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Novel recombinant anti-mycolic acid immunoglobulin tools for improved understanding and management of tuberculosis

by

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Summary

Tuberculosis (TB) is an infectious disease that is caused by the *Mycobacterium tuberculosis (M.tb)* species of bacteria. Despite this being discovered for over 100 years the disease continues to cause epidemics worldwide. The diagnosis of TB is a challenging aspect, with the current tests exhibiting many problems including: long time period between testing and accurate diagnosis, not enough sensitivity, not always accurate, and, in some cases, expensive. Thus novel biomarkers are urgently needed to aid in the management of the disease. Mycolic acids (MAs) are complex lipid molecules that are found in the cell wall of mycobacteria and in particular in *M.tb*. The fact that these lipids are species-specific makes them a key component for understanding the *M.tb* organism. While the isolation of these compounds from the organism can be a complex and costly process, the production of stereo-controlled, chemically synthetic tuberculous, mycobacterial MAs can now be conducted in the laboratory. In TB patient sera, antibody immune activity to MA is always accompanied by antibody immune activity to cholesterol. Investigations into the cholesteroid nature of MA have been underway for many years. Researchers have tried to find the MA subclass responsible for this characteristic of the lipids. In this study, we apply recombinant monoclonal antibodies to MA to correlate the cholesteroid functionality of mycolic acids to their structure by means of cross-reactivity of antigen recognition. This thesis reports the generation of three different chicken-derived phage-displayed single-chain variable fragments (scFv) that reacted similarly towards the natural mixture of MA, but differently to the three individual classes. The first antibody recognized all three classes of chemically synthetic MAs, the second only the two oxygenated types of MAs and the third only methoxy MA. The cholesterol cross-reactivity was investigated after grafting each of the three scFv types onto two configurations of constant chain domains – CH1-4 and CH2-4. Weak but significant cross-reactivity with cholesterol was found only with CH2-4 versions, probably due to the enhanced binding cooperativity, aiding in the

sensitivity. Cholesterol was only recognized by the two monoclonal antibodies that also were capable of recognizing *trans*-keto-mycolic acids, suggesting that the cholesteroid nature of mycolic acids is determined by the conformation of MA that is induced by this stereo-isomer of keto-mycolic acids. The 2017 World Health Organisation ministerial conference held in Moscow emphasized the dire need for faster acting drugs and technologies that quickly diagnose TB and determine the degree of drug resistance to improve the management of TB worldwide. It is anticipated that the gallibody tools developed here will find application in quick, affordable TB screening tests aimed at regular monitoring of people at risk of infection as well as to determine how TB patients respond to their anti-TB therapies.

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Abbreviations

AIDS: Acquired immunodeficiency syndrome

BCG: Bacillus Calmette-Guérin

BSA: Bovine serum albumin

Cas: Casein

CCD: Counter current distribution

CD: System of nomenclature for cell surface protein on white blood cells

cDMEM: Complete culture media

CDR: Complementarity determining regions

CFP-10: 10 kDa culture filtrate antigen

DBSA: Denature bovine serum albumin

DNA: Deoxyribonucleic acid

E. coli: *Escherichia coli*

ELISA: Enzyme linked immunosorbent assay

ESAT-6: 6kDa early secretory antigenic target

ETH: Ethambutol

Fab: Fragment for antigen binding

HEK: Human embryonic kidney

HIV: Human immunodeficiency virus

HRP: Horse radish peroxidase

IFN-γ: Interferon gamma

IGRA: Interferon gamma release assay

IgY: Immunoglobulin Y

INH: Isoniazid

IPTG: Isopropyl-β-D-thiogalactoside

LAM: Lipoarabinomannan

LB: Luria-Bertani

LFI: Lateral flow immunoassay

M. avium: Mycobacterium avium

M.tb: Mycobacterium tuberculosis

MA: Mycolic acid

MALIA: Mycolic acid lateral-flow immunoassay

MARTI: Mycolic acid real time inhibition

MDR: Multi-drug resistant

NBSA: Native bovine serum albumin

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

POC: Point of care

PP: Periplasmic

PPD: Purified protein derivative

PZA: Pyrazinamide

RIF: Rifampicin

scFv: Single chain variable fragment

SNF: Supernatant fluid

TAE: Tris-acetate-EDTA

TB: Tuberculosis

Th: T helper

TLC: Thin layer chromatography

TST: Tuberculin skin test

VH: Variable heavy

VL: Variable light

WHO: World Health Organisation

XDR: Extensively drug resistant

Chapter 1: Introduction

1.1. Tuberculosis

Tuberculosis (TB) is an infectious disease that is caused by the *Mycobacterium tuberculosis (M.tb)* species of bacteria (1). This disease dates back to Neolithic times where it was previously referred to as Pott spondylitis, scrofula and consumption (2). While evidence shows that the disease has been in existence for over 10000 years (3-5), the causative agent of TB was only discovered by Robert Koch in 1882 (1). Despite this being discovered already for over 100 years the disease continues to cause epidemics worldwide. It was estimated that approximately 11 million people in Africa were killed by the disease between the year 2000 and 2016. While the mortality rate (regarding TB) remains high in Africa, one should take note that the disease is a worldwide problem. The estimated TB incidence rates can be seen in Figure 1 (6).

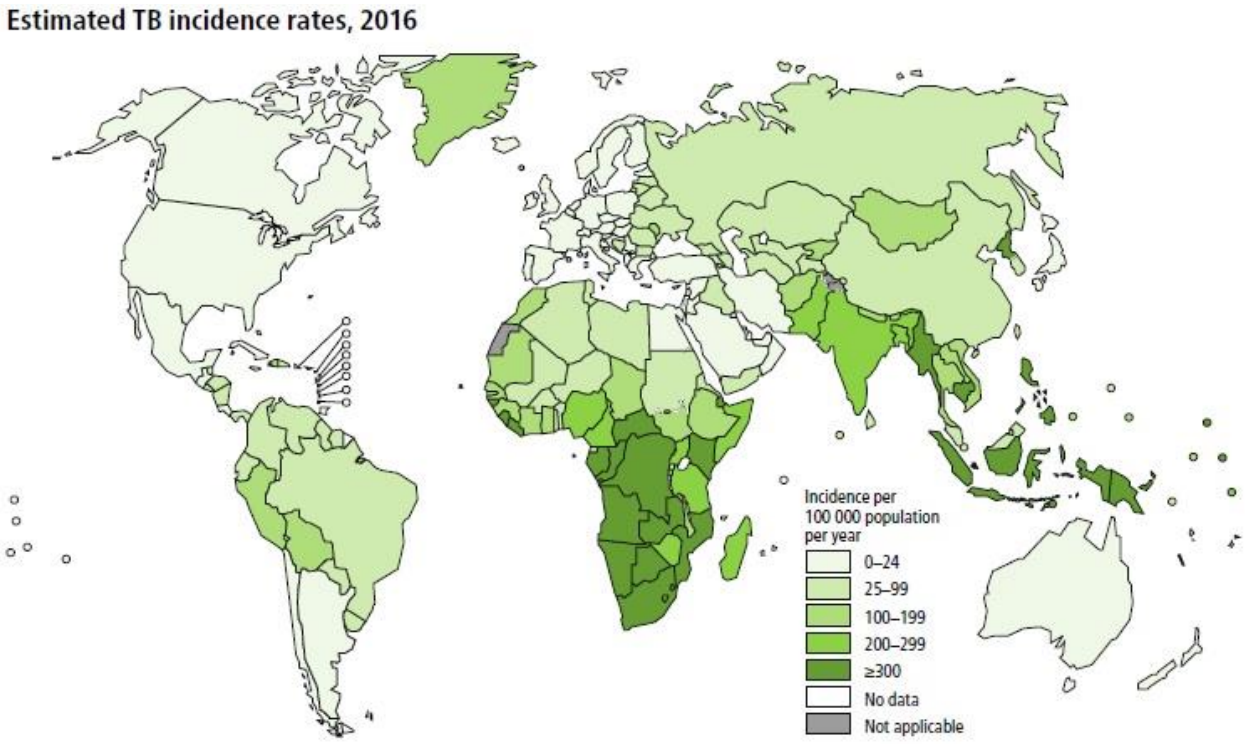


Figure 1: World map showing incident rate of TB infected people worldwide (6)

Figure 1 illustrates the incidence rate of the people infected with TB worldwide in 2016. Just as TB is a problem, there are a large number of people in the world that are infected with the human immunodeficiency virus (HIV). This disease has the ability to alter an individual's immune system and often these patients then contract TB also. In the year 2016 the World Health Organisation (WHO) reported that approximately 1.1 million people were living with a TB and HIV co-infection worldwide (6). This total contributes largely to the incidence rate of TB around the world. While the TB-HIV co-infection is a major problem worldwide it was reported in 2016 that more people were killed by TB disease than HIV alone (6). TB is a growing epidemic and is expected to expand if the disease is not curbed soon.

1.1.1. Disease progression in the host

Due to the fact that the pathogen is coughed up in an aerosol, it can be spread from person to person through the air by inhalation of droplets from the atmosphere (7). Infection with TB occurs when the bacterium enters the individual's body through the host's respiratory pathway (8, 9). The infection may spread through blood or other transporter fluids from the host's lungs to other parts of the host's body. In many cases the infection remains in the host's lungs where the infection first begins with phagocytosis of *M.tb* by alveolar macrophages. Within these macrophages the bacteria replicate and induce an inflammatory response in the infected area (8). The infected area becomes flooded with lymphocytes and macrophages, with these forming granulomas containing the pathogenic bacilli (8). The above mentioned process takes between two to six weeks to happen and is the deciding factor with regard to the establishment of TB. This can be seen in Figure 2 (7).

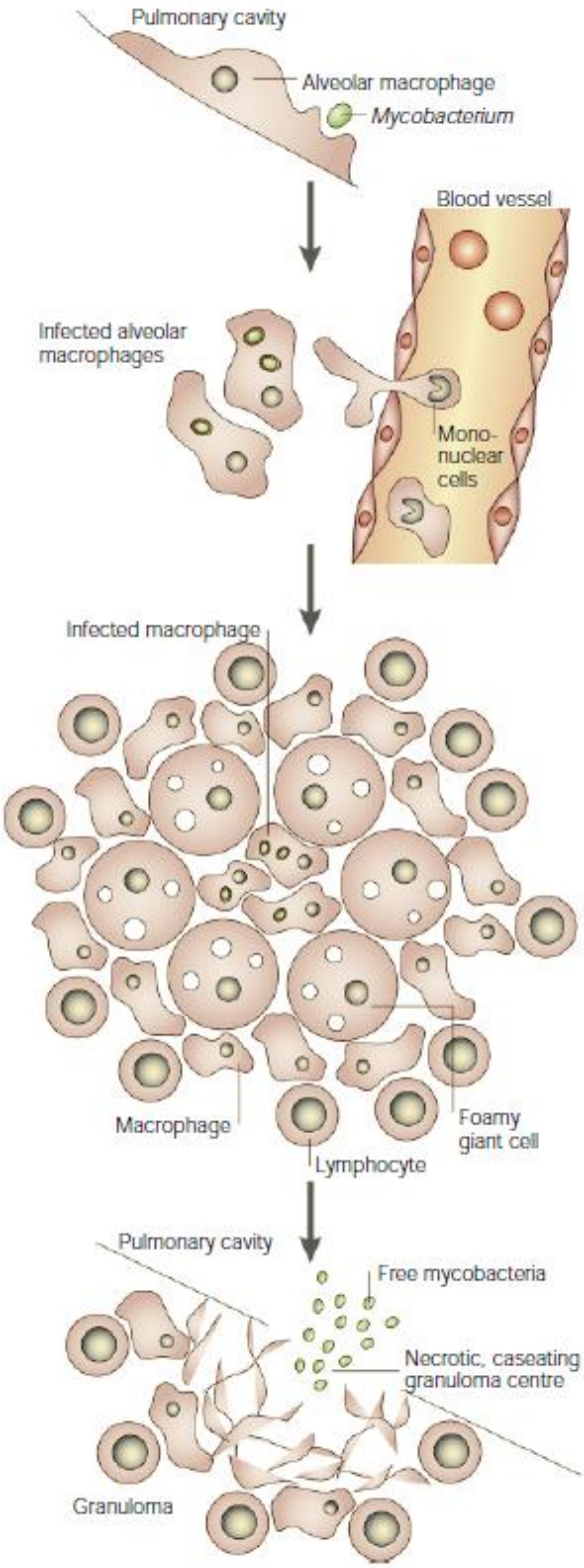


Figure 2: Initial stage of infection by *M.tuberculosis* bacteria (7). Obtained with permission

From Figure 2 we can see that free mycobacteria are present in the centre of the granuloma. Depending on whether these bacilli remain in the granuloma or are released into the host's body, determines whether the patient will develop pulmonary or miliary TB (8). These two forms of TB can further be classified into active TB, latent TB and persistent TB.

1.1.2. Active TB versus Latent TB

Active TB occurs when the bacilli are released from the granuloma into the host's airways. Active TB is a form of the disease that affects the person's prevailing health status. People living with this form of TB (active) are therefore at risk of dying from the illnesses depending on the strength of their immune system. Patients with active TB may undergo treatment in an attempt to cure the disease. However, if the treatment is stopped prematurely, the patient will develop persistent TB and is subjected to death by the disease. On the other hand, latent TB is the form of the disease that is in remission. This occurs when the bacilli remain in the granuloma. The person is therefore infected with the disease causing organism *M.tb*, but the body has triggered an immune response that controls and forces the disease into remission (10). It is important to take note that people with the latent persistent form cannot transmit the disease. One should take note that although the bacteria are not released from the granuloma immediately, they may be released at a (much) later stage. This may cause the individual's immune system to relapse and the host may become infected with the disease.

1.2. Treatment of Tuberculosis

There are four first line drugs available for treatment of TB and these are used when a person is diagnosed with active TB (11). The drugs are Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), and Ethambutol (EMB). Although treatment is available for the disease, the disease still remains a problem due to the lengthy treatment regime involved resulting in

patient non-compliance. There are two phases of drug susceptible TB treatment: a short initial phase of around two months followed by an extended continuous phase, which lasts approximately 4 months. During the initial phase, patients are required to take all four of the drugs, for a two month period. This is followed by the continuous phase where patients are only subjected to taking RIF and INH for four months. The problem of non-compliance comes into play in the initial phase, when after two weeks of treatment patients start to feel better and stop taking the medication, which in turn can lead to the development of drug resistant forms of the disease in the host. Although the issue of patient non-compliance is one problem, transmission of the disease and the development of drug resistant forms can continue to take place if patients do not obtain an accurate diagnosis in the "early" stage.

TB drug resistance can vary from mono-drug resistance to multidrug resistance (MDR) and even extensively resistant (XDR) (12-14). MDR-TB can be defined as a form of TB where the disease causing organism does not respond to the two most powerful TB drugs: INH and RIF. XDR-TB, as the name suggests, occurs when the bacteria develops resistance against all four of the available drugs. Treatment of the resistant TB strains is a costly and challenging process as the treatment time is longer (9-12 months), drugs are not always available and patients experience adverse side effects. As a result of this patients are often hospitalised to avoid spreading of these almost incurable forms of the disease (12-14).

1.3. Diagnosis of TB

1.3.1. Sputum microscopy

There are two gold standards available for the diagnosis of active TB, namely the sputum smear microscopy and culture methods. The sputum smear microscopy method is conducted by staining sputum samples collected from patients with the Ziehl-Neelsen stain, and inspecting

the sample microscopically for the presence of acid-fast bacilli (15). These mycobacteria, *M.tb*, are termed acid-fast as they resist destaining with acid alcohol. This is due to the highly lipid-rich component of their cell wall, which retains the stain and colours the organisms. The sputum smear method has shown to be relatively quick, inexpensive and highly specific, but displays low sensitivity in general, and notoriously so in TB patients that cannot produce sputum with detectable amounts of mycobacteria, such as children, patients co-infected with HIV or those with extra pulmonary TB. As a result of the low sensitivity found in the test, modifications have been made in an attempt to improve the sensitivity. An alternative method makes use of fluorescent stains, such as auramine-O, and a microscope with a mercury vapour bulb for visualizing the organisms. Although this modified method was shown to have increased sensitivity, it requires more specialized laboratory equipment; thereby making it a more expensive option.

1.3.2. Sputum culture

The other gold standard technique is the mycobacterial culture method (1, 16). Here, sputum samples are cultured on plates using Löwenstein-Jenson solid media and grown during a period ranging from 6-8 weeks. The plates are then inspected for the presence of mycobacterial colonies. Cord formation is characteristic of *M.tb* bacteria and therefore patients' sputa that display colonies with this feature are classified as being TB positive. This method is used to determine the morphology of the cultures in an attempt to get an idea of the species of mycobacteria present and any drug resistance that may have developed in the organisms. One may note that the method requires the use of a biosafety level 3 laboratory, highly trained staff and a lengthy time period that is usually increased in the case of HIV co-infected patients. While the time period to yield results can be improved by replacing the solid media with a

liquid form, the method is still highly complex, making it inappropriate for a point of care (POC) setting.

1.3.3. Nucleic acid amplification

The restrictions found using above mentioned techniques led to the investigation of using the Polymerase Chain Reaction (PCR) as analytical tool for detecting TB related nucleic acids. This idea was first explored by Forbes and Hicks (1993) where they used PCR to analyse patients' sputa (17). The results of their experiment displayed that this method had a sensitivity of 87.2% and a specificity of 97.7%. They also indicated that this method was safe for routine laboratory use for the following reasons: "First, dissemination of infectious aerosols is minimised since the tubes remain closed during processing. Second, infectious organisms are rendered nonviable following boiling and beating." Although these positive finding were established, there were limitations found to this method: PCR is a laboratory based method that requires highly skilled personnel as contamination to any substance can affect the entire reaction and its results. There is also a limit to the number of samples that can be analysed in 1 day (17). As a result the method is not accepted for use as a POC application.

1.3.4. GeneXpert

The production of the newly developed GeneXpert machinery from Cepheid eliminated the above mentioned limitations (18). This technology consists of a single-use, disposable cartridge and the GeneXpert machine. The cartridge, in which sample is placed, contains all the buffers and enzymes needed for the reaction. It also contains a PCR tube and is able to retain the wastes from the reactions taking place. The machine is software based and can perform both the nucleic acid isolation and real time PCR in an automated, hands-free machine process. This technology was first applied for detection of cancer in patients (18, 19). The

technology was then converted into the form of an MTB/RIF resistance test and used for TB diagnosis, currently known as the Cepheid Xpert MTB/RIF assay. It was shown to produce results in less than 2 hours to diagnose TB and to determine whether the infectious mycobacterial strain shows resistance to the TB drug RIF. GeneXpert requires sputum samples to be collected from the patients and which are then placed into cartridges that are, in turn, inserted into the GeneXpert machinery. This method of diagnosis was shown to be suitable for testing in clinical facilities outside the laboratory, as it does not require skilled personnel, is a hands-free test, and does not generate any aerosolized viable bacilli other than during sputum collection (18). Although proven to have made great strides towards more efficient TB diagnosis, the major drawbacks of the test are that it is expensive and can only detect resistance to one of the TB drugs, RIF. The latter was addressed by the development of a modified version of the machine known as the MTBDR plus assay, which is known to be able to detect resistance to three TB drugs (RIF, INH and ETH), thereby improving the diagnosis of MDR and XDR TB. Initial trials of this technology were promising; however, the technology is too expensive to be applied in POC settings.

1.3.5. Radiography

Radiography has been used extensively over the years for diagnosing pulmonary TB. This technique has been proven useful as researchers have identified key differences between healthy and diseased lungs over the years (20). In addition the technique provides an alternative method for diagnosing TB patients who have difficulty producing sputum – such as children and HIV co-infected patients. While shown to be effective for diagnosing TB, shortfalls of the technique have surfaced. The decline in sensitivity and specificity of the method concomitant with the rise of the acquired immunodeficiency syndrome (AIDS) epidemic, has resulted in clinicians recommending that the technique is used in combination with alternative diagnostic

tools to provide an accurate diagnosis, specifically for patients living with a HIV co-infection (21, 22).

1.3.6. Tuberculin skin test

The tuberculin skin test (TST) is another method that may be used for detection. In this case a sample of protein purified derivative (PPD), a protein isolate from the *M.tb* bacteria, is injected into the patient's skin and allowed to aggregate for 24-72 hours, after which results of inflammatory skin response are read (23). The results of the test are obtained by measuring the degree of induration produced by the administration of PPD. The measurements, expressed in millimetres, can be used to assess the presence of infection in different subpopulations. The interpretation of the indurance measurements are described in the Centres for Disease Control and Prevention guidelines on TB control. Although the TST was one of the first tests discovered for the diagnosis of TB, it is still used today for the diagnosis of latent TB infection. One of the major drawbacks of the TST is its lack of specificity. This is due to the fact that the test cannot distinguish between exposure to or infection with *M.tb* or other forms of mycobacteria.

1.3.7. Shortfalls related to current diagnosis

The above mentioned techniques have been successful in diagnosing adult patients with uncomplicated pulmonary TB alone. However, the problem arises when children or HIV co-infected patients need to be diagnosed. There are two problems arising in these cases. The first being the fact that these patients often have difficulty in providing sputum samples with detectable quantities of mycobacteria for the test. This is most problematic among children who then need to be diagnosed by mycobacterial growth from stomach lavages, after testing positive with the skin test method (24). The second problem that arises is the issue of false

negative diagnosis. This often occurs in the patients co-infected with HIV. These patients have a low CD4 cell count that influences the quality of their sputum samples, often leading to a false negative result. This indicates that these tests are not adequately accurate for the diagnosis of TB. In order to eliminate this problem, one would need to develop a test that works with a method that does not involve the use of sputum samples.

1.4. Blood-based diagnosis

Many serological diagnostics have been proposed in the past for the testing of TB. These tests mainly make use of Enzyme linked immunosorbent assay (ELISA), using antibody detection, as a method of testing. The methods suggested making use of components of the cell wall of *M.tb* as antigens for the detection of biomarker antibodies of active TB. The cell wall of *M.tb* bacteria consists of lipids, proteins and carbohydrates, but is dominated by the lipid component. This can be seen in Figure 3 (25).

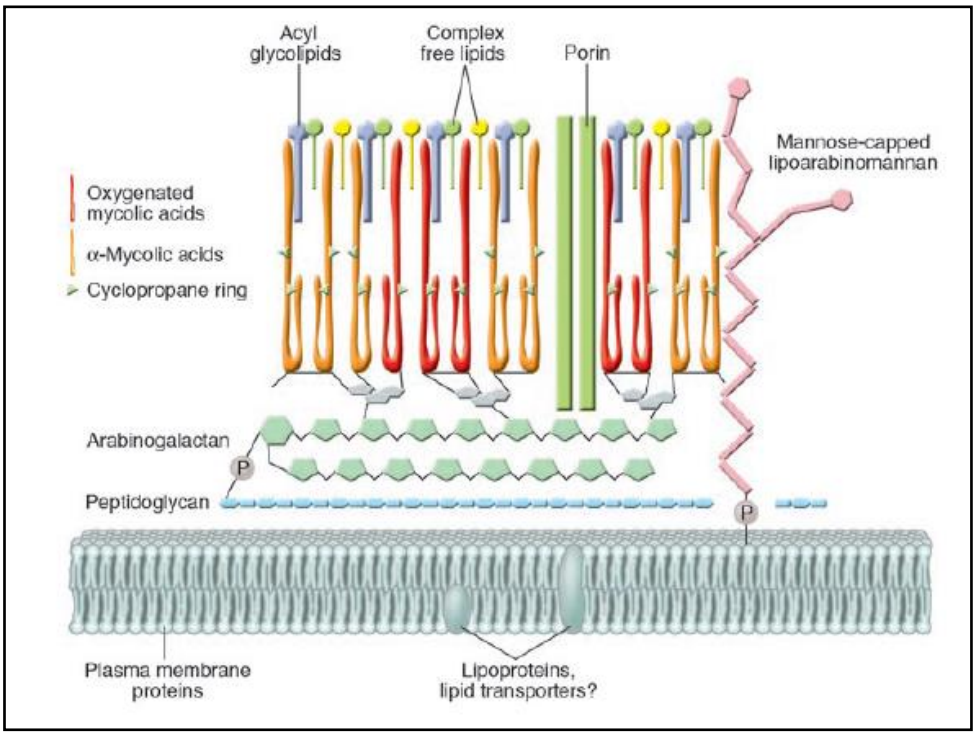


Figure 3: Schematic diagram depicting cell wall of *M. tuberculosis* (22). Obtained with permission.

1.4.1. 38 kDa antigen

Wilkinson *et al.* (1997) explored the option of using the 38 kDa antigen for the serodiagnosis of TB (26). The study conducted tested the 38 kDa antigen in an *in vitro* assay of T cell function and serology. The results showed that this test had a sensitivity of 72.6% and specificity of 94.9%. Although these findings suggest that the test will be useful for future use, a problem was identified as the test could not detect the difference between inactive TB versus latent TB. Another problem identified was that the test showed a sensitivity and specificity of approximately 50% for HIV co-infected patients, in which detection of TB still remains a problem (26).

1.4.2. Lipoarabinomannan

Another article published by Sada *et al.* (1990) suggested the use of the lipoarabinomannan (LAM) antigen in an ELISA experiment aimed at the serological diagnosis of TB (27). This method used the LAM compound in an acylated and highly antigenic state. The results collected displayed that the test had a sensitivity of 72% and a specificity of 91%. This study was the first to highlight the potential for LAM in TB diagnostics. One such test that exploits this potential is Determine™ TB LAM Ag test available from Alere Healthcare (Alere Inc., Waltham, MA, USA), which is discussed in more detail in section in 1.6.1.

1.4.3. Interferon gamma release assay

Due to the shortfalls observed with the TST, a new *in vitro* assay has been developed to better diagnose latent TB infection. This assay, termed the interferon gamma release assay (IGRA), is a blood based test that measures interferon gamma (IFN-γ) release after stimulation with TB specific antigens (28). Previously PPD was used as the TB antigen which elicited IFN-γ release by blood exposed to the antigen. However, past studies have shown that using this antigen

decreased the specificity of the test, especially in patients that were previously vaccinated with Bacillus Calmette-Guérin (BCG), because PPD is present in multiple forms of mycobacteria. This led to a revised version of the test that makes use of antigens exclusively present in the *M.tb* organisms. The new IGRA test involves the incubation of whole blood samples with a mixture of *M.tb* specific peptides. A study conducted by Mori *et al* illustrated the use of ESAT-6 and CFP-10, antigens specific to *M.tb*, in an adapted version of IGRA (29). These authors' modified IGRA displayed improved sensitivity and specificity as results obtained were not affected by a patient's prior BCG vaccination.

1.4.4. Anti-mycolic acid antibodies

Finally, a method is explored from an idea suggested by Pan *et al.* (1999), which makes use of mycolic acids (MA) as precursor molecules for the detection of TB (30). This idea was further explored in an experiment done by Schleicher *et al.* (2002), where an ELISA was done using MA as antigen to detect anti-mycolic acid antibodies found in patient sera (31). The results showed that there was a larger amount of anti-mycolic acid antibodies present in TB positive patients compared to TB negative. Due to this result, further experiments are being conducted to produce a diagnostic test that uses mycolic acid and its respective antibodies, anti-mycolic acid antibodies, as detectors for the presence of TB (32). The Mycolic acid Antibody Real Time Inhibition (MARTI) assay, makes use of this notion (33). This method makes use of immobilized liposomes (carrying MA) on IAsys biosensor cuvettes, which are then used to monitor the binding of anti-mycolic acid antibodies (32). The proposed MARTI test showed positive results for detection of TB using this principle. However, it was shown that the biosensor technique is very difficult and expensive to perform in the laboratory and the suggested technology is not yet suitable for screening large numbers of patients' sera in POC settings (33). Recently a follow up study on the use of anti-mycolic acid antibodies for TB

detection was published by Jones *et al.* (2017) (34). In their study the authors make use of synthetic MA linked to trehalose for detection of the biomarker antibodies, thereby illustrating that the sensitivity and specificity of the ELISA based test can be improved by using a range of mycolic acid antigens linked to trehalose.

The promising results gained by the detection of biomarker antibodies to MA led our group to conduct further research into the antigen and its potential for improving TB diagnosis.

1.5. Mycolic acids

Mycolic acids are complex lipid molecules that are found in mycobacteria in general and therefore also in *M.tb* (35, 36). These compounds have been shown to be present as a major component of the cell wall of mycobacteria and to be associated with an insoluble matrix that is esterified to carbohydrate-containing molecules (35). There are three types of MA, namely α-meromycolate, keto-meromycolate and methoxy-meromycolate (37). The structures of these β-hydroxy-α-alkyl branched lipids are shown in Figure 4 (38).

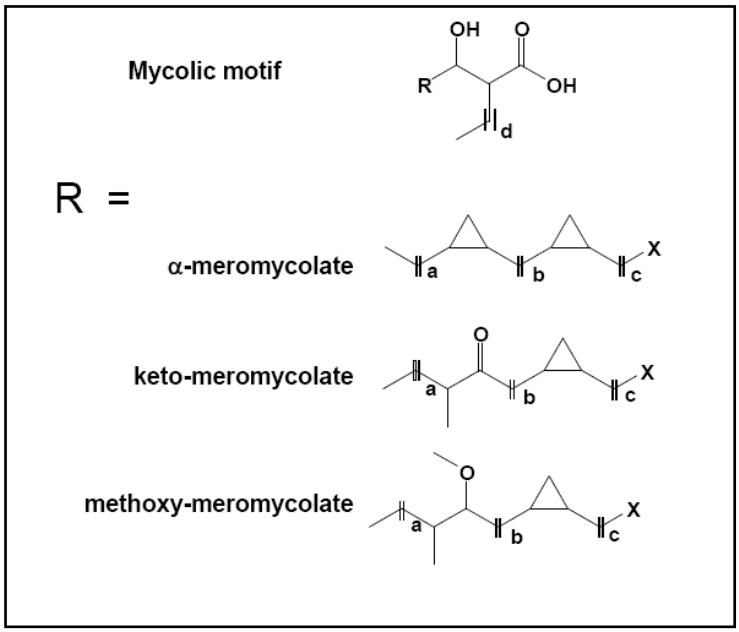


Figure 4: Graphical representation of the structures of mycolic acids (38). Obtained with permission.

In addition to these forms, MA are present in a compound named Trehalose 6, 6'-dimycolate that is a characteristic of *M.tb* in both its cell wall and culture supernatants grown with the bacterium (35, 39). Trehalose 6, 6'-dimycolate, commonly known as the cord factor, is responsible for inhibiting *in vitro* endogenous pulmonary surfactant activity and thus aids in the progression of the TB disease. Mycolic acids can be obtained through isolation from cultures of the *M.tb* organism (40) or by the production of synthetic derivatives in the laboratory (41-48).

1.5.1. Benefits of MA antigen

While the MA lipids are found in all mycobacteria, the combinations of the lipid classes present allow for distinguishing between the different bacterial strains. For example, the combination of all three lipid classes – alpha, keto, and methoxy MA – have been shown to be present in *M.tb,* whereas *Mycobacterium avium* (*M. avium)* only exhibits the alpha- and keto-subclasses. These lipid molecules make use of an alternative pathway of elimination in the immune system compared to proteins. The CD1 family of leukocyte proteins, discovered in the late 1970s, are able to present lipid and glycolipid antigens to T cells (49, 50). The human CD1 family is composed of five genes located on chromosome 1, with four of the genes having the ability to form protein products. These products, namely CD1a, CD1b, CD1c and CD1d, are able to work together or individually in the presentation of lipids and glycolipids as antigens (49, 50). In particular, it was found that CD1b in humans presented MA to CD4-, CD8- double negative T cells (51), which were subsequently shown to play a role in the regulation of auto-immunity (52). As a result of this the lipid molecules follow a different pathway of antigen processing and presentation to that of proteins, in particular by avoiding major histocompatibility complex (MHC) presentation in antigen presenting cells and CD4 mediated recognition by T cells.

This allows the immune response to MA to remain unaffected by any degree of a patient's HIV co-infection.

1.5.2. Cholesterol cross-reactivity

While MA was shown to be a promising antigen for the detection of biomarker antibodies for TB, these compounds have also shown to have a shortfall in the form of cholesterol cross-reactivity. The cholesteroid nature of MA was first reported by Benadie *et al*. (2008) who were able to show that TB positive patient sera bound equally well to MA and cholesterol (53). Furthermore they were able to demonstrate the structural similarity that can occur between the MA and cholesterol compounds. Later, Beukes *et al.* (2010) confirmed the antibody cross reactivity that occurs between cholesterol and MA by demonstrating the effect with monoclonal recombinant single chain variable fragments (scFv) that could recognize both MA as well as cholesterol (54).

