The Effects of Lipids from *Mycobacterium bovis* on Bovine Innate and Acquired Immune Responses

By

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Declaration

The work presented in this thesis was carried out in the Bovine TB Research Team at the Animal Health and Veterinary Laboratories Agency KT15 3NB, during the period September 2009 to September 2014. The work in this thesis is original except where acknowledged by references.

No portion of the work is being, or has been submitted for a degree, diploma or any other qualification at any other University.

Published Work Declaration

This thesis contains work which has been accepted to and / or published in peer - reviewed journals. Any publications arising from the work presented in this thesis were written and prepared by Christopher Pirson who is listed as the publications primary author and the contributions of other authors to the text in this thesis are not substantial.

Thesis content which is similar to that contained in associated peer - reviewed publications is listed below:

Pirson *et al*., (2012) "**Differential effects of *Mycobacterium bovis* - derived polar and apolar lipid fractions on bovine innate immune cells**" Veterinary Research **43**:54

- Chapter One: pages 6, 10 and 31
- Chapter Two sections:
    - Preparation of Bacterial Isolates for Lipid Extraction
    - Extraction of Crude Free Mycobacterial Lipids
    - Analysis of Lipid Fractions by 2D Thin Layer Chromatography
    - Preparation of Lipid Antigen Suspensions
    - Uninfected Cattle
    - Isolation of Bovine PBMC from Whole Blood
    - Isolation of $CD14^+$ Monocytes from Bovine PBMC
    - Generation of Bovine Cultured Monocytes and MDDC
    - Multiplex Measurement of Cytokine Production
    - Innate Cell Labelling & Analysis by Flow Cytometry
    - Mixed Lymphocyte Reaction
- Chapter Three, Discussion: page 70
- Chapter Four sections:
    - Results
    - Discussion

Pirson *et al*., (2015) "**Highly purified mycobacterial phosphatidylinositol mannosides drive cell mediated responses and activate NKT cells in cattle**" Clinical and Vaccine Immunology **22**:2

- Chapter Two sections:
    - Preparation of Lipid Antigen Suspensions
    - *M. bovis* Infected Cattle
    - Isolation of Bovine PBMC from Whole Blood
    - Measurement of IFNγ by Bovigam™ ELISA
    - Lymphocyte Transformation Assay
    - Lymphocyte Labelling & Analysis by Flow Cytometry
- Chapter Six: pages 130, 131 and 137 - 138

Signed:

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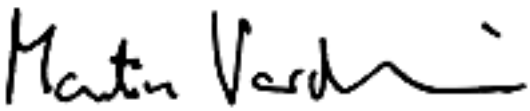

H. M. Vordermeier

Abstract

The interaction between the host and the pathogen is critical in defining the outcome of an infection. For cattle with bovine tuberculosis (BTB), this interaction is likely to occur between antigen presenting cells in the lung and the lipid rich surface of the causative agent *Mycobacterium bovis* (*M. bovis*). It is well documented that lipid molecules from mycobacteria are capable of modulating immune responses however previous work has made use of model animal systems or lipids from avirulent bacteria. The aim of this study was to extract lipids from virulent *M. bovis* and assess any immunomodulatory ability of these molecules in cattle with a view to aiding in the development of control measures for BTB.

To this end, lipids were extracted from *M. bovis* AF 2122/97 and AN5 and the fractions characterised. Upon thin layer chromatography analysis, polar and apolar fractions from both bacterial strains were found to be broadly similar in their lipid constitution although quantitative differences were noted. Lipopeptide was also identified in both polar fractions. Stimulation of bovine antigen presenting cells with the lipid fractions showed polar lipids mediated increases in IL - 10 and IL - 12 production and reductions in cell surface expression of MHCII and CD1b. Further investigation of the polar lipid fraction was performed by subfractionation but no individual lipid could be found responsible for the responses of antigen presenting cells to these subfractions.

The ability of the polar and apolar lipid fractions to be recognised by cells of the adaptive immune system was assessed and the polar fraction was found to drive production of IFNγ and strong proliferation of bovine lymphocytes. The role of lipopeptide in the polar fraction was evaluated by enzymatic degradation with Proteinase K and blockade of either MHCII or CD1. While lipopeptide was found to play a role in the generation of lymphocyte responses, these treatments did not abrogate the effects completely suggesting a lipid mediated component as well. Screening of highly purified individual lipid molecules led to the selection of one molecule ($AcPIM_6$), which was found to be capable of driving antigen specific proliferation of NKT cells.

Dedication

For the cows…

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Table of Contents

List of Figures

List of Tables

Publications Associated with this Thesis

C. Pirson, G. J. Jones, S. Steinbach, G. S. Besra & H. M. Vordermeier, (2012). "**Differential effects of *Mycobacterium bovis* - derived polar and apolar lipid fractions on bovine innate immune cells**" Veterinary Research **43** : 54

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Posters Associated with this Thesis

VI International *M. bovis* Conference

Differential effects of *Mycobacterium bovis* - derived polar and apolar lipid fractions on bovine innate immune cells

List of Abbreviations

BTB	Bovine Tuberculosis
CIITA	Class II Major Histocompatibility Complex Transactivator
CD	Cluster of Differentiation
CFP - 10	Culture Filtrate Protein - 10 kDa
CM	Cultured Monocyte
CR	Complement Receptor
DAT	Diacyl Trehalose
DC	Dendritic Cell
DC - SIGN	Dendritic Cell - Specific Intercellular Adhesion Molecule - 3 Grabbing Nonintegrin
DPG	Diphosphatidyl Glycerol
DPH	1, 6 - diphenyl - 1, 3, 5 - hexatriene
ESAT - 6	Early Secreted Antigenic Target 6 kDa
GB	Great Britain
GM - CSF	Granulocyte - Macrophage Colony Stimulating Factor
IFN	Interferon
IL	Interleukin
kDa	Kilodalton
LAM	Lipoarabinomannan
ManLAM	Mannose Capped Lipoarabinomannan
MAPK	Mitogen - Activated Protein Kinase
MDDC	Monocyte Derived Dendritic Cell
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MMG	Monomycolyl Glycerol
MPA	Molybdophosphoric Acid
MQ	Menaquinone
MR	Mannose Receptor
NFκB	Nuclear Factor kappa - Light Chain Enhancer of Activated B Cells
NIRViD	Near Infra - Red Viability Dye
NLR	Nucleotide Oligomerisation Domain - Like Receptor
NOD	Nucleotide Oligomerisation Domain
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PDIM	Phthiocerol Dimycocerosate

PE	Phosphatidyl Ethanolamine
PGL	Phenolic Glycolipid
PI	Phosphatidylinositol
PIM	Phosphatidylinositol Mannoside
PPD - A	Purified Protein Derivative - Avian
PPD - B	Purified Protein Derivative - Bovine
PRR	Patern Recognition Receptor
PWM	Pokeweed Mitogen
R - PE	R - Phycoerythrin
RLU	Relative Light Units
RPMI	Roswell Park Memorial Institute
SGL	Sulphoglycolipid
SL	Sulpholipid
TAG	Triacyl Glycerol
TCR	T Cell Receptor
TDB	Trehalose Dibehenate
TDM	Trehalose Dimycolate
Th	T Helper
TLC	The Layer Chromatography
TLR	Toll - Like Receptor
TMM	Trehalose Monomycolate
TNF	Tumour Necrosis Factor
ViViD	Violet Viability Dye

Chapter One

Introduction

The Mycobacteria

Mycobacteria are members of the Actinomycete branch of the Gram - positive bacteria. Other Actinomycete genera include the *Nocardia*, *Corynebacterium* and *Streptomyces*[1]. The name Mycobacterium stems from the mould-like appearance of strains when grown as pellicles on nutrient broth. There are now at least 49 recognised species of mycobacteria[1], however most of the energies associated with research into mycobacteria are devoted to perhaps the best known members of the genus: *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis (TB) in humans; *Mycobacterium bovis* (*M. bovis*), the causative agent of tuberculosis in cattle (and a range of mammalian species, including humans) and *Mycobacterium leprae*, the agent responsible for the disease of leprosy. The mycobacteria have a genome of approximately 4.5 million base pairs which contains a high G+C content (65.6%) and around 4,000 genes, many of which encode enzymes involved in lipogenesis, lipolysis and lipid transport[2]. In fact, lipids may be responsible for one of the most characteristic properties of the mycobacteria; that of acid - fastness[3]. The ability to resist acid mediated decolourisation after red fuchsin is not a property unique to mycobacteria but

was critical in the first identification of the bacilli[4, 5]. Originally using Bismarck brown and methylene blue, Robert Koch noted that "*Under the microscope all the constituents of animal tissue, that is, the cell nuclei and their products of disintegration appear brown, while the tubercle bacilli, on the other hand, stain a beautiful blue*"[4, 5]. This technique was modified by Ehrlich[6] who used red fuchsin and a mineral acid but was further refined by the eponymous Ziehl (who optimised the acid decolourisation)[7] and Neelsen (who combined Ehrlich's red stain with Ziehl's acid)[8].

The *Mycobacterium tuberculosis* Complex

The isolation of the tubercle bacillus in 1882 by Robert Koch[4, 5] was one of the single most significant points in the history of microbiology, as it was also the first isolation of any micro - organism in pure culture. However, it soon became apparent that there were characteristic differences between the strains isolated from man, cattle and birds. During the allocation of species in the 1890s, the mammalian tubercle bacillus was named *M. tuberculosis* specifically to reflect its significance within the genus[9]. Around the same time it was noted that there were distinct morphological and cultural differences between human and bovine isolates and that the bovine strains were more virulent in rabbits[10].

In 1946, a further mammalian derivative of the tubercle bacillus was isolated from voles[11] and became known as *Mycobacterium microti*[12]. *M. microti* is primarily pathogenic for rodents and demonstrates little or no pathogenicity in man or domestic animals.

Further reclassification of the genus occurred in the 1960's, when it was discovered that strains initially thought to be *M. tuberculosis* isolated from patients in tropical Africa resembled a heterogeneous group of isolates. These strains were phenotypically different from *M. tuberculosis* and *M. bovis* and were named *Mycobacterium africanum*[13].

Due to this close relationship, *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. microti* are known as the *M. tuberculosis* complex. A recent addition to the complex was a strain isolated from human TB patients in Africa which proved to have a distinct phenotype, different from the other members of the complex. This strain has been named *Mycobacterium canetti*[14]. Other members of the complex include *M. caprae*, *M. pinnipedii*, *M. suricattae*, and *M. mung*.

Mycobacterium tuberculosis

Tuberculosis (TB) has been referred to as "*one of a few severe communicable diseases that may be described as true pestilences of mankind*"[15]. Often described as an ancient disease, *M. tuberculosis* has been detected in the remains of Bison dating from 18,000 years ago[16] as well as in Neolithic human remains[17] and as Potts disease in the spines of Egyptian mummies[18].

Today the tubercle bacillus continues to claim more lives than any other single infectious agent with an estimated 8.7 million new cases of TB and 1.4 million deaths in 2011 alone[19]. In recent years, the incidence of TB has risen drastically in both industrialised

and developing countries. Until 60 years ago, there were no anti - TB drugs, as was the case for virtually all infectious diseases, yet in a recent survey by the World Health Organisation (WHO), strains of *M. tuberculosis* resistant to at least a single anti - tuberculous agent were documented in every country surveyed[20]. This is compounded by the emergence of strains resistant to both primary anti - TB drugs (isoniazid and rifampicin), known as multi drug resistant TB (MDR - TB).

M. tuberculosis displays many characteristic features such as its slow growth, dormancy, intracellular lifecycle and pathogenesis, and a complex cell envelope. With its generation time of approximately 24 hours in synthetic medium or infected animals it is a difficult organism to study and its long doubling time also contributes to the chronic nature of the disease. This slow growth may also provide a selective advantage; where other organisms outgrow their intracellular environment and kill their habitat, mycobacteria slow their growth and convert into a so called “dormant” phase, thereby further limiting their chances of destroying their hosts[21, 22].

M. tuberculosis is spread predominantly by the aerosol route, where droplet nuclei containing 3 to 5 bacilli in a particle of <5 μm are inhaled and gain access to the alveoli[23-25]. Once inside the alveoli, the bacilli are engulfed by alveolar macrophages which attempt to kill the organism via the respiratory burst and phagolysosome fusion[26, 27]. Virulent mycobacteria remain within phagosomes which fail to fuse with lysosomes, even though mycobacterial phagosomes are almost identical to other early phagosomes[28], and thereby avoid lysosomal degradation. This ability is dependent on viable bacilli, as killed cells are rapidly destroyed[29, 30]. By remaining in so - called “mycobacterial

phagosomes" the bacilli avoid destruction and also hinder the induction of adaptive immune responses[31, 32].

Mycobacterium bovis

In his speech upon receiving the Nobel Prize in 1901, Emil von Behring stated "*as you know, tuberculosis in cattle is one of the most damaging infectious diseases to affect agriculture*"[33]. Bovine tuberculosis (BTB) caused by *M. bovis* is a zoonotic disease that has posed major animal health problems for the farming industry in England and Wales since the 1930s[34] and affects cattle and other mammals worldwide.

M. bovis causes a disease in man which is clinically indistinguishable from *M. tuberculosis* infection and it was thought previously that tuberculosis in domestic animals appeared prior to its recognition in humans[35] and that *M. tuberculosis* was derived from *M. bovis*[36]. However, thanks to the completion of the genomes of *M. tuberculosis*[2] and *M. bovis*[37], it has been shown that the pattern of deletions amongst members of the *M. tuberculosis* complex suggests a derivation from a common ancestral organism[38, 39].

Many studies were performed comparing human and bovine strains, fuelled by the incorrect statement "*the human subject is immune against infection with bovine bacilli or is so slightly susceptible that it is not necessary to take any steps to counteract risk of infection*" made by Koch to the British Congress on Tuberculosis in 1901[40]. This supposition was arrived at due to the relatively non - pathogenic nature of human tubercle isolates in cattle and Koch assumed the strains were species - specific. The most

fervent voice of dissent came from the veterinary profession and the British Royal Commission was appointed to study the issue. Research was funded by the Commission between 1901 and 1911 and provided irrefutable proof that humans could be infected by the bovine tubercle bacillus[41]. The bovine bacilli became known as *M. bovis*; although this name only became recognised in the literature considerably more recently[42].

The zoonotic importance of *M. bovis* cannot be underestimated. As far back as 1810, it was reported that the incidence of tuberculous cervical lymphadenitis (also known as scrofula) was higher in those children who were fed cow's milk rather than breast milk[43]. The importance of *M. bovis* infection in humans was further highlighted in 1927 when a study of 906 pulmonary and 202 extra - pulmonary cases of tuberculosis revealed that 67% of extra - pulmonary tuberculosis was caused by *M. bovis*[44]. This zoonotic potential is still of great concern today. Consumption of raw or unpasteurised animal products, or direct contact with infected animals in slaughterhouses, plays a large role in zoonotic *M. bovis* infection of humans in Africa and South America[45-47]. Yet this is not just a problem associated with less developed countries; recently Rodwell *et al*. have shown that as much as 45% of all culture positive TB in children from Hispanic populations in San Diego was caused by *M. bovis*[48].

Control of BTB In Great Britain

The control programme for BTB in Great Britain (GB) is a test and slaughter policy which relies on the ability to identify *M. bovis* infected animals and their subsequent removal and slaughter. This policy is in accordance with European Law which specifies the requirements for testing and diagnosis of BTB[49]. Previously, regular testing of cattle occurred at intervals based on the prevalence of BTB in their geographical location (parish testing intervals) which were determined each year based on the level of BTB in the parish in the previous 6 years. However, on the 1st of January 2013, a new system was introduced which replaced the use of parishes with county borders and split the country into high and low risk areas based on disease prevalence. Animals within the high risk area are tested annually whilst those in the low risk area are tested every four years. Dividing the high and low risk areas is the 'edge' area, where BTB is not endemic but animals are tested annually to enable any early detection of spread from the high risk area. The high risk, low risk and edge areas are shown in figure 1:1.

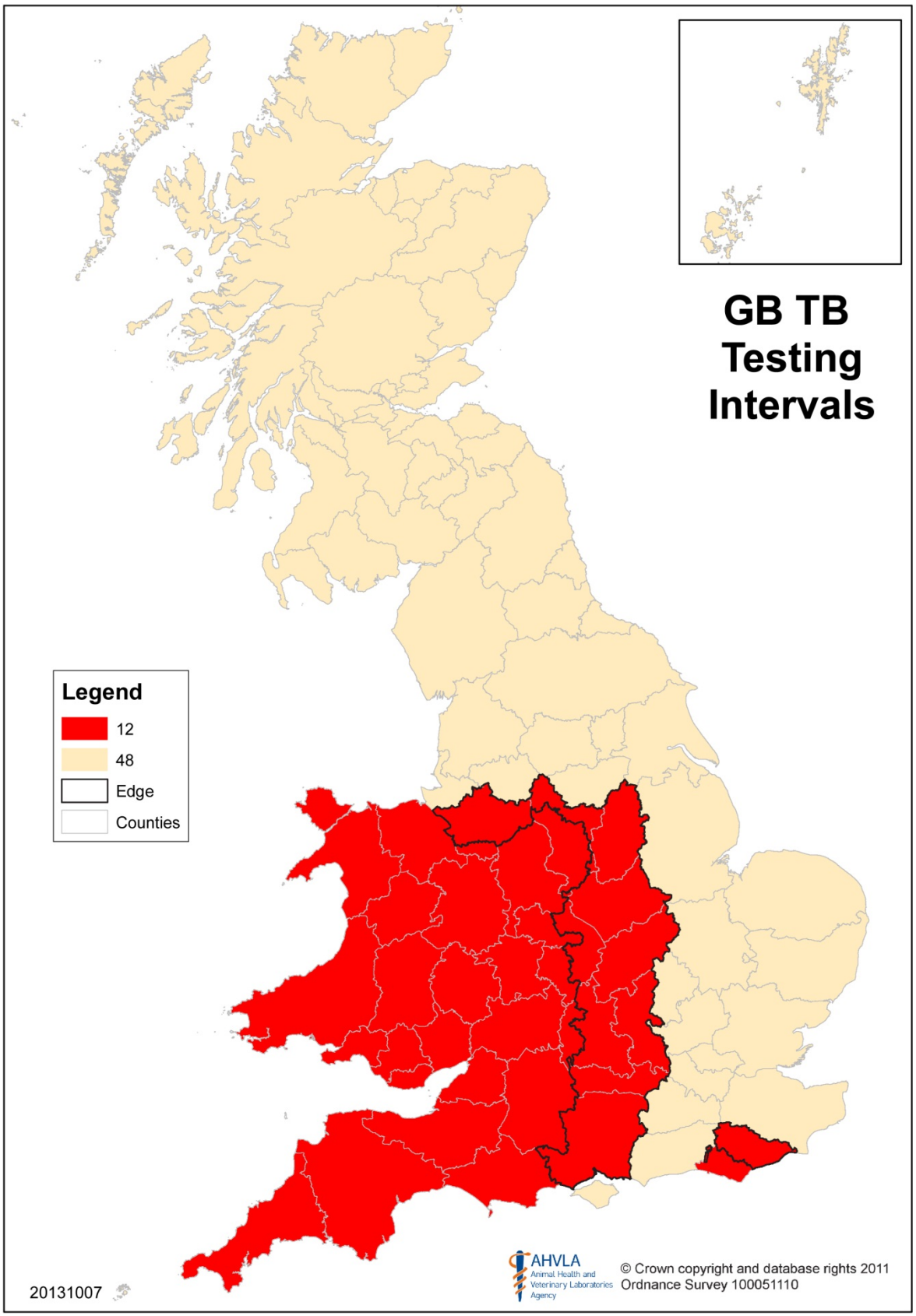

Figure 1:1 - BTB testing areas shown for the year 2014
Available from http://ahvla.defra.gov.uk/documents/bovine-tb/pti-map.pdf.

Infection is identified using the single intradermal comparative tuberculin test (SICTT) which enables the veterinarian to measure the delayed type hypersensitivity (DTH) response to an intradermal injection of a crude preparation of mycobacterial antigens known as purified protein derivative (PPD) or tuberculin.

In the early 1890's, Bernhard Bang introduced the intradermal test as a diagnostic tool in the control of BTB in Denmark. The 'Bang method' of testing consisted of repetitive six-monthly use of the intradermal assay combined with physical separation of test - positive and test - negative cows, and only culling cattle with "*tuberculosis of the udder*"[50]. Bang also introduced the pasteurisation of milk, buttermilk, and whey, to prevent transmission of BTB via milk and milk products to calves long before pasteurisation of these products for human consumption became standard[50]. Following reports on the achievements of the Bang method, it was accepted worldwide as the major tool in the control of BTB and is still the basis of all control programs for BTB today.

It was Koch who developed what is now known as 'old' tuberculin, the reagent used as the stimulating antigen in the early intradermal tests. While attempting to isolate the "*active principle of tuberculin*"[51] for use as a treatment for human TB infection, Koch subcutaneously injected heat killed samples of cultures into guinea pigs and noted the presence of a characteristic skin reaction 24 - 48 hours later[52, 53]. Despite not recognising the importance of this reaction as a diagnostic tool, he introduced this method to assess the potency of tuberculin preparations.

In the 1930's a major improvement in the quality of the tuberculins, replacing Koch's Old Tuberculin, was achieved by the work of Florence Seibert. The use of a synthetic medium

and precipitation methods, facilitated a more reproducible and a large scale production leading to the PPD reagents used today[54].

The advent of a compulsory skin test, after the voluntary Attested Herd Scheme of the 1950's and 1960's, coupled with compulsory slaughter of all test positive animals, resulted in a significant drop in the levels of BTB in GB from an estimated 40% of all cattle infected in 1934[34] to 0.41% in 1996[55]. However, the original skin testing regimen was not without its flaws.

The use of *M. tuberculosis* to produce tuberculin for use in cattle lead to a high proportion of test - positive cattle which displayed no clinical evidence of disease when examined *post mortem*. The introduction of the comparative test, using a tuberculin derived from the avian mycobacterial species (*M. avium*), was based on pioneering studies performed in the late 1930's[56, 57]. The use of avian tuberculin (PPD - A) adds a significant degree of specificity to the skin test by allowing an assessment of any immune priming caused by exposure to environmental mycobacteria. The skin test has remained unchanged since with the exception of a switch from mammalian tuberculin, which was produced using the *M. tuberculosis* strains DT, C and PN[58], to bovine tuberculin (PPD - B) produced from an *M. bovis* strain isolated in England in 1948[59]. It was found that the tuberculin generated from the *M. bovis* AN5 strain was both more potent when used in cattle and more specific[58].

The incidence of BTB in cattle in GB has shown a steady and continual increase since 1996 (figure 1:2), despite the unremitting implementation of control measures, possibly due to

the presence of a wildlife reservoir consisting primarily of the badger[55] (*Meles meles*). Large scale trials have been performed to study the effect of culling badgers on the incidence of BTB[55] which suggest that, while culling can reduce disease incidence in cattle in the area of the cull, perturbation of the badger population leads to an increase in disease occurrence in the surrounding areas[60]. Within GB, BTB has spread drastically since the Foot & Mouth disease outbreak in 2001 with the annual number of animals slaughtered rising from a mean of 5,646 animals between 1996 and 2001 to an annual mean of 29,504 between 2002 and 2012 inclusive[61].

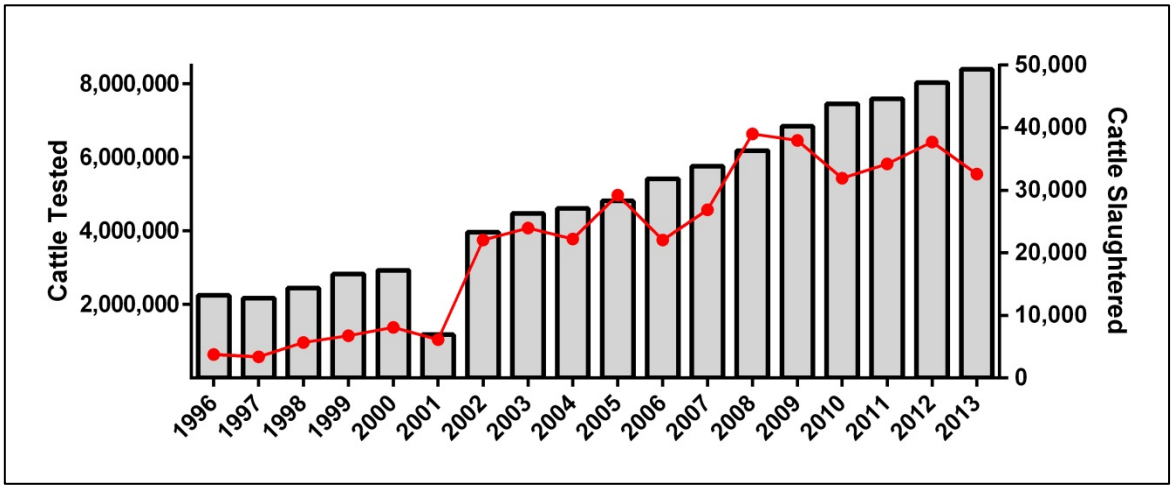

Figure 1:2 - Incidence of BTB in GB since 1996
Bars represent number cattle subjected to SICTT; line shows number of cattle slaughtered. Available from: http://www.defra.gov.uk/animal-diseases/a-z/bovine-tb/.

New Strategies for BTB Control

In 1996 Douglas Hogg MP, the then Minister of Agriculture, Fisheries and Food commissioned an independent scientific review into Government policy on BTB. Chaired by the Chief Executive of the Natural Environment Research Council, Professor John

Krebs, the review's final report contained many recommendations, the first of which was the development of a cattle vaccine; a strategy which was considered to be the best long term option to control BTB[55].

The only licensed vaccine for use against TB (in any host species) is the attenuated *M. bovis* strain bacille Calmette - Guérin (BCG). BCG is the most widely used vaccine in the world today with more than 3 billion individuals immunised[62]. The development of BCG was the result of pathogenesis experiments at the Pasteur Institute in France where Albert Calmette and Camille Guérin discovered that for successful infection of guinea pigs the bovine tubercle bacilli needed to be emulsified[63].

While BCG is the only available vaccine for use against TB infection, reports of its usefulness have varied. Overall, it is widely accepted that BCG vaccination significantly reduces the risk of TB in humans by 50 %[64]. However BCG daughter strains are not identical[65, 66] and, across a variety of populations, the protective efficacy of BCG has been reported anywhere between 0 % and 80 %, and usually in children and adolescents[67]. More recently it has been reported that BCG - induced protection may last for up to 60 years in certain human populations[68] but other reports have shown that 2 doses of BCG are required to confer protection to children in Turkey[69] and a large study performed in India involving patients covering a wide age range showed no protective effect of 2 different BCG vaccines when compared with a placebo[70]. The reasons for this variable efficacy are not understood although many theories have been proposed, such as interference of the immune response to BCG due to exposure to environmental mycobacteria; differences between the BCG daughter strains used for

vaccination; the loss of protective antigens from BCG during passage and potential failure in stimulating adequately balanced $CD4^+$ and $CD8^+$ T cell responses[64, 71]. Other potential confounders include variability in dose, route of administration, age of patients, genetic variance between vaccinees and storage methods of the vaccine such as lyophilisation[64, 71]. Clearly a better defined and tailored approach is needed to develop more efficacious and promising vaccines.

A variety of strategies have been adopted in an effort to improve the efficacy of BCG vaccination. One major route of investigation is the modification of BCG at the genomic level such that it overexpresses known tuberculoid antigens such as 85B (Ag85B). For example, the vaccine candidate rBCG30, which overexpresses Ag85B, has been shown to be more protective than it's parental strain in animal challenge studies[72, 73].

A second improvement strategy has been to enhance priming of $CD8^+$ T cells, rather than primarily targeting $CD4^+$ T cells. An elegant strategy developed by Kaufmann has been to enhance the ability of BCG to escape from phagocytic endosomes, thus allowing endogenous antigen processing and presentation in the context of the major histocompatibility complex (MHC) I. Inclusion of a cytolysin gene (*hly*) from *Listeria monocytogenes* into the BCG genome, along with deletion of the BCG urease gene *ureC* enables the vaccine to puncture the membrane of the early endosome and escape into the cytoplasm[74]. Not only does this enable greater endogenous antigen processing and MHCI mediated presentation, but bacterial escape from the endosome can lead to apoptosis in the infected cell and hence allow cross presentation of exogenous antigen to

naïve $CD8^+$ T cells[75]. This approach has led to enhanced protection over the parental strain and was also safer in immunocompromised SCID mice[76].

Other potential improvements to BCG include the addition of superoxide dismutase to the genome to enhance BCG survival and granuloma formation[77] and the reintroduction of genes that were lost from BCG during its attenuation such as the RD1 locus[78]. This latter approach is not without criticism, for fears that it may increase the virulence of BCG[79]. Alternatives to modification or enhancement of BCG itself are also being assessed. Generation of a live attenuated *M. tuberculosis* vaccine is a possibility, although there is concern over the potential for these strains to revert to a virulent phenotype[80].

The use of antigens which activate T cells in previously infected subjects may also provide novel vaccines. The search for antigens has identified a raft of potential vaccine candidates, some of which have been formulated into recombinant fusion proteins. The vaccine candidate Hybrid-I, consisting of Ag85B and the immunodominant Early Secreted Antigenic Target 6 kDa (ESAT - 6), has been shown to be as protective as BCG when formulated with an adjuvant[81, 82]. The use of ESAT - 6 as an immunising antigen is controversial as it is also the primary basis of the new generation of diagnostic tests, which may be compromised by its use[83].

Antigenic proteins such as Hybrid-I are promising vaccine candidates but there is little improvement in protection over traditional BCG vaccination. However, these candidates may be of significant use in a highly productive area of research - the heterologous prime

- boost strategy. Using an initial priming vaccine and boosting with a more defined subunit has the advantage that the boost preferentially expands TB - specific memory T cells generated by the initial priming immunisation. Using BCG as a primary vaccine allows the potential to boost with any of the subunit candidates currently under research, such as Hybrid-I, however these boosting antigens may not stimulate sufficient $CD8^+$ T cell responses.

A popular trial strategy used a recombinant, non - replicating virus presenting antigenic subunits on its surface as the boost to the BCG prime. The use of the poxvirus Modified Vaccinia Ankara (MVA) which expresses Ag85A, known as MVA85A, as a boosting agent, after priming with BCG, has been shown to induce greater levels of antigen specific $CD4^+$ and $CD8^+$ T cells as well as greater protection against challenge when compared to either BCG or the MVA85A alone[84]. Similar responses have been seen in human trials with this regimen where immunisation with one of the vaccines generated only short - lived antigen specific immune responses but the prime - boost strategy generated longer lived, stronger responses[85]. However in a phase 2B clinical trial where 2,797 BCG vaccinated South African infants were boosted with MVA85A, only 2 % of participants were protected against *M. tuberculosis* infection[86]. The authors note that the levels of antigen specific $CD4^+$ T cells in the study participants was as much as 90 % lower than in previous studies performed with adults[87, 88] and suggest that understanding the mechanisms behind expanding the appropriate T cell populations may be critical to the development of an efficacious human vaccine.

