Chemically synthetic mycolic acids as vaccine adjuvants

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Summary:

The benefit of using adjuvants in vaccines is well established, but not without some controversy. Typical problems associated with new candidate human and animal adjuvants include inefficiency, interference from adjuvant antigenicity and unwanted inflammatory reactions. Alum, a well-established adjuvant with a good safety record, has displayed shortfalls in its induction of protective immunity and its longevity in storage. Another adjuvant MF59, used in anthrax and influenza vaccines, has been clouded with controversy in the past years by the USA army. These problems have led to investigation of new adjuvant candidates. Purified mycolic acids (MAs), synthetic MAs or derivatives thereof have shown potent immune steering activity. This project set about to determine if one or more synthetic MA may be a beneficial compound as a vaccine adjuvant. Various synthetic MAs carrier systems and controls (positive and negative) were safety tested in rabbits with no severe inflammatory reactions at the site of injection, paving the way for the next phase of testing in cattle. Twenty male calves of ages five to six months were intradermally injected with an anti-TB antigen (Ag85A) in combination with different synthetic MA adjuvants, while MF59 served as positive, and phosphate buffered saline (PBS) as negative adjuvant controls. None of the animals revealed severe inflammatory reactions at the site of injection. Blood samples collected for the isolation of peripheral blood mononuclear cells (PBMCs) and sera were used to perform T-cell proliferation assays and enzyme-linked immunosorbent assays (ELISAs), respectively. It was concluded from the T-cell proliferation assays that the concentration of Ag85A used in the synthetic MA mix adjuvant may have been too low to elicit a cellular immune response resulting in the absence of proliferating cells stimulated with Ag85A. In the case of the synthetic MAs no antibody responses were found, putatively as a result of the harsh methods required to prepare the vaccine solution at the desired concentration and volume for injection. Although all adjuvant formulations used in this study proved to be safe, the ELISA assessment revealed the impact that timing of vaccinations may have on the humoral response. Thus, MF59 appeared to be safe and efficient to use if elicited with two primary vaccinations, administered two weeks apart, but that the interval between primary and booster injection is critical for the antibody levels to be obtained. Although a booster vaccination six months or a year later were reported to give adequate results in humans, the results revealed here showed the negative effects of administering the booster too early. While MF59 has been shown to be a successful adjuvant in many vaccines, this study teaches the effects of short time periods between primary and booster immunizations. This research contributes to understanding on how new adjuvant compounds can be tested for safety and efficiency in inducing the desired protective cellular and humoral immune responses in vaccine formulations.

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Abbreviations:

Ag:	Antigen
BCG:	*Mycobacterium bovis* Bacille Calmette Guérin
CD:	System of nomenclature for cell surface proteins on white blood cells
CFSE:	5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester
ConA:	Concanavalin A
cRPMI:	Complete culture media
CSIR:	Council of Scientific and Industrial Research
DC:	Dendritic cells
DCM:	Dichloromethane
DMSO:	Dimethylsulphoxide
DOPC:	1, 2-Dioleoyl Phosphatidylcholine
DOTAP:	1, 2-dioleoyl-3-trimethylammonium-propane
ELISA:	Enzyme-Linked Immunosorbent Assay
FACS:	Fluorescent activated cell sorting
FBS:	Foetal bovine serum
FCA:	Freund's complete adjuvant
FIA:	Freund's incomplete adjuvant
FSC:	Forward-scatter
HRP:	Horseradish peroxidase
IL:	Interleukin
MA:	Mycolic acid
MHC:	Major histocompatibility complex
M.tb:	*Mycobacterium tuberculosis*
OVI:	Onderstepoort Veterinary Institute
PBMC's:	Peripheral blood mononuclear cells
PBS:	Phosphate buffered saline
PC:	Phosphatidylcholine
PLGA:	Poly (lactic-co glycolic acid)
PPD:	Protein purified derivatives
PSN:	Penicillin- Streptomycin- Neomycin
SOP:	Standard operating procedure
SSC:	Side-scatter
SST:	Serum separating tubes

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TB:	Tuberculosis
TCR:	T-cell receptor
Th:	T helper
Tregs:	Regulatory T-cells
UPBRC:	University of Pretoria's Biomedical Research Centre
UP:	University of Pretoria
WHO:	World Health Organization
w/v:	weight/volume
w/o:	water in oil
w/o/w:	water in oil in water

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1. Introduction

The immune system is the defense mechanism of the human body that acts in response to invasion by foreign encounters (Chaplin, 2006; Parkin and Cohen, 2001). The type of defense response the body will produce is dependent of the type of attack on the body. 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 defense, is a rapid non-specific reaction produced in response to pathogens that have crossed the physical barriers of the body (Beutler, 2004; Turvey and Broide, 2010). These pathogens, which may be in the form of bacteria, are attacked by leukocytes that make use of phagocytosis for elimination (Chaplin, 2010). Phagocytosis is the process whereby foreign molecules are engulfed by leukocytes and presented in the form of phagosomes, which fuse with lysosomes before undergoing degradation (Beutler, 2004). The above mentioned process is only one of many that are involved in innate immune responses. Inflammatory responses are also non- specific responses executed due to innate immunity as a result of physical damage to the tissues of the body (Beutler, 2004; Parkin and Cohen, 2001; Roitt, 1998; Turvey and Broide, 2010).

The adaptive immune response is a more specific and specialized response elicited when the body recognizes the presence of a pathogen as being foreign (Bonilla and Oettgen, 2010; Chaplin, 2010; Parkin and Cohen, 2001). After a pathogen has been identified as being foreign the response begins with the presentation of the pathogen's proteins, also known as antigens (Edgar, 2011), on membrane proteins such as the major histocompatibility complex (MHC) molecules (Bonilla and Oettgen, 2010; Chaplin, 2010; Parkin and Cohen, 2001). There are two types of MHC molecules, namely MHC class 1 and MHC class 2, which are responsible for the presentation of short peptides to T-cells. The type of T-cell activated is dependent of the MHC class that the antigen is presented on. If the antigen is presented on a MHC class 1 molecule, then CD8+ T- cells will be able to respond and produce a cytotoxic response (Bonilla and Oettgen, 2010; Chaplin, 2006; Roitt, 1998). This is carried out by the CD8+ T-cells, which are also known as cytotoxic T-cells that puncture any cells in the body that are identified as being infected or affected. However, if presentation is on the MHC class 2 molecule, then CD4+ T-cells will react and produce T-helper cells (Th cells) to carry out the response (Beutler, 2004; Bonilla and Oettgen, 2010; Chaplin, 2006).

Up to this point only the T-lymphocyte aspect of the adaptive immune response has been discussed. There is however another component of this system that plays an active role, namely the

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B-lymphocytes (Beutler, 2004; Bonilla and Oettgen, 2010; Chaplin, 2006). 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 (Bonilla and Oettgen, 2010). B-cells may also produce plasma cells in response to antigen in combination with signals received from T-cells and other cells (Shanker, 2010). These plasma cells are responsible for the production of antibodies, which are also known as immunoglobulins. Antibodies aid in the response by attaching to the antigenic portion of the pathogen and rendering it harmless (Bonilla and Oettgen, 2010). This process may take place in response to T-cells, where the antigens are T-cell dependent, or in the absence where antigens are independent of the T-cells (Bonilla and Oettgen, 2010; Chaplin, 2006; Chaplin, 2010).

Tuberculosis (TB) is an infectious disease that is caused by the *Mycobacterium tuberculosis (M. tuberculosis)* species of bacteria (Grange, 1988). Due to the fact that the infectious agent is coughed out as an aerosol, it can be spread from person to person through inhalation of droplets from the atmosphere (Russell, 2001). Infection with TB occurs when the mycobacterium enters the individual's body through the host's respiratory pathway (Flynn and Chan, 2001; Raja, 2004). The infection spreads through blood or other transporter fluids from the lungs of the host to other parts of the body. In many cases the pathogen travels to the host's lungs where the infection first begins with phagocytosis of *M. tuberculosis* by alveolar macrophages. Within these macrophages the bacteria replicate and induce an inflammatory response in the infected area (Flynn and Chan, 2001). The infected area becomes flooded with lymphocytes and macrophages, which form a granuloma containing the bacilli (Flynn and Chan, 2001). The above mentioned process takes between two to six weeks to happen and is the deciding factor with regard to infection with the bacterium.

Depending on whether these bacilli remain in the granuloma or are released into the host's body, the patient will develop pulmonary or miliary TB (Flynn and Chan, 2001). These two forms of TB (pulmonary and miliary) can further be classified into active TB, latent TB and persistent TB. Active TB occurs when the bacilli are released from the granuloma into the host's airways (Raja, 2004). Thus, active TB is a form of the disease that presents characteristic TB symptoms, affecting the person's health status. People living with active TB are therefore at risk of dying from the illnesses depending on the strength of their immune systems. Patients with active TB may undergo treatment in an attempt to cure the disease. However, if the nine months treatment is stopped prematurely, the patient will still be subject to persistent TB that can recur to active

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disease, with a high risk of drug resistance (Raja, 2004). On the other hand, latent TB is the form of the disease that tends to remain in remission and does not develop granulatomous lesions (Parrish *et al.,* 1998). It only becomes re-activated under extreme conditions such as immune compromise or malnutrition. The person is therefore infected with the disease causing organism *M. tuberculosis* but their body has triggered an immune response that controls and forces the disease into remission (Parrish *et al*., 1998). It is important to take note that people with the latent or persistent form cannot transmit the disease. In literature, latent and persistent TB are often used to describe the same syndrome – the separate definitions are not generally accepted yet (Flynn and Chan, 2001).

A vaccine is a solution, emulsion or compound, prepared in the laboratory, which is used to improve a person's immunity to a particular disease. Vaccines can contain a portion of the disease- causing agent's proteins, nucleic acid or cellular components as antigens or immunogens. The effect a vaccine will have on the host's body all depends on what the vaccine consists of. Thus its components will determine the mode of action to provide protection. In the case of a vaccine against tuberculosis one would want a cellular immunity to be developed in the host as *M. tuberculosis* is an intracellular pathogen. As a result the direct action of helper ($CD4^+$) and/or cytotoxic ($CD8^+$) T-cells would be involved. Research conducted recently has illustrated that CD4 T- cells, which are of T helper 1 (Th1) immunity producing interferon γ, tumor necrosis factor α and interleukin 2 (IL-2) mainly, are also able to give rise to interleukin 17 (IL-17) and aid in T helper 17 (Th17) type immunity (Kauffmann *et al.*, 2005). This is important as IL-17 elicits long term immune memory and one would want a vaccine to express this cell type. The presence of CD8 T-cells is also important as these cells are responsible for lysing antigen- presenting cells (Lewinsohn *et al*., 2003). Therefore patients injected with a good vaccine against *M. tuberculosis* would produce high levels of $CD4^+$ and $CD8^+$ T-cells in the blood.

The current vaccine for TB, Bacillus Calmette-Guérin (BCG), is a live attenuated vaccine that was derived from the bovine TB bacillus by Calmette and Guerin in the early 1900s (Grange *et al*., 1983). Although it is the most commonly used vaccine in the world, studies have shown that BCG can only be credited with an overall efficacy of 50% (Brewer and Colditz, 1995). BCG has shown to be efficient in protecting children from contracting dangerous forms of TB but fails to protect adults against pulmonary TB. The vaccine has also shown to be contra-indicated for immunocompromised individuals, such as those living with HIV, where the vaccine itself can cause

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a disseminated form of TB and sometimes even death (Cohn, 1997). A review report indicated that the vaccine is not entirely effective in preventing TB contraction as the strains of BCG being produced, differ significantly, while the functionality of the vaccine in various geographical regions also vary enormously (Fine, 1995).

There are currently two main groups of vaccines being investigated: those that are to be used to replace BCG and booster vaccines that are to be administered in addition to BCG. The main three new vaccine candidates discussed below will only be those to replace BCG. The first is a recombinant strain of BCG known as rBCGΔ*ureC::hly* which is a new candidate that has been developed in Germany (Grode *et al*., 2005). This vaccine is shown to be more efficacious than BCG as it activates a broader immune response, one which includes Th17, CD8 and Th1 responding cells (Grode *et al*., 2005). The vaccine possesses listeriolysin, a compound with membrane-perforating activity, which induces apoptosis in the host's infected cells; thereby allowing for improved immunity (Winau *et al.*, 2006). The second candidate is also a recombinant strain of BCG known as rBCG(*mbtB*)30 that provides more potent cell mediated and protective immunity in guinea pigs (Tullius *et al*., 2008). The third candidate is known as Aeras-422 (Sun *et al.*, 2009). This vaccine is a combination of the 2 above mentioned recombinant strains of BCG. The vaccine expresses a compound called perfringolysin, which works in a similar way to listeriolysin; thereby inducing apoptosis of the host's infected cell. It also expresses antigen 85A (Ag85A) and Rv3407, in addition to Antigen 85B (Ag85B); thereby improving the immunogenicity of Ag85B alone (Mollenkopf *et al*., 2004; Sun *et al.*, 2009).

Adjuvants can be described as additive compounds that are used in combination with vaccines in order to enhance the host's immune responses to the vaccine, and thereby to protect the host to the disease for which the host is vaccinated (Ott and Van Nest, 2006; Schijns, 2000). The beneficial effect of the use of adjuvants was discovered in the 1920s and is still used today to aid vaccines against all diseases. One of the most successful adjuvants used in a number of vaccines are the aluminum salt based adjuvants, collectively known as Alum (Mbow *et al.*, 2010; Reed *et al*., 2009). The process of formation of these adjuvants consists of adsorbing antigens onto highly charged alum particles (Reed *et al*., 2009). Alum adjuvants are believed to act through depot formation of the vaccine at the site of injection, promotion of antigen phagocytosis, and recruitment of macrophages (Ulanova *et al.*, 2001). Although this group of adjuvants has a well-documented safety record (Mbow *et al.*, 2010; Reed *et al*., 2009), there are a few shortcomings too. Alum has been shown to promote mainly Th2 cell responses thereby inducing mainly humoral

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immunity and making it a poor initiator of T-cell responses (Brewer *et al.*, 1999; Mbow *et al.*, 2010). Due to this alum is unable to elicit Th1 and cytotoxic T-lymphocyte responses thereby limiting its ability to control intracellular pathogens (Edelman, 2002). Lastly, when frozen, alum loses its potency, thus eliminating the chance of long term storage of this adjuvant. As a result of these shortfalls the investigation of new adjuvants is underway.

A number of different substances have been used in adjuvants with components of the *M. tuberculosis* organism being most important in the view of the topic of this dissertation. The most well- known adjuvants containing components of *M. tuberculosis* is Freund's complete adjuvant (FCA) (Sadelain *et al.*, 1990). It is a water in oil emulsion containing inactivated and dried mycobacteria. It is prohibited for use in humans and nowadays also for veterinary vaccines due to its adverse side effects. Freund's incomplete adjuvant (FIA) is often used as a carrier substance as it is just the water in oil emulsion that doesn't contain the mycobacterial portion (Billiau and Matthys, 2001). It was usually used as the adjuvant in booster injections in vaccination programmes that started with Freund's Complete Adjuvant. However, although it did have reduced inflammatory side-effects when compared to FCA, the discomfort caused by FIA was still of such an intensity that its use was terminated and is now even considered unethical to use in experimental animals (MacLennan *et al.*, 1965; Stuart-Harris, 1969).

Other examples of emulsion adjuvants include MPL® and MF59. MPL® is a non-toxic derivative of LPS obtained from *Salmonella minnesota* composed of a hydrophilic polysaccharide and hydrophobic lipid, known as Lipid A (Baldridge and Crane, 1999; Brandenburg and Wiese, 2004). Research conducted on MPL has revealed that the different components perform individual functions, with Lipid A inducing most of the endotoxic activity elicited by this compound and the polysaccharide enhancing its solubility (Johnson, 2008). This compound is believed to be a potent stimulator of Th1 cells (Reed *et al.*, 2009). MF59 is an adjuvant that contains a low amount of biodegradable squalene oil, is safe and potent and has illustrated its ability in a number of licensed human influenza vaccines (O'Hagan *et al.*, 2007). This adjuvant has shown to elicit helper T cell responses with vaccination and an increased antibody titre with a balance between IgG1:IgG2a responses (Ott *et al.*, 1995; Podda and Del Giudice, 2003). Its mechanism of action does not include depot formation but rather the direct stimulation of cytokine and chemokine production (O'Hagan *et al.*, 2012; Podda and Del Giudice, 2003; Seubert *et al.*, 2008). Although MF59 has displayed an effective activity when used in influenza vaccine, this adjuvant has been clouded with controversy by the US Army for a number of years. Research into this revealed that vaccines

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containing varying amounts of squalene oil were tested on these soldiers resulting in a number of them experiencing toxic effects, which in many cases ultimately resulted in death (Matsumoto, 2005).

Saponins have also been investigated for their ability to function as vaccine adjuvants. A prime example of these compounds is QS-21, a purified fraction of the extract Quil-A derived from the bark of *Quillaja saponaria* (Sun *et al*., 2009). While QS-21 has been shown to induce strong cellular immune responses against pathogen-derived antigens, its toxicity has prohibited it from being used in humans (Kensil and Kammer, 1998; Sun *et al*., 2009)). Another group of extracts used as adjuvants are those isolated from microorganisms which include LPS and MDP (Aguilar and Rodriguez, 2007). Although shown to mediate their activity successfully through TLRs, these compounds have also exhibited toxicity thereby preventing their use in humans (Audibert *et al*, 1976; Parant *et al*., 1980).

The study of nucleic acids over the years has given rise to the immunostimulatory effects they possess, particularly in derivatives such as CpG motifs and ODNs (O'Hagan *et al*., 2001). A review article by Aguilar and Rodriguez describe CpG motifs as being "six deoxynucleotides-long DNA sequences with a central CpG dinucleotide" that are recognized by TLR-9 when initiating an innate immune response (Cornelie *et al*., 2004). ODNs are short synthetic single-stranded DNA molecules with a modified backbone that have been shown to exhibit activation of APC and/or cytokine secretion (Weiner et al., 1997). While the above mentioned nucleic acids have been successful in initiating strong immune responses, their toxicity has prohibited the continuation of their investigation as adjuvants for vaccines (Aguilar and Rodriguez, 2007; O'Hagan *et al*., 2001).

It is evident that most of the adjuvants mentioned above are only capable of eliciting one aspect of immunity, i.e. either humoral or cellular activity. As a result scientists are now exploring the use of combination adjuvants for increased function. The adjuvants AS01, AS04 and MPL-SE are just a few of those utilising MPL® in their composition. AS01 is composed of MPL® in liposomes and has displayed both strong humoral and cell mediated responses (Mbow *et al.*, 2010; Reed *et al.*, 2009). AS04, a combination of MPL® and Alum in an aqueous formulation, elicits high levels of specific antibodies with fewer injections (Mastelic *et al.*, 2010; Mbow *et al.*, 2010). The combination of MPL and squalene oil, known as MPL®-SE, has been shown to promote Th1 responses (Alving *et al*., 2012; Reed *et al.*, 2009).