1.5.3. Mycolic acid Lateral-flow Immunoassay

While the above mentioned methods for the detection of biomarker antibodies to MA display potential for improving TB diagnosis, the technologies are still too advanced to be used in a POC setting. This led our group to expand our research and, in turn, explore the option of using the principle to develop a new lateral flow diagnostic test for POC facilities. The test, to be named the Mycolic Acid Lateral-flow Immuno Assay (MALIA) test, was developed from the MARTI test concept (55). The test will take the form of a lateral flow test, similar to a pregnancy test, in which TB can be diagnosed in a time period of less than 1 hour. As the name MALIA suggests, MA are used in the test to indicate the presence of active TB in a patient with the aid of monoclonal as well as patient anti-MA antibodies. The MALIA method is

anticipated to be more successful than other methods as it is designed to be capable of screening high numbers of patient sera in a small time period.

1.6. Immunochromatographic based diagnostics

The use of lateral flow immunoassays originated in the 1980s but remains ever popular in recent times (56). This diagnostic device first illustrated its potential in the form of a lateral flow pregnancy test that was able to detect human chorionic gonadotropin, a hormone secreted from the placenta during pregnancy that can be detected in a woman's urine sample (56, 57). Lateral flow devices consist of the following components: a sample pad, conjugate pad, nitrocellulose membrane and an absorbent pad (58). These are set up as illustrated in Figure 5 below.

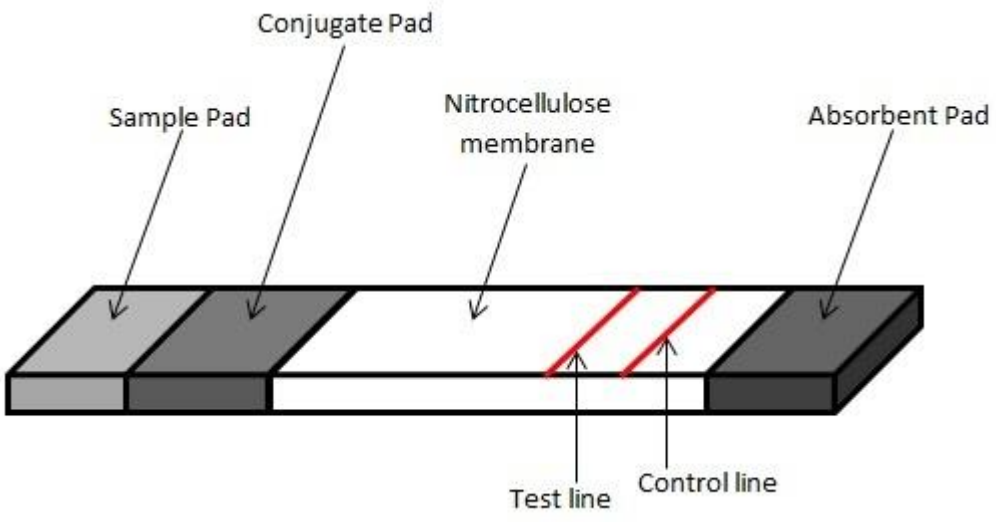


Figure 5: Diagrammatic representation of a lateral flow device.

The device is able to transport the sample fluid (item to be tested), from the sample pad using microfluidic technology known as capillary action (56, 58). The active site of the lateral flow device, typically known as the nitrocellulose membrane, contains the test strip. The purpose of this membrane is to capture the antigen or antibody of interest, usually done at the test line.

The control line is designed to capture unbound conjugate antibodies from the conjugate pad in an attempt to illustrate that the test is functional.

1.6.1. Determine™ TB LAM

The use of lateral flow devices is not a foreign concept in TB diagnostics. One prime example is the Determine™ TB LAM antigen test available from Alere Healthcare (Alere Inc., Waltham, MA, USA). This test is designed to detect the *M.tb* cell wall component LAM in patients' urine samples. An anti-LAM monoclonal antibody labelled with gold is deposited in the conjugate pad of the test to capture and carry samples presenting the LAM antigen. The antigen-antibody complex then travels up the test to the antigen placed on the test line resulting in a colour change (plasmon resonance induced by clustered gold particles) when a result is positive. The major drawback of the above mentioned test is that it has been shown to have an extremely low sensitivity in patients that are not in an advanced stage of AIDS (59). As a result it has been suggested in review articles that the test be used in combination with the more established tests, such as the Xpert MTB/RIF assay, in order to obtain more accurate results (60).

1.6.2. Capilia TB-Neo Assay

The Capilia TB-Neo Assay is another example of an immunochromatographic test for the detection of TB. This test has been developed to detect the MPB64 protein found in *M.tb* culture samples by making use of anti-MPB64 monoclonal antibodies (61). When a sample is applied to the test the gold labelled anti-MPB64 antibody binds to the MPB64 antigen present in the sample, and travels with it to detector antibody immobilized on the test line. The production of a red band at the test line indicates a positive result (61). This test has been shown to have a sensitivity of 99.2%, a specificity of 96.4%, and a reproducibility of 89.3% (62). While the

potential of this test is shown by its sensitivity and specificity, the test makes use of *M.tb* cultures as the sample input – a sample type that can only be handled in a biosafety 3 laboratory (63). Thus this technology is not ready for POC settings.

1.6.3. Antibodies in immunochromatographic assays

In the examples of immunochromatographic assays mentioned above it can be noted that antibodies and their detection are a key component of this technology. Lateral flow tests make use of antibodies for the detection of antigen or antibodies in samples. These antibodies are labelled with a detector molecule to provide a means of visualising the molecule of interest in a sample.

Antibodies are very specific compounds that bind to their respective molecules through a complementary binding site. These molecules can be obtained by isolating them from the serum of blood or through antibody engineering techniques in the laboratory. These methods will be discussed later on in the chapters that follow.

Due to the fact that the antibody molecules are key components in lateral flow devices, MA specific antibodies are required for MALIA. Previous work conducted by Dr Lindokuhle Ndlandla, a former PhD student in our research group, yielded the expression of phage-displayed scFvs that are either MA mono-specific (for the methoxy MA acid sub-classes) or cholesterol-cross reactive (64). These monoclonal antibody fragments are only scFvs, and as such are not suitable for use in the proposed lateral flow device. This project will therefore focus on the genetic engineering and expression of the antibody fragments such that they may be conjugated to colloidal gold as well as applied for use in a lateral flow set up.

1.7. Problem Statement

Current TB diagnostic tests have been shown to exhibit many problems including: long time period between testing and accurate diagnosis, not enough sensitivity for a particular type of TB (e.g. pulmonary or miliary) or patient (e.g. children or HIV co-infected), not always accurate, and, in most cases, too expensive and sophisticated for POC applications. Most of these methods make use of sputum samples obtained from the patient, a procedure that holds infection risk for the clinical staff attending to the patient. At present new methods are being explored that will not only be able to diagnose patients using blood samples, thereby avoiding risky sampling methods, but also covering child TB, extrapulmonary TB and TB in HIV burdened populations. It should also reduce the time period between sampling and diagnosis of TB. One of these methods employs MA and anti-mycolic acid antibodies as the indicators of presence of the disease. The Mycolic-Acid Real Time Inhibition assay makes use of MA and antibodies against mycolic acid for the detection of TB using a biosensor. Although this method has been successful in the serological diagnosis of TB, the biosensor equipment requires highly-skilled personnel for operation and costs of the unit are extremely high; thereby making this system not feasible for simple diagnosis. As a result, a new device needs to be developed employing the use of MA and anti-mycolic acid antibodies in a simple process that can be used with minimal effort in a POC environment.

1.8. Hypothesis

Labelled recombinant monoclonal antibodies to mycolic acid can be made to be displaced by TB patient biomarker antibodies in an affordable, effective and fast serodiagnostic assay for active TB.

1.9. Aims

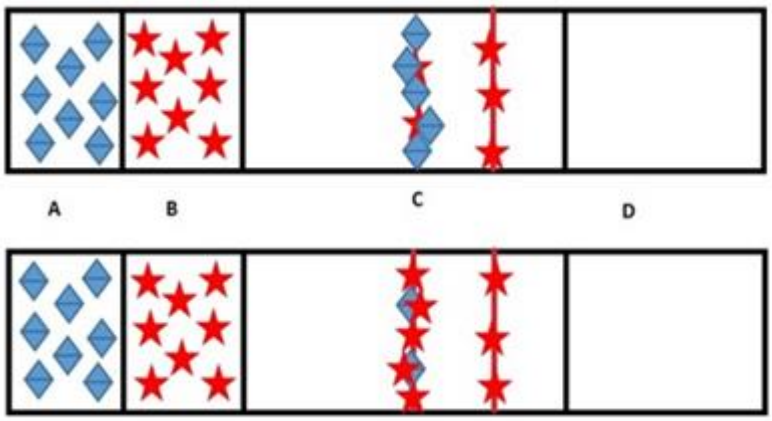
- Production and purification of MA from *M. tuberculosis* organism
- Development and characterisation of a set of recombinant monoclonal chicken antibodies. (Gallibodies) against MA and proof of principle of their potential application in lateral flow TB immunodiagnosis.
- Determining the feasibility of a mycolic-acids-antigen based lateral flow device to diagnose TB.

Chapter 2: Aims and strategies of the study

Current Tuberculosis (TB) diagnostic tests have been shown to exhibit many problems including: long time period between testing and accurate diagnosis, not enough sensitivity, not always accurate, and, in some cases, expensive. Most of these methods make use of sputum samples obtained from the patient. At present new methods are being explored aiming to diagnose patients using blood samples, which may improve diagnosis of paediatric TB, extrapulmonary TB and TB in HIV burdened populations, while at the same time reduce the time period between testing and diagnosis of TB. One of these methods employs MA and anti-mycolic acid antibodies as the indicators of presence of the disease. The Mycolic-Acid Real Time Inhibition assay makes use of MA and antibodies against mycolic acid for the diagnosis of active TB using a biosensor. Although this method has been successful in the serological diagnosis of TB, the biosensor equipment requires highly-skilled personnel for operation and costs of the unit are extremely high; thereby making this system unfeasible for simple diagnosis.

As a result, a new device needs to be developed employing the use of MA and anti-MA antibodies in a simple process that can be used with minimal effort in a POC set up. This gave rise to a project working towards developing MALIA. MALIA has been designed as POC negative-predictor screening test that will take a form similar to a pregnancy test, enabling the patient to obtain their results in less than 4 hours. The development of MALIA begun within the TB research group of the Department of Biochemistry, University of Pretoria in 2016 where Beukes and Verschoor patented the proposed technology (55).

The principle of the test is based on a competition assay whereby patient serum antibodies will compete with a gold labelled monoclonal antibody displacement probe for binding to MA. The test set up can be seen in Figure 6.



TB positive = no colour development at test line.
Colour only at control line
Serum antibodies/free MA in blood outcompete displacement probe

TB negative = colour development at both test line and control line.
Displacement probe outcompetes serum antibodies/free MA in blood

Figure 6: Composition of the MALIA lateral flow test. A - Sample pad with patient antibodies (♦), B - Conjugate pad containing gold labelled displacement probe (★), C – Nitrocellulose membrane with test line and control line, D – Wick.

Figure 6 illustrates the proposed MALIA technology. Patient antibodies added to the sample pad will travel along the strip from the start point in the sample pad through to the wick. As the patient serum antibodies travel through the conjugate pad they will come into contact with the gold labelled displacement probe thereby starting the binding competition for antigen between the two antibody types. The patient serum anti-MA antibodies and the displacement probe are then to compete for binding to the MA deposited at the test line. In addition to the test line, a control line will also be present on the nitrocellulose membrane to capture the unbound displacement probe, thereby illustrating the correct functionality of the test. Due to the fact that the test is designed as being a competition assay, the results can be interpreted by the colour change taking place. A TB positive case will be represented by a colour development occurring at the control line only. This will indicate that the patient serum anti-MA antibodies were able to outcompete the gold labelled displacement probe in binding to the MA, thus no signal will

be present at the test line. In contrast a colour change taking place at both the test and control line will indicate a negative result illustrating the ability of the gold labelled displacement probe to outcompete the patient serum anti-MA antibodies.

The work on this project began with Beukes *et al*. where the authors were able to successfully pan for MA-specific antibodies from a recombinant chicken antibody library (54). While the team could successfully isolate both cholesterol cross-reactive and non-cross-reactive anti-MA phage antibodies, their stability could not be retained over time. In a follow up attempt, PhD student Lindokuhle Ndlandla was able to select a new set of anti-MA phage antibodies, using a similar approach, by selecting for thermally stable scFvs (64).

Although the original MALIA patent was described making use of the so-called scFv antibody fragments, results have shown that scFvs released from phages have the ability to naturally form dimers and trimers (65, 66). As a result the valency and concentration of antibody could not be controlled easily – the control of this parameter being essential in competition assays. Thus the aim of this study was to continue the development of the displacement probe for use in the proposed MALIA test.

Chapter 3 of this thesis is focused on the conversion of the anti-MA phage antibody fragments of Ndlandla (64) into full chain bivalent immunoglobulin Y (IgY) antibodies, referred to as gallibodies. This chapter explores the technology of antibody engineering, which includes methods for the manipulation of genetic material, cloning, gel analysis techniques, mammalian cell culture and antibody characterisation. The successful production of the gallibodies formed the basis of the work in the remaining chapters.

The specificities of the anti-MA phage antibody fragments has brought to light their importance for use in MALIA. Although the antigen binding characteristics of these scFvs were conducted while displayed on phages, their specificities had to be confirmed after the conversion from antibody fragments to gallibodies. Thus, Chapter 4 describes the integration of my work with that of the PhD work of Lindokuhle Ndlandla in characterising the monoclonal antibodies. This resulted in a manuscript that was submitted for publication, which provided deeper insight into the cholesteroid nature of the MA, by the way that the two antigens are recognised by the different monoclonal anti-MA gallibodies.

In the final Chapter 5 the potential of these newly developed recombinant monoclonal antibodies as diagnostic tools for the detection of active TB is discussed. The application of the gallibodies in various other new and existing products for improved management of TB is discussed.

Chapter 3: Development of antibody displacement probe

3.1. Introduction

The immune system is the defence mechanism of the human body that acts in response to invasion by foreign encounters (67, 68). The type of defence response produced by the body is dependent on the type of attack. As a result there are two types of immune responses that may be formed, namely the innate and adaptive immune responses. The innate immune response, also known as the first line of defence, is a rapid non-specific reaction produced in response to pathogens that have crossed the physical barriers of the body (69, 70). These pathogens, which may be in the form of bacteria, are attacked by leukocytes that make use of phagocytosis for elimination (71). Inflammatory responses are also non- specific responses executed due to innate immunity as a result of physical damage to the tissues of the body (68-70, 72).

The adaptive immune response is a more specific and specialized response elicited when the body recognises the presence of a pathogen as being foreign (68, 71, 73). Once a pathogen has been identified as being foreign the response begins with the presentation of the pathogen's proteins, also known as antigens (74), on membrane proteins such as the MHC molecules (68, 71, 73). There are two types of MHC molecules, namely MHC class I and MHC class II, which are responsible for the presentation of short peptides to T-cells. The type of T-cell activated is dependent on the MHC class that the antigen is presented on. If the antigen is presented on a MHC class I molecule, then CD8+ T-cells will respond and produce a cytotoxic response (67, 72, 73). Cytotoxic T-cells may puncture any cells in the body that are identified as being infected or affected. However, if presentation is on the MHC class II molecule, then CD4+ T-cells will react and produce T-helper cells (Th cells) to carry out the response (67, 69, 73).

Besides T-lymphocytes, there is another component of the adaptive immune response that plays an active role, namely the B-lymphocytes (67, 69, 73). These cells are responsible for the adaptive humoral response mediated by the action of antibodies. B-cells are derived from hemopoietic stem cells in the bone marrow, where they later mature and acquire their antigen specificity (73). B-cells differentiate into plasma cells and memory B cells in response to antigen in combination with signals received from T-cells and other cells (75). The plasma cells are responsible for the production of antibodies, which are also known as immunoglobulins. Antibodies aid in the adaptive immune response by attaching to the antigenic portion of the pathogen and rendering it harmless (73). This process may take place with the help of T-cells, where the antigens are known to be T-cell dependent, or without T cell help, when the antigens are known to be T-cell independent (67, 71, 73).

Antibodies are Y-shaped protein molecules that are formed by the pairing of two heavy and two light chains. The heavy chain makes up that part of the constant region [fragment crystallisable (Fc)] of the antibody which defines the immunoglobulin isotype – i.e. IgA, IgE, IgD, IgG or IgM (76). The remaining part of the constant region is composed of two identical types of light chains, either lambda or kappa. The selection of light chain type is determined by the Fc region. The ends of the Y shaped molecules are comprised of a combination of the heavy chain and light chain, which have the ability to bind to antigen. Thus this region, consisting of a portion of the constant region as well as a variable section, is termed the fragment for antigen binding (Fab) portion. The structure and components of an antibody can be seen in Figure 7.

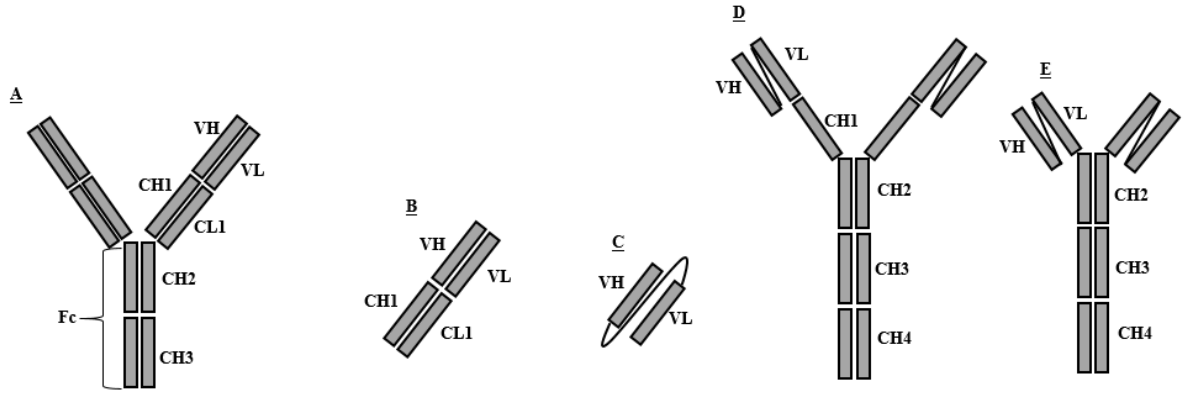


Figure 7: Structure of an antibody molecule. (A) Full antibody; (B) Fragment for antigen binding (Fab); (C) Single chain variable fragment (scFv); (D) Gallibody scFvIgY$_{(CH1-4)}$; (E) Gallibody scFvIgY$_{(CH2-4)}$; CH – constant heavy chain, VH – variable heavy chain , CL – constant light chain, VL – variable light chain, Fc – fragment crystallisable.

The antigen binding surface of the Fab region is comprised of three complementarity determining regions (CDR 1, 2 and 3) each of the heavy and the light chain that come together (77). While the CDR1 and CDR2 regions may contribute somewhat to the diversity of an antibody, it is the CDR3 region that plays the major the role. This is due to the differences in the way the CDRs are produced, where CDR1 and CDR2 are formed by point mutations occurring in the CDR, whereas CDR3 comes about by point mutations as well as several mechanisms of gene recombination (78).

Due to the role that antibodies play in the recognition of pathogens in the immune system, their use for detection of disease is greatly applicable. While antibodies are naturally produced by the immune system to aid in its response to foreign molecules, antibody can also be generated in the laboratory. Monoclonal antibody engineering commenced in 1975 (79), and has rapidly grown since for their use in research and due to their increasing commercialisation as antibody based therapeutics (80). Monoclonal antibodies can be prepared using either the traditional

hybridoma technology or the more modern phage display. The production of antibodies through hybridoma technology was first illustrated in 1975 by Köhler and Milstein where they were able to produce (*in vivo*) murine monoclonal antibodies of defined specificity (79). Transgenic mice are used for the production of humanised antibodies by means of hybridoma technology. While shown to be useful for generating monoclonal antibodies, hybridoma technology can become a challenging process for a number of reasons, such as species limitations, dependence on immunisations, constraints imposed by ethics of animal experimentation, cost and commercial requirements for various applications. Thus the *in vitro* method of phage display has become increasingly popular in recent times. Phage display, as the name suggests, is a process whereby proteins of interest are expressed on the surface of a phage (81, 82). In order for this to occur a portion of the protein's nucleotide sequence must be inserted into the phage in the form of a gene encoding a phage coat protein. This ensures that when the phage replicates the protein will be presented on the phage's surface while the nucleic acid will remain inside the phage. Phage display can be for the production of full chain antibodies or antibody fragments such as scFv or Fab fragments (83). While it has been shown that these fragments can be screened for their specific binding of antigens, their applications in other areas of research such as diagnostics and therapeutics require their conversion and engineering into alternative recombinant forms, i.e. whole antibody, diabodies or tetramers (84). The first human antibody gene library to be made for this purpose was reported in 1991, by the group of Greg Winter (85). By randomly combining the heavy and light chain variable genes, the authors were successfully able to generate a library of over 10^7 members that can be displayed on phages.

Progression in this field has led to the development of a number of gene libraries of different host origin. One such library is the *Nkuku*® chicken V-gene library produced in Onderstepoort,

South Africa (81). This chicken gene library differs from that of Winter's group in that it is a semi-synthetic library, meaning it was produced by combining two different phage display library – the naïve repertoire of chicken immunoglobulins and a synthetic repertoire generated by combining variations of the heavy and light chain variable genes. Using this method the authors were able to generate a library of 10^9 members that can be used in phage display.

The expression of scFvs in alternative recombinant formats involves the manipulation and rearrangement of genes as in proteomics. Phage displayed antibodies in different formats can be preliminary assessed for functionality and suitability in lateral flow immunoassay (LFI) by means of the more user friendly method of ELISA. ELISA is a method used here to detect the antibody of interest by making use of an antigen immobilized by adsorption to the surface of a micro plate well. There are many types of ELISAs: direct, indirect, sandwich and competitive (86, 87). In all cases a micro titre plate is coated with a specific antigen. Direct ELISA is conducted when enzyme labelled specific antibodies are bound to the plate directly. Indirect ELISA is conducted when primary antibodies from an antiserum sample binds first to the coated antigen and are then detected indirectly via a secondary enzyme labelled anti-immunoglobulin antibody. A sandwich ELISA is a method which uses the process described for both direct and indirect ELISAs. Initially an antibody is bound directly to the plate. An antigen is then added which the initial antibody binds to. Next a second antibody is added which binds to a different site of the antigen. The final layer of the sandwich is built with the addition of the enzyme labelled anti-immunoglobulin antibody. While the setup of a competitive ELISA can be similar to the other types mentioned, here, two types of primary antibodies compete for binding (86). In this project, only indirect ELISA is used. This process is outlined in Figure 8.

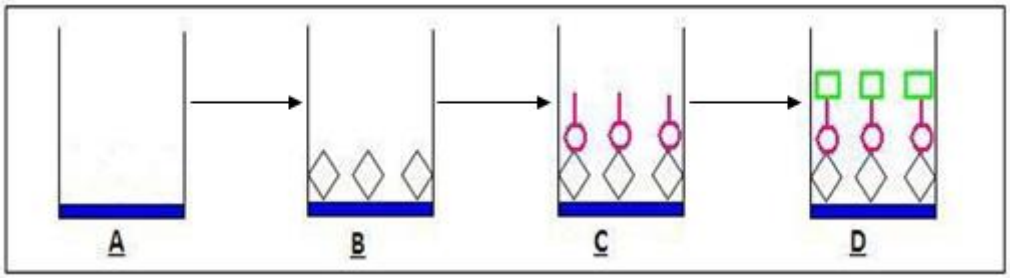


Figure 8: Graphical representation of Indirect ELISA (88). (A) Coating of plate with antigen; (B) Binding of primary antibodies from serum; (C) Binding of secondary enzyme labelled antibody; (D) Binding for conversion of substrate for colour reaction at the end.

The experiment to be conducted will make use of MA as the antigen in the indirect ELISA to test the binding of the recombinant antibodies to antigen coated wells. Subsequently, the displacement of recombinant antibodies from the test line by MA coated nanoparticles will be investigated, thereby giving an indication of the sensitivity that may be eventually achieved in a LFI, where antibodies in human sera will be the displacement agent competing for binding to the mycolic acid antigen on the test line.

Previous work conducted by Lindokuhle Ndlandla, a PhD student in our research group, yielded the stable expression of three chicken-derived monoclonal phage-displayed scFvs with different CDR3 amino acid sequences as seen in Table 1 (64).

Table 1: Monoclonal chicken derived phage-displayed scFvs with varying specificities (64). V_H - variable heavy chain, V_L - variable light chain

Clone	V_H CDR3	V_L CDR3
Anti-MA mAb 12	MNYRRRQ	TEDSTY
Anti-MA mAb 16	RRITNK	RDSGAP
Anti-MA mAb 18	RKTNKHRIDAWGHGTEV	GSYEASNSAGIFG

It is clear from the sequences shown in Table 1 above that the amino acid sequences of the three monoclonal antibodies vary in the type and length of the amino acids present in both chains of the CDR3 region. Researchers have shown previously that the heavy chain of the CDR3 region plays is hugely responsible for the binding specificity of antibodies. Thus the differences seen in the V_HCDR3 seen in Table 1 confirm the various sequence possibilities by which the three mAbs may exert their MA-binding specificity.

While the method of phage display technology is useful for selecting scFvs with different binding specificities, it has been reported that scFvs released from their phage proteins have the ability to naturally form dimers and trimers (65, 66). As a result the use of these released scFv antibody fragments often results in the formation of multivalent non-covalently bound antibody fragments, which may give false-positive cross-reactive binding due to the uncontrolled valency for antigen binding. Moreover, using conglomerated phage displayed scFv in a lateral flow set up would not be feasible, as the bulky complex may have trouble flowing through the test strip towards the wick by capillary force. Ideally then, the scFvs needed to be re-grafted on a normal antibody framework, providing it with the characteristics that are proven to work in a LFI.

This chapter is focused on the conversion of the monoclonal scFvs into full chain IgY antibodies illustrated in figure 7 D and E.

Aim: Development of a set of recombinant monoclonal chicken antibodies (Gallibodies) against MA.

3.2. Materials and Methods

LB medium: 10 g Tryptone, 5 g yeast extract, 10 g sodium chloride per 1 L double distilled deionized water, adjusted to pH 7 using 1 M NaOH. Make up 1 L volume, autoclaved and stored at RT until further use. For LB-agar, 15 g agar for every 1 L was added. Plates were stored at 4 °C until further use.

20X PBS: 160 g NaCl, 4 g KCl, 28.8 g Na_2HPO_4, 4.8 KH_2PO4 per 1 L double distilled deionized water, adjusted to pH 7.4. Make up 1 L volume, autoclaved and stored in 50 ml aliquots until further use.

1X PBS: 50 ml 20X PBS added to 950 ml double distilled deionized water, adjusted pH to 7.4 before volume made up to 1 L.

1M H_2SO_4: In a fume hood, 1 ml concentrated sulphuric acid was added to 17 ml dddH_2O while stirring continuously.

3.2.1. Screening of library for MA binders

The naïve semi-synthetic chicken phage-displayed scFv (*Nkuku*®) library containing 2 x 10^9 phage particles (81) was obtained from ARC-Onderstepoort Veterinary Institute (South Africa). This library was panned as previously described for AMAA (54). Thermally stable expressed phage displayed antibodies were selected for and characterised by sequencing and ELISA as described in the thesis of Lindokuhle Ndlandla (2017) (64).

3.2.2. Isolation of scFvs

Individual clones were grown overnight at 37 °C with shaking (220 rpm) in LB medium containing 2% glucose and 100 µg/ml ampicillin. The following day, these cultures were inoculated 1:100 into 10 ml fresh medium containing glucose and ampicillin and grown to midlog (OD_{600} = 0.9). The cultures were then centrifuged and the supernatant fluid (SNF) discarded. Cell pellets were resuspended in 2 ml LB containing ampicillin and 1 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated overnight with shaking at 30 °C to induce expression of soluble fragments. Secreted scFv fragments remained in the SNF and were isolated by centrifugation at 3000 rpm for 10 min at 4 °C. Cell pellets were retained to harvest periplasmic (PP) scFvs by resuspending them in one-tenth volume of 1 × PBS supplemented with 1 M NaCl and 1 mM EDTA and incubating on ice for 30 min. The fractions were centrifuged at 6000 × *g* for 10 min at 4 °C and the SNFs containing the scFvs were transferred to fresh tubes.

3.2.3. Evaluation of binding as scFvs

ELISA with MA was conducted to confirm the functionality of the scFvs. Briefly, Nunc-Maxisorp ELISA plates were coated with 50 µl of natural mixture of MAs (Sigma-Aldrich, USA) dissolved in freshly distilled hexane to a final concentration of 250 µg/ml. The coated

plates were allowed to have the hexane evaporate from them at room temperature and were then stored until further use. The following day, the ELISA plates were washed three times with 300 µl per well of PBS/0.05% Tween 20. Non-specific binding sites were blocked with 300 µl per well of 2% milk powder/PBS for 1 hr at 37 °C and then washed three times with PBS/ 0.05% Tween 20. Isolated scFvs were diluted with 4% milk powder/PBS+0.1% Tween 20 (1:1, v/v) and 50 µl of this mixture were added to each well. Plates were incubated for 1 h at 37°C. The test solution was discarded and plates washed three times with 300 µl per well of PBS/ 0.05% Tween 20. Mouse anti-c-*myc* tag mAb 9E10 (Onderstepoort, South Africa) diluted 1:1 in 4% milk powder-PBS/0.1% Tween 20 was added to the wells (50 µl per well) and the plates incubated at 37 °C for 1 h. The Ab solution was discarded and the plates washed three times with 300 µl of PBS/0.05% Tween 20 per well to remove any unbound Abs. Polyclonal rabbit anti-mouse immunoglobulin-HRP (Dako, Denmark) diluted 1:1000 in 2% milk powder/PBS-0.05% Tween 20 was added to the wells (50 µl per well) and the plates incubated at 37 °C for 1 h. The conjugate solution was discarded and the plates washed three times with 300 µl of PBS/0.05% Tween 20 per well. The signal was developed by adding 50 µl of TMB Single Solution Chromogen for ELISA (Invitrogen™, Thermo Scientific, USA) to each well and then incubated at room temperature for 15 min. To stop the reaction, 50 µl of 2 N H_2SO_4 was added to each well. Plates were read at 450 nm (Thermo Electron Corporation Multiskan EX plate reader).

3.2.4. Plasmid isolation and purification

Bacterial cultures were grown overnight as described in 3.2.2. The following day cells were harvested by centrifugation at 3000 rpm for 10 min at 4 °C. Plasmids were then isolated and purified using the QIAGEN® Plasmid Midi Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions. The concentration of the DNA isolated was determined by

analysing the sample with a NanoDrop® ND 1000 spectrophotomer (NanoDrop Technologies Inc., Wilmington, USA).

3.2.5. Plasmid amplification

In an attempt to increase the amount of plasmid available to work with as well as adding sites to enable the restriction digest to take place, the extracted DNA of the clones were amplified with a PCR reaction using the VH*BsiW* (5' GATCCGTACGGCCGTGACGTTGGACG 3') and VL*AscI* (5' GATCGGCGCGCCACCTAGGACGGTCAGGG 3') primers (Inqaba Biotech, South Africa). The *Ex Taq*™ DNA polymerase kit (Takara, Shiga, Japan) was used for the PCR reaction run for 25 cycles at a Tm of 54 °C.

3.2.6. Agarose gel electrophoresis

The DNA amplification process was confirmed using agarose gel electrophoresis. A 1% agarose gel was prepared by adding an agarose gel tablet (Bioline, London, UK) to 50 ml 1 X Tris-acetate-EDTA (TAE) buffer and adding 1 drop of 1 µg/ml ethidium bromide to the gel before pouring into a mould and allowing to set. Samples were prepared by adding 5 µl Blue/Orange loading dye (Promega, USA) to 25 µl sample before loading into the gel. The Hyperladder I DNA molecular mass standard (Bioline, London, UK) was included as a reference. Gels were run in 1 X TAE buffer according to standard procedure (89).