As reported in humans[67], estimates of protective efficacy of BCG in cattle have ranged between 0 % and 70 %[89-94] and heterologous prime - boost strategies also show promise in cattle[95, 96]. Similar to work in humans, a variety of alternative strategies have been tried in cattle. DNA vaccines expressing mycobacterial antigens (including MPB70, MPB83, Hsp60 and Hsp70) have been developed and trialled with generally inadequate results[97, 98]. However, one study reported significant protection using a DNA vaccine which encoded ESAT - 6 as well as the co - stimulatory molecules CD80 and CD86, usually found on dendritic cells (DC)[99]. DNA vaccines have proven more successful when applied in a heterologous prime - boost strategy based upon a BCG prime[97]. Interestingly, a subsequent follow up study from the same authors demonstrated no difference in protection if the DNA vaccine was used to prime and BCG used as the boosting antigen[100]. Vital work performed by Vordermeier *et al.* compared several vaccination strategies in cattle using BCG and boosting with either MVA85A or a recombinant adenovirus also expressing Ag85A (Ad85A) and demonstrated greater protective efficacy with the heterologous prime - boost strategies[96]. Critically, this was the first time any enhanced protective effect was shown in a natural host of mycobacteria.

Another potential method for successful vaccination of cattle is administration of BCG via the oral route. Studies performed in New Zealand demonstrated that this method conveyed similar protection to experimental challenge as subcutaneous immunisation, however the dose had to be increased and the vaccine had to be administered in a lipid formulation[101]. Unfortunately, reducing the dose of BCG used in these experiments renders this system unprotective[102] and the protective efficacy cannot be increased by

co - administration of mycobacterial proteins as heterologous prime - boost antigens[103]. Interestingly, all oral vaccines trialled have required formulation in a lipid matrix to confer protection[104-107]. Not only do these lipid formulations enhance BCG survival in the gut, but they can play a role in enhancing the host immune response[108]. Several commercial lipid adjuvants exist which have been shown to enhance Th1 responses or vaccine efficacy[109-111] and the addition of mycobacterial lipids into these adjuvant formulations has been further shown to enhance the protective activity of vaccines. The most studied mycobacterial adjuvants have been the cord factors. Trehalose dimycolate (TDM) has been shown to enhance long term protection of mice when administered as an adjuvant for the boost in a heterologous prime - boost strategy[112] and use of the synthetic analogue trehalose dibehenate (TDB) as an adjuvant has been shown to activate Th1 and Th17 responses via the macrophage inducible Ca^{2+} - dependant C - type lectin (Mincle)[113]. The polar lipid mono - mycolyl glycerol (MMG) has also been formulated into an adjuvant which has been shown to be capable of activating DC and driving Th1 responses[114, 115]. These advances clearly highlight the role of lipids in modulating immune responses and their potential applications as adjuvants or vaccine candidates.

Mycobacterial Lipids

Biologically, the mycobacteria are considered to be Gram positive as they possess a single peptidoglycan cell wall. What is unusual about the mycobacterial cell wall is that, rather than proteins and carbohydrates found in many other bacteria, most of the molecules attached to it are lipids[116]. In fact, it has been estimated that as much as 60% of the dry weight of mycobacteria is cell wall associated lipid[117] and these lipids are

widely believed to be important in directing the interactions between the pathogen and its host[118, 119]. Fractionation of the mycobacterial cell envelope is difficult as the envelope is physically strong and fractions tend to adhere to one another[120], however pioneering work by Brodie *et al*., using rapid growing mycobacterial species, lead to the ability to isolate the plasma membrane and fractionate the cell wall[121].

While the plasma membrane appears essentially the same as found in other Gram positive bacteria, there are some distinct membrane bound components not found in other bacterial genera, primarily lipomannan, lipoarabinomannan (LAM) and phosphatidylinositol mannosides (PIMs)[120]. Freeze fracture studies of the mycobacterial plasma membrane show a typical fracture plane between the membrane surfaces, with integral membrane proteins embedded in these layers[122], and no evidence of a second outer membrane, such as those found in Gram negative bacteria. However a second plane of fracture has been identified which is associated with carbohydrate molecules[123] that may include LAM and PIMs[120].

Much time and effort has been invested in developing a comprehensive model of the mycobacterial cell envelope. Thin - section transmission electron microscopy of mycobacteria reveals a plasma membrane surrounded by a thick electron - transparent periplasmic space. External to this is an electron - dense layer which, unlike the peptidoglycan found in most Gram positive bacteria, is believed to be composed of a peptidoglycan and arabinogalactan complex. Attached to this is another electron - transparent layer of mycolic acids[124]. A final electron - dense outer layer composed of carbohydrates and protein surrounds the bacterium and may also contain lipids[125-127].

This lipid - rich envelope is unusual in that it forms a permeability barrier and may be a bilayer in composition, similar to the outer membrane seen in Gram negative bacteria[128].

The outer membrane, and the arrangement of the lipids therein, has been the study of enormous research and many models have been proposed[128-132]. It is believed that mycolic acids are covalently bound to the external arabinogalactan portion of the cell wall and an outer membrane of various lipids surrounds this. Lipids found in this outer membrane may be glycolipids, such as trehalose monomycolate (TMM) and TDM, phospholipids, phthiocerol dimycocerosates and other species - specific lipids[128-132]. The model is illustrated in figure 1:3 and the structures of some of the outer lipids are shown in figure 1:4.

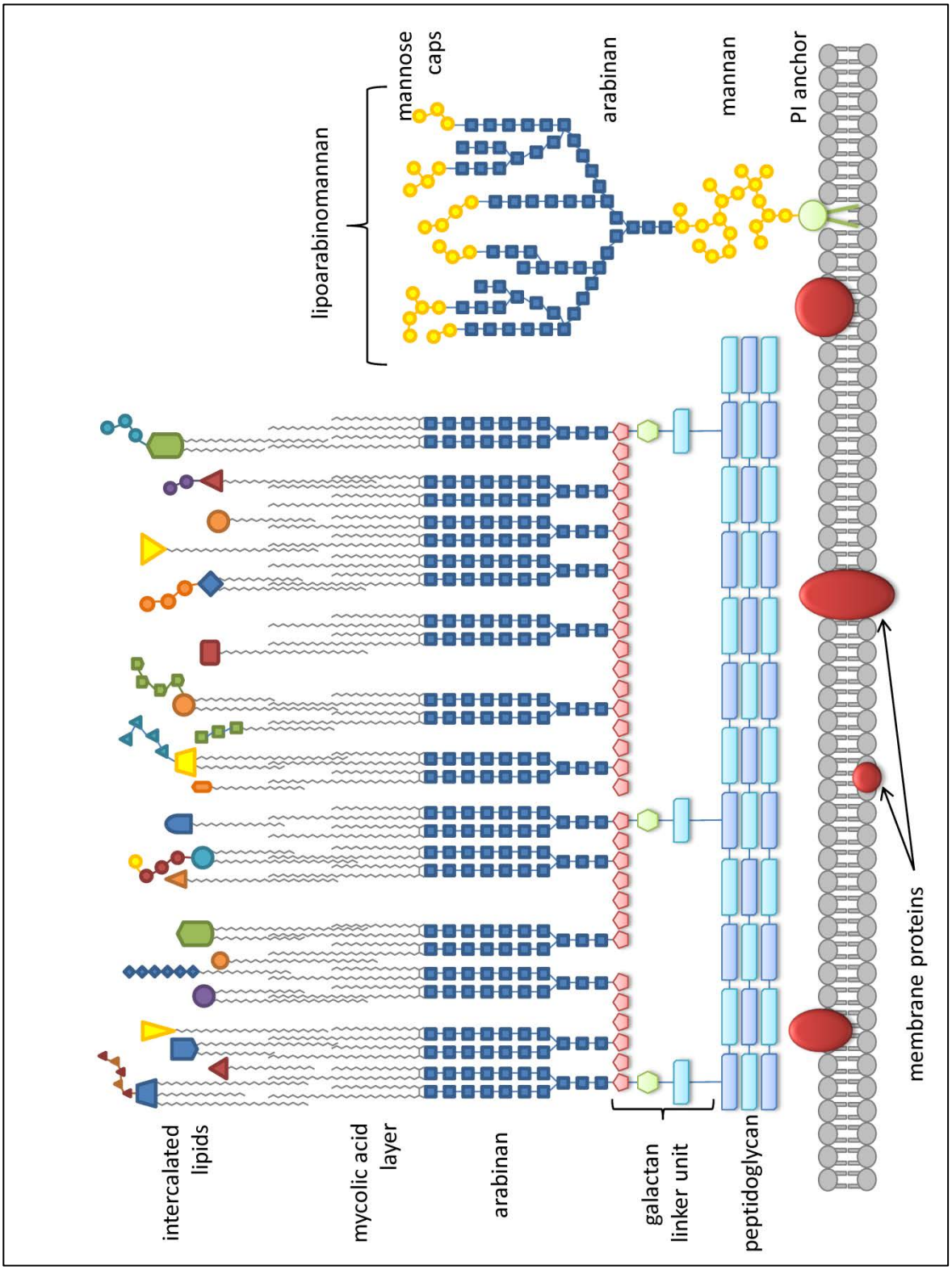

Figure 1:3 - Model of the mycobacterial cell wall.
Symbols do not denote specific molecules. PI: phosphatidylinositol.

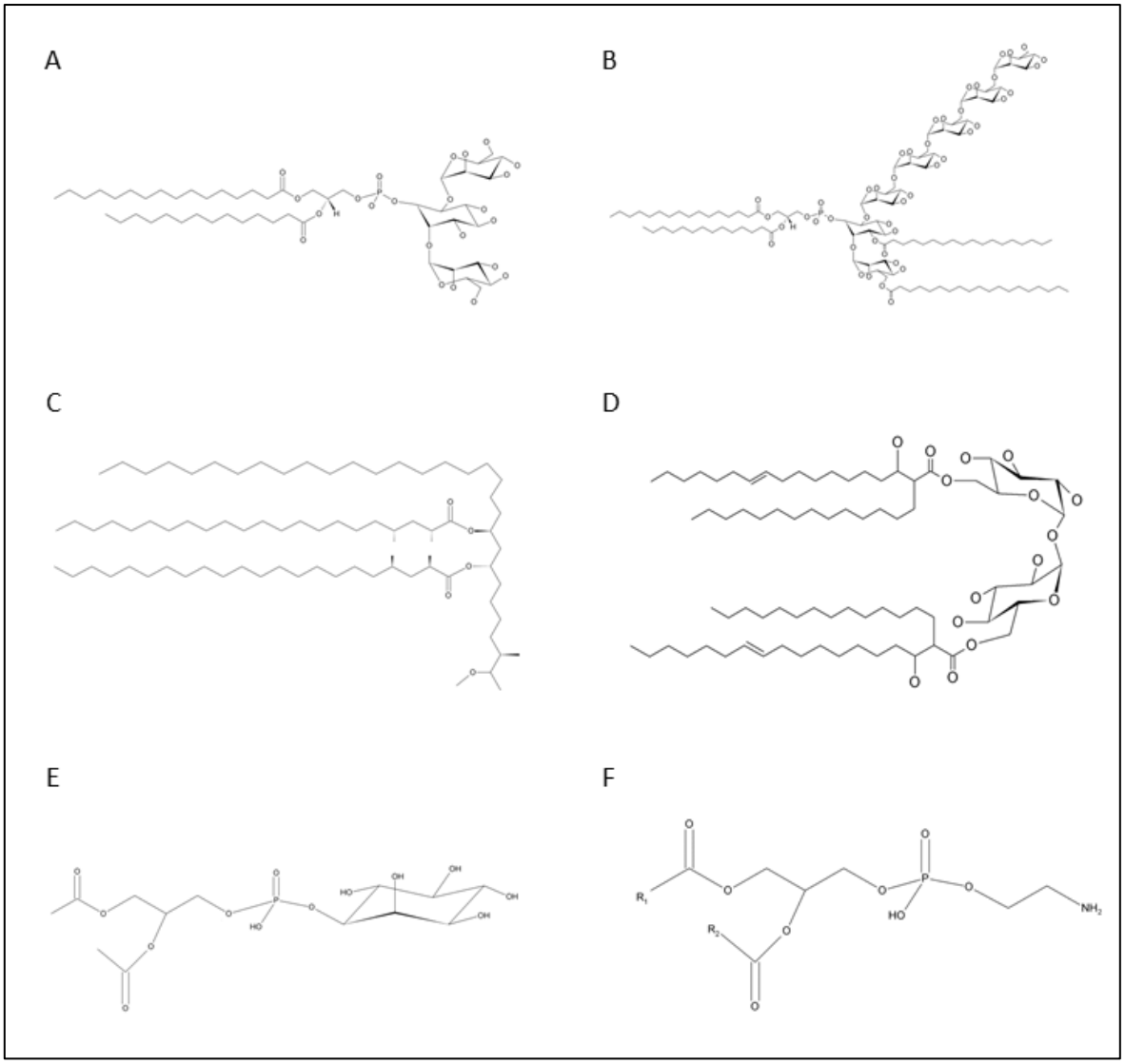

Figure 1:4 - Structures of some common outer envelope lipids found in virulent mycobacteria (A) Phosphatidylinositol dimannoside (PIM_2); (B) Diacylated phosphatidylinositol hexamannoside (Ac_2PIM_6); (C) Phthiocerol dimycocerosate (PDIM); (D) Trehalose-6-6'-dimycolate (TDM); (E) Phosphatidylinositol (PI); (F) Phosphatidylethanolamine (PE).

The structure of the cell wall and its outer envelope are critical to the stability of the cell. It is well documented that mutations affecting either the length of mycolic acids[133] or their modification[134-136] can alter the phenotype of the bacterial cell leading to altered colony morphology and persistence in host cells[137] or alter the permeability and lability of the outer membrane[138]. In fact, genetic removal of mycolic acids from *Corynebacterium glutamicum* caused complete removal of the outer membrane[127, 139] and even renders the mutant bacteria more permeable to drug entry[140].

Immune Recognition of Lipids

Dendritic cells (DC) are a group of bone marrow derived leucocytes, found in many tissues which serve several functions but, perhaps most importantly, initiate adaptive immune responses[141]. Originally identified in 1973 by Steinman & Cohn[142], DC are highly specialised for antigen uptake, processing and presentation to T cells and are considered to be the only antigen presenting cells (APC) capable of presenting antigen to, and activating, naïve T cells[143]. These cells are considered to epitomise the so called "professional" APCs[142]. Their dendritic membrane extensions maximise the surface area of the cell and the membrane is replete with MHC, adhesion and costimulatory molecules[141, 142]. Tissue localisation and different routes of derivation from the bone marrow mean that there are several types of highly specialised DC[144].

So - called immature DCs, primarily resident in peripheral tissues, are highly efficient phagocytes capable of engulfment and presentation of a huge variety of antigens[145]. Activation of these "sentinel" cells is driven via a signal, which may consist of physical tissue insult (and the consequent release of TNFα and IL - 1), antigen uptake or contact with molecules containing pathogen - associated molecular patterns (PAMPs)[142, 146, 147]. Activated DCs exit tissue via the draining lymphatic system and migrate to the draining lymph nodes. Migration is mediated by chemotactic gradients and this migratory stage is often characterised by increased expression of the chemokine receptor CCR7[148-150]. The chemokines CCL19 (MIP - 3β) and CCL21 (6Ckine) are ligands for CCR7 and their production by lymph node resident stromal cells is known to be important in directing

naïve T cells and migratory DCs to the lymph node[151]. However, whether expression of both of these chemokines is essential for DC migration remains unclear[152-154].

Upon arrival at the lymph node, DCs are capable of stimulating and modulating the T cell response, a process believed to be driven by the innate interaction between DCs and pathogen and the subsequent production of IFNα and IL - 12[155-157]. These DCs are primarily characterised by their increase in expression of MHCII molecules on the cell surface[158], as well as other co - stimulatory molecules such as CD40, CD80 and CD86[141, 159].

Lipid Modulation of Innate Immune Responses

The role of macrophages in protection against tuberculous infection was discovered over 60 years ago[160-162]. Much work was performed in the 1980s which lead to a greater understanding of the interaction between the macrophage and other host immune components including a method of macrophage mediated bacterial killing using hydrogen peroxide[163] and the critical role of IFNγ in the activation and enhancement of macrophages and their killing[164, 165]. Since then the interaction between mycobacterial species, usually *M. tuberculosis* or BCG, and APCs has been heavily studied. Both DCs and macrophages can interact with mycobacteria using an array of receptors such as complement receptors (CR) 1, 3 and 4; and pattern recognition receptors (PRRs) including the scavenger receptor, the Toll - like receptors (TLRs), nucleotide binding oligomerisation domain containing (NOD) - like receptors and surface bound lectins such as the mannose receptor (MR) and the DC-specific intercellular adhesion molecule - 3 grabbing

nonintegrin (DC - SIGN)[166]. Ligation of different receptors drives different effector functions, some specifically promoting phagocytosis (e.g. scavenger receptors) and others triggering non - phagocytic maturation or activation (such as the TLRs). It is the selective and multiple ligation of these receptors which dictates the effector function of the APC[167, 168].

Of course, a successful pathogen is one which is capable of, in part at least, avoiding or subverting the host immune response. It was originally hypothesised in the 1970s that *M. tuberculosis* had the ability to avoid killing by the macrophage[29, 169]. In fact it has been suggested that the typical immunopathology associated with pathogenic mycobacterial infection (the granuloma) may be beneficial to the bacilli[170]. Nevertheless, the interface between the bacilli and their host is pivotal in defining the progress of infection, and those cells which interact with the pathogen both initiate and shape the response. Immune recognition of *M. bovis* (as well as *M. tuberculosis*) is mediated by receptor : ligand interaction and lipids, being so abundant in the mycobacterial cell envelope, are likely to be central in this contact.

Interaction between mycobacteria and a variety of mammalian PRRs has been well documented[171-174]. Both macrophages and DC have been shown to recognise a variety of lipid antigens via TLR2 including AraLAM[175], lipomannan (from both *M. tuberculosis* and BCG)[176], PIM_2[175] and PIM_6[177]. Other PRRs, especially C - type lectins such as the mannose receptor (CD207), DC - SIGN (CD209) and Dectin - 1, have also been documented as playing important roles in sensing tuberculous infection.

Macrophage phagocytosis of *M. tuberculosis* is primarily mediated through the mannose receptor and usually leads to an anti - inflammatory response[178, 179]. *M. tuberculosis* derived mannosylated LAM (ManLAM) has been shown to bind to the mannose receptor and is capable of inhibiting IL - 12 production[180] and this interaction is also a key step in blocking phagosome - lysosome fusion[181].

DC - SIGN is usually found on the surface of DC and is known to bind a range of mycobacterial lipids including ManLAM and lipomannans[182, 183]. This has been shown *in vivo* and it has been suggested that interaction with DC - SIGN may allow mycobacterial entry into a DC, where it remains during DC migration to the lymph nodes[182]. More recently, Doz *et al*.[171] have shown that diacylated lipomannans are capable of inhibiting both cytokine and nitric oxide production in LPS - activated macrophages and this property was assigned to interaction with either DC - SIGN or the mannose receptor[171].

Although Dectin - 1 is more commonly associated with pattern recognition during fungal infection, recent studies have suggested a role in TNFα production in *M. avium* - infected macrophages[184]. In another study, Dectin - 1 dependant signalling was been shown to be involved in the production of IL - 12p40 and IL - 12p70 by splenic DC in response to *M. tuberculosis*[185]. Despite these studies no specific mycobacterial lipid ligands for Dectin - 1 have been found.

Innate recognition of mycobacterial antigens may not be limited to the cell surface. NOD like receptor (NLR) 2 has been shown to synergise with TLR2 to increase nitric oxide and TNFα production[186] whilst removal of NOD2 from murine macrophages and DC impairs cytokine and nitric oxide production in response to live *M. tuberculosis*[187]. This study

also showed that the arabinogalactan - peptidoglycan cell wall core of *M. tuberculosis* induced TNFα and IL - 12p40 production via NOD2 in murine macrophages [187].

Lipid Recognition by Adaptive Immunity

Lipids have been shown to mediate a range of effects on lymphocytes. Molecules such as LAM are known to be chemotactic for T cells when purified from either virulent or attenuated *M. tuberculosis*[188]. Interestingly, supernatants from macrophage cultures only retain this chemotactic activity when infected with virulent *M. tuberculosis* suggesting differences between the strains in their ability to export LAM[188]. ManLAM has also been shown to supress T cell proliferation when added to T cell clones specific for other antigens[189] and also to supress T cell activation as measured by reduced mRNA expression of IL - 2, IL - 3, GM - CSF and the IL - 2 receptor α chain[190]. The discovery that T cells could recognise lipids if the antigen is presented in the context of CD1 was a significant breakthrough[191]. It is now known that, within the human genome at least, there are 5 CD1 molecules: CD1a - e. These are split into 3 groups based on their sequence homology; group 1 consists of CD1a, b and c, group 2 just CD1d and group 3 contains CD1e.

CD1a is found on DC and differs from other group 1 CD1 molecules in that it is constitutively expressed at high levels on human Langerhans cells[192] and has been shown to be able to present some mycobacterial lipids to $CD8^+$ αβ T cells[193]. CD1a has also been found to present the mycobacterial lipopeptide didehydroxymycobactin[194] and is capable of activating $CD8^+$ αβ T cells[195]. As the precursor to mycobactin, an iron

chelating agent critical for survival within the macrophage, levels of didehydroxymycobactin increase during intracellular growth[196] and this may be an important mechanism for recognition of infected cells.

The second group 1 molecule CD1b is expressed by activated monocytes and DC and presents most of the identified mycobacterial lipid antigens[197] and is unique amongst CD1 molecules in that the structure of the binding cleft allows for binding of lipids of a large range of sizes[198-200]. The first CD1b restricted mycobacterial antigen identified was mycolic acid, which was shown to stimulate T cells[191], and all subsequent mycolic acid based lipid antigens are presented through CD1b thanks to its ability to accept long acyl chains into the binding pockets.

In fact, the mycolic acid structure may be responsible for the immunstimulatory activity of other lipids as many are based on the same backbone. For example, GMM consists of a glucose residue attached to a mycolic acid and is capable of stimulating specific T cells[201]. GMM loaded CD1b tetramers have been shown to label T cells from *M. tuberculosis* infected patients[202] suggesting an expansion of lipid responsive T cells in response to infection. These tetramer labelled cells were found to be $CD4^+$ and express limited diversity in their TCR repertoire and further analysis demonstrated expansion of these cells in patients latently infected with *M. tuberculosis*[203]. Interestingly, the authors also identified that the restricted TCR repertoire was associated with high levels of tetramer labelling and found that tetramer - intermediate labelled cells expressed a more diverse TCR repertoire suggesting the presence of other CD1b restricted mycobacterial lipids[203].

GroMM is another example and consists of a glycerol moiety attached to a mycolic acid structure and has been shown to drive T cell proliferation and IL - 2 production[204]. Another family of CD1b presented lipids are those containing PI such as PIM and LAM[205]. Both LAM and LM, which lacks the arabinose molecules, are capable of stimulating specific T cells through CD1b although some discrimination is made between molecules from different bacteria[205]. Despite the large binding pockets of CD1b, lipoglycans require internalising and processing before they are bound[206]. For example, the hexamannsoylated PIM_6 is known to stimulate T cells in the context of CD1b but only after partial digestion involving CD1e[207]. It has since been shown that CD1e selectively assists an α - mannosidase based on the degree of acylation of the PIM molecule[208]. Sulphoglycolipids are also presented by CD1b[209] but these molecules are not present in *M. bovis* [210, 211].

The final group 1 CD1 molecule is CD1c which is unique in that the binding cleft allows the hydrophilic component of a bound antigen to protrude and it is thought that this may interact with the T cell receptor[212]. While CD1c cycles through deep endosomal compartments where lipid binding proteins are present in a similar fashion to CD1b and CD1d, it also cycles through early endosomal compartments where the pH is higher and no lipid chaperone proteins are present[197] and it is thought that the partially open structure of CD1c may allow for antigen binding to happen in this environment[197]. CD1c has been shown to present a variety of lipids of a range of structures[213-215] and the role of CD1c in mycobacterial infection has been well characterised. Experiments with T cell lines meant that it was initially thought that CD1c presented branched chain phospholipids such as mannosylated phosphomycoketide[213] however use of the DN6 T

cell line showed responses to unglycosylated molecules revealing the specificity of the cell line to be naked phosphmycoketide[216]. Further, CD1c tetramer analysis showed only unglycosylated molecules were recognised despite the ability of cells to respond to glycosylated molecules when presented by APC suggesting that antigen processing must be removing the glycosyl residues[216].

Unusually, CD1 molecules are also known to be ligands for both αβ and γδ T cells. Initially γδ T cells were found to be CD1c responsive[217] but studies with duodenal cells have since shown this population to be responsive to all group 1 and group 2 CD1 molecules[218] despite a limited TCR repertoire. The use of CD1d tetramers has identified a sulphatide responsive γδ population in peripheral blood which express the same limited TCR repertoire[219]. While little is known about γδ T cell ligands, CD1d has been shown to be expressed in locations where this restricted γδ T cell population exists[220-222] and activated cells have a Th1 effector phenotype and produce granulysin[217] hence CD1d may constitute a legitimate γδ T cell ligand.

Found on the surface of most haematopoietic cells, CD1d is the only group 2 molecule and one of the best studied of the CD1 family. Crystal structure analysis performed using antigen - loaded CD1d bound to a TCR has shown that the molecule binds lipids with acyl chains of specific lengths[223]. Levels of CD1d are highly variable and alter in response to a range of microorganisms, the receptors ligated and the presence of different soluble factors. *M. tuberculosis* infection or exposure to mycobacterial lipids has been shown to upregulate CD1d expression on bone marrow - derived macrophages[224] whilst infection of monocytes prevents the upregulation of CD1d in cells that differentiate into DC[225].

CD1d restricted T cells are classified into 2 groups; the invariant NKT cells and the non - invariant CD1d restricted T cells. Invariant NKT cells in mice and humans express only a single TCR α chain and only a small selection of β chains and are characterised by their high affinity recognition of α galactosylceramide (αGalCer)[226] but these cells are highly conserved in other species[197]. CD1d restricted invariant NKT cells are known to recognise certain non - microbial lipids including PI and PE, albeit with low affinity in comparison to αGalCer[227], and one study has suggested that these cells are also capable of recognising mycobacterial PIM_4 extracted from BCG[228].

The non - invariant CD1d restricted T cells resemble other, more traditional T cells and are involved in classical adaptive responses[197]. These cells have been shown to recognise DPG and PI from *M. tuberculosis*[229].

Group 3 CD1 molecules consist solely of CD1e which remains within the cytosol of DC and is not externalised[230]. CD1e acts as a lipid binding protein and mediates the transfer of lipids to CD1b[231] and also in the degradation of some large lipid molecules, such as PIM_6, as discussed above[207, 208].

The Aims of this Study

Acquired cellular immune responses are utterly dependent on the initial interaction between the pathogen and the host's innate immune system and this first point of contact for *M. bovis* is likely to be an interaction with either alveolar macrophages or dendritic cells in the host lung. It is widely accepted that the interaction between the phagocyte and the external molecules of the bacilli play a significant role in

the establishment of infection and, hence, the outcome of disease[118, 143, 159, 166, 232-238]. Cell - mediated immunity is essential in developing both protective and pathological immunity to tuberculosis, as pulmonary macrophages alone are insufficient to control infection. Rather, they act as a refuge for the bacilli which are capable of inhibiting phagosome - lysosome fusion and thereby avoiding destruction[239, 240]. In humans, granuloma formation usually contains the infection[241] leading to a state of so - called dormancy, or latency, although infection is rarely eliminated. However, in cattle there is considerable debate about the presence of latent infection[242].

As previously discussed, the production of IL - 12 by DCs and the subsequent Th1 polarisation of the cell - mediated response is essential for IFNγ production and macrophage activation[243] and the bacilli : antigen presenting cell interaction is perhaps the most important event that occurs during the infection process[170]. Given the high levels of lipid found on the mycobacterial cell wall, it is highly likely that these lipids will play an important role in immune recognition of the pathogen. In fact, many lipids have already been shown to stimulate potent immune responses[115], some have even been associated with immune suppression and hypervirulence[244] and immunostimulatory lipids have even been used to develop Th1 polarising adjuvants[114].

Yet there is a distinct lack of published information regarding the interaction of the virulent pathogen and it's hosts APCs. A search of PubMed using the relevant terms returns very few results and none of them discuss pathogen derived lipid in host - derived APCs. For example, Andersen *et al*.[115] demonstrated upregulation of CD40, CD86 and HLA - DR and the production of IL - 6 and TNFα by MDDC derived from human

macrophages but either extracted lipids from *M. bovis* BCG rather than the virulent human pathogen *M. tuberculosis* or used chemically synthesised MMG. Bovine specific work performed by Hope *et al*. made use of bovine monocytes in the generation of MDDC but these cells were stimulated with a synthetic lipopeptide[233] rather than a pathogen - derived lipid antigen. In the study performed by Reed *et al.*[244], the authors showed that blockage of synthesis of the phenolic glycolipid (PGL) correlated with increased secretion of TNFα, IL - 6 and IL - 12 by the host, and removed the "hyperlethal" phenotype displayed by the bacilli, however this was shown only in the murine model.

Immunologically active bacterial lipids play a major role in the outcome of tuberculous infection and have a range of potential practical uses. The use of specific lipids from BCG and *M. tuberculosis* to enhance protective efficacy or duration of immunity of vaccines has been demonstrated in a variety of model systems[112-115].

Further, given the ability of lipids to traffic more freely than proteins between macrophage intracellular compartments[245] and the ability of lipids to enter directly into the membranes of APC[246], it is possible that lipid molecules themselves could confer protective immunity. A potential advantage of this approach to vaccine development is that CD1 molecules are highly non - polymorphic and CD1 - presented lipids are not subject to large structural changes[247] as well as the fact that CD1 - restricted T cells are known to exhibit anti - microbial activity[213, 248]. These data suggest that specific lipid molecules could be used as adjuvants or as vaccine candidates for cattle however more needs to be known about how lipids interact with bovine immune cells.

Lipids such as mycolic acids and LAM have also been investigated as highly specific diagnostic reagents in humans[249-251] and similar methods could be applied to cattle.

Therefore, this study addresses the hypothesis that lipids from *M. bovis* constitute a source of molecules that could be developed into tools which could contribute to the development of control measures for BTB. These may include subunit vaccine candidates acting as antigens, or biological adjuvants, immunostimulators and specific diagnostic reagents. As a first step towards these goals, this thesis aims to identify and immunologically characterise *M. bovis* - derived lipid preparations and compounds by pursuing the following objectives:

- Develop methods to isolate and characterise lipid moieties from *M. bovis*,
- Assess the effect of *M bovis* lipids on bovine APC to identify potential immunomodulatory activity which could be exploited in the development of novel adjuvants,
- Identify the lipid targets of the bovine adaptive immune response which could be used as innovative vaccine candidates.

Chapter Two

Materials & Methods

Preparation of Bacterial Isolates for Lipid Extraction

Mycobacterum bovis strain AF 2122/97 was grown in Middlebrooks 7H9 medium, prepared using 4.7 g 7H9, 2 ml glycerol and 0.5 g Tween 80 in 800 ml of reverse osmosis purified water (roH_20). This mixture was stirred until homogeneous and autoclaved at 121 ^oC for 30 minutes. After cooling, 100 ml of a sterile 10x sodium pyruvate stock was added along with 100 ml of sterile Middlebrooks albumin / dextrose / catalyse (ADC) enrichment broth.

Initial starting cultures were performed in 10ml volumes in static flasks at 37 ^oC within an ACDP Containment Level 3 (CL3) facility. Large quantities of bacterial cells were grown in 100 ml rolling culture flasks inoculated with 1ml of starting culture and incubated until mid - log phase, defined as an optical density (at 600 nm wavelength) of 0.6 - 0.8.

To harvest cells, mid - log phase cultures were decanted into sterile 50 ml Falcon tubes and spun at 2,500 g for 5 minutes. Supernatants were decanted and cell pellets were

washed twice in sterile milliQ purified water. Pellets were suspended in 5ml sterile milliQ water and stored at -80 ^{o}C until being killed and removed from the CL3 facility. Cell pellets were thawed before killing and heat killed in a water bath at 80 ^{o}C to 90 ^{o}C for between 1 and 2 hours. Finally, heat killed cell pellets were freeze dried prior to extraction of lipids.