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Mycolic acids (MA) are complex lipid molecules that are found in mycobacteria and a few other genera of oil feeding organisms, in particular in *M. tuberculosis* (Minnikin and Polgar, 1966; Barry III *et al*., 1998; Colston, 1996). These compounds have been shown to be present as a major component of the cell wall of mycobacteria where it is mainly esterified to carbohydrate-containing molecules (Barry III *et al*., 1998). There are 3 types of MAs in *M. tuberculosis*, namely α-meromycolate, keto-meromycolate and methoxy-meromycolate (Minnikin and Polgar, 1966). The structures of these β-hydroxy-α-alkyl branched lipids are shown in figure 1.

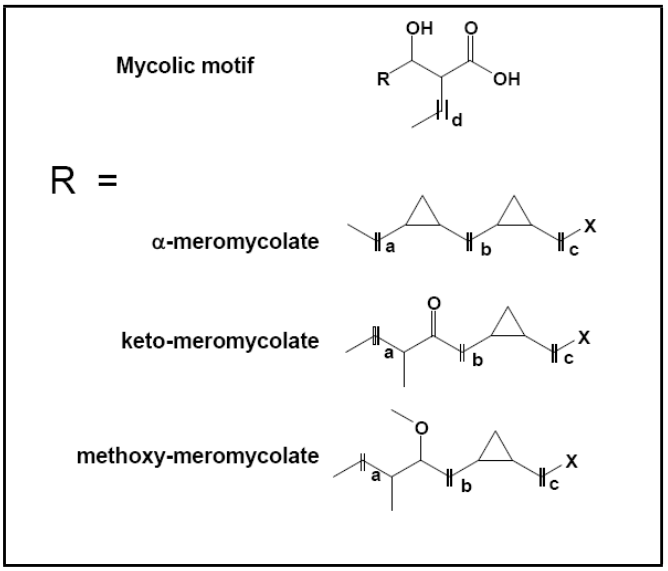

Figure 1: Graphical representation of the structures of mycolic acids (Benadie, 2007).

In addition to these free forms, MAs are present as esters of trehalose, such as Trehalose 6, 6'-dimycolate, which is a characteristic component of *M. tuberculosis* in both its cell wall and in the supernatants of cultures grown with the bacterium (Barry III *et al*., 1998; Wang *et al*., 2008). Trehalose 6, 6'-dimycolate, commonly known as cord factor, is responsible for inhibiting *in vitro* endogenous pulmonary surfactant activity and thus aids in the progression of pulmonary TB disease. Stereo-controlled, chemically synthetic MAs have been chemically produced recently (Al Dulayymi *et al*., 2005; Al Dulayymi *et al*., 2006; Al Dulayymi *et al*., 2007) that were tested to be representative of the original mycolic acids found in the organism *M. tuberculosis* (Beukes *et al*, 2010), thereby allowing the study of structure-function relationships of MAs for the various classes of MA that may exist.

It is well known in literature that the MHC molecules are responsible for the presentation of short peptides through their antigen binding groove to either $CD4^+$ or $CD8^+$ T cells (Bonilla and Oettgen, 2010). These molecules, MHC, are similar to the CD1 family as they are both involved in

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antigen presentation. Although the CD1 family of molecules was discovered in the late 1970s, it is now known that the molecules are able to present lipid and glycolipid antigens to T cells (Sugita *et al.,* 1998; Sugita *et al.,* 2000). 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 (Sugita *et al.,* 1998; Sugita *et al.,* 2000). In particular, it was found that CD1b in humans presented MA to CD4-, CD8- double negative T cells (Beckman *et al.,* 1994), which were subsequently shown to play a role in the regulation of auto-immunity (Goodrum *et al*., 2001).

In 2005 Korf and co-workers showed that MA purified from the cell wall of *M. tuberculosis* were able to mimic certain major features of the *M .tuberculosis* infection. In particular, they showed that interaction of MA with macrophages gave rise to foamy macrophages similar to those seen in tuberculous granulomas (Korf *et al.,* 2005). These findings were interesting as they suggested that MAs could act as a direct trigger of innate immunity. The presence of these foamy macrophages were reported again a year later in another paper written by Korf and her co-authors, where they were able to illustrate that these macrophages exhibited an increased pro-inflammatory macrophage steering function by an increased cytokine release (Korf *et al.,* 2006). The results indicated the potential of MA to control pulmonary allergic asthma responses in the mouse, brought about by suppressive effects of regulatory T-cells (Treg cells) (Shevach *et al.,* 2001; Shevach, 2002). The combination of the results obtained from the above data suggests that MAs have potential immune steering properties.

The immune steering properties of MAs were again reported some years later by the same group, this time with the use of chemically synthetic MAs. Different isomers of the different MA classes were being explored for protective function (Vander Beken *et al.,* 2011). These isomers in liposomes were administered to mice and the responses recorded. The results of these experiments revealed that *cis*-methoxy MA and *cis*-keto MA elicited an inflammatory response, with that of the *cis*-methoxy MA being stronger than the *cis*-keto MA response. It was also found that keto MA in the *trans* configuration illustrated an anti-inflammatory response which could inhibit the inflammatory response of the *cis*-keto MA (Vander Beken *et al.,* 2011). Due to the results found in the above mentioned experiment the use of these stereo-controlled chemically synthetic MAs was further explored in rabbits and cattle to test for their safety, immunity and immune memory in mammals.

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1.2 Problem Statement

The use of adjuvanted vaccines, although shown to be successful in most cases, has exhibited decreased efficiency in recent times. Alum, a well-established adjuvant with a good safety record, has displayed shortfalls in its functional immunity and storage (Reed *et al.*, 2009). Another adjuvant MF59, used in anthrax and influenza vaccines, has been clouded with controversy in the past years by the USA army (Matsumoto, 2005). These problems have led to investigation of new adjuvant candidates. Mycolic acids have been shown to possess potential immune steering properties that are being explored in diagnostics, therapeutics and prevention (Korf *et al*., 2005; Korf *et al*., 2006; Vander Beken *et al*., 2011). In this study individual, chemically synthetic MAs were investigated as possible vaccine adjuvants to investigate their immune steering properties for launching an immune response against a tuberculosis protein antigen. In particular, the stereo-controlled *cis*- configurations of methoxy MA and keto MA were explored, due to their previous success in eliciting desirable immune responses in mice.

1.3. Hypothesis

One or more synthetic *M .tuberculosis* type MA is a beneficial compound to be used as a vaccine adjuvant, with optimal immune potentiation and minimum side effects.

1.4. Aim

Analysis of antibody- and T-cell responses elicited by synthetic MA adjuvanted antigen 85A (Ag85A) for comparison to MF59 as adjuvant in vaccinated cattle.

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2. Exploring the inflammatory, humoral and cellular immune responses of synthetic mycolic acids as vaccine adjuvants

2.1 Introduction

Mycolic acids (MAs) are complex lipid molecules that are found in mycobacteria and in particular in *M. tuberculosis* (Minnikin and Polgar, 1966; Barry III *et al.*, 1998; Colston, 1996). These compounds have been shown to be present as a major component of the cell wall of these mycobacteria or to be associated with an insoluble matrix that is esterified to carbohydrate-containing molecules (Barry III *et al.*, 1998). Synthetic MAs are chemically produced- in the laboratory- as compounds that are representative of the original mycolic acids found in the organism *M. tuberculosis*.

Research conducted on MA, purified from the cell wall of *M. tuberculosis*, has illustrated its ability to mimic certain features of the *M. tuberculosis* infection. One such feature seen was the presence of foamy macrophages similar to those seen in tuberculous granulomas thereby suggesting the potential of MAs to directly trigger the innate immune response (Korf *et al*., 2005). Further research into these foamy macrophages, brought about by MAs, exhibited an increased pro-inflammatory function indicating the potential of MAs to control pulmonary allergic responses (Korf *et al.*, 2006).

The positive results of the natural MAs showing immune steering properties led to the investigation of chemically synthetic MAs for the same properties. This research, conducted in Belgium, saw the different isomers of the different MA classes explored for their immune steering function (Vander Beken *et al.*, 2011). These compounds, administered to mice through liposomes, revealed the following results presented in table 1.

Table 1: Immune-steering properties of isomers of different mycolic acid classes. Results from Vander Beken and co-workers (Vander Beken *et al*., 2011)

Synthetic mycolic acid isomer	Type of response elicited
cis-methoxy	Inflammatory
cis-keto	Inflammatory
trans-keto	Anti-inflammatory

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Due to the results found in table 1, the potential of the immune stimulating properties of these compounds has focused this research on their ability to act as new vaccine adjuvants. However, because mycolic acids are lipid compounds, the presentation of these antigens to T cells needs to be done with the aid of the CD1 family of proteins (Sugita *et al.,* 1998; Sugita *et al.,* 2000). Although the potential of the synthetic MAs was first discovered in mice, these organisms (mice) do not express the CD1b protein whereas cattle and humans do (Van Rhijn *et al*., 2008). This protein (CD1b) has become known for its ability to elicit CD4/CD8 double negative T cells when loaded with MAs (Beckman *et al*, 1994). As a result of this and other examples presented in literature, it is believed that cattle would be a better model than mice to study the effects of MAs as vaccine adjuvants (Van Rhijn *et al*., 2008).

Before any research can begin one would need to establish the feasibility of experiments in order to produce quality results. In order to do this it is important to become familiar with the different techniques that are to be used as well as the principles and safety of the procedures. This is especially important in the case of vaccine adjuvants where a compound is injected into an animal, where ethics clearance is also required. Therefore, before we can determine how a compound will function as an adjuvant, protocols need to be drawn up to perform the animal work and measure the inflammatory, cellular and antibody responses brought about by the compound in question (Cox and Coulter, 1997; Mastelic *et al.*, 2010).

As defined by Ferrero-Miliani and co-workers "Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants" (Ferrero- Miliani *et al.*, 2007). The inflammatory response brought about by the adjuvants as well as their carrier systems need to be investigated before any other responses can be determined. This is often achieved by intradermally injecting the compound and observing the site after injection for any physical effects on the skin. It is also important to keep a record of any other changes that may take place in the body resulting from the compound. These changes may include body mass, body temperature, blood pressure, heart rate and animal behaviour.

Although there are many techniques that may be used to measure antibody responses elicited in an organism's body, an enzyme linked immunosorbent assay (ELISA) with serum is quite convenient and accurate. ELISA is a method used here to detect the antibody of interest by making use of an antigen (Ag85A) immobilized by adsorption to the surface of a micro plate well. There are two types of ELISAs: direct ELISA and indirect ELISA (Butler, 2000; Engvall and Perlmann,

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1971). In both cases a micro titre plate is coated with a specific antigen. Direct ELISA is conducted when enzyme labeled 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. In this dissertation, only indirect ELISA is used. This process is outlined in figure 1.

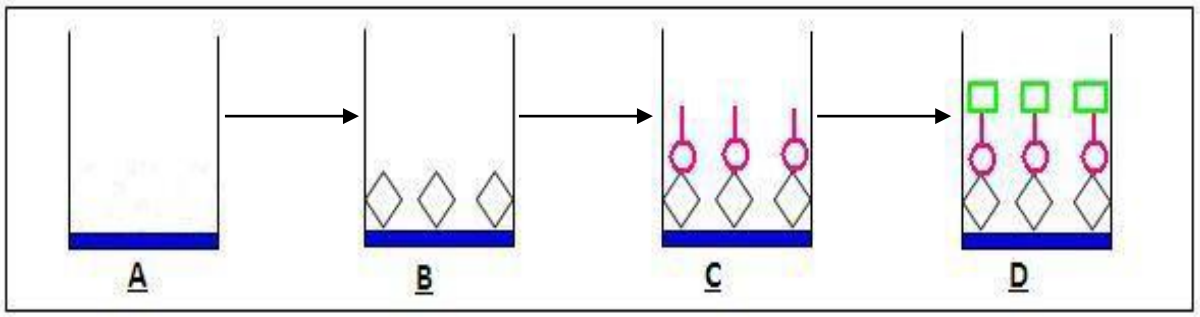

Figure 2: Graphical representation of Indirect ELISA. (A) Coating of plate with antigen; (B) Binding of primary antibodies from serum; (C) Binding of secondary enzyme labelled antibody; (D) Binding of substrate for colour reaction at the end.

The ELISA technique may also be used to determine which antibody isotype plays the most dominant role in the response elicited. This process, known as immunoglobulin isotyping, can provide an indication of the type of immunity the compounds give rise to, i.e. T helper (Th) 1 or Th2 or T independent immunity. This will be explored in more detail in the next chapter.

Exploring the antibody responses elicited by a vaccine adjuvant helps us to investigate only one aspect of the immune system. It is therefore important to go one step further and determine the effects of the adjuvant on the cellular level. This can be achieved by conducting T-cell proliferation assays with the technology of flow cytometry. Before the different activated cell types can be characterized, peripheral blood mononuclear cells (PBMCs) from the blood samples need to be cultured. 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. As a result all work with cells is done in a biological safety cabinet. The cell culture laboratory is mainly used for maintaining, cloning and sub-culturing mammalian cell lines, but biological testing experiments were also conducted here. These experiments included antigenic challenge, staining and cell counting procedures and

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preparation for analysis of cells. Cell counting is done to monitor the confluency of cells and how well the cells are growing, i.e. to determine the percentage viability of the cells.

To explore the cattle T-cell responses to Ag85A reinforced by the synthetic MAs as adjuvants, proliferation of the T-lymphocytes was measured from blood samples taken from the animals before and after vaccination. In order to do this a proliferation marker was used known as CFSE. CFSE is a dye used to stain the cytoplasm of proliferating mammalian cells (Lyons, 1999; Lyons, 2000; Lyons *et al*., 2001). The entry method of CFSE into the cell as proposed by Parish (1999) is described below. The dye enters the cell as a non-fluorescent, highly permeable version of the compound, known as CFDASE (Parish, 1999; Wang *et al.*, 2005). Once inside the cell esterases remove the two acetate groups from CFDASE converting it to CFSE (Parish, 1999). The removal of these two groups renders the dye membrane impermeant thereby "trapping" it in the cell. As a result, the dye remains in the cell and is passed on and diluted into daughter populations as it divides (Lyons, 1999; Lyons, 2000; Lyons *et al*., 2001). Although an appropriate proliferation dye was selected, a stimulant needed to be added to the cells to enhance the cells' proliferation. As a result a positive control was chosen in the form of ConA, a compound known to trigger T-lymphocytes proliferation (Palacios, 1982).

After the cells had been antigenically challenged and cultured for a few days they were stained with various fluorescent antibodies in preparation for analysis by flow cytometry. A flow cytometer is able to measure the size and internal granularity of cells as well as sort and separate the different cell types present in a sample (Haynes, 1988; Mandy *et al*., 1995). The technology does this with the use of three different subsystems: fluidics, optics and electronics. The fluidics system ensures that the sample is carried to the point of interrogation so it may be analysed. The optics system is responsible for generating light signals from interrogation of the sample cells, to characterise their size, surface/cytoplasmic labels and internal complexity. Once light signals have been generated from the optics system, the electronics system converts these signals into data that can be analysed by means of a computer programme. A number of graphs may then be generated to illustrate the cells' sizes, internal granularities, thereby separating them into different populations of cell types present in the sample. Cells can also be sorted in the process by charge assignment to different cell types resulting in the cells being separated based on their deflection in a magnetic field. This flow cytometric process is called **F**luorescent **A**ctivated **C**ell **S**orting (FACS) (Haynes, 1988; Mandy *et al*., 1995).

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In this section the methods are explored and optimised that were used to determine the inflammatory, antibody and cellular responses elicited by the vaccine in combination with the proposed vaccine adjuvants. This chapter describes the attempts to find workable experimental methods and conditions to be used for the duration of the investigation. In particular, this chapter aimed to:

- Explore the safety of a number of carrier systems, either alone or containing the synthetic MA in question
- Investigate the functionality of the chosen flow cytometry stains for use in T-cell proliferation assays
- Determine and set up a standardized ELISA protocol for comparing results across different plates

2.2. Materials and Methods

2.2.1. Inflammatory side effects of experimental adjuvants in rabbits

2.2.1.1. Animal model

Clearance was obtained for this study from the Faculty of Veterinary Science, University of Pretoria (V036/10) to use six New Zealand White rabbits to evaluate and compare five different synthetic MAs to a commercial mixture of natural MAs. This was a pilot study and therefore only one animal per MA was needed. Animals were housed in the rabbit unit at the University of Pretoria's Biomedical Research Centre (UPBRC) according to internal standard operating procedure (SOP). Before the study commenced animals had a five day period to acclimatize. Rabbits received a normal maintenance diet and water *ad lib* provided by the facility. At the end of the study, animals were euthanized according to internal SOP of the UPBRC.

2.2.1.2. Vaccinations

MAs are not water soluble and each preparation was administered with three different carrier systems: liposomes, microcapsules and poly (lactic-co-glycolic acid) (PLGA). Prior to vaccination hair was removed from a region on the dorsum of each rabbit, ~15 cm x 7 cm, by shaving with electrical clippers. The preparations of the carrier systems as well as vaccines are described in addendum A. The animals were vaccinated as follows: six rabbits were injected at eight sites per rabbit. The vaccines were administered in two symmetrical rows (4 sites/row) on the back of each

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rabbit. To account for possible interference from the carriers, each carrier was administered at two different sites – one injection site received the carrier in combination with the specific MA and a corresponding site received only the carrier. Freund's Incomplete adjuvant (FIA) and phosphate buffered saline (PBS) served as positive and negative controls respectively. Animals that showed no significant side effects at the injection sites received a second vaccination on day 21. Figure 3 below indicates the different sites.