3.2.7. Restriction enzyme digestion

Restriction endonucleases were used for digesting as follows: Initially both the scFv inserts and vectors were digested with *AscI* in a reaction carried out in a thermocycler (Eppendorf, Hamburg, Germany). Prior to the second digest the samples were purified using the QIAGEN® Plasmid Midi Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions.

This was then followed with a second digest using *BSiW* for the scFv inserts and *Acc651* for the vectors, respectively. All restriction endonucleases were obtained from New England Biolabs and reactions were carried out as per the manufacturer's instructions. Agarose gels were run, as described in 3.2.6, after both the first and second digestion to confirm the process was successful.

3.2.8. DNA Precipitation

DNA precipitation was initiated by adding 2.5 volumes of absolute ethanol and one tenth volume of sodium acetate to the samples. These solutions were incubated at -20 °C overnight. The precipitated DNA was recovered the next day by centrifugation at 13 000 rpm at 4 °C. The supernatants were poured off and pellets resuspended in a 40 µl volume of dddH_20 for storage until further use.

3.2.9. Crystal violet gel

DNA of the digested vectors and scFvs were precipitated using the method described above. To improve the efficiency of cloning isolated DNA of the vectors and scFvs were purified using a crystal violet gel following the methods of Rand (90). Briefly, a crystal violet gel was prepared by adding 100 ul crystal violet to a 0.1% agarose gel mixture and allowing to set. Samples were prepared by adding 5 µl 2% Ficoll 400+10% glycerol, vortexing and pelleting before loading into a crystal violet gel to allow for further confirmation and purification after the digestion. DNA was recovered by using a gel extraction kit (QIAGEN QIAquick Gel extraction kit) following the manufacturer's instructions. The concentration of the DNA recovered was determined as described above in 3.2.4.

3.2.10. Ligations

Ligations were performed using T4 DNA Ligase enzyme (Roche). Vectors and scFv inserts were mixed in suitable molar ratios and made up to the appropriate final volume (60 µl or 90 µl) depending on the amount of vector needed. The reaction was left to incubate for 3 days at 4 °C, allowing enough time for the ligation to take place.

Following the incubation time DNA was precipitated as described above. The DNA precipitated was further extracted using the MSB Spin PCRpace Kit (Invitek, Berlin, Germany) following the manufacturer's instructions. Concentrations of the isolated DNA was determined using the method mentioned above in 3.2.4.

3.2.11. Transformations

Following the clean-up, ligations were subjected to a standard *Escherichia coli* (*E. coli)* transformation procedure using JM109 competent cells (Promega, USA). DNA from the ligations was added to the cells (in a suitable ratio) using the heat-shock method as per the manufacturer's instructions. Cells were plated undiluted, 1:10 and 1:100 on agar plates and grown overnight at 37 °C.

3.2.12. Colony PCR

Picked colonies were suspended in 25 µl dddH_2O before cells were lysed by heating samples at 95 °C for 5 min. Lysed cells were centrifuged at 14 000 rpm for 5 min. Supernatants were used in combination with the GoTaq® Green master mix (Promega, USA) for the PCR reaction. Samples were prepared and run as per manufacturer's instructions. T7for (5' TAATACGACTCACTATAGGG 3') was used as the forward primer and CH2-4rev (5' AGGAGGAGGG GTGGAGGACC 3') was used as the reverse primer. PCR end products were prepared and run on agarose gel as described in 3.2.6.

3.2.13. Sequencing

Liquid cultures of bacterial colonies were prepared and allowed to grow at 37 °C for 5 hours with shaking (230 rpm). Plasmid extractions and concentration were performed as described in section 2.2.4. Sequences obtained from analysis (Inqaba Biotech, South Africa) of plasmid DNA were compared to the original templates using BioEdit (91).

3.2.14. Resuscitation of HEK cells

Complete media (cDMEM) was prepared by adding 5 ml of FBS (FBS Superior heat inactivated, Biochrom) to culture medium [Dulbecco's Modified Eagle's Media (DMEM with GlutaMAX, GIBCO®] to a total volume of 50 ml. The tubes containing the cDMEM were placed in a water bath at 37 °C. Stored human embryonic kidney (HEK) cells were removed from the liquid nitrogen stores and defrosted at 37 °C until only a small piece of ice remained. The cells were then slowly added to 9 ml of pre-warmed cDMEM in a T-25 flask before being stored in the incubator overnight at 37 °C, 5% CO_2. The following day the media was replaced with new cDMEM and the cells incubated for a further 3-4 days or until a cell confluency of 80-100% was obtained.

3.2.15. Transfections

Prior to transfections cells were transferred to 6-well plates and grown to >80% confluency. A media change was performed on the day if it appeared to yellow. Samples for transfections were prepared by adding 250 µl DMEM, 2.5 µg DNA and 8 µl *Trans*IT®-293 transfection reagent (Mirus, Madison, USA) together in an Eppendorf tube and triturated to mix. The samples were incubated for 30 min at RT before being added to the cells dropwise.

3.2.16. Zeocin selection

Cells were incubated for 24 hours before media was replaced with cDMEM containing 100 µg/ml Zeocin (Invitrogen™, Carlsbad, USA). Culturing of cells continued in this way for approximately 2 weeks to allow for selection of transfected cells, noticeable as small islands of cells that remained. Cells that were not transfected underwent cell death during the process. Remaining islands were continuously cultured and sub-cultured until sufficient stocks were obtained.

3.2.17. Gallibody functionality

The functionality of the newly produced gallibodies was tested with ELISA. Media collected from the cells were used as the primary antibody as this is where the antibodies were contained. Non-sterile, 96-well micro-titres plates (NUNC®) were used for all of the experiments. Plates were coated with solutions of 0.06 µg/µl or 250 µg/ml MA/Hexane. The hexane was allowed to evaporate before the plates were covered with foil and stored at 4 °C overnight or until use. A volume of 300 µl/well of 2% milk powder/PBS was added to the plates and left to incubate at RT for 2 hr for blocking. Once the 2 hr blocking period had passed, the wells were washed three times with 0.05% Tween 20/PBS. Media solutions (containing gallibodies), diluted 1:1 with 4% milk powder/PBS + 0.01% Tween 20 were then loaded into wells (total volume 50 µl/ well) and the plates left at 37 °C to incubate for 1 hr. Following the incubation period, wells were again washed as above and 50 µl/well of the conjugate solution (Goat anti-chicken IgG (Fc)-HRP diluted 1:10 000 with 2% milk powder/PBS + 0.05% Tween 20) was added to the plate, allowing the plate to incubate at 37 °C for 1 hour. After 1 hour of incubation the wells were washed as above and 50 µl of TMB substrate solution were loaded into each well. After 10 min 1 M H_2SO_4 (50 µl/well) was added to the plates to stop the reaction and a final reading was done using a 450 nm filter.

3.2.18. Protein purification

Gallibodies were purified using columns loaded with nickel-nitrilotriacetic acid agarose resin (QIAGEN®, Hilden, Germany) specific for capturing histidine tagged proteins. An amount of 5 ml of cell culture media containing the gallibodies was diluted 1:3 in lysis buffer (0.05 M Na_2HPO_4, 0.03 M NaCl) before being passed through the column. The flow through was passed through the column a second time and then retained. The column was washed twice with lysis buffer and proteins eluted in 1 ml fractions using the elution buffers as follows: 1) 10 mM Imidazole, 0.05 M Na_2HPO_4, 0.03 M NaCl; 2) 20 mM Imidazole, 0.05 M Na_2HPO_4, 0.03 M NaCl; 3) and 4) 250 mM Imidazole, 0.05 M Na_2HPO_4, 0.03 M NaCl. All fractions were collected and characterised by ELISA (section 2.2.17), SDS-PAGE and protein concentration determination.

3.2.19. SDS-PAGE

Samples were prepared by mixing 20 µl samples with 20 µl protein-sample buffer before heating at 100 °C for 5 min. Denatured samples were run on Mini-PROTEAN® TGX precast protein gels loaded in a Criterion™ Cell vertical gel system (both Bio-Rad, Hercules, USA) and run in 1 X TGS buffer at 200V for 1 hour. PageRuler prestained Protein ladder (Thermofischer,) was included on all gels as a molecular weight marker. Protein bands were stained with Aquastain® for 10 min at room temperature prior to destaining in dddH_2O at overnight at RT.

3.2.20. Protein concentration determination

The concentration of the purified gallibodies was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, USA), which is based on the Bradford method (92). A volume of 200 µl diluted Dye Reagent was added to 10 µl protein sample or standard before incubating at room temperature for 5 min as per the manufacturer's instructions. Following the incubation time the absorbance was read at 595 nm using a BioTek PowerWave HT Microplate Spectrophotometer (BioTek intruments, Inc., Winookski, USA).

3.2.21. Gallibody concentration

Purified gallibodies were concentrated in spin columns with a concurrent buffer change (0.1 M Borate buffer, pH 7.4) using Vivaspin 10 000 MW PES centrifugation columns (VivaScience, Satorius Group, United Kingdom). Final gallibody concentrations were determined using the method described in section 3.2.20.

3.3. Results

This work is a continuation of the work conducted over the past 10 years within our research group. Most recently the results of PhD student Lindokuhle Ndlandla yielded the stable expression of three monoclonal phage displayed antibody fragments with varying specificity – shown in the introduction to this chapter (64). While the scFvs were shown to bind to the MA antigen when displayed, their ability to bind when separated from phages needed to be determined. Thus the functionality of the scFvs released from the phages were evaluated using ELISA. The results obtained from the evaluation can be seen in Figure 9.

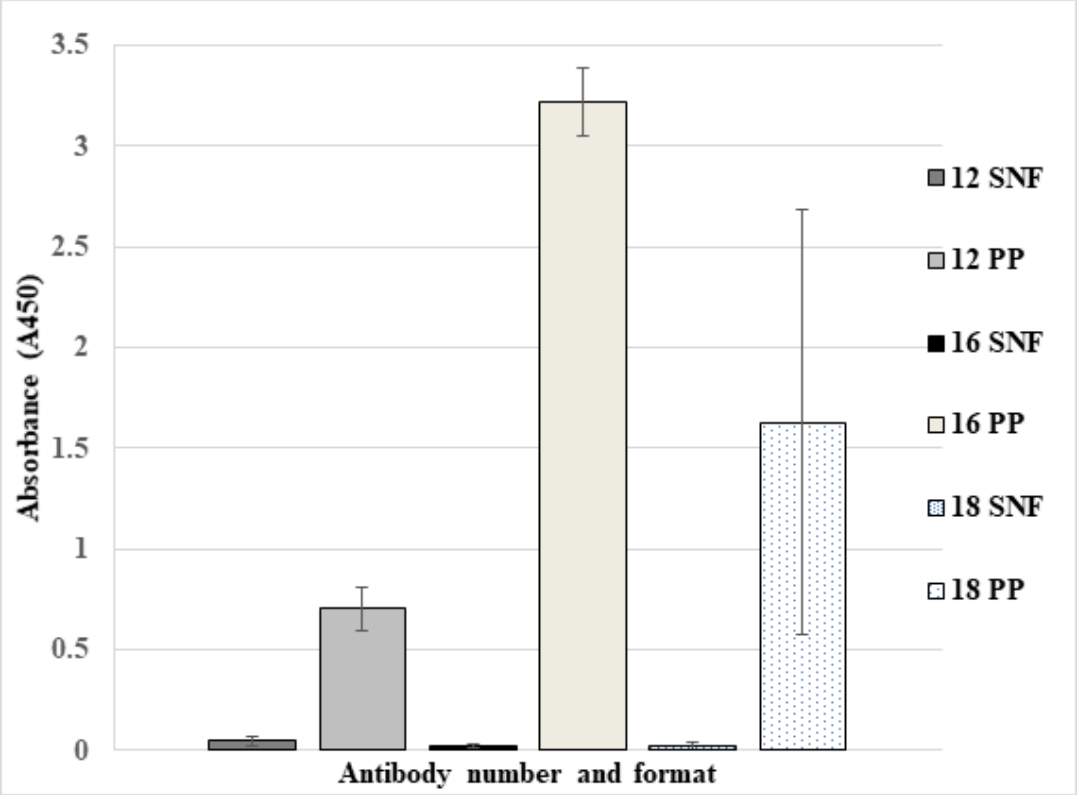


Figure 9: Functionality of phage released scFvs in binding to mycolic acid antigen measured by ELISA. scFv preparations used here were all of unknown protein concentration. Hexane coated wells served as the control antigen. 12 = anti-MA 12; 16 = anti-MA 16; 18 = anti-MA 18; SNF = supernatant fluid; PP = periplasmic membrane. Results indicate that the binding of SNF scFvs to MA is significantly different to that of PP scFvs (n = 4; p value < 0.005; Error bars represent standard deviation).

The results from Figure 9 show that the scFvs were able to bind to MA. Although the signals obtained for SNFs were extremely low, scFvs obtained from the periplasmic membrane gave much better readings.

The conversion of the antibody fragments into full chain IgY antibodies began with a modification and amplification process of the scFv DNA using PCR. This was conducted to ensure that sites were available on the scFv fragment genes to allow them to be cloned into the

bivalent IgY vector as well as producing enough start product for the experiments to follow. An agarose gel was run to determine the success of the process. The results can be seen in Figure 10.

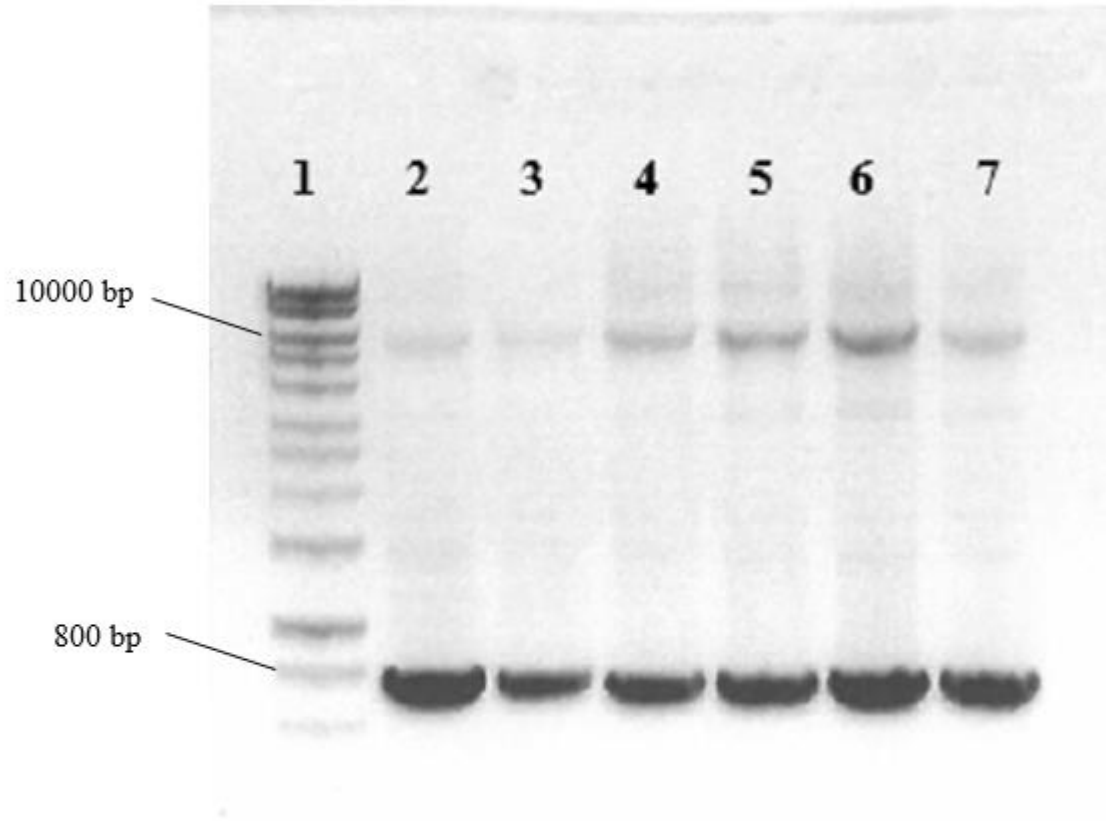


Figure 10: Agarose gel electrophoresis for analysis of amplified DNA of scFv inserts 12, 16 and 18. Lane 1 = Hyperladder I molecular weight marker, 2-3 = anti-MA 12, 4-5 = anti-MA 16, 6-7 = anti-MA 18; samples loaded in duplicate.

From the agarose gel seen in Figure 10 it was observed that a band is present at 800 bp for each of the scFv fragments. This data confirmed that the fragments had successfully been amplified during PCR.

The vectors selected for cloning were provided to our collaborators at OVI, Onderstepoort by Greunke *et al.* from the University of Hamburg. These vectors, named scFvIgY (CH_{1-4})His and

scFvIgY(CH_{2-4})His, were designed containing the constant region of the chicken antibody chain (93). An additional oligonucleotide, coding for a peptide called scFv 27, was included in the vector to substitute the scFv regions of the chain. This region, known as the stuffer region, was excised during the restriction enzyme process. The design of the vectors can be seen in Figure 11 below.

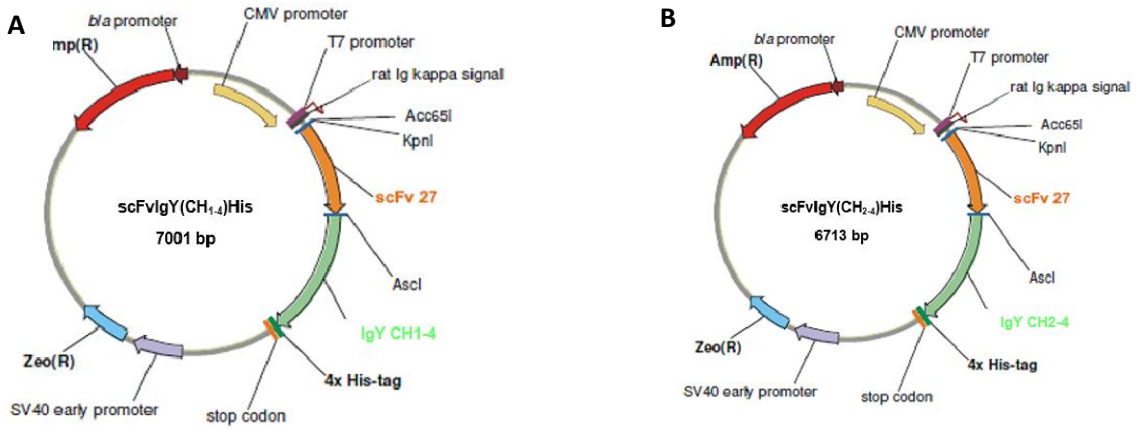


Figure 11: Maps of vectors used for expression of recombinant chicken antibodies (93, 94). A = scFvIgY(CH_{1-4})His , B = scFvIgY(CH_{2-4})His. Adapted from information received by Dr. E. Spillner.

In the maps shown in Figure 11 the stuffer region can be seen coloured in yellow. At the ends of this region one may note the presence of the *Acc65I* and *AscI* sites at the start and end of the scFv 27, respectively. The presence of these sites enabled us to use the restriction endonucleases *Acc65I* and *AscI* for the vector digestion process, which prepared the vector for cloning by excising the stuffer region. Similarly, because the scFv clones were modified during the PCR with additional sites, the restriction endonucleases *BsiWI* and *AscI* were used in their digests. In order to ensure the digests were successful, an agarose gel was run. The results can be seen in Figure 12.

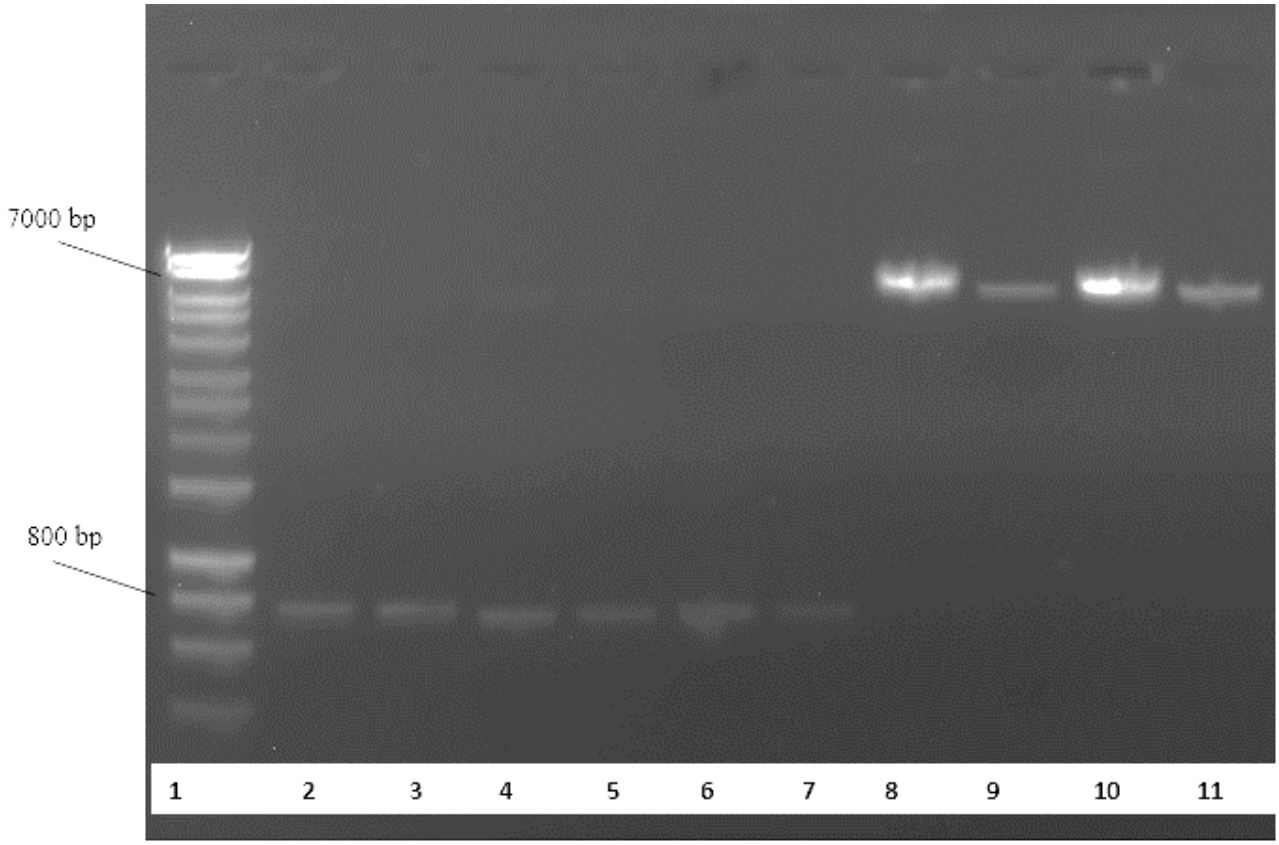


Figure 12: Agarose gel electrophoresis results of restriction enzyme digests of scFv inserts 12, 16 and 18, and vectors CH1-4 and CH2-4. Lane 1 = HyperLadder I molecular weight marker, 2 = anti-MA 12 digest 1, 3 = anti-MA 12 digest 2, 4 = anti-MA 16 digest 1, 5 = anti-MA 16 digest 2, 6 = anti-MA 18 digest 1, 7 = anti-MA 18 digest 2, 8 = vector CH1-4 digest 1, 9 = vector CH1-4 digest 2, 10 = vector CH2-4 digest 1, 11 = vector CH2-4 digest 2

The results in Figure 12 show that the digests of the scFv inserts yield bands of smaller sizes (800 bp) than those of the vectors (7000 bp). This is expected as the scFv fragments are meant to be inserted into the stuffer region of the vectors. It can also be noted that in each case the band obtained from digest 2 in both the vectors and scFv clones appear sharper and smoother than the bands from digest 1. This suggest that a “cleaner” version has been produced during the digests.

Once a successful digest had been achieved the scFv inserts and vectors were combined in suitable molar ratios with the DNA ligase and incubated over a time period of 3 days to allow the ligations to take place. DNA was then concentrated to ensure that enough sample was available for the transformation process in *E. coli* cells. From the vector maps shown in Figure 11 it can be seen that the vectors contain a region that contains an ampicillin resistance gene. Due to this a selection process can take place if the antibiotic ampicillin is included in the media. As a result any scFv codes that have been inserted into the vector during the ligation process will be successfully expressed by the bacteria, while those floating freely will be degraded. Upon observation of the plates it was noted that colonies only grew in the undiluted sample and at 1:10. As a result colonies were picked at these dilutions and subjected to colony PCR and analysis on gel electrophoresis to ensure the bacteria were producing DNA for the newly formed gallibodies. Agarose gels of the colony PCR samples can be seen in Figure 13.

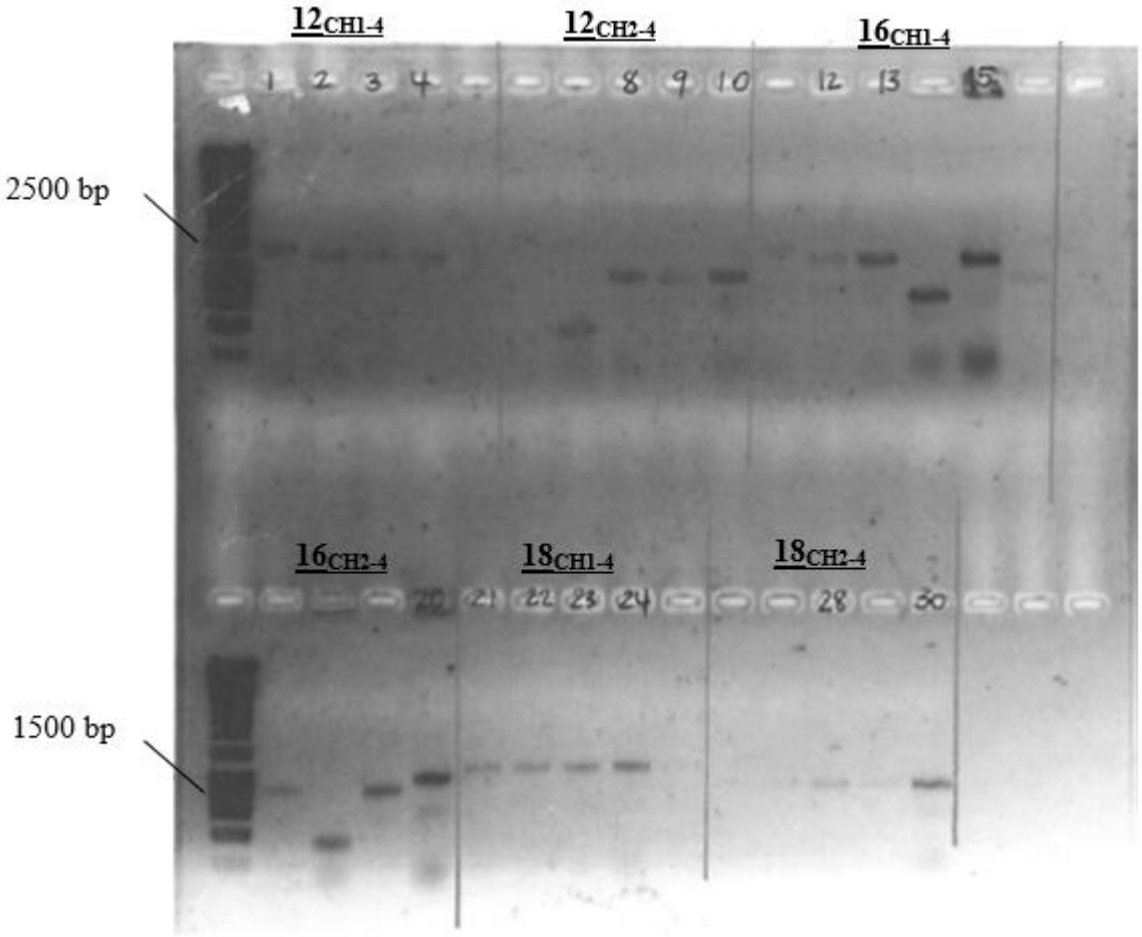


Figure 13: Agarose gel reflecting colony PCR results of newly produced gallibodies. 12 = anti-MA 12, 16 = anti-MA 16, 18 = anti-MA 18, CH1-4 = scFvIgY(CH_{1-4})His, CH2-4 = scFvIgY(CH_{2-4})His. The PCR product for the CH_{1-4} gallibodies can be observed around 2250 bp where as those for the CH_{2-4} gallibodies is seen around 1650 bp.

From the results seen in Figure 13 it can be noted that bands were observed around 2250 bp for gallibodies containing the scFvIgY(CH1-4)His vector and around 1650 bp for the scFvIgY(CH2-4)His vector, respectively. This is expected as in the results mentioned above the starting size of the scFv fragments was 800 bp. During the colony PCR reaction primers are used that will amplify the entire scFv fragment inserted and a small section of the vector. As a result the size of the two scFv fragments (2 x 800 bp) will increase by 640 bp (2 x 320 bp) for the CH_{1-4} and 40 bp (2 x 20 bp) for the CH_{2-4} vector.

The selected colonies were grown up as liquid cultures and the DNA extracted in preparation for sequencing. Samples were sent to Inqaba Biotech for sequencing reactions. Results obtained were interrogated using the BioEdit program and can be seen in Figure 14.

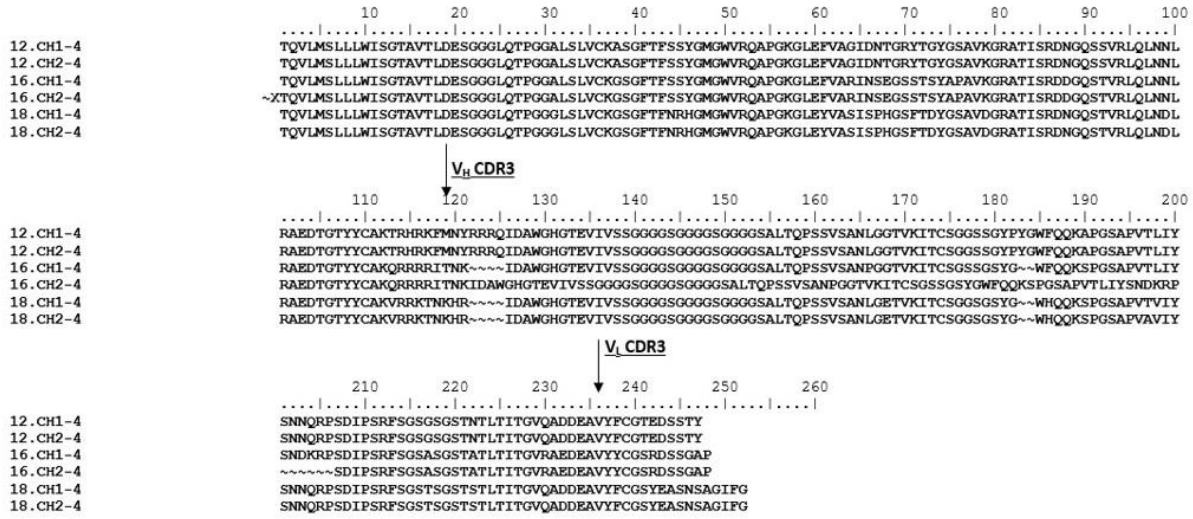


Figure 14: Amino acid sequences of the scFv aminoterminals of the full gallibody clones produced by recombinant engineering. 12) Anti-MA 12, 16) Anti-MA 16, 18) Anti-MA 18, CH1-4 = full length constant region, CH2-4 = truncated constant region, V_H = variable heavy chain, V_L = variable light chain

From the results seen in Figure 14 it can be noted that up to the 15th amino acid codon of the vector is present. From the amino acid 16 codon, we start to see the appearance of the scFv inserts; thereby illustrating that the cloning process was successful and gallibodies were produced. Furthermore the unique sequences of the V_HCDR3 and V_LCDR3 can be seen on the labels illustrated above, confirming the success of the antibody engineering process. Up to this point the successful DNA cloning of the gallibodies was demonstrated. An additional step of expression is required thereafter for transcription and translation of the antibodies in eukaryote cells. In order to achieve this, a human embryonic kidney cell culture line, HEK293, was used. If one refers back to the vector maps in figure 11, there is a gene of Zeocin resistance present

in the vectors. This allows for a similar selection process as that used for the transformation reaction in *E. coli*, substituting the bacterial cell type for mammalian HEK cells and the ampicillin antibiotic for Zeocin, respectively. As a result the HEK cells were transfected with DNA of the gallibodies produced and subjected to Zeocin selection over time. The process is illustrated in Figure 15.