M. bovis AN5 was sourced from the Tuberculin Production Unit at AHVLA Weybridge. Bacterial cells were grown in an ACDP CL3 laboratory as a surface pellicle on glycerol rich Bureau of Animal Industry (BAI) medium containing 14 g of L - asparagine, 1.5 g of dipotassium hydrogen phosphate, 0.74 g of sodium citrate, 1.5 g of magnesium sulphate, 0.3 g of ferric sulphate, 0.08 g of zinc sulphate, 0.008 g of manganese chloride, 0.00138 g of cobaltous chloride, 10 g of glucose and 100 g of glycerol litre^{-1}.
The pellicle was autoclaved at 134 ^{o}C for 1 hour before being freeze dried prior to extraction of lipids.

Extraction of Crude Free Mycobacterial Lipid

The extraction method used in this thesis has been previously described[252].
Freeze dried bacterial cells were added to a mixture of 400 ml CH_3OH and 40 ml 0.3 % aqueous NaCl. A further 440 ml of petroleum ether was added, the top of the vessel covered and mixed for 12 to 16 hours. The mixture was decanted into centrifuge bottles and spun at 4000 g for 10 minutes to pellet the bacterial cells. The upper, non - aqueous phase was removed by pipetting and stored. To the lower, aqueous phase a further 440 ml of petroleum ether was added and the mixture stirred for 2 hours before being spun

again, the non - aqueous layer removed and pooled with the first. The petroleum ether was removed from these extracts using a rotary evaporator with cold finger condenser and the lipid transferred to a pre - weighed glass tube in 4 : 1 $CHCl_3$: CH_3OH. The $CHCl_3$: CH_3OH mixture was evaporated using a heating block and a stream of N_2 gas leaving dried lipid in the pre - weighed tube which was subsequently weighed again to determine the mass of apolar lipids extracted.

Extraction of the polar lipids was performed by adding 212 ml $CHCl_3$, 236 ml CH_3OH and 72 ml 0.3 % aqueous NaCl (520 ml of 9 : 10 : 3 mixture of $CHCl_3$: CH_3OH : NaCl). This mixture was stirred for 12 to 16 hours before being passed through double Whatman 91 240 mm filters papers to collect the cells. Once dried on the filter, the cells were re - extracted twice using 170 ml of a 5 : 10 : 4 mixture of $CHCl_3$: CH_3OH : NaCl. After a final filtration to remove cells and cell debris from the polar phase, 290 ml of both $CHCl_3$ and NaCl were added to polar phase which was mixed for one hour and allowed to split in a separating funnel. The lower, aqueous, phase was removed and dried in a rotary evaporator. Final polar lipid mass was ascertained as described for the apolar petroleum ether extracted lipid fraction. The process is illustrated in figure 2:1.

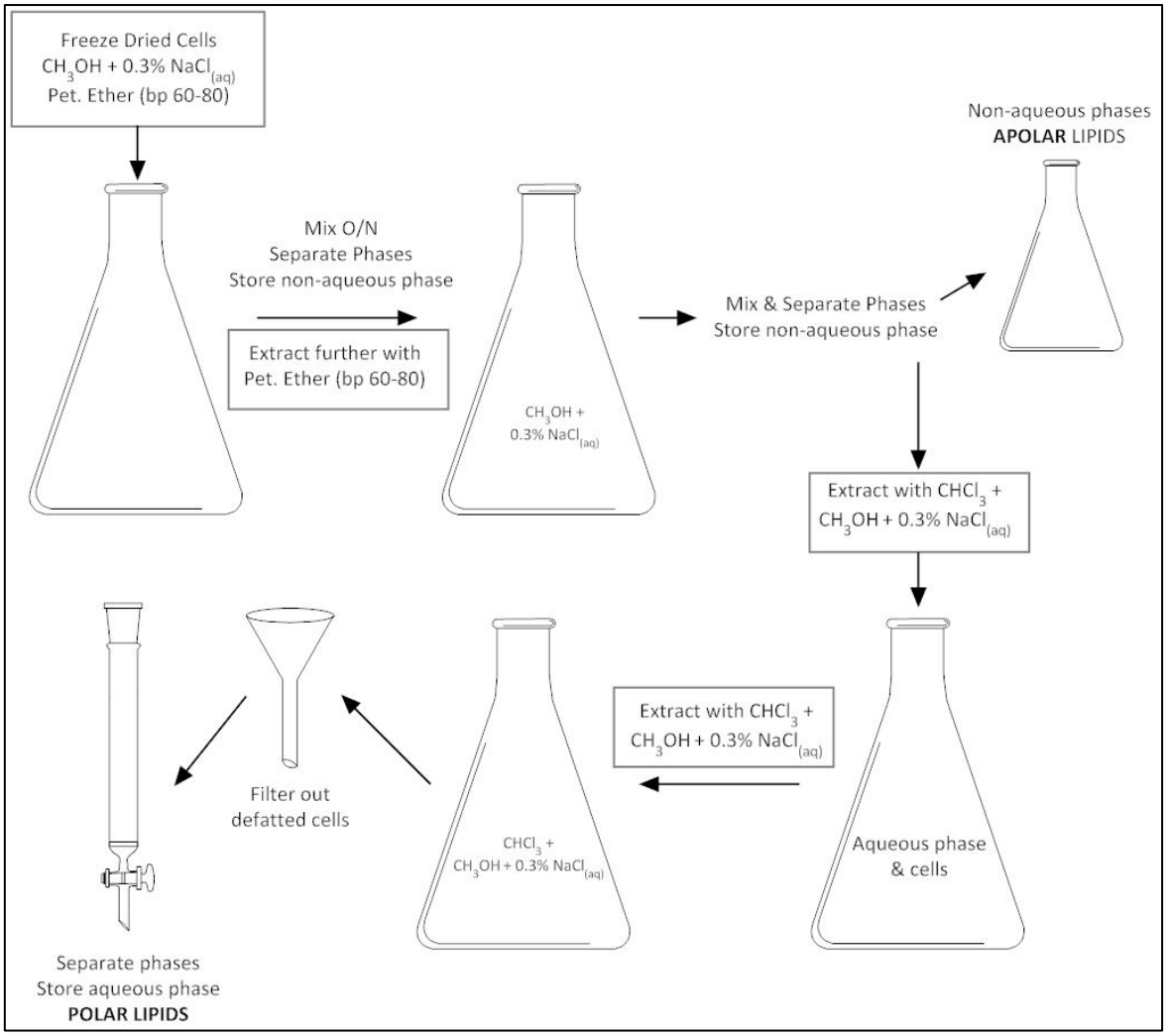

Figure 2:1 - Schematic representation of the extraction of free mycobacterial lipids.

Analysis of Lipid Fractions by 2D Thin Layer Chromatography

Aluminium backed silica gel 60 F_{254} TLC plates were cut into approximately 6 cm squares and 100 µg of lipid extract was spotted onto the plates using glass micro - capillary pipettes. Plates were dried at 80 ^{o}C in an oven for 10 minutes before running.

Appropriate solvent mixtures were prepared in 100 ml volumes as previously described[(252)] and TLC tanks were equilibrated using blotting paper before plates were

run. The solvent systems used in all 5 TLC systems are shown in table 2:1 and plates were run as indicated.

TLC plates were dried for 10 minutes at 80 ^{o}C between each run and before staining to ensure that all residual solvent was removed.

Staining was performed using either a 5 % solution of molybdophosphoric acid (MPA) in 100 ml of 95 % v/v ethanol in water (MPA) or a 1.5 % w/v solution of ninhydrin dissolved in 100 ml *n* - butanol to which 3 ml glacial acetic acid was added. Stains were sprayed onto TLC plates which were subsequently charred using a hot air gun before being photographed or scanned.

Table 2:1 - Solvent systems for TLC analysis of mycobacterial lipids (adapted from Dobson *et al.*[252])

Solvent System	Run Direction	Components	Runs	Fractions Analysed	Lipids Resolved
A	1	petroleum ether : ethyl acetate (98 : 2)	3	Apolar	PDIM, TAG, MQ
	2	petroleum ether : acetone (98 : 2)	1		
B	1	petroleum ether : acetone (92 : 8)	3	Apolar	ATs, PGL, MMG
	2	toluene : acetone (95 : 5)	1		
C	1	chloroform : methanol (96 : 4)	1	Apolar	PGL, MMG
	2	toluene : acetone (80 : 20)	1		
D	1	chloroform : methanol : water (100 : 14 : 0.8)	1	Apolar & Polar	CF, SL, GMM
	2	chloroform : acetone : methanol : water (50 : 60: 2.5 : 3)	1		
E	1	chloroform : methanol : water (60 : 30 : 6)	1	Polar	DPG, PE PI, PIM
	2	chloroform : acetic acid : methanol : water (40 : 25 : 3 : 6)	1		

Thin Layer Chromatography Densitometrical Analysis

High resolution uncompressed TIFF images were loaded into Quantity One analysis software and the gradient tool used to select individual lipid spots. Automatic and manual separation of the spots from the background colour was found to be optimal when the TLC images were false coloured using the "spectrum" option. A block selection of the entire plate was also made to include all lipids, including those present either at the origin or solvent front (figure 3:5). Calculation of the volume of each spot was performed by multiplying the intensity of the spot by its area in mm^2. The intensity of each spot was calculated as a percentage of the total plate.

Preparation of Lipid Antigen Suspensions

Suspensions of all lipid antigens were prepared in an aqueous phase for use in cell culture experiments after first removing any $CHCl_3$: CH_3OH by evaporation using an N_2 gas stream. Complete RPMI was added to the dried lipid and the mixture was then heated to 80 ^oC for 5 minutes followed by sonication for 5 minutes. This heating and sonicating cycle was repeated twice. A visual check was made of lipid preparations; were any lipid visible to the naked eye either on the glass or in solution, another round of heat and sonication was performed. Apolar and polar lipids were used at 20 µg ml^{-1} for 12 - 16 hours.

Lipid Purification by 1D Thin Layer Chromatography

Thirty milligrams of crude free mycobacterial lipid was streaked onto the long side of 10 cm x 20 cm plastic backed silica gel 60 F_{254} TLC plate. Plates were run in an equilibrated 14 : 6 : 0.8 mixture of $CHCl_3$: CH_3OH : H_2O and dried with warm air before being sprayed with a solution of 0.01 % 1, 6 - diphenyl - 1, 3, 5 - hexatriene (DPH) in a 9 : 1 mixture of petroleum ether : acetone. Staining was visualised under long wavelength ultra - violet light (366 nm; UV - A) and visible bands were marked with pencil. A representative plate is shown in figure 2:2.

Once bands had been marked, plates were washed in toluene to remove DPH and confirmation of removal was made by visualising the plate under 366 nm UV light. Bands were then scraped from the plate and collected into individual glass tubes containing 5 ml of a 2 : 1 mixture of $CHCl_3$: CH_3OH and mixed on a rotor for 10 minutes. After pelleting the samples at 2000 g for 5 minutes, the supernatants were removed and filtered through glass wool. The silica pellet re - extracted a further 2 times before being dried under a stream of N_2.

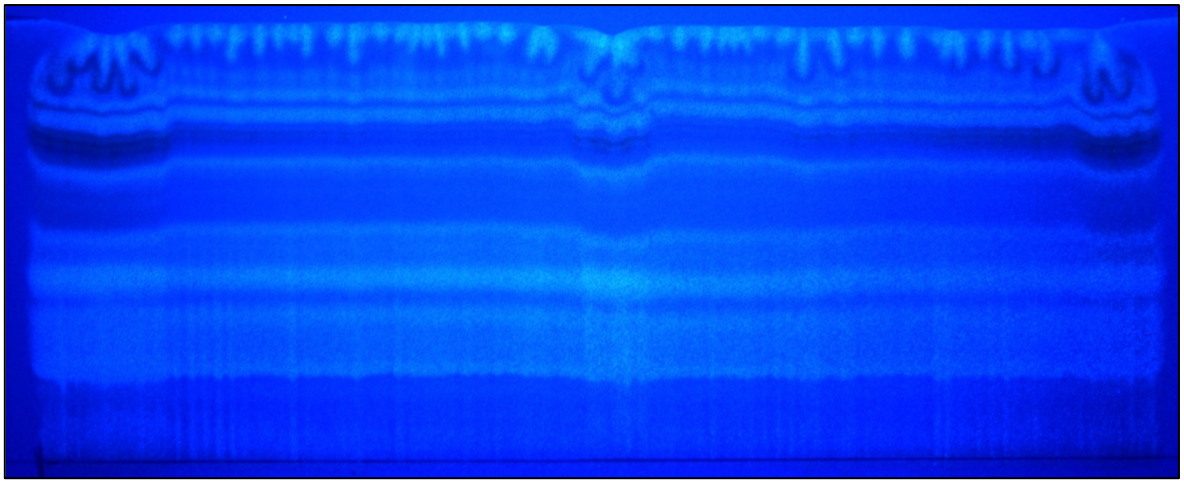

Figure 2:2 - Representative 1D TLC of crude free polar mycobacterial lipids
Plate was run in 14 : 6 : 0.8 CHCl3 : CH3OH : H2O, stained with 0.01 % 1, 6 - diphenyl - 1, 3, 5 - hexatriene in 9 : 1 petroleum ether : acetone. Plate visualised under long wavelength (366 nm) UV light.

Finely suspended and colloidal silica was removed from the samples by dissolving in 6 ml of a 10 : 10 : 3 mixture of $CHCl_3$: CH_3OH : H_2O. Samples were mixed on a rotor for 30 minutes before the addition of 2.625 ml of $CHCl_3$ and 1.125ml of H_2O for a further 10 minutes. Separation of the phases was performed by centrifugation at 2000 g for 5 minutes and the upper, aqueous, phase and silica interface was removed. The remaining lower organic phase was treated with 3 ml of a 3 : 47 : 48 mixture of $CHCl_3$: CH_3OH : H_2O and mixed on a rotor for a further 10 minutes before centrifugation and removal of the silica interface and upper layer. This process was repeated a further 3 times.

Finally, the silica free lower phase was dried under a stream of N_2, resuspended in 5 ml of a 2 : 1 mixture of $CHCl_3$: CH_3OH and transferred to a clean, pre - weighed tube for weighing and suspension.

Uninfected Cattle

Cattle between the ages of 6 and 36 months were obtained from herds within 4 - yearly testing parishes with no history of a BTB breakdown in the past 4 years as previously described[253]. These animals were purchased when around 6 months old and transported to AHVLA. Whilst at AHVLA they tested negative in both Bovigam IFNγ assay and the SICCT. Further, they were also tested in the IFNγ test with the specific antigens ESAT - 6 and culture filtrate protein - 10 kDa (CFP - 10) used as a combined peptide cocktail and results confirmed the animals as negative.

M. bovis Infected Cattle

Cattle between the ages of 6 and 36 months were obtained from herds within 1 - yearly testing parishes with a confirmed BTB breakdown within the past year as previously described[253]. These animals were purchased at the disclosing skin test and transported to AHVLA. Whilst at AHVLA they regularly tested positive in both Bovigam IFNγ assay and the SICCT. Further, they were also tested in the IFNγ test with the specific antigens ESAT - 6 and CFP - 10 used as a combined peptide cocktail and results confirmed the animals as *M. bovis* infected.

Isolation of Bovine PBMC from Whole Blood

The isolation of PBMC has been previously described[253]. Briefly, whole blood was mixed in equal amounts with sterile HBSS containing 10 U ml $^{-1}$ heparin. This mixture was overlaid onto Histopaque 1077 (Sigma) and centrifuged at 800 g for 40 minutes. The PBMC interface was removed using a pastette and washed twice in HBSS containing heparin. Cells were identified via trypan blue exclusion and enumerated using a haemocytometer.

Isolation of $CD14^+$ Monocytes from Bovine PBMC

Isolation of $CD14^+$ cells was performed according to the manufacturer's instructions. PBMC were counted and suspended in 80 µl of MACS rinsing buffer (sterile PBS containing 2mM EDTA and 035 % BSA or FCS) per 10^7 cells before the addition of 10 µl of MACS anti - CD14 MicroBeads (Miltenyi) per 10^7 cells. After a 15 minute incubation at +4 ^{o}C on a rotator, cells were pelleted and resuspended in 500 µl per 10^8 cells and passed through MACS LS columns as per the manufacturer's instructions. The $CD14^+$ fraction was counted and cells diluted to 1.5 x10^6 ml $^{-1}$ in complete RPMI 1640.

Generation of Bovine Cultured Monocytes and MDDC

$CD14^+$ monocytes were plated in 1 ml volumes of complete RPMI 1640 at 1.5 x10^6 ml^{-1} in 24 well plates (Nunc Nunclon) before adding either 1000 U ml $^{-1}$ equine GM - CSF

(Kingfisher Biotech; RP0022E - 005) (cultured monocytes - CM) or 1000 U ml $^{-1}$ equine GM - CSF and 4 ng ml $^{-1}$ bovine IL - 4 (AbD Serotec; PBP006) (monocyte derived DC - MDDC). Cells were incubated at 37 ^{o}C + 5 % CO_2 for 3 days.

After 3 days cells were harvested using a cell scraper and enumerated before being plated out at 1.5 x10^{6} ml $^{-1}$ in fresh complete RPMI and lipid solution was added to the wells. Cells were incubated for a further 12 - 16 hours before supernatants were collected and cells harvested for subsequent flow cytometric analysis.

Measurement of IFNγ by Bovigam^{TM} ELISA

Levels of IFNγ were determined using the Bovigam^{TM} ELISA kit (Prionics AG, Switzerland) in accordance with the manufacturer's instructions. For the assessment of adaptive immune responses to individual PIM molecules (see Chapter Six) responses were considered positive if the OD450 exceeded the mean + 2 times the standard deviation of OD450 for nil antigen stimulated cultures from all 10 animals.

Multiplex Measurement of Cytokine Production

Fresh and cultured monocytes and MDDC were derived as described above. After 3 days in culture, cells were stimulated with 20 µg ml $^{-1}$ of either the polar or apolar lipid fraction dissolved in RPMI as previously described. After 12 - 16 hours of stimulation, supernatants were harvested and assayed for cytokine levels using the MSD multiplex

platform (MSD Systems, Washington) as described previously[254, 255]. Briefly, supernatants generated were analysed using a custom multiplex electrochemiluminescent system which allows simultaneous detection of IL - 1β, IL - 6, IL - 10, IL - 12, MIP - 1β and TNFα (Meso Scale Discovery, Maryland, USA). Multiplex 96 well plates were supplied with target capture antibodies spotted onto 6 separate carbon electrodes in each well (commercially available antibodies: TNFα (Endogen, Rockford, IL, USA); IL - 10 and IL - 12 (AbD-Serotec); IL - 1β, IL - 6 and human cross-reactive MIP - 1β (MSD)).

Plates were blocked with MSD assay buffer for 30 minutes at room temperature before the addition of samples or standards for 1 hour at room temperature. Standards were prepared by serial dilution.

After incubation, plates were washed and combined biotinylated secondary detector antibodies were added for a further hour. Finally, plates were washed, loaded with MSD read buffer T and analysed using an MSD Sector Imager 6000.

Innate Cell Labelling & Analysis by Flow Cytometry

Cells were harvested from the well and labelled with the live / dead indicator ViViD (Invitrogen) in PBS. Cells were then plated at approximately 50,000 stain^{-1} and washed using 150 µl MACS rinse buffer before being stained for 15 minutes using either anti - bovine CD14 (ccG33; Institute for Animal Health; 1 : 50), anti - equine MHCII (MCA1085; AbD Serotec; 1 : 50), anti - bovine CD40 (IL - A156; cell supernatant, AHVLA; 1

: 10), anti - bovine CD80 (IL - A159; cell supernatant, AHVLA; 1 : 10), anti - bovine CD86 (IL - A190; cell supernatant, AHVLA; 1 : 10), anti - bovine CD1b (CC14; AbD Serotec MCA831G; 1 : 10) or an IgG1 isotype control (Av20; Institute for Animal Health; 1 : 50).

Labelled cells were washed using 150 µl MACS rinse buffer and secondary labelling was performed using a 1 : 400 dilution of anti - IgG1 conjugated to R - Phycoerythrin (R - PE) (Invitrogen; P21129) in 50 µl volumes for 10 minutes. After incubation, cells were washed by the addition of 150µl PBS, pelleted and resuspended in 100 µl of 2 % paraformaldehyde (Cytofix; BD Biosciences) for at least 30 minutes at 4 ^{o}C before analysis on a CyAn ADP analyser.

For capture and analysis, initial gating was on the ViViD^{lo} (live) cells into a subsequent small cell / lymphocyte exclusion gate. An example of the gating strategy is shown in figure 2:3. Normalisation of the median fluorescence intensity (MFI) was performed by subtraction of the MFI of the Av20 isotype control from the MFI of the specific antibody labelling (Δ MFI).

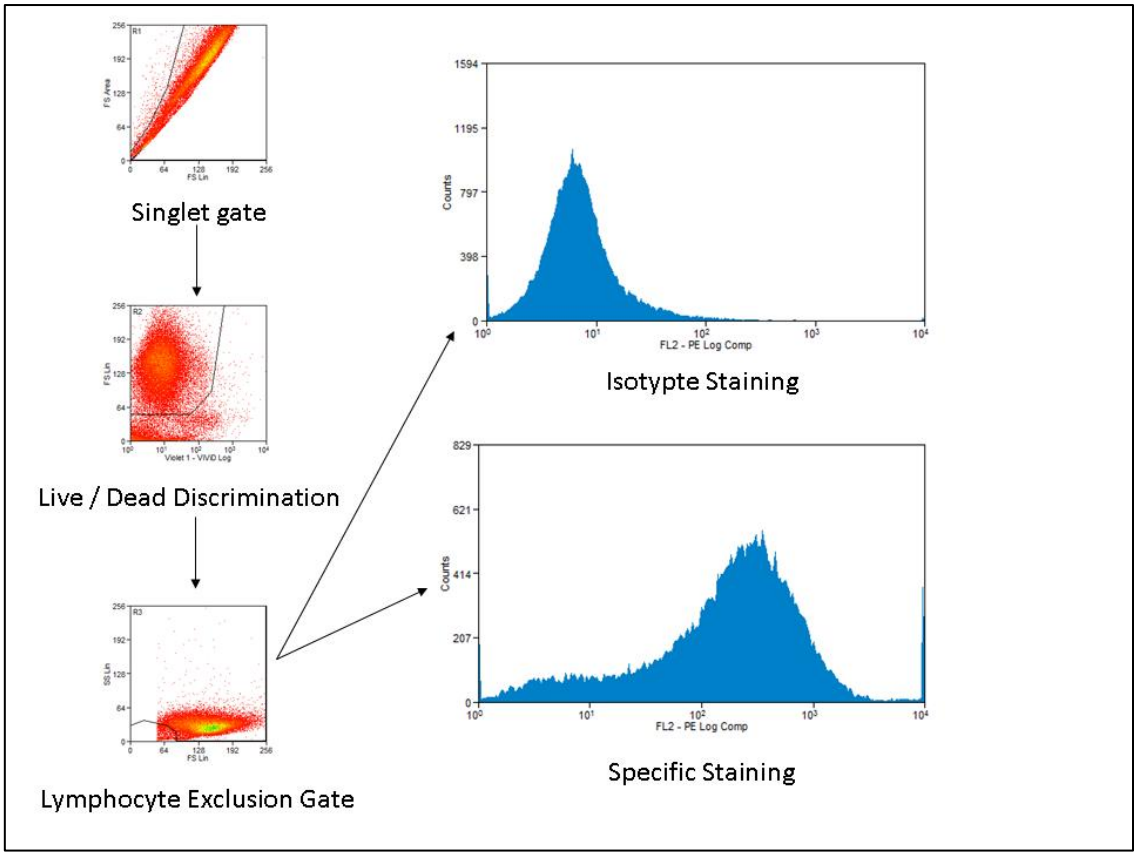

Figure 2:3 - Example of gating strategy for analysis of monocytes, MDM and MDDC
A singlet gate (FSC lin v FSC area) removes debris and clusters of cells; ViViD staining allows isolation of live / viable cells; a FSC vs SSC plot previously reverse gated on PBMC acts as a lymphocyte exclusion gate. Typical CD1b labelled MDDC are shown with their concurrent isotypte labelling.

Mixed Lymphocyte Reaction

Bovine MDDC and cultured monocytes were prepared as described above. Following 3 days in culture, cells were pulsed with lipid antigen overnight before being enumerated, washed to remove any cytokines and lipid from the media and incubated at 10^7 cells ml^{-1} in the presence of Mitomycin C at 100 µg ml^{-1} for 30 minutes at 37 ^oC + 5 % CO_2. Lipid pulsed, Mitomycin C treated MDDC or cultured monocytes were cultured in 1 ml at 37 ^oC + 5 % CO_2 at 2 x10^5 with 1 x10^5 PBMC isolated from a second, allogeneic animal. After 5 days, cells were pulsed overnight with 1 µCi $well^{-1}$ of ^{3}H-thymidine before

being harvested using a Harvester 96 Mach III (TomTec Inc, Hamden, CT, USA) as described previously[256]. Lymphocyte proliferation was assessed by the increased cellular incorporation of ^{3}H-thymidine which was measured using a MicroBeta^{2} 2450 (Perkin Elmer, Waltham, MA, USA).

Lymphocyte Transformation Assay

PBMC were isolated from heparinised whole blood using Histopaque 1077 (Sigma) gradient centrifugation (as described above) and 2 x10^{5} cells well $^{-1}$ plated out in triplicate wells after resuspension in complete RPMI 1640. Cells were mixed with lipid antigen, complete RPMI as a negative control, pokeweed mitogen (PWM) at 10 µg ml $^{-1}$ as a positive control, or a 1 : 100 dilution of PPD - B.

Measurement of proliferation by the incorporation of ^{3}H - Thy has been previously described[256]. Briefly, after 4 days of incubation at 37 ^{o}C + 5% CO_2, 30 µl of supernatant was harvested for cytokine analysis and cells were pulsed with 1 µCi ^{3}H - Thy well $^{-1}$ in 30 µl to replace the removed supernatant. After a further 16 - 24 hours incubation the cells were washed and harvested using a semi - automated harvester (Tomtec) and incorporation of ^{3}H - Thy was read using MicroBeta^{2} (Perkin Elmer).

For the assessment of adaptive immune responses to individual PIM molecules (see Chapter Six) responses were considered positive if the counts per minute (CPM) exceeded the mean + 2 times the standard deviation of the CPM for nil antigen stimulated cultures from all 10 animals.

Lymphocyte Labelling & Analysis by Flow Cytometry

Bovine PBMC were isolated as described above and labelled with CellTrace Violet (Invitrogen Molecular Probes, Paisley, UK) in accordance with the manufacturer's instructions. Briefly, PBMC were suspended at 1 $x10^7$ ml^{-1} in pre - warmed PBS and 5 mM CellTrace Violet was added to a final working concentration of 1 µM. Cells were incubated at 37 ^oC for 20 minutes before unbound dye was quenched with 5 times the labelling volume of complete cell culture medium at 37 ^oC for 5 minutes. Finally, cells were pelleted and washed in pre - warmed complete cell culture medium, plated at 2 $x10^5$ cells $well^{-1}$ and incubated at 37 ^oC + 5 % CO_2 for 5 days in the presence of antigen. Cultured cells were harvested and resuspended in flow cytometry buffer (PBS containing 2 % FCS and 0.05 % NaN_3) and labelled for 15 mins with near infrared live / dead viability dye (NIRViD, Invitrogen Life Technologies, Paisley, UK) and mouse anti - bovine CD335 (AKS1, AbD Serotec, Oxfordshire, UK). Cells were washed in flow cytometry buffer and secondary labelling of anti - CD335 was performed using a 1 : 400 dilution of rat anti - mouse IgG2a conjugated to allophycocyanin for a further 15 minutes. After a subsequent wash, cells were further labelled with combinations of R - PE Zenon labelled (Invitrogen Life Technologies, Paisley, UK) mouse anti - bovine CD3 (MM1A; WSU Monoclonal Antibody Centre, Pullman, Washington, USA), mouse anti - bovine CD4 conjugated to alexafluor 647 (CC30, AbD Serotec, Oxfordshire, UK), mouse anti - bovine CD8 conjugated to alexafluor 647 (CC63, AbD Serotec, Oxfordshire, UK) and alexafluor 488 Zenon labelled (Invitrogen Life Technologies, Paisley, UK) mouse anti - bovine γδ TCR1 (GB21a; WSU Monoclonal Antibody Centre, Pullman, Washington, USA). Finally, labelled cells were washed in flow cytometry buffer and resuspended in 150 µL of 2 % paraformaldehyde

(Cytofix; BD Biosciences, Oxfordshire, UK) for at least 30 min at 4 °C before analysis on a CyAn ADP analyser. For capture and analysis, initial gating was on single, NIRViD^{lo} (live) cells into a subsequent lymphocyte gate before gating on CellTrace Violet^{lo} cells and assessing surface phenotype (figure 2:4).

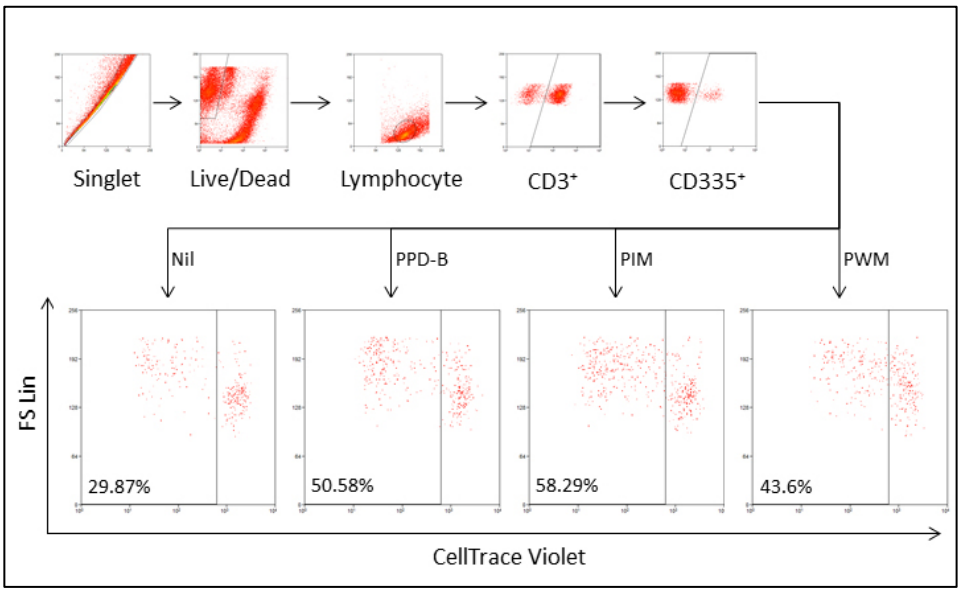

Figure 2:4 - Example of gating strategy for analysis of proliferative cells
Flow cytometric gating strategy: single, live CD3$^+$ CD335$^+$ lymphocytes are assessed for CellTrace Violet labelling and CellTrace^{lo} cells gated for phenotyping. Numbers represent the percentage of proliferating cells in response to each antigen.