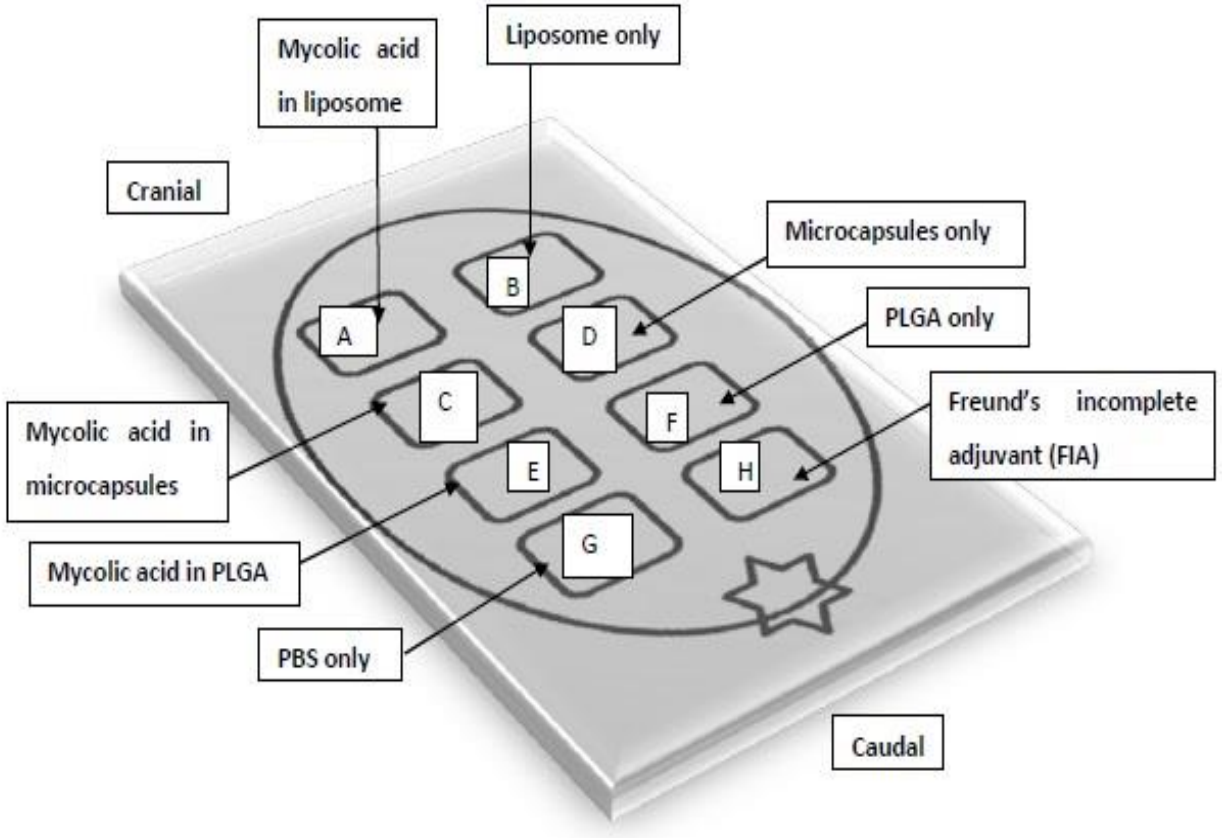

Figure 3: Schematic representation of the administration of the different MAs and their carriers to the backs of rabbits.

2.2.1.3. Monitoring of inflammation

The animals were monitored during the first 30 minutes after vaccination (for signs of anaphylactic reaction) and then twice daily, for the next 3 days, for signs of inflammation (swelling, increased skin temperature, erythema, induration and ulceration) at the respective injection sites. Thereafter all injection sites were examined once a day for the remainder of the study. The following scale was used to assist with recording of lesions at the various injection sites:

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0 = no visible change

1 = discrete or patchy erythema

2 = moderate and confluent erythema

3 = intense erythema and swelling

4 = ulceration

As a reference, records of the following were also collected: body mass was monitored on a weekly basis, body temperature was monitored on a daily basis and animal behaviour was checked twice daily for signs of discomfort, pruritis, self-mutilation, abnormal posture, generalised decrease in grooming and abnormal appearances, like rough hair coat.

2.2.2. Testing of adjuvants in cattle

2.2.2.1. Animal model

Ethical clearance was obtained for this study (V013/11) where twenty bull calves of ages five to six months were selected as an experimental model for this project. On arrival clinical investigation was performed during which the cattle were dewormed and treated against *Coccidia* infection. Animals were treated with antibiotics according to a standard veterinary operating procedure. The cattle were housed in conventional stables at the UPBRC according to internal SOPs. Special confinement was not necessary, because the animals were identified with a permanent identification system (ear tags). The animals had a period of five weeks to acclimatize to their new environment before the study commenced. A normal maintenance diet containing eragrostis, Lucerne, grower meal and water was provided to the animals by the UPBRC. At the end of the study animals were clinically assessed and cleared by the state veterinarian before being returned to the food chain.

2.2.2.2. Preparation of MA/PC liposomes in combination with Ag85A for injection

MA/PC liposomes were prepared in autoclaved glass vials (Separations Pty Ltd) with screw caps (Chromatography Research Supplies Inc.). Synthetic MAs (School of Chemistry, University of Bangor, UK; See addendum B for structures) were alliquoted in 0.3 mg quantities into glass vials either as such (methoxy-MA; keto-MA) or a MA mixture consisting of 0.15 mg alpha-MA, 0.075 mg methoxy-MA and 0.075 mg keto-MA. These synthetic MAs were dissolved in chloroform (Merck) by heating on a heating block for 30 seconds at 90°C and vortexing until the solution was

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clear. The clear solutions were alliquoted in 50 µg MA quantities to a new set of glass vials and put onto the heat block for the chloroform to evaporate (Lemmer 2010).

Phosphatidylcholine (PC) (Sigma) was dissolved in chloroform at a concentration of a 100 mg/ml of which 90 µl was added to each of the above MA samples (50 ug/dose). This MA/PC solution was dried on a heating block (90°C) under nitrogen gas. Saline (0.9% NaCl) was added (2 ml) and the solution vortexed. It was placed on the heating block for 20 minutes and was vortexed every 5 minutes for the duration of this period. At the end of 20 minutes the solution was vortexed for 1 minute, after which it was sonicated using a Virsonic sonifier (Virtis, Gardiner, N.Y.) for 2 minutes, 30 cycles at an output of 5.

The MA/PC liposomes were aliquoted into glass vials (200 µl per vial) and placed in the -70 °C freezer for 1 hour. The liposomes were lyophilised overnight and stored in the -70 °C freezer until use.

The positive control was prepared by combining 540 µl Ag85A (112 µg/ml; Protein Service Facility, Gent) with 60 µl MF59, while the negative control was prepared by combining 540 µl Ag85A with 60 µl PBS, both triturated with a pipette at room temperature to obtain a smooth emulsion (positive control) or clear solution (negative control). A volume of 60 µl of PBS pH 7.2 (Invitrogen) and 540 µl of Ag85A was added to vials containing methoxy- MA/PC liposomes, keto-MA/PC liposomes and natural MA mix/PC liposomes. Here, the poor solubility of synthetic MAs provided a challenge. The vials had to be placed onto a heating block for 30 minutes at 90 °C, vortexed for 1 minute and sonicated for 1.5 minutes at 30 cycles, output 3 in order to get smooth suspensions. This probably destroyed the conformation of the Ag85A, but was nevertheless done to at least get an indication of stimulation of the cellular immunity, which is known to be independent of the conformation of the protein antigens.

2.2.2.3. Vaccinations and skin thickness measurements

Cattle were secured in a crush with a neck clamp and an appropriate head restraint before each vaccination procedure. The hair was clipped at the vaccination site in the lateral mid- cervical region and the skin-fold thickness was measured after vaccination (MTI stainless Germany). A McLintock tuberculin syringe (SGVac) was used to administer 0.1 ml of each formulation via the intra-dermal route. The skin-fold thickness was measured again, 72 hours after vaccination. This was done to determine if there is any degree of swelling at the site of injection. This was repeated

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for every booster immunization. The animals were vaccinated in the neck on week 0; 3 and 12 according to the scheme in table 2.

Table 2: Vaccination schedule of the cattle.

Group nr.	Number of animals	Bull Numbers	Adjuvant	Antigen
1	4	3,13,15,18	methoxy MA	Ag85A
2	4	4,7,14,19	keto MA	Ag85A
3	4	1,11,16,17	α-: methoxy-: keto MA (in the ratio 2:1:1)	Ag85A
4	4	2,5,12,20	MF59	Ag85A
5	4	6,8,9,10	PBS	Ag85A

2.2.2.4. Serum collection and Peripheral Blood Mononuclear Cell isolation

Blood samples were obtained from each animal both before and during the study according to the time line shown in figure 3.

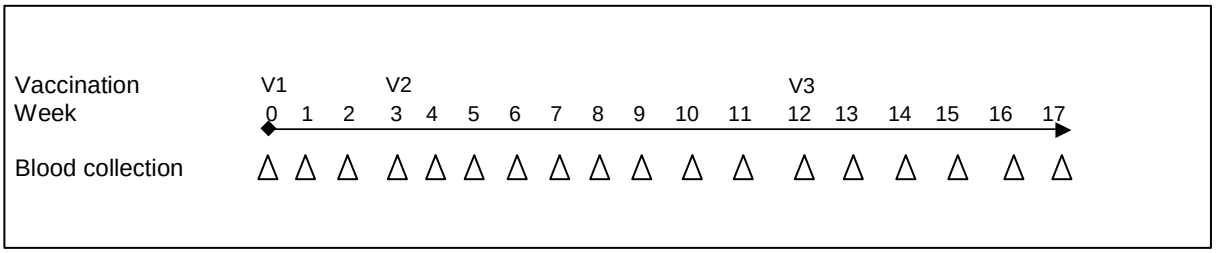

Figure 4: Time line representing vaccination (Vn) and bleeding (Δ) events on cattle.

Blood was collected from the bull calves using 20G×1-1/2 needles (SGVac) and syringes (SGVac) and samples for serum were collected in 9 ml Serum Gel SST Gold tubes (Lasec SA) from the jugular vein. Heparinised blood samples (3 tubes of 9 ml each) were collected from the jugular vein at week 0; 2; 4; 11 and 13 for the isolation of PBMCs. All collections were performed by qualified personnel according to internal SOPs from the UPBRC at Onderstepoort Veterinary Institute (OVI).

For serum collection the blood was allowed to clot by placing the tubes at room temperature for 2 hours after which it was placed at 4°C overnight. The tubes were centrifuged (Heraeus Labofuge 300 centrifuge) at 1107 × g for 35 minutes at RT. The serum was transferred to a sterile 13.5 µl

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centrifuge tube (Whitehead Scientific), after which 1.5 ml was liquated into 1.8 ml cryo-vials with an external thread (AEC Amersham) and stored at -70 °C until use.

The Sodium Heparin tubes, used for the isolation of PBMCs, were inverted to allow the red blood cells to mix with the heparin in the tube, thereby preventing clotting. These samples were transported from OVI to the Council for Scientific and Industrial Research (CSIR). Separation medium or Histopaque (Sigma) was warmed-up to room temperature (RT) and protected from light, because of its light sensitivity. Leucosep tubes (50 ml; Lasec SA) were each filled with 15 ml separation medium and centrifuged (Beckman Coulter X-22R centrifuge) for 30 seconds at 1000 × g at RT.

The Leucosep tubes (2 tubes per calve) were gently filled with the anti-coagulated blood and the sample material was diluted (1:1) with calcium and magnesium free PBS (Invitrogen), pH 7.2, to improve separation. The Sodium Heparin tubes were washed with PBS to obtain a maximum number of PBMC's. The Leucosep tubes were centrifuged at RT for 15 minutes at 1000 × g. The breaks of the centrifuge were switched off to prevent the disruption of the porous barrier of the Leucosep tube.

After centrifugation the plasma layer was collected up to a minimum remnant of 5-10 mm above the interphase, using a plastic Pasteur pipette (J-PLAST), and discarded. The enriched cell fraction (lymphocytes/PBMCs) was harvested by means of a plastic Pasteur pipette or by pouring off the supernatant from the Leucosep tube into another 15 ml tube. This enriched cell fraction was washed with 10 ml PBS and centrifuged for 10 minutes at 300 × g.

The supernatant was poured off and the pellet resuspended in 5 ml ACK lysis buffer (Life Technologies). This suspension was incubated for 5 minutes after which 7 ml complete medium was added to quench the reaction and centrifuged at 300 × g for 10 minutes at RT. The complete medium was prepared by filtering 50 ml foetal bovine serum (FBS; 10%; Invitrogen) and 5 ml Penicillin Streptomycin (1%; Invitrogen) into 500 ml RPMI medium 1640 (Invitrogen).

The supernatant was removed using a vacuum pump (Vacusafe, IBS Integra Biosciences, Switzerland) and the washing step was repeated. The cells were resuspended in 1 ml PBS and pooled in a total volume of 10 ml PBS (pH 7.2). Cells (100 ul) were transferred to an Eppendorf tube to perform a cell count, using a haemocytometer, and 50 µl 0.1 % Trypan blue/PBS was

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added to the cells in the Eppendorf tube. The 15 ml tubes were centrifuged at 300 × g for 10 minutes at RT after the cell count was performed.

The supernatant was removed using a vacuum pump, the pellet loosened and resuspended in freeze medium. Freeze medium was prepared by filtering 200 ml FBS (80%) and 25 ml dimethylsulphoxide (DMSO; 10%; Sigma or Invitrogen) into 25 ml complete medium. The cells (1 ml) were transferred to cryo-vials with an internal thread (AEC Amersham), and sealed in a polystyrene container to be transferred directly to the -70 °C freezer at University of Pretoria (UP). The cells were left in the -70 °C freezer overnight and were transferred to a liquid nitrogen tank the next day.

2.2.2.5. T-cell proliferation assays

Peripheral blood mononuclear cell resuscitation

Stored PBMCs from method 2.2.2.4 were removed from the liquid nitrogen stores and transported on dry ice from the UP to the CSIR. Complete media (cRPMI) was prepared by adding 5 ml of FBS (FBS Superior heat inactivated, Biochrom) and 1ml of Penicillin-Streptomycin-Neomycin (PSN) antibiotic mixture (PSN, GIBCO®) to culture medium (RPMI 1640 medium, GIBCO®) to a total volume of 50 ml. The tubes containing the cRPMI were placed in a water bath at 37°C. The vials of PBMCs were defrosted at 37°C until only a small piece of ice remained. The cells were then slowly added to 10 ml of pre-warmed cRPMI and the tubes inverted slowly twice and centrifuged at 200 x g for 5 min at RT. Supernatants were aspirated using a vacuum pump and cell pellets re- suspended in 20 ml pre-warmed cRPMI.

Cell culture, cell counting and CFSE staining

The cells were then split into two tubes, one containing 5ml cells in media (tube A) and the other 15ml cells in media (tube B). A 50 µl aliquot of cells was removed from each tube for cell counting. The tubes were once again centrifuged as above. While the cells were being centrifuged, a cell count was performed using the 50 µl cell aliquot by adding 50 µl 0.1% Trypan blue/PBS to record the numbers of live and dead cells. Once again the supernatants were aspirated after centrifugation and the pellets were now re-suspended in 1 ml pre-warmed cRPMI. In tube B 2 µl 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was added and the tube's contents incubated for 10 min at 37°C in the dark. During this time 4 ml of cRPMI was added to a T-25 flask along with the 1 ml of cells in tube A. The flask was then placed in the incubator at 37°C and 5% CO_2 overnight. After the incubation the CFSE staining (tube B) was then quenched by

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adding 5 volumes of ice cold cRPMI and the tube placed on ice for 5 min. Following quenching, the cells were washed and centrifuged 3 times, at 300 x g for 10 min at RT for each wash. The supernatants were then resuspended in 3 ml cRPMI after the last centrifugation step. Three T-25 flasks were prepared by adding 4 ml cRPMI, adding 20 µl Concavalin A (ConA; 1 mg/ml) into one flask and Ag85A (112µg/ml) into another, leaving the last flask containing media only. From tube B 1 ml of cells was added to each of the 3 flasks, which were then incubated at 37°C and 5% CO_2. Following 2 days of incubation, cells were transferred from their respective flasks into individual 15 ml centrifuge tubes and a 50 µl aliquot was collected from each tube for cell counting. The tubes were then centrifuged at 300 x g at RT for 10 min. During this time a cell count was performed. After centrifugation supernatants were removed and pellets re- suspended in 4 ml pre-warmed cRPMI. Cells were then plated (300 µl/well) in round bottom plates (Costar®, NY.) and plates incubated at 37°C and 5% CO_2 overnight.

Titration test of CD marker antibodies and blocking agents

PBMCs were resuscitated from the frozen state as described above, except that medium had no antibiotic supplementation. A 50 µl aliquot of cells was removed from each tube for cell counting. The tube was once again centrifuged at 200 x g for 5 min at RT. While the cells were being centrifuged, a cell count was performed using the 50 µl cell aliquot by adding 50 µl 0.1% Trypan blue/PBS and recording the numbers of live and dead cells. Once again the supernatant was removed after centrifugation and the pellet now re-suspended in 1 ml pre warmed cRPMI. The 1 ml PBMC/cRPMI was then added to 19 ml pre-warmed cRPMI in a T-25 flask, which was incubated at 37°C and 5% CO_2 for overnight.

The following day a 50 µl aliquot of cells was removed from the flask to perform a cell count. The content of the flask was then placed in a 50 ml tube which was centrifuged at 300 x g for 10 min at RT. In parallel with the centrifugation a cell count was performed. After centrifugation the supernatant was removed and pellet resuspended in cRPMI to a total volume allowing for 300 µl/well to be pipetted into a round bottom plate (Costar®, NY.) Cells were plated and plates centrifuged at 300 x g for 10 min at 4°C. Supernatants were then removed and pellets resuspended in 100 µl/well 0.1% BSA/PBS. Plates were again centrifuged as above. In parallel with the centrifugation solutions of Fc block/BSA-PBS (1/200, 1/100, 1/50, 1/20 and 1/10; either compatible Rat IgG1 Negative control antibody or Mouse IgG2a Negative control antibody; both Serotec AbD) were prepared. After centrifugation supernatants were removed and pellets were resuspended in the compatible Fc block/BSA- PBS solution, IgG1 for CD3 and IgG2a for CD4 and

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CD8. Plates were incubated for 30 min at 4°C in the dark. In parallel with the incubation the antibody/BSA-PBS solutions (1/200, 1/100, 1/50, 1/20 and 1/10; either Rat Anti-human CD3: Pacific Blue®, Mouse Anti-bovine CD4: Alexa Fluor®647 or Mouse Anti-bovine CD8: RPE; All Serotec AbD) were prepared in the dark. Following incubation, plates were centrifuged as above at 300 x g for 10 min at 4°C. Supernatants were once again aspirated and pellets resuspended in the appropriate labelled cell marker antibody/BSA-PBS solution, allowing the plates to incubate for 30 min at 4°C in the dark. After incubation, plates were centrifuged as above and pellets resuspended in 100 µl 0.1% BSA/PBS. Cells were then transferred to FACS tubes containing 200 µl 0.1%BSA/PBS and transported on ice from CSIR to UP for measurement on the FACS Aria® (BD Biosciences).

FACS experiment

The next day plates were centrifuged at 300 x g for 10 min at 4°C. Supernatants were then removed and pellets resuspended in 100 µl/ well 0.1% BSA/PBS. Plates were again centrifuged as above. During the centrifugation a solution of Fc block/BSA-PBS (1/200 Rat IgG1 negative control; AbD Serotec) was prepared. After centrifugation supernatants were removed and pellets were resuspended in 100 µl/well of the Fc block/BSA-PBS solution. Plates were incubated for 20 min at 4°C in the dark. During the incubation the antibody/BSA- PBS solution (1/200 Rat Anti-human CD3: Alexa Fluor®647; AbD Serotec) was prepared in the dark. Following incubation, plates were centrifuged as above at 300 x g for 10 min at 4°C. Supernatants were once again removed and pellets resuspended in 100 µl/well of the antibody/BSA-PBS solution and incubated for 30 min at 4°C in the dark. After incubation plates were centrifuged as above and pellets resuspended in 100 µl/well 0.1% BSA/PBS. Cells were then transferred to FACS tubes containing 200 µl 0.1%BSA/PBS and transported on ice from CSIR to the UP Campus for measurement on the flow cytometer (Beckman Coulter).