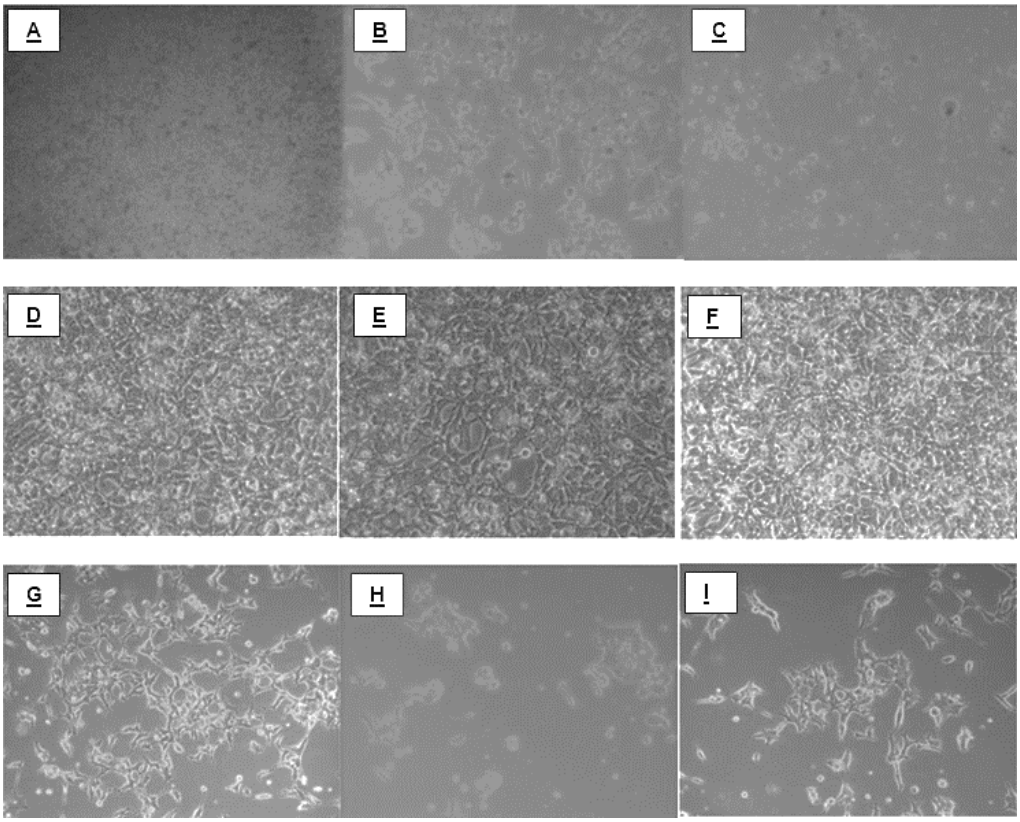


Figure 15: Cell morphology of transfected HEK cells undergoing Zeocin selection. During the initial stages the cells morphology is that shown in A. Following the transfection process cells that have not taken up the plasmid DNA start to die leaving a smaller percentage of confluent cells shown in B. As shown in C cells that have not been transfected will continue to die as they do not have the Zeocin resistant gene present. A = No Zeocin control, B = Transfected cells, C = Zeocin control, D = $12_{CH2\text{-}4}$ transfection, E = $16_{CH2\text{-}4}$ transfection, F = $18_{CH2\text{-}4}$ transfection, G = $12_{CH1\text{-}4}$ transfection, H = $16_{CH1\text{-}4}$ transfection, I = $18_{CH1\text{-}4}$ transfection. All pictures were taken at 10X magnification.

From the results shown in Figure 15 one can take note of how the Zeocin selection process occurs in cells. Cells start off looking healthy with a good morphology, similar to the no Zeocin control in picture A. After the transfection had occurred and the cells continued to undergo selection they started to resemble picture B. Cells that had not been transfected were usually included as a negative control, as seen in C. These cells do not contain the vector with Zeocin resistance and therefore cannot withstand the exposure to Zeocin. As a result the cells start to shrink, appearing more rounded, and eventually die over time. Cells that have been transfected successfully continue to grow and resemble the no Zeocin control over time, growing at different rates. This can be seen in pictures D-I.

As the cells continue to grow after transfection, they continuously produce and secrete antibodies into the culture medium. Due to the fact that the cells are of mammalian origin they possess the necessary cell components that allow them to produce antibodies where the proteins are correctly folded and decorated with oligosaccharides. These antibodies, readily available in the culture media, were tested for their functionality in ELISA. During this process MA was used as the antigen to test the ability of the antibodies to perform and an anti-chicken conjugate was utilized to confirm that the antibodies binding to the antigen were of chicken origin. The results can be seen in Figure 16.

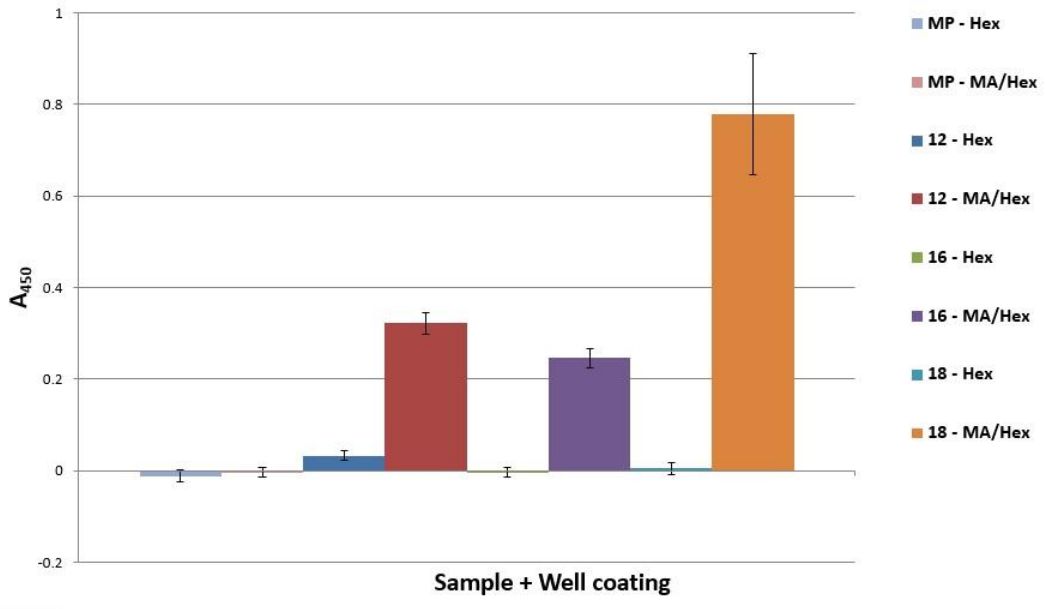


Figure 16: ELISA results to confirm that gallibodies bind to mycolic acid antigen. Gallibodies used here were all of unknown protein concentration. Milk powder (MP) was used as the gallibody control and Hexane (Hex) coated wells served as the control antigen. Gallibodies with the CH2-4 vector were tested for functionality. 12 = $12_{CH2\text{-}4}$, 16 = $16_{CH2\text{-}4}$ and 18 = $18_{CH2\text{-}4}$. Binding of gallibodies to antigen (MA/Hex) is significantly different of gallibodies to antigen control (n = 4, p value < 0.005).

The results shown in Figure 16 illustrate that the gallibodies were able to bind to the MA antigen successfully. We can see from the bars corresponding to 12-Hex, 16-Hex and 18-Hex that a smaller signal was obtained indicating that a small percentage of background binding occurs. However, the results obtained on the MA coated well (12 – MA/Hex, 16 – MA/Hex and 18 – MA/Hex) were significantly different, indicating that the gallibodies have been successfully produced. The protein concentration of the gallibodies used here had not yet been determined.

Following purification using the nickel affinity columns the fractions collected were characterized with SDS-PAGE to determine whether there was any protein loss during the process. The results from the gel runs can be seen in Figure 17.

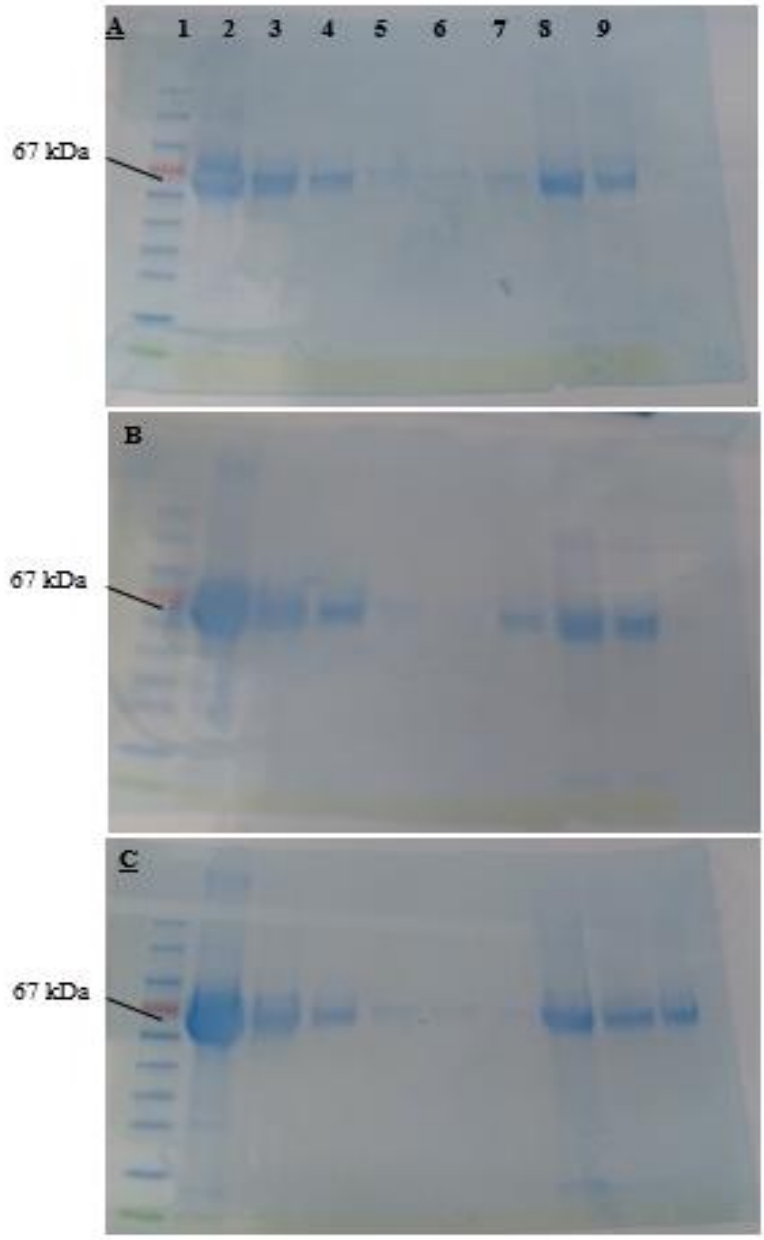


Figure 17: SDS-PAGE analysis illustrating gallibody purification using Ni-NTA affinity columns. A) $12_{CH2\text{-}4}$, B) $16_{CH2\text{-}4}$, C) $18_{CH2\text{-}4}$. Gel lanes 1) Marker, 2) Culture supernatant, 3) Flow through 1, 4) Flow through 2, 5) Washes, 6) Elution 1, 7) Elution 2, 8) Elution 3, 9) Elution 4. Successful purification is demonstrated by the comparable thickness of the 67 kDa band obtained with the culture supernatant (2) and the elutions (6-9).

The results in Figure 17 indicate that a large proportion of protein can be found in the start sample. This is expected as the cell culture media is made up a number of proteins, besides the secreted antibodies, that provide nutrients to the cell. The quantity of protein decreases in the flow through, washes. First elutions gave lower protein concentrations due to the low concentration of imidazole present in elution buffers 1. Finally in the three elutions we see a peak in the protein band of desired size in the second elution, indicating that majority of the purified gallibody is collected in these three fractions. While the results shown in Figure 16 and Figure 17 represent the characterisations of the CH2-4 gallibody types, similar results were obtained with the CH1-4 subtypes (data not shown).

To further characterize these fractions, the concentration of the samples was determined. A chicken antibody of known concentration was used as a positive control for the assay. From the results obtained it could be seen that the concentration of the start samples were comparable to the concentration of the elutions when the dilution factors were taken into account (data not shown). This illustrates that the process used for protein purification was optimal for obtaining the maximum yield of antibodies.

Once again MA was used as the antigen to test the biological activity of the antibodies. An anti-chicken enzyme conjugate was utilized to confirm that the antibodies binding to the antigen where of chicken origin. The results can be seen in Figure 18.

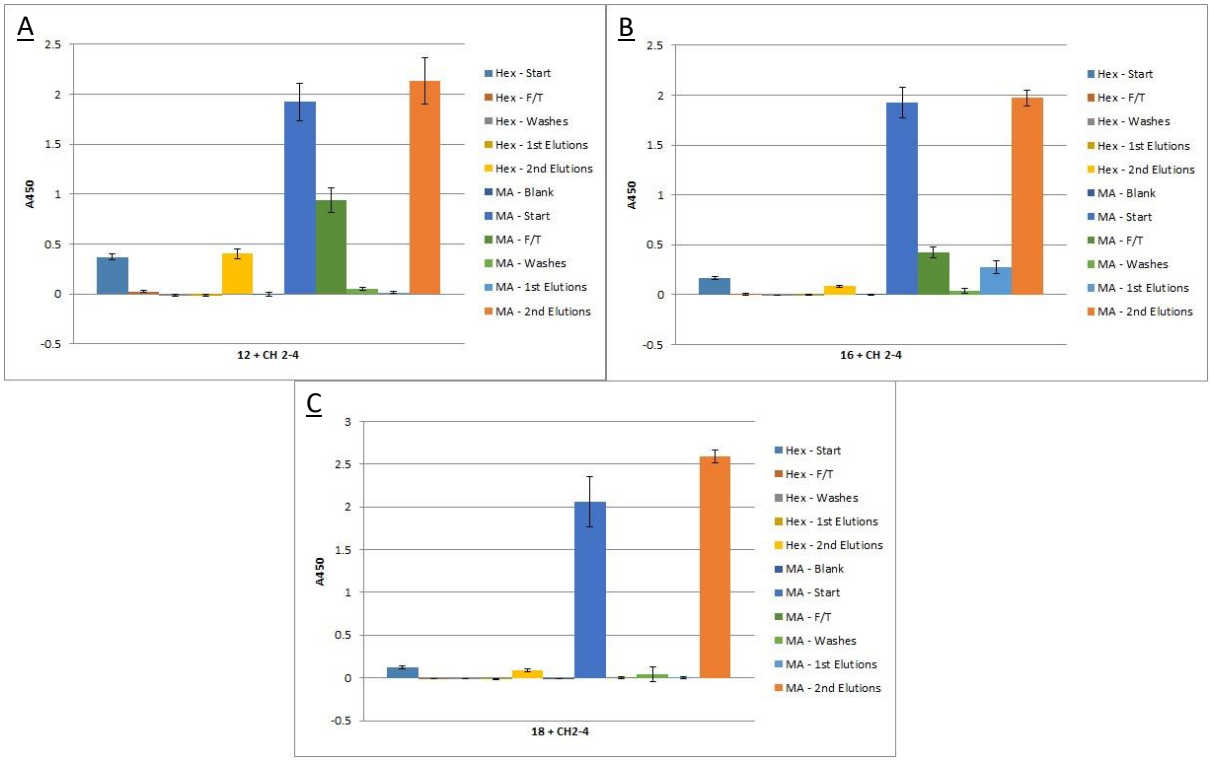


Figure 18: ELISA results to confirm the biological activity of gallibodies to bind to mycolic acid antigen after purification. Milk powder was used as the negative antibody control sample and hexane coated wells served as the negative antigen control. Gallibodies with the CH2-4 vector were tested for functionality. A = $12_{CH2\text{-}4}$, B = $16_{CH2\text{-}4}$, C = $18_{CH2\text{-}4}$, Hex = negative antigen control, MA – antigen, F/T = flow through. Binding of purified fractions is significantly different on MA antigen versus negative antigen control ($n = 4$; p value < 0.005). Start sample binding to MA is not significantly different to 2^{nd} elutions (p value > 0.005) indicating that maximum yield was obtained during purification.

From the results shown in Figure 18 it can be noted that all three types of purified gallibodies were able to bind to the MA antigen successfully. The bars corresponding to samples tested for binding to the control antigen (Hex – Start, Hex – F/T, Hex – Washes, Hex – 1^{st} elutions and Hex – 2^{nd} elutions) exhibited a smaller signal, indicating that a small percentage of background binding occurs. However, the results presented on the MA coated wells (MA – Start, MA – F/T, MA – Washes, MA – 1^{st} elutions and MA – 2^{nd} elutions) are significantly different

indicating that the gallibodies are still functional after purification. As expected the signal of the start sample binding to MA elicited a significant signal compared to the other fractions. However, the bars corresponding to the 2nd elutions illustrate a comparable signal to the start sample suggesting that the purification process was optimal in its current form. The purification and functionality tests conducted for gallibodies produced with the CH1-4 yielded similar results to those shown above (data not shown).

3.4. Discussion

Previous work conducted within our research group, yielded the expression of stable monoclonal phage-displayed scFvs with varying specificities. Two of these scFvs, termed 16 and 18, were shown only to bind MA while 12 was shown to be cross-reactive with cholesterol. While the discovery of these monoclonal antibody fragments is not novel, their stable expression and binding specificities shed light on their uniqueness (64). Although phage-display is a useful technique for the selection of monoclonal antibodies with differing specificities, the use of phage expressed antibodies in lateral flow immunoassays is generally avoided due to the bulky nature of the phage-antibody complex. Thus these antibody fragments are usually engineered into alternative antibody formats.

The expression of scFvs in alternative recombinant formats involves the manipulation and rearrangement of genes as in protein genomics. However, because these monoclonal scFvs were still attached to phagemids they had first to be rescued using helper phages, before any genetic manipulation could take place (95). Thereafter the monoclonal scFvs could be recombinantly expressed as such, or engineered into alternative formats using appropriate expression vectors. The scFvs used in this study were rescued as described previously in the thesis of Ndlandla (64). Thereafter only fusion phage (phages presenting scFvs) remain. Multiple copies of the antibody fragments may then be produced by IPTG induction, a process

in which the phage is forced into protein production mode. Although the resulting antibody fragments remain presented on the phages, scFvs could be isolated by centrifugation following the induction process. Once obtained in isolation, the scFvs were subjected to an ELISA to confirm their functionality to bind to MA. There was a possibility that this would not succeed, as once isolated, scFvs can become non-detectable due to their loss of avidity attained with multi-valency. The results with ELISA illustrated that the scFvs were all still able to bind to MA, even with very little background binding. While the signals obtained with the scFvs present in the supernatant fluid (SNF) were of extremely low magnitude, the scFvs isolated from the periplasmic membrane (PP) produced a strong signal. When monoclonal antibodies are expressed using phages, the phages are forced into a mode allowing only the expression of the specific antibody sequence you implant. As a result the antibody fragments produced can often be trapped in the space between the cell wall and intercellular membrane, known as the periplasmic membrane. That is why the scFvs are always prepared by one of two methods. ScFv fragments loosely bound to the cell wall of the phages can be detached in a simple centrifugation step when they were floating freely in the SNF, while the phage cells will deposit in the pellet. The second method involves lysing the pellet and "rescuing" the scFvs trapped in the PP. Although the latter method involves a little more work, the results of the ELISAs conducted with the monoclonal scFv fragments revealed that these scFvs from the PP represented a much higher yield than those present freely in SNF. Due to this finding the antibody engineering process continued using PP scFv fragments.

The process of antibody engineering was achieved through sub-cloning the antibody fragments into a suitable full chain antibody framework vector. The process described in the Methods section included quality checks to ensure the process ran smoothly. These quality checks were in the form of agarose gel analysis, concentration determination, SDS-PAGE analysis and ELISA. These checks enable one to go back and fix an error before moving on to the next step.

However, one should keep in mind that the success of the entire cloning process may only be determined once the sequence alignment has been conducted. For example, within this study we previously attempted to subclone the scFv DNA inserts into the vectors. While the quality checks revealed that the process was on track, the end step of sequence alignment analysis revealed that the antibody fragments had not been successfully inserted into the vector (data not shown). As a result the entire process had to be re-planned and executed to achieve the correct sequences reported in Results.

An additional challenge faced with this component of the project was the production of the gallibodies through transfection of a mammalian cell line using cell culture. Cell culture is a technique used to grow cells in a clean and sterile environment to ensure successful growth of the cells to be investigated. Cells are grown in an incubator set at 37 °C and 5% CO_2, as these have shown to be optimal conditions for general mammalian cell culture. All work in the laboratory, specifically with cells, needs to be conducted in a sterile manner to prevent contamination with bacteria and fungi. While sterility of the environment is a major concern within this process, an unsuccessful transfection can also hinder the production of the desired antibodies. Thus each method needed to be optimised for the end goal.

Once the sub-cloning procedure had been completed and the gallibodies were successfully produced by means of cell culture, their ability to bind to MA had to be determined once again. Using ELISA we were able to illustrate that the newly produced gallibodies retained their specificity for binding to MA, with limited background binding to the antigen negative control. Furthermore this technique could be applied for ensuring the antibodies' binding specificity after the purification process. By following the methods described above we were able to successfully produce two antibody frameworks for each of three different monoclonal chicken antibodies.

In the chapters that follow, the binding affinities of the newly produced gallibodies are characterized to inform some scope for the areas of application for these monoclonal antibodies.

Chapter 4: Manuscript

4.1. Preface:

Mycolic acids (MAs) are complex lipid molecules that are found in the cell wall of mycobacteria and in particular in *M.tb* (96). The fact that these lipids are species-specific makes them a key component for understanding the *M.tb* organism. While the isolation of these compounds from the organism can be a complex and costly process, the production of stereo-controlled, chemically synthetic tuberculous, mycobacterial MAs can now be conducted in the laboratory (41-48, 97, 98). In TB patient sera, antibody immune activity to MA is always accompanied by antibody immune activity to cholesterol. Investigations into the cholesteroid nature have been underway for many years, where researchers have tried to find the MA subclass responsible for this characteristic of the lipids (99). In the manuscript here submitted, we apply recombinant monoclonal antibody technology to correlate the cholesteroid functionality of MA to the MA subclasses by means of cross-reactivity of antigen recognition.

This work began in the PhD study of Ndlandla, where the researcher was successful in the selection a new set of anti-MA phage antibodies, using a similar approach to Beukes *et al*. (54), but by selecting for only thermally stable scFvs (64). While the antibodies could be used as antibody fragments for determining their binding specificities towards cholesterol and MAs, their sensitivity could only be determined by keeping their valency and concentration under control. Thus by converting the scFv fragments into full chain bivalent antibodies we could better determine the sensitivity as well as further characterize the specificities of the antibodies towards cholesterol and MA. My work comprised the grafting of the three different scFvs prepared by Ndlandla each onto two different chicken antibody (gallibody) frames, resulting in six different gallibody immune tools to investigate the cholesteroid nature of MA, its

antigenic nature after immobilization or derivatization and the potential application of the gallibody set for the development of new TB diagnostics.

The integration of my work with that of the PhD work of Lindokuhle Ndlandla in characterising the monoclonal antibodies resulted in a manuscript that was accepted for publication (100). The publication provides deeper insight into the cholesteroid nature of the MA, by the way that the two antigens are recognised by the different monoclonal anti-MA gallibodies.

The antigenicity and cholesteroid nature of mycolic acids determined by recombinant chicken antibodies

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Running title: Antigenicity and cholesteroid nature of mycolic acids

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Footnotes (abbreviations used): MA, mycolic acids; TB, tuberculosis; scFv, single chain variable fragment; AMAA, anti-mycolic acid antibodies; mAb, monoclonal antibody; ACHA, anti-cholesterol antibodies; *E. coli*, *Escherichia coli*; CD, cluster domain; V_H, variable heavy chain; V_L, variable light chain; Cas, casein; IPTG, isopropyl-β-D-thiogalactoside; SNF, supernatant fluid; NBSA, native BSA; DBSA, denatured BSA; CDR, complementarity-determining regions. Monoclonal antibodies used in this study are available in the form of cell cultures supernatants and may be supplied to other investigators by signing a material transfer agreement with the CSIR. Interested parties may contact Dr Yolandy Lemmer (ylemmer@csir.co.za).

ABSTRACT

Mycolic acids (MA) are major, species-specific lipid components of Mycobacteria and related genera. In *Mycobacterium tuberculosis,* it is made up of alpha-, methoxy- and keto-MA, each with specific biological functions and conformational characteristics. Antibodies in tuberculosis (TB) patient sera respond differently towards the three MA classes and were reported to cross-react with cholesterol. To understand the antigenicity and cholesterol cross-reactivity of MA, we generated three different chicken -derived phage-displayed single-chain variable fragments (scFv) that reacted similarly towards the natural mixture of MA, but the first recognized all three classes of chemically synthetic MAs, the second only the two oxygenated types of MAs and the third only methoxy MA. The cholesterol cross-reactivity was investigated after grafting each of the three scFv types onto two configurations of constant chain domains – CH1-4 and CH2-4. Weak but significant cross-reactivity with cholesterol was found only with CH2-4 versions, notably those two that were also able to recognize the *trans*-keto MA. The cholesteroid nature of mycobacterial mycolic acids therefore seems to be determined by the *trans*-keto MA subclass. The significantly weaker binding to cholesterol in comparison to MA confirms the potential TB diagnostic application of these antibodies.

Keywords: Mycolic acids, cholesterol, *Mycobacterium tuberculosis*, monoclonal antibodies, gallibodies, Diagnostic tools

INTRODUCTION

Tuberculosis (TB) is an infectious disease that is caused by the *Mycobacterium tuberculosis* species of bacteria (1). Despite this being discovered already for over 100 years the disease continues to cause epidemics worldwide. TB is the leading cause of death due to infectious disease globally, ranking higher than HIV/AIDS according to the latest World Health Organisation report of 2017 (2). It maintains a heavy burden on economies and human health, not only in the developing countries but also throughout the world. The latest statistics released by WHO in 2017 reported that approximately 1.1 million people were living with TB and HIV co-infection worldwide. The diagnosis of TB is a challenging aspect impacting on the management of the disease. Current TB diagnostic tests have been shown to still exhibit problems including: long time period between testing and accurate diagnosis, not enough sensitivity, not always accurate, and, in some cases, expensive. The 2017 WHO report stated that the diagnostic pipeline is progressing fast enough (2). TB is a growing epidemic and will expand if the disease is not curbed as soon as possible.

The mycobacterial cell envelope is made up of a variety of antigens of which mycolic acids (MA) represent the major lipid component (3). They occur either as free acids, linked to glycolipids such as trehalose dimycolate or bound to arabinogalactan of the peptidoglycan layer (4-6). It is known that TB patients produce anti-mycolic acid antibodies (AMAA). The AMAA levels are maintained in sera of HIV-infected TB patients regardless of a declining $CD4^+$ T cell count (7,8). This enables a biomarker test based on detection of AMAA to detect active TB disease regardless of the HIV status of the patient, which is often the challenge with antibody (Ab) biomarker tests. Although AMAA are known to exist in TB patients, the antigen moiety of MAs that is recognized by the Abs is not known and the molecular basis that governs MA-specific Ab-MA interactions is not well understood. A well-studied case of lipid antigen

recognition by Abs is that of cholesterol, where it has been demonstrated that Ab-lipid interactions do not follow the classical Ab recognition mechanism defined for general proteins. A specific protein antigenic epitope binds to a single Ab specificity, similar to enzyme-substrate recognition, but monoclonal Abs (mAbs) generated against cholesterol recognize the structural arrangement of several cholesterol molecular moieties, rather than a single defined epitope. Monoclonal Abs against cholesterol do not recognize it as a monomeric ligand or hapten, but in its crystalline form, or when in monolayers (9,10). Interestingly, a mAb that recognizes cholesterol with the hydroxyl functional group in the 3β-position cannot recognize epicholesterol where the hydroxyl group is in the 3α-position, suggesting different packing of cholesterol molecules in monolayers under different molecular arrangements. Moreover, the same mAb cannot recognize ergosterol that has the same stereochemistry of the hydroxyl functional group as cholesterol (11,12). This suggests that the specificity and/or selectivity of these structure-recognizing mAbs depend more on overall structural arrangement of particular steroid molecules than on the specific antibody contact with a spatial orientation of single functional groups of the steroid.

Unlike immobilized cholesterol, which forms from a homogeneous structure, naturally occurring MAs (Fig 1) exist as a chemically heterogeneous mixture of three major classes (alpha-, keto- and methoxy-MA).

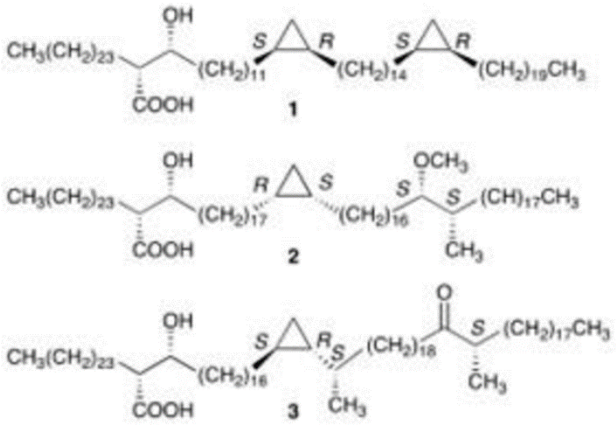


Fig 1. Structures of the three major MA classes from *Mycobacterium tuberculosis*, alpha-MA (1), methoxy-MA (2) and keto-MA (3).

These classes are characterized by the presence of chemical functional groups, viz. proximal and distal *cis*-/*trans*-cyclopropane rings for alpha-MA, proximal *cis*-/*trans*-cyclopropane and distal keto for keto-MA and proximal *cis*-/*trans*-cyclopropane and distal methoxy for methoxy-MA (5,6,13,14).

The chemical variations in MA molecule compositions are also present in the cell wall of *M.tb* and differ remarkably between mycobacterial species (5,6,13). Conformational studies of MAs in Langmuir monolayers have shown that the three major classes adopt a four-chain folded conformation that can be seen as a W-shape in two dimensions, with the molecules folding at their proximal and distal functional groups. While keto-MA favored a rigid, fully folded W-shape conformation, alpha- and methoxy-MA had partially folded conformations (15-17). It was also shown that the folding and packing of MAs are influenced by the classes, with oxygenated MAs containing α-methyl *trans*-cyclopropane groups folding more readily than those with *cis*-cyclopropane rings (18). This suggests that depending on constituents of natural MAs mixture, the MA antigen will fold differently. The structural relatedness of cholesterol and MAs is supported by (i) the fact that Amphotericin B binds to both MAs and cholesterol,

(ii) patient serum Abs produced against MAs cross-react with cholesterol, and (iii) a single mAb against MA was found that binds both cholesterol and MAs (19,20). Notably, a mAb against MA was also found that did not cross-react with cholesterol. The structural relatedness between cholesterol and MAs was reportedly responsible for the low accuracy in the serodiagnosis of TB aimed at detecting AMAA using the ELISA assay (8). It is well established that all humans have anti-cholesterol antibodies (ACHA) in their blood (21), which may in part explain the low Ab activity to MAs in TB negative patients. When designing an AMAA biomarker based test it will be necessary to avoid the detection of cholesterol-binding cross-reactive Abs.

Previously, researchers successfully generated MA-specific mAbs using phage display technology (19,22). The rationale for using phage display technology to generate MA-specific mAb is that Abs can be generated against any antigen target, including non-immunogenic or poorly immunogenic antigens such as lipids without the need for immunization (23,24). However, in order for mAbs to be used as research tools in diagnostics and in therapeutics, they need to meet certain criteria: (i) be able to bind with adequate affinity and specificity to the target, (ii) be stable and (iii) be readily produced in an affordable expression system such as *E. coli* (25). Although single chain variable Ab fragments (scFv) remain the most used mAb formats (26,27), they have stability and aggregation problems (28). A number of different strategies are used to increase scFv stability including Ab engineering after bio-panning/selection and stress-guided selection, i.e. selective pressures such as high temperatures, low pH or high concentrations of guanidinium chloride (25,29,30).