Monoclonal Antibody Blocking of MHCII and CD1

Bovine PBMC were isolated as described above and incubated for 2 hours in the presence of either anti - equine MHCII (MCA1085; AbD Serotec), anti - ovine CD1 (MCA2212; AbD Serotec), anti - galline Bu - 1a/b as an isotype control (MCA5764; AbD Serotec) or no monoclonal antibody. All antibodies were sterile and contained no

preservatives. After 2 hours, antigens were added and proliferation measured as described for the Lymphocyte Transformation Assay

Removal of Lipopeptide by Proteinase Treatment

The use of Proteinase K to remove lipopeptide has been previously described[257]. For treatment, 20 µg of lipid was combined with 5 µg of Proteinase K from *Tritirachium album* (Sigma Alrdich; P6556) in PBS in a PCR plate. Using a thermocycler, the mixture was incubated at 50 ^{o}C for 30 minutes and the Proteinase subsequently inactivated by increasing the temperature to 80 ^{o}C for a further 30 minutes. After inactivation, the samples were chilled to 4 ^{o}C. Mock treatment was performed by incubating the 5 µg of Proteinase K at 80 ^{o}C for 30 minutes before adding 20 µg of lipids and continuing the treatment as above. Proteinase K treated and mock treated lipid antigens were suspended as described above and used to stimulate PBMC as described for the Lymphocyte Transformation Assay.

Data & Statistical Analysis

All data representation and statistical analysis was performed using GraphPad Prism version 5 and GraphPad InStat version 3. Densitometry analysis of TLCs was performed using the gradient selection tools in Quantity One version 4.6.9. All flow cytometric data was captured using a 9 colour Beckman Coulter CyAn ADP analyser and data was captured and analysed using Summit v4.3.

Chapter Three

Preparation & Characterisation of Crude Lipid Extracts

Background

To enable analysis of specific *M. bovis* - derived lipids, and subsequently the host response to them, lipids first need to be extracted from bacterial cells. Furthermore, the content of these extracts must be analysed to show the presence of lipids and allow for basic identification of specific lipid moieties.

The extraction of bacterial lipids was first described in 1959 by Bligh and Dyer[258] and was further pioneered by Minnikin[128, 259, 260]. Extraction of free lipids essentially removes the outer - membrane or leaflet of the mycobacterial cell wall, leaving the long chain molecules, such as the mycolic acids, intact in the defatted cells[259, 260]. The process (illustrated in figure 2:1) involves treatment of freeze dried bacterial cells with a mixture of methanol, saline and petroleum ether. From this mixture, the cells and aqueous phase are removed using chloroform and methanol leaving the extractable free apolar lipids. Further treatment of the secondary extract with chloroform and saline yields the remaining polar lipids.

Primary analysis of these lipid extracts is usually performed using 5 specially developed thin - layer chromatography (TLC) systems (as detailed in table 2:1). Four of these systems (A - D) are used to analyse the apolar lipids, while the polar fraction was analysed with using System D and E.

These systems cover the polarity range of the extracted lipids; system A is used to detect triacyl glycerols (TAG), menaquinones (MQ) and the phthiocerol family of mycocerosic acids (phthiocerol dimycocerosates; PDIMs)[261]. Identification of unesterified fatty acids and acyltrehaloses is performed using system B, the second least polar system[252]. The third, slightly more polar, system C enables detection of more fatty acids and some glycosides, which are structurally related to the PDIMs. System D, the most polar system used in the analysis of the non - aqueous petroleum ether extracts, resolves the trehalose based mycolates (such as trehalose dimycolate [TDM] or cord factor), the acylated trehaloses such as diacyl trehalose (DAT) and the sulpholipids (such as SL - 1).

As well as the petroleum ether extracts, system D is also used to analyse the least polar entities in the chloroform : methanol extracts and enables identification of the glycopeptidolipids and small amounts of sulpholipids. The most polar TLC system is only useful in the analysis of the chloroform : methanol extracts and was introduced to the standard array of systems in 1966[262]. The system primarily resolves glycolipids and phosphatide based lipids such as diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) and its mannosides (PIMs).

TLC plates may be stained to further aid identification of individual lipid classes and functional residues. Treatment with MPA, usually dissolved in ethanol or another polar solvent, stains phenolics, hydrocarbon based waxes and alkaloids. Used as a general stain, it allows identification of any lipid molecules which appear brown after staining and subsequent heating. Upon charring, reduction of MPA (Mo^{6+}) to Mo^{5+} and Mo^{4+} compounds by organic compounds causes the production of dark spots against a light green background. Alternatively, plates may be sprayed with a solution of ninhydrin dissolved in *n* - butanol. Originally discovered accidentally by Siegfried Ruhemann in 1910[263] who observed the ability of the compound to react with amine residues forming a purple coloured product[264], ninhydrin was originally used to detect trace amounts of amino acids in biological samples[265, 266] and has been used to detect amino acids in TLC since the 1950s[267] and has been used in the detection of fingerprints since 1959[268]. The formation of either Ruhemann's Purple upon reaction of ninhydrin with a primary amine or a light yellow compound upon reaction with a secondary amine allows for the identification of lipopeptides co - extracted with the lipid fractions.

While initial experiments were performed using AF 2122/97 - derived lipids (see Chapter Four), biosafety concerns over the high cell mass required for large scale lipid extraction meant that an alternative source of high volume mycobacterial culture was required. To this end, pellicle grown *M. bovis* AN5 was sourced and used for subfractionation work (see Chapter Five). This chapter documents the extraction of lipids from both the reference strain of *M. bovis*, AF 2122/97 and the PPD - B production strain, AN5 and the identification of the individual lipid components by 2D TLC.

This chapter addresses the hypothesis that lipids from the virulent *M. bovis* strains AF 2122/97 and AN5 could be extracted based on their solubility in aqueous and non - aqueous solvents. These fractions could be subsequently characterised, quantified and their main components identified using previously published analysis of mycobacterial lipids[252].

Results

Extraction & Analysis of Lipids from *M. bovis* AF 2122/97

Lipids were extracted from the reference strain of *M. bovis* (AF 2122/97) using the methods described above and were stained using both MPA and ninhydrin. AF 2122/97 - derived lipids are shown in figure 3:1 (MPA stained) and figure 3:2 (ninhydrin stained).

Analysis of the petroleum ether extract (containing the apolar fraction) with the least polar solvent system (figure 3:1 A) enabled the identification of TAG, MQ and the PDIMs. System B (figure 3:1 B) resolved pentacyl trehalose (PAT) and the co - located monomycolyl glycerol (MMG) and phenolic glycolipid (PGL), a lipid unique to *M. bovis*. The use of solvent system C (figure 3:1 C) allowed further discrimination of MMG and PGL whilst the most polar constituents of the petroleum ether extract, the cord factors TDM and TMM and glucose monomycolate (GMM) were seen using system D (figure 3:1 D).

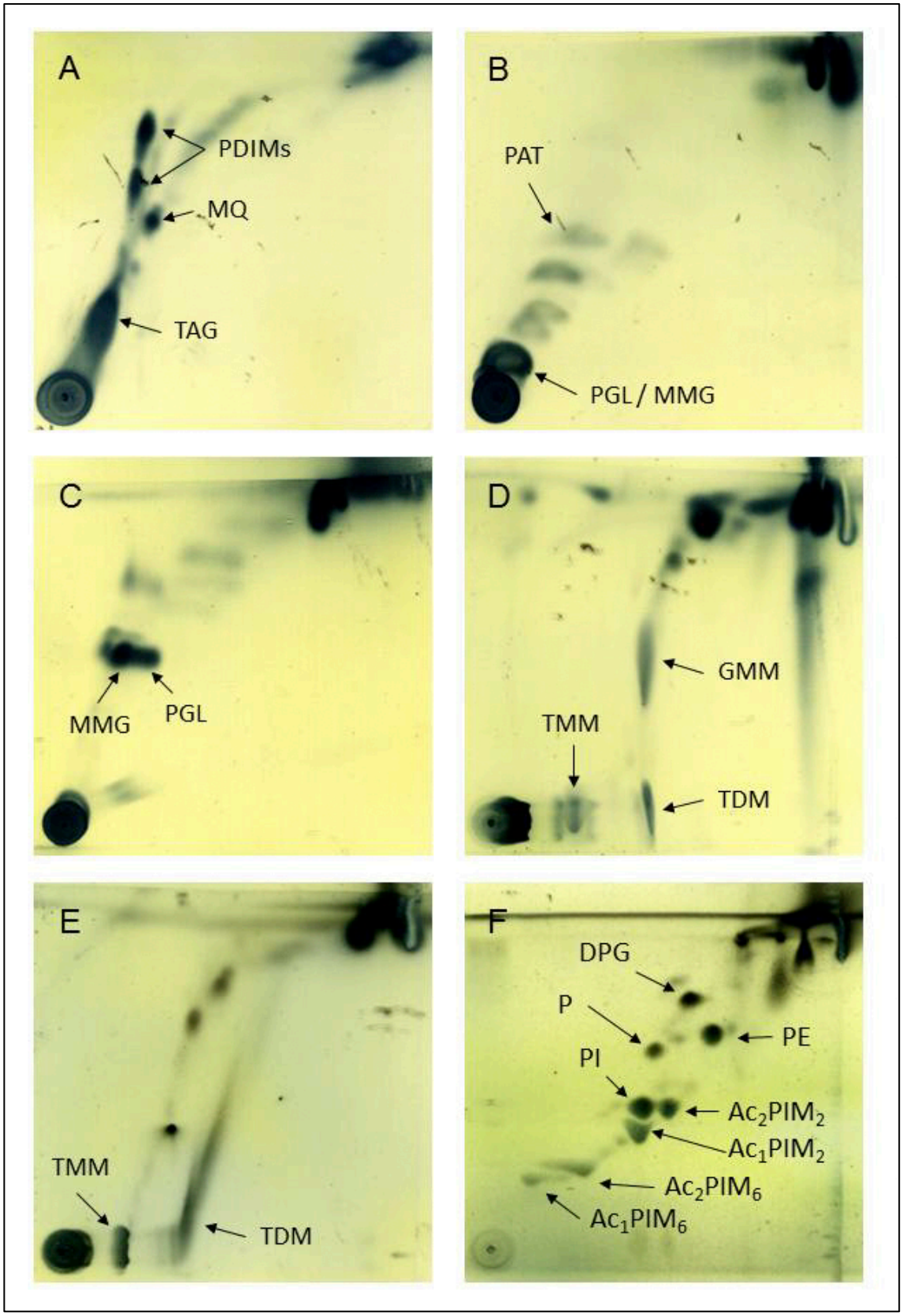

Figure 3:1 - 2D TLC analysis of crude, free lipids extracted from *M. bovis* AF 2122/97 and stained with MPA (A - D): Apolar fraction analysed with TLC systems A, B, C and D. (E and F): Polar fraction analysed with systems D and E.

Analysis of the chloroform : methanol extract (which contains the polar lipid fraction) with system D (figure 3:1 E) resolved TDM and TMM. More individual lipids were seen using the most polar system (figure 3:1 F) which mainly resolved a variety of PIMs and miscellaneous phospholipids.

Staining of plates with ninhydrin allows for the discrimination of amines and amino acids and is commonly used to assess the presence of lipopeptide in lipid preparations. As can be seen in figure 3:2 A - D, no ninhydrin staining can been seen in the petroleum ether fraction.

Analysis of the polar, chloroform : methanol extract, stained with ninhydrin showed no lipopeptide present in TLC system D (figure 3:2 E). In contrast, lipopeptide was resolved using system E (figure 3:2 F). A primary amine containing molecule was found co - located with the dimannoslylated PIMs (figure 3:2 F, red arrow) and an unknown secondary amine containing molecule was seen to have migrated near to the PE spot (figure 3:2 F, yellow arrow).

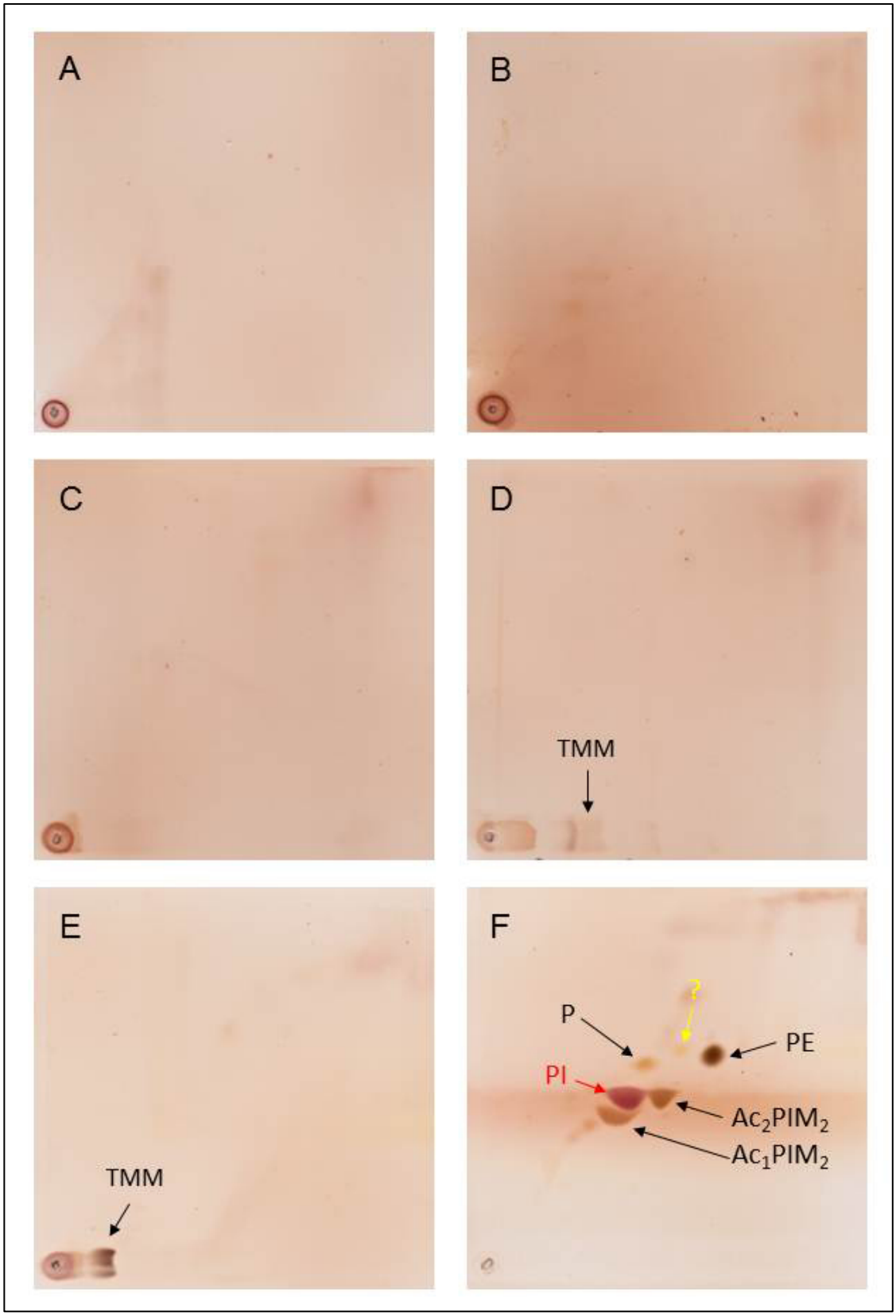

Figure 3:2 - 2D TLC analysis of crude, free lipids extracted from *M. bovis* AF 2122/97 and stained with ninhydrin (A - D): Apolar fraction analysed with TLC systems A, B, C and D. (E and F): Polar fraction analysed with systems D and E. Red arrows indicate primary amines; yellow arrows indicate secondary amines; black arrows indicate charred lipid for reference. Identifiable spots are named.

Extraction & Analysis of Lipids from *M. bovis* AN5

To perform larger scale lipid extraction for subsequent subfractionation, a far greater bacterial cell mass was required than had been used thus far (see Chapter Five). From a biosafety perspective, culturing such large volumes of *M. bovis* AF 2122/97 was considered unreasonable as high yield, pellicle grown *M. bovis* AN5 was already available for use. *M. bovis* AN5 - derived crude lipid extracts were analysed using the same solvent systems (table 2:1) and stained with the same reagents (MPA and ninhydrin) as shown for AF 2122/97 lipids.

As was seen for AF 2122/97 - derived lipids, the apolar fraction, resolved using system A, contained TAG, MQ and the PDIMs (figure 3:3 A). PAT, MMG and PGL were identified using system B (figure 3:3 B) and MMG and PGL further separated with the use of system C (figure 3:3 C). TMM, TDM and GMM were again resolved using system D (figure 3:3 D).

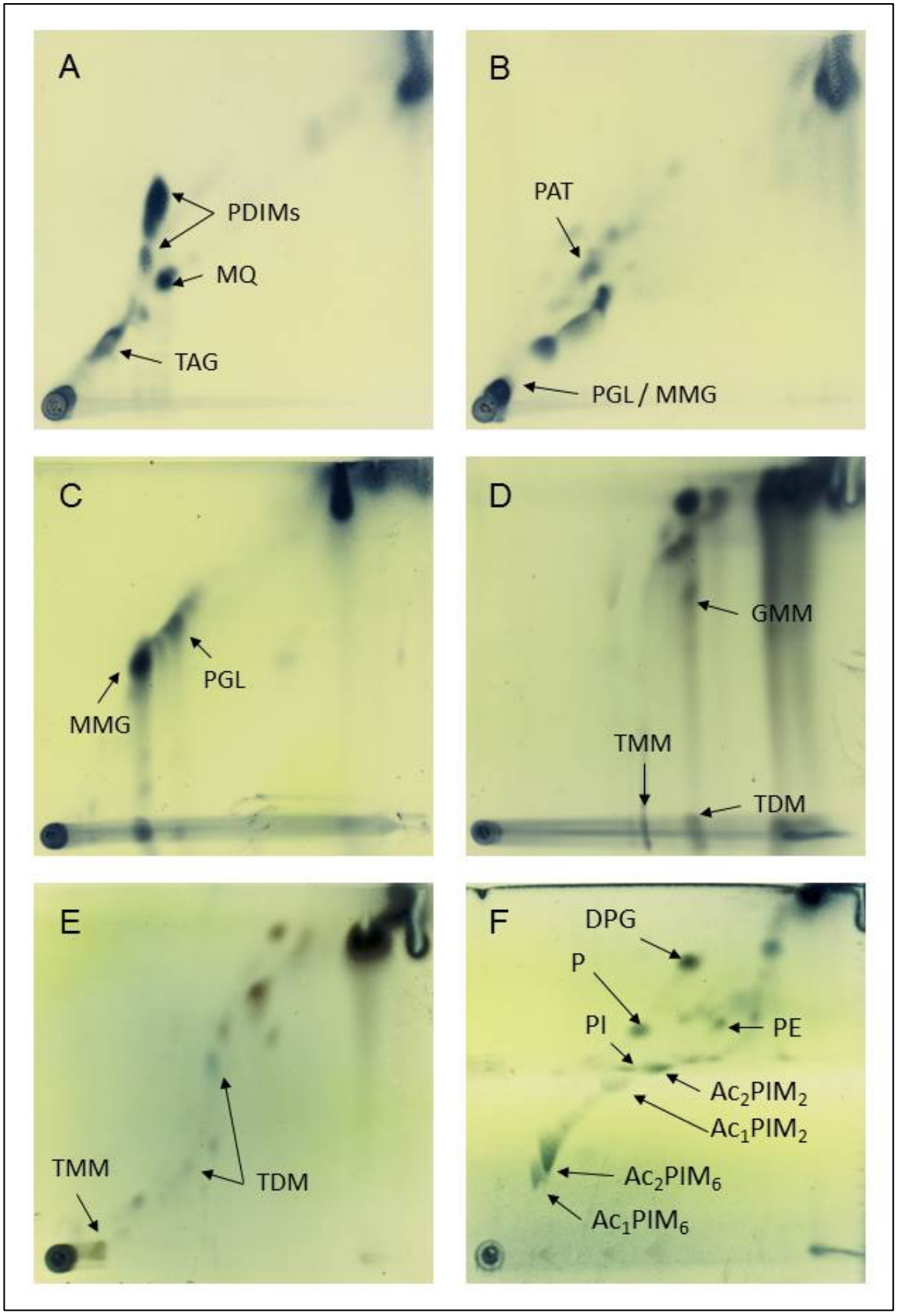

Figure 3:3 - 2D TLC analysis of crude, free lipids extracted from *M. bovis* AN5 and stained with MPA
(A - D): Apolar fraction analysed with TLC systems A, B, C and D. (E and F): Polar fraction analysed with systems D and E.

Similarly, the polar fraction from *M. bovis* AN5 contained TMM and TDM (figure 3:3 E) and the same array of PIMs, phospholipids, PE and PI (figure 3:3 F) as the *M. bovis* AF 2122/97 - derived lipids (figure 3:1 E - F).

As was seen with AF 2122/97 - derived lipids, ninhydrin staining of the *M. bovis* AN5 - derived lipid fractions showed no lipopeptide present in the apolar fraction (figure 3:4 A - D) and no ninhydrin staining was seen in the polar fraction separated with system D (figure 3:4 E).

Finally, ninhydrin staining of system E revealed amine containing molecules co- locating with the dimannoside PIMs (figure 3:4 F, red arrow) and an unknown secondary amine located close to the PE spot (figure 3:4 F, yellow arrow).

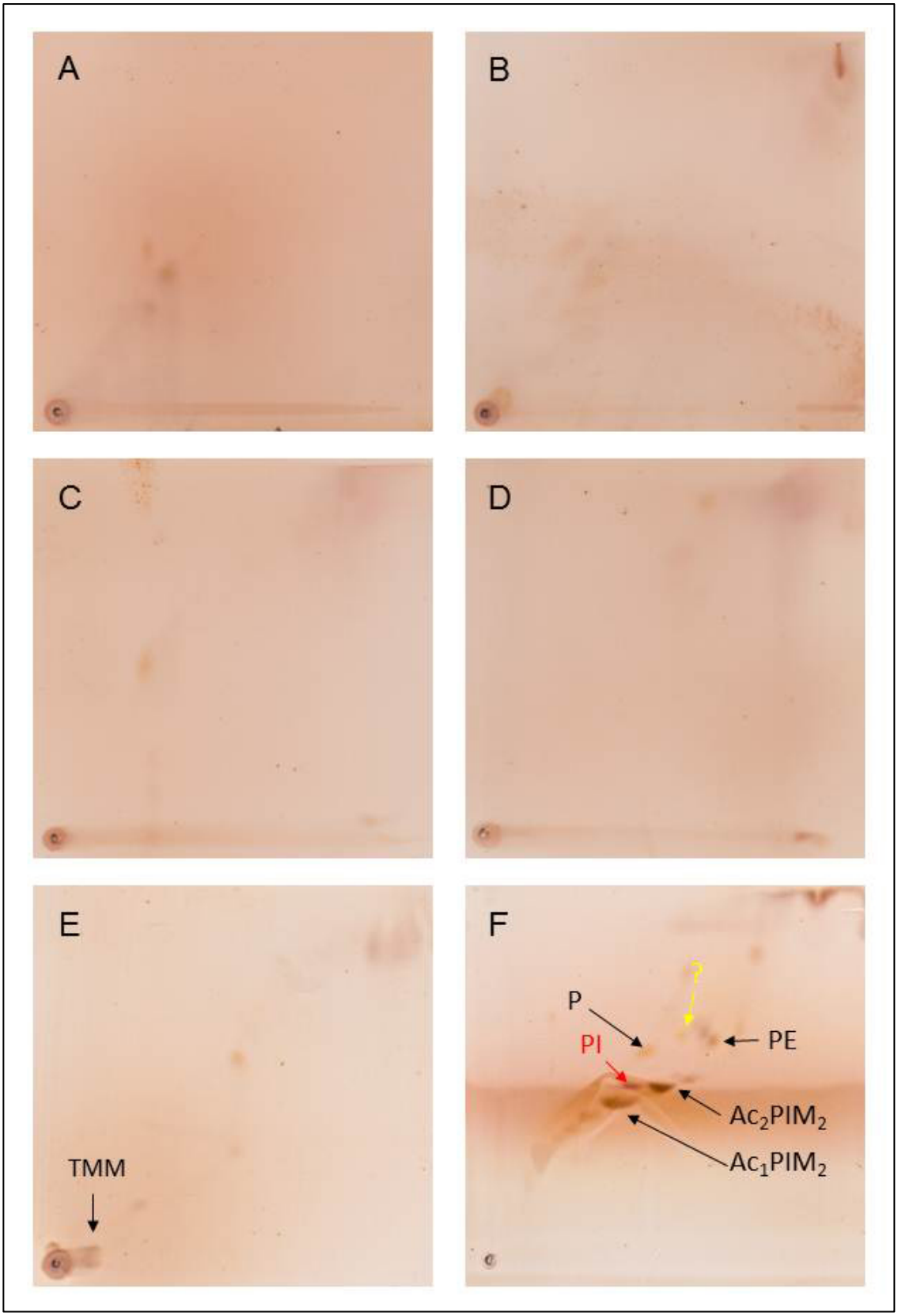

Figure 3:4 - 2D TLC analysis of crude, free lipids extracted from *M. bovis* AN5 and stained with ninhydrin (A - D): Apolar fraction analysed with TLC systems A, B, C and D. (E and F): Polar fraction analysed with systems D and E. Red arrows indicate primary amines; yellow arrows indicate secondary amines; black arrows indicate charred lipid for reference. Identifiable spots are named.

Abundance Analysis of the Crude Lipid Fractions

The use of densitometry analytical techniques allows for the quantification of individual lipids on the TLC plates and thus enables calculation of the relative abundance of each lipid. As the same quantity of each crude lipid fraction (100 µg) was loaded onto each TLC plate, it is possible to directly compare all of the plates containing lipids from either the polar or apolar fractions. Of note, differences in the separation of the spots made the selection of individual spots challenging which introduced a degree of variability into the quantification.

Using false coloured TLC images of the AF 2122/97 - derived polar fraction (figure 3:5 A - D) the abundance of the individual lipids is shown in table 3:1.

In total, around 16 % of the apolar lipid fraction can be identified by this method. The largest proportion of identifiable lipid is made up of TAG (4.77 % of the apolar fraction) followed by the PDIMs (3.81 %). In system B (figure 3:5 B) PGL and MMG co - locate on the plate and cannot be resolved individually, however in system C (figure 3:5 C) they can be resolved separately. MMG makes up the larger proportion of these lipids (2.15 % of the total apolar fraction) while PGL accounts for 1.2 % of the total lipid preparation. GMM, visible in system D (figure 3:5 D) makes up 1.62 % of the total fraction and the remaining lipids all account for less than 1 % of the fraction each (MQ: 0.89 %, PAT: 0.79 %, TDM: 0.72 % and TMM: 0.60 %).

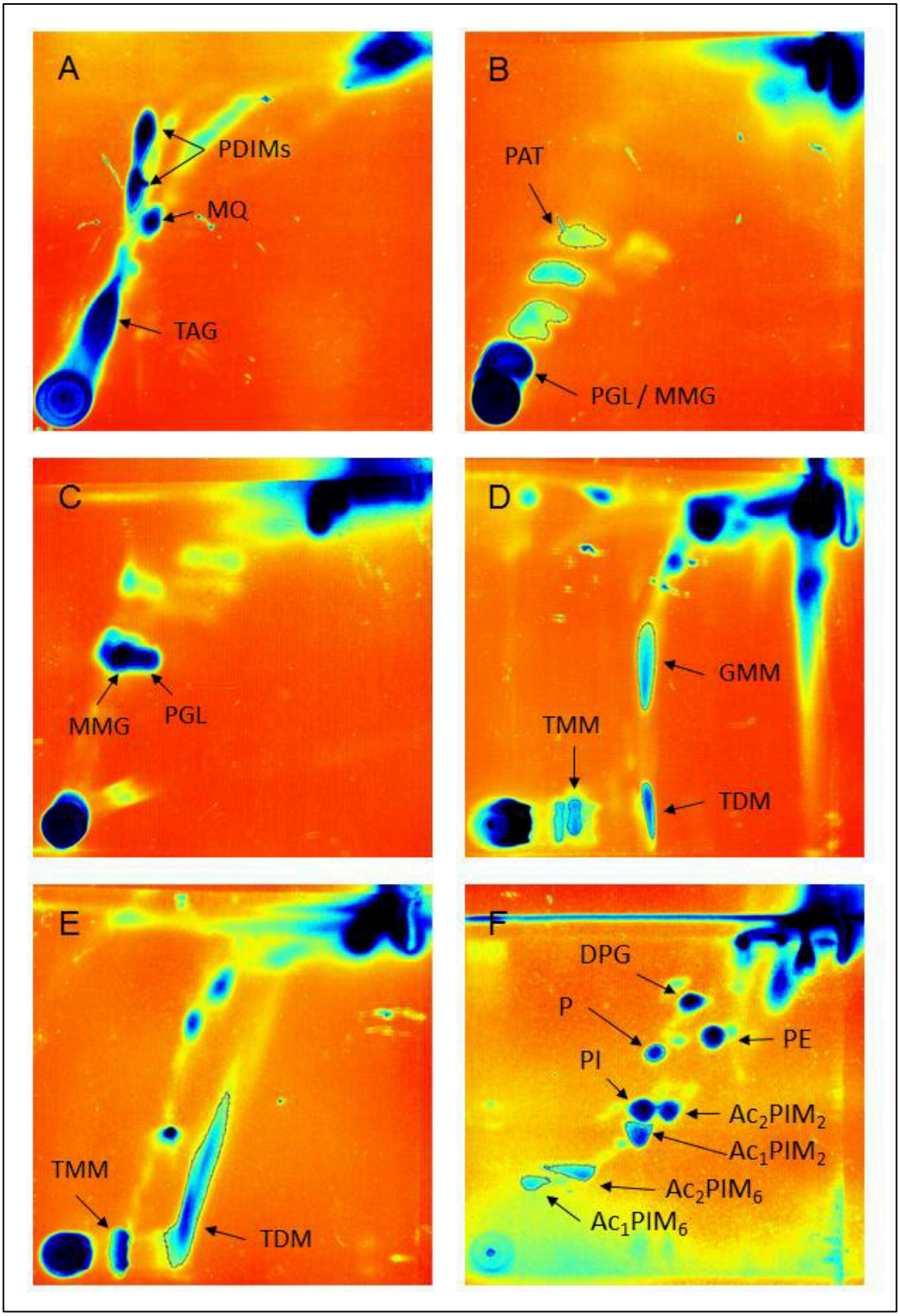

Figure 3:5 - False coloured densitometry analysis of lipids extracted from *M. bovis* AF 2122/97 and analysed by 2D TLC (A - D): Apolar fraction analysed with TLC systems A, B, C and D. (E and F): Polar fraction analysed with systems D and E.

As shown in table 3:2, within the AF 2122/97 - derived polar lipid fraction visible in systems D and E (figure 3:5 E and F) TDM is the most abundant identifiable lipid species (4.04 %) with TMM second comprising 1.22 % of the fraction. The lipids resolved by system E (figure 3:5 F) make up less than 1 % of the total polar fraction each, however in total these 8 lipid moieties account for 4.09 % of the fraction.

Analysis of the AN5 - derived polar fraction (figure 3:6 A - D) was also performed and the abundance of the individual lipids is shown in table 3:1.

In total, around 9 % of the apolar lipid fraction can be identified by this method. The largest proportion of identifiable lipid is made up of the PDIMs (3.36 % of the apolar fraction) followed by MMG (1.36 %), TAG (1.1 %) and MQ (0.94 %). The remaining 2 % of identifiable lipids consisted of PAT (0.58 %), PGL (0.5 %), TDM (0.43 %), GMM (0.37 %) and TMM (0.15 %).

In total, only 4.5 % of the AN5 - derived polar fraction could be identified (figure 3:6 E and F; table 3:2). The largest component was Ac_1PIM_6 (1.59 %) followed by TDM (0.93 %). Ac_2PIM_6 and DPG were present in similar quantities (0.46 %) and the remaining 1 % was accounted for by P (0.28 %), Ac_2PIM_2 (0.26 %),TMM (0.21 %), PE (0.16 %), Ac_1PIM_2 (0.08 %) and PI (0.07 %).

