for statistical significance.

2.6 Results

2.6.1 Fluorescent labeling of MA

In order to study the binding of modified MA to AmB on the biosensor, the mycolic acids were labeled with 5-Bromomethylfluorescein (5- BMF) [161], the product formed tested on TLC and the Rf value determined for labeled MA (Rf = 0.89 in $CHCl_3$:MeOH:H_2O, 30:14:2). 5-BMF is a sensitive fluorescent reagent that is used for derivatization via carboxylic acids [159]. The reaction mechanism involves the conversion of the free acid to a salt and then reacting with the 5-BMF, using crown ether as the phase transfer catalyst to form the conjugated product [159].

By making use of thin layer chromatography (TLC), the coupling of the fluorescein to the MA was assessed. A reconstruction of the two dimensional TLC results are as shown in figure 2.1 and table 2.1. The Fluorescein-MA sample migrated in the 1st dimension, whereas a slight streak was visible in the 2nd dimension indicating a small fraction of label not linked to the MA. The 5BMF alone migrated in the 1st and 2nd dimension. Repeated washes (16 times) with acid water were used to remove any unbound fluorescent label from the Fluorescein-MA. The washed, labelled MA product was incorporated into liposomes for the biosensor studies.

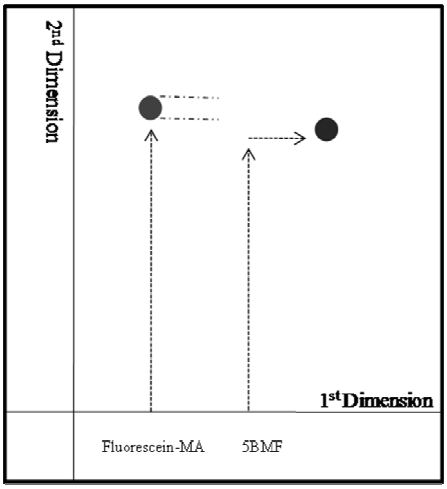

Table 2.1

	1st Dimension Rf value	2nd Dimension Rf value
Fluorescein-MA	**0.89**	**0**
5BMF	**0.86**	**0.93**

Figure 2.1 *Reconstruction of the two dimensional TLC. The Fluorescein-MA represent the coupled MA to 5BMF and the 5BMF the fluorescent label alone. The 1st dimension had a mobile phase of $CHCl_3$:MeOH:H_2O, 30:14:2 and the 2nd dimension had a mobile phase of methanol. Table 2.1 gives the Rf values obtained. The plates were visualized by staining with 5% molybdatophosphoric acid solution.*

2.6.2 Comparative Amphotericin B recognition of MA, labelled MA or cholesterol

A structural relationship and attraction between free MA and cholesterol was shown in our laboratories in a previous study [121]. This was demonstrated on the IAsys biosensor system, where the interaction between MA and Amphotericin B - an antifungal macrolide agent known to bind to cholesterol [162] – was shown. On the ESPRIT biosensor, the same principle was confirmed (Figure 2.2). In an attempt to determine what the effect of labelling of MA would be on its manifestation of cholesteroid nature, the binding interaction between Amphotericin B and either cholesterol-, MA-, or fluorescein labeled MA-containing immobilized liposomes were tested. The results confirmed the ability of the ESPRIT biosensor (SPR) to demonstrate that Amphotericin B recognizes both cholesterol and MA and shows for the first time how altering the structure of MA by adding a bulky label on its carboxylic acid group affects the binding to Amphotericin B (Figure 2.2).