The current study was designed to investigate whether the antigenicity and cholesteroid nature of MA is dependent on a particular MA subclass type rather than a combination thereof. To confirm the cholesteroid nature of MAs and to determine the nature of its observed cross-reactivity, a scFv chicken antibody gene library was screened for specific binders to MA and

cross-reactivity with cholesterol (31). The rationale for using chicken antibodies is that, like humans, chickens express specialized MA-presenting CD1 proteins for lipid antigen presentation. It is well-known that lipid antigens can be presented to T cells using CD1 proteins on antigen presenting cells as presenter molecules. Although the CD1 family of molecules was discovered and described in the late 1970s, their function as lipid and glycolipid antigen presenting proteins was only recognized in the early 1990's, when Beckman *et al.* showed that CD1 proteins present mycobacterial MA on human dendritic cells to CD4/CD8 double negative T cells, causing these to become activated (30). Mice were found not to have the specific CD1 member that presents MA, but chickens do (32).

Three monoclonal chicken scFv Ab fragments with three different specificities were selected: one recognizing all three MA subclasses and cholesterol, another only keto- and methoxy-MA subclasses and a third only the methoxy-MA subclass. In addition, these scFv fragments were engineered into two types of bivalent IgY formats (33) which we refer to as gallibodies, one a theoretically flexible CH1-4 construct and the other a truncated and hypothetically more rigid CH2-4 type as shown in Fig 2.

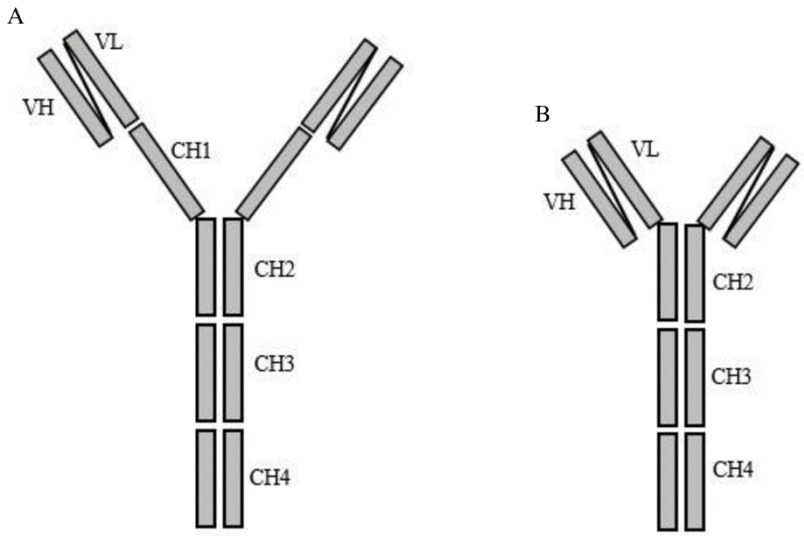


Fig 2. Structures of two types of vectors used for antibody engineering. A: scFvIgY $_{(CH1-4)}$ and B: scFvIgY $_{(CH2-4)}$. Figure adapted from Greunke *et al*. (33).

MATERIALS AND METHODS

Screening of the library for MA binders

The naïve semi-synthetic chicken phage-displayed scFv (*Nkuku*®) library containing 2 x 10^9 phage particles (31) was obtained from ARC-Onderstepoort Veterinary Institute (South Africa). The library contains scFv Ab fragments displayed on recombinant M13 bacteriophage. The scFv Ab fragments were derived from combinatorial pairings of chicken variable heavy chain (V_H) and variable light chain (V_L) immunoglobulin domains. V_H and V_L domains are linked by an interpeptide segment consisting of the sequence $(GGGGS)_3$ enabling a fold typical of scFvs. This library was panned as previously described for AMAA (19).

Briefly, Nunc-Maxisorp ELISA plates (Thermo Scientific, USA) were coated with 50 µl of a natural isolated mixture of MAs (Sigma-Aldrich, USA) dissolved in freshly distilled hexane to a final concentration of 250 µg/ml. The coated plates were allowed to evaporate at room temperature and then incubated overnight at 4 °C. Plates were washed three times with 300 µl per well of phosphate buffered saline (PBS) containing 0.1% Tween 20. Two blocking agents, 2% Casein (Cas)/PBS pH 7 and 3% bovine serum albumin (BSA)/PBS pH 7 were used alternately during four consecutive rounds of panning to reduce the possibility of enriching for blocking agent-specific binders. Plate wells were blocked with 300 µl of a blocking agent and incubated for 2 h. The *Nkuku*® phage library was pre-incubated with the blocking buffer for 30 min at room temperature and then added to the MA-coated wells and incubated for 2 h at room temperature. Plate wells were then washed twenty times with 300 µl per well of PBS/ 0.1% Tween 20 to remove unbound phages. Stringency of washing was increased during subsequent rounds of panning by increasing the number of washes from three to twenty and simultaneously increasing the wait period between washed from 30 s to 1 min. To elute phages

displaying Ab fragments that bound to MA, 150 µl of 100 mM trimethylamine was added to each well and the plate was allowed to shake at (150 rpm) for 10 min. Eluted phage were then neutralised with 150 µl of 1 M Tris, pH 7.4 and used to infect exponentially growing cultures of *E. coli* TG1 cells (New England Biolabs Inc., UK). After overnight growth, the bacteria were collected by centrifugation. Phage particles were rescued using M13KO7 helper phage (New England Biolabs Inc., UK), precipitated with PEG/NaCl (20 % polyethylene glycol 6000, 2.5 M NaCl) and used as input for the next round of panning. Biopanning was sequentially repeated four times.

Phage-ELISA

Polyclonal populations of fusion phage produced after each round of selection were tested by ELISA to confirm enrichment of MA-specific phage binders. To identify MA-specific monoclonal phage binders, individual bacterial clones were selected from each round of panning. The MA-specific monoclonal phage binders were further assessed for their long-term storage (four weeks) ability under different temperatures, cross-reactivity with cholesterol and non-specific binding to hydrophobic ligands using the BSA system (previously described; (34)). Nunc-Maxisorp ELISA plates were coated with 50 µl of natural mixture of MAs dissolved in freshly distilled hexane to a final concentration of 250 µg/ml (for polyclonal phage) or 62.5 µg/ml (for monoclonal phage). Cholesterol (Sigma-Aldrich, USA) was dissolved in hexane and coated at both 0.250 mg/ml and 1 mg/ml. Bovine serum albumin was coated at 1 mg/ml. The MA and cholesterol coated plates were allowed to have the hexane evaporate from them at room temperature and were then incubated overnight at 4 °C. The following day, plates were washed three times with 300 µl per well of PBS/0.1% Tween 20. The non-specific binding sites were blocked with 300 µl per well of 2% Cas/PBS pH 7 for 2 h whereafter the plates were washed three times with PBS/ 0.1% Tween 20. The PEG

precipitated phages were mixed with 2% Cas/PBS-0.2% Tween 20 (1:1, v/v) and 50 µl of this mixture (test solution) was added to each well and incubated for 1 h at 37 °C. The test solution was discarded and plates washed three times with 300 µl per well of PBS/ 0.1% Tween 20. A mouse monoclonal anti-M13 mAb B62-FE2 (PROGEN Biotechnik, Germany) was diluted 1:1000 in 2% Cas-PBS/0.1% Tween 20, added to the wells (50 µl per well) and incubated at 30 °C for 60 min. The Ab solution was discarded and the plates washed three times with 300 µl of PBS/0.1% Tween 20 per well to remove the unbound Abs. Rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Sigma Aldrich, USA) was diluted 1:1000 in 2% Cas-PBS/0.1%Tween 20, was then added to each well and incubated at 30°C for 60 min. The Ab solution was discarded and the plate was washed three times with 300 µl PBS/0.1% Tween 20 per well. The signal was developed by adding 50 µl TMB Single Solution Chromogen for ELISA (Invitrogen™, Thermo Scientific, USA) to each well and then incubated at room temperature for 30 min. To stop the reaction, 50 µl of 2 N H_2SO_4 was added to each well. Plates were read using a Multiskan Ascent plate reader (Thermo Scientific, USA) set at a measurement of 450 nm and a reference of 620 nm wavelengths.

Characterization of mAbs binding activities against chemically synthetic MAs

Synthetic, stereo-controlled single MAs were used to test the binding affinity of the phage clones towards the different classes of MAs. The details on their synthesis have been reported (35-42). The ELISA plates were coated with the different synthetic MAs, namely a 50/50 (v/v) mixture of synthetic MA (alpha- and methoxy-MA or alpha-and keto-MA). Natural MAs served as antigen positive control. The lipids were dissolved in hexane to a final concentration of 62.5 µg/ml and carrier hexane only coated wells served as antigen negative controls. After coating, hexane was allowed to evaporate at room temperature and plates were incubated

overnight at 4°C. Phage mAb clones of the same concentration (10^3 CFU per mL determined by phagemid titre) were tested, using ELISA as described above.

DNA sequencing

To assess the uniqueness of individual phage clones, the nucleic acids were sequenced on an Applied Biosystems ABI PRISM® 3100 Genetic Analyser (Foster City, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA), which is based on the dideoxy chain-termination DNA sequencing method of Sanger *et al*., 1997 (43). Sequence alignment was carried out using BioEdit (44).The following primers were used for amplification of scFv gene inserts from recombinant pHEN1 phagemid vector; forward OP52 primer: 5' CCCTCATAGGTTAGCGTAACG 3' and reverse M13rev primer: 5' CAGGAAACAGCTATGAC 3' (Inqaba Biotech, South Africa).

Evaluation of binding as single-chain fragments

Individual clones were grown overnight at 37°C with shaking (220 rpm) in LB medium containing 2% glucose and 100 µg/ml ampicillin. The following day, these cultures were inoculated 1:100 into 50 ml fresh medium containing glucose and ampicillin and grown to midlog (OD_{600} = 0.9). The cultures were then centrifuged and the supernatant fluid (SNF) discarded. Cell pellets were resuspended in 10 ml LB containing ampicillin and 1 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated overnight with shaking at 30 °C to induce expression of soluble fragments. Secreted scFv fragments remain in the SNF and were isolated by centrifugation at 3000 rpm for 10 min at 4°C. Cell pellets were retained to harvest periplasmic scFvs by resuspending them in one-tenth volume of 1 × PBS supplemented with 1 M NaCl and 1 mM EDTA and incubating on ice for 30 min. The fractions were centrifuged at 6000 × *g* for 10 min at 4 °C and the SNFs containing the scFvs were transferred to fresh tubes.

ELISA with MA was conducted to confirm the functionality of the scFvs. Briefly, Nunc-Maxisorp ELISA plates were coated with 50 µl of natural mixture of MAs (Sigma-Aldrich, USA) dissolved in freshly distilled hexane to a final concentration of 250 µg/ml. The coated plates were allowed to have the hexane evaporate from them at room temperature and were then stored until further use. The following day, the ELISA plates were washed three times with 300 µl per well of PBS/0.1% Tween 20. Non-specific binding sites were blocked with 300 µl per well of 4% milk powder/PBS 1 hr at 37 °C and then washed three times with PBS/ 0.1% Tween 20. Isolated scFvs were diluted with 2% milk powder/PBS-0.05% Tween 20 (1:1, v/v) and 50 µl of this mixture was added to each well. Plates were incubated for 1 h at 37°C. The test solution was discarded and plates washed three times with 300 µl per well of PBS/ 0.1% Tween 20. Mouse anti-c-*myc* tag mAb 9E10 (Onderstepoort, South Africa) diluted 1:1 in 4% milk powder-PBS/0.1% Tween 20 was added to the wells (50 µl per well) and the plates incubated at 37°C for 1 h. The Ab solution was discarded and the plates washed three times with 300 µl of PBS/0.1% Tween 20 per well to remove any unbound Abs. Polyclonal rabbit anti-mouse immunoglobulin (Dako, Denmark) diluted 1:1000 in 2% milk powder/PBS-0.05% Tween20 was added to the wells (50 µl per well) and the plates incubated at 37°C for 1 h. The conjugate solution was discarded and the plates washed three times with 300 µl of PBS/0.1% Tween 20 per well. The signal was developed by adding 50 µl of TMB Single Solution Chromogen for ELISA (Invitrogen™, Thermo Scientific, USA) to each well and then incubated at room temperature for 15 min. To stop the reaction, 50 µl of 2 N H_2SO_4 was added to each well. Plates were read at 450 nm (Thermo Electron Corporation Multiskan EX plate reader).

Antibody engineering

Single chain variable fragments antibody inserts were prepared for cloning following methods previously described (45). Briefly, scFv inserts were amplified from plasmid templates by PCR using primers to introduce a *BsiW* (5' GATCCGTACGGCCGTGACGTTGGACG 3') and an *AscI* (5' GATCGGCGCGCCACCTAGGACGGTCAGGG 3') cleavage site (Inqaba Biotech, South Africa). Restriction digests were performed on scFv inserts (Anti-MA 12, 16 and 18) and IgY-format expression vectors (scFvIgY $_{(CH1-4)}$ and scFvIgY $_{(CH2-4)}$) (33) prior to sub cloning. Ligated plasmids were transformed into JM109 chemically competent *E. coli* (Promega, Madison, USA) and amplified under ampicillin resistance. Five clones from each ligation was picked for colony PCR using the forward primer (5' TAATACGACTCACTATAGGG 3') and reverse primer (5' AGGAGGAGGGGTGGAGGACC 3') to check for the presence of the scFv inserts. Sequences obtained from analysis (Inqaba Biotech, South Africa) of plasmid DNA were compared to the original templates using BioEdit (44).

Gallibody production and purification

Human embryonic kidney (HEK) 293-H cells (Invitrogen™, Carlsbad, USA) were grown to a confluency of 80-100% in Dulbecco's Modified Eagle Medium (DMEM)(ThermoFischer Scientific, USA), supplemented with 10% (v/v) foetal bovine serum (FBS) and transfected with 2.5 µg plasmid DNA using TransIT®-293 Transfection Reagent (Mirus Bio Products, Madison, USA). Cultures were grown at 37 °C and 5% CO_2 gas. Successfully transfected cells continued to grow under antibiotic selection (Zeocin ™, Invitrogen™, Carlsbad, USA) and were expanded in fresh tissue culture flasks containing DMEM, 10% FBS and > 50 µg/ml Zeocin. Gallibodies were purified from cell culture supernatants using nickel-nitrilotriacetic acid agarose according to the manufacturer's (QIAGEN®, Hilden, Germany) instructions.

Purity and specificity of the purified gallibodies was checked using SDS-PAGE (See S2 Fig), western blot and ELISA. Purified gallibodies were concentrated in spin columns with a concurrent buffer change (0.1 M borate buffer, pH 7.4) using Vivaspin 10 000 MW PES centrifugation columns (VivaScience, Satorius Group, United Kingdom).

Gallibody ELISA

Nunc-Maxisorp ELISA plates were coated with 50 µl of natural mixture of MAs (Sigma-Aldrich, USA) dissolved in freshly distilled hexane to a final concentration of 250 µg/ml. Cholesterol was dissolved in hexane and coated at 1 mg/ml. The coated plates were allowed to have the hexane evaporate from them at room temperature and were then stored until further use. The following day, the ELISA plates were washed three times with 300 µl per well of PBS/0.1% Tween 20. Non-specific binding sites were blocked with 300 µl per well of 2% Cas/PBS pH 7 for 2 hrs at 37 °C and then washed three times with PBS/ 0.1% Tween 20. Purified gallibodies were diluted to 1 mg/ml with 2% Cas/PBS-0.2% Tween 20 (1:1, v/v) and 50 µl of this mixture was added to each well. Two-fold serial dilutions of each gallibody were prepared on the antigen coated plates, with the concentration range varying from 2 µg/ml to 1000 µg/ml. Plates were incubated for 1 h at 37°C. The test solution was discarded and plates washed three times with 300 µl per well of PBS/ 0.1% Tween 20. Goat anti-chicken Fc: HRP (AbD Serotec, Kidlington, UK) diluted 1:1000 in 2% Cas-PBS/0.1% Tween 20 was added to the wells (50 µl per well) and the plates incubated at 30°C for 1 h. The Ab solution was discarded and the plates washed three times with 300 µl of PBS/0.1% Tween 20 per well to remove any unbound Abs. The signal was developed by adding 50 µl of TMB Single Solution Chromogen for ELISA (Invitrogen™, Thermo Scientific, USA) to each well and then incubated at room temperature for 5 min on MA and 20 min on cholesterol, respectively. To

stop the reaction, 50 µl of 2 N H_2SO_4 was added to each well. Plates were read at 450 nm (Thermo Electron Corporation Multiskan EX plate reader).

Statistical analyses

In order to normalize antibody binding signals between different plates, the ratio differences of antibody binding signals relative to casein blocked non-antigen coated wells was determined and data adjusted accordingly. Data analyses was performed using the student's t-test at a confidence level of 95%.

RESULTS

Affinity enrichment of chicken antigen specific phage binders by bio-panning

To evaluate fusion phage specificity, individual clones were selected from the pooled outputs of all four rounds of panning. Only phage binders that retained reactivity to MA after 1 week of storage at 4°C were used as a pool for selecting stably expressed monoclonal phage binders (25). The next step was to distinguish true binders from false binders in the stable pool of phage binders. Approximately 270 individual clones were screened by phage-ELISA assay. Although a large number of unspecific phages remained after numerous panning rounds, these were reduced to five which were shown to be stable after one week of incubation at 4 °C (46). Two bound specifically to MA, another reacted with both MA and cholesterol and the remaining bound non-specifically.

Cholesterol and MA cross-reactive phage-displayed scFv mAb

The specificities of the three MA reactive phage clones were further evaluated by ELISA. Denatured BSA system was used with exposed hydrophobic patches that are hidden in native BSA (34). If the monoclonal phage-fused scFv Abs are not specific to MA, it is expected that they would also bind to hydrophobic patches of BSA. Our results show that anti-MA 12 reacted with both cholesterol (at the higher concentration of 1 mg/ml) and MAs. The binding to these molecules is selective, as anti-MA 12 did not bind to either native BSA (NBSA) or denatured BSA (DBSA) through hydrophobic interactions. Anti-MA 16 and anti-MA 18 showed slight binding to cholesterol only at the higher concentration ($p < 0.005$) but were not reactive to BSA (Fig 3).

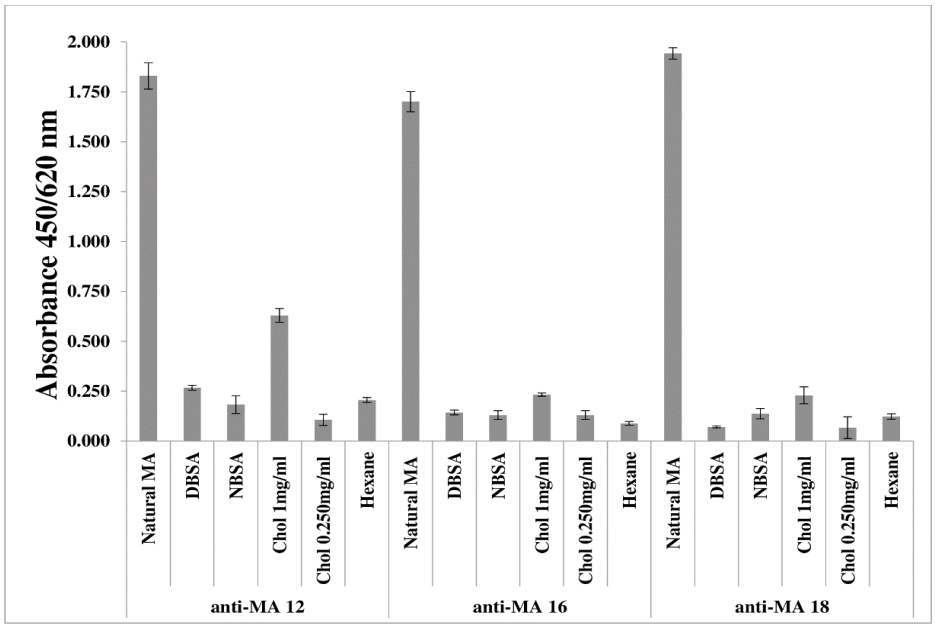


Fig 3. Evaluation of the selectivity and specificity of MA-reactive fusion-phage clones in ELISA. Natural MA was used as a positive control, while denatured bovine serum albumin (DBSA), native bovine serum albumin (NBSA) and hexane were used as negative controls. MAs were coated at 0.250 mg/ml while cholesterol was coated at both 0.250 mg/ml and 1 mg/ml. The concentration of bovine serum albumin used was 1 mg/ml. Error bars= Standard error of mean, n=5 (biological repeats).

MA-specific phage-displayed scFvs have different fine specificities of MA binding

The fine specificities of the isolated mAbs to MAs were then investigated using stereo-controlled chemically synthetic MA classes (alpha-, keto- and methoxy-) in ELISA (Fig 4).

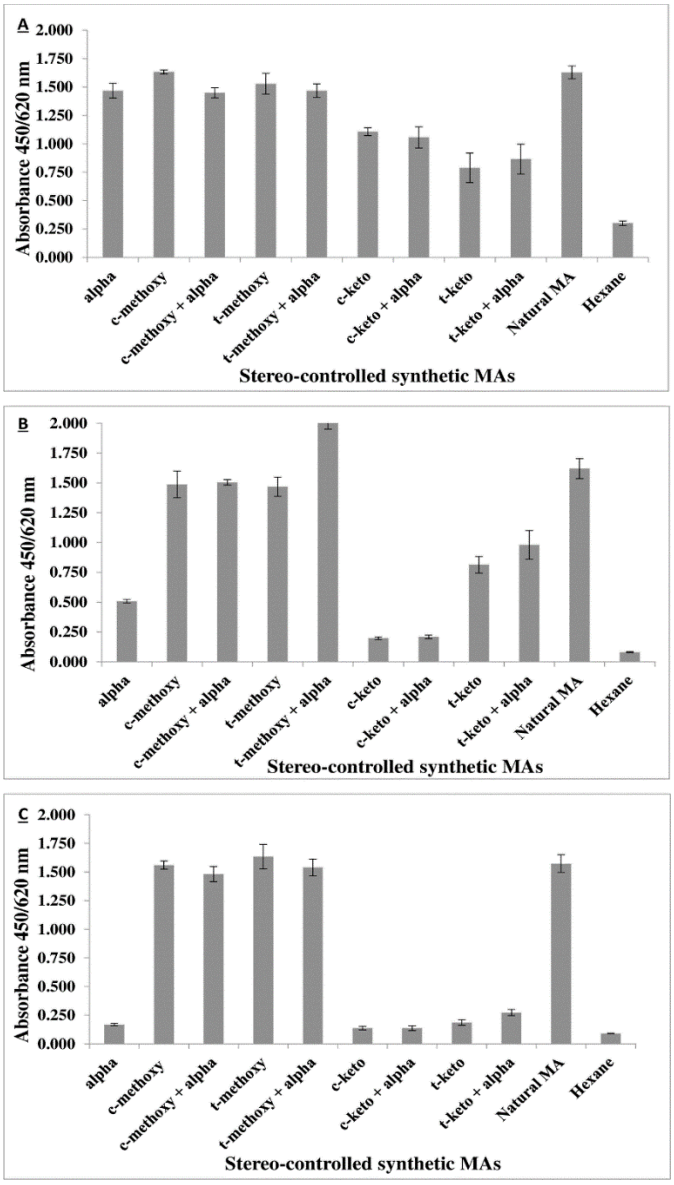


Fig 4. Characterization of the binding specificities of MA-specific phage-displayed Abs with ELISA using single stereo-isomers of stereo-controlled synthetic MAs, their 1:1 mixtures with α-MA and natural MA mixture. In this monoclonal phage ELISA assay, the

coating of MA antigens was done by adding 50 µl per well of MA (62.5 µg/ml) dissolved in hexane. Hexane alone was used as a negative control. A) Anti-MA 12, B) Anti-MA 16, C) Anti-MA 18. Error bars= Standard error of mean, n=8 (biological repeats) t=*trans*, c=*cis*. The experiments on all synthetic MAs were performed with the three mAbs in parallel under the same conditions. This result is a representative of more than three biological repeats with six technical repeats for each mAb.

Anti-MA 12 recognized all classes of MAs. Anti-MA 16 reacted with all classes of MAs except *cis*-keto. Anti-MA 18 bound strongly and exclusively to methoxy MA of either *cis*- or *trans*-configuration and irrespective of whether associated with 50% alpha-MA composition. Alpha-MA did not influence the antigenic properties of MA, since a combination of either keto- or methoxy- with alpha-MA did not result in reduced or enhanced recognition of MA antigen by the selected mAbs.

Amino acid sequences of the hypervariable regions of the MA-specific phage-displayed scFvs

Since anti-MA 12, 16 and 18 were shown to bind with three unique specificities, the Abs were sequenced to test whether their amino acid sequences are also different. The alignment (Table 1) showed the expected homology between the three monoclonal scFv Abs for the framework regions as reported in literature (31), while the complementarity-determining regions (CDRs) differed.

Table 1. Deduced amino acid sequences of CDR 3 regions for phage displayed monoclonal scFv Abs isolated against MA

Clone	V_H CDR3	V_L CDR3
Anti-MA mAb 12	MNYRRRQ	TEDSTY
Anti-MA mAb 16	RRITNK	RDSGAP
Anti-MA mAb 18	RKTNKHRIDAWGHGTEV	GSYEASNSAGIFG

V_H variable heavy chain, V_L variable light chain

It is known that the heavy chain CDR3 plays the dominant role in the observed binding specificity of Abs. The specificity conferred by the heavy chain is preserved regardless of its pairing with various light chains (47). The CDR1 and CDR2 regions of both heavy and light chains showed minimal sequence diversity, but the CDR 3 regions of both heavy and light chains confirmed that the three monoclonal scFv Abs are unique (Table 1). The most striking result was that the anti-MA mAb 18 showed significantly longer CDR3 regions in both the heavy and the light chain. Whereas CDR1 and CDR2 are known to come about by point mutation only, the larger CD3 additionally comes about by rearrangement of the V genes with D genes (H- chain) and J genes (H- and L- chains) with intermittent random nucleotide insertions.

Mycolic acid specific monoclonal gallibodies

The clones produced during the antibody engineering process were sequenced to determine the success of the subcloning procedure. Three of the newly generated clones for each of the two gallibody constant chain frames (CH1-4 and CH2-4) were selected for the subsequent gallibody production (see S1 Fig).

To confirm the specificity of the monoclonal antibodies following the conversion from phage-displayed scFvs to purified monoclonal IgY-like antibodies (See S2 Fig), gallibodies were analysed with ELISA on MA. Samples were loaded at a starting concentration of 1000 µg/ml of gallibody and titrated down to 2 µg/ml on the antigen coated plates.

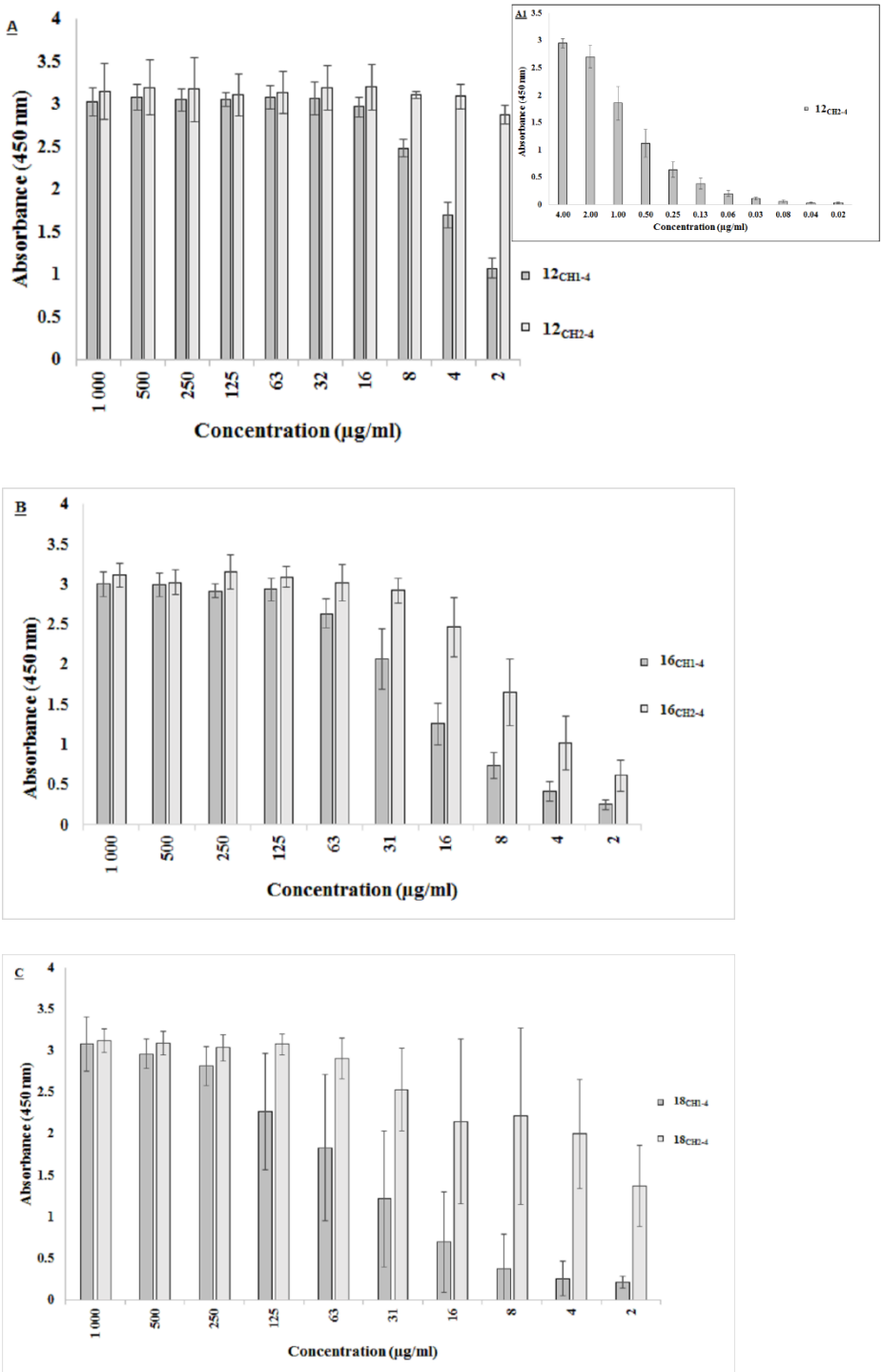


Fig 5. Gallibody binding to natural MAs in ELISA. Coating of MA antigens was done at 250 µg/ml in hexane. Hexane alone was used as a negative antigen control for background correction of the data. A) Anti-MA 12, B) Anti-MA 16, C) Anti-MA 18. Error bars = Standard deviation, n = 8 (biological repeats), CH1-4 = full length constant region, CH2-4 = truncated constant region. This result is representative of two biological repeats with four technical

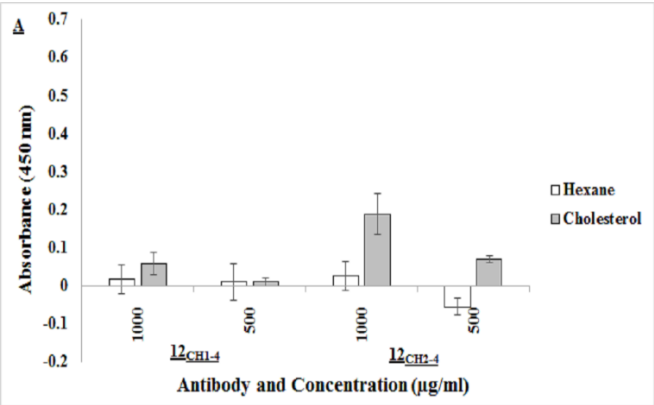
repeats for each concentration of gallibody. Insert A1) represents gallibody 12_{CH2-4} titration at lower concentrations. No non-specific binding of gallibodies was observed when the antigen negative control was used.