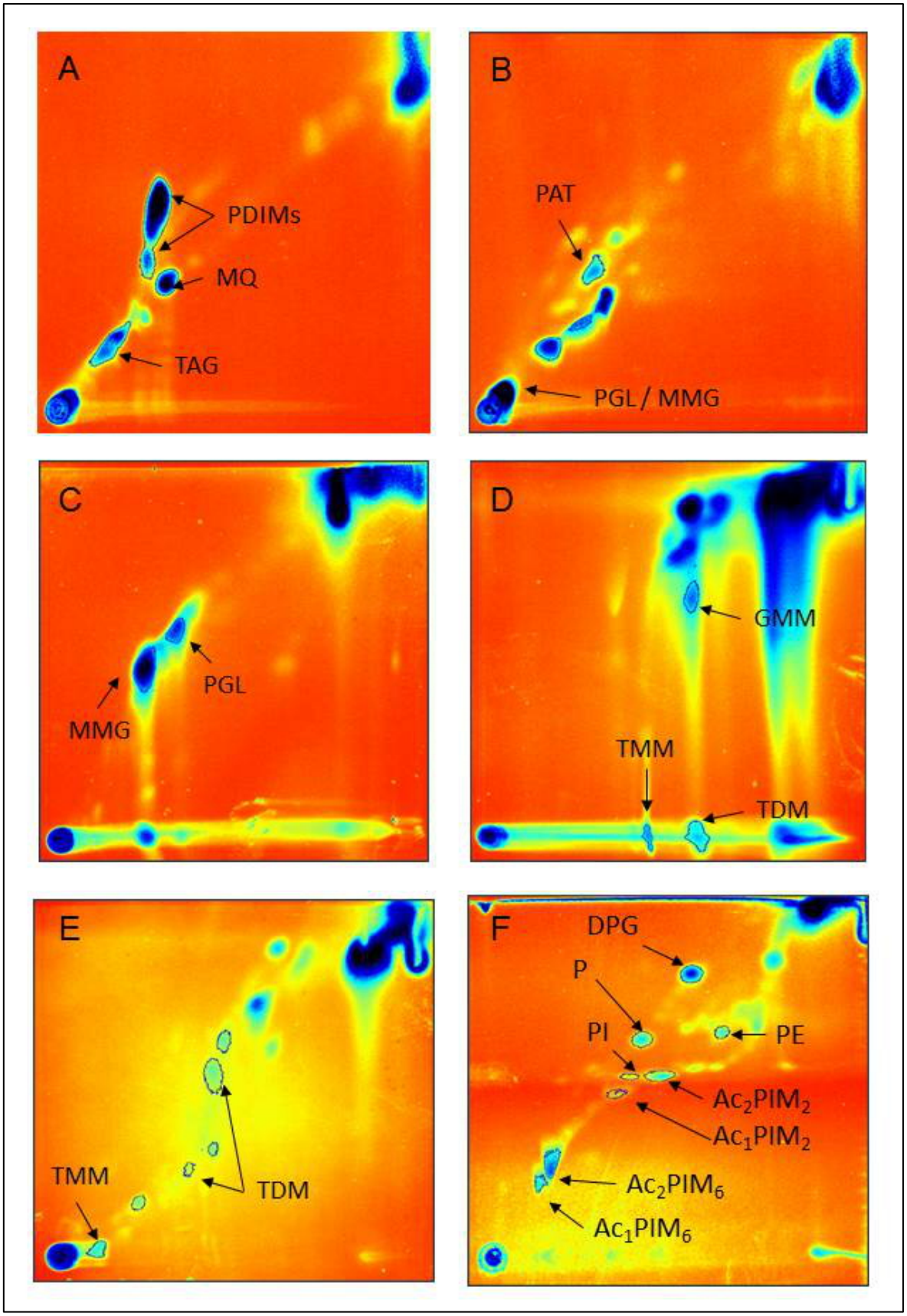

Figure 3:6 - False coloured densitometry analysis of lipids extracted from *M. bovis* AN5 and analysed by 2D TLC (A - D): Apolar fraction analysed with TLC systems A, B, C and D. (E and F): Polar fraction analysed with systems D and E.

Table 3:1 - Densitometry of the apolar lipid fractions.
Volumes (intensity x area) are used to calculate to amount of total identifiable lipid within the apolar fractions.
*Lipids are co - located and inseparable in this system and have not been used as part of the total calculation.

Solvent System	Identified lipids	% Total lipid AF 2122/97	% total lipid AN5
A	TAG	4.77	1.10
	MQ	0.89	0.94
	PDIMs	3.81	3.36
B	PGL / MMG	3.44*	0.93*
	PAT	0.79	0.58
C	MMG	2.15	1.36
	PGL	1.20	0.50
D	TMM	0.60	0.15
	TDM	0.72	0.43
	GMM	1.62	0.37
Total identifiable lipids		16.54 %	8.79 %

Table 3:2 - Densitometry of the polar lipid fractions.
Volumes (intensity x area) are used to calculate to amount of total identifiable lipid within the polar fractions.

Solvent System	Identified lipids	% Total lipid AF 2122/97	% total lipid AN5
D	TMM	1.22	0.21
	TDM	4.04	0.93
E	Ac_1PIM_6	0.28	1.59
	Ac_2PIM_6	0.53	0.46
	Ac_1PIM_2	0.62	0.08
	Ac_2PIM_2	0.43	0.26
	PI	0.69	0.07
	P	0.37	0.28
	DPG	0.52	0.46
	PE	0.66	0.16
Total identifiable lipids		9.35 %	4.50 %

Using these methods it was possible to identify approximately 25 % of the total lipids extracted from *M. bovis* AF 2122/97 and approximately 13 % of those from *M. bovis* AN5. Whilst identical compounds could be identified qualitatively in the lipid profiles of both strains, there were obvious quantitative differences. Thse will be discussed in the following section.

Discussion

Extraction of mycobacterial lipids and their subsequent analysis by 2D TLC has been previously described for *M. tuberculosis* [269], but little data exists on total lipid profiling of *M. bovis*. Previous work by Dandapat *et al.*[270] attempted to characterise *M. bovis* AN5 based on the expression of PGL and PDIMs, but only as a tool for identification of the organism. Further, the use of *M. bovis* AN5 may not have been representative of the AF 2122/97 reference strain. Presented here is the first biphasic extraction and complete analysis of the lipids of *M. bovis* AF 2122/97.

In figure 3:1, the use of MPA staining allowed the identification of a broad range of characteristic mycobacterial lipids including PDIMS (figure 3:1 A), the *M. bovis* characteristic PGL[271] (figure 3:1 B and C), TDM (figure 3:1 D and E) and PIMs (figure 3:1 F). As expected, no sulphoglycolipid was found (figure 3:1 D)[210, 211]. Interestingly, TDM was found in both the polar and apolar extracts (figure 3:1 D and E). This may be related to its particularly amphipathic nature[272] and variable acylation states which are known to alter its hydrophobicity[273, 274] and may cause the molecule to split differentially across the biphase interface during lipid extraction.

No systematic lipopeptide profiles have been published for any mycobacterial species, despite the fact that lipopeptides from a variety of mycobacterial species have been shown to modulate immune responses[275-277] and that some responses attributed to lipids may be driven by lipopeptide instead[257].

Analysis of the AF 2122/97 - derived lipid fractions by ninhydrin indicated no lipopeptide presence in the apolar fraction (figure 3:2 A - D) but both primary and secondary amine containing molecules in the polar lipid fraction (figure 3:2 F). Interestingly, one primary lipopeptide co - locates precisely with PI suggesting that the amine containing molecule may be physically associated with the lipid. A secondary amine was seen which located near the PE spot (figure 3:2 F). The identity of both these molecules is unknown, as is the reason for their co - location with the lipid molecules although it may be that any lipopeptide present is chemically bonded with the lipids.

Analysis of the *M. bovis* AN5 - derived lipids with the standard TLC systems and stained with MPA (figure 3:3) allowed for a comparison between the 2 bacterial strains. Overall, the same lipid molecules were present in the apolar (figure 3:3 A - D) and polar (figure 3:3 E - F) lipid fractions. However, it is clear that the lipids derived from *M. bovis* AN5 do not separate in exactly the same manner as those from AF 2122/97 and form spots of different shapes. From the analysis performed here it is not possible to tell if these differences are due to the synthesis of inherently different forms of these lipids in the 2 strains, or if the differing conditions of bacterial culture have affected lipid synthesis; a factor known to play a role in the lipid production of mycobacteria[278, 279].

Lipids from *M. bovis* AN5 were also stained with ninhydrin (figure 3:4). Although not as clear as for AF 2122/97 - derived lipids, the primary amine staining co - located with PI is present in AN5 - derived lipids, as is the secondary amine seen located near the PE spot (figure 3:4 F).

It is worth noting that the lipids from *M. bovis* AN5 separated less cleanly than those from AF 2122/97. This is most clearly demonstrated in the most polar solvent systems used to analyse the apolar lipids (figure 3:3 C - D) where lipids dissolved in the mobile phase have left streaks or smears in both directions of the TLC. Such smearing or streaking is often due to overloading of the analyte on the TLC plates, however this is unlikely in this instance. Each plate shown in figure 3:1, figure 3:2 and figure 3:4 has 100 µg of lipid loaded at the origin and spots are consistently located and well defined. Perhaps more likely is that damage was caused to some of the AN5 - derived lipid molecules by autoclaving at 134 ^{o}C for 1 hour. For technical reasons, it was not possible to heat kill the pellicle grown *M. bovis* AN5 by the same method as the AF 2122/97, which was heated to between 80 ^{o}C and 90 ^{o}C for 1 - 2 hours, and this extra heat may have denatured some of the lipid structures causing them to dissolve into or precipitate out of the mobile phase less efficiently.

The effect of growth as a pellicle upon lipid composition is not well understood, despite the fact that growth at the interface between medium and air plays an important role in many other bacterial genera. *Vibrio cholerae*, some streptococci and staphylococci are known to form biofilms which have been implicated in their virulence[(280-283)]. It has been suggested that *M. tuberculosis* is capable of forming biofilms and some evidence has been found that biofilm - like structures form in guinea pig lung lesions which enhance antimicrobial resistance and virulence of the bacteria[(284)]. However little information exists on changes in lipid profile, synthesis or metabolism when mycobacteria are cultured in their planktonic form. It has been shown that the use of carbon limiting chemostats to replicate bacterial growth rates of active infection and a slower dormant

phenotype led to significant changes in total lipid composition of BCG[285]. However, this study used a single lipid extraction and subsequent elemental analysis by combustion to ascertain the authors' findings. Other work has demonstrated an alteration of the transcriptional response which led to the identification of a novel wax ester when *M. tuberculosis* was grown in iron limited conditions[278]. It is also known that keto - mycolic acids are essential for pellicle formation by *M. tuberculosis*[286] and that pellicle formation can allow drug resistant bacteria to replicate[287].

Overall, the lipid profiles obtained by extraction of the apolar and polar fractions from both AF 2122/97 and AN5 are remarkably similar. All lipids migrated to the same locations upon 2D TLC analysis and all lipids of known identities were present in both strains.

In an attempt to further characterise the lipid fractions, densitometry analysis was performed to allow relative quantification of the lipids present in the polar and apolar subfractions. This technique is more commonly used for imaging gels analysed using a gel documentation system, however it was possible to load in high resolution images of the TLC plates, false colour them and select individual spots for analysis (figure 3:5 and figure 3:6).

Using the images of the AF 2122/97 - derived lipids, each spot of a known identity was selected, along with the entire plate. As the plates shown in figure 3:5 A - D all contain 100 µg of the apolar lipid fraction it is possible to collate the information for the total lipid on each plate and calculate the volume of each individual spot. In total, the

identifiable apolar lipids from AF 2122/97 constitute just over 16 % of the total, MPA stained lipid in the fraction (table 3:1). Similarly, just over 9 % of the polar lipids could be identified (table 3:2).

Interestingly, a smaller proportion of the AN5 - derived lipid fractions could be quantified despite the presence of the same lipid molecules. As discussed above, growth conditions could have played a significant role in the different relative abundances of the lipid molecules. It is also likely that autoclave treatment of the AN5 pellicle, and the subsequent damage to the lipid molecules, affected the ability to perform the densitometry analysis. More spreading and smearing of lipid was present in the AN5 - derived lipids upon TLC analysis which compromised the ability of the analysis software to select the edges of the spots. Therefore it is possible that more identifiable lipid is present than can be reported here.

These data demonstrate that considerably more lipid is present in these fractions than can be identified with these TLC systems. Further evidence of this can be seen in the solvent front and origins of the TLC plates. The least polar solvent system used (system A) allows only the least polar lipids to dissolve into the mobile phase and migrate over the plate. However, some extremely apolar lipid material is contained within the solvent front and can be seen in the apolar lipids from both AF 2122/97 (figure 3:1 A) and AN5 (figure 3:3 A).

Similarly, system E is the most polar solvent system currently in use and allows the most polar lipids to dissolve. However MPA stained lipid material is still present at the origin

(figure 3:1 F and figure 3:3 F) suggesting that lipid, or lipid containing, material of greater polarity is present in the fraction which requires further analysis in more polar solvents.

In conclusion, this chapter demonstrates the ability to chemically extract aqueous and non - aqueous lipid fractions from virulent strains of *M. bovis*. Further, it was possible to characterise these fractions by MPA stained 2D TLC and identify the main lipid families contained within the fractions of both strains of *M. bovis*. Ninhydrin staining of these TLCs also enabled the discovery of lipopeptides present in the polar fraction from both strains. Finally, the application of densitometrical analysis allowed the quantification of the lipid species within the fractions. Having generated and characterised these fractions, both qualitatively and quantitatively, they could then be used to stimulate bovine innate immune responses.

Chapter Summary

- **Objective**

  The extraction of polar and apolar lipid fractions from *M. bovis strains* AF 2122/97 and AN5 and their subsequent characterisation by 2D TLC and densitometry

- **Results**

  Polar and apolar lipid fractions were successfully extracted from both *M. bovis* strains. A broad range of lipids was identified in both polar and apolar fractions and qualitative TLC analysis showed that AF 2122/97 and AN5 fractions consist of similar lipid species. Lipopeptide was identified in the polar fractions from both strains and these were qualitatively similar. Densitometrical analysis showed that identifiable lipids composed approximately 25 % of the AF 2122/97 - derived fractions. The AN5 - derived fractions were composed of 13 % identified lipids.

- **Conclusions**

  Polar and apolar lipid fractions can be chemically extracted from virulent *M. bovis* and have been characterised and quantified here for the first time. Further, the polar fraction has been shown to contain lipopeptides. Much of the content of the fractions is unknown and requires the development of new analytical techniques as the current TLC systems will not resolve these molecules.

Chapter Four

Effects of Crude Lipids on Bovine Innate Immune Cells

Background

It is widely accepted that the interaction between host and pathogen is critical in establishing an appropriate and effective immune response which controls the invading pathogen and protects the host from the effects of infection. The role of macrophages in protection against tuberculous infection was discovered over 60 years ago[160-162]. Much work was performed in the 1980s which lead to a greater understanding of the interaction between the macrophage and other host immune components including a method of macrophage mediated bacterial killing using hydrogen peroxide[163] and the critical role of IFNγ in the activation and enhancement of macrophages and their killing[164, 165]. Since then the interaction between mycobacterial species, usually *M. tuberculosis* or BCG, and APCs has been heavily studied.

Ligation of different receptors drives different effector functions, some specifically promoting phagocytosis (e.g. scavenger receptors) and others triggering non - phagocytic maturation or activation (such as the TLRs). It is the selective and multiple ligation of these receptors which dictates the effector function of the APC[167, 168]. Further, it has

been reported that *M. tuberculosis* interaction with TLR2 mediates TNFα release[174]. Genetic removal of TLR2 in murine models leads to impaired immune responses to *M. avium*[172] and recent work has shown impaired anti - mycobacterial activity of macrophages and reduced TNFα production in TLR2 deficient mice[173]. It has also been shown that triacylated lipomannans bound to the TLR2 - TLR1 heterodimer induced the production of IL - 12p40 and nitric oxide and that tetra - acylated lipomannans exerted the same effect but through TLR4[171]. In contrast, mannose receptor mediated phagocytosis of *M. tuberculosis* has been shown to generate Th2 T cell responses characterised by production of IL - 10, CCL22 and CCL17[178, 179] and ligation of the MR by mannosylated LAM (ManLAM) inhibits IL - 12 production[180].

The ability to derive DCs and macrophages from blood monocytes *in vitro* and harness their antigen presenting ability was first described by Sallusto & Lanzavecchia[288]. Previous to this, *in vitro* derivation of DCs required either cord blood or bone marrow precursor cells and was performed using GM - CSF and TNFα[289]. In their ground breaking work, Sallusto & Lanzavecchia demonstrated not only the ability to culture DC from peripheral blood monocytes, but that TNFα inhibits DC development and that IL - 4 enhanced it. This has since proven to be a major advancement, thanks to the simplicity of their method, and has led to a huge increase in the study of DCs.

It is clear that innate recognition of mycobacteria is critical in generating an appropriate immune response and that the mammalian immune system has many ways of sensing mycobacteria. This chapter addresses the hypothesis that the lipid fractions generated

and characterised in Chapter Three would be capable of driving functional and phenotypic responses in bovine antigen presenting cells.

Results

Characterisation of Cultured Monocytes and Monocyte Derived DC

$CD14^+$ monocytes were isolated from uninfected cattle and cultured for 3 days in the presence of either GM - CSF (cultured monocytes) or GM - CSF and IL - 4 (MDDC). Cultured monocytes displayed little morphological change from the freshly isolated cells (figure 4:1 A). However, after 3 days of culture in the presence of GM - CSF and IL - 4, the cells displayed characteristic DC - like morphology primarily characterised by flattening and the extension of dendritic processes (figure 4:1 B).

$CD14^+$ cells were also cultured for 3 days without the addition of cytokines to the medium. These cells did not display any flattening or process extension and appeared almost morphologically indistinguishable to those cultured with GM - CSF (not shown). The majority of the untreated cells did not survive 3 days of culture and, upon enumeration using trypan blue to discriminate live cells, yields were typically very low with only 10 - 15 % the cells viable. Further, upon subsequent flow cytometric analysis, up to 95 % of these trypan blue counted cells were considered dead after staining with ViViD, therefore these cells were not used in any further studies.

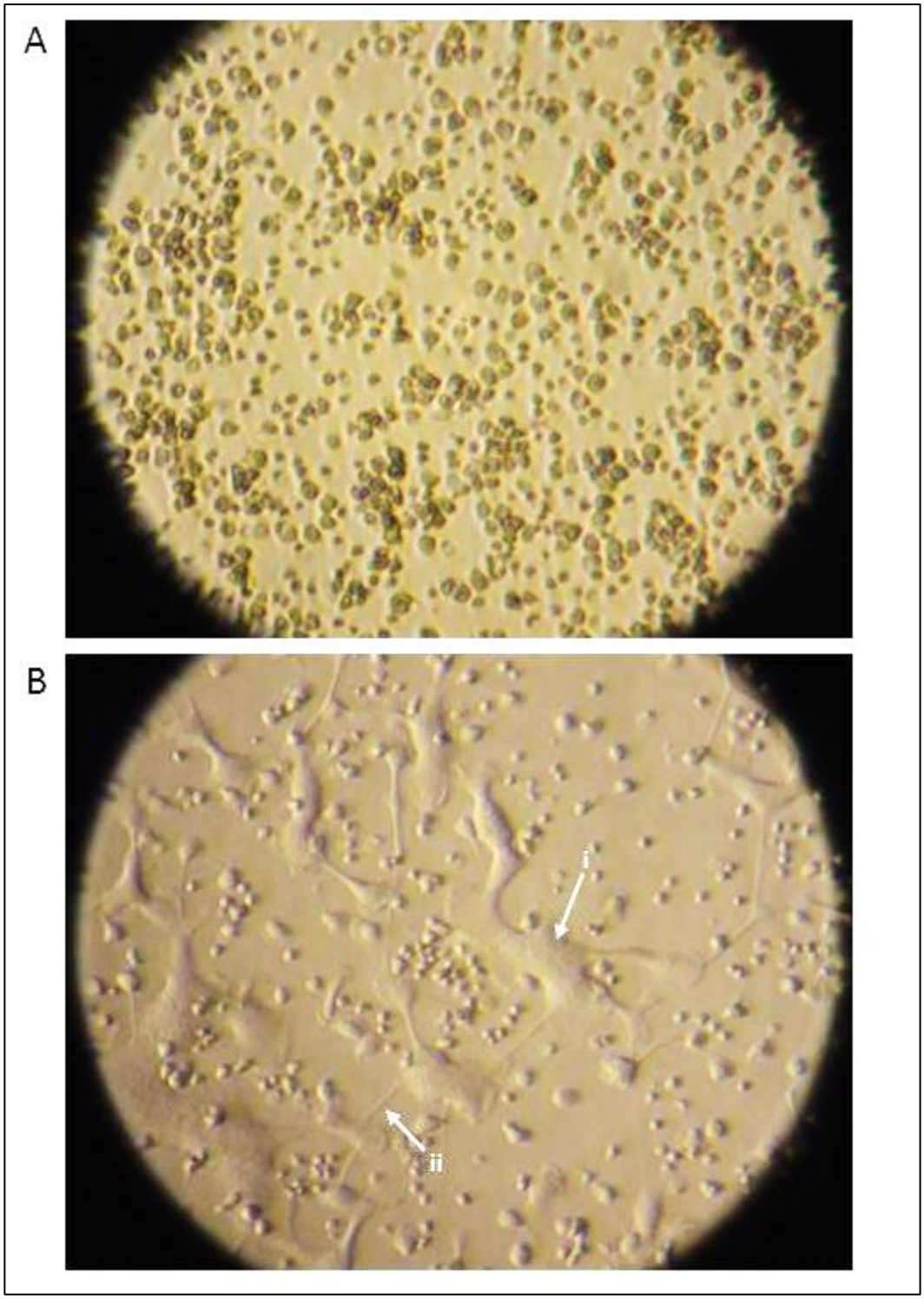

Figure 4:1 - $CD14^+$ cells after culture for 3 days in the presence of either GM - CSF or GM - CSF and IL - 4. (A) bovine GM - CSF; (B) bovine GM - CSF and IL - 4. Arrow (i) indicates flattening and enlarging of cells. Arrow (ii) indicates a dendritic cellular processes. Magnification 400x using phase contrast microscopy.

Having shown morphologic differences between the culture conditions, the cells were further characterised using flow cytometry to assess the expression of several key molecules to define their individual phenotypes.

Figure 4:2 summarises the normalised expression of the surface molecules MHCII (A), CD86 (B), CD40 (C), CD80 (D), and CD1b (E) on freshly isolated monocytes, GM - CSF treated cells (regarded as cultured monocytes [CM]) and GM - CSF and IL - 4 treated cells (regarded as MDDC). When comparing monocytes with CM, a trend was evident for lower expression of MHCII on cultured monocytes (figure 4:2 A). This small reduction in MHCII expression was accompanied by a similar trend for a reduction in CD86 expression. No difference was seen in the levels of CD40 expressed by either monocytes or CM (figure 4:2 C). CM expressed significantly more CD80 than monocytes expression when compared to monocytes (figure 4:2 D). Finally, neither monocytes nor CM expressed any CD1b (figure 4:2 E).

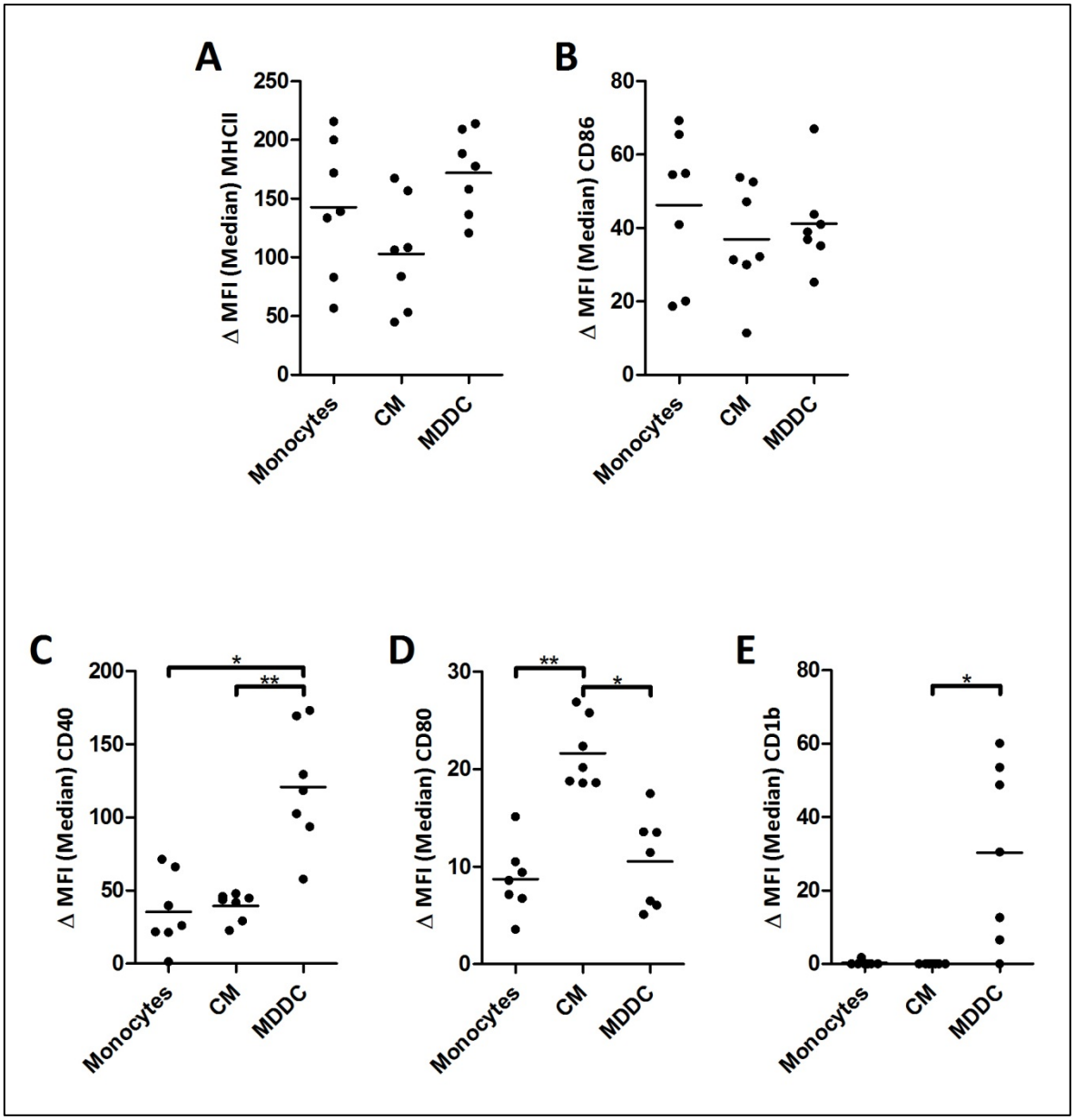

Figure 4:2 - Phenotype of fresh $CD14^{+}$ monocytes, cultured monocytes (CM) and cultured DC (MDDC) Median fluorescence intensity of (A) MHCII, (B) CD86, (C) CD40, (D) CD80, (E) CD1b. Points represent individual animals; bar represents mean Δ MFI. * $p < 0.05$, ** $p < 0.01$ using repeated measures ANOVA with Bonferroni multiple comparisons test.

Comparison of MDDC with monocytes showed that there was a trend for increased levels of MHCII expression on MDDC (figure 4:2 A). No difference was seen in the levels of CD86 expressed by either MDDC or monocytes (figure 4:2 B) or CD80 (figure 4:2 D). However, MDDC were found to express significantly higher levels of CD40 than

monocytes (figure 4:2 C) and, perhaps most strikingly, MDDC were the only cells to express the lipid - specific antigen presentation molecule CD1b (figure 4:2 E).

Finally, comparison of CM with MDDC showed a strong trend for higher levels of MHCII expression on MDDC (figure 4:2 A) and similar levels of CD86 expression on both cell types (figure 4:2 B). MDDC expression of CD40 was significantly higher than that seen on CM (figure 4:2 C) while CM expression of CD80 was significantly higher than that by MDDC (figure 4:2 D). As was seen with monocytes, MDDC expressed significantly increased levels of CD1b in comparison to CM, which expressed no CD1b (figure 4:2 E).

Overall, clear differences were apparent between bovine monocytes, cultured monocytes and MDDC. Cultured monocytes were characterised by their decreased expression of MHCII (Δ MFI = 114 compared to Δ MFI = 172 on monocytes) and CD86 (Δ MFI = 35 compared to Δ MFI = 50 on monocytes) combined with the subsequent significant increase in CD80 expression (Δ MFI = 23 compared to Δ MFI = 10 on monocytes; $p < 0.0005$).

MDDC were defined by their significantly higher levels of CD40 (Δ MFI = 126 compared to Δ MFI = 41 on monocytes; $p < 0.001$) and the presence of CD1b (Δ MFI = 30 compared to Δ MFI = 0 on monocytes and MDM). Furthermore, MDDC displayed a characteristic flattened morphology and the extension of long cellular process.

Cytokine Responses to Crude Mycobacterial Lipids

In order to assess the effect of *M. bovis* - derived lipids on these bovine innate cells, cytokine production was measured after overnight stimulation with the lipid fractions (figure 4:3).

Significantly increased IL - 10 secretion was seen from all 3 cell types following stimulation with the polar lipid fraction (figure 4:3 A). Strong IL - 10 responses were seen for 3 animals, while only modest increases were noted for the remaining cattle (figure 4:3 A). In contrast, little or no significant increase in IL - 10 production was seen following stimulation with the apolar lipid fraction, although apolar lipids induced some IL - 10 production by cultured monocytes and MDDC from 3 animals. All responses to apolar lipids were by far lower than those induced by the polar lipid fraction

IL - 12 levels in the culture supernatants were measured simultaneously and the results are shown in figure 4:3 B. Stimulation with the polar lipid fraction induced a large increase in IL - 12 production by MDDC, while lower responses were also observed from monocyte and cultured monocyte populations. In contrast to the polar lipids, stimulation with the apolar lipid fraction resulted in minimal increases in IL - 12 production by monocytes, cultured monocytes or MDDC.