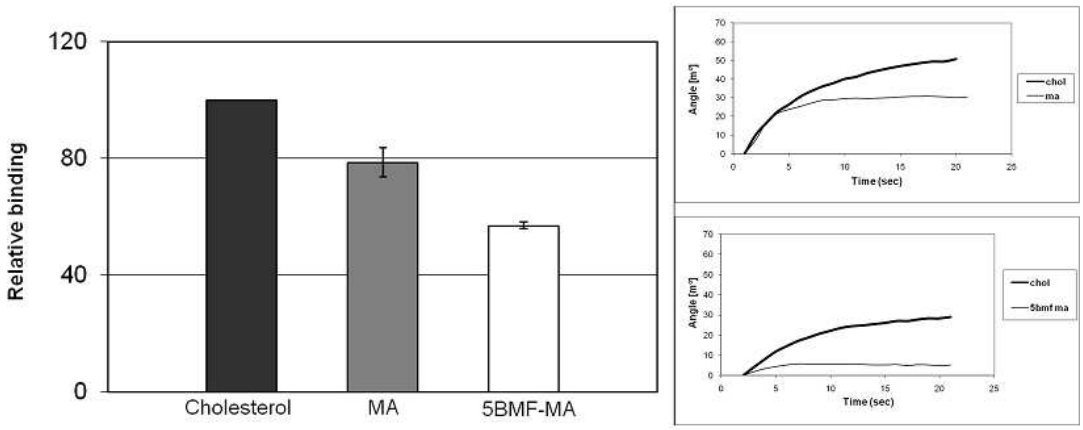

Figure 2.2 *Normalised AmB binding capacity (left) on immobilized lipid antigens: cholesterol, MA and Fluorescein-MA. Typical AmB binding curves (right) on immobilized MA or Fluorescein-MA compared to cholesterol. The error bars indicate the standard deviation, n = 3 for each set.*

A reduction in the relative binding for the labelled MA was observed, compared to the binding of MA and cholesterol. From these findings it could not be unequivocally established whether the fluorescent label on the MA destroyed, or merely affected the biological activity, as it is difficult to define the point of zero-activity with this application of the evanescent field biosensor. The ELISA experiments that follow were designed to shed more light on the matter.

2.6.3 Effect of a modified carboxylic group of MA on antibody binding and recognition

The cholesteroid nature of modified MA was implied in 2.6.2 by its ability to bind with AmB in an SPR biosensor. It was not clear whether derivatization of the carboxylic acid destroyed, or merely weakened its recognition by Amphotericin B, i.e. the cholesteroid nature of MA. To shed more light on this, the antibody recognition of modified MA was tested in an ELISA experiment. Pan *et al.* (1999) reported that methylesters of mycolic acids were recognised by TB positive patient sera in an ELISA assay [119]. Previously, our group showed that free mycolic acids were antigenic [118]. Here we tested the antigenicity of both the methyl- and Fluorescein-esters of MA to determine what the role of the carboxylic acid is in maintaining an

antigenic conformation. The ELISA plates were coated with the three different lipid antigens and TB patient sera were allowed to bind to it. The binding was measured at 450 nm with an indirect ELISA. Coating the antigens from hexane solution gave better results than when coating was done from hot PBS, but both methods served the purpose (Figure 2.4). When the carboxylic acid of MA was derivatised with 5- BMF (Figure 2.3), the TB positive and TB negative patient serum antibodies were still able to bind and recognise the molecule (Figure 2.4). This could be due to the carboxyl and hydroxyl group present on the fluorescein molecule that could substitute for the role of the carboxyl group of MA to stabilise the functionally active conformation by hydrogen bonding [102, 103, 121]. The methylated MA showed negligible binding compared to the labelled MA, despite the fact that both modifications entailed esterification of the carboxylic acid of the molecule. The results suggest that the binding of antibodies to mycolic acid relies on the availability of a free carboxylic acid in the vicinity of the mycolic motif. TB negative pooled sera also gave strong antibody binding signals to the natural MA as well as the Fluorescein-MA, possibly due to cholesterol cross-reactivity with mycolic acids as suggested previously [117, 118, 121].