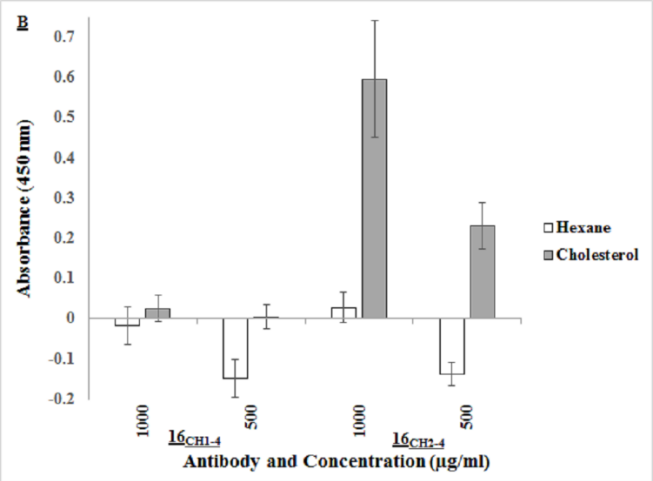
Fig 5A shows that gallibody 12_{CH2-4} was able to bind MA across the entire concentration range, with the amount of binding remaining the same even when an antibody concentration as low as 2 µg/ml was used. The sensitivity for gallibody 12CH2-4 was reached when the antibody was used at a concentration of 1 µg/ml (Fig 5 A1). The sensitivity for gallibody 12_{CH1-4} on MA was weaker at $A_{50\%}$ = 4 µg/ml. $A_{50\%}$ was determined by identifying the concentration of gallibody where absorbance was approximately 50% of the highest signal.

Gallibody 16 showed a slightly weaker sensitivity of MA binding at $A_{50\%}$ of 25 µg/ml and 8 µg/ml for gallibody 16_{CH1-4} and 16_{CH2-4}, respectively. Gallibody 18 showed the weakest binding to MA with $A_{50\%}$ for gallibody18_{CH1-4} at 75 µg/ml and gallibody 18_{CH2-4} at 16 µg/ml.

Nature of cholesterol cross-reactivity

In an attempt to probe the cholesterol cross-reactivity of MA, the gallibodies' ability to bind to cholesterol was analysed with ELISA. Fig 6 illustrates the cholesterol cross-reactivity for all the gallibodies.





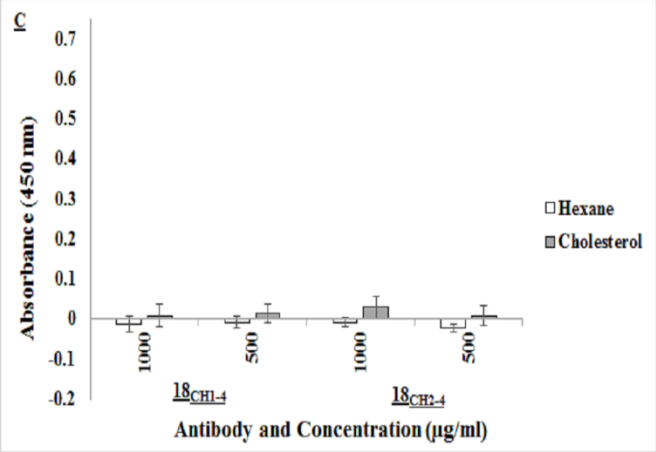


Fig 6. Cholesterol cross-reactivity of gallibodies confirmed with ELISA using hexane solvent alone as negative control. Coating of cholesterol was done at 1 mg/ml in hexane. A) Anti-MA 12, B) Anti-MA 16, C) Anti-MA 18. Error bars = Standard deviation, n = 8 (biological repeats) CH1-4 = full length constant region, CH2-4 = truncated constant region. The results each represent two biological repeats with four technical repeats for each

concentration of gallibody. The data were extracted from the same experiment that was performed in Fig. 5, making the absorbance readings from both figures directly comparable.

The results show that all of the CH1-4 gallibody types (12_{CH1-4}, 16_{CH1-4} and 18_{CH1-4}) and one of the CH2-4 type (18_{CH2-4}) did not produce signals that were significantly different from the antigen negative control, indicating that these antibodies were not able to cross-react with cholesterol. In contrast, the truncated gallibody 12_{CH2-4} and 16_{CH2-4} cross-reacted with cholesterol. The binding signal of gallibody 16_{CH2-4} was more than two fold higher than the 12_{CH2-4} version, suggesting that the former has a higher affinity for cholesterol.

DISCUSSION

Our group previously demonstrated that it is feasible to generate MA-specific recombinant scFv antibody fragments from a semi-synthetic chicken antibody-gene library (19). We used the approach that Beukes *et al*., (2010) used previously to pan for MA-specific Abs from the recombinant chicken antibody library, but selected for thermally stable scFvs. Similarly to Beukes and colleagues (2010), both cholesterol cross-reactive and non-cross-reactive anti- MA phage antibodies were isolated (19).

It appeared as if all the anti-MA phage antibodies recognised cholesterol, with anti-MA 12 giving the highest binding. The binding to cholesterol was much weaker than the binding to MA. We confirmed the specificity of cholesterol binding by using denatured BSA as a negative antigen control. The phage-displayed antibodies showed increased binding to a higher concentration of cholesterol, confirming that cholesterol is more antigenic at the higher concentration as reported by other authors (10).

While the method of phage display technology is useful for selecting scFvs with different binding specificities, it has been reported that soluble scFvs have the ability to naturally form

dimers and trimers (48,49), forming multivalent non-covalently bound antibody fragments. This may give false-positive cross-reactive binding due to the uncontrolled valency for antigen binding. While the soluble scFvs retained their ability to bind to MA, the data obtained revealed their low binding affinity (data not shown). Thus in order to truly define the binding specificities of the selected mAbs, the three scFvs were subcloned into bivalent IgY expression vectors. Results of the gallibody characterization were in agreement with those obtained with phage-displayed Abs, illustrating that all gallibodies bound to MA with approximately equal strength. Cholesterol binding results revealed that only the CH2-4 gallibody subtypes of anti-MA 12 and anti-MA 16 bound to cholesterol at high antibody concentrations. Furthermore, in contrast to the results obtained with phage antibodies, where phage antibody 12 bound strongest to cholesterol, gallibody $16_{CH2\text{-}4}$ now displayed the highest binding. This emphasizes the importance of controlling both protein concentration and antigen binding valency for quantitatively comparing cross-reactivity of anti-MA antibodies. Gallibody $18_{CH2\text{-}4}$ qualified the result found with phage antibody 18 in that it showed no affinity of binding for cholesterol. Methoxy MA is known to be the most antigenic for sensitive detection of anti-MA detection in TB patients (19,22). Anti-MA 18, which recognizes methoxy-MA but not keto-MA, has a very unique and long CDR3 region compared to anti-MA12 and anti-MA 16. The CDR3 region is the main carrier of diversity and specificity in the Ab binding site. Other researchers have shown that not only are the differences in sequence of the CDR3 heavy chains between Abs important in determining specificities but also the length of CDR3 heavy chains (50).

In a study by Chan and colleagues (2013) aimed at generating anti-MA Abs from a human antibody gene library using phage-display technology, the authors reported that they could not find a cholesterol cross-reactive anti-MA Ab (22). This observation is corroborated by our findings of all three CH1-4 gallibodies. However, cross-reactivity with cholesterol was observed with gallibody $12_{CH2\text{-}4}$ and gallibody $16_{CH2\text{-}4}$ at high antibody concentrations (1000

µg/ml and 500µg/ml). Gallibody 12 and 16 both have specificity for binding to *trans*-keto MA as such or in combination with alpha- or methoxy- MAs. In the study of Chan *et al.,* all anti-MA Ab generated could not recognize keto-MA but had weak binding to alpha-MA. Our study corroborates this finding as the $18_{CH2\text{-}4}$ gallibody – which also does not recognize keto MA – similarly did not cross-react with cholesterol. Anti-MA 18 only recognizes methoxy mycolate as such or in combination with alpha-MA (Fig 4). Anti-MA 16 recognizes all the synthetic MAs and combinations thereof, except *cis*-keto MA (Fig 4). Notably the lack of specificity of anti-MA 16 towards binding of *cis*-keto MA had no influence on the cholesterol cross-reactivity. Due to these findings, it seems likely that the cholesteroid nature of the MA antigen manifests in the fine specificity towards the *trans*-keto MA subtype.

Interestingly, an involvement of keto MA with cholesterol *in vivo* has recently been reported by Vermeulen *et al.* (51). These authors wanted to determine the effect that the different MA subclasses have on the growth of the mycobacteria in macrophages, particularly the accumulation of cholesterol brought about by the *M. tuberculosis* organism during disease progression. Their results revealed that the keto MA is responsible for the accumulation of intracellular lipid droplets packed with cholesterol. Furthermore, when mouse macrophages were treated with keto MA, bacterial growth was largely unrestricted compared to those treated with alpha- or methoxy- MA.

Notably, from the results obtained it was only selective CH2-4 gallibody types that displayed cholesterol cross-reactivity. None of the CH1-4 gallibodies possessed this ability. This finding can be related to the structures of the two IgY framework constructs used for antibody engineering. The IgY immunoglobulin class of antibodies has been described in the literature as the avian population's equivalent of the IgG subclass. While these antibodies are similar in structure, one of the most notable differences is the IgY subclass' lack of a hinge region (52). These antibodies (IgY) rely on their CH1 and CH2 domains for flexibility, with the CH1

domain contributing largely in this respect (53). As a result of this and the fact that the CH2-4 construct lacks the CH1 domain, one may refer to the two gallibody subtypes as the flexible CH1-4 and the rigid truncated CH2-4. The CH2-4 versions of the three antibody specificities were generally more sensitive for antigen binding than the CH1-4 versions. This may explain why only the rigid CH2-4 anti-MA monoclonal antibodies cross-react to cholesterol. It is probably due to divalent binding to repeating cholesteroid epitopes on clusters of immobilized MA, which is enhanced by better positive cooperative binding of the two more closely cropped Fab domains on CH2-4 antibody constructs. Mathebula *et al.* (2009) previously showed how mycolic acids cluster into small islands on a self-assembled monolayer of octadecane thiol (54). The cholesteroid epitopes in the clusters may be too close, and the MA clusters too far apart to be recognized divalently by the more flexible and larger CH1-4 type of gallibody. Thus the binding of the two CH2-4 gallibodies is probably due to the enhanced co-cooperativity aiding in the sensitivity.

The weak affinity of these recombinant mAbs relates to the binding activity of natural antibodies to MA found in TB patient sera. Previously research conducted within our research group demonstrated this property of AMAA – their low affinity. While these antibodies can be detected using ELISA, we revealed how cases can be missed when this standard technique is used for detection (8,55). As a result of this more sensitive biosensor techniques for detecting these AMAA, are applied for the diagnosis of TB patients (7).

It has been shown before that the three MA major classes (alpha-, keto- and methoxy-MA) of *M. tuberculosis* have varying antigenicity with respect to antibody recognition and that cross-reactive antibodies binding to both MA and cholesterol exist in human sera. This has now been investigated using recombinant anti-MA mAbs derived from a recombinant chicken Ab repertoire. The major findings of this study substantiate the dominant antigenic nature of

methoxy-MA reported before, while now demonstrating that the cholesteroid nature of MAs seems to depend on the ability of the AMAA to recognize the *trans*-keto MA subtype.

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Supporting information

S1 Fig. Sequences of gallibody clones produced by antibody engineering. 12) Anti-MA 12, 16) Anti-MA 16, 18) Anti-MA 18, CH1-4 = full length constant region, CH2-4 = truncated constant region, V_H = variable heavy chain, V_L = variable light chain.

S2 Fig. SDS-PAGE analysis illustrating gallibody purification using Ni-NTA affinity columns. A) 12_{CH1-4}, B) 16_{CH1-4}, C) 18_{CH1-4}, D) 12_{CH2-4}, E) 16_{CH2-4}, F) 18_{CH2-4}. Gel lanes 1) Marker, 2) Culture supernatant, 3) Flow through 1, 4) Flow through 2, 5) Washes, 6) Elution 1, 7) Elution 2, 8) Elution 3, 9) Elution 4. Successful purification is demonstrated by the comparable thickness of the 67 kDa band obtained with the culture supernatant (2) and the elutions (6-9).

NOTE: All supplementary figures contained within chapter 3 and the appendix of this thesis. S1 Fig is presented in chapter 3, figure 14; S2 Fig A-C is presented in appendix A and S2 Fig D-F is presented in chapter 3, figure 17.

4.2. Post-script

To date methods for characterisation of MA, and its subclasses, make use of highly specialized technology (such as LC-MS, NMR and biosensor) and/or antibody probing where patient serum is used as the source. The above mentioned characterisation methods are not only costly, but require skilled personnel. The use of TB patient sera requires ethics approval for the research, is limited by the small volumes obtainable from each patient and is subject to the highly variable nature of the antibodies from patient to patient. This may be due to the demonstrated broad range of IgM/IgG ratios among patients as well as the various degrees of cholesterol cross-reactivity (101). The article in this chapter that was submitted for publication changed this situation for good, providing six very powerful tools to be applied to the immunochemical characterization of the various types of mycolic acid that exist in *M.tb* and other tuberculous and non-tuberculous mycobacteria.

Table 2 demonstrates how the monoclonal gallibodies reported here can be applied to characterize the chemical composition of naturally isolated MAs. The presence of keto-MA in an immobilised sample can be quantified by comparing the ELISA values of Gallibody 12 with that of Gallibody 18. The higher the ratio of 12/18, the more keto-MA is in the sample. The type of keto-MA can then be further characterized by determining the Gallibody 12/16 binding ratio in ELISA. The ELISA binding signal 12/16 ratio should be proportional to the cis-/trans-keto-MA ratio of its content in the sample. These ratios can be investigated at higher sensitivity with the CH2-4 gallibodies.

Table 2: Binding specificities of monoclonal chicken antibodies.

Monoclonal antibody / MA specificity	12	16	18
Natural MA	✓	✓	✓
Methoxy + Alpha MA	✓	✓	✓
Methoxy MA	✓	✓	✓
trans-Keto MA	✓	✓	--
cis-Keto MA	✓	--	--
Alpha MA	✓	--	--
Cholesterol	✓	✓	--

Tracking of keto-MA may be useful for determining the growth stage of *M.tb.* In a paper published by Yuan e*t al*. (102), the authors were able to show that the production of keto-MA appears more prominent during the exponential growth stage of *M.tb*. Furthermore these authors were able to demonstrate the essential role keto-MA plays in *M.tb* in host cells (102). The authors illustrated the increase of keto MA during the *M.tb* growth stage within macrophages. While the use of keto-MA has been shown to be essential during the growth stages of *M.tb*, researchers have shown this oxygenated lipid group also plays a role in bacterial organization. It is well known that the *M.tb* organism arranges itself in an ordered structure, known as biofilms, which helps to sustain its survival within the host (103). Recently researchers have been able to show the effect that keto-MA has in the development of these ordered structures (104).

This work supports recent work published by our group, in particular that of Vermeulen *et al*., which provided further motivation for the link between cholesterol and the keto-MA subclass (105). In their study the authors show that while methoxy-MA was responsible for vacuole formation, this subclass did not enhance or sustain the growth of the *M.tb* organism in murine peritoneal macrophages. It was, in fact, the keto-MA that was responsible for accumulating cholesterol in lipid droplets in the macrophages that remained in the lipid droplets unless methoxy MA allowed the cholesterol to be sequestered into the vacuoles. In an infected macrophage, this may well relate to how cholesterol is sequestered for the purpose of feeding the pathogen in its phagosomal locus. Is it the cholesteroid nature of keto-MA that allows it to interfere in the cholesterol homeostasis of the cell? Our work in this chapter would certainly support that. Notably, the state of persistent TB is characterized by Mtb in the cell becoming almost exclusively dependent on a cholesterol diet. The analysis of keto-MA type and content of various strains of Mtb may therefore well predict the virulence of such strains to cause and sustain the state of TB in a patient.

The research presented above provides key information into how the organism supports its growth during progression of the disease. Using this information researchers can design drugs that target specific points within the growth of the organism. The emerging field of nanomedicines may aid in this aspect. Progress in this field has highlighted the benefits of using MA as a drug targeting ligand (106). In the paper of Lemmer *et al*., (2015) the authors show that by coating the encapsulated TB drugs with MA the particles exhibit significantly improved uptake by macrophages Thus by making use of the MA specific gallibodies one can ensure that the right type and amount of MA coating is present on the capsule surface of nanoencapsulated anti-TB drugs during their production process.

The development of recombinant monoclonal antibodies against MA provides the potential for improving the management of the TB epidemic, specifically in the field of diagnostics and treatment. In this chapter we add to the knowledge base of understanding the *M.tb* organism by sharing information relating to the role of the cell wall lipid components of the organism for sustaining itself during disease progression. We anticipate more exciting applications of our gallibody tools in future research aimed at improving existing technologies for assisting the management of TB worldwide.

Chapter 5: Concluding Discussion

Tuberculosis (TB), an infectious disease caused by the *Mycobacterium tuberculosis* organism, continues to cause an epidemic despite it having co-evolved with *Homo sapiens* for over 10 000 years (107). On the 17th November 2017 TB FIND released a letter entitled: "Ending TB means investing in R&D", which emphasised the urgent need for government investments in curbing the epidemic. In the report the board stated that while there were 10.4 million cases of active TB in 2016, only 6 million were diagnosed and notified. Furthermore, the number of drug-resistant infections continue to increase. As such it pointed out that: "there remains a dire need for better, faster-acting drugs, a new vaccine, and technologies that quickly diagnose TB and determine the degree of drug-resistance" (108). The focus of this thesis is the development of novel recombinant immunoglobulin tools that can aid in improving the understanding and management of TB.

Mycolic acids are complex lipid molecules found in mycobacteria and therefore also in *M.tb.* While these lipids are found in all mycobacteria, the combinations of selected classes of them in cell walls allow for distinguishing between the different mycobacterial strains, as if by fingerprinting. For example, the combination of all three lipid classes – alpha, keto, and methoxy MA – are shown to be present in *M.tb* whereas *M. avium* only exhibits the alpha and keto subclasses. The above mentioned properties of MA as well as the discovery of anti-mycolic acid antibodies have shed light on the potential of detecting TB using biomarker antibodies against lipid antigens (30, 31, 33, 109). Natural MA can be obtained commercially or prepared by isolating and purifying the compounds manually. Some of the steps of the process needs to be conducted in a specialised laboratory, because the source of the compounds, the *M.tb* organism, can be released as an inhalable aerosol. As a result the process can become costly. Our collaborators at the School of Chemistry at Bangor University have

assisted in addressing this concern by being the first to produce stereo-controlled, chemically synthetic tuberculous, mycobacterial MAs in the laboratory (41-48). These synthetic derivatives were shown to function as well as the natural source, thus providing a useful antigen that can be used to detect TB specific biomarkers antibodies. In their statement the CEOs of TB FIND made the following comments related to TB diagnostics: "At first glance, the TB diagnostics pipeline looks healthy. However, emerging game-changers are at risk due to underfunding at the clinical trial stage. In addition, very few diagnostic candidates would address the most critical need—a POC test for primary care facilities. Diversification of the POC pipeline, and identification of new biomarkers are urgently needed" (108). The Mycolic Acid Real Time Inhibition assay (MARTI) makes use of MA and antibodies against mycolic acid for the detection of TB using a biosensor (109). Although this method has been successful in the serological diagnosis of TB, the biosensor equipment requires highly skilled personnel for operation and costs of the unit are extremely high, thereby making this system impractical for simple diagnosis. While developments have been made in converting the biosensor method into a more user friendly process using electro-impedance spectroscopy (EIS), challenges have surfaced in the process. Thus there is a need for a new device employing the use of MA and anti-MA antibodies in a POC device. The potential that MA has in improving TB diagnosis has been published recently in a paper by Jones *et al*. (34). In their study the authors show that with the use of seven synthetic MA antigens their diagnostics tool had a sensitivity and specificity of 96% and 95%, respectively. Moreover, these results are based on a serological assay making use of an antigen from the *M.tb* organism, which may improve previous research conducted in this field (15, 26, 27, 110).

TB patient antibodies to MA appear to be ideal biomarkers for the diagnosis of active TB for the various reasons mentioned below. These antibodies originate from the subset of natural

antibodies aimed at lipid antigen and as such are not affected by the CD4 count of the TB-HIV patient population (49, 51). In addition these natural antibodies are not affected by prior vaccination, as with alternative methods of testing such as the TST. As such this makes them ideal analyte candidates for the diagnosis of TB as their detection: (i) is not a sputum-based test but can be conducted using one drop of blood, (ii) can distinguish between active and latent TB, (iii) allows one to follow a patient's response to treatment (32), and (iv) will aid in diagnosing extrapulmonary and paediatric TB. While there are a number of benefits for the use of MA in improving TB diagnostics, there are also a number of limitations of the molecules. These include: the anti-MA antibodies' low affinity of antigen binding, the cholesterol cross-reactivity of anti-MA immunoglobulins (53), the unpredictable ratio of IgG/M in patients sera (111, 112) and the observation that false negative results may be unavoidable under certain conditions (32). In addition to the above mentioned limitations, preliminary work has highlighted the difficulties associated with the incorporation of this lipid into a lateral flow test, in particular the art of reproducibly applying MA on the test line in an antigenic form and coping with the low avidity of the monoclonal antibodies for binding to the antigen.

The current project works towards developing MALIA. This test will take the form of a competition assay in which patient serum antibodies will compete with a gold labelled displacement probe for predicting a negative TB outcome (55). The work on MALIA began with the selection of a stable set of recombinant anti-MA phage antibodies by PhD student Ndlandla (64) from the *Nkuku*® gene library following methods similar to that of Beukes *et al*. (2010) (54). These anti-MA phage antibodies reacted similarly towards the natural mixture of MA, however, differences in the amino acid sequences in the CDR3 region highlighted the possibility of their differing specificities to MA. While these anti-MA phage antibodies provided the starting point for the development of the displacement probe needed in MALIA,

these reagents are only antibody fragments (scFv) and as such require further modification for application in the proposed test, at least to overcome the inherent low affinity of binding to MA.

The work in chapter 3 of this thesis focused on the conversion of the three different anti-MA scFvs into full chain bivalent IgY antibodies. These bivalent antibodies, termed gallibodies, were developed by gene recombinant grafting of the scFv fragments onto two constant chain domain frames – CH1-4 and CH2-4. This gave rise to six recombinant monoclonal chicken antibodies that retained their ability to bind to the natural mix of MA. With the use of protein purification and characterisation methods we could develop an optimised method of producing a maximum yield of the gallibodies for use in MALIA.

Preliminary work on determining the fine specificities of the anti-MA phage antibodies began with Ndlandla (64) where she showed the difference in their specificity towards cholesterol and MA. While the phage antibodies displayed varying strengths of binding to cholesterol, the differences in their specificities were prominent in their binding to the MA subclasses – alpha-, methoxy- and keto-MA. Anti-MA 12 recognized all three classes of chemically synthetic MAs, anti-MA 16 only the two oxygenated types of MAs and anti-MA 18 only methoxy MA. Further characterization of the antibodies conducted on the newly grafted gallibodies revealed that while all six gallibody types retained their specificity in binding to MA, only two ($12_{CH2\text{-}4}$ and $16_{CH2\text{-}4}$) retained cross-reactivity with cholesterol at high antibody concentrations. The integration of the results obtained in this study and those of Ndlandla allowed us to compare the specificities of the antibodies. Anti-MA 12 displayed specificity for the natural mix of MA, including the various synthetic subclasses listed, and cholesterol. Anti-MA 16 bound to cholesterol as well as most MA subclasses except the *cis*-keto MA and alpha MA subclasses.

Anti-18 did not show specificity for cholesterol but only bound to the natural MA, the combination of methoxy and alpha MA, and methoxy MA. While comparing the similarities in the specificity of the antibodies it could be noted that all three bound to the natural MA, the combination of methoxy and alpha MA, and methoxy MA alone. Furthermore by relating the cholesterol cross-reactivity to the antibodies' MA specificities we could establish that the cholesteroid nature of the MA seems likely to lie in the fine specificity for the trans-keto MA subclass. The integration of the findings from various studies allowed for the production of a manuscript – presented in chapter 4 - that has been accepted for publication.

An interesting observation made during the gallibody characterisation experiments related to the cholesterol cross-reactivity. Of the six gallibodies produced, only the $12_{CH2\text{-}4}$ and $16_{CH2\text{-}4}$ subtypes displayed cholesterol cross-reactivity, with this only occurring at very high antibody concentration (low avidity). While the gallibodies are known to be of low avidity, these findings highlight their much lower avidity for cholesterol compared to their MA avidity. Furthermore, it was noted that of the two that bound cholesterol, it was only evident when they were grafted on the CH2-4 IgY constant framework. One theory for this occurrence may be related to the structure of the two IgY frames shown in Figure 19.

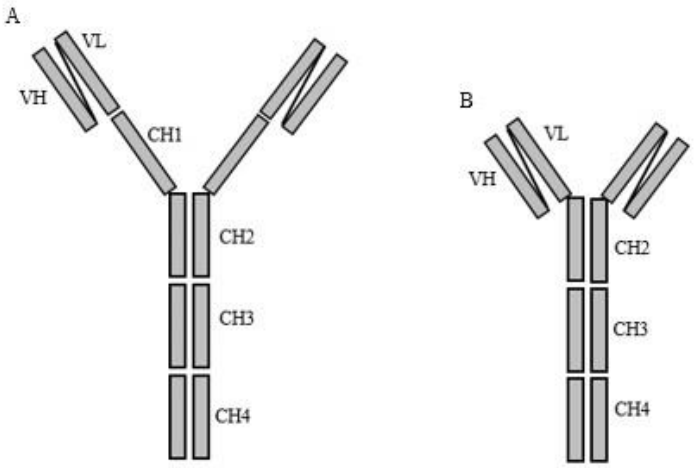

Figure 19: Structures of two types of IgY frames used for antibody engineering. A: scFvIgY (CH1–4) and B: scFvIgY (CH2–4). Figure adapted from Greunke *et al.,* (93).

From the IgY structures shown in Figure 19 it can be seen that the main difference between CH1-4 and CH2-4 vectors is the lack of the CH1 domain in the latter vector. As a result of this difference the CH2-4 gallibodies may have their Fab regions more closely cropped than the CH1-4 types. As a result the CH2-4 gallibodies may be able to exert a stronger positive cooperativity for multivalent binding of the antibodies to a surface coated with MA, in which epitopes are repeated. Thus this characteristic binding may be responsible for the CH2-4 having the ability to detectably bind to cholesterol. The much lower avidity of the gallibodies for binding to interfering cholesterol shows potential for the specificity of MALIA as it may help us to overcome a major drawback of using the MA antigen for TB diagnostics. In addition, while MAs are found in all mycobacteria, the combinations of the lipid classes present allow for distinguishing between the different bacterial strains – thus providing an advantage of using the gallibodies. The specificity of MALIA can be predicted according to the gallibody type incorporated into the final product. By making use of the gallibody types that do not recognise cholesterol one may overcome one the major drawbacks associated with using MA for TB diagnostics – cholesterol cross-reactivity of anti-mycolic acid antibodies. With the use of the

18_{CH1-4} or 18_{CH2-4} in the MALIA device the specificity of the assay can be further enhanced by making use of a gallibody subtype that recognises the MA subclass most commonly associated with M.tb – methoxy MA.

While the incorporation of these novel diagnostic tools in MALIA may help to address the current issues related to TB diagnosis, it will be important to keep in mind other tests under development and on the market that are already capable of this. The development of an aptamer based technology is one example. In a study by Rotherham *et al*. (2012) the authors reported on the stable selection of ssDNA aptamers against CFP-10 and ESAT-6 antigens that were shown to be feasible tools for detecting active TB (113). While preliminary studies of these diagnostics tools were shown to be effective for diagnosing TB in both HIV-infected and HIV-uninfected patients, the assay was not feasible for commercialisation.

The application of the gallibodies in a MALIA test may be expected to face several challenges typical for developing a test that incorporates lipid antigens. Due to the fact that most lateral flow immunoassays make use of protein antigens, the nitrocellulose membranes are designed to have a high protein binding capacity, thus making them compatible with protein antigens and antibodies. While there is limited information available on LFTs with lipid antigens, the process has been attempted by other authors. Castro *et al*. (2013) were successful in developing a lateral flow immunoassay making use of lipoidal antigens in a test for diagnosing syphilis (114). Although the authors shared their difficulties of immobilizing the lipoidal antigen onto the nitrocellulose membrane, they were able to overcome this problem by building an antibody-antigen complex. This method would not be possible in the case of MA as the compound does not have many antigen binding sites as is the case of the lipoidal antigen used by Castro *et al*. As a result alternative antibody labels, antibody engineering and/or antigen modification will

need to be explored going further. Due to the proven low avidity of the bivalent monoclonal antibodies developed in this study one can predict that incorporating them into the MALIA test would result in an assay with a poor sensitivity. However, by the further development of the displacement probe – into multivalent bodies or oligomers – the avidity of the antibodies may be increased thereby increasing the sensitivity of MALIA.

These gallibodies may be applied to supplement other areas of research aimed at fighting the TB epidemic. The treatment regime for TB consists of two phases: the initial phase and the continuous phase, combinedly lasting approximately 6 months. During the initial phase patients are require to take all four of the drugs - Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), and Ethambutol (EMB) - for a two month period. This is followed by the continuous phase, where patients are only subjected to taking RIF and INH for four months. While the regime has been shown to be effective in curing the disease, patients often stop taking their medication early because: (i) they may feel better after two weeks of treatment, or (ii) the side effects of the drugs are too intense to manage. In an attempt to resolve the issues regarding treatment, specifically those related to the latter, researchers have begun exploring the use of nanomedicines. Advances in this field have shown the potential of nanomedicines for improving treatment with decreased dosages, drug modification and targeted drug delivery. In a study by Lemmer *et al*. (2015) the authors showed that by coating encapsulated TB drugs with MA enhanced targeted drug delivery could be achieved (106). Upon consideration of the results of this study it may be suggested MA is a good targeting ligand for the following reason: because both the nanoparticles and *M.tb* are surrounded by a MA layer they are able to follow the same path of entry into macrophages. As a result they end up in the same phagosomes where the drugs can be released from the nanoparticles by phagosomal enzymes thus enabling targeted drug delivery (106). Due to the fact that the above mentioned nanoparticles are

decorated with MA, a tool that can detect the presence of the lipids would be useful in the production methods. The gallibodies developed in this study could be used as a probe to detect the MA on the nanoparticles – thus ensuring that the nanoparticles include the targeting ligand in the correct conformation needed for improved drug delivery.

The MARTI TB diagnostic process conducted using EIS was patented by our group in 2016 (115, 116). The method describes a process for coating the MA antigen onto a gold screen printed electrode, checking for the presence of MA by measuring a change in the cyclic voltammetry profile from uncoated to coated. While this process has been effective in obtaining an antigenic coating of MA onto the electrodes, a quality control check at the point of coating would enhance the technology. The development of monoclonal anti-mycolic acid antibodies, with confirmed specificity to MA, may provide a solution. Due to the fact that these antibodies are specific for MA they may be applied as a quality control check for the presence of antigenic MA on a surface or in a sample. Students within the TB research group at the University of Pretoria are currently exploring methods for the application of these gallibodies in MA-probing, specifically in the process of MA isolation and purification. The current counter current distribution (CCD) purification method is usually coupled with thin layer chromatography (TLC) to ensure the compound of interest, MA, is pure. While the use of TLC is an ideal platform for ensuring the hydrophobic compound is retained during the purification, methods for determining the antigenicity of the MA may only be done by detection of patient serum anti-MA antibodies - a costly process. Thus by employing the gallibodies during the MA production process, a quality check can be introduced to ensure the compound's antigenicity is retained during the purification process.

Another idea to consider in future research for the application of the anti-MA gallibodies is for the detection of the *M.tb* organism. The current methods of TB detection with microscopy employs the use of dyes that stain acid-fast bacilli. The process, known as Ziehl-Neelsen staining, was designed specifically for the mycobacteria genus due to the large content of lipids present in their cell walls. While this technique may be used to detect a variety of mycobacteria, it is not specific enough to differentiate between different organisms within the group. In addition a minimum amount of bacteria is required to ensure accurate staining occurs, which therefore affects the sensitivity of the method. The advantage of the differences in the MA specificities of the six gallibody subtypes, shown in table 2, provides the benefit for detecting different mycobacterial organisms. As mentioned the combinations of the MA classes present in mycobacteria may allow for distinguishing the different bacterial strains. Hence by coupling a suitable label, such as a fluorescent tag, the gallibodies can be used to attempt to resolve the above mentioned shortfalls with microscopy. The development of such a fluorescent antibody tool will not only be useful for the detection of *M.tb* in patients, but will also provide a means for detecting TB in corpses – a means for accurately detecting the cause of death in the deceased. In addition, post-mortem autopsy on TB infected corpses poses a huge infection risk to pathologists and their assistants. A prior screening of corpses with MALIA to exclude TB will provide a safer environment for performing autopsies, especially in urgent cases (117).