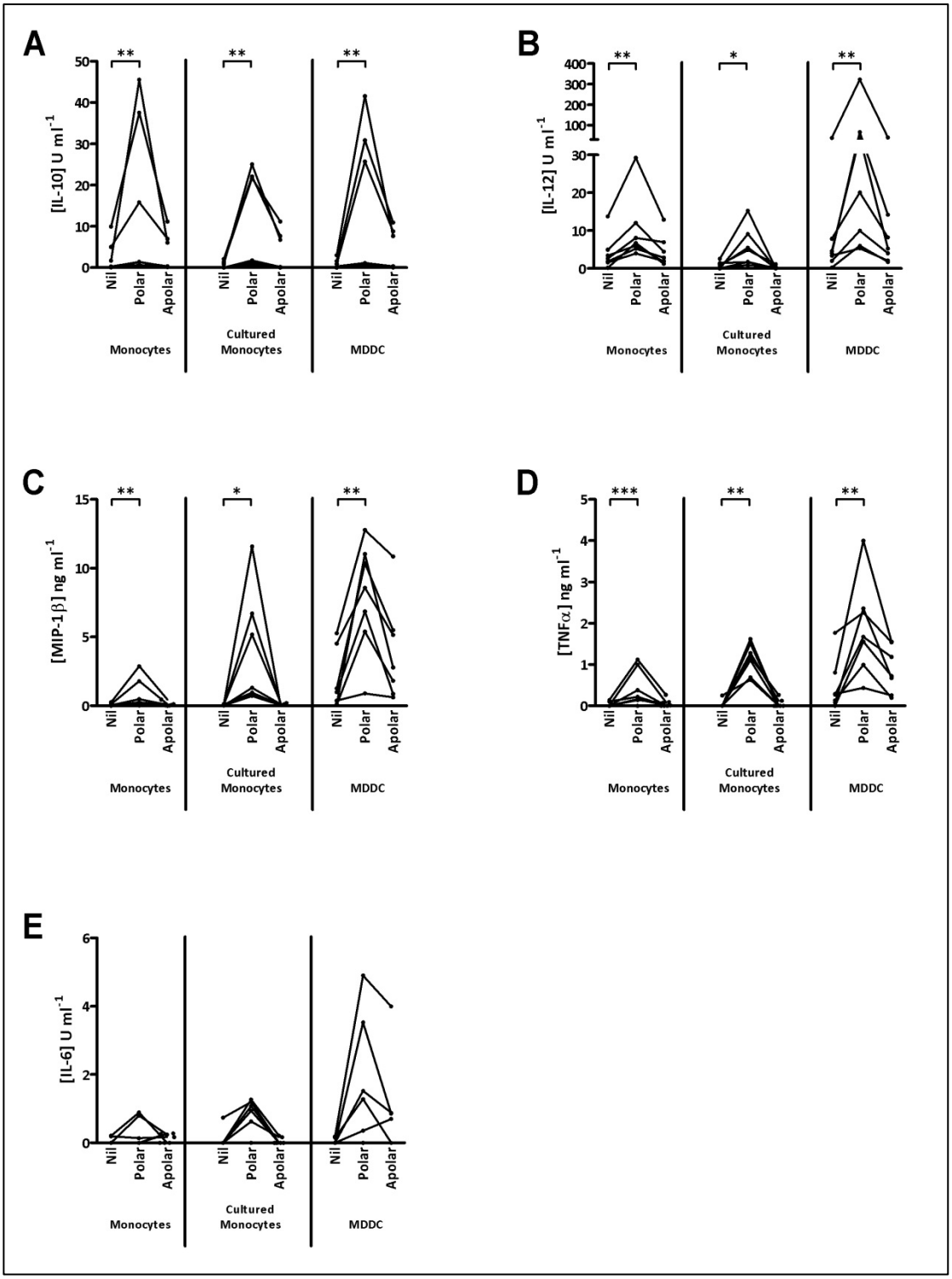

Figure 4:3 - Effect of stimulation with the polar and apolar lipid fractions on cytokine production by bovine innate immune cells.

(A) IL - 10, (B) IL - 12, (C) MIP - 1β, (D) TNFα & (E) IL - 6. Points represent mean responses from duplicate wells for each of 7 animals tested. Lines indicate that cells were derived from the same animal; * $0 < 0.05$; ** $p < 0.01$; *** $p < 0.001$ using Friedman repeated measures ANOVA with Dunn's multiple comparisons test.

Levels of MIP - 1β were also found to be significantly increased after exposure to the polar lipid fraction with no significant increase seen after apolar lipid stimulation (figure 4:3 C). Both cultured monocytes and MDDC produced noticeably more MIP - 1β than fresh monocytes, with MDDC from 6, and cultured monocytes from 3, of the 7 animals responding strongly (figure 4:3 C).

Significant increases in TNFα production were also seen, again in response to the polar lipid fraction (figure 4:3 D). While polar lipid treated cultured monocytes from all 7 cattle produced significant levels of TNFα, considerably more TNFα was produced by MDDC (figure 4:3 D). Further, the level of TNFα production was similar between fresh and cultured monocytes (figure 4:3 D).

The production of IL - 6 (figure 4:3 E) followed a broadly similar pattern to that of TNFα (figure 4:3 D) although statistical significance was not achieved. Fresh monocytes from 2 cattle produced more IL-6 after exposure to the polar lipids, which also drove increased IL-6 production in cultured monocytes from 6 cattle (figure 4:3 E). Polar lipid driven IL - 6 production by MDDC was noted in 5 of the 7 animals screened, with one of these animals producing more IL - 6 to the apolar lipid fraction than the polar. While statistical significance was not achieved, the levels of IL - 6 produced by MDDC are notably higher than from fresh or cultured monocytes (figure 4:3 E).

These data clearly demonstrate that the polar lipid fraction drives the production of significant amounts of IL - 10, IL - 12, MIP - 1β and TNFα from all cell types. Furthermore, it is clear that MDDC produced more IL - 12, TNFα and IL - 6 than fresh or cultured

monocytes and MIP -1β production is greater from both cultured monocytes and MDDC than in fresh monocytes.

Phenotypic Responses to Crude Mycobacterial Lipids

Exposure of bovine antigen presenting cells to the polar lipid fraction led to significant increases in the production of a variety of cytokines (figure 4:3 A - E), all of which can play important roles in directing the subsequent cell - mediated response. In order to further assess the effect of *M. bovis* - derived lipids and the local cytokine milieu on these cells, analysis of the expression of key antigen presentation related molecules was assessed by flow cytometry (figure 4:4).

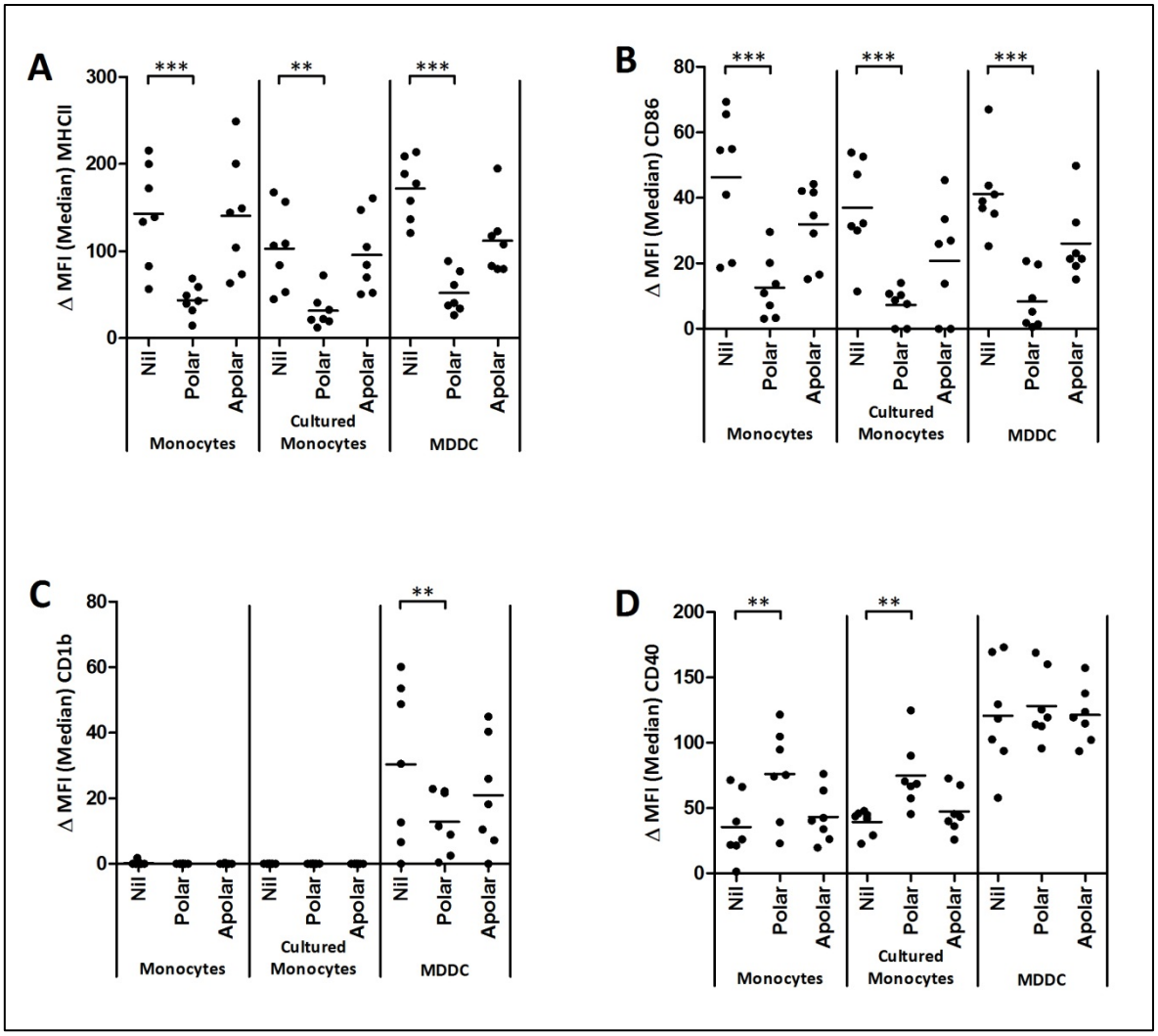

Figure 4:4 - Effect of stimulation with the polar and apolar lipid fractions on phenotype of bovine innate immune cells. (A) MHCII, (B) CD86, (C) CD1b and (D) CD40. A single point represents the median fluorescence intensity of the specific stain after subtraction of an isotype control (ΔMFI) for each of 7 animals tested; ** $p < 0.01$; *** $p < 0.001$ using repeated measures ANOVA with Bonferroni multiple comparisons test.

Stimulation with the polar lipid fraction resulted in a significant reduction in the cell surface expression of MHCII on all three cell types (figure 4:4 A). Furthermore, MHCII expression was also lower on MDDC following stimulation with apolar lipids, although this did not achieve statistical significance (figure 4:4 A). CD86 expression was also significantly reduced on all three cell types following stimulation with the polar lipid fraction (figure 4:4 B). While there was a trend for lower CD86 expression on all three cell types following stimulation with the apolar lipid fraction, this again did not achieve

statistical significance (figure 4:4 B). Similarly to unstimulated cells, monocytes and CM stimulated with either lipid fraction did not express CD1b (figure 4:4 C). However, incubation of MDDC with the polar lipid fraction resulted in a significant reduction in CD1b surface expression (figure 4:4 C). Not all cell surface molecules were down - regulated following treatment with lipids. CD40 expression on both monocytes and cultured monocytes increased significantly following stimulation with the polar lipid fraction (figure 4:4 D), although no effect was seen on MDDC. Finally, no significant difference was seen in CD80 levels following stimulation with either the polar or apolar lipid fractions (data not shown).

In summary, these data demonstrate that *M. bovis* - derived lipids, and in particular the polar fraction, downregulate the expression of several key cell surface molecules involved in antigen presentation.

Consequence of MDDC Exposure to *M. bovis* - Derived Lipids

To identify and assess any functional consequence of the lipid induced reduction in molecules related to antigen presentation the ability of lipid treated innate cell types to stimulate an alloreactive response was assessed. Co - culture of the responder PBMC population with either untreated MDDC or cultured monocytes resulted in a 5 - fold increase in their proliferation (figure 4:5). No proliferation was noted for either MDDC or cultured monocytes in the absence of responder cells (data not shown). Polar lipid treated MDDC retained their ability to induce proliferation in the responder population despite the downregulation of important costimulatory molecules. In contrast, allo -

stimulation of the responder population by polar lipid treated cultured monocytes resulted in significantly reduced proliferative responses to levels comparable with the unstimulated responder control cells (figure 4:5).

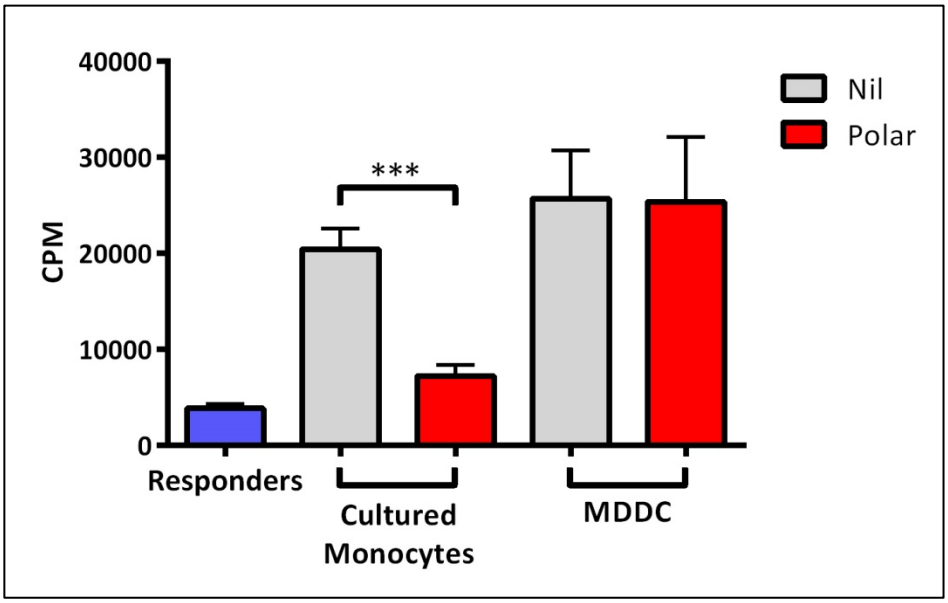

Figure 4:5 - Proliferative responses of PBMC stimulated with polar lipid treated allotypic cultured monocytes and MDDC Red bars - polar lipid treated allotypic cultured monocytes or MDDC; light grey bars - untreated allotypic cultured monocytes or MDDC. Bars represent the mean of triplicate wells ± standard error of the mean; *** $p < 0.001$ using repeated measures ANOVA with Bonferroni multiple comparisons test.

Discussion

To assess whether crude lipid fractions were capable of mediating the responses of bovine innate cells, stimulation experiments were performed and the level of a range of cytokines was analysed. Cells were stimulated with either polar and apolar fractions, however it was only after stimulation with the polar fraction that significant increases in the production of various cytokines were detected. Perhaps most striking is the significant increase in the production of both the Th - 1 polarising IL - 12 and the anti-inflammatory cytokine IL - 10 (figure 4:3 A - B). Initially this seems contradictory, but it is important to note that the fractions used are complex mixtures of a variety of lipids some of which, such as MMG[115], are known to induce potent immunostimulatory cytokine profiles while others, such as glycerol monomycolate (GroMM), are known Th2 polarisers[290]. Further, many lipid molecules from *M. tuberculosis* have been shown to mediate a range of pleiotropic effects on innate cells in murine models[291, 292].

There is little information in the published literature discussing cytokine production by lipid treated antigen presenting cells. Instead, much work concentrates on assessing $CD4^+$ and $CD8^+$ T cells responses either after direct exposure of the lymphocytes to lipid antigens or by stimulating lymphocytes with infected or treated antigen presenting cells. For example, TDM, which is present in the polar and apolar fractions (figure 3:1 D - E), has been shown to induce both Th1 and Th2 cytokines. The induction of IFNγ and IL - 12 and the depletion of IL - 4 producing NK cells has been attributed to TDM[293, 294] as well as a role, along with IL - 6 and TNFα, in stable granuloma formation[295]. Yet TDM is also implicated in the production of IL - 5 and IL - 10 in a CD1 - dependant manner[296].

Furthermore, GroMM has been implicated in the induction of Th2 polarising responses[290] whilst the closely related GMM has been shown to induce Th1 cytokine responses in T cells[297]. Similarly, anti - inflammatory effects have also attributed to PIM_2 and PIM_6 where, upon lipid treatment of LPS activated macrophages, Doz *et al*. measured downregulation of TLR4, TNFα, IL - 12p40, IL - 6, KC and IL - 10 as well as MyD88 mediated NO release[298]. However, PIM_2 has also been shown to activate MDDC, inducing significant increases in IL - 12 production[299, 300].

Given the significant increase in IL - 10 production by all innate cell types assessed, and the important role these cells play in generating and directing the immune response, the expression of antigen presentation associated cell surface molecules after lipid exposure was analysed. Lipid treatment of APCs lead to a significant decrease in the levels of costimulatory molecules associated with antigen presentation including MHCII and CD86 on all cell types studied and CD1b on MDDC (figure 4:4 A - C). Negative regulation of these molecules by a variety of lipid components has been noted previously, especially MHCII in human and murine systems. Similar to the data presented here, the 19 - kDa lipoprotein is capable of downregulating MHCII expression on human THP - 1 macrophages by inhibiting activation of the IFNγ - induced CIITA[301, 302]. Downregulation of MHCII, as well as TLR2 and TLR4, has also been reported on human MDDC after lipid exposure[303] and a further study also found impaired expression of CD1a, MHCII, CD80 and CD83 on human MDDC[304].

Downregulation of CD1 molecules has also been shown previously. Gagliardi *et al*. demonstrated that MDDC generated *in vitro* from monocytes which had been treated

with BCG did not express CD1 and showed reduced MHCII, CD40 and CD80[305] and this has since been shown to be due to cell wall associated carbohydrate α - glucan[225] and mediated through the p38 MAPK pathway[306]. However these experiments have all been performed in human or murine systems and with specific lipids, often from avirulent bacterial isolates.

Interestingly, treatment of fresh and cultured monocytes with the polar lipid fraction significantly increased the level of CD40 expression (figure 4:4 D) and this effect is not seen on MDDC. This finding seems contradictory to the published literature[305, 307] however these studies used avirulent BCG or TDM alone, rather than the complex and more biologically representative lipid preparations derived from virulent mycobacteria used here, as well as being performed in human or murine macrophage models. Bovine MDDC expression of CD40 does not alter after stimulation with either polar or apolar lipids which may be due to its constitutively higher levels of expression than on fresh or cultured monocytes.

Finally, no difference was seen in the expression of CD80 after lipid treatment, although this has also been reported in other systems using virulent *M. tuberculosis* or avirulent BCG derived lipids[304, 305, 307].

The significant reduction in the levels of MHCII, CD86 and CD1b is consistent with the phenotype of an impaired antigen presenting cell[308]. Given the effect of the polar lipids on the expression of these molecules and the concurrent increase in IL - 10 production, it was hypothesised that the polar lipid fraction, or one of its components, hampers the

ability of the cells to successfully present antigen to T cells and may be able to suppress the induction of a Th1 response during infection. To assess any functional deficit in these cells, especially due to the loss in MHCII, lipid treated and untreated cells were used to drive allotypic proliferative responses.

Cultured monocytes drove proliferation of allogeneic PBMC (figure 4:5) and treatment of cultured monocytes with the polar lipid fraction significantly abrogated these responses as suggested by the downregulation of MHCII and other costimulatory molecules. Proliferative responses were also seen when allogeneic PBMC were combined with untreated MDDC (figure 4:5) however no difference in proliferation was seen using lipid treated MDDC despite flow cytometric analysis revealing characteristic reduction in the level of MHCII on the MDDC (data not shown). While these results seem at odds with each other, it is possible that the loss of MHCII may be overcome by the high level of CD40 expressed by MDDC (figure 4:4 D) or the constitutively higher levels of IL - 12 produced by these cells which further increases significantly after lipid stimulation (figure 4:3 B). Also, some evidence exists that the presence of CD80 is enough to stimulate allogeneic T cells in the absence of CD86 signalling[309]. Given the significant reduction in CD86 expression on MDDC, the maintenance of CD80 may play a role. Finally, it is possible that, due to constitutively higher levels of MHCII and CD40 present on MDDC, as well as their expression of CD1b, the levels of MHCII and CD86 on these cells remains sufficient to drive an allotypic reaction.

These data demonstrate that *M. bovis* derived lipid fractions are capable of stimulating responses in bovine innate cells and that these different cell types respond in distinct ways.

Interestingly, the alteration in cell surface phenotype of both cultured monocytes and MDDC seen after polar lipid stimulation is also evident after exposure to the apolar lipid fraction, albeit to a lesser, not statistically significant, extent. This may be due to specific lipid components present in both the polar fraction and the apolar preparation, such as TDM. However, it may also be due to the insolubility of less polar lipids in the aqueous environment an *in vitro* culture system which may limit lipid bioavailability.

Despite the presence of individual compounds known to have Th1 inducing properties, the overall effect of these fractions appears to be down modulation of APC function. This may be a strategy employed by the bacilli to delay protective immune responses. The effects presented here confirm similar to observations seen in humans[302, 310-315]. In conclusion, this chapter demonstrates the ability of the lipid fractions to mediate effects on bovine innate cells. These lipids, especially those contained within the polar fraction are capable of interacting with the host's innate immune cells such that the cells ability to initiate an adequate T cell response may be compromised, although this effect could only be demonstrated for cultured monocytes and not MDDC. The effects mediated by these lipids may play a pivotal role in the outcome of infection and subfractionation may help to differentiate down - modulatory compounds from those that may have adjuvant or Th1 stimulatory properties.

Chapter Summary

- **Objective**

  To measure any effects mediated by the lipid fractions on bovine innate immune cells, characterise the innate cell cytokine and phenotypic responses and assess if the function of the cell was affected.

- **Results**

  MDDC and CM were derived and characterised. CM were found to express significantly increased levels of CD80 and no CD1b; MDDC were found to express high levels of CD1b and CD40.

  The polar fraction was found to drive significant increases in the levels of IL - 10, IL - 12, MIP - 1β and TNFα from all cell types. MDDC were seen to produce more IL - 10 than other cells. Polar lipids were also shown to significantly decrease expression of MHCII and CD86 on all cell types as well as CD1b on MDDC. Polar lipids also significantly increased CD40 expression by monocytes and CM.

  Polar lipid treated CM drove significantly less proliferation in allogeneic PBMC than untreated CM. This effect was not seen with MDDC.

- **Conclusions**

  The polar lipid fraction has been shown to modulate both the cytokine production and potential antigen presentation ability of bovine innate immune cells. These lipids may play a critical role in defining the outcome of infection.

Chapter Five

Effects of Lipid Subfractions on Bovine Innate Immune Cells

Background

It is clear that the components in the polar lipid fraction are capable of interacting with bovine innate immune cells in a manner which may impair the cells effectiveness in modulating immune responses. These effects could mean that the innate cells ability to initiate an adequate T cell response may be compromised. However, the use of crude lipid fractions containing the total free extractable lipid from *M. bovis* does not allow for any discrimination of effects mediated within the antigen mixtures and the responses seen so far cannot be attributed to any specific lipid entities.

Individual lipids have been shown to bind with a large array of innate cell receptors and mediate a broad range of effects. Various mycobacterial lipids have been shown to be recognised directly by TLR2 including AraLAM[(175)], lipomannan[(176)], PIM_2[(175)], and PIM_6[(177)] or in association with TLR1 / 6, TLR4[(316, 317)] or other molecules.

For example, Means *et al.* demonstrated the TLR2 mediated recognition of LAM but found that CD14 was required for signalling and effector function modulation[(318)] whilst Jones *et al*. showed that TLR2 mediated recognition of PIM_2 generated increases in TNFα

as well as activation of the NF - κB and MAP kinases without the requirement for accessory molecules[175]. In another study, Means *et al.* also demonstrated CD14 independent, TLR4 - mediated activation of both CHO cells and murine macrophages by a heat labile, protease resistant cell wall component able to induce TNFα production[319]. Despite further research demonstrating that TNFα production can be blocked by a TLR4 antangonist, this cell wall component remains unidentified[320] and most TLR - activating purified mycobacterial antigens tested so far signal through TLR2[317].

Polar lipids such as PIMs are also known to mediate a range of effects based on differential receptor binding. Although both PIMs and LM contain mannose and have been shown to regulate cytokine, NO and T cell responses[321-323], LM has been shown not to bind to the mannose receptor but to DC - SIGN and mediate a responses through TLR2[324]. Through this mechanism, LM induces apoptosis[325] and IL - 12 production by macrophages[326]. In contrast to LM, the higher PIMs (PIM_{5-6}) have been shown to bind to the mannose receptor and can interfere with phagosome : lysosome fusion[183] whilst the lower mannosylated PIMs (PIM_{1-4}) preferentially bind to complement receptor 3 (CR3) and facilitate phagosome fusion with the early endosomal compartment[327-329]. To further complicate the situation, more recent work has shown that the higher mannosylated PIMs are also bound by DC - SIGN[330].

Unsurprisingly, lipid recognition and receptor interaction exhibits a degree of cellular specificity. For example, human MDDC have been repeatedly shown to bind *M. tuberculosis* through DC - SIGN[182, 331, 332] whilst the major *M. tuberculosis* receptors on macrophages are CR3 and the mannose receptor[183, 327-329].

The huge complexity highlighted here demonstrates both the variety of potential antigenic structures on the bacterial surface as well as the broad array of host molecules which play a role in host - pathogen interaction. The crude lipid extracts used previously are enormously complex and contain an array of lipid molecules shown here to mediate a broad range of effects on the host, as well as some lipopeptide and unidentified lipids.

This chapter addresses the hypothesis that the polar lipid fraction could be separated into smaller and more defined subfractions which could be used to stimulate bovine MDDC to identify an individual or subset of lipids responsible for driving the responses seen in Chapter Four. A variety of strategies were employed to separate this fraction into smaller fractions or individual components. These subfractions were then used to stimulate bovine innate immune cells and subsequently assessing the cells for changes in cytokine production and surface phenotype.

Results

The Effect of Crude Polar Lipids from *M. bovis* AN5

Due to the biosafety constraints of culturing large volumes of *M. bovis* AF 2122/97 and the substantial length of time required to grow the large amount of cell mass required for subfractionation, an alternative source of material was used for these studies. Given that *M. bovis* AN5 lipids appear qualitatively very similar to those from AF 2122/97 (see Chapter Three), a pellicle of AN5 was obtained. Used in the production of bovine PPD, *M. bovis* AN5 cultured in this way yields very high bacterial mass. However, as *M. bovis* AN5 is genotypically distinct from AF 2122/97, and it is known that conditions of bacterial culture can affect the overall lipid profile of the cells[278, 333], the effects mediated by the polar lipid fraction isolated from *M. bovis* AF2122/97 on bovine MDDC was compared to the polar lipid fraction from *M. bovis* AN5.

The polar lipids from AN5 were assessed to see if they generated similar phenotypic changes to those seen with AF 2122/97 - derived lipids (figure 5:1). As was seen previously, treatment of bovine MDDC with AF 2122/97 - derived lipids lead to a significant reduction in the expression of both MHCII (figure 5:1 A) and CD1b (figure 5:1 B). Despite the quantitative differences in the lipid profiles of both strains, polar lipids extracted from *M. bovis* AN5 also generated a significant reduction in the expression of MHCII (figure 5:1 A) and CD1b (figure 5:1 B). These data therefore suggested that the AN5 - derived lipid preparation contained the same immunologically active lipid species

as the AF 2122/97 - derived lipid fraction and could, therefore, serve as starting material for large scale subfractionation.

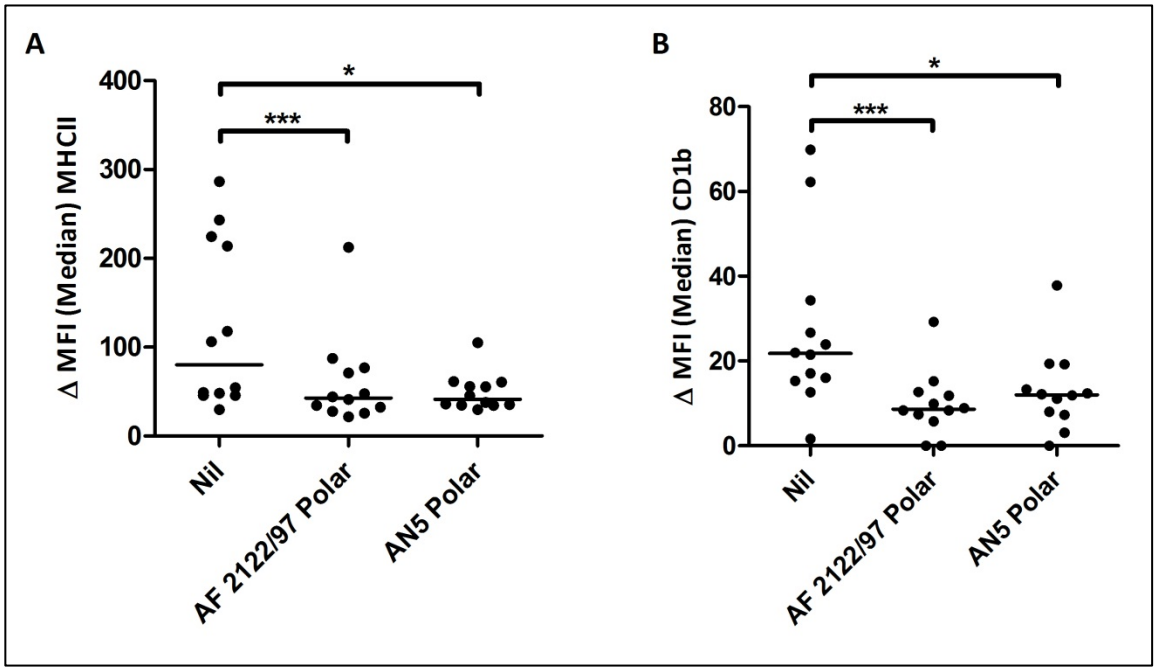

Figure 5:1 - Effect of polar lipids from *M. bovis* AF 2122/97 and AN5 on phenotype of bovine MDDC. (A) MHCII, (B) CD1b. Points represent mean responses from duplicate wells for each of 14 animals tested. Lines indicate the sample median; * $p < 0.05$; *** $p < 0.001$ using Friedman repeated measures ANOVA with Dunn's multiple comparisons test.

Subfractionation of the Crude Polar Lipids from *M. bovis* AN5

Having shown that the polar lipids derived from *M. bovis* AN5 mediate comparable responses in bovine MDDC as those from AF 2122/97, AN5 - derived lipids were used for subfractionation experiments. Several subfractionation methods were trialled. Firstly, silica columns packed in $CHCl_3$ with a mobile phase increasing in polarity by the addition of increasing levels of CH_3OH yielded no separation of the polar fraction. As can be seen in figure 5:2 A, most lipid was eluted from the column with no polar

solvent added (0 % CH_3OH) whilst the remaining lipid eluted as soon as any CH_3OH was present (figure 5:2 B). Further, the efficiency of this method was poor. Columns were initially loaded with 50 mg of crude lipid fraction and less than 800 µg of total lipid was eluted, leading to an efficiency of only 1.6 %.