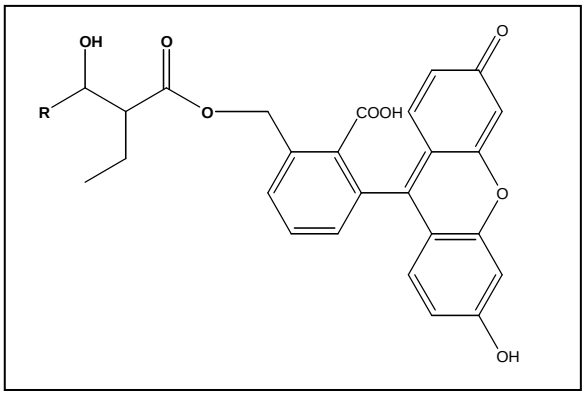

Figure 2.3 *Chemical structure of fluorescein labelled MA.*

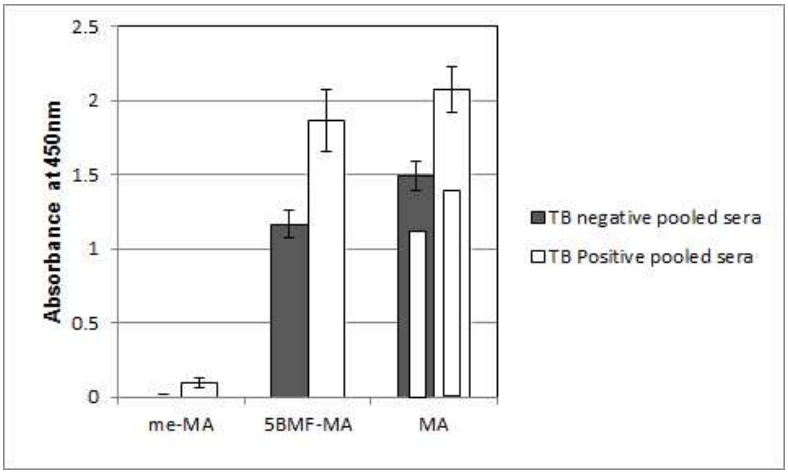

Figure 2.4 *Comparison of antibody recognition to MA, ME-MA [160] and Fluorescein-MA. The ELISA signals of pooled TB positive and pooled TB negative sera was measured at an absorbance of 450 nm to the whole fraction of MA, Fluorescein-MA and ME-MA [160] antigens coated onto ELISA plates from hexane. Inner bars indicated within the MA mix bars indicate the signals when coating was done from hot PBS instead of hexane. The error bars indicate the standard deviation.*

2.6.4 Response of patient sera to natural and synthetic mycolic acids

Mycolic acids in *M.tb* contain a mycolic motif, which is an α-alkyl, β-hydroxy acid. Stereochemically, the α and β positions relative to the carboxylic group present in all mycolic acids have been shown to be in the R-configuration. The main branch, known as the meromycolate moiety, contains two functionalities, which are differently substituted around the distal and proximal positions [97]. The proximal position is usually either a cis- or an alpha-methyl-trans-cyclopropane, while the distal functionality is usually a cis-cyclopropane or one of several oxygenated functional groups including hydroxyl-, methoxy- and alpha-methyl-keto- groups.

The relationship between the structure and the cholesteroid nature of MA was investigated by testing different stereo-controlled synthetic MA to antibodies in TB patient serum. The difference in binding to TB negative and TB positive patient sera was examined as well as the extent of binding that could be achieved with every synthetic MA with both types of sera. The hypothesis tested here was that a specific

synthetic MA structure can be found that represents the cholesteroid nature and that another synthetic MA structure manifests as the stronger antigen for antibody binding in TB positive patient sera.

The binding signals of TB positive and TB negative pooled sera towards the different synthetic MA subclasses were compared to that obtained towards natural free MA in ELISA. The importance of the stereochemistry of the merochains of mycolic acids for antigenic activity was studied by using different isomers of stereo-controlled chemically synthesized methoxy-, keto-, alpha- and hydroxy-MAs as antigens. Hexane was used as solvent to coat the plates with the MAs, similar to the approach of Pan *et al*. (1999) [119]. The four major subclasses of mycolic acids produced different results indicating the importance of the main functional groups on the merochain (Figure 2.5). Methoxy-MA bound strongest, followed in descending order by hydroxy-, keto- and alpha-MA. The exact stereochemistry of each subtype, i.e. the precise spatial arrangement of the functional groups, also appeared to be important.