2.2.2.6. Enzyme Linked Immuno-Sorbent Assay

Coating of micro-titre plates

Non-sterile, 96-well micro-titres plates (Costar®, NY.) were used for all of the experiments. Plates were coated with either the antigen 85A (Ag85A; 112 µg/ul; Protein Service Facility, Gent) solution or PBS, serving as the experiment and control, respectively. A 10 µg/ml solution of Ag85A in PBS was prepared, ensuring the antigen was dissolved properly in the PBS. The wells were then coated with the Ag85A/PBS solution (100 µl/well) using a multi- channel pipette. Plates

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were covered with foil and stored at 4°C overnight, until use the next morning.

Preparation of buffers

Solutions of 1% BSA/PBS (w/v), 3% BSA/PBS (w/v) and 0.05% Tween®20/PBS (v/v) were prepared as the dilution buffer, block buffer and washing buffer respectively, to be used on the morning of the experiment. The BSA/PBS solutions were prepared by weighing out the desired amount of BSA and sprinkling it on top of the PBS, allowing it to dissolve into solution before use.

Enzyme Linked Immunosorbent Assay (ELISA)

The antigen coated plates were washed 3 times with 0.05% Tween®20/PBS (300 µl/ well) at RT. After the last wash step, the wells were aspirated and sucked dry using a vacuum pump. Then 300 µl of 3% BSA/PBS was added to the wells and the plates left to incubate at RT for 2 hr for blocking. During this period the serum sample dilutions (1:40, 1:80, 1:160 and 1:320 in 1% BSA/PBS, total volume of 400 µl) were prepared in a round bottom plate. A 1 M H_2SO_4 solution was also prepared by adding the H_2SO_4 drop wise to dddH_20 while stirring. Once the 2 hr blocking period had passed, the wells were again washed 3 times with 0.05% Tween®20/PBS (as above), wells were aspirated and sucked dry and then serum dilutions were loaded into wells (100 µl/ well). The plates were left at RT to incubate for 1 hr. A solution of conjugate [Rabbit anti-Bovine IgG (H/L): Horseradish peroxidase (HRP); AbD Serotec] was prepared in a 1:1000 dilution during this period by adding the relevant amount to the blocking buffer. Following the incubation period, wells were again washed as above, sucked dry and 100 µl/well of the conjugate solution (as described above) was added to the plate, allowing the plate to incubate at RT for 30 min. A TMB substrate solution (kit consisting of solutions A and B; BD Biosciences) was prepared for the colour development by adding solution A to solution B in a 1:1 ratio in a petri dish and covering the dish with foil. After 30 min of incubation the wells were washed as above, sucked dry, and 100 µl of substrate solution from the petri dish were loaded into each well. The absorbance of the plates was then read after 30 min using a 620 nm filter at 10 min intervals with the aid of the SLT 340 ATC photometer (Labsystems, Finland) reader. After the 30 min reading, 1 M H_2SO_4 (50 µl/well) was added to the plates and a final reading was done using a 450 nm filter and 690 nm reference filter for the stopped reaction.

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2.3 Results

2.3.1. Investigating inflammatory responses of carrier systems in rabbits

Before the use of chemically synthetic MAs could be explored for their potential adjuvant properties in cattle, the safety of the compounds had to be investigated in rabbits. A number of carrier systems were explored, alone or containing the MA in question, to determine the inflammatory response elicited individually by the carrier as well as the combination of the carrier and MA. The results are shown in table 3 below.

Table 3: Degree of inflammation incurred in rabbits with different mycolic acids in different carrier systems. Inflammation was measured on a scale ranging from 0-4 to represent the following: 0 = no visible change, 1 = discrete or patchy erythema, 2 = moderate and confluent erythema, 3 = intense erythema and swelling, and 4 = ulceration.

Administration of mycolic acid	Type of Mycolic acid			
	Sigma natural mix	Synthetic cis-Methoxy	Synthetic cis-Keto	Synthetic trans-keto
FIA	2	2	2	2
PBS	0	0	0	0
PLGA only	0	0	0	0
MA in PLGA	0	0	0	0
Microcapsules only	0	0	0	0
MA in microcapsules	0	0	0	0
Liposomes only	0	0	0	0
MA in liposomes	0	0	0	0

The results show that FIA, but not any of the natural or synthetic MAs elicited an inflammatory response at the site of the injection. It is clear from the remaining results that any of the carrier systems are safe to use for the administration of MA. However, the microcapsules containing the respective MA did give a slight swelling effect at the sites of injection (data not shown).

The reference record of changes in body mass, body temperature and animal behaviour collected revealed that no significant difference was noted in any of these parameters (data not shown).

2.3.2. Testing of adjuvants in cattle

2.3.2.1 Safety evaluation of adjuvants in cattle

Due to the satisfactory safety results of the liposomal carrier systems in rabbits shown in 2.3.1,

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cattle were separated randomly into four groups and subjected to intra-dermal vaccinations of liposomal preparations of MA and Ag85A and standard controls of Ag85A in either MF59 adjuvant or just in PBS. Inflammatory responses were determined by means of skin thickness measurements taken at the site of injection and plotted for the different groups. This can be seen in figure 5.

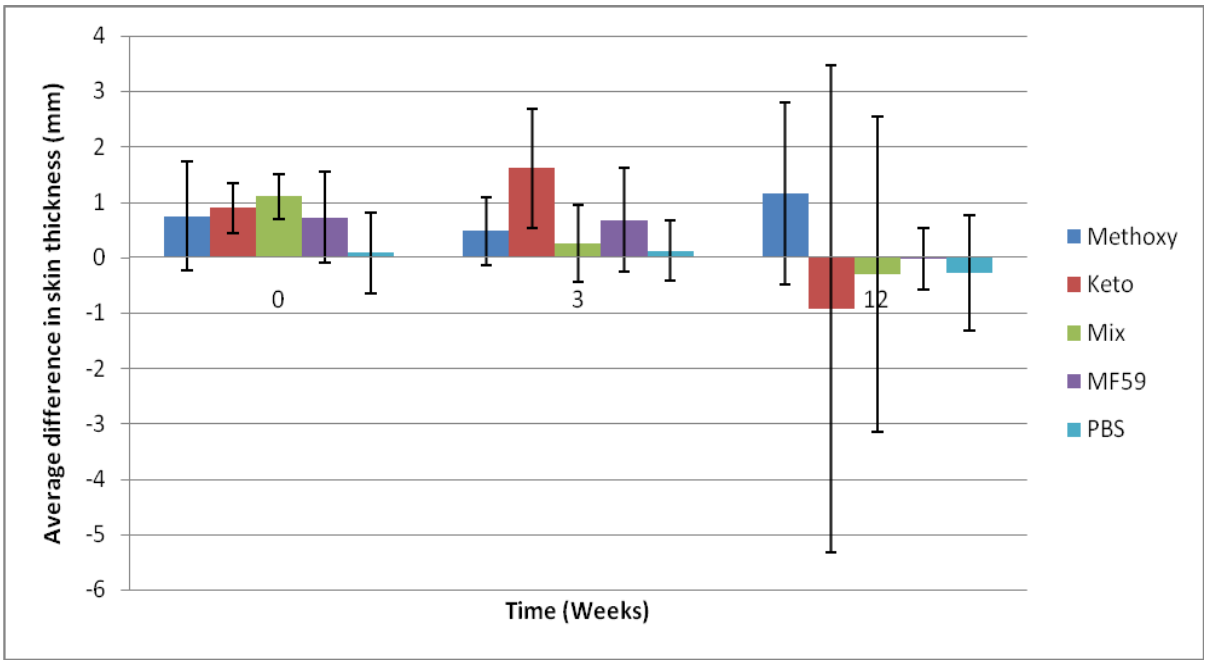

Figure 5: The average difference in skin thickness (mm) after each booster immunization (week 0; 3 and 12). The difference in skin thickness was obtained by subtracting the skin thickness measured at 0 hours from the skin thickness measured at 72 hours. The error bars represent the standard deviation (SD) for each group of animals (n=4).

From the results obtained, it was clear that no significant difference in skin thickness between the different groups of cattle was observed. At the end of the study, the animals were assessed for mycobacterial disease by means of the PPD skin test. Bovine mycobacterial protein purified derivatives (PPD) was administered intra-dermally and skin thickness (mm) was measured within 1-3 days. The results revealed that none of the cattle were found positive for the disease (results not shown).

2.3.2.2. Analyses of T-cell responses to adjuvants

Flow cytometry is a technique that allows one to detect the presence of cells using fluorescently labelled antibodies. Although the technology has been used for a while, new methods are emerging utilising novel applications. One such application is the concept of multi-colour analyses for a more in depth analysis of cells. Here several fluorescent labelled cell markers are used

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simultaneously in order to measure different cells in one analysis (Lanier and Recktenwald, 1991). Multicolour flow cytometry may be beneficial for more in depth analyses of cells, however, the use of different fluorochromes may result in a spectral overlap. Due to this, colour compensation may be necessary to account for the wavelengths that overlap (Shapiro, 2003). In the current study three different colours were used in a hierarchy to separate cells: first based on the T-cell CD3 marker and later on dividing the cells into the CD4 and CD8 T-cell subtypes.

An experiment was therefore set up to determine the optimal concentrations of these fluorescently labelled antibodies (CD3, CD4 and CD8 markers) as well as their compatible Fc blocking reagents (IgG1 or IgG2a). This was done in a titration experiment and the results can be seen in figures 6, 7 and 8 below. Figure 6 shows the results of the titration experiment of the fluorescent labelled CD3 antibody used in combination with its compatible IgG1 Fc blocking antibody.

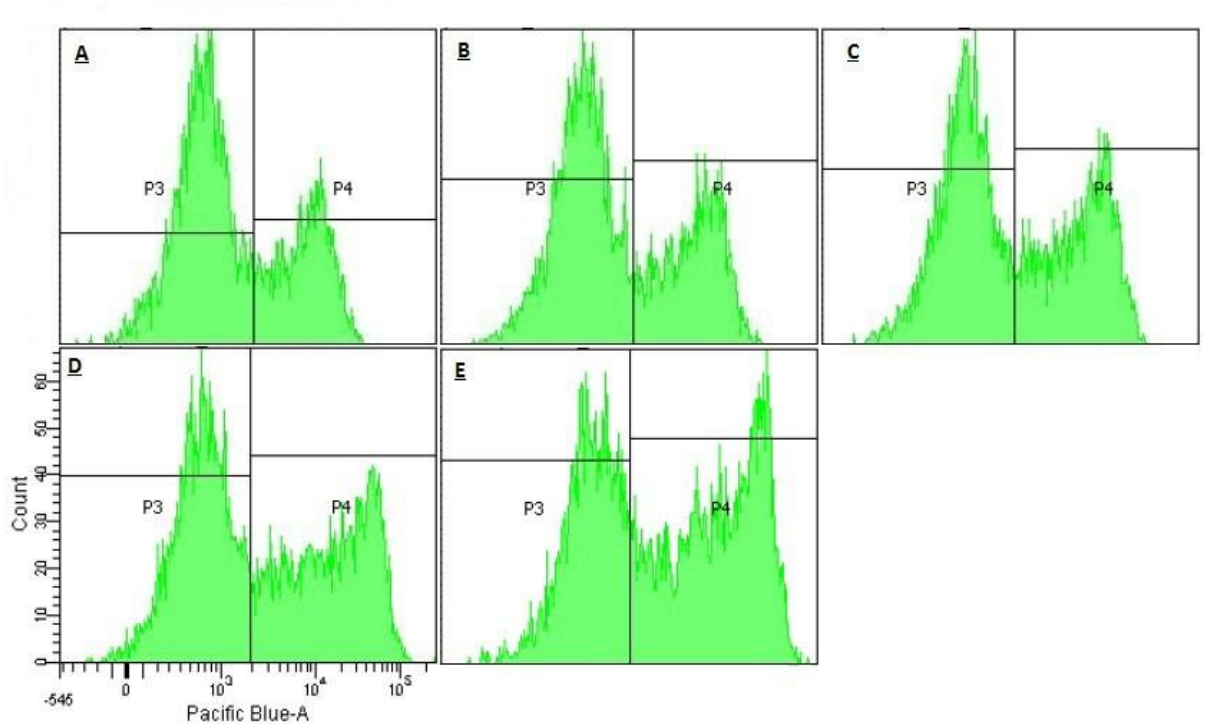

Figure 6: Titration experiment of the CD3 marker antibodies measuring CD3 cells among total PBMCs using the Pacific Blue fluorochrome. Fluorescently labelled CD3 antibody was used in combination with IgG1 Fc receptor blocking antibody. Experiment was conducted using different antibody concentrations as follows: A = 1/200; B = 1/100; C = 1/50; D = 1/20; and E = 1/10. Negative staining population is presented in gate P3 and the positive population in gate P4.

The results shown in figure 6 indicate that at an antibody dilution of as high as 1/200 one is already able to get a clear separation of fluorescent and non-fluorescent peaks in the histograms. It may be seen that as the concentration of antibody increases, the degree of non- specific staining also increases as the low fluorescent peak gradually moves to the right. One can conclude from the

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range of concentrations tested that the 1/200 concentration is best suited for the CD3 stain within the given set of reagents and samples.

Figure 7 shows the results of the titration experiment of the fluorescent labelled CD4 antibody used in combination with IgG2a Fc blocking antibody.

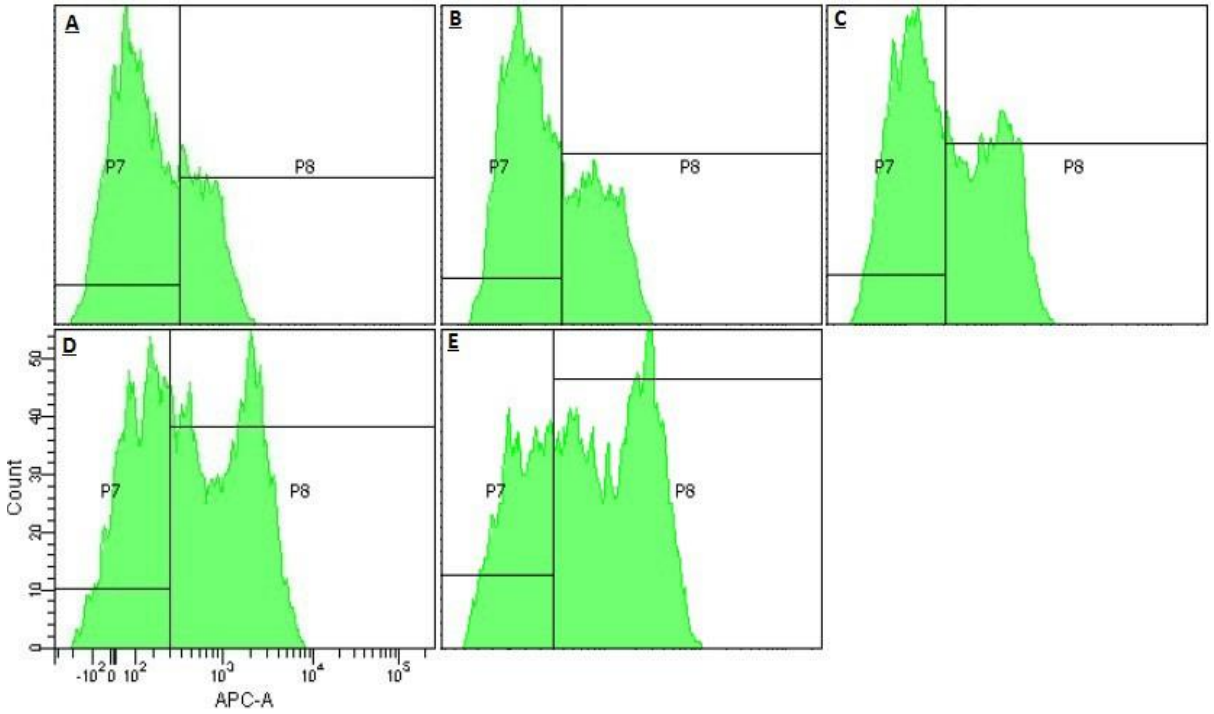

Figure 7: Titration experiment of the CD4 marker antibodies measuring CD4 cells among total PBMCs using the APC fluorochrome. Fluorescent labelled CD4 antibody was used in combination with IgG2a Fc receptor blocking antibody. Experiment was conducted using different antibody concentrations represented as follows: A = 1/200; B = 1/100; C = 1/50; D = 1/20; and E = 1/10. The negative population is presented in gate P7 and the positive population in gate P8, respectively.

As can be seen from figure 7 the separation of the fluorescent peak from the non-fluorescent peak in the histogram only becomes clear at the 1/50 dilution of antibody. At the 1/20 and 1/10 dilutions, the degree of non-specific staining increases with the increasing antibody concentration. It is therefore concluded that the optimal concentration of antibody is 1/50 for the CD4 stain used here.

The titration results of the fluorescently labelled CD8 antibody, used in combination with IgG2a negative control, can be seen in figure 8 below.

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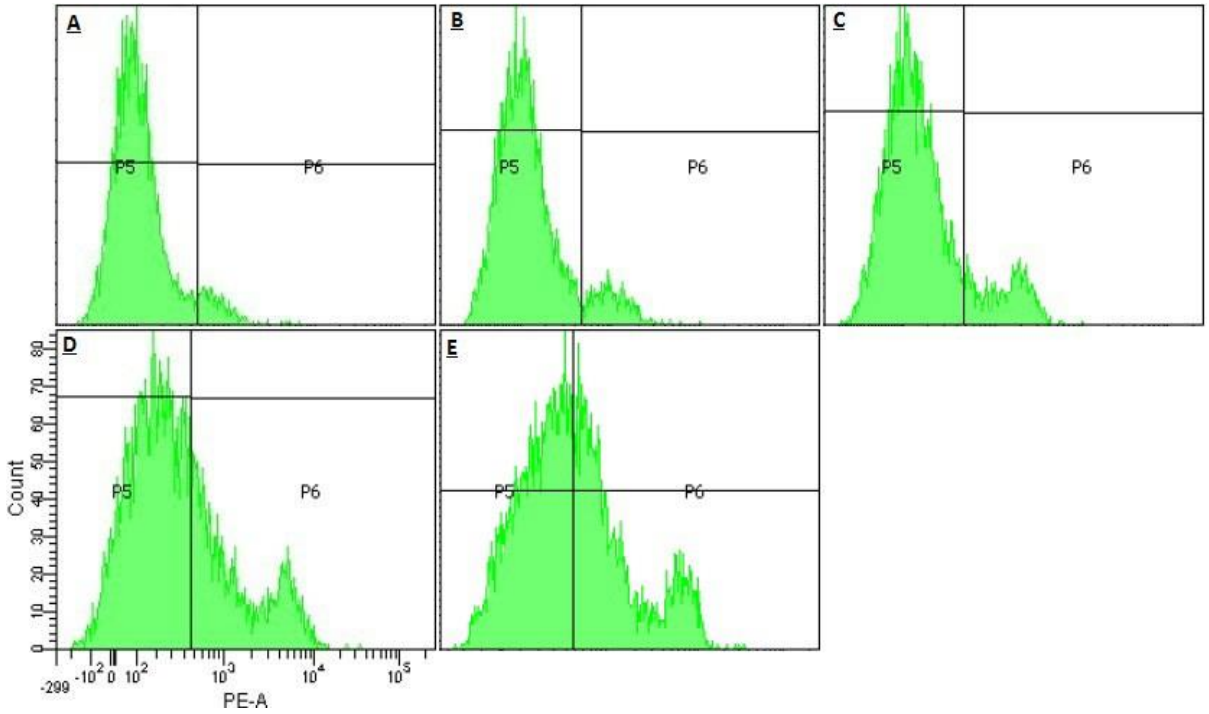

Figure 8: Titration experiment of the CD8 marker antibodies measuring CD8 cells among total PBMCs using the PE fluorochrome. Fluorescent labelled CD8 antibody was used in combination with IgG2a Fc receptor blocking antibody. Experiment was conducted using different antibody concentrations represented as follows: A = 1/200; B = 1/100; C = 1/50; D = 1/20; and E = 1/10. The negative population is presented in gate P7 and the positive population in gate P8, respectively.