The work in this thesis contributes to the biggest need in TB diagnosis today: the development of a POC device that can be used for screening children, HIV-positive people, hospital staff as well as others at risk for contracting TB. The proposed test, MALIA, will serve as a negative predictor test and will take the form of a lateral flow immunoassay. The inability of a human serum sample to be displaced by labelled antibody probes on a test strip coated with MA will indicate that the person can be ruled out from having contracted TB. Here we report on the

development of six recombinant monoclonal antibody tools that cannot only detect MA but serve as a displacement tool for anti-mycolic acid antibodies and MA in patient serum samples. With the use of a suitable label these mAbs can be used for indicating active TB disease. Although the final product is yet to be assembled and validated, the development of the essential monoclonal antibody displacement tools are described here. Furthermore their functionality and characterisation is demonstrated - providing the evidence to predict that the assay will have a low sensitivity but high specificity. Proposals for increasing the sensitivity were discussed. In addition to the incorporation of these recombinant tools into the proposed MALIA device for TB screening, we discussed the potential of these antibodies for other applications contributing to the fight against TB.

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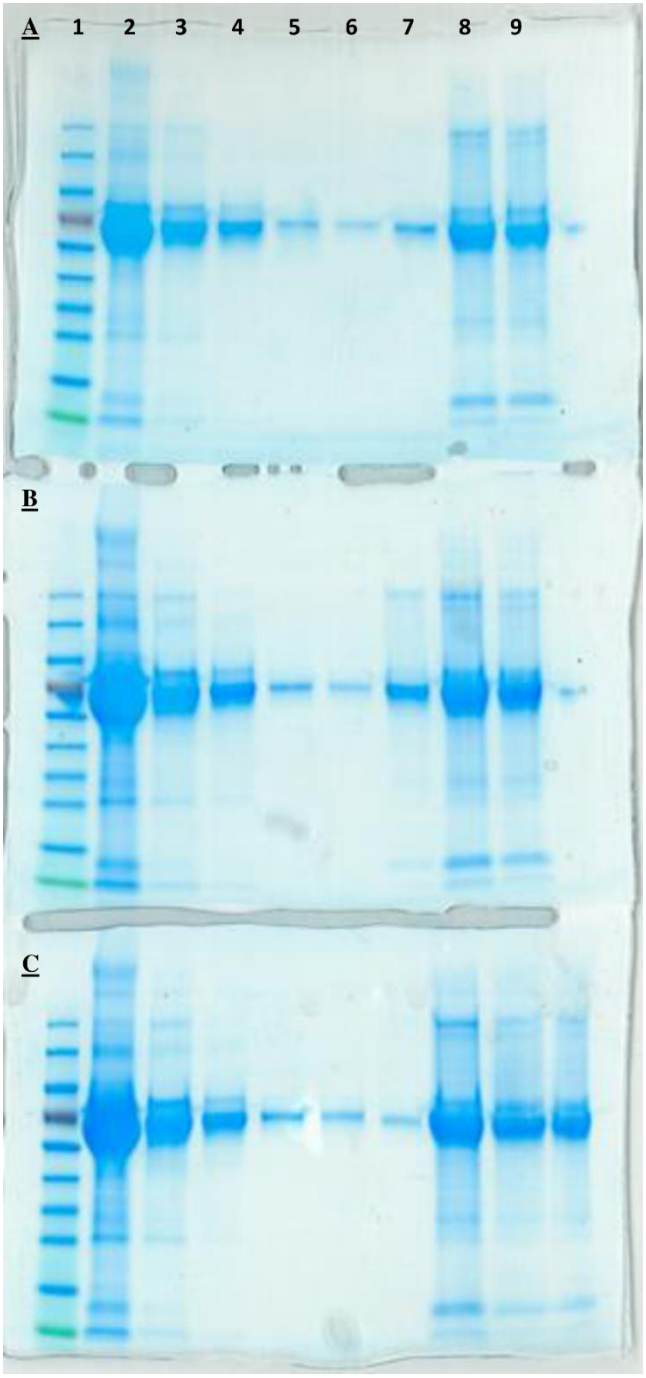
Appendix A



S2 Fig. SDS-PAGE analysis illustrating gallibody purification using Ni-NTA affinity columns. A) 12_{CH1-4}, B) 16_{CH1-4}, C) $18_{CH1-4.}$ Gel lanes 1) Marker, 2) Culture supernatant, 3) Flow through 1, 4) Flow through 2, 5) Washes, 6) Elution 1, 7) Elution 2, 8) Elution 3, 9) Elution 4. Successful purification is demonstrated by the comparable thickness of the 67 kDa band obtained with the culture supernatant (2) and the elutions (6-9).

Appendix B

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

CERTIFICATE OF REGISTRATION

Registration Number: **39.2/ARC: ONDERSTEPOORT VETERINARY INSTITUTE - 15/009**

It is hereby certified that the facility situated at:

ARC: ONDERSTEPOORT VETERINARY INSTITUTE
PRIVATE BAG X5
ONDERSTEPOORT
0110

has been registered in the name(s) of: **DR D B WALLACE**

on behalf of: **ARC: ONDERSTEPOORT VETERINARY INSTITUTE**

in terms of Regulation 8 of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997) in respect of the following-

type of facility: **LABORATORY AND ANIMAL CONTINMENT**

containment level: **ONE (1), TWO (2) AND THREE (3)**

for period: **31 JULY 2015 – 31 JULY 2018**

Date issued: 31 JULY 2015

DEPT. AGRICULTURE FORESTRY & FISHERIES
DIRECTORATE GENETIC RESOURCES
2015 -07- 31
PRIVATE BAG X973 PRETORIA 0001
REGISTRAR - GMO ACT

Registrar for Genetically Modified Organisms

Date

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Genetic Resources, Private Bag X973, PRETORIA, 0001
Harvest House Room 166, 30 Hamilton Street, Arcadia, Pretoria, 0002

From: Ms N L Mkhonza
Tel: (012) 319-6165 • **Fax:** (012) 319-6296 • **E-mail:** BathobileM@daff.gov.za
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Dr D. B Wallace
ARC: Onderstepoort Veterinary Institute
Private Bag X5
Onderstepoort
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+27 (0) 12 529 9442 (Tel)
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Dear Dr D. B Wallace

RE: REGISTRATION OF FACILITY

With reference to the application to register a facility, submitted in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997). Registration number **39.2/ARC: Ondersterpoort Veterinary Institute-15/009.**

The facility is hereby registered; please find attached your certificate which serves as proof of registration. Please familiarize yourself with the Standard Operating Procedure approved for Regulation 2(2) to determine whether your current activities or any future activities would require an additional contained use permit or not.

Please consult the website of the Department at www.daff.gov.za (Branches, Agricultural Production, Health & Food Safety/ Genetic Resources/ Biosafety) for the latest application forms and the SOP document referred to in the above paragraph.

If any of the provisions of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997), including any condition of any permit issued in terms of the GMO Act, is not complied with at all times, you will be subject to prosecution in terms of Section 21 of the GMO Act, 1997.

Yours sincerely

DEPT AGRICULTURE FORESTRY & FISHERIES
DIRECTORATE GENETIC RESOURCES
2015 -07- 31
PRIVATE BAG X973 PRETORIA 0001
REGISTRAR - GMO ACT

Ms N L Mkhonza
Registrar: Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997)

PLOS ONE

RESEARCH ARTICLE

The antigenicity and cholesteroid nature of mycolic acids determined by recombinant chicken antibodies

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Abstract

Mycolic acids (MA) are major, species-specific lipid components of Mycobacteria and related genera. In *Mycobacterium tuberculosis*, it is made up of alpha-, methoxy- and keto-MA, each with specific biological functions and conformational characteristics. Antibodies in tuberculosis (TB) patient sera respond differently towards the three MA classes and were reported to cross-react with cholesterol. To understand the antigenicity and cholesterol cross-reactivity of MA, we generated three different chicken -derived phage-displayed single-chain variable fragments (scFv) that reacted similarly towards the natural mixture of MA, but the first recognized all three classes of chemically synthetic MAs, the second only the two oxygenated types of MAs and the third only methoxy MA. The cholesterol cross-reactivity was investigated after grafting each of the three scFv types onto two configurations of constant chain domains–CH1-4 and CH2-4. Weak but significant cross-reactivity with cholesterol was found only with CH2-4 versions, notably those two that were also able to recognize the *trans*-keto MA. The cholesteroid nature of mycobacterial mycolic acids therefore seems to be determined by the *trans*-keto MA subclass. The significantly weaker binding to cholesterol in comparison to MA confirms the potential TB diagnostic application of these antibodies.

Introduction

Tuberculosis (TB) is an infectious disease that is caused by the *Mycobacterium tuberculosis* species of bacteria [1]. Despite this being discovered already for over 100 years the disease continues to cause epidemics worldwide. TB is the leading cause of death due to infectious disease globally, ranking higher than HIV/AIDS according to the latest World Health Organisation

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Abbreviations: MA, mycolic acids; TB, tuberculosis; scFv, single chain variable fragment; AMAA, anti-mycolic acid antibodies; mAb, monoclonal antibody; ACHA, anti-cholesterol antibodies; E. coli, *Escherichia coli*; CD, cluster domain; V_H, variable heavy chain; V_L, variable light chain; Cas, casein; IPTG, isopropyl-β-D-thiogalactoside; SNF, supernatant fluid; NBSA, native BSA; DBSA, denatured BSA; CDR, complementarity-determining regions.

report of 2017 [2]. It maintains a heavy burden on economies and human health, not only in the developing countries but also throughout the world. The latest statistics released by WHO in 2017 reported that approximately 1.1 million people were living with TB and HIV co-infection worldwide. The diagnosis of TB is a challenging aspect impacting on the management of the disease. Current TB diagnostic tests have been shown to still exhibit problems including: long time period between testing and accurate diagnosis, not enough sensitivity, not always accurate, and, in some cases, expensive. The 2017 WHO report stated that the diagnostic pipeline is progressing fast enough [2]. TB is a growing epidemic and will expand if the disease is not curbed as soon as possible.

The mycobacterial cell envelope is made up of a variety of antigens of which mycolic acids (MA) represent the major lipid component [3]. They occur either as free acids, linked to glycolipids such as trehalose dimycolate or bound to arabinogalactan of the peptidoglycan layer [4–6]. It is known that TB patients produce anti-mycolic acid antibodies (AMAA). The AMAA levels are maintained in sera of HIV-infected TB patients regardless of a declining $CD4^+$ T cell count [7, 8]. This enables a biomarker test based on detection of AMAA to detect active TB disease regardless of the HIV status of the patient, which is often the challenge with antibody (Ab) biomarker tests. Although AMAA are known to exist in TB patients, the antigen moiety of MAs that is recognized by the Abs is not known and the molecular basis that governs MA-specific Ab-MA interactions is not well understood. A well-studied case of lipid antigen recognition by Abs is that of cholesterol, where it has been demonstrated that Ab-lipid interactions do not follow the classical Ab recognition mechanism defined for general proteins. A specific protein antigenic epitope binds to a single Ab specificity, similar to enzyme-substrate recognition, but monoclonal Abs (mAbs) generated against cholesterol recognize the structural arrangement of several cholesterol molecular moieties, rather than a single defined epitope. Monoclonal Abs against cholesterol do not recognize it as a monomeric ligand or hapten, but in its crystalline form, or when in monolayers [9, 10]. Interestingly, a mAb that recognizes cholesterol with the hydroxyl functional group in the 3β-position cannot recognize epicholesterol where the hydroxyl group is in the 3α-position, suggesting different packing of cholesterol molecules in monolayers under different molecular arrangements. Moreover, the same mAb cannot recognize ergosterol that has the same stereochemistry of the hydroxyl functional group as cholesterol [11, 12]. This suggests that the specificity and/or selectivity of these structure-recognizing mAbs depend more on overall structural arrangement of particular steroid molecules than on the specific antibody contact with a spatial orientation of single functional groups of the steroid.

Unlike immobilized cholesterol, which forms from a homogeneous structure, naturally occurring MAs (Fig 1) exist as a chemically heterogeneous mixture of three major classes (alpha-, keto- and methoxy-MA).

These classes are characterized by the presence of chemical functional groups, viz. proximal and distal *cis*-/*trans*-cyclopropane rings for alpha-MA, proximal *cis*-/*trans*-cyclopropane and distal keto for keto-MA and proximal *cis*-/*trans*-cyclopropane and distal methoxy for methoxy-MA [5, 6, 13, 14].

The chemical variations in MA molecule compositions are also present in the cell wall of *M. tuberculosis* and differ remarkably between mycobacterial species [5, 6, 13]. Conformational studies of MAs in Langmuir monolayers have shown that the three major classes adopt a four-chain folded conformation that can be seen as a W-shape in two dimensions, with the molecules folding at their proximal and distal functional groups. While keto-MA favored a rigid, fully folded W-shape conformation, alpha- and methoxy-MA had partially folded conformations [15–17]. It was also shown that the folding and packing of MAs are influenced by the classes, with oxygenated MAs containing α-methyl *trans*-cyclopropane groups folding more

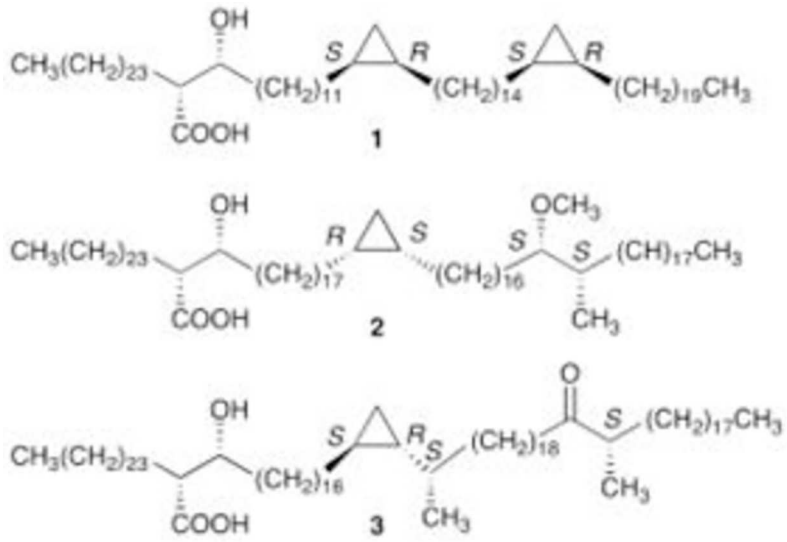

Fig 1. Structures of the three major MA classes from *Mycobacterium tuberculosis*, alpha-MA (1), methoxy-MA (2) and keto-MA (3).

https://doi.org/10.1371/journal.pone.0200298.g001

readily than those with *cis*-cyclopropane rings [18]. This suggests that depending on constituents of natural MAs mixture, the MA antigen will fold differently. The structural relatedness of cholesterol and MAs is supported by (i) the fact that Amphotericin B binds to both MAs and cholesterol, (ii) patient serum Abs produced against MAs cross-react with cholesterol, and (iii) a single mAb against MA was found that binds both cholesterol and MAs [19, 20]. Notably, a mAb against MA was also found that did not cross-react with cholesterol. The structural relatedness between cholesterol and MAs was reportedly responsible for the low accuracy in the serodiagnosis of TB aimed at detecting AMAA using the ELISA assay [8]. It is well established that all humans have anti-cholesterol antibodies (ACHA) in their blood [21], which may in part explain the low Ab activity to MAs in TB negative patients. When designing an AMAA biomarker based test it will be necessary to avoid the detection of cholesterol-binding cross-reactive Abs.

Previously, researchers successfully generated MA-specific mAbs using phage display technology [19, 22]. The rationale for using phage display technology to generate MA-specific mAb is that Abs can be generated against any antigen target, including non-immunogenic or poorly immunogenic antigens such as lipids without the need for immunization [23, 24]. However, in order for mAbs to be used as research tools in diagnostics and in therapeutics, they need to meet certain criteria: (i) be able to bind with adequate affinity and specificity to the target, (ii) be stable and (iii) be readily produced in an affordable expression system such as *E. coli* [25]. Although single chain variable Ab fragments (scFv) remain the most used mAb formats [26, 27], they have stability and aggregation problems [28]. A number of different strategies are used to increase scFv stability including Ab engineering after bio-panning/selection and stress-guided selection, i.e. selective pressures such as high temperatures, low pH or high concentrations of guanidinium chloride [25, 29].

The current study was designed to investigate whether the antigenicity and cholesteroid nature of MA is dependent on a particular MA subclass type rather than a combination thereof. To confirm the cholesteroid nature of MAs and to determine the nature of its observed cross-reactivity, a scFv chicken antibody gene library was screened for specific binders to MA and cross-reactivity with cholesterol [30]. The rationale for using chicken antibodies is that,

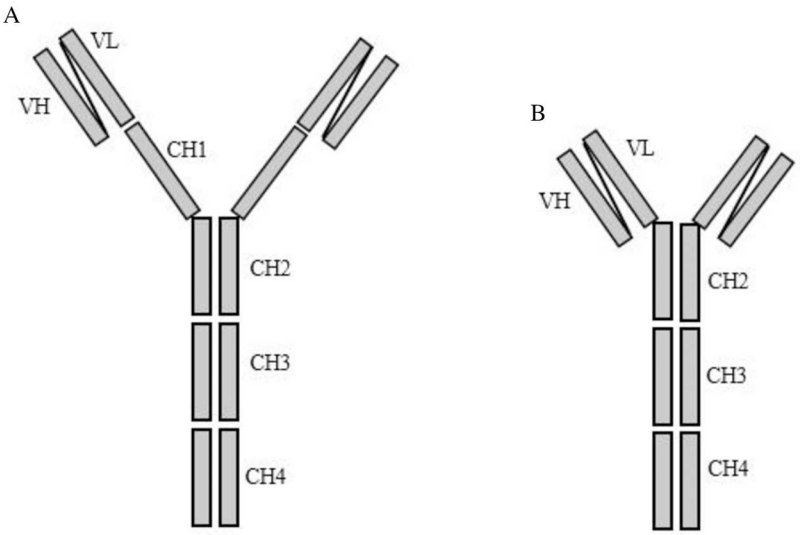

Fig 2. Structures of two types of vectors used for antibody engineering. A: scFvIgY $_{(CH1-4)}$ and B: scFvIgY $_{(CH2-4)}$. Figure adapted from Greunke *et al.* [33].

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like humans, chickens express specialized MA-presenting CD1 proteins for lipid antigen presentation. It is well-known that lipid antigens can be presented to T cells using CD1 proteins on antigen presenting cells as presenter molecules. Although the CD1 family of molecules was discovered and described in the late 1970s, their function as lipid and glycolipid antigen presenting proteins was only recognized in the early 1990's, when Beckman *et al.* showed that CD1 proteins present mycobacterial MA on human dendritic cells to CD4/CD8 double negative T cells, causing these to become activated [31]. Mice were found not to have the specific CD1 member that presents MA, but chickens do [32].

Three monoclonal chicken scFv Ab fragments with three different specificities were selected: one recognizing all three MA subclasses and cholesterol, another only keto- and methoxy-MA subclasses and a third only the methoxy-MA subclass. In addition, these scFv fragments were engineered into two types of bivalent IgY formats [33] which we refer to as gallibodies, one a theoretically flexible CH1-4 construct and the other a truncated and hypothetically more rigid CH2-4 type as shown in Fig 2.

Materials and methods

Screening of the library for MA binders

The naïve semi-synthetic chicken phage-displayed scFv (*Nkuku*®) library containing 2 x 10^9 phage particles [30] was obtained from ARC-Onderstepoort Veterinary Institute (South Africa). The library contains scFv Ab fragments displayed on recombinant M13 bacteriophage. The scFv Ab fragments were derived from combinatorial pairings of chicken variable heavy chain (V_H) and variable light chain (V_L) immunoglobulin domains. V_H and V_L domains are linked by an interpeptide segment consisting of the sequence $(GGGGS)_3$ enabling a fold typical of scFvs. This library was panned as previously described for AMAA [19].

Briefly, Nunc-Maxisorp ELISA plates (Thermo Scientific, USA) were coated with 50 μl of a natural isolated mixture of MAs (Sigma-Aldrich, USA) dissolved in freshly distilled hexane to a final concentration of 250 μg/ml. The coated plates were allowed to evaporate at room temperature and then incubated overnight at 4˚C. Plates were washed three times with 300 μl per

well of phosphate buffered saline (PBS) containing 0.1% Tween 20. Two blocking agents, 2% Casein (Cas)/PBS pH 7 and 3% bovine serum albumin (BSA)/PBS pH 7 were used alternately during four consecutive rounds of panning to reduce the possibility of enriching for blocking agent-specific binders. Plate wells were blocked with 300 μl of a blocking agent and incubated for 2 h. The *Nkuku*® phage library was pre-incubated with the blocking buffer for 30 min at room temperature and then added to the MA-coated wells and incubated for 2 h at room temperature. Plate wells were then washed twenty times with 300 μl per well of PBS/ 0.1% Tween 20 to remove unbound phages. Stringency of washing was increased during subsequent rounds of panning by increasing the number of washes from three to twenty and simultaneously increasing the wait period between washed from 30 s to 1 min. To elute phages displaying Ab fragments that bound to MA, 150 μl of 100 mM trimethylamine was added to each well and the plate was allowed to shake at (150 rpm) for 10 min. Eluted phage were then neutralised with 150 μl of 1 M Tris, pH 7.4 and used to infect exponentially growing cultures of *E. coli* TG1 cells (New England Biolabs Inc., UK). After overnight growth, the bacteria were collected by centrifugation. Phage particles were rescued using M13KO7 helper phage (New England Biolabs Inc., UK), precipitated with PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) and used as input for the next round of panning. Biopanning was sequentially repeated four times.

Phage-ELISA

Polyclonal populations of fusion phage produced after each round of selection were tested by ELISA to confirm enrichment of MA-specific phage binders. To identify MA-specific monoclonal phage binders, individual bacterial clones were selected from each round of panning. The MA-specific monoclonal phage binders were further assessed for their long-term storage (four weeks) ability under different temperatures, cross-reactivity with cholesterol and non-specific binding to hydrophobic ligands using the BSA system (previously described; [34]). Nunc-Maxisorp ELISA plates were coated with 50 μl of natural mixture of MAs dissolved in freshly distilled hexane to a final concentration of 250 μg/ml (for polyclonal phage) or 62.5 μg/ml (for monoclonal phage). Cholesterol (Sigma-Aldrich, USA) was dissolved in hexane and coated at both 0.250 mg/ml and 1 mg/ml. Bovine serum albumin was coated at 1 mg/ml. The MA and cholesterol coated plates were allowed to have the hexane evaporate from them at room temperature and were then incubated overnight at 4˚C. The following day, plates were washed three times with 300 μl per well of PBS/0.1% Tween 20. The non-specific binding sites were blocked with 300 μl per well of 2% Cas/PBS pH 7 for 2 h whereafter the plates were washed three times with PBS/ 0.1% Tween 20. The PEG precipitated phages were mixed with 2% Cas/PBS-0.2% Tween 20 (1:1, v/v) and 50 μl of this mixture (test solution) was added to each well and incubated for 1 h at 37˚C. The test solution was discarded and plates washed three times with 300 μl per well of PBS/ 0.1% Tween 20. A mouse monoclonal anti-M13 mAb B62-FE2 (PROGEN Biotechnik, Germany) was diluted 1:1000 in 2% Cas-PBS/0.1% Tween 20, added to the wells (50 μl per well) and incubated at 30˚C for 60 min. The Ab solution was discarded and the plates washed three times with 300 μl of PBS/0.1% Tween 20 per well to remove the unbound Abs. Rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Sigma Aldrich, USA) was diluted 1:1000 in 2% Cas-PBS/0.1%Tween 20, was then added to each well and incubated at 30˚C for 60 min. The Ab solution was discarded and the plate was washed three times with 300 μl PBS/0.1% Tween 20 per well. The signal was developed by adding 50 μl TMB Single Solution Chromogen for ELISA (Invitrogen™, Thermo Scientific, USA) to each well and then incubated at room temperature for 30 min. To stop the reaction, 50 μl of 2 N H_2SO_4 was added to each well. Plates were read using a Multiskan Ascent plate reader

(Thermo Scientific, USA) set at a measurement of 450 nm and a reference of 620 nm wavelengths.

Characterization of mAbs binding activities against chemically synthetic MAs

Synthetic, stereo-controlled single MAs were used to test the binding affinity of the phage clones towards the different classes of MAs. The details on their synthesis have been reported [35–42]. The ELISA plates were coated with the different synthetic MAs, namely a 50/50 (v/v) mixture of synthetic MA (alpha- and methoxy-MA or alpha-and keto-MA). Natural MAs served as antigen positive control. The lipids were dissolved in hexane to a final concentration of 62.5 μg/ml and carrier hexane only coated wells served as antigen negative controls. After coating, hexane was allowed to evaporate at room temperature and plates were incubated overnight at 4˚C. Phage mAb clones of the same concentration (10^3 CFU per mL determined by phagemid titre) were tested, using ELISA as described above.

DNA sequencing

To assess the uniqueness of individual phage clones, the nucleic acids were sequenced on an Applied Biosystems ABI PRISM® 3100 Genetic Analyser (Foster City, USA) using the Big-Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA), which is based on the dideoxy chain-termination DNA sequencing method of Sanger *et al*., 1997 [43]. Sequence alignment was carried out using BioEdit [44].The following primers were used for amplification of scFv gene inserts from recombinant pHEN1 phagemid vector; forward OP52 primer: `5′ CCCTCATAGGTTAGCGTAACG 3'` and reverse M13rev primer: `5′ CAG GAAACAGCTATGAC 3'` (Inqaba Biotech, South Africa).

Evaluation of binding as single-chain fragments

Individual clones were grown overnight at 37˚C with shaking (220 rpm) in LB medium containing 2% glucose and 100 μg/ml ampicillin. The following day, these cultures were inoculated 1:100 into 50 ml fresh medium containing glucose and ampicillin and grown to midlog (OD_{600} = 0.9). The cultures were then centrifuged and the supernatant fluid (SNF) discarded. Cell pellets were resuspended in 10 ml LB containing ampicillin and 1 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated overnight with shaking at 30˚C to induce expression of soluble fragments. Secreted scFv fragments remain in the SNF and were isolated by centrifugation at 3000 rpm for 10 min at 4˚C. Cell pellets were retained to harvest periplasmic scFvs by resuspending them in one-tenth volume of 1 × PBS supplemented with 1 M NaCl and 1 mM EDTA and incubating on ice for 30 min. The fractions were centrifuged at 6000 × *g* for 10 min at 4˚C and the SNFs containing the scFvs were transferred to fresh tubes. ELISA with MA was conducted to confirm the functionality of the scFvs. Briefly, Nunc-Maxisorp ELISA plates were coated with 50 μl of natural mixture of MAs (Sigma-Aldrich, USA) dissolved in freshly distilled hexane to a final concentration of 250 μg/ml. The coated plates were allowed to have the hexane evaporate from them at room temperature and were then stored until further use. The following day, the ELISA plates were washed three times with 300 μl per well of PBS/0.1% Tween 20. Non-specific binding sites were blocked with 300 μl per well of 4% milk powder/PBS 1 hr at 37˚C and then washed three times with PBS/ 0.1% Tween 20. Isolated scFvs were diluted with 2% milk powder/PBS-0.05% Tween 20 (1:1, v/v) and 50 μl of this mixture was added to each well. Plates were incubated for 1 h at 37˚C. The test solution was discarded and plates washed three times with 300 μl per well of PBS/ 0.1% Tween 20. Mouse anti-c-*myc* tag mAb 9E10 (Onderstepoort, South Africa) diluted 1:1 in 4% milk powder-PBS/0.1% Tween 20

was added to the wells (50 μl per well) and the plates incubated at 37˚C for 1 h. The Ab solution was discarded and the plates washed three times with 300 μl of PBS/0.1% Tween 20 per well to remove any unbound Abs. Polyclonal rabbit anti-mouse immunoglobulin (Dako, Denmark) diluted 1:1000 in 2% milk powder/PBS-0.05% Tween20 was added to the wells (50 μl per well) and the plates incubated at 37˚C for 1 h. The conjugate solution was discarded and the plates washed three times with 300 μl of PBS/0.1% Tween 20 per well. The signal was developed by adding 50 μl of TMB Single Solution Chromogen for ELISA (Invitrogen™, Thermo Scientific, USA) to each well and then incubated at room temperature for 15 min. To stop the reaction, 50 μl of 2 N H_2SO_4 was added to each well. Plates were read at 450 nm (Thermo Electron Corporation Multiskan EX plate reader).

Antibody engineering

Single chain variable fragments antibody inserts were prepared for cloning following methods previously described [45]. Briefly, scFv inserts were amplified from plasmid templates by PCR using primers to introduce a *BsiW* (5′ GATCCGTACGGCCGTGACGTTGGACG 3′) and an *AscI* (5′ GATCGGCGCGCCACCTAGGACGGTCAGGG 3′) cleavage site (Inqaba Biotech, South Africa). Restriction digests were performed on scFv inserts (Anti-MA 12, 16 and 18) and IgY-format expression vectors (scFvIgY $_{(CH1-4)}$ and scFvIgY $_{(CH2-4)}$) [33] prior to sub cloning. Ligated plasmids were transformed into JM109 chemically competent *E. coli* (Promega, Madison, USA) and amplified under ampicillin resistance. Five clones from each ligation was picked for colony PCR using the forward primer (5′ TAATACGACTCACTATAGGG 3′) and reverse primer (5′ AGGAGGAGGGGTGGAGGACC 3′) to check for the presence of the scFv inserts. Sequences obtained from analysis (Inqaba Biotech, South Africa) of plasmid DNA were compared to the original templates using BioEdit [44].

Gallibody production and purification

Human embryonic kidney (HEK) 293-H cells (Invitrogen™, Carlsbad, USA) were grown to a confluency of 80–100% in Dulbecco's Modified Eagle Medium (DMEM)(ThermoFischer Scientific, USA), supplemented with 10% (v/v) foetal bovine serum (FBS) and transfected with 2.5 μg plasmid DNA using TransIT®-293 Transfection Reagent (Mirus Bio Products, Madison, USA). Cultures were grown at 37˚C and 5% CO_2 gas. Successfully transfected cells continued to grow under antibiotic selection (Zeocin ™, Invitrogen™, Carlsbad, USA) and were expanded in fresh tissue culture flasks containing DMEM, 10% FBS and $>$ 50 μg/ml Zeocin. Gallibodies were purified from cell culture supernatants using nickel-nitrilotriacetic acid agarose according to the manufacturer's (QIAGEN®, Hilden, Germany) instructions. Purity and specificity of the purified gallibodies was checked using SDS-PAGE (See S2 Fig), western blot and ELISA. Purified gallibodies were concentrated in spin columns with a concurrent buffer change (0.1 M borate buffer, pH 7.4) using Vivaspin 10 000 MW PES centrifugation columns (VivaScience, Satorius Group, United Kingdom).

Gallibody ELISA

Nunc-Maxisorp ELISA plates were coated with 50 μl of natural mixture of MAs (Sigma-Aldrich, USA) dissolved in freshly distilled hexane to a final concentration of 250 μg/ml. Cholesterol was dissolved in hexane and coated at 1 mg/ml. The coated plates were allowed to have the hexane evaporate from them at room temperature and were then stored until further use. The following day, the ELISA plates were washed three times with 300 μl per well of PBS/0.1% Tween 20. Non-specific binding sites were blocked with 300 μl per well of 2% Cas/PBS pH 7 for 2 hrs at 37˚C and then washed three times with PBS/ 0.1% Tween 20. Purified gallibodies

were diluted to 1 mg/ml with 2% Cas/PBS-0.2% Tween 20 (1:1, v/v) and 50 μl of this mixture was added to each well. Two-fold serial dilutions of each gallibody were prepared on the antigen coated plates, with the concentration range varying from 2 μg/ml to 1000 μg/ml. Plates were incubated for 1 h at 37˚C. The test solution was discarded and plates washed three times with 300 μl per well of PBS/ 0.1% Tween 20. Goat anti-chicken Fc: HRP (AbD Serotec, Kidlington, UK) diluted 1:1000 in 2% Cas-PBS/0.1% Tween 20 was added to the wells (50 μl per well) and the plates incubated at 30˚C for 1 h. The Ab solution was discarded and the plates washed three times with 300 μl of PBS/0.1% Tween 20 per well to remove any unbound Abs. The signal was developed by adding 50 μl of TMB Single Solution Chromogen for ELISA (Invitrogen™, Thermo Scientific, USA) to each well and then incubated at room temperature for 5 min on MA and 20 min on cholesterol, respectively. To stop the reaction, 50 μl of 2 N H_2SO_4 was added to each well. Plates were read at 450 nm (Thermo Electron Corporation Multiskan EX plate reader).