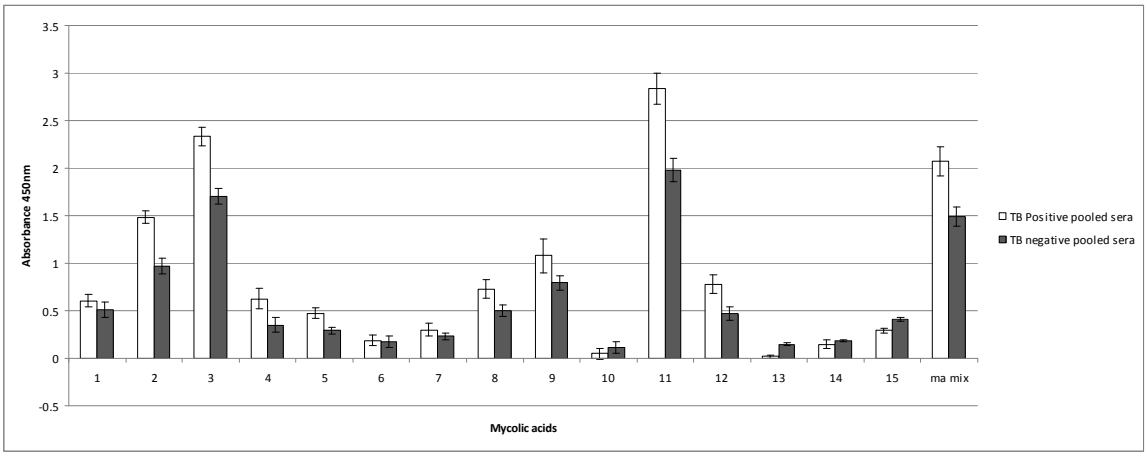

Figure 2.5 *ELISA signals of antibody binding in TB positive and TB negative pooled patient sera to synthetic (1 – 14), natural alpha- (15) and the natural mixture mycolic acids (ma mix). Methoxy-MAs: 1-4, 11; Hydroxy-MAs: 5, 7-9; Keto-MAs: 6, 10, 12; Alpha-MAs: 13-15. The error bars indicate the standard deviation. The 2.5 d rule was applied to remove outliers. n = min 7, max 16.*

The natural MA mixture as well as several of the synthetic MAs showed antibody binding with both TB positive and TB negative patient sera, while some MAs

appeared to have little or no antigenic activity. Strikingly, however, was the fact that the ratio of binding signal between TB positive and TB negative patient sera remained comparable among all the various types and isomers of MA. Although TB positive patient sera always gave better binding to the antigenic mycolic acids than TB negative patient sera, there generally was, in fact, no single antigenic mycolic acid that was significantly better able to differentiate between TB positive and TB negative patient sera than the natural mixture of mycolic acids could. This could in principle mean that if a MA is antigenic, then it assumes the cholesteroid functional structure that cross-reacts with anti-cholesterol antibodies. The question of whether a particular MA structure assumes a cholesteroid functional character then simply reduces to whether the particular MA is antigenic or not. The fact that TB positive patient sera statistically score higher than TB negative patient sera in recognition of MA in ELISA may then be explained simply by a higher concentration of the reactive antibodies in TB patient sera. Whether a single antibody would be able to recognize both cholesterol and MA, or whether cross-reactivity is due to the presence of a mixture of anti-cholesterol and anti-MA antibodies remains unanswered at this stage, although the observation that Amphotericin B can bind both cholesterol and MA (Figure 2.2) argues in favour of the former.

Each subtype's ELISA antibody binding signal (antigenicity) was subsequently compared to that obtained from the natural MA mixture and analyzed for its significance in defining the structure function relationship of MA.

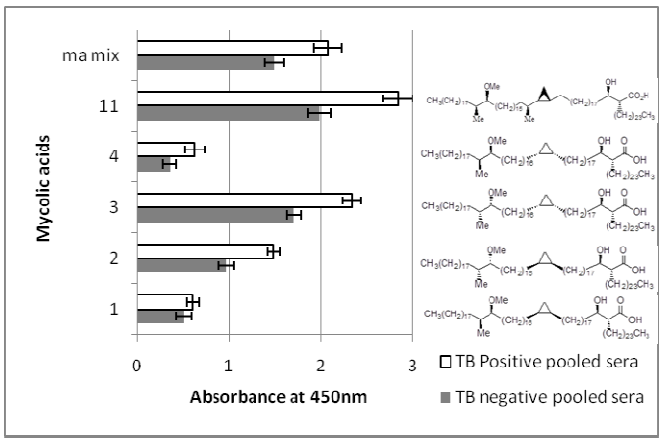

Figure 2.6 *ELISA antibody binding signals of TB positive and TB negative patient sera to synthetic methoxy mycolic acids. The error bars indicate the standard deviation. The 2.5 d rule was applied to remove outliers. n = min 14, max 16.*