From the results shown in figure 8 above it is evident that a small peak is seen at the 1/200 concentration. This peak continues to grow as the concentration of antibody increases, where it reaches its peak at the 1/50 concentration. Once again the degree of non-specific staining increases as the concentration of antibody increases. Therefore it is noted that the 1/50 concentration of antibody is best suited for the CD8 stain.

In order to investigate the effect the proposed adjuvants had on the cellular response, T-cell proliferation assays were set up as described in 2.2.2.5. Concavalin A (ConA) was used as a positive control for T cell proliferation (Palacios, 1982). Cells stimulated with ConA were stained with a combination of CD3 and Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) to track the proliferation. CFSE is a fluorescent dye that stains the cytoplasm of cells. This dye may be used to measure proliferation as half of the dye is carried over to the daughter cells when the cells divide (Hawkins *et al.*, 2007; Lyons, 1999; Lyons, 2000; Lyons *et al.*, 2001). A CD3 antibody conjugated with a fluorescent label was used to track the T-cells as CD3 is present on all of these types of cells. Data pertaining to these assays were collected by means of flow cytometry and had

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to be processed before results could be interpreted. The following steps were undertaken to achieve this:

The cells (unstimulated, Ag85A stimulated or ConA stimulated) were first analysed in terms of forward scatter and side scatter to determine the size and granularity of the cells, respectively. The results appear in figure 9.

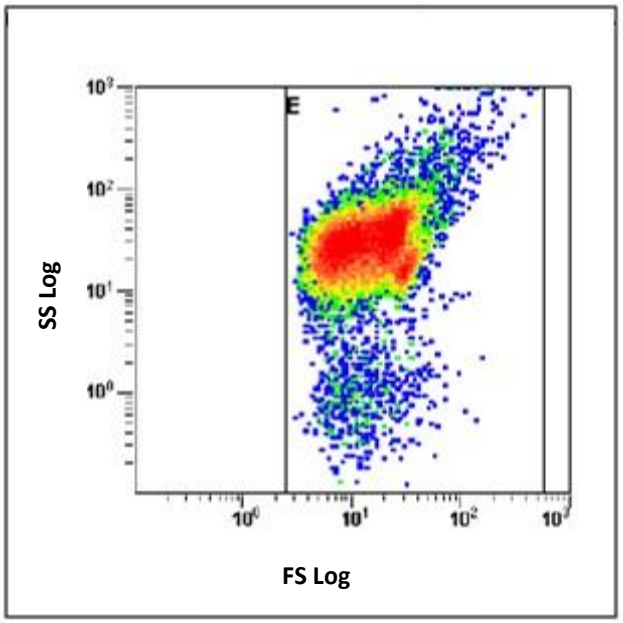

Figure 9: Flow cytometry data of forward scatter versus side scatter for PBMCs from MF59 vaccinated bull 5 cells obtained from week 4. SS Log = side scatter; FS = forward scatter. Cells gated in E represent live cells.

From figure 9 it can be seen that there is a large spread of cells, both in size and granularity. However, the majority of the cells lies in the centre of the plot and may be seen in orange. A gate was set to include all the cells and is shown as gate E. This was done in such a way that all the cells could be interrogated based on the CD3 stain. The next step was to determine the cells that were double positive for both CFSE (shown as FITC) and CD3. These results are shown in figure 10.

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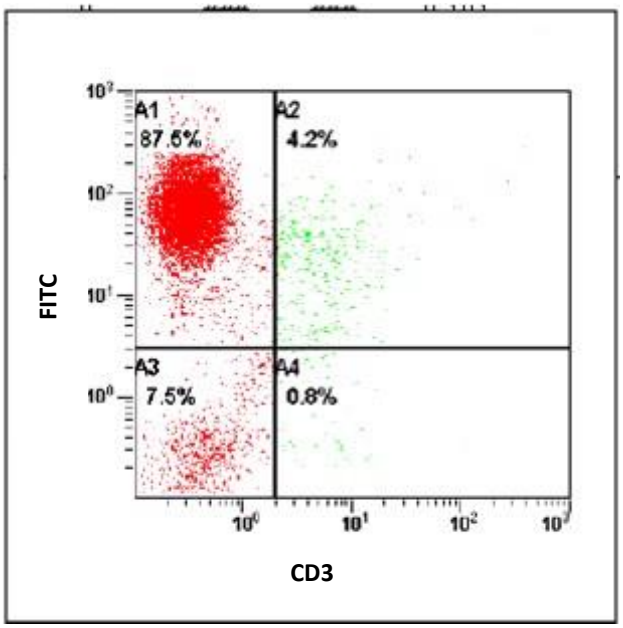

Figure 10: Flow cytometry experiment measuring CFSE versus CD3 of PBMCs from MF59 vaccinated bull 5 cells obtained from week 4. FITC = CFSE; CD3 = T cells. Cells present in each quadrant indicate the following: A1= $CFSE^{+}/CD3^{-}$, A2= $CFSE^{+}/CD3^{+}$, A3= $CFSE^{-}/CD3^{-}$, and A4= $CFSE^{-/}CD3^{+}$.

Figure 10 shows a dot plot of cells stained for CFSE and CD3. A quadrant was constructed where quadrant A3 was determined by means of the results obtained for unstained control cells (results not shown) and set to show the cells that are CFSE (FITC) positive (A1), CD3 positive (A4) and positive for both parameters (A2). A new gate was then constructed using forward scatter to only focus on the live CD3 positive cells. This can be seen in the figure 11.

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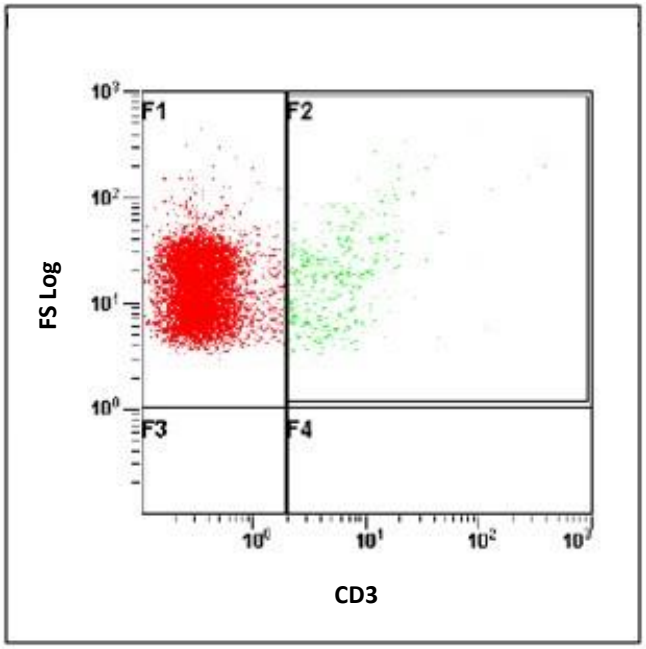

Figure 11: Flow cytometry results for forward scatter versus CD3 of PBMCs from MF59 vaccinated bull 5 cells obtained from week 4. FS = forward scatter; CD3 = T cells. Cells represent in the following: F1= $CD3^-$, F2= $CD3^+$, F3 and F4 indicate cell debris.

In figure 11 it is confirmed that the new gate setting selects better for the live (normal sized) cells from the sample. Quadrant F2 (shown in green) now clearly shows the target cells for the T cell proliferation assay, namely that subpopulation of the cells that are positive for both CD3 and CFSE, indicating the presence of proliferating T-cells in the population. The cells gated in quad F2 were then presented in a histogram (figure 12).

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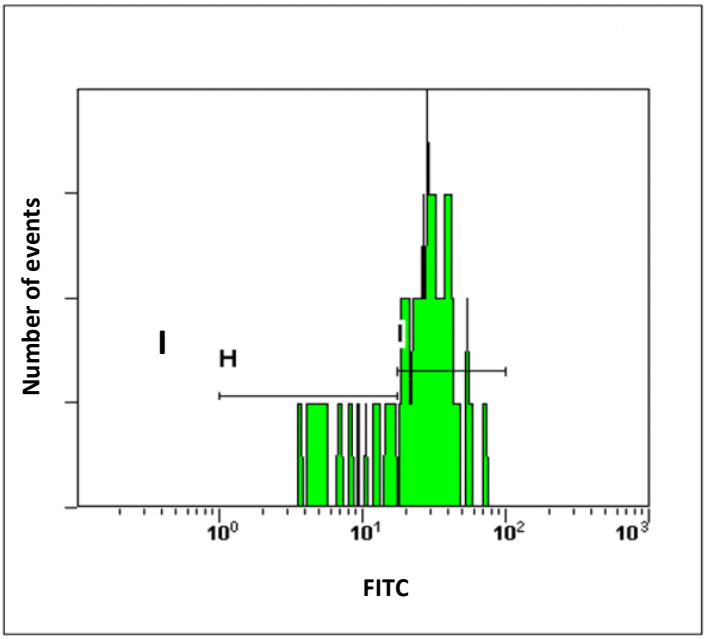

Figure 12: T-cell proliferation in MF59 bull 5 PBMC cells at week 4, stimulated with ConA. FITC = CFSE. Cells gated indicate the following: H= proliferated/dividing cells, I= undivided cells.

When measuring the proliferation that a sample of cells undergoes one should look for the daughter cells (H) that arise from the main peak (I) in Fig 12. Due to the fact that proliferation was always seen using the positive control, ConA, and little or no proliferation was seen in the unstimulated samples it was decided that the positive control would represent 100% proliferation and the negative control 0% for each set of cells, respectively. Using this as a guideline a formula was drawn up to determine the percentage of proliferation taking place in the cells stimulated with Ag85A as follows:

% Proliferation = ratio difference in controls X Ag85A proliferation,

where: ratio difference in controls = week 0 control diff/week 4 control difference

Ag85A proliferation = difference in proliferation between week 4 and 0

*week 0 control difference = ConA value – Unstimulated cells value at week 0

**week 4 control difference = ConA value – Unstimulated cells at week 4

Using the formula shown above the percentage proliferation of the T cells was calculated with respect to stimulation with Ag85A and the results revealed the following. It was seen that no proliferation takes place in any of the cell samples stimulated with Ag85A, with the exception of bull 8 (results not shown). In order to confirm these results student's t-tests were conducted to identify if any differences may be seen between the different groups of animals. These results may be seen in the table below.

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Table 4: Results of paired student t-tests related to percentage proliferation of Ag85A stimulated cells between different groups of vaccinated animals. Values are calculated based on an average of 3 values obtained from animals in the respective groups shown in table 2. All animals were vaccinated with Ag85A in combination with either MF59, PBS or Synthetic MA mix. Significant values ($p \geq 0.05$) are shown in bold.

Groups	P-values
MF59 versus Synthetic mix	0.76
MF59 versus PBS	0.52
Synthetic mix versus PBS	0.49

The results in the table above indicate no significant difference between the cell samples of the different groups of animals. This thereby confirms the results that no increased proliferation was present in cells stimulated with Ag85A.

2.3.2.3. Optimised ELISA controls and methods

To best investigate the antibody responses elicited from the different "adjuvant" compounds ELISAs were conducted. Due to the large number of samples (sera collected weekly from 20 bull calves divided in four groups) that had to be analysed, a normalization protocol had to be designed that would allow us to compare results obtained of samples analyzed on the many different plates. To begin the process we first analysed the sera from week 13 of the four bull calves vaccinated with a combination of Ag85A and MF59. The results are shown in figure 13.

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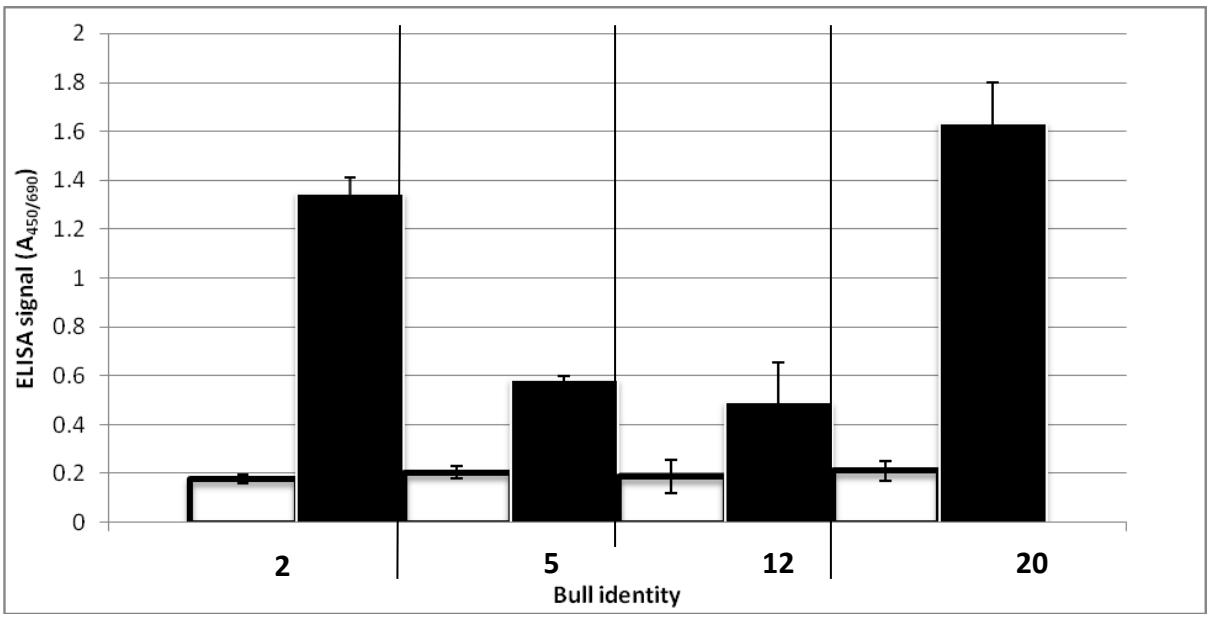

Figure 13: Anti-Ag85A antibody detection by ELISA of serum samples obtained at week 0 (pre-vaccination; open bars) and week 13 (filled bars) of Ag85A/MF59 vaccinated bulls. Error bars represent standard deviation of four repeat measurements.

All sera obtained from week 2 gave positive antibody responses to Ag85A, compared to those from week 0. Bull 2 and 20 presented with the highest responses and therefore either of the two is suitable as the positive control reference on every ELISA plate for normalisation of ELISA results between plates. Due to the larger volumes of serum collected from bull 2 at week 0 and 13, this animal was selected for use as the negative and positive controls, respectively. The plate plan shown in figure 14 was used for further analyses.

		1	2	3	4	5	6	7	8	9	10	11	12
A	1:40	Coat wells with 1X PBS only	Bull 2 Week 0 sera added	Bull 2 Week 13 sera added									
B	1:80												
C	1:160												
D	1:320												
E	1:40		Bull 2 Week 0 sera added	Bull 2 Week 13 sera Added									
F	1:80												
G	1:160												
H	1:320												

Figure 14: ELISA plate layout used for all the anti-Ag85A antibody response determinations. The second column indicates the serum dilutions used. Column numbers 4 – 12 were used to test bull sera from other animals among the four experimental groups.

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Figure 14 gives the plate layout of all analyses of the anti-Ag85A serum antibody responses. The first three columns of each plate represent the standards across each plate so that "inter- plate" comparisons may take place. Therefore wells coated with 1 X PBS will serve as a negative control for the plate to ensure that no false responses other than background ELISA signals are being obtained from buffers and reagents. Wells present in columns 2-12 will be coated with a 10 ug/ml Ag85A/PBS solution. Columns 2 and 3 then respectively received bull 2 pre-vaccination sera and sera obtained at week 13 into the vaccination schedule at the indicated dilutions. This served as the respective negative and positive controls for the cattle sera to be analysed, and will be added to the wells in duplicate. Although these standards were only added in duplicate, values that hugely deviated from one another were rejected and the experiment was repeated. As already mentioned, positive and negative controls were set in certain columns; therefore sera from the bull being analysed was added in triplicate in the remaining columns (4-12). Therefore sera from each bull were added in triplicate and analysed in a dilution series (1:40, 1:80, 1:160, and 1:320).

2.4. Discussion

Adjuvants are useful substances that are used in combination with vaccines to enhance their functionality in immune responses (Ott and Van Nest, 2006; Schijns, 2000). Due to the beneficial effects of these substances to improve vaccines, scientists are constantly searching for novel adjuvant compounds. However, before a substance may be classified as such one needs to investigate the inflammatory, antibody and cellular responses elicited from it. This requires the setup of relevant protocols to determine the effects in an efficient way. The methods and results of this chapter address the setup of suitable technology to investigate chemically synthetic mycolic acids as potential adjuvants in cattle vaccines.

Mycolic acids constitute the most abundant lipid component of the cell wall in *M. tuberculosis* and are particularly insoluble in water (Barry III *et al.,* 1998; Colston, 1996). Due to this property of the MAs, different carrier systems were investigated to determine how to administer the compounds as adjuvants. From the results shown above it is clear that the use of microcapsules as MA carrier systems is sub-optimal, as an inflammatory responses was present at the site of injection of the two compounds, but not with empty microcapsules. Although FIA was earlier regarded as safe for use in humans, studies have shown that these compounds actually have potential toxic side effects. For example, FIA was frequently shown to cause severe inflammation at

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the site of injection (MacLennan *et al*., 1965; Stuart-Harris, 1969). As a result the use of FIA as adjuvant and microcapsules as MA carrier were ruled out for use in the cattle vaccination experiment performed here. The results indicate that no inflammatory responses are elicited by PBS, PLGA and liposomes as carriers for administration of the synthetic MAs. In this experiment PBS was used as a negative control for the adjuvant testing. PLGA is a biodegradable polymeric nanoparticle that is under investigation as a carrier system (Rawat *et al*., 2006). PLGA particles have proved safe for human pharmacological use, e.g. as a potential delivery system for drugs, genes and DNA (Panyam and Labhasetwar, 2004). Of importance in the vaccine field is the fact that the particles have illustrated their ability to enhance the delivery of antigens to dendritic cells (Elamanchili *et al.,* 2004), important mediator cells of innate immunity. Another carrier that was considered was liposomes. Liposomes are bilayered vesicles that are mainly composed of phospholipids (Rawat *et al*., 2006). As with PLGA, liposomes have shown potential in a number of fields including drug delivery; thereby illustrating their safe use (Chen *et al.*, 1999; Peer *et al.,* 2007). Due to their proven potential as MA carriers (Lemmer *et al.*, 2009) we used liposomes as the carrier systems for the vaccination experiments in cattle.