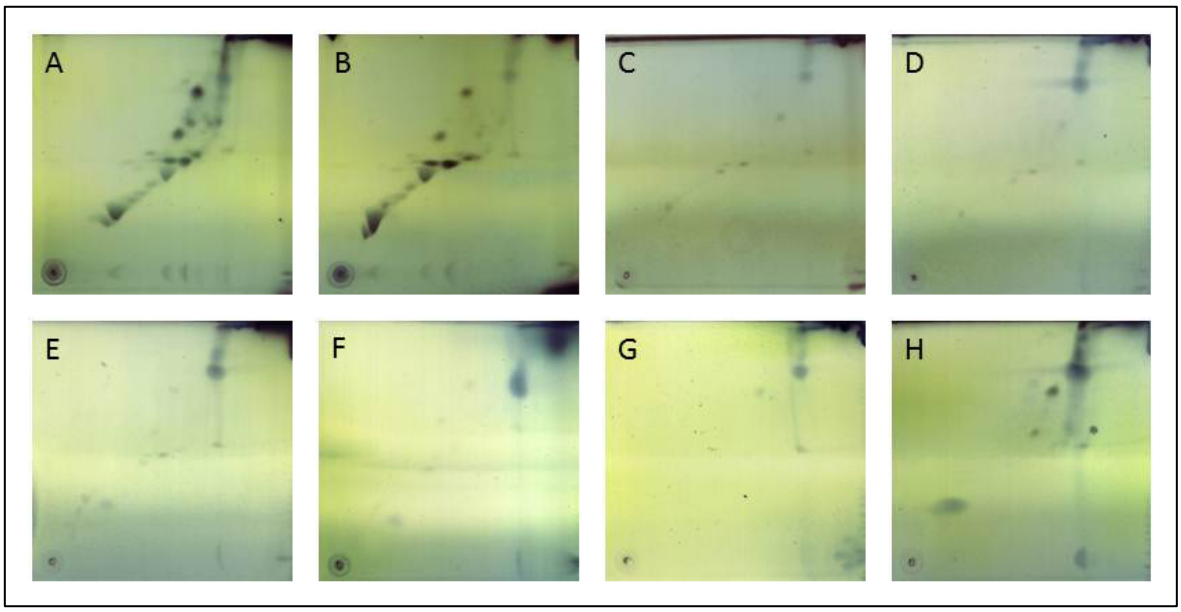

Figure 5:2 - Subfractionation by glass column chromatography.
Crude polar lipid was loaded onto a glass column packed with silica in $CHCl_3$ before being eluted with a mobile phase consisting increasing levels of CH_3OH. TLCs run in solvent system E and stained with MPA.
(A) 0 % v/v; (B) 2 % v/v; (C) 5 % v/v; (D) 10 % v/v; (E) 15 % v/v; (F) 20 % v/v; (G) 30 % v/v; (H) 50 % v/v.

This method was refined with the use of syringe mounted solid phase extraction columns. Columns were loaded with 5 mg of AN5 - derived crude polar lipid and the same mobile phases applied. Some lipid material was washed from the column even in the presence of no polar solvent (figure 5:3 A). Once CH_3OH was present, increasing the polarity of the mobile phase had little effect until the level of solvent reached 5 % where phosphatidylinositol and diphosphatidyl glycerol began to elute off the column (figure 5:3 C). However, once the polarity of the mobile phase was increased to 15 % CH_3OH (figure 5:3 E) all the components of the crude fraction were eluted from the column.

Increasing the levels of CH_3OH beyond this point increased the elution of PIMs and the more polar components of the fraction (figure 5:3 F - H).

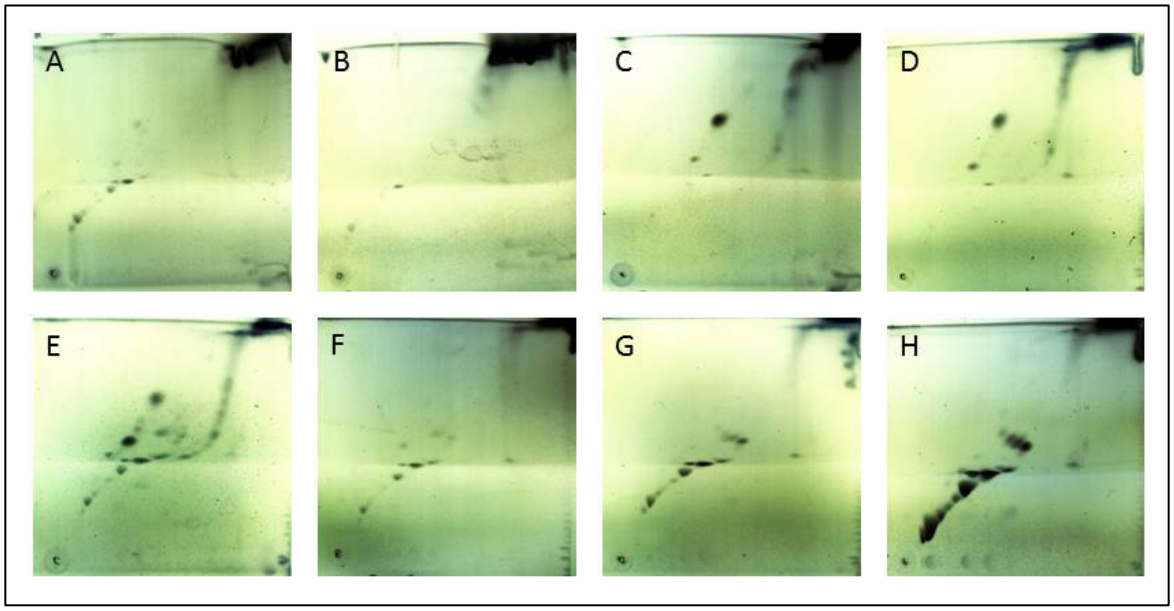

Figure 5:3 - Subfractionation by solid phase extraction chromatography.
Crude polar lipid was loaded onto a solid phase extraction column packed with silica in $CHCl_3$ before being eluted with a mobile phase consisting increasing levels of CH_3OH. TLCs run in solvent system E and stained with MPA.
(A) 0 % v/v; (B) 2 % v/v; (C) 5 % v/v; (D) 10 % v/v; (E) 15 % v/v; (F) 20 % v/v; (G) 30 % v/v; (H) 50 % v/v.

Whilst this method allowed for some discrimination of the lipids within separate fractions, the carryover of lipids between subfractions was quite marked (figure 5:3). The use of SPE columns was found to be more efficient that silica column chromatography. Of the 5 mg used for each experiment, around 0.5 mg of lipid was eluted in the combined subfraction. This equates to an efficiency of around 10 %.

To try to further separate the polar fraction, and decrease loss of the crude lipid fraction, the lipids were separated by 1 dimensional TLC, visualised under UV light after staining with 1, 6 - diphenyl - 1, 3, 5 - hexatriene (figure 2:2). Individual bands were scraped from the plate and the lipid dissolved into a 2 : 1 mixture of $CHCl_3$: CH_3OH. Using this

approach, it was possible to isolate 6 individual subfractions as can be seen in figure 5:4. Each scraped band corresponds to a region of the polar lipid fraction and little carryover between subfractions is visible (figure 5:4).

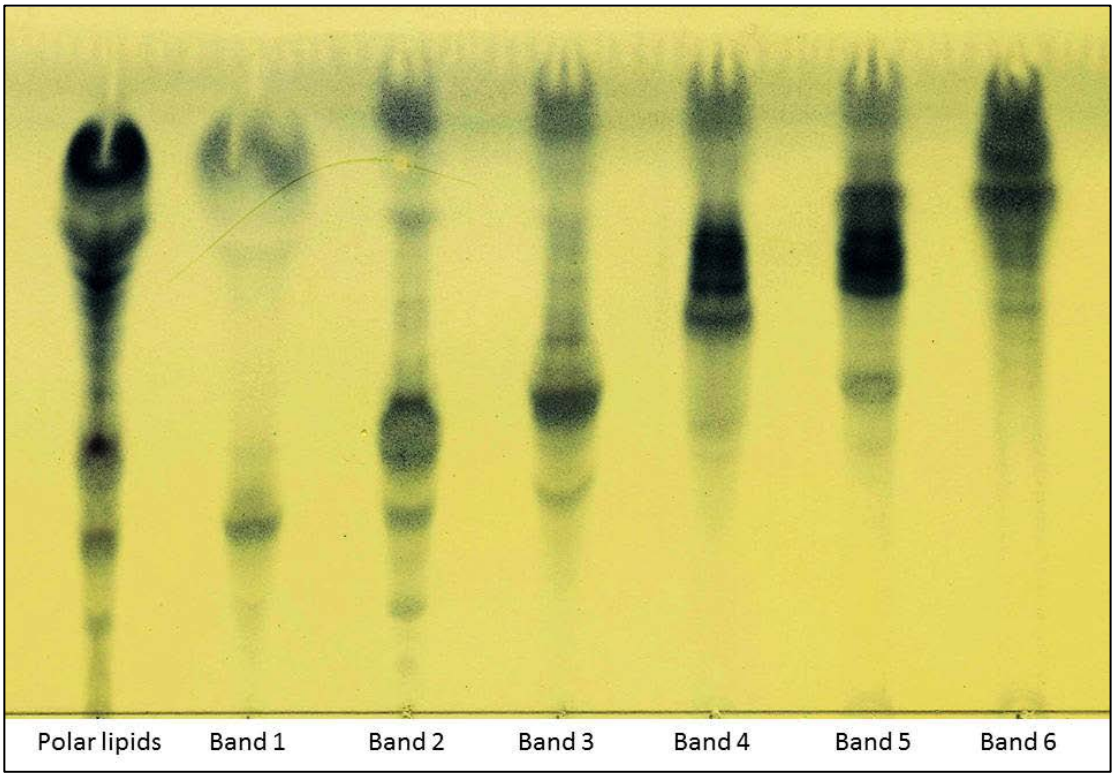

Figure 5:4 - One dimensional TLC of the polar lipid subfractions.
Crude polar lipids were separated by TLC and 6 bands scraped off TLC plates and pooled into individual subfractions. Each individual band can be seen here run in the same 1D TLC system next to the polar fraction for comparison.

Further analysis of these subfractions by 2D TLC was performed using 2D TLC system E (figure 5:5). Band 1 was found to contain phospholipid, phosphatidylinositol, Ac_1PIM_2 and Ac_2PIM_2 (figure 5:5 A). P, PI and Ac_2PIM_2 were also found in band 2, as well as DPG (figure 5:5 B). Band 3 contained Ac_2PIM_2, DPG and PE (figure 5:5 C) whilst bands 4 and 5 both Ac_2PIM_2 and PE and DPG respectively (figure 5:5 D and E). Band 6 was initially scraped from the solvent front of the 1D TLCs and contained no identifiable lipids (figure 5:5 F).

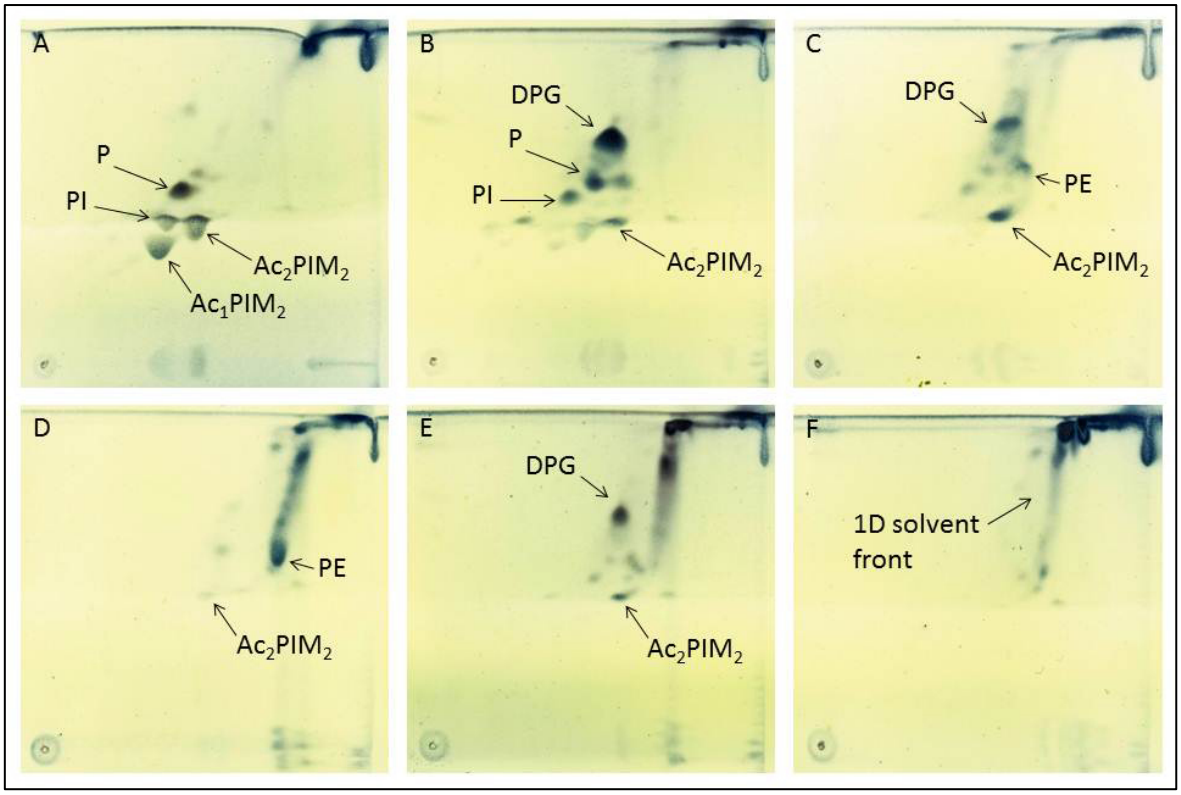

Figure 5:5 - 2D TLC analysis of the 6 polar lipid subfractions stained with MPA
(A) Band 1, (B) Band 2, (C) Band 3, (D) Band 4, (E) Band 5, (F) Band 6. Each subfraction was run on 2D TLC system E.

The subfractions generated by 1D TLC were demonstrably different in their lipid compositions and exhibited less carryover between subfractions than using either of the column based methods. Furthermore, the efficiency of subfractionation was increased greatly. Of the 30 mg loaded onto each 1D plate, the combined subfractions contained at least 6.5 mg which equates to an efficiency of approximately 21 %. Given the greater separation between the subfractions and increase in efficiency, 1D TLC subfractionation was chosen as the method to generate subfractions for further biological analysis.

Cytokine & Phenotypic Responses to Lipid Subfractions

Having successfully separated the polar lipid fraction, the 6 discrete subfractions were tested for their ability to modulate MDDC functions as seen with unfractionated polar lipids (see Chapter Four). MDDC were generated from 3 uninfected cattle and stimulated with the AN5 - derived polar lipid fraction and each of the 6 subfractions at 20 $\mu g\ ml^{-1}$ and the levels of IL - 10 and IL - 12 were measured as described above.

The AN5 - derived polar lipid fraction generated robust IL - 10 responses from the MDDC of all 3 animals tested with a median IL - 10 level of 66,404 RLU compared to a median Nil of 26,553 RLU (figure 5:6 A). All 6 subfractions also drove increases in IL - 10 production with bands 3 and 4 generating the highest responses while bands 1 and 6 generating the lowest responses although this trend was not significant (figure 5:6 A). Similarly, an increase in IL - 12 production was seen after lipid stimulation of the MDDC (figure 5:6 B). The AN5 - derived polar lipid fraction increased the median IL - 12 response and responses of a similar magnitude were seen after stimulation with bands 2, 3 and 4 while bands 1, 5 and 6 drove more modest increases in IL - 12. Again, this trend was not statistically significant.

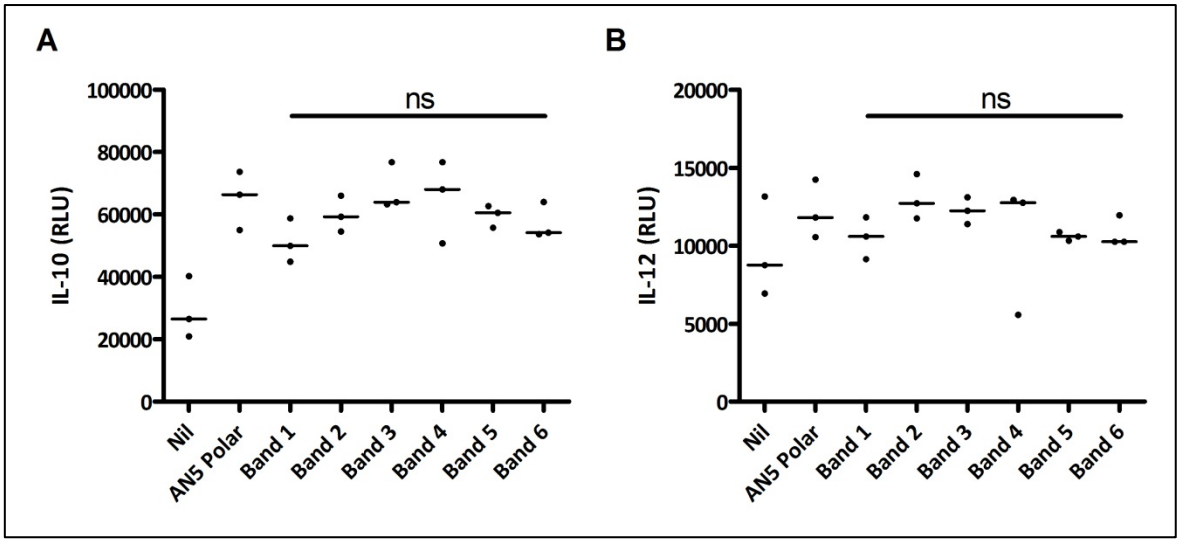

Figure 5:6 - Effect of polar lipids from *M. bovis* AN5 on cytokine production by bovine MDDC (A) IL - 10, (B) IL - 12. Points represent mean responses from duplicate wells for each of 3 animals tested. Measurements in Relative Light Units (RLU). Lines indicate the sample median; ns = not significant using Friedman repeated measures ANOVA with Dunn's multiple comparisons test.

MDDC expression of MHCII and CD1b after stimulation with the lipid subfractions was also assessed (figure 5:7). Stimulation of MDDC with AN5 - derived polar lipid fraction generated a notable loss of MHCII expression with the median Δ MFI decreasing from 243.08 to 55.55 (figure 5:7 A). Again, all the subfractions mediated a similar effect in reducing the level of MHCII expression to almost the same level with the median Δ MFI ranging from 59.71 for band 5 to 68.15 for band 6 (figure 5:7 A).

MDDC expression of CD1b after stimulation with the lipid subfractions followed a similar pattern to that seen for MHCII. Exposure of the cells to the polar lipids generated a noticeable loss of surface CD1b (figure 5:7 B) where the median Δ MFI of 16.03 for untreated cells dropped to 3.13. Treatment of MDDC with any of the subfractions also caused a loss of CD1b expression with the median Δ MFI ranging from 4.89 for band 4 down to 2.23 for band 5 (figure 5:7 B).

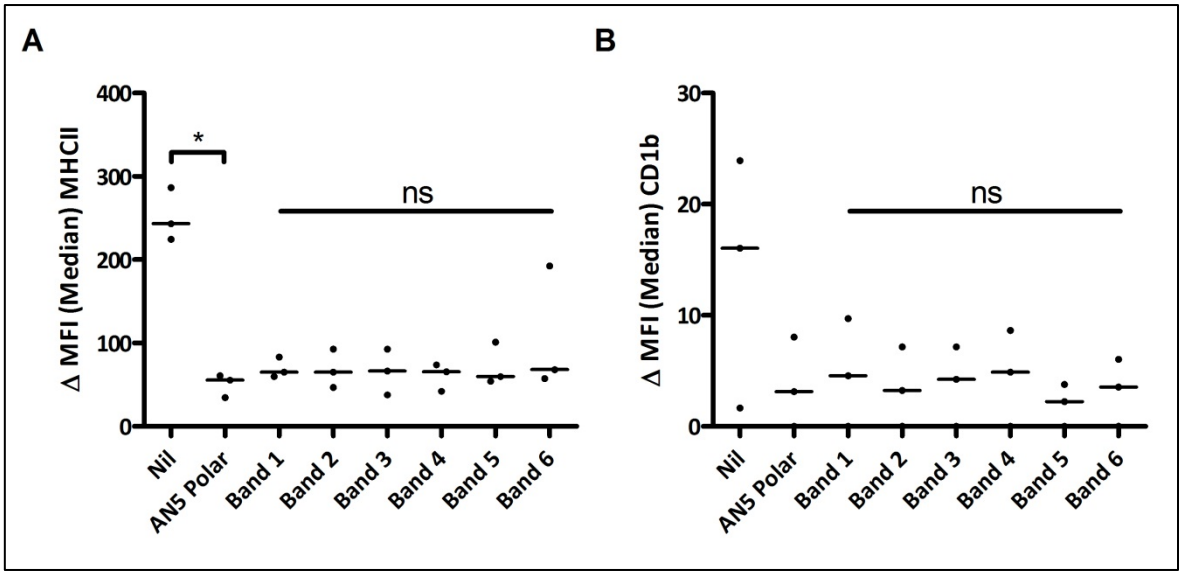

Figure 5:7 - Effect of lipid subfractions from *M. bovis* AN5 on phenotype of bovine MDDC (A) MHCII, (B) CD1b. Points represent mean responses from duplicate wells for each of 3 animals tested. Lines indicate the sample median; * $p < 0.05$; ns = not significant using Friedman repeated measures ANOVA with Dunn's multiple comparisons test.

As each subfraction contained fewer lipid moieties than the crude fraction, it was possible that their use at the same concentration as the crude fraction (20 µg ml^{-1}) meant that individual lipids were present at higher concentrations in the subfractions than in the crude fraction, and this could have been one possible reason for the lack of discrimination between the subfractions. To assess this, MDDC from 2 animals were exposed to the subfractions at a series of concentrations. Again, exposure of bovine MDDC to the polar lipid fraction from AN5 generated elevated levels of IL - 10 (figure 5:8).

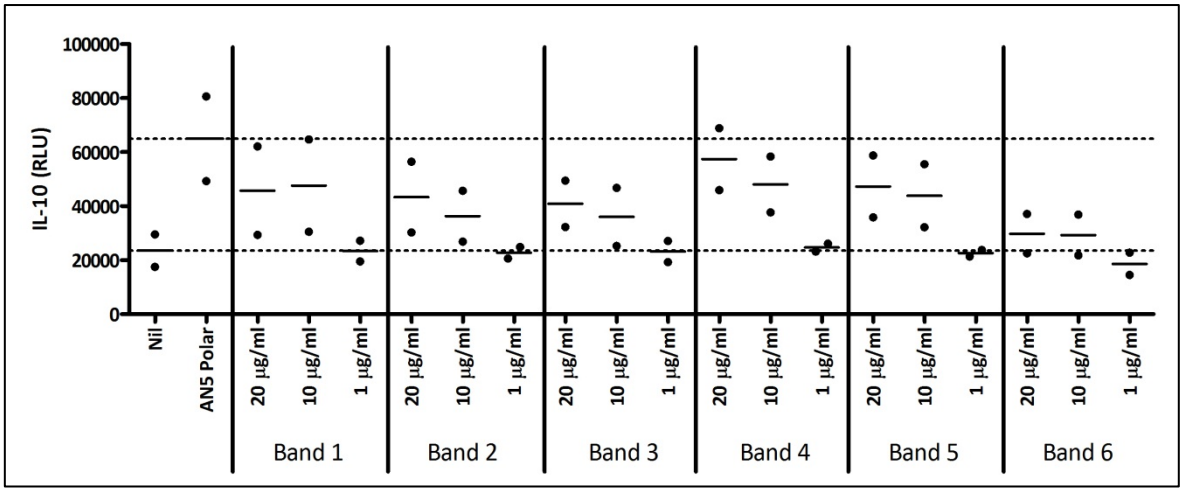

Figure 5:8 - Effect of serially diluted polar lipids from *M. bovis* AN5 on IL - 10 production by bovine MDDC Points represent mean responses from duplicate wells for each of 2 animals tested. Solid lines indicate the sample mean; dashed lines indicate the Nil or AN5 Polar mean.

Stimulation of bovine MDDC using bands 1 - 5 also drove dose dependant increase in IL - 10 production (figure 5:8). Band 6 did not increase in the level of IL - 10 produced regardless of the concentration at which it was used (figure 5:8). Treatment of cells with band 1 - 5 at 20 µg ml^{-1} drove IL - 10 production but, in general, not to the same level as the AN5 - derived polar lipid fraction alone (figure 5:8). Of the subfractions, band 4 drove the largest IL - 10 response (figure 5:8). Overall no increases were seen when any of the subfractions were used at 1 µg ml^{-1} (figure 5:8).

An increase in production of IL - 12 was also evident after stimulation with the crude AN5 - derived polar fraction and bands 1, 2 and 4 (figure 5:9). Again, the increase in IL - 12 production in response to the lipid subfractions was dose dependant and the level of IL - 12 detected when bands 1, 2 and 4 were used at 1 µg ml^{-1} had returned to the background level (figure 5:9). Unlike IL - 10 production, those subfractions which stimulated IL - 12 production did so to at least a similar level as the complete AN5 - derived polar lipid fraction (figure 5:9). In fact, the mean level of IL - 12 driven by band 4

was higher than that for the AN5 polar fraction (figure 5:9). Bands 3, 5 and 6 induced limited increases in IL - 12 from bovine MDDC (figure 5:9).

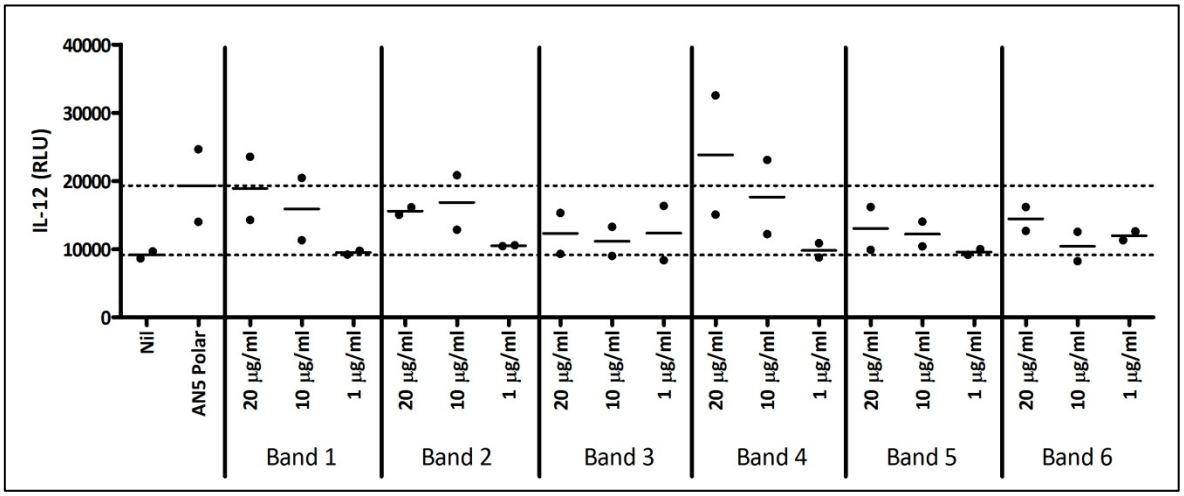

Figure 5:9 - Effect of serially diluted polar lipids from *M. bovis* AN5 on IL - 12 production by bovine MDDC
Points represent mean responses from duplicate wells for each of 2 animals tested. Solid lines indicate the sample mean; dashed lines indicate the Nil or AN5 Polar mean.

As the decrease in MHCII expression had been a consistent indicator of lipid activity on cell phenotype, this was also assessed with the diluted lipid subfractions. As expected, a large reduction in MHCII expression was seen after stimulation with the crude polar fraction from AN5 (figure 5:10). Further, all of the lipid subfractions also caused a decrease in the levels of MHCII expressed, although this reduction was not of the same magnitude as the crude lipid fraction (figure 5:10). The reduction of MHCII levels was also dose dependant, with the expression of MHCII increasing as the concentration of lipid subfraction decreased. When the subfractions were used at 1 µg ml^{-1} the levels of MHCII were similar to that of unstimulated cells (figure 5:10).

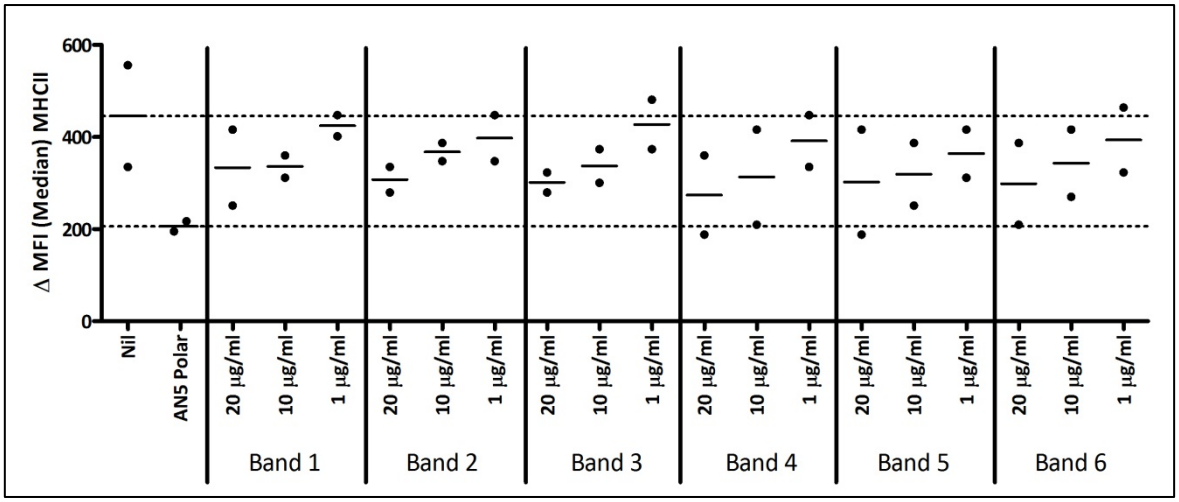

Figure 5:10 - Effect of serially diluted polar lipids from *M. bovis* AN5 on MHCII expression by bovine MDDC
Points represent mean responses from duplicate wells for each of 2 animals tested. Solid lines indicate the sample mean; dashed lines indicate the Nil or AN5 Polar mean.

Despite the differences in lipid composition of the subfractions, when used to stimulate MDDC, little difference was seen in their ability to drive cytokine responses or affect levels of MHCII expression. Band 4 drove the highest mean levels of IL - 10 and IL - 12 production and had the strongest effect on mean MHCII expression, however the responses generated by each subfraction were broadly similar. As the biological activity of the subfractions was dose dependant the subfractions were assessed for the presence of other potentially stimulatory molecules.

Assessment of Lipopeptide presence in Lipid Subfractions

Subfractionation of the *M. bovis* AN5 - derived polar fraction generated 6 subfractions which contained demonstrably different lipid compositions (figure 5:5). When bovine MDDC were treated with these subfractions, bands 1 - 5 were able to generate dose - dependent increases in the levels of IL - 10 (figure 5:8) and all bands

generated increased levels of IL - 12 (figure 5:9). Further, all bands led to a dose dependant reduction in the expression of MHCII (figure 5:10). However there was little difference between the responses to the different subfractions despite the differing lipid contents of the subfractions.

To try to explain the lack of variation in biological responses to these subfractions, each was re - analysed by 2D TLC and stained with ninhydrin to assess the presence and distribution of any lipopeptide.