Chemically synthetic MA derivatives were made that were representative of four MA subclasses, namely methoxy-MA, hydroxy-MA, keto-MA and alpha-MA. The synthetic methoxy MA subclass had the highest binding to the antibodies from the four synthetic subclasses tested, followed by hydroxy-, keto- and alpha MA (Figure 2.6). The stereochemistry of the methoxy group is important for the recognition by antibodies in the sera. Even small changes in the stereochemical arrangement of the groups influenced the amount of binding observed. As seen from figure 2.6 the antibody binding signal of RR-cis-cyclopropane, RS-methoxy MA configuration (no 3) most closely resembles the response towards the natural mixture of MA. A change of the stereochemistry of either the cyclopropane (no 2) to SR-cis, or the methoxy-group (no 4) to SS reduced the binding signal by approximately half. If the weaker SR-cis configuration of the cyclopropane is combined with the weaker SS-methoxy configuration (no 1), the signal is once again halved, as may be expected. However, when the cyclopropane group assumed the methyl-trans configuration (no 11), an antigenicity signal was obtained that was even higher than that obtained for the natural MA mixture, despite its association with the weaker SS configuration around the methoxy-group ($P < 0.01$). This proves beyond a doubt that the stereochemical configuration of the two functional groups on the mero-chain of the most antigenic methoxy-MA influences the way in which they are recognised by patient antibodies.

If the assumption holds that antigenicity and the cholesteroid configuration are one and the same functional property, then a synthetic methoxy MA with a methyl-trans cyclopropane configuration would provide the strongest targeting ligand to steer towards cholesterol-rich TB infection foci, at least when the methoxy group is in the SS-configuration. It remains to be determined whether the RS-configuration of the methoxy group combined with the methyl-trans cyclopropane will provide an even more antigenic/cholesteroid MA.

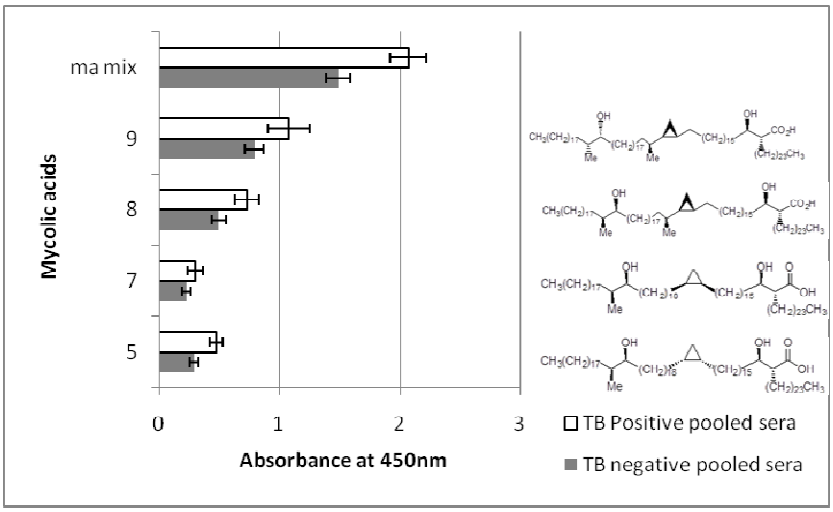

Figure 2.7 *ELISA antibody binding signals of TB positive and TB negative patient sera to synthetic hydroxyl mycolic acids. The error bars indicate the standard deviation. The 2.5 d rule was applied to remove outliers. n = min 15, max 16.*

The synthetic hydroxy mycolates (Figure 2.7), which are the likely precursors of both methoxy- and keto-mycolates [106, 115, 163] all attracted weaker antibody binding compared to the natural MA mixture. The methyl-trans configuration of the proximal cyclopropane group appears to be a pre-requisite for antigenicity of the hydroxyl MAs (no.8 and 9, compared to the RS-, or SR-cis-cyclopropaned no 5 and 7). The hydroxyl group in the RR conformation (no 9) is preferred over the SS configuration (no 8) with statistical significance, $P < 0.01$.