Although liposomes were found to be an ideal carrier system in the rabbit experiment, one of the major challenges that needed to be overcome was getting the MAs into a protein antigen containing solution to be used for vaccinations. The nature of MAs has been described in literature as being wax compounds and therefore they are insoluble in water (Barry III *et al.*, 1998; Colston, 1996). Although MAs can be brought easily into a liposomal suspension by heat, it is a different matter altogether to attempt to suspend/ dissolve them in water at room temperature. With both the protein Ag85A and the MAs of extremely limited solubility, heat could not be avoided to prepare the vaccine mixture of MA and protein Ag85A. Thus, the method used by Lemmer for the preparation of MA liposomes was applied, that entailed heating the MA in hot buffer (Lemmer, 2010) that contained the Ag85A. Thus, Ag85A used in combination with the various proposed MA adjuvants were probably denatured, while this was not the case in the positive (MF59 adjuvant) or negative (PBS) controls. Although this process may seem irregular it was carried out to determine whether the MAs could at least elicit a T cell mediated response; such a response does not rely on intact protein conformation of Ag85A.

A challenging aspect of these experiments was finding antibodies specific for cell markers of cattle. Most experiments using flow cytometry are conducted on cells obtained from humans and mice. As a result many companies do not produce fluorescent-conjugated antibodies aimed specifically at

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cattle. Due to this shortcoming some of the stains chosen were specific for humans but displayed cross reactivity with bovine cells. In order to measure the T-cell responses accurately three types of antibodies were selected for use in staining. These three Serotec AbD antibodies were Rat Anti-human CD3: Pacific blue, a Mouse Anti-bovine CD8:RPE, and a Mouse Anti-bovine CD4: Alexa fluor® 647. The three T-cell antibodies were chosen to be used in conjunction with one another. It is well known in literature that CD3 is a marker found on T-lymphocytes (Robertson and Ritz, 1990). The presentation of peptide antigens is usually conducted via the MHC molecules (Bonilla and Oettgen, 2010; Chaplin, 2010; Parkin and Cohen, 2001). During this process cells are either presented on the MHC class 1 molecules, triggering $CD8^+$ T-cell responses, or on the MHC class 2 molecules, which trigger the $CD4^+$ T-cells (Bonilla and Oettgen, 2010; Chaplin, 2010). Due to the characteristics of the T-lymphocytes the methods involved staining the cells with all three antibodies so that the CD3 marker would first identify the T-cells, after which they were to be classified as being $CD8^+$ or $CD4^+$ T-cells. The results showed that the T cell proliferation test could be done in cattle, when the optimum concentration of antibody was used. These were found to be at dilutions of 1/200, 1/50 and 1/50 for the CD3, CD4 and CD8 stains, respectively. Fc blockers were used in the same concentrations as the antibodies.

The cellular response, measured by means of T-cell proliferation assays, revealed that no proliferation was achieved in the cells stimulated with Ag85A. It is clear from our results that the system does work as both the positive control, ConA, and negative control, unstimulated, worked. In each case it was shown that the ConA stimulated cells produced a larger percentage of proliferative cells compared to the stimulation with Ag85A. These results suggest that the sensitivity of the T cell proliferation test was out of range, probably due to the low concentration of the Ag85A in the prepared doses of vaccination. Although this may be the case, it cannot be excluded that the $CD4^+$, $CD25^+$ T-cells, also known as Tregs are involved. Tregs are a naturally occurring population of cells that suppress immune responses both *in vivo* and *in vitro*. It was reported previously that these $CD25^+$ T cells often work in conjunction with $CD4^+$ T cells to down-regulate immune responses (Shevach *et al.*, 2001; Shevach, 2002). The most outstanding characteristic of these Treg cells, however, is their inability to proliferate in response to T-cell receptor (TCR) stimulation alone (Kruisbeek *et al.*, 2001). In this study the CD4 T cells did not proliferate probably due to the low concentration of protein present in cells stimulated with Ag85A resulting in the lack of proliferation. This inability may be overcome with the addition of IL-2 which aids in proliferation of this cell subtype (Kruisbeek *et al.*, 2001). Here, IL-2 could not be

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incorporated into the proliferation assays due to lack of time and financial constraints. As a result the experiment may have been out of range. Future experiments in this respect should include the use of IL-2 in the stimulation of the cells to boost the sensitivity of the assay, as well as investigate the possible role of the Treg cells (CD4+ and CD25+) to explore the suppressive effects on the cellular level. The latter can be achieved by selective suppression of the Treg activity with the use of monoclonal antibodies directed to CD25 (Korf *et al.*, 2006).

Although ELISAs are not considered difficult assays to carry out one should ensure that the results obtained are reproducible and comparable on separate plates. For the purpose of these experiments we first determined that the positive control serum samples tested positive for antibodies binding to Ag85A. The results showed that anti-Ag85A antibody responses could be detected in all four bulls injected with MF59 at week 13 compared to week 0. The responses were weaker in animals that were not vaccinated with adjuvanted preparations, but just Ag85A in PBS (data not shown). Thus, serum samples from week 0 and week 13 of bull 2 were selected to be used as a standard on each plate acting as the positive serum controls. As negative control for the antigen, a column of wells coated only with PBS on every plate was introduced to ensure that there was no non-specific antibody binding activity due to the buffers or the plate used. How this panned out will be described in the chapter that follows.

2.5. Conclusion

New adjuvants should be safe and efficient to use, while simultaneously showing the expected benefits. This requires careful experimental testing. Therefore as a starting point we illustrate in this chapter how we may go about determining the inflammatory, antibody and cellular responses elicited by the novel mycolic acids as potential vaccine adjuvants. Liposomes and PLGA nanoparticles proved not to be inflammatory agents in themselves and liposomes were selected for further experiments. Microparticles left some trace of swelling at the site of injection, especially in the presence of FIA, which was therefore excluded for further work. None of the synthetic and natural mycolic acids gave any undesirable inflammatory side effects, which allowed all of them to be tested in cattle. The results of the T-cell proliferation assays indicated the importance of using Fc blocking agents in the final T cell staining and specified the optimal concentrations at which both the Fc blockers and fluorescent marker antibody stains should be used for the experiments to be conducted. The lack of proliferation observed in response to *in vitro* Ag85A stimulation for all vaccine preparations, was probably due to too low vaccine doses of Ag85A due to solubility

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restrictions imposed by the recombinant Ag85A. For investigating the humoral antibody response to MA-adjuvanted Ag85A vaccines in cattle, the positive controls were found and characterised, the reaction conditions optimised and the experimental plate layouts planned for a comprehensive assessment of the many serum samples that were collected from all five groups of cattle that were vaccinated. The next chapter applies these protocols in an attempt to determine how MAs may function as vaccine adjuvants.

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3. Comparing the anti-Ag85A antibody responses of synthetic mycolic acid and MF59 adjuvanted vaccines

3.1. Introduction

The immune system is the defense mechanism present in the human body that acts in response to invasion by foreign encounters (Chaplin, 2006; Parkin and Cohen, 2001). Up to this point mainly the T-lymphocyte aspect of the adaptive immune response has been discussed and covered in the previous chapter. There is however another component of this system that plays an active role, namely the B-lymphocytes (Beutler, 2004; Bonilla and Oettgen, 2010; Chaplin, 2006). These cells are responsible for the adaptive humoral response carried out by the action of antibodies. B-cells are developed from hemopoietic stem cells in the bone marrow where they later mature and acquire their antigen specificity (Bonilla and Oettgen, 2010). B-cells may also produce plasma cells in response to signals received from T-cells and other cells. These plasma cells are responsible for the production of antibodies, which are also known as immunoglobulins. Antibodies aid in the immune defense by attaching to the antigenic portion of the pathogen and rendering it harmless (Bonilla and Oettgen, 2010). This process may take place in parallel with response of T-cells, when the antigens are T-cell dependent, or in the absence when the antigens are independent of the T-cells (Bonilla and Oettgen, 2010; Chaplin, 2006; Chaplin, 2010).

The different responses of the immune system are important when looking for new vaccines and adjuvants. The use of adjuvants was invented in the 1920s and is still used today to aid vaccines against all diseases. A number of different substances have been used in adjuvants. Components of the *M. tuberculosis* organism were among the first to be recognized as important in this context. Thus, Freund's complete adjuvant (FCA) was one of the first formulations that relied on the immune steering properties provided by whole, inactivated and dried mycobacteria (Sadelain *et al.*, 1990). Contained in a water in oil emulsion, it was shown to prevent the juvenile onset of diabetes in mice. Although successful as an adjuvant in mice and many other mammals, FCA caused adverse local inflammatory side effects, making it unfit for use in humans and eventually also not for commercial veterinary vaccines. In early animal vaccine research, an initial vaccination with FCA was followed up by booster vaccinations of the antigen with FIA, which is just the water in oil emulsion without the mycobacterial components. This served to reduce the inflammatory side effects.

Nowadays, improved safe and potent adjuvants are available, of which a prime example is

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MF59, an oil-in-water emulsion adjuvant that contains a low amount of biodegradable squalene oil, making use of two non-ionic surfactants for stabilization and a low ionic citrate buffer as the continuous phase (O'Hagan *et al.,* 2007). It has demonstrated its ability in a number of licensed vaccine applications, including human anti-influenza vaccines (O'Hagan *et al.,* 2007). Views on the mechanism of action employed by MF59 have been well documented in literature. These mechanisms include targeting of human immune cells (including monocytes, macrophages and granulocytes), increased antigen uptake, the release of chemoattractants, and cell differentiation (Dupuis *et al.,* 1998; Dupuis *et al.,* 2001; Seubert *et al.,* 2008). Although there is evidence of the mechanism of action available, it is now known in literature that all the components of the MF59 adjuvant need to be present in order to achieve optimal activity (Calabro *et al.,* 2013).

Current data on human trials relate to annual injections of MF59 Flu vaccines and is only allowed for over-60 year old individuals. As a result there is still a huge void of knowledge around the frequency of vaccination and time periods between vaccinations for ethical trialling of adjuvanted vaccines. Here we set out to determine any possible undesired effects of short periods between adjuvanted vaccinations, in an attempt to help guide the current design of ethics formulation for the trialling of adjuvanted vaccines with experimental evidence, in support of the mission of the WHO's Global Vaccine Safety Initiative (WHO, 2012).

In this chapter the following aims will be explored:

1. Analyse the antibody responses of cattle sera collected from the three weeks following vaccinations
2. Investigate the antibody isotypes present in sera obtained post-vaccination

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3.2. Materials and Methods

3.2.1. Antibody response determination

3.2.1.1. Coating of micro-titre plates

Non-sterile, 96-flat-bottom polystyrene plates (Costar®, NY.) were used for the duration of the experiments conducted. Plates were coated with either the antigen 85A (Ag85A; 112 µg/ul; Protein Service Facility, Gent) or Phosphate buffered saline (PBS), serving as the experiment and control, respectively. A 10 µg/ml solution of Ag85A in PBS was prepared, ensuring the antigen was dissolved properly in the PBS. The wells were then coated with the Ag85A/PBS solution (100 µl/well) using a multi-channel pipette. Plates were covered with foil and stored at 4°C overnight, until use the next morning.

3.2.1.2. Preparation of buffers

Solutions of 1% BSA/PBS (w/v), 3% BSA/PBS (w/v) and 0.05% Tween®20/PBS (v/v) were prepared as the dilution buffer, block buffer and washing buffer respectively, to be used on the morning of the experiment. The BSA/PBS solutions were prepared by weighing out the desired amount of BSA and sprinkling it on top of the PBS, allowing it to dissolve into solution before use.

3.2.1.3 Enzyme Linked Immunosorbent Assay (ELISA)

The following day wells were washed with 0.05% Tween20/PBS, blocked with 3% BSA/PBS (300 µl/well) for 2 hours, and incubated with 100 µl/well diluted sera (collected as described in previous chapter) ($\frac{1}{40}$, $\frac{1}{80}$, $\frac{1}{160}$ and $\frac{1}{320}$) for 1 hour. A secondary antibody of rabbit anti-bovine IgG (H/L): Horse radish peroxidase (HRP; AbD Serotec) was used at a 1:1000 dilution in 3% BSA/PBS at 100 µl per well and the plate incubated for 30 minutes at 23 °C. Following incubation the wells were emptied and washed with 0.05% Tween20/PBS before 100 µl/well TMB substrate (Solutions A and B mixed 1:1; BD Biosciences, South Africa) were added for colour development. The results were measured with a Multiscan Ascent Plate reader (AEC Amersham, South Africa) at 620 nm at times 10; 20 and 30 minutes. The reaction was stopped after 30 minutes by addition of 50 µl per well 1 M sulphuric acid solution and the final colour reaction was measured immediately at a wavelength of 450/690 nm, where 690 nm was used a reference filter.

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3.2.2. Comparative immunoglobulin isotyping ELISA

3.2.2.1. Coating of micro-titre plates

Coating was done as mentioned in section 3.2.1.1 above.

3.2.2.2. Preparation of buffers

Refer to method in 3.2.1.2 above for preparation.

3.2.2.3. Enzyme Linked Immunosorbent Assay (ELISA)

On the day of analysis wells were washed with 0.05% Tween20/PBS, blocked with 300 µl/well 3% BSA/PBS for 2 hours, and incubated with 100 µl per well of diluted sera (collected as described in previous chapter) ($\frac{1}{80}, \frac{1}{160}, \frac{1}{320}$ and $\frac{1}{640}$) for 1 hour. Secondary antibody conjugates of rabbit anti-bovine IgG (H/L): HRP (AbD Serotec, United Kingdom), sheep anti-bovine IgM: HRP (AbD Serotec), sheep anti-bovine IgG1: HRP (AbD Serotec) and sheep anti-bovine IgG2: HRP (AbD Serotec) were used at 100µl per well of a 1:1000 dilution in 3% BSA/PBS and the plate incubated for 30 minutes at 23°C. Following the incubation the wells were emptied and washed with 0.05% Tween20/PBS. TMB substrate (100 µl/well of solutions A and B mixed 1:1; BD Biosciences, South Africa) was added for colour development and the results measured with a Multiscan Ascent Plate reader (AEC Amersham, South Africa) at 620 nm at times 10, 20 and 30 minutes. The reaction was stopped after 30 minutes by adding 50 µl 1 M sulphuric acid solution and the final colour reaction was measured at a wavelength of 450/690 nm.

3.3. Results

3.3.1. Antibody responses elicited by different adjuvants

Blood was collected weekly for the duration of the study, as shown in figure 15, and the sera obtained were subjected to analysis by ELISA to detect antibodies to Ag85A.

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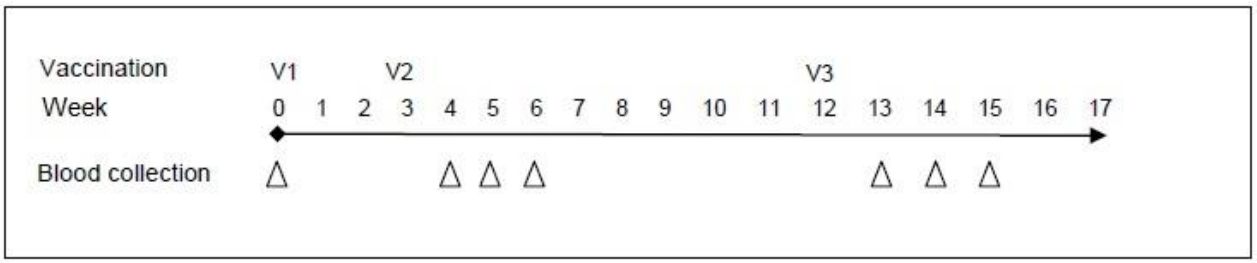

Figure 15: Time line representing vaccination (V) and bleeding times. Animals were bled weekly. Indicated bleedings (Δ) relate to samplings for which results are reported.

The preliminary results obtained illustrated the following: MF59 (the positive control) produced the highest absorbance signals with the Sigma MA mix and methoxy MA following closely behind it (data not shown). The absorbance observed with the keto MA was slightly lower than this with PBS, the negative control, being the lowest (data not shown). These results were gathered from samples at week 13.

It was seen in the previous chapter that the T-cell responses were only investigated on cells obtained from cattle immunized with Ag85A in combination with either MF59, PBS or the synthetic MA mixture. The antibody responses were also measured in samples from these animals to investigate if a link could be found between the cellular and humoral responses. Due to the uncertainty produced with the preliminary results and the absence of standards on each plate, the experiment was optimised as described in the previous chapter. In an attempt to directly compare the antibody responses obtained to those described in previous trails of MF59 present in literature, the same terminology was adopted to describe the vaccinations elicited (Banzhoff *et al.*, 2009; Hatz *et al.*, 2012). Therefore the first two vaccinations (V1 and V2 shown in figure 15) will be termed the primary vaccinations and V3 (figure 15) termed the booster vaccine.

The ELISA experiment analysis was therefore conducted on weeks 4, 5 and 6 so that we could determine the antibody response in the three weeks that followed the primary vaccinations. Initially only raw data was collected which had to be normalized using standards present on all plates to allow for interplate comparison. S' values were calculated to relate an ELISA reading for a particular sample (S) to a positive control reference sample (Ref). ELISA values for this reference were obtained in an initial analysis (RefI) and, in addition, present on each sample plate (RefS). S^X represents the absorbance value of the sample obtained for the week (superscripts X; 0 or 13) into the vaccination and sampling programme being analysed. These parameters were used to

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calculate S'values in the formula:

$$S' \text{ value} = (Refl^{13} - Refl^{0} / RefS^{13} - RefS^{0})(S^{X} - S^{0})$$

The early response was determined on serum obtained four, five and six weeks after the commencement of the experiment, i.e. a serial analysis starting one week after the second primary vaccination at week 3 as can be seen in figure 16 below.