Statistical analyses

In order to normalize antibody binding signals between different plates, the ratio differences of antibody binding signals relative to casein blocked non-antigen coated wells was determined and data adjusted accordingly. Data analyses was performed using the student's t-test at a confidence level of 95%.

Results

Affinity enrichment of chicken antigen specific phage binders by bio-panning

To evaluate fusion phage specificity, individual clones were selected from the pooled outputs of all four rounds of panning. Only phage binders that retained reactivity to MA after 1 week of storage at 4˚C were used as a pool for selecting stably expressed monoclonal phage binders [25]. The next step was to distinguish true binders from false binders in the stable pool of phage binders. Approximately 270 individual clones were screened by phage-ELISA assay. Although a large number of unspecific phages remained after numerous panning rounds, these were reduced to five which were shown to be stable after one week of incubation at 4˚C [46]. Two bound specifically to MA, another reacted with both MA and cholesterol and the remaining bound non-specifically.

Cholesterol and MA cross-reactive phage-displayed scFv mAb

The specificities of the three MA reactive phage clones were further evaluated by ELISA. Denatured BSA system was used with exposed hydrophobic patches that are hidden in native BSA [34]. If the monoclonal phage-fused scFv Abs are not specific to MA, it is expected that they would also bind to hydrophobic patches of BSA. Our results show that anti-MA 12 reacted with both cholesterol (at the higher concentration of 1 mg/ml) and MAs. The binding to these molecules is selective, as anti-MA 12 did not bind to either native BSA (NBSA) or denatured BSA (DBSA) through hydrophobic interactions. Anti-MA 16 and anti-MA 18 showed slight binding to cholesterol only at the higher concentration ($p < 0.005$) but were not reactive to BSA (Fig 3).

MA-specific phage-displayed scFvs have different fine specificities of MA binding

The fine specificities of the isolated mAbs to MAs were then investigated using stereo-controlled chemically synthetic MA classes (alpha-, keto- and methoxy-) in ELISA (Fig 4).

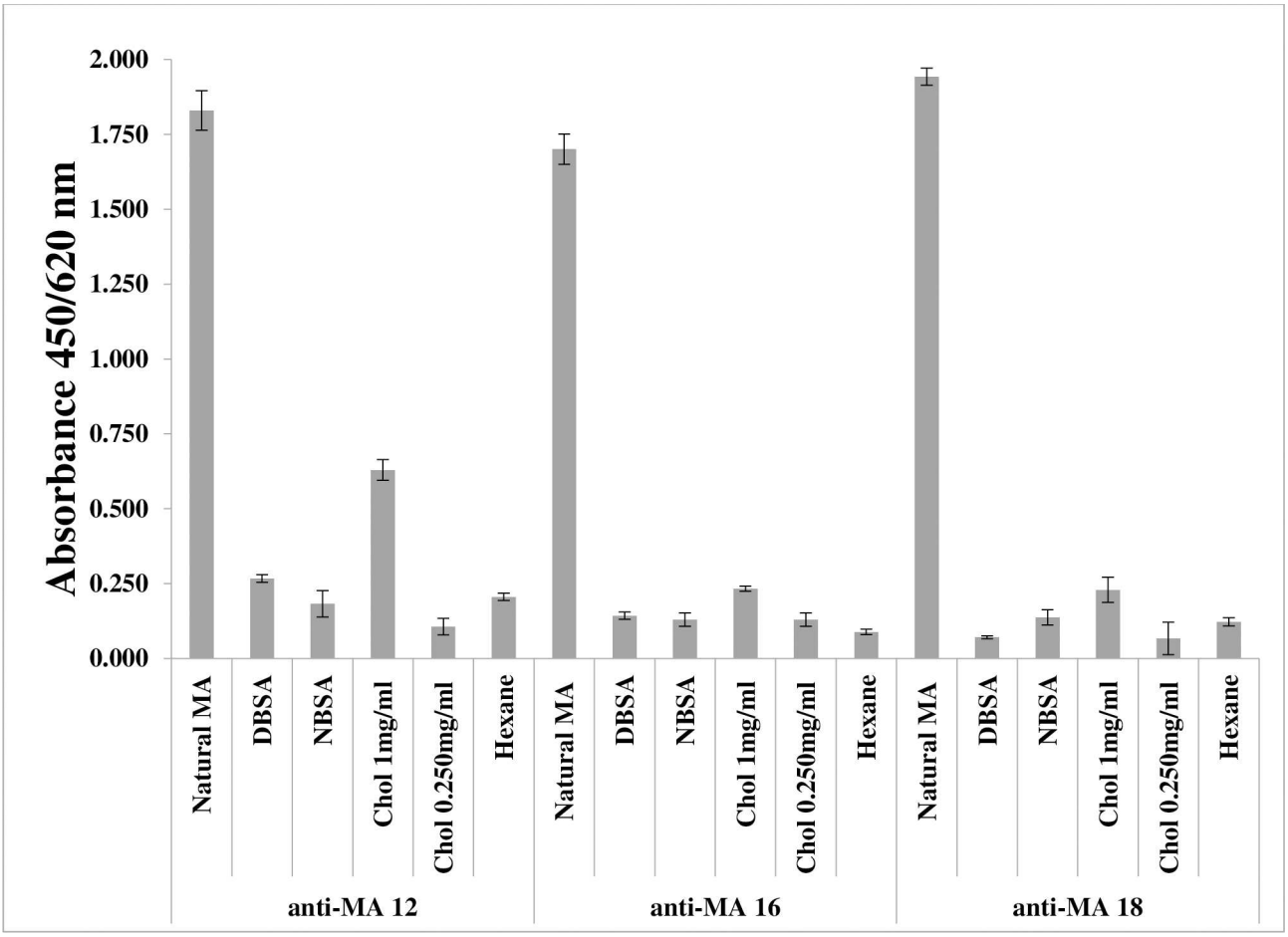

Fig 3. Evaluation of the selectivity and specificity of MA-reactive fusion-phage clones in ELISA. Natural MA was used as a positive control, while denatured bovine serum albumin (DBSA), native bovine serum albumin (NBSA) and hexane were used as negative controls. MAs were coated at 0.250 mg/ml while cholesterol was coated at both 0.250 mg/ml and 1 mg/ml. The concentration of bovine serum albumin used was 1 mg/ml. Error bars = Standard error of mean, n = 5 (biological repeats). See S1 Dataset.

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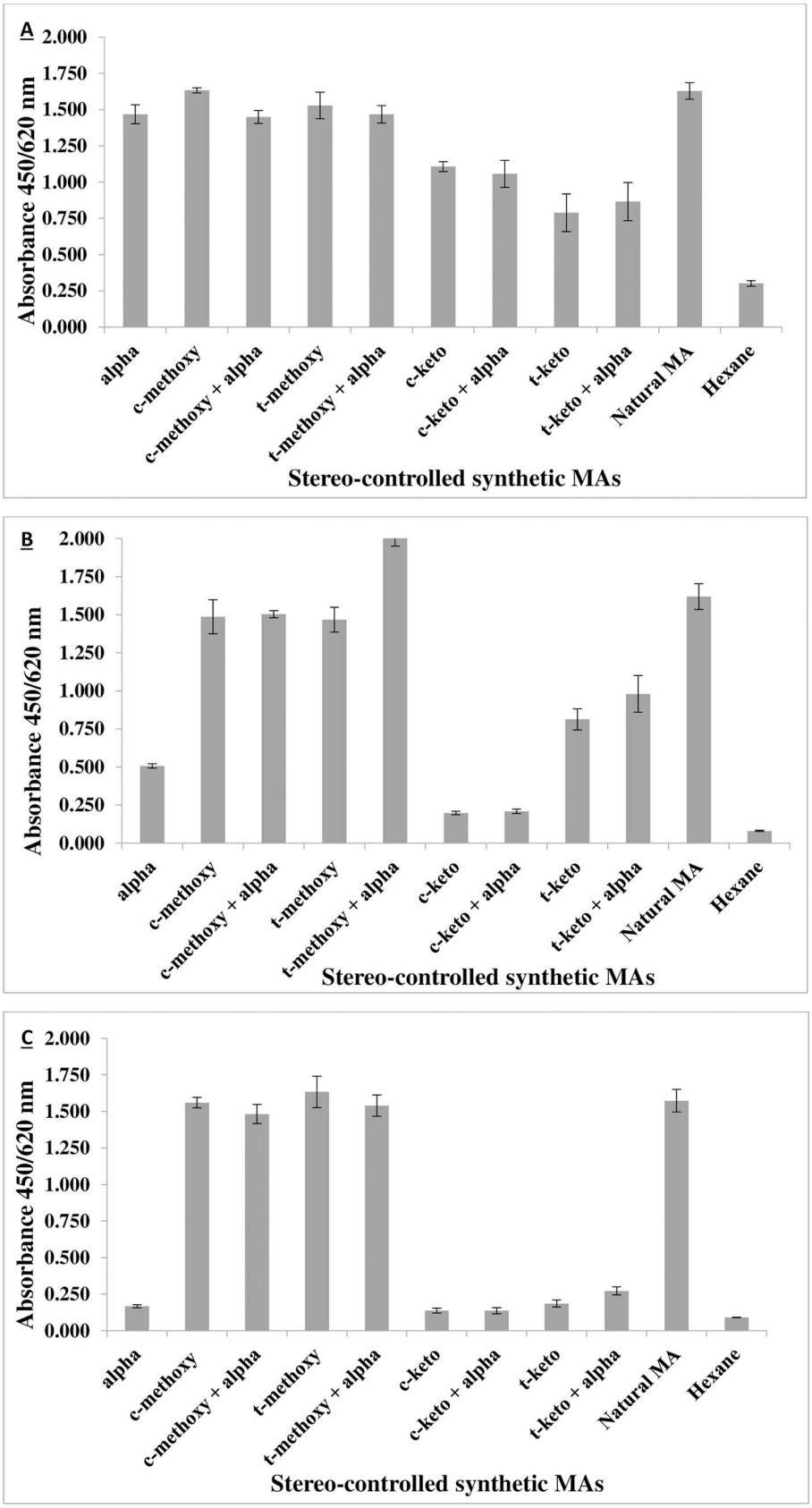
Anti-MA 12 recognized all classes of MAs. Anti-MA 16 reacted with all classes of MAs except *cis*-keto. Anti-MA 18 bound strongly and exclusively to methoxy MA of either *cis*- or *trans*- configuration and irrespective of whether associated with 50% alpha-MA composition. Alpha-MA did not influence the antigenic properties of MA, since a combination of either keto- or methoxy- with alpha-MA did not result in reduced or enhanced recognition of MA antigen by the selected mAbs.

Amino acid sequences of the hypervariable regions of the MA-specific phage-displayed scFvs

Since anti-MA 12, 16 and 18 were shown to bind with three unique specificities, the Abs were sequenced to test whether their amino acid sequences are also different. The alignment (Table 1) showed the expected homology between the three monoclonal scFv Abs for the framework regions as reported in literature [30], while the complementarity-determining regions (CDRs) differed.

It is known that the heavy chain CDR3 plays the dominant role in the observed binding specificity of Abs. The specificity conferred by the heavy chain is preserved regardless of its


A
Absorbance 450/620 nm
2.000
1.750
1.500
1.250
1.000
0.750
0.500
0.250
0.000
alpha
c-methoxy
c-methoxy + alpha
t-methoxy
t-methoxy + alpha
c-keto
c-keto + alpha
t-keto
t-keto + alpha
Natural MA
Hexane
Stereo-controlled synthetic MAs
B
Absorbance 450/620 nm
2.000
1.750
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1.000
0.750
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alpha
c-methoxy
c-methoxy + alpha
t-methoxy
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c-keto
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t-keto + alpha
Natural MA
Hexane
Stereo-controlled synthetic MAs
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Absorbance 450/620 nm
2.000
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0.750
0.500
0.250
0.000
alpha
c-methoxy
c-methoxy + alpha
t-methoxy
t-methoxy + alpha
c-keto
c-keto + alpha
t-keto
t-keto + alpha
Natural MA
Hexane
Stereo-controlled synthetic MAs

Fig 4. Characterization of the binding specificities of MA-specific phage-displayed Abs with ELISA using single stereo-isomers of stereo-controlled synthetic MAs, their 1:1 mixtures with α-MA and natural MA mixture. In this monoclonal phage ELISA assay, the coating of MA antigens was done by adding 50 μl per well of MA (62.5 μg/ml) dissolved in hexane. Hexane alone was used as a negative control. A) Anti-MA 12, B) Anti-MA 16, C) Anti-MA 18. Error bars = Standard error of mean, n = 8 (biological repeats) t = *trans*, c = *cis*. The experiments on all synthetic MAs were performed with the three mAbs in parallel under the same conditions. This result is a representative of more than three biological repeats with six technical repeats for each mAb. See S1 Dataset.

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pairing with various light chains [47]. The CDR1 and CDR2 regions of both heavy and light chains showed minimal sequence diversity, but the CDR 3 regions of both heavy and light chains confirmed that the three monoclonal scFv Abs are unique (Table 1). The most striking result was that the anti-MA mAb 18 showed significantly longer CDR3 regions in both the heavy and the light chain. Whereas CDR1 and CDR2 are known to come about by point mutation only, the larger CD3 additionally comes about by rearrangement of the V genes with D genes (H- chain) and J genes (H- and L- chains) with intermittent random nucleotide insertions.

Mycolic acid specific monoclonal gallibodies

The clones produced during the antibody engineering process were sequenced to determine the success of the subcloning procedure. Three of the newly generated clones for each of the two gallibody constant chain frames (CH1-4 and CH2-4) were selected for the subsequent gallibody production (see S1 Fig).

To confirm the specificity of the monoclonal antibodies following the conversion from phage-displayed scFvs to purified monoclonal IgY-like antibodies (See S2 Fig), gallibodies were analysed with ELISA on MA. Samples were loaded at a starting concentration of 1000 μg/ml of gallibody and titrated down to 2 μg/ml on the antigen coated plates.

Fig 5A shows that gallibody $12_{CH2\text{-}4}$ was able to bind MA across the entire concentration range, with the amount of binding remaining the same even when an antibody concentration as low as 2 μg/ml was used. The sensitivity for gallibody 12CH2-4 was reached when the antibody was used at a concentration of 1 μg/ml (Fig 5A1). The sensitivity for gallibody $12_{CH1\text{-}4}$ on MA was weaker at $A_{50\%}$ = 4 μg/ml. $A_{50\%}$ was determined by identifying the concentration of gallibody where absorbance was approximately 50% of the highest signal.

Gallibody 16 showed a slightly weaker sensitivity of MA binding at $A_{50\%}$ of 25 μg/ml and 8 μg/ml for gallibody $16_{CH1\text{-}4}$ and $16_{CH2\text{-}4}$, respectively. Gallibody 18 showed the weakest binding to MA with $A_{50\%}$ for gallibody$18_{CH1\text{-}4}$ at 75 μg/ml and gallibody $18_{CH2\text{-}4}$ at 16 μg/ml.

Nature of cholesterol cross-reactivity

In an attempt to probe the cholesterol cross-reactivity of MA, the gallibodies' ability to bind to cholesterol was analysed with ELISA. Fig 6 illustrates the cholesterol cross-reactivity for all the gallibodies.

Table 1. Deduced amino acid sequences of CDR 3 regions for phage displayed monoclonal scFv Abs isolated against MA.

Clone	V_H CDR3	V_L CDR3
Anti-MA mAb 12	MNYRRRQ	TEDSTY
Anti-MA mAb 16	RRITNK	RDSGAP
Anti-MA mAb 18	RKTNKHRIDAWGHGTEV	GSYEASNSAGIFG

V_H variable heavy chain, V_L variable light chain

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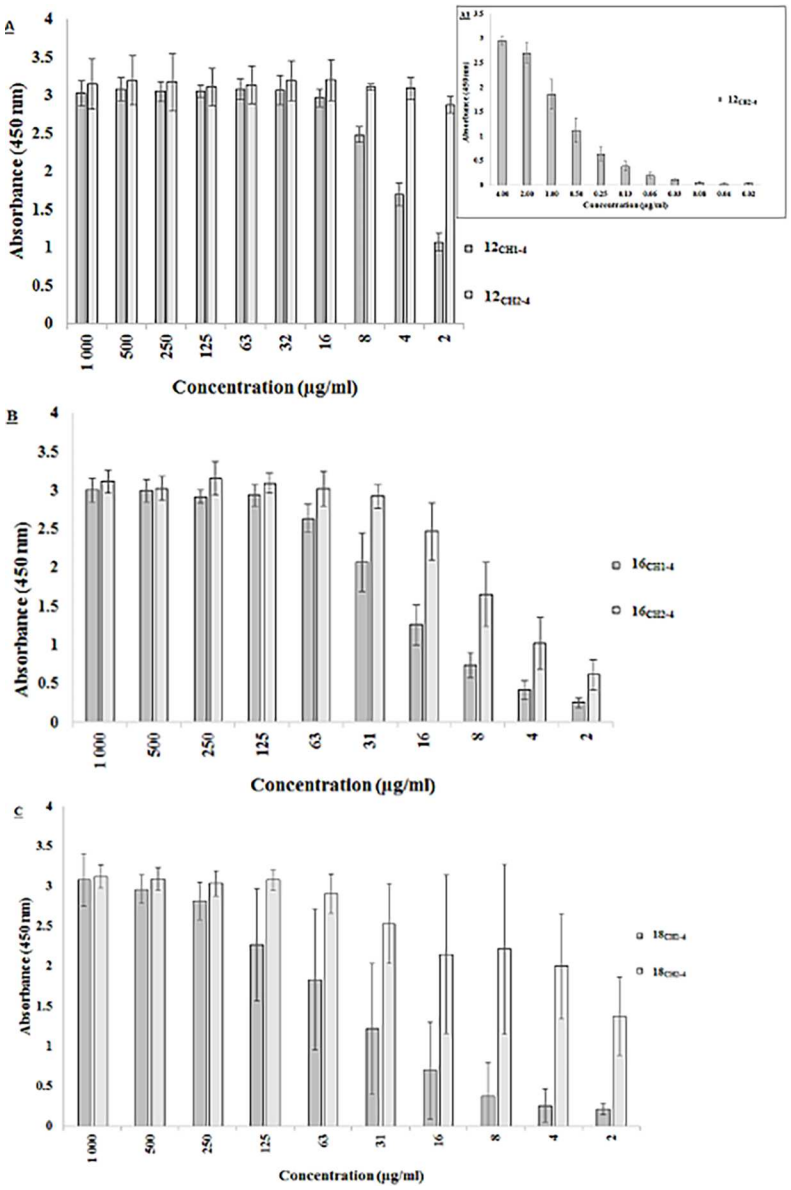

Fig 5. Gallibody binding to natural MAs in ELISA. Coating of MA antigens was done at 250 μg/ml in hexane. Hexane alone was used as a negative antigen control for background correction of the data. A) Anti-MA 12, B) Anti-MA 16, C) Anti-MA 18. Error bars = Standard deviation, n = 8 (biological repeats), CH1-4 = full length constant region, CH2-4 = truncated constant region. This result is representative of two biological repeats with four technical repeats for each concentration of gallibody. Insert A1) represents gallibody 12_{CH2-4} titration at lower concentrations. No non-specific binding of gallibodies was observed when the antigen negative control was used. See S2 Dataset.

https://doi.org/10.1371/journal.pone.0200298.g005

The results show that all of the CH1-4 gallibody types (12_{CH1-4}, 16_{CH1-4} and 18_{CH1-4}) and one of the CH2-4 type (18_{CH2-4}) did not produce signals that were significantly different from the antigen negative control, indicating that these antibodies were not able to cross-react with cholesterol. In contrast, the truncated gallibody 12_{CH2-4} and 16_{CH2-4} cross-reacted with cholesterol. The binding signal of gallibody 16_{CH2-4} was more than two fold higher than the 12_{CH2-4} version, suggesting that the former has a higher affinity for cholesterol.

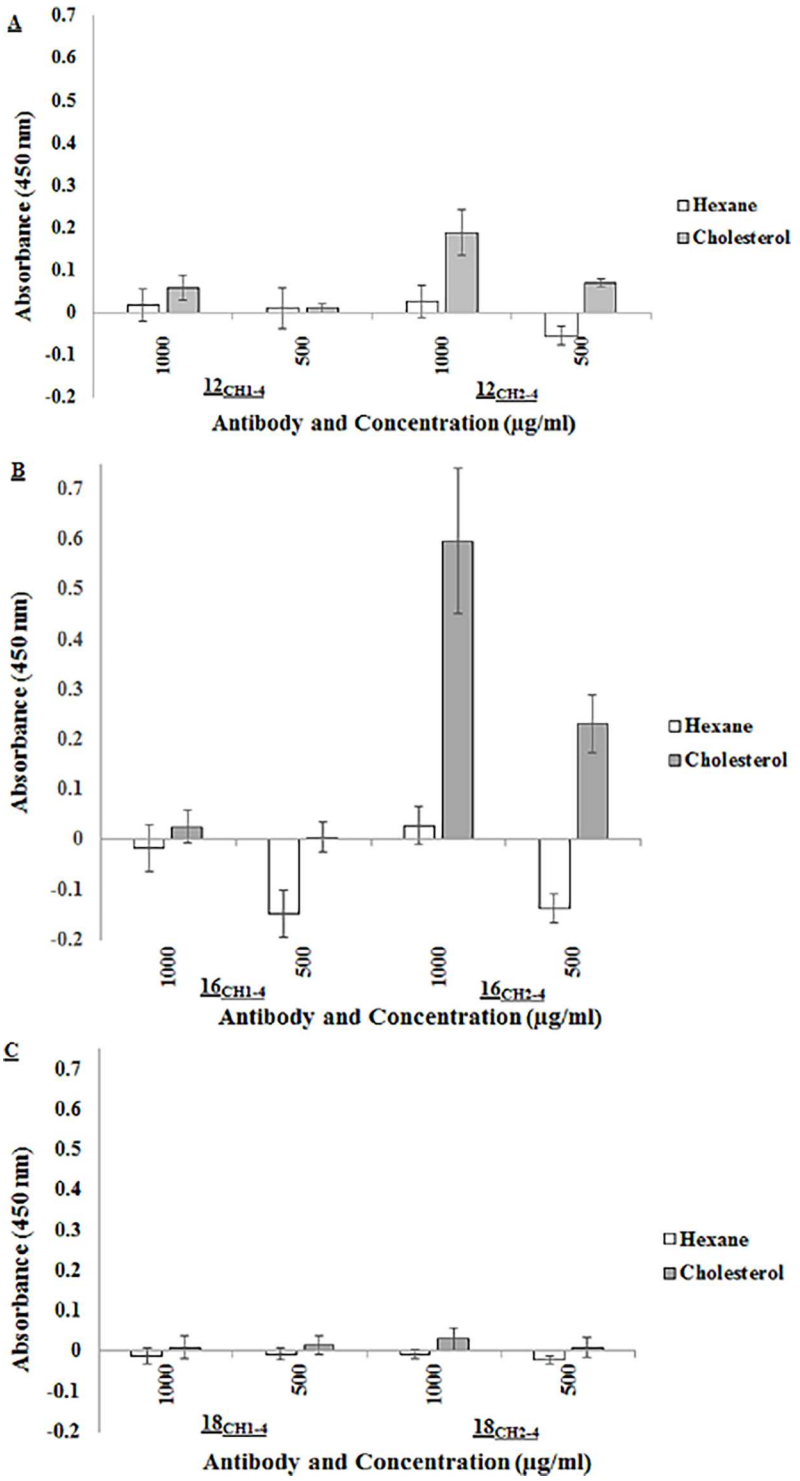

Fig 6. Cholesterol cross-reactivity of gallibodies confirmed with ELISA using hexane solvent alone as negative control. Coating of cholesterol was done at 1 mg/ml in hexane. A) Anti-MA 12, B) Anti-MA 16, C) Anti-MA 18. Error bars = Standard deviation, n = 8 (biological repeats) CH1-4 = full length constant region, CH2-4 = truncated constant region. The results each represent two biological repeats with four technical repeats for each concentration of gallibody. The data were extracted from the same experiment that was performed in Fig 5, making the absorbance readings from both figures directly comparable. See S3 Dataset.

https://doi.org/10.1371/journal.pone.0200298.g006

Discussion

Our group previously demonstrated that it is feasible to generate MA-specific recombinant scFv antibody fragments from a semi-synthetic chicken antibody-gene library [19]. We used the approach that Beukes *et al*., (2010) used previously to pan for MA-specific Abs from the recombinant chicken antibody library, but selected for thermally stable scFvs. Similarly to Beukes and colleagues (2010), both cholesterol cross-reactive and non-cross-reactive anti- MA phage antibodies were isolated [19].

It appeared as if all the anti-MA phage antibodies recognised cholesterol, with anti-MA 12 giving the highest binding. The binding to cholesterol was much weaker than the binding to MA. We confirmed the specificity of cholesterol binding by using denatured BSA as a negative antigen control. The phage-displayed antibodies showed increased binding to a higher concentration of cholesterol, confirming that cholesterol is more antigenic at the higher concentration as reported by other authors [10].

While the method of phage display technology is useful for selecting scFvs with different binding specificities, it has been reported that soluble scFvs have the ability to naturally form dimers and trimers [48, 49], forming multivalent non-covalently bound antibody fragments. This may give false-positive cross-reactive binding due to the uncontrolled valency for antigen binding. While the soluble scFvs retained their ability to bind to MA, the data obtained revealed their low binding affinity (data not shown). Thus in order to truly define the binding specificities of the selected mAbs, the three scFvs were subcloned into bivalent IgY expression vectors. Results of the gallibody characterization were in agreement with those obtained with phage-displayed Abs, illustrating that all gallibodies bound to MA with approximately equal strength. Cholesterol binding results revealed that only the CH2-4 gallibody subtypes of anti-MA 12 and anti-MA 16 bound to cholesterol at high antibody concentrations. Furthermore, in contrast to the results obtained with phage antibodies, where phage antibody 12 bound strongest to cholesterol, gallibody $16_{CH2\text{-}4}$ now displayed the highest binding. This emphasizes the importance of controlling both protein concentration and antigen binding valency for quantitatively comparing cross-reactivity of anti-MA antibodies. Gallibody $18_{CH2\text{-}4}$ qualified the result found with phage antibody 18 in that it showed no affinity of binding for cholesterol. Methoxy MA is known to be the most antigenic for sensitive detection of anti-MA detection in TB patients [19, 22]. Anti-MA 18, which recognizes methoxy-MA but not keto-MA, has a very unique and long CDR3 region compared to anti-MA12 and anti-MA 16. The CDR3 region is the main carrier of diversity and specificity in the Ab binding site. Other researchers have shown that not only are the differences in sequence of the CDR3 heavy chains between Abs important in determining specificities but also the length of CDR3 heavy chains [50].

In a study by Chan and colleagues (2013) aimed at generating anti-MA Abs from a human antibody gene library using phage-display technology, the authors reported that they could not find a cholesterol cross-reactive anti-MA Ab [22]. This observation is corroborated by our findings of all three CH1-4 gallibodies. However, cross-reactivity with cholesterol was observed with gallibody $12_{CH2\text{-}4}$ and gallibody $16_{CH2\text{-}4}$ at high antibody concentrations (1000 μg/ml and 500μg/ml). Gallibody 12 and 16 both have specificity for binding to *trans*-keto MA as such or in combination with alpha- or methoxy- MAs. In the study of Chan *et al*., all anti-MA Ab generated could not recognize keto-MA but had weak binding to alpha-MA. Our study corroborates this finding as the $18_{CH2\text{-}4}$ gallibody–which also does not recognize keto MA–similarly did not cross-react with cholesterol. Anti-MA 18 only recognizes methoxy mycolate as such or in combination with alpha-MA (Fig 4). Anti-MA 16 recognizes all the synthetic MAs and combinations thereof, except *cis*-keto MA (Fig 4). Notably the lack of specificity of anti-MA 16 towards binding of *cis*-keto MA had no influence on the cholesterol cross-

reactivity. Due to these findings, it seems likely that the cholesteroid nature of the MA antigen manifests in the fine specificity towards the *trans*-keto MA subtype.

Interestingly, an involvement of keto MA with cholesterol *in vivo* has recently been reported by Vermeulen *et al*. [51]. These authors wanted to determine the effect that the different MA subclasses have on the growth of the mycobacteria in macrophages, particularly the accumulation of cholesterol brought about by the *M*. *tuberculosis* organism during disease progression. Their results revealed that the keto MA is responsible for the accumulation of intracellular lipid droplets packed with cholesterol. Furthermore, when mouse macrophages were treated with keto MA, bacterial growth was largely unrestricted compared to those treated with alpha- or methoxy- MA.

Notably, from the results obtained it was only selective CH2-4 gallibody types that displayed cholesterol cross-reactivity. None of the CH1-4 gallibodies possessed this ability. This finding can be related to the structures of the two IgY framework constructs used for antibody engineering. The IgY immunoglobulin class of antibodies has been described in the literature as the avian population's equivalent of the IgG subclass. While these antibodies are similar in structure, one of the most notable differences is the IgY subclass' lack of a hinge region [52]. These antibodies (IgY) rely on their CH1 and CH2 domains for flexibility, with the CH1 domain contributing largely in this respect [53]. As a result of this and the fact that the CH2-4 construct lacks the CH1 domain, one may refer to the two gallibody subtypes as the flexible CH1-4 and the rigid truncated CH2-4. The CH2-4 versions of the three antibody specificities were generally more sensitive for antigen binding than the CH1-4 versions. This may explain why only the rigid CH2-4 anti-MA monoclonal antibodies cross-react to cholesterol. It is probably due to divalent binding to repeating cholesteroid epitopes on clusters of immobilized MA, which is enhanced by better positive cooperative binding of the two more closely cropped Fab domains on CH2-4 antibody constructs. Mathebula *et al*. (2009) previously showed how mycolic acids cluster into small islands on a self-assembled monolayer of octadecane thiol [54]. The cholesteroid epitopes in the clusters may be too close, and the MA clusters too far apart to be recognized divalently by the more flexible and larger CH1-4 type of gallibody. Thus the binding of the two CH2-4 gallibodies is probably due to the enhanced co-cooperativity aiding in the sensitivity.

The weak affinity of these recombinant mAbs relates to the binding activity of natural antibodies to MA found in TB patient sera. Previously research conducted within our research group demonstrated this property of AMAA–their low affinity. While these antibodies can be detected using ELISA, we revealed how cases can be missed when this standard technique is used for detection [8, 55]. As a result of this more sensitive biosensor techniques for detecting these AMAA, are applied for the diagnosis of TB patients [7].

It has been shown before that the three MA major classes (alpha-, keto- and methoxy-MA) of *M*. *tuberculosis* have varying antigenicity with respect to antibody recognition and that cross-reactive antibodies binding to both MA and cholesterol exist in human sera. This has now been investigated using recombinant anti-MA mAbs derived from a recombinant chicken Ab repertoire. The major findings of this study substantiate the dominant antigenic nature of methoxy-MA reported before, while now demonstrating that the cholesteroid nature of MAs seems to depend on the ability of the AMAA to recognize the *trans*-keto MA subtype.

Supporting information

S1 Fig. Sequences of gallibody clones produced by antibody engineering. 12) Anti-MA 12, 16) Anti-MA 16, 18) Anti-MA 18, CH1-4 = full length constant region, CH2-4 = truncated

constant region, V_H = variable heavy chain, V_L = variable light chain.
(TIF)

S2 Fig. SDS-PAGE analysis illustrating gallibody purification using Ni-NTA affinity columns. A) 12_{CH1-4}, B) 16_{CH1-4}, C) 18_{CH1-4}, D) 12_{CH2-4}, E) 16_{CH2-4}, F) 18_{CH2-4}. Gel lanes 1) Marker, 2) Culture supernatant, 3) Flow through 1, 4) Flow through 2, 5) Washes, 6) Elution 1, 7) Elution 2, 8) Elution 3, 9) Elution 4. Successful purification is demonstrated by the comparable thickness of the 67 kDa band obtained with the culture supernatant (2) and the elutions (6–9).
(TIF)

S1 Dataset. Experimental data used for producing Figs 3 and 4.
(XLSX)

S2 Dataset. Experimental data used for producing Fig 5.
(XLSX)

S3 Dataset. Experimental data used for producing Fig 6.
(XLSX)

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Monoclonal antibodies used in this study are available in the form of cell cultures supernatants and may be supplied to other investigators by signing a material transfer agreement with the CSIR. Interested parties may contact Dr Yolandy Lemmer (ylemmer@csir.co.za).

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