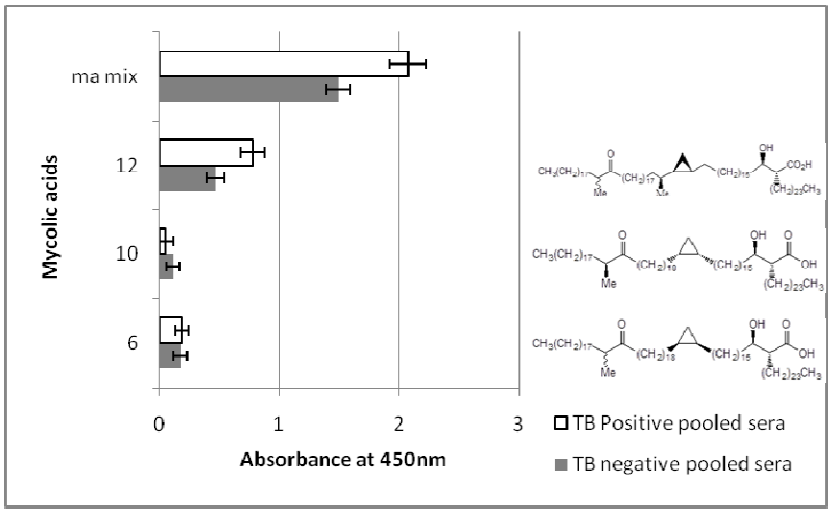

Figure 2.8 *ELISA antibody binding of TB positive and TB negative patient sera to synthetic keto mycolic acids. The error bars indicate the standard deviation. The 2.5 d rule was applied to remove outliers. n = min 15, max 16.*

Similar to the hydroxy-MAs, keto-MAs also require a proximal cyclopropane in the methyl-trans configuration (no 12, Figure 2. 8) to be antigenic, compared to the two (RS- and SR-) cis-cyclopropane configurations (no 10 and 6, Figure 2.8) that did not show any significant antigenic activity. In all the oxygenated MAs (i.e. methoxy-, hydroxyl- and keto-MA), the methyl-trans cyclopropane configuration provides for the best antigenic/cholesteroid functionality.

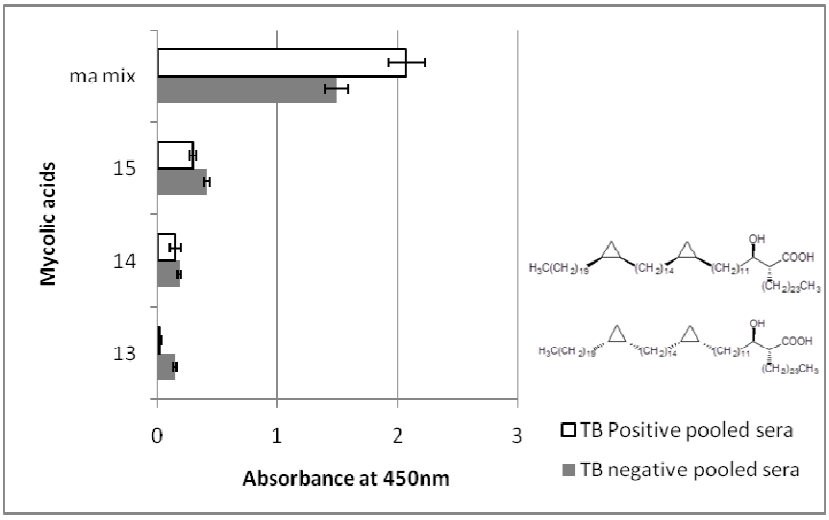

Figure 2.9 *ELISA antibody binding signals of TB positive and TB negative sera to synthetic alpha mycolic acids. The error bars indicate the standard deviation. The 2.5 d rule was applied to remove outliers. n = min 8, max 16.*

Two synthetic alpha MAs (no.13 and 14) were compared to natural alpha-MA (no.15) and the natural MA mix to determine their relative antigenicity. The antibody binding signal to either the natural, or the synthetic alpha MA failed to distinguish between TB positive and TB negative patient sera compared to the natural MA mix. The synthetic MA gave such low antibody binding signals, that nothing could be learned from the variations in the stereochemistries of the distal and proximal cyclopropanes on the antigenicity of alpha-MA. It could well be that the natural alpha MA gains its higher antigenicity from the presence of methyl-trans alpha MAs in the isolated mixture.

For the first time it is demonstrated here that synthetic, stereochemically and diastereomerically pure mycolic acids show differential biological activity in terms of recognition by TB positive patient serum. The results seem to favour methoxy mycolic acid of *M.tb* as the best cholesteroid functional entity or antigen to use in TB serodiagnostic devices, while a proximal methyl-trans cyclopropane enhances these functionalities.