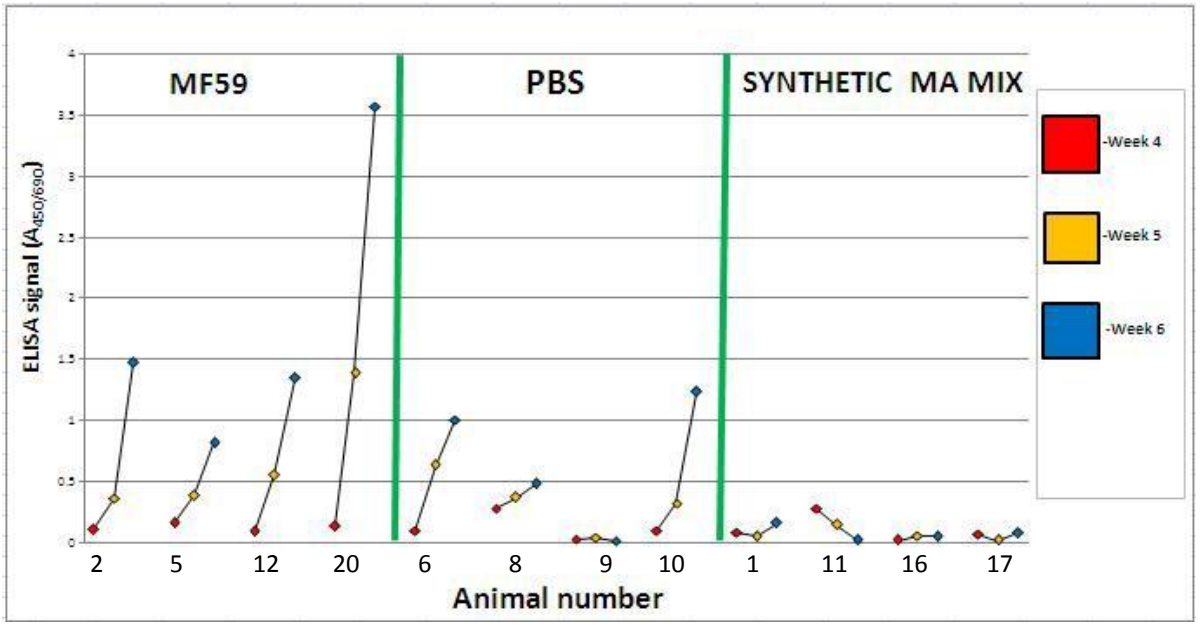

Figure 16: Prevalence of antibodies to Ag85A in synthetic MA mix and MF59 adjuvanted vaccinated cattle at weeks 4, 5 and 6 measured by ELISA. The response for each of the four animals per group is shown. ELISA signals are normalized S' values – see text.

The results in figure 16 show that antibody responses to Ag85A were elicited by all the group of Ag85A immunized animals treated with the MF59 vaccine, and three Ag85A immunized animals where the Ag85A was dissolved in PBS. The antibody binding signals were strongest at week 6, i.e. three weeks after the primary injections. The strongest increasing trend of appearance of anti-Ag85A antibodies occurred in the MF59 adjuvant group. This demonstrates a superior effect of the MF59 adjuvant. The results indicate that the response elicited by the animals of the synthetic MA mixture is much lower than that of both the positive control and PBS control.

The experiment was extended to see if the same trends would manifest in the late response, after the booster vaccination. Sera obtained at weeks 13, 14 and 15 of the experiment, i.e. at the first, second

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and third week after the booster vaccination, were therefore also analysed for anti-Ag85A antibody responses. The results are shown in figure 17.

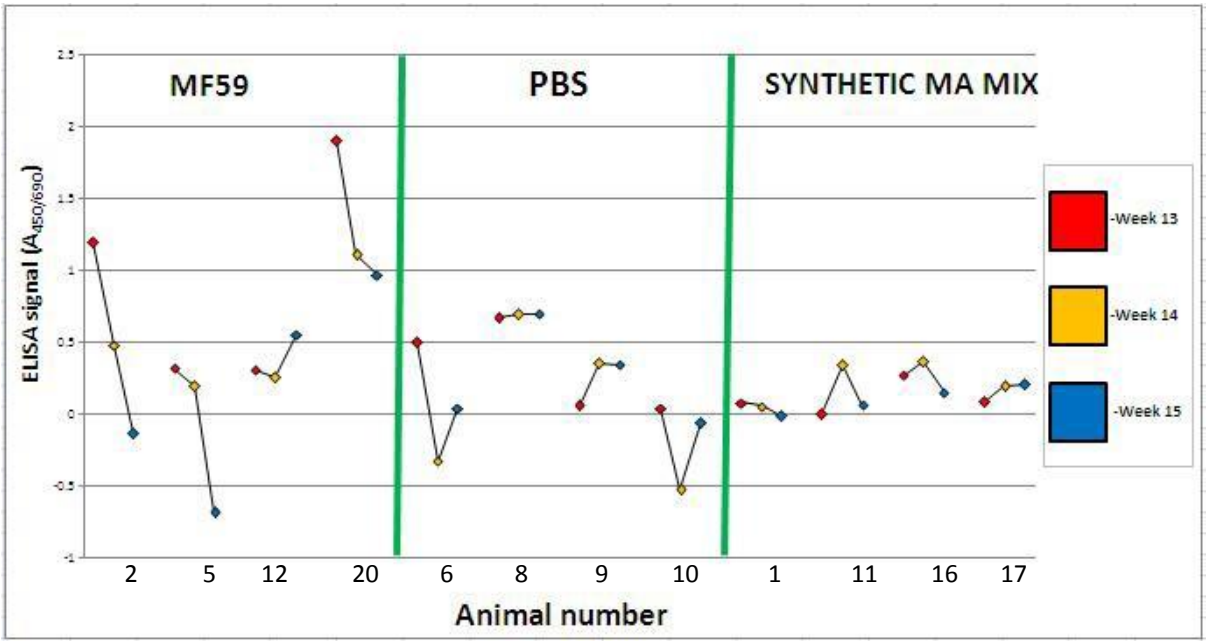

Figure 17: Prevalence of antibodies to Ag85A in synthetic MA mix and MF59 adjuvanted vaccinated cattle at weeks 13, 14, and 15 measured by ELISA. The response for each of the four animals per group is shown in the same sequence as in the early response (Fig. 16). ELISA signals are normalized as S' values – see text.

The results obtained with the late antibody responses indicate opposite trends to those seen in the early response. The MF59 group now shows the highest antibody responses in the first week after the booster injection (week 13) for three of the animals, going down in subsequent weeks. In the animal that showed the lowest antibody signal in week 13, the signal tended to maintain itself over the three weeks of measurement. In the PBS control, only two animals tested positive for anti-Ag85A antibody activity in week 13, with only one animal showing a weakly defined, irregular downward trend over the three weeks measured. The synthetic MA mixture group exhibited the lowest antibody response compared to the others and therefore confirmed the results obtained following the vaccine booster. Due to the absence of antibody response by the synthetic MA mix group these results were not subjected to further analyses.

Previous research has shown that the primary antibody response to antigen generally rises quickly, followed by a steep decline (Edgar, 2011). Here, the results from the early response (Fig.

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16) showed the opposite effect with an increasing Ag85A antibody binding activity over the three weeks following the primary vaccinations in both the MF59 and PBS groups. A student t-test was conducted on the rates of increased antibody activity of the two groups to determine the significance of the difference between the MF59 adjuvanted and non-adjuvanted vaccinated groups. The more pronounced increasing tendency of antibody titres over the three weeks in the MF59 vaccinated cattle compared to PBS vaccinated cattle was shown to be significant to a confidence level of better than 90% (p value = 0.08).

The secondary response, as described in literature, is shown to be stronger than the primary response when measured one week after the booster injection, which was also found to be the case here. Generally, the secondary response is then maintained better than the primary response (Edgar, 2011), but this was found to be radically different here in the adjuvanted vaccine treated group. We actually saw the opposite effect (Fig. 17) with a steep decline in Ag85A binding activity over the three weeks following the booster vaccination. A student t-test was again used to determine the significance of the difference in antibody level between the MF59 adjuvanted and non-adjuvanted vaccinated groups. The downward trend of antibody titres over the three weeks is most prominent in MF59 vaccinated cattle, compared to the PBS vaccinated cattle, at a confidence level of 95% (p value = 0.05). One can conclude that the MF59 adjuvanted vaccine performed better than the non-adjuvanted vaccine after two injections, but a third injection with the adjuvanted vaccine was counterproductive towards obtaining a sustainable antibody response to Ag85A. It even indicated the risk that a sustained antibody response may be weakened by a third injection of adjuvanted vaccine within three months of the vaccination regime.

3.3.2. Immunoglobulin isotyping responses

The early response results shown in figure 16 show an increasing antibody level over the three weeks following the primary vaccinations. The opposite can be seen after the booster vaccination where a steep decline in the antibody activity is observed in the three weeks following this booster injection. One would expect an early IgM response to be relatively unstable and to decline fast, while a late IgG response should be better sustained in the circulation (Edgar, 2011). To investigate why we observe an unexpected opposite effect between the early and late stage antibody responses to adjuvanted Ag85A vaccinations (compared to the above mentioned example from literature), an experiment was set up to determine which antibody isotype dominated in the early and late stages.

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The results are shown in figure 18.

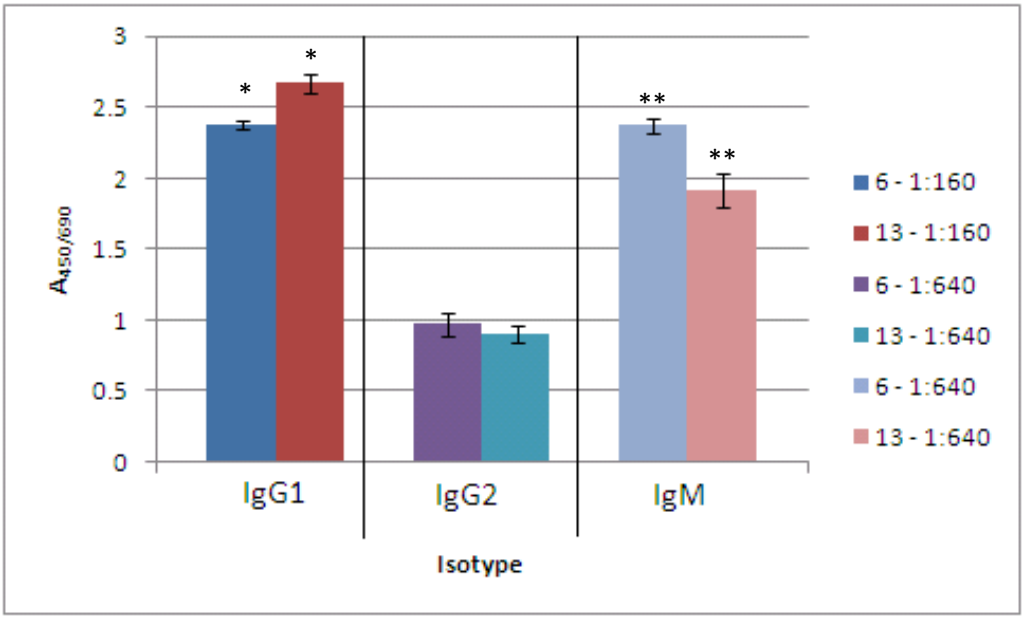

Figure 18: Comparative immunoglobulin isotyping ELISA results obtained from MF59 adjuvanted vaccinated animal at week 6 and week 13. Serum from bull 2 in the MF59 vaccinated cohort was obtained at week 6 and 13 and isotyped as described. Results are the average value of three replicates, with error bars representing standard deviation. Dilutions in the right hand legend relate to how serum was diluted to obtain the best isotyping signals. Significant differences are indicated with stars above the relevant bars.

The results in figure 18 above indicate the differences present between sera from weeks 6 and 13 relating the IgG1, IgG2 and IgM. In terms of the IgG2 antibody it is clear that there is no significant difference between the sera obtained at the two time points. However, the results indicate that a higher level of IgG1 antibodies are present at week 13 and a higher level of the IgM class of antibodies present at week 6, respectively. These differences were shown to be significant when calculated by means of student t-tests, the results of which are presented in the table below.

Table 5: Results of paired student t-tests related to isotyping data. Significant values ($p \geq 0.05$) are shown in bold.

	IgG1	IgG2	IgM
1 in 80	0.562973	0.197474	0.208698
1 in 160	**0.009998**	0.524526	0.74414
1 in 320	0.343107	0.433564	0.057007
1 in 640	0.815251	0.456221	**0.009576**

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It is clear from table 5 that a significant difference between MF59 bull 2 serum of week 6 and week 13 is seen only in with the IgG1 and IgM class of antibodies. This implies that the unexpected non-sustained late antibody response cannot be explained by the relative stability of the prevailing immunoglobulin isotypes, but should be sought for in the mechanisms of antibody regulation by means of lymphocyte activation and suppression discussed below.

3.4. Discussion

The current project was set up in an attempt to identify if chemically synthetic MAs have the ability to function as vaccine adjuvants in cattle. MF59 is an adjuvant that has shown to be a successful candidate in improving vaccine activity in previous research and therefore served as a positive control in the experiments conducted (O'Hagan *et al.,* 2012). Trial experiments into the use of MF59 have shown that two primary doses of 7.5 µg protein antigen in the adjuvanted vaccine separated by three weeks are sufficient to obtain a good antibody response, but needed to be followed up by a booster injection either six months or a year later to provide significant immune protection (Banzhoff *et al.,* 2009; Hatz *et al.,* 2012). The current study followed a similar antigen dosage of 9.3 µg protein antigen per animal given as two primary injections separated by two weeks, but was then followed by a booster injection two and a half months later – two times shorter than was reported for trials with humans.

The results shown above with regards to the ELISA analysis illustrate the antibody response to the different compounds being explored. It is clear from the results that Ag85A together with MF59 produces higher antibody responses than when using PBS buffer only as antigen carrier, thereby confirming why it is a well-established adjuvant (O'Hagan *et al.*, 2007; O'Hagan *et al.*, 2012). The increasing tendency of antibody titres in the three weeks following the two primary injections was more pronounced in the MF59 vaccinated cattle, compared to PBS vaccinated cattle. This most likely reflects improved, progressive antibody production in the MF59 vaccinated cattle, typical when IgM dominates (Hjelm *et al.,* 2006), a desirable outcome in support of the use of the adjuvant. In the late stage, however, the downward trend of antibody titres over three weeks following the booster injection is most pronounced in MF59 vaccinated cattle, compared to unadjuvanted vaccinated cattle, an outcome that is not desired and that argues against the use of the adjuvant in this stage of the response. This is most likely due to active B cell down-regulation that may occur if IgG prevails and dominates the antibody response in the circulation at the time of the final booster vaccination (Hjelm *et al.,* 2006). The ELISA

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results showed that the synthetic MAs mixture was inactive as an adjuvant. These results confirm what was seen with regards to the cellular response shown in the previous chapter. The method provided in the previous chapter describes the process used to get the MAs into a suitable solution to be used for vaccination. Due to the lipid property of the compounds, the solution has to be heated to extensive temperature which possibly degraded the Ag85A used in combination for the vaccines. As a result the degraded protein conformation may possibly have had an impact on both the cellular and antibody responses, with both of them being inactive and absent.

As mentioned in the results section it is clear that Ag85A combined with the positive control, MF59, produces the highest antibody body response as expected. This is seen in both the early response (rapid increase) and late response (steep decrease). These results suggest a possible feedback mechanism which was explored in the form of the comparative immunoglobulin isotyping ELISA (Hjelm *et al.,* 2006). Although the results did not show an overwhelming domination of IgG in the late sera of adjuvanted vaccinized cattle, the IgG/IgM ratio was more than 1, compared to smaller than 1 for the early stage sera at statistical significance ($P<0.05$). Thus, an IgM isotype mediated positive feedback mechanism may have been involved in the observed progressively increasing antibody response to Ag85A in MF59 adjuvanted vaccine treated cattle in the early immune response, while an IgG isotype mediated negative feedback mechanism may have been involved in the mechanism of action of down-regulation of antibody responses in the late stage of the vaccination programme, after the booster injection. Although it is the IgG isotype of antibody that confers the desired immune memory of vaccinated animals (Wack *et al.*, 2008), these should not be detectably present when the booster injection is administered.

Although shown to function well in influenza vaccines administered only once annually, there is no evidence relating to the effects that may arise due to decreased time periods between immunizations.

Studies into the effect of different adjuvants in mice have enabled scientists to learn more about the mode of action of MF59 (Mastelic *et al.,* 2010). Data obtained revealed MF59 to be a strong immune potentiator at the injection site by targeting muscle tissue directly, thereby resulting in the early recruitment of leukocytes. In particular, CD11b blood cells may differentiate into active local dendritic cells (DC) by facilitation of muscle cells that are activated by MF59 (Mosca *et al.,* 2008). This endows dual adjuvant properties for MF59 in that it combines antigen delivery with

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immune potentiator activity at the injection site (Mastelic *et al.*, 2010). These results demonstrate what may happen if a booster injection of MF59 adjuvanted vaccine is administered too soon after the primary vaccinations in cattle. It then appears to overturn the beneficial effects of the adjuvant, by actively down-regulating the secondary antibody response in cattle.

The down-regulation of specific antibody production is reminiscent of what happens in the process of desensitizing patients towards particular allergens, e.g. Honeybee venom. To effect desensitization, human patients receive repeated intradermal injections of the allergen in increasing doses in the microgram range at a weekly frequency to a maximum final dose of 100 µg, approximately ten-fold higher than the dose used in this cattle study. The total desensitization programme is typically condensed into a five week regime (Abkiewicz *et al.*, 1979; Goldberg and Confino-Cohen, 2010). The mechanism of attaining this state of "high zone immune tolerance" is not elaborated on, nor whether any advantage may be sought in administering the allergens in an adjuvanted way.

Of relevance to this study is that it seems possible to induce specific immune tolerance in individuals, when periods in between primary and booster injections of adjuvanted vaccines are too short. We therefore suggest that adjuvants for vaccines are preferably tested in large animals to determine the optimal doses in relation to the intervals between booster injections. Small experimental rodent animals are too short lived for experiments to test the optimal dose frequency of adjuvanted vaccines. In addition, it is not practical to extract blood on a weekly basis from mice over an extended period in order to determine the progressive up- and down-regulatory effects of positive and negative feedback in antibody response to vaccination. This study demonstrates the importance of these parameters by using cattle as experimental animals for testing a shorter than usual time period between primary and booster vaccinations with an adjuvanted vaccine, showing both the advantageous antibody up regulation after primary vaccinations and the danger of specific antibody down-regulation that occurs when the booster injection is given too early after the primary injections.

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3.5. Conclusion

Although MF59 is an established vaccine adjuvant that is already in use in influenza vaccination, knowledge is lacking surrounding the ideal frequency of immunizations with this adjuvant. The research conducted above provides some insight into the effects that may take place when more than two MF59 adjuvanted vaccinations are administered. As a result in this chapter we suggest reasoning as to why these adjuvanted influenza vaccines are only provided once annually. Future research into this field should provide details of processes occurring in the cells when vaccinations exceed the required amount thereby highlighting the adverse effects that may accompany immunization.

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4. Concluding discussion

Adjuvants are generally used in combination with vaccines to elicit an effective, longer- lasting immune response with less antigen and fewer vaccinations (Gupta and Siber, 1995; Schijns, 2000). The use of adjuvants was invented in the 1920s and is still used today to aid vaccines against a variety of diseases. Freund's complete adjuvant (FCA) was one of the first formulations that relied on the immune steering properties provided by whole, inactivated and dried mycobacteria (Sadelain *et al.*, 1990). Although successful as an adjuvant in mice and many other mammals, FCA caused adverse local inflammatory side effects, making it unfit for use in humans and eventually also not for commercial veterinary vaccines. In early animal vaccine research, an initial vaccination with FCA was followed up by booster vaccinations of the antigen with Freund's incomplete adjuvant (FIA), which is just the water in oil emulsion without the mycobacterial components (Billiau and Matthys, 2001). This served to reduce the inflammatory side effects. However, although it did have much lesser inflammatory side-effects, the discomfort caused by FIA was still of such an intensity that its use was terminated and is now even considered unethical to use in experimental animals.

The aluminum salt based adjuvants, collectively known as Alum, have been used successfully in a number of vaccines (Mbow *et al.*, 2010; Reed *et al.*, 2009). Although this group of adjuvants has a well-documented safety record (Mbow *et al.*, 2010; Reed *et al.*, 2009), there are a few short comings too. Alum has been shown to promote mainly Th2 cell responses thereby inducing mainly humoral immunity and making it a poor initiator of T-cell responses (Brewer *et al.*, 1999; Mbow *et al.*, 2010). Due to this, Alum is unable to facilitate T helper (Th) 1 and cytotoxic T-lymphocyte responses, which makes their use not suitable in vaccines aiming to control or eliminate intracellular pathogens (Edelman, 2002). Lastly, Alum loses its potency upon freezing, thus eliminating this as a method involving long term storage of such adjuvanted vaccines.

A prime example of an improved, safe and potent adjuvant is MF59, an oil-in-water emulsion adjuvant that contains a low amount of biodegradable squalene oil, making use of two non-ionic surfactants for stabilization and a low ionic citrate buffer as the continuous phase (O'Hagan *et al.*, 2007). It has demonstrated its ability in a number of licensed vaccine applications, including human anti-influenza vaccines. Although MF59 has displayed effective activity when used in influenza vaccines, this adjuvant has been clouded with controversy through its use by the US Army for a number of years. Research into this revealed that vaccines containing varying

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amounts of squalene oil were used on soldiers, resulting in a number of them experiencing toxic effects, which in many cases ultimately resulted in death (Matsumoto, 2005). It is evident from this that adjuvants may sometimes display undesirable side-effects. It is important to define for each adjuvant the type of immunity they are capable of promoting, i.e. either humoral or cellular activity, depending on what is known to provide protection against the particular disease. As a result the search for new adjuvants is a continuous process.

Mycolic acids (MAs) are complex lipid molecules that are found in the cell wall of mycobacteria and in particular in *M. tuberculosis* (Minnikin and Polgar, 1966; Barry III *et al.*, 1998; Colston, 1996). Synthetic tuberculous, mycobacterial MAs can now be chemically produced in the laboratory (Al Dulayymi *et al*., 2005; Al Dulayymi *et al*., 2006; Al Dulayymi *et al*., 2007; Verschoor *et al.*, 2012). These compounds appeared to be representative of the original mycolic acids found in the organism *M. tuberculosis*. Research conducted on natural MA, purified from the cell wall of *M. tuberculosis*, has illustrated its immune steering properties (Korf *et al.*, 2005; Korf *et al.*, 2006). Further research, conducted in Belgium, saw the different chemically synthetic isomers of the different MA classes, and explored these for their immune steering properties that may attribute them with a protective immune function when used in vaccines as adjuvants (Vander Beken *et al.*, 2011). These MA classes, administered to mice through liposomes, revealed for example that *cis*-methoxy MA and *cis*-keto MA were both able to elicit an inflammatory response, with that of the *cis*-methoxy MA being more effective than the *cis*-keto MA. It was also found that the isomer of keto MA in the *trans* configuration illustrated an anti-inflammatory response, which could inhibit the inflammatory response of the *cis*-keto MA isomer (Vander Beken *et al.*, 2011). The potential of these compounds as vaccine adjuvants is the focus of this research.

The use of some of these stereo-controlled, chemically synthetic MAs was first explored in rabbits to test the safety of their use in mammals. Six New Zealand White rabbits were used to evaluate and compare five different synthetic MAs to a commercial mixture of natural MAs. The results from these experiments indicated that the use of these compounds administered in liposomes produced no inflammatory side effects. In this way, they were safer to use than when the compounds were delivered in microcapsules or as an emulsion with the positive FIA control. This led to the development of the current project where the use of chemically synthetic MAs as adjuvant for an Ag85A protein subunit vaccine was investigated for their safety, type of immunity and immune memory towards Ag85A in cattle.

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Twenty male calves of ages five to six months were intradermally injected with an anti-TB antigen, Antigen 85A (Ag85A), in combination with different synthetic MA adjuvants, while MF59 and phosphate buffered saline (PBS) served as a positive and negative adjuvant control, respectively. Ag85A was provided as a PBS solution at the maximum protein concentration allowed by its water solubility. This could be added to the MF59 adjuvant at room temperature and vortexed to prepare the oil in water emulsion, but required heating and sonication to emulsify it with the waxy MA in liposomes at the equivalent protein concentration. The Ag85A protein antigen was therefore probably heat denatured in the MA adjuvanted vaccines, allowing only their T cell responses against the protein antigen to be assessed as a reasonable indicator of the adjuvant qualities. Antibody production requires the intact conformation of the injected protein antigen, while T cells respond to peptides that are processed from the protein antigen by the antigen presenting macrophage or dendritocyte. The T cell response is therefore usually not affected by denaturation of the antigen. None of the animals revealed severe inflammatory side-reactions at the site of injection. Blood samples were collected weekly from all the animals for the duration of the experiment. These samples were then used for the isolation of peripheral blood mononuclear cells (PBMCs) and serum, and stored until further use. This preparatory part of the work falls outside the scope of this dissertation, but is an important part of the immediate project history to recall for understanding the research aims and outcomes. For this MSc study the stored samples were used to perform T-cell proliferation assays and antibody responses against Ag85A to determine the vaccine adjuvant potential of synthetic MAs.

T-cell proliferation assays on the cattle blood used to determine the effect that MAs would have on the cellular response were done after *in vitro* culture of the PBMCs, during which they were exposed to either Concavalin A (ConA; positive control), Ag85A (experiment) or non-stimulated (negative control). With positive and negative controls responding as expected for each sample analysed by flow cytometry, the results showed no induced proliferation in any of the samples upon in vitro stimulation with Ag85A. It was concluded from these assays that the concentration of Ag85A used with either MF59 or the synthetic MA mix as adjuvants may have been too low to detect an effect on the cellular immune response with the flow cytometric technology used. The lack of sensitivity of the assay could also have been caused by the bio reagents used in the flow cytometry, as fluorescently labelled antibodies specific for bovine cells are not generally available. As a result, cross-reactive antibodies to the correlating cell markers of other animal species had to be used in most cases in this study, which may have resulted in

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decreased sensitivity of detection of T cell proliferation.

Whereas the MF59 adjuvanted primary injections of Ag85A gave a pronounced antibody response in cattle, no such response was found at all in the MA mix adjuvanted injections in which the Ag85A antigen was heat denatured to enable making it up to the same concentration in the vaccine as with the MF59 positive control. This demonstrated the importance of intact protein antigen conformation to elicit active antibodies.

The ELISA results of anti-Ag85A antibodies in the MF59 adjuvanted vaccinated cattle revealed the serious impact that timing can have in administering the adjuvanted vaccine boosters. Here it was seen that administering the booster too soon may have toxic effects rather than increasing the antibody levels. Although shown to function well in influenza vaccines administered only once annually, there is no evidence relating to the effects that may arise due to decreased time periods between immunizations. The results of this study illustrate possible undesired effects that short periods between adjuvanted vaccinations may have. These findings may help guide the current design of ethics formulation for the trialling of adjuvanted vaccines with experimental evidence, in support of the mission of the WHO's Global Vaccine Safety Initiative (WHO, 2012).

Trials involving the use of MF59 in humans have shown that two primary doses of protein antigen in the adjuvanted vaccine separated by three weeks are sufficient to obtain an antibody response, but needed to be followed up by a booster injection either six months or a year later to provide significant immune protection (Banzhoff *et al.,* 2009; Hatz *et al.,* 2012). The current study followed a similar antigen dosage plan given as two primary injections separated by two weeks, but was then followed by a booster injection two and a half months later – half the time interval that was reported for trials with humans. This experimental set-up illustrated that booster vaccines, which are administered too soon after primary vaccinations, may be counteractive towards inducing long term immune memory against a pathogen related antigen. The increasing tendency of antibody titres in the three weeks following the two primary injections was more pronounced in the MF59 adjuvanted vaccinated cattle, compared to non-adjuvanted vaccinated animals. This is typical when IgM dominates (Hjelm *et al.,* 2006) and is a desirable outcome in support of the use of the adjuvant. In the late stage, however, the undesirable downward trend of antibody titres over three weeks following the booster injection is most pronounced in MF59 adjuvanted vaccinated cattle, an outcome that, in this specific set-up argues against the use of the adjuvant. This is most likely due to active B cell down-regulation that may occur if IgG prevails

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and dominates the antibody response in the circulation at the time of booster vaccination (Hjelm *et al.,* 2006). Although it is IgG that confers the desired immune memory of vaccinated animals (Wack *et al.,* 2008), this should probably not be detectably prevalent when the booster injection is administered.

Although in this study the use of the liposome carrier system was not ideal for the preparation of MA vaccines, a different carrier system in the future may prove to be better. A suitable carrier is probably required to integrate intact, un-denatured poorly soluble protein Ag85A and MA in sufficient amounts and concentration to ensure a measurable antibody and cellular response. One such system that may be considered is the use of Poly (lactic-co glycolic acid) (PLGA) nanocapsules as delivery vehicles. These particles have already proven to be suitable for use in combination with synthetic MAs as shown in the rabbit experiment in chapter 2. Research into the use of these PLGA nanocapsules has revealed that the different size particles are responsible for eliciting different responses (Akagi *et al.,* 2012). If one aims to elicit a cellular immune response, smaller particles (<200 nm) would be preferred as it has already been shown that these are specifically taken up by antigen presenting macrophages only (Akagi *et al.,* 2012). Alternatively one may use big particles (2-8 µm) to produce an antibody response, as at this size range they are too big to be taken up by macrophages and will gradually release their antigen contents as they degrade (Akagi *et al.,* 2012). This in turn will stimulate B cells to produce antibodies.

Another idea to be considered in future research on the adjuvant properties of MAs is to explore a method that improves the water solubility of recombinantly expressed Ag85A. The poor solubility of this protein forced the preparators of this project into using vaccine composition method that ultimately denatured the Ag85A in the MA adjuvanted Ag85A vaccines. A better soluble Ag85A may allow it to be mixed with prepared and cooled suspensions of the MA at the desired concentration. Alternatively, PLGA particles may serve as a better carrier for both the lipid and protein components, allowing them to simply be mixed, vortexed and injected.

The major finding of this study was the demonstration of how counterproductive booster vaccination is when the period between primary and booster injections of adjuvanted vaccines are too short. It is suggested here that new adjuvants for vaccines are first tested in large animals to determine the optimal vaccine dose and the optimal interval between primary vaccinations and booster injections, evaluated by following the antibody responses over several weeks after the

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individual injections and booster. It has been illustrated in the literature that the optimal vaccine dose and optimal interval between primary and booster vaccinations are interrelated (Benner *et al*., 1974). The relationship between these two parameters would be worthwhile investigating in the search for new vaccine adjuvants. Small experimental rodent animals are too short lived for experiments to test the optimal dose frequency of adjuvanted vaccines. In addition, it is not practical to extract blood on a weekly basis from mice over an extended period in order to determine the progressive up- and down-regulatory effects of positive and negative feedback in the antibody response to vaccination. This study served to demonstrate the importance of these parameters by using cattle as experimental animals for testing a shorter than usual time period between primary and booster vaccinations with an adjuvanted vaccine, showing both the advantageous antibody up-regulation after primary vaccinations and the counterproductive specific antibody down-regulation that occurs when the booster injection is given too early after the primary injections.

Current data on human trials of MF59 adjuvanted influenza vaccines relate to annual injections of the vaccine in over-60 year old individuals only. As a result there is still a huge void of knowledge around the optimal frequency of vaccination and time periods between vaccinations for trialling of adjuvanted vaccines in an ethical way. This study provides insight into the possible undesired effects of short periods between adjuvanted vaccinations. Whereas the conditions did not yet allow the proper assessment of synthetic MAs as adjuvants in this study, experimental evidence has been provided to help guide the design of vaccination protocols for future clinical trials of adjuvanted vaccines, in support of the mission of the WHO's Global Vaccine Safety Initiative (WHO, 2012).

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Addendum A

Vaccine preparations for rabbit study

Microcapsule vaccine preparation (De Koker *et al*., 2010)

Mycolic acids (0.5 mg), either individual synthetic derivatives (cis-methoxy-MA; cis-keto-MA; trans-keto-MA; all from School of Chemistry, University of Bangor, UK; See addendum B for structures) or a natural MA mixture (Sigma) were dissolved in chloroform by heating on a heating block for 30 seconds at 90°C and vortexing until the solution was clear. These MAs were then mixed with DOTAP: DOPC at a ratio of 3:7 to form a lipid mixture. The lipid mixtures were well dried before 200 µl sterile endotoxin free PBS was added to each. These MA mixtures were then vortexed shortly, sonicated for 30 min at 40°C, and vortexed again until a milky emulsion (free of particles) was formed.

A quantity of 200 µl capsules (5X) were diluted with 800 µl sterile endotoxin free PBS and 200 ul liposome preparation simultaneously. This newly formed solution was then vortexed for 1 min and then incubated for 1 hour at RT while gently shaking.

Following incubation the solution with centrifuged at 1000 x g for 10 min. Supernatants were then discarded and pellets resuspended in 200 µl sterile endotoxin free PBS to form the vaccine solution.

Liposome vaccine preparation (Lemmer, 2010)

MA/PC liposomes were prepared in autoclaved glass vials (Separations Pty Ltd) with screw caps (Chromatography Research Supplies Inc.). Synthetic MAs (School of Chemistry, University of Bangor, UK; See addendum B for structures) were alliquoted in 0.3 mg quantities into glass vials either as such (cis-methoxy-MA; cis-keto-MA; trans-keto-MA) or a natural MA mixture (Sigma). These MAs (individual synthetic and natural mix) were dissolved in chloroform (Merck) by heating on a heating block for 30 seconds at 90°C and vortexing until the solution was clear. The clear solutions were alliquoted in 50 µg MA quantities to a new set of glass vials and put onto the heat block for the chloroform to evaporate.

Phosphatidylcholine (PC) (Sigma) was dissolved in chloroform at a concentration of a 100 mg/ml of which 90 µl was added to each of the above MA samples (50 ug/dose). This MA/PC solution was dried on a heating block (90°C) under nitrogen gas. Saline (0.9% NaCl) was added (2 ml) and the solution vortexed. It was placed on the heating block for 20 minutes and was vortexed every 5 minutes for the duration of this period. At the end of 20 minutes the solution was vortexed for 1 minute, after which it was sonicated using a Virsonic sonifier (Virtis, Gardiner, N.Y.) for 2 minutes, 30 cycles at an output of 5. The MA/PC liposomes were aliquoted into glass vials (200 µl per vial) and placed in the -70 °C freezer for 1 hour. The liposomes were lyophilised overnight and stored in the -70 °C freezer until use. A volume of 60 µl of PBS pH 7.2 (Invitrogen) was

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added to vials containing methoxy-MA/PC liposomes, keto-MA/PC liposomes and synthetic MA mix/PC liposomes. These vials were placed onto a heating block for 30 minutes at 90 C, vortexed for 1 minute and sonicated for 1.5 minutes at 30 cycles to form the vaccine.

PLGA vaccine preparation (Lemmer, 2010)

Poly DL, lactic-co-glycolic acid (PLGA; Sigma Chemical co., St Louis, USA) 50:50 (MW:45000-75000) was dissolved in 6 ml of dichloromethane (DCM) at a concentration of 1.25% weight/volume (w/v). MAs, either individual synthetic derivatives (cis-methoxy-MA; cis-keto-MA; trans-keto-MA; all from School of Chemistry, University of Bangor, UK; See addendum B for structures) or a natural MA mixture (Sigma) were dissolved in dichloromethane (Merck, Germany) and added whilst stirring. For the first water in oil (w/o) emulsion, aqueous PBS (pH 7.4, 2 ml) was added to the mixture and homogenization was done at 5000 rpm for 3 min. The second water in oil in water (w/o/w) emulsion was formed by homogenization at 8000 rpm for 3 min. The second emulsion formed was stirred at 500 rpm overnight at atmospheric pressure to evaporate the organic solvent. The PLGA nanoparticles were recovered by centrifugation at 19000 rpm for 15 min. The resulting particles were freeze dried by lyophilisation in a Vitrus Benchtop freeze dryer. Freeze dried MA containing nanoparticle were then added to PBS to form the final vaccine.

Characterisation of carriers and vaccine preparation

All particles were characterised before use. Each prepared vaccine was made up to a volume of 10 µl with MA at its highest load. All particles contained MA at the same concentration of 250 ug/ml MIE.

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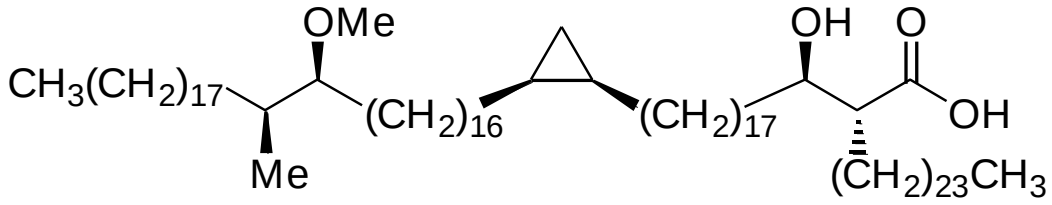
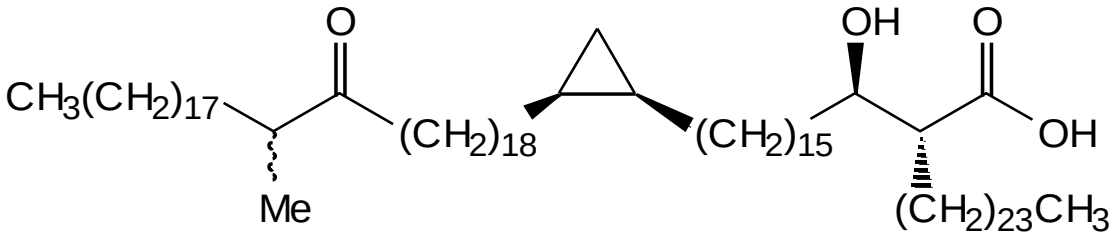
Addendum B

Synthetic mycolic acid structures

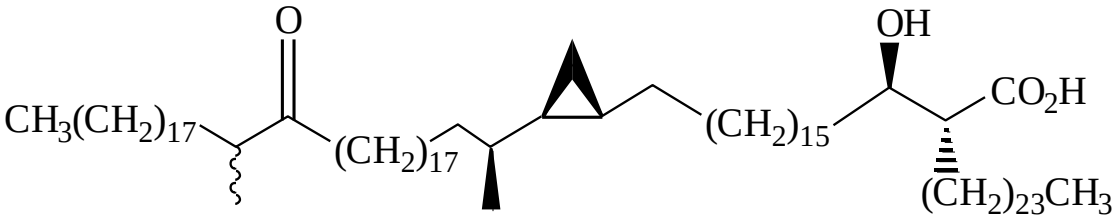
cis-Methoxy MA:



cis-Keto MA:



trans-Keto MA:



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