2.7 Discussion

An antibody binding cross-reactivity between cholesterol and MA was first suggested by Schleicher *et al.,* (2002) [118] and subsequently shown to exist [121]. AmB is an antifungal macrolide that is able to bind cholesterol [162] through hydrogen bond formation between the amine or hydroxyl group of the mycosamine moiety of AmB and the hydroxyl group of the sterol [164, 165], as well as hydrophobic interactions [162]. It was shown also to bind to immobilized mycolic acids by means of a wave-guide evanescent field biosensor [121], thereby giving the first clue to a cholesteroid functionality of MAs. The same principle was applied and confirmed in the more popularly used surface plasmon resonance (SPR) variation of an evanescent field biosensor [117]. The antibody binding to mycolic acids with TB negative patient sera was most likely due to anti-cholesterol antibodies that are known to exist in all

humans [166, 167]. The cholesteroid nature of MA was previously shown to manifest also as an attraction between cholesterol and MA [168].

The structure-function relationship study of MAs was initiated by determining the role of the carboxylic acid. Methyl-esterification destroyed all functional activity, but esterification with fluorescein merely decreased the affinity towards AmB and antibody somewhat. The structure of fluorescein reveals that it has a free carboxylic acid that may remain available for hydrogen bonding to oxygenated functional groups in the mero-chain, probably to support a fold that determines antigenicity or a cholesteroid functional nature. Methyl-esterification of MA destroys a carboxylic acid that seems to be essential for hydrogen bonded stabilization of a functional oxygenated MA. Oxygenated (at least methoxy- and keto-) mycolic acids play a major role in the pathogenesis of the bacteria [108, 109]. Methoxy MA is the major free mycolic acid present in mycobacterial biofilms [141] that may be associated with the dormant stage of TB to create a cholesterol rich environment that may be utilized as a source of carbon nutrient. The attraction of cholesterol to MA that was previously demonstrated by our group [121] would fit well in such a hypothesis of nutrient provision to the dormant TB bacilli in an unvascularized tuberculous lesion. Cholesterol is able to cross bilayer membrane barriers by diffusion, independently of receptor mediated transport [169].

Mycolic acids might be attracted to cholesterol by means of their hydrophobic nature (Van der Waals forces) or through a more specific hydrogen bonding by the structural features of both molecules [121]. By Langmuir monolayer studies it was shown that a structural fold of the mycolic acids present itself in a “W” or a “Z” conformation giving a hydrophobic surface that could resemble a shape of cholesterol [97, 103]. The packing of mycolic acids in a Langmuir monolayer has previously been shown to differ among alpha-, keto- and methoxy-MA subclasses. Keto MA tended more towards a W-shaped configuration with exceptional rigidity in monolayers, whereas methoxy- and alpha-MA exhibited a more flexible conformation towards variation of experimental parameters [102, 103]. Thus, the packing of MA appears to be influenced by the orientation of the functional groups, and thus assumes different conformations for interaction with antibodies in sera.

For the first time it was demonstrated here that synthetic, stereochemically and diastereomerically pure mycolic acids show differential biological activity in terms of recognition by TB positive patient serum. The results seem to favour methoxy-mycolic acid of *Mycobacterium tuberculosis* as the best cholesteroid antigen for cholesterol attraction, based on its binding response with the TB negative patient sera. The results do not suggest with certainty what the stereochemical specifications are for the naturally produced mycobacterial mycolic acids found in the cell walls of *M.tb*. The best antigenicity seen in synthetic MA does not necessarily indicate the most likely structure of the mycolic acid antigen(s) in nature. In particular, it seems unlikely that a particular MA molecule can constitute an antibody binding site filling antigen or hapten. Rather, the surface created by packed mycolic acids is likely to be the structure that is recognized by antibodies, similar to the case of monoclonal antibody recognition of cholesterol [170]. Different from cholesterol, which is homogeneous of structure, MA exists as a mixture in *Mycobacterium tuberculosis*. It may well be that mycolic acid folding and packing is influenced by the presence of different types of MA subclasses and variants thereof together, such that the natural mycolic acid antigen(s) may never be recreated synthetically by the use of a single species of pure synthetic mycolic acid. Exact specifications for an optimal mycolic acid mixture to assume cholesteroid nature and be attracted to cholesterol may in future be determined by mixing various ratios of different MA subclasses as coating antigen in ELISA, or as cholesterol capturing entity in evanescent field biosensing. In summary then, the hypothesis that one particular mycolic acid class represents cholesteroid nature, while another is more antigenic in TB positive patient sera is hereby rejected. Antigenicity and cholesteroid nature appears to correlate with one another in mycolic acid structure.