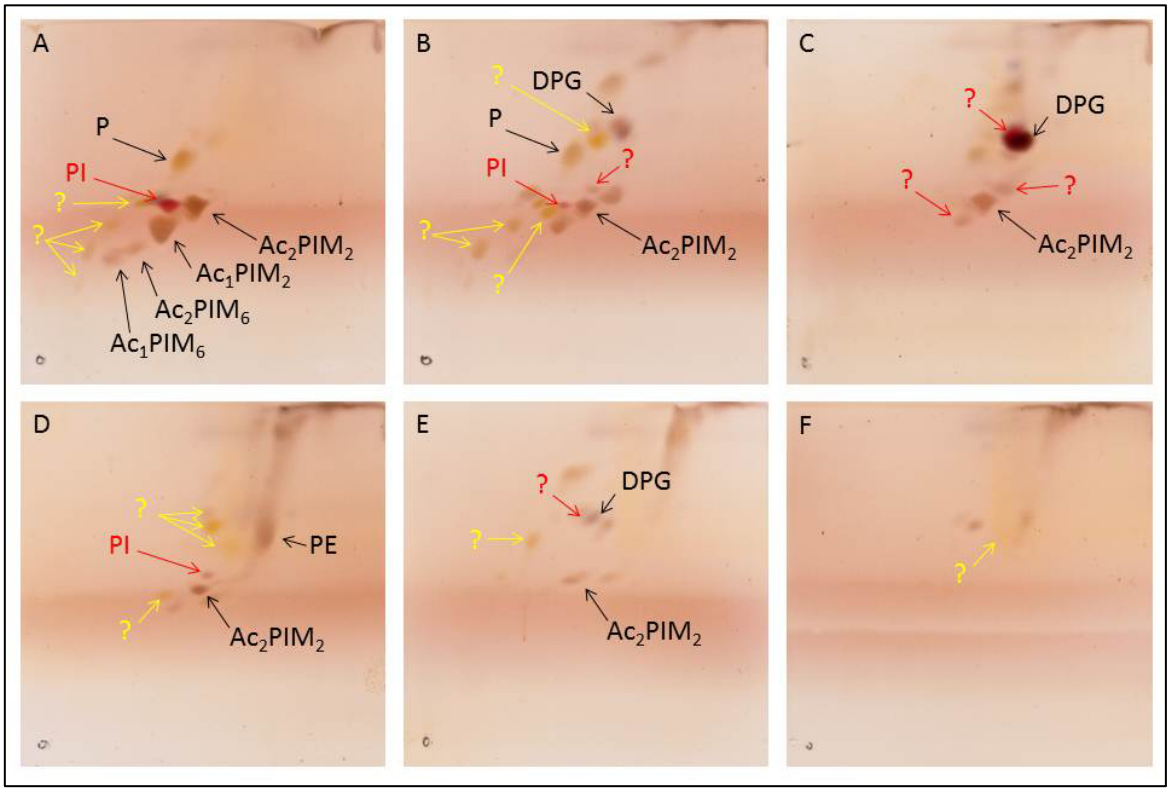

Figure 5:11 - 2D TLC analysis of the 6 polar lipid subfractions stained with ninhydrin
(A) Band 1, (B) Band 2, (C) Band 3, (D) Band 4, (E) Band 5, (F) Band 6. Each subfraction was run on 2D TLC system E. Red arrows indicate primary amines; yellow arrows indicate secondary amines; black arrows indicate charred lipid for reference. Identifiable spots are named.

Amine residues were found throughout the subfractions. As was seen in the crude lipid fractions (figure 3:2 and figure 3:4) a primary amine containing molecule was identified co - located with PI and was visible in bands 1, 2 and 4 (figure 5:11 A, B and D). Band 1 also contained 4 unidentified secondary amines (figure 5:11 A). As well as the primary amine located at the PI spot, another primary amine of unknown identity and several unknown secondary amines were found (figure 5:11 B). In band 3, a primary amine was identified located very close to the DPG spot along with 2 more unknown molecules (figure 5:11 C). Band 4 contained the primary amine located at the PI spot and 4 unidentified secondary amine residues (figure 5:11 D). Band 5 also contained amine residues, 1 primary and 1 secondary, neither of which were co - located with lipid molecules (figure 5:11 E). Finally, band 6 contained no clear amine resides, however a secondary amine residue was identified as a smudge (figure 5:11 F).

Discussion

In an attempt to identify individual lipid components responsible for the effects mediated by the polar fraction in bovine innate immune cells, the polar fraction from *M. bovis* AN5 was subfractionated and the subfractions analysed and tested. The use of AN5 for subfractionation was primarily due to biosafety concerns over the cultivation of large volumes of AF 2122/97 when a pellicle of AN5 was already available. Further, analysis of the lipids from *M. bovis* AN5 showed little difference when assessed by MPA (figure 3:1 and figure 3:3) and ninhydrin stained TLCs (figure 3:2 and figure 3:4).

Despite the similarities when analysed by TLC, it was important to assess the ability of the AN5 - derived fraction to mediate similar responses seen to the AF 2122/97 - derived lipids. To this end, both polar lipid fractions were used to stimulate bovine MDDC and the effect on expression of MHCII and CD1b was assessed.

As was seen previously (figure 4:4), stimulation of bovine MDDC with both the AF 2122/97 and AN5 - derived polar lipids generated a significant reduction in the expression of MHCII (figure 5:1 A). Only the AF 2122/97 - derived polar fraction was capable of driving a significant reduction in the levels of CD1b (figure 5:1 B), however a clear trend for reduced CD1b is evident after stimulation with the AN5 - derived polar fraction. Overall both the effector and phenotypic responses to the AN5 - derived polar lipid fraction were similar to that seen with the AF 2122/97 - derived lipids and the AN5 - derived fraction was used for subfractionation.

Initially subfractionation was attempted using column chromatography. Silica packed columns were run in $CHCl_3$ with increasingly polar eluents, made by the addition of increasing proportions of CH_3OH. This method of fractionation, and variations there on, have been used in a variety of settings and with much success[334-336], however this method failed to separate the AN5 - derived polar lipid fraction. Despite the lack of any polar solvent in the first eluent, the entire fraction was visible in this elute (figure 5:2 A). The addition of 2 % CH_3OH caused the elution of the rest of the fraction (figure 5:2 B) and no further lipid material appeared in any subsequent elutions (figure 5:2 C - H). The lack of separation could be related to the flow rate of the column. If this was too fast, the column would not have fully equilibrated and the lipid fraction would be forced through the column. This could also explain the fact that of the 50 mg of polar fraction loaded onto the column, less than 1 mg of material was eluted from the column.

Although large scale column chromatography failed to generate any discriminate subfractions and was found to be extremely inefficient, many authors have reported successful column chromatographic separation of polar lipids[334-336]. As a refinement, silica packed SPE columns were used with smaller starting quantities of AN5 - derived polar fraction. While this method did allow for the elution of PI and DPG in 5 % CH_3OH (figure 5:3 C) and the PIMs, PI and PE at 50 % (figure 5:3 H) there was no discrimination between the subfractions. Again, other authors have reported successful use of SPE columns to separate lipids, including those from *M. bovis* BCG[337] and *M. smegmatis*[338], however separation of *M. bovis* BCG lipids required magnesium silicate columns, more complex solvent systems and further purification by anion - exchange chromatography[338]. The separation of *M. smegmatis* polar lipids using SPE columns also

used a magnesium silicate stationary phase followed by separation by 2D TLC and reverse - phase chromatography[337].

Rather than pursue more complex column separation techniques, the separation of lipids by TLC and their subsequent removal from the plate was ultimately used for separation. A variety of TLC systems have been employed for purification, many with great success[339-342]. One dimensional TLC allowed the identification of 6 bands which could be individually collected, purified and analysed (figure 5:4). Subsequent 2D analysis of each subfraction demonstrated discrete fractions with minimal carryover of lipid molecules between subfractions (figure 5:5).

Having generated 6 individual polar lipid subfractions, these were tested in the same innate cell systems described previously. Measurement of IL - 10 and IL - 12 showed increased levels of both cytokines (figure 5:6). Assessment of the levels of MHCII and CD1b after stimulation with the subfractions also showed reductions in the expression of both molecules but no differences between the subfractions were observed (figure 5:7).

The use of the subfractions at a single concentration could mask responses as some lipid species were present in more than one subfraction, and at different concentrations. To address this, the subfractions were serially diluted and tested again. Interestingly, measurement of IL - 10 production by bovine MDDC showed that after stimulation with the 6 serially diluted subfractions, all except band 6 (the solvent front from the 1D TLC separations) drove dose dependant increases which returned to the background level as the fractions were diluted to 1 $\mu g\ ml^{-1}$ (figure 5:8). None of the subfractions drove IL - 10

production as high as the AN5 polar fraction, suggesting that the response driven by the complete fraction may be at least partly synergistic one, requiring components in different subfractions (figure 5:8). No differences in the levels of IL - 10 produced was seen between the subfractions suggesting something present in all fractions may be responsible for driving the production of IL - 10.

In contrast, differential IL - 12 production was seen after stimulation of bovine MDDC with the serially diluted subfractions. As seen previously, the AN5 - derived polar fraction drove increased IL - 12 production as did bands 1, 2 and 4. Further, the responses seen after exposure to these bands were dose dependant and IL - 12 levels returned to the background when the subfractions were used at 1 μg ml^{-1} (figure 5:9). The highest levels of IL - 12 were seen in response to subfractions 1 and 4. Band 1 consisted of mostly the lower mannosylated PIMs and other phospholipids (figure 5:5 A) which are known to drive IL - 12 production from DC *in vitro*[330]. Interestingly, the only identifiable lipid in band 4 was PE (figure 5:5 D) and no inflammatory effects have been attributed to this molecule.

Although 2 subfractions generated slightly different IL - 12 responses, the lack of discrimination between the subfractions based on their ability to drive cytokine production was disappointing. To try to further differentiate the subfractions, the levels of MHCII expression were assessed after exposure of bovine MDDC to the serially diluted subfractions. Again, each subfraction drove a reduction in the levels of MHCII present on the cells and expression was regained as the subfraction was diluted, but no differences in the magnitude of the effect were seen between the subfractions (figure 5:10).

Overall, no differences between the subfractions were seen in their ability to mediate the responses characterised originally using the crude polar fraction, despite the fact that each fraction clearly consisted of different lipid molecules (figure 5:5). These data suggested some other component, or components, present in the subfractions which had not been identified. As lipopeptide had been identified in the crude polar fractions from both AF 2122/97 (figure 3:2) and AN5 (figure 3:4) , ninhydrin stained 2D TLCs were performed to assess the presence of any remaining lipopeptide in the subfractions (figure 5:11).

As can be seen in figure 5:11, lipopeptide could be identified in all 6 subfractions, albeit to a lesser extent in band 6 (figure 5:11 F). Interestingly ninhydrin staining residues could be seen which were not apparent when the crude fractions had been analysed (indicated with red arrows in figure 5:11 A - F). It is most likely that these molecules were present in the crude fraction but not visible due to their lower concentration. The degree of lipopeptide overlap between the subfractions suggests that the lipopeptide molecules migrated across the entire 1D TLC plate during the initial separation. Lipopeptides are known to mediate a range of effects on the immune system which may account for the apparent lack of lipid specificity seen here. Despite knowledge of mycobacterial lipopeptides, no classification or categorisation has ever been performed, hence there is no reference work from which to identify lipopeptides. The best characterised mycobacterial lipopeptide is the 19 kDa lipoprotein, which has been shown to mediate a broad range of effects on innate immune cells. It is known to inhibit antigen processing in murine macrophages by decreasing the synthesis and expression of MHCII[301, 313, 343] and MHCI[344] as well as increasing IL - 12 production by human macrophages[345]. These

are similar to the effects seen when using the crude lipid fractions to stimulate bovine MDDC in Chapter Four. Conversely the same lipoprotein, when cloned into *M. smegmatis*, has been associated with immune suppression by a reduction in the levels of IL - 12, IL - 10 and TNFα[346] and lipopeptides from other bacterial genera have been shown to stimulate maturation of DC as measured by increasing expression of MHCII, CD80 and CD86[347].

The lipopeptides present in these lipid subfractions have not been identified but considering the degree of overlap between fractions, and the consistency of the biological effects seen, it is not unreasonable to suggest that they may play a significant role in the responses seen to the subfractions.

These data demonstrate that selective purification of individual lipid fractions from a crude mixture is challenging. While the lipid content of each subfraction is demonstrably different from the others, the biological effects they mediate on bovine MDDC do not reflect this and no response can be attributed to any particular lipid. Lipopeptides, surprisingly not removed by modified Bligh and Dyer extraction used to remove the silica contamination from the subfractions, can be identified in each fraction. Further, the high degree of lipopeptide carryover between subfractions could explain the similar biological responses seen to the subfractions. In addition, it appears that more lipopeptide is present in band 1 and band 4, hinting at a possible cause for the slightly stronger responses seen to band 4, whilst less lipopeptide was present in band 6 where responses to which were lower in magnitude or non - existent. That Ac_2PIM_2 was present in all

subfractions apart from band 6 further highlights the difficulty in purifying individual lipid molecules and this may also be important in the lack of differential responses.

Also of note is the absence of the higher mannosylated PIMs from these subfractions. Two possible explanations the their absence exist: firstly it is possible that the 1D solvent system did not allow for their movement from the origin, although this is unlikely as almost no lipid remains visible at the origin; or secondly that the high degree of mannosylation caused the molecules to locate at the interface of the modified Bligh and Dyer extraction during the removal of colloidal silica and the PIMs could have been removed with the aqueous phase.

In conclusion, this chapter demonstrates the complexities and challenges associated with selective purification of individual lipid species from complex mixtures. Despite the generation of 6 subfractions of differing lipid compositions, probably due to the extensive overlap of lipid species across the fractions thus preventing the purification of individual lipid species, no differences were seen in the biological responses to the subfractions. Further analysis of the subfractions suggested that the presence of Ac_2PIM_2, lipopeptides or both, could explain the consistency of the biological responses.

Chapter Summary

- **Objective**

  To separate the polar lipid fraction into smaller discrete subfractions which could be characterised and used to identify which lipid or subset of lipids were driving the responses seen previously.

- **Results**

  The polar fractions from AF 2122/97 and AN5 were shown to mediate similar effects on MHCII and CD1b expression by MDDC and AN5 lipids were used for subfractionation. A variety of methods were trialled but suffered from a lack of discrimination and poor efficiency. Finally, 1D TLC was used to generate 6 subfractions which were used for testing.

  All subfractions mediated similar effects with no significant differences seen in their ability to drive IL - 10 and IL - 12 production or the reduction of MHCII or CD1b on MDDC. This effect was found to be dose dependent.

  Further analysis of the subfractions highlighted the presence of lipopeptide in all subfractions.

- **Conclusions**

  The selective purification of lipid molecules from complex mixtures is challenging. The presence of lipopeptide in the subfractions, the carryover of Ac_2PIM_2 and the absence of the high mannosylated PIMs confounds the biological responses meaning no particular response can be assigned to any specific lipid molecule or family.

Chapter Six

Effect of Lipids on Bovine Acquired Cell - Mediated Immunity

Background

Despite the demonstrably different lipid compositions of the lipid subfractions and the dose dependant effects they mediated on bovine MDDC in both cytokine production and phenotype, no differential effects were seen between the fractions. However, a variety of amine containing molecules were identified in all of the subfractions which suggested the presence of lipopeptide. As both lipid and lipopeptide molecules have been shown to modulate cell - mediated immunity, the crude fractions were assessed for the ability to generate responses in PBMC.

The identification of CD1 proteins as non - polymorphic MHCI - like molecules, and their role in the presentation of lipid antigens to T cells[191] has led to much greater understanding of the role of lipids in adaptive immunity. Early work demonstrated the role of the Group 1 molecule CD1b in the presentation of mycolic acids[191] however many other lipids have since been shown to be presented through CD1b including sulphoglycolipids[199, 209] and glycolipids like GMM[202]. CD1b is the most well understood member of the Group 1 CD1 family thanks to work in humans but little is known about

the other Group 1 molecules due to their genomic deletion in common laboratory mice[348]. However there has been recent interest in CD1c since the discovery that phosphomycoketide[216] and lipopeptides containing an N - terminal acylation[215] are CD1c restricted.

CD1d is the only Group 2 CD1 molecule, and is another well - characterised CD1 protein. Ever since the realisation that NKT cells could respond to lipid antigens[191, 205, 349], and the subsequent discovery of the CD1d restricted lipid antigen α - Galactosylceramide (αGalCer)[226, 350], much research effort has been concentrated on understanding lipid antigens. Although work was initially focussed on invariant NKT cells it has since been shown that great diversity exists in the lipid - responsive TCR repertoire[351-353] and that these diverse NKT cells contribute to the Th1 / Th2 balance[351, 354]. It has been shown that CD1d restricted NKT cells are capable of recognising a variety of lipid antigens including phospholipids[227].

Recently, a CD1b restricted subset of T cells has been found[355]. These cells require the *CD1B* gene for their development and have been shown produce proinflammatory cytokines in response CD1b expressing DCs[355]. It is clear that NKT cells have a significant role to play in lipid mediate responses and the hunt for their antigens has continued[224, 356, 357].

One of the most important groups of phospholipids are the PIMs. Interest in PIMs was stimulated since it was shown that phosphatidylinositol dimannoside (PIM_2) forms the

phosphoglycolipid anchor which tethers a large array of glycolipids and lipoglycans, including lipomannan and lipoarabinomannan, to the cellular membrane[358].

Previous analysis of the lipid fractions by ninhydrin stained 2D TLC showed the presence of lipopeptide in the polar fractions (figure 3:2 and figure 3:4). These molecules are known to modulate adaptive immune responses[275, 277] and have been shown to be important targets of the MHC - restricted T cell response to *M. tuberculosis*[257]. Further, many lipids are known to modulate adaptive immunity[202, 203, 216, 355, 359, 360], particularly the PIMs[208, 228, 361-363]. Therefore, the polar and apolar fractions were used to assess the role of both lipid and lipopeptide in the generation of adaptive immune responses.

This chapter addresses the hypothesis that the crude lipid fractions could be used to generated cell - mediated immune responses and thus have potential as subunit vaccine candidates. Further, it was hypothesised that these responses could be characterised phenotypically and the potential role of lipid, lipopeptide or a combination of both, could be assessed.

Results

Lymphocyte Responses to Crude Mycobacterial Lipids

Given that both lipid and lipopeptide are known to affect cell - mediated immunity, the crude lipid fractions were screened for their ability to drive adaptive immune responses. The AF 2122/97 - derived polar lipid fraction drove significantly increased proliferation (figure 6:1 A) and IFNγ production (figure 6:1 B) when compared to the nil control. Furthermore, polar lipid stimulation drove significantly more proliferation and IFNγ production than stimulation with the apolar lipid fraction (figure 6:1). The apolar fraction generated no increase in either proliferation or IFNγ production (figure 6:1).

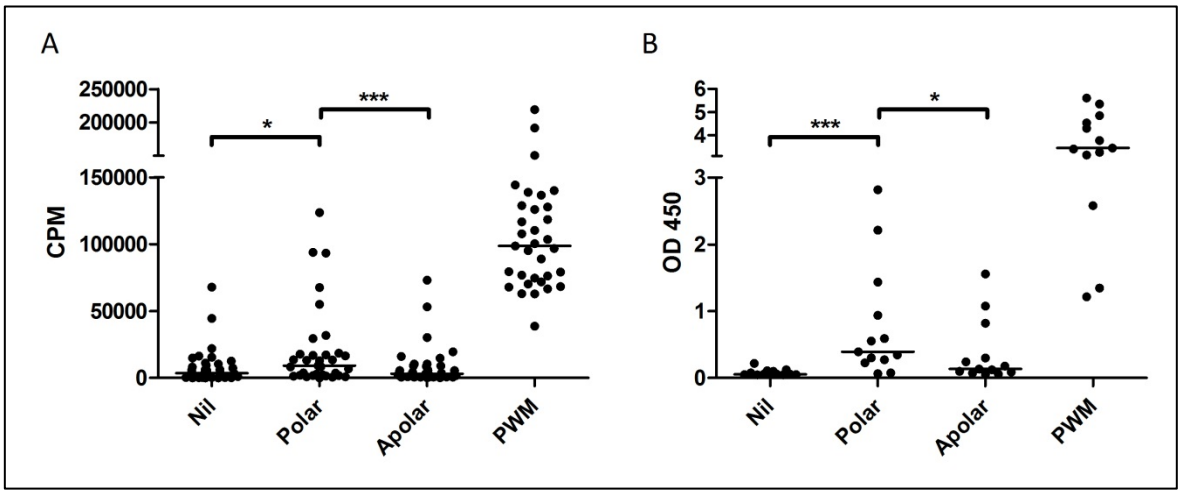

Figure 6:1 - Effect of stimulation with the AF 2122/97 polar and apolar lipid fractions on bovine PBMC. (A) Proliferation measured by ^{3}H - Thy incorporation; (B) IFNγ production measured by Bovigam. Each point represents the mean of triplicate wells; horizontal lines represent the sample median; * $p < 0.05$, *** $p < 0.001$ using Friedman repeated measures ANOVA with Dunn's multiple comparisons test. PWM - Pokeweed Mitogen. Lipids used at 20 µg ml^{-1} for (A) 5 days or (B) 12 - 16 hours.

The polar lipid fraction from *M. bovis* AF 2122/97 was also compared to the polar fraction from *M. bovis* AN5, as had been done previously for phenotype (figure 5:1) on bovine MDDC. As seen previously, the AF 2122/97 - derived polar fraction drove a significant increase in proliferation (figure 6:2 A), whilst the AN5 - derived polar fraction drove an even greater increase in proliferation (figure 6:2 A). A significant increase was also seen in IFNγ production after PBMC were exposed to the AF 2122/97 - derived polar lipids or the AN5 - derived polar lipids (figure 6:2 B).

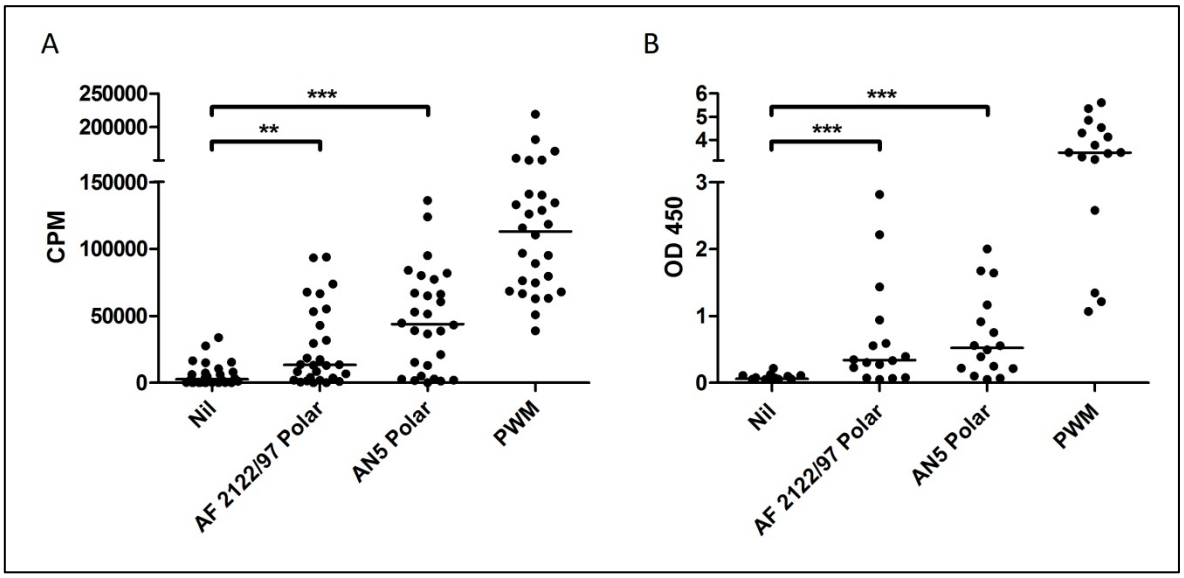

Figure 6:2 - Effect of stimulation with the AF 2122/97 polar and AN5 polar lipid fractions on bovine PBMC. (A) Proliferation measured by ^{3}H - Thy incorporation; (B) IFNγ production measured by Bovigam. Each point represents the mean of triplicate wells; horizontal lines represent the sample median; ** $p < 0.01$, *** $p < 0.001$ using Friedman repeated measures ANOVA with Dunn's multiple comparisons test. PWM - Pokeweed Mitogen. Lipids used at 20 µg ml^{-1} for (A) 5 days or (B) 12 - 16 hours.

Lipopeptide Activity in the Crude Polar Fraction

Having shown that the polar lipid fraction from *M. bovis* AN5 was capable of driving both IFNγ production and cellular proliferation in PBMC from TB infected cattle, the effect of lipopeptide in the lipid fraction was assessed by using antibodies to block either MHCII or CD1.

As expected, strong proliferative responses were seen to the AN5 polar fraction (figure 6:3 A, hatched bar). The addition of an isotype control antibody generated a slight reduction in proliferation irrespective of the level of dilution of the antibody (figure 6:3 A, grey bars). Interestingly, the addition of an anti - CD1 antibody did not affect proliferation and responses were similar to that seen when the isotype control antibody was added (figure 6:3 A, red bars). Further, the addition of an antibody which blocked MHCII molecules generated a dose dependant reduction in proliferation (figure 6:3 A, blue bars). The addition of antibody did not increase the background level of stimulation or the ability of the cells to proliferate (data not shown).

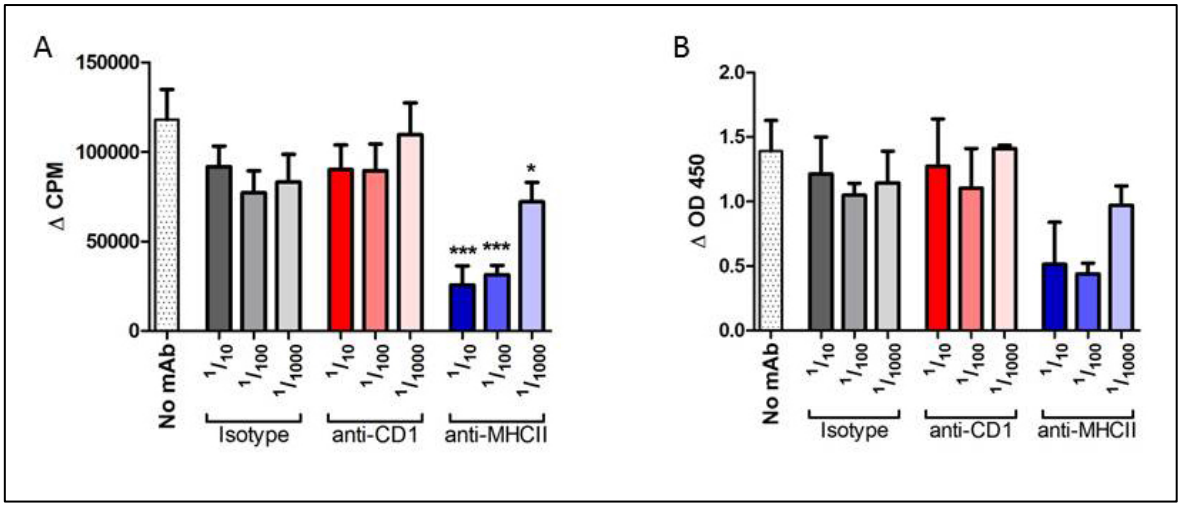

Figure 6:3 - Effect of blocking CD1 and MHCII on AN5 polar fraction driven cell - mediated responses. (A) Proliferation measured by ^{3}H - Thy incorporation and (B) IFNγ production after stimulation with AN5 - derived polar lipids in the presence of serial dilutions of anti - CD1 (red bars), anti - MHCII (blue bars) or isotype control (grey bars) monoclonal antibodies. Hatched bars indicate response with no antibody added. Each bar represents the mean of triplicate responses in 3 animals tested with the nil value subtracted (Δ); * $p < 0.05$, *** $p < 0.001$ using repeated measures ANOVA with Dunnett's multiple comparison test. Lipids used at 20 μg ml^{-1} for (A) 5 days or (B) 12 - 16 hours.

Measurement of the level of IFNγ produced in the same experiment is shown in figure 6:3 B. Again, a strong IFNγ response was seen to the AN5 - derived polar fraction in the absence of any antibody (figure 6:3 B, hatched bar) and the addition of an isotype control or the anti - CD1 monoclonal antibody had no effect on the levels of IFNγ produced (figure 6:3 B, grey bars and red bars respectively). Addition of the anti - MHCII monoclonal antibody did cause a small, dose dependant reduction in IFNγ production (figure 6:3 B, blue bars). No antibody - mediated effect was seen with the background level of IFNγ production or the ability of the cells to respond to PWM (data not shown).

Although blockade of MHCII appeared to reduce proliferative and cytokine responses it was still unclear what sort of molecule might be driving these responses. To this end, the polar lipid fraction was treated with Proteinase K to degrade any lipopeptide present in the fraction. Further, to ensure that the heating required during Proteinase K treatment, or the presence of inactivated Proteinase K itself, did not affect the responses, a mock

treatment was also performed. Treatment of the AN5 polar fraction with Proteinase K led to reduced proliferation in PBMC (figure 6:4) when compared to the untreated polar fraction. Mock Proteinase K treatment did not alter the ability of the fraction to drive strong proliferative responses (figure 6:4).

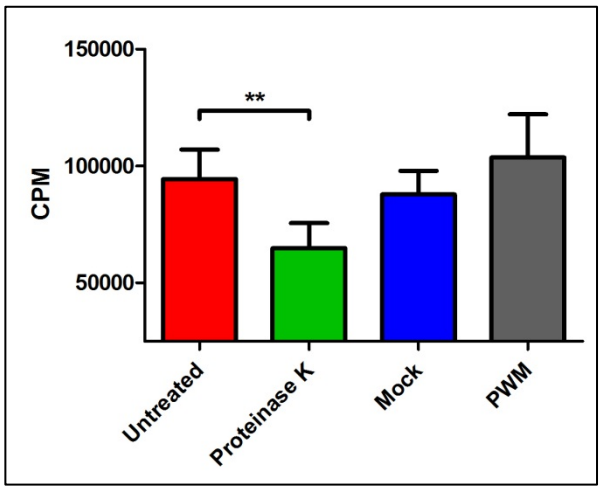

Figure 6:4 - Effect of Proteinase K treatment on proliferative ability of the AN5 polar lipid fraction. Proliferation measured by ^{3}H - Thy incorporation after stimulation of PBMC with the AN5 polar lipid fraction. Each bar represents the mean of triplicate responses in 3 animals tested; ** $p < 0.01$ using Friedman repeated measures ANOVA with Dunn's multiple comparisons test.

Together these data demonstrate a large degree of MHCII restriction within the AN5 - derived polar fraction and it is clear that some of the proliferative response driven by this fraction can be degraded by treatment with Proteinase K. However, neither of these methods completely abrogate the response suggesting that a lipid factor may be involved in driving proliferation.

As the densitometry analysis performed on the AN5 polar fraction (figure 3:6) had showed that over half of the total AN5 derived polar lipid fraction consisted of PIMs (table 3:2), it was considered a possibility that the non - lipopeptide mediated responses

seen could be due to PIM activity. To address this, 5 highly purified PIMs were obtained through a collaborator and used to stimulate bovine PBMC.

Adaptive Immune Responses to Purified PIM Molecules

PBMC from 10 naturally *M. bovis* infected cattle were cultured for 5 days in the presence of each PIM molecule and proliferative responses were measured. Both the frequency and strength of proliferative responses differed depending on the nature of the PIM molecule (figure 6:5 A). PIM_2 failed to induce a proliferative response in any of the animals studied, while $AcPIM_2$ induced responses in only 3 out of 10 animals. In contrast, Ac_2PIM_2, $AcPIM_6$ and Ac_2PIM_6 were more frequently recognised, inducing proliferative responses in 6 (Ac_2PIM_2 and $AcPIM_6$) and 7 (Ac_2PIM_6) out of 10 animals. Overall, significantly greater PBMC proliferation was detected in the Ac_2PIM_2, $AcPIM_6$ and Ac_2PIM_6 treatment groups compared to nil stimulated controls, with median values tending to be greater following $AcPIM_6$ stimulation (figure 6:5 A).

In addition to measuring proliferative responses, the ability of the PIM molecules to induce IFNγ responses in the same animals was assessed (figure 6:5 B). Again, the frequency of responding animals differed depending upon the nature of the PIM molecule. $AcPIM_2$ was least recognised, inducing responses in only 2 of the 10 animals. PIM_2, Ac_2PIM_2 and Ac_2PIM_6 were more frequently recognised, with responses detected in 3 (PIM_2) and 4 (Ac_2PIM_2 and Ac_2PIM_6) out of 10 animals. $AcPIM_6$ was most frequently recognised, inducing responses in half of the animals studied. Furthermore, $AcPIM_6$ was

the only PIM molecule to induce significantly greater levels ($p < 0.01$) of IFNγ overall when compared to nil stimulated controls (figure 6:5 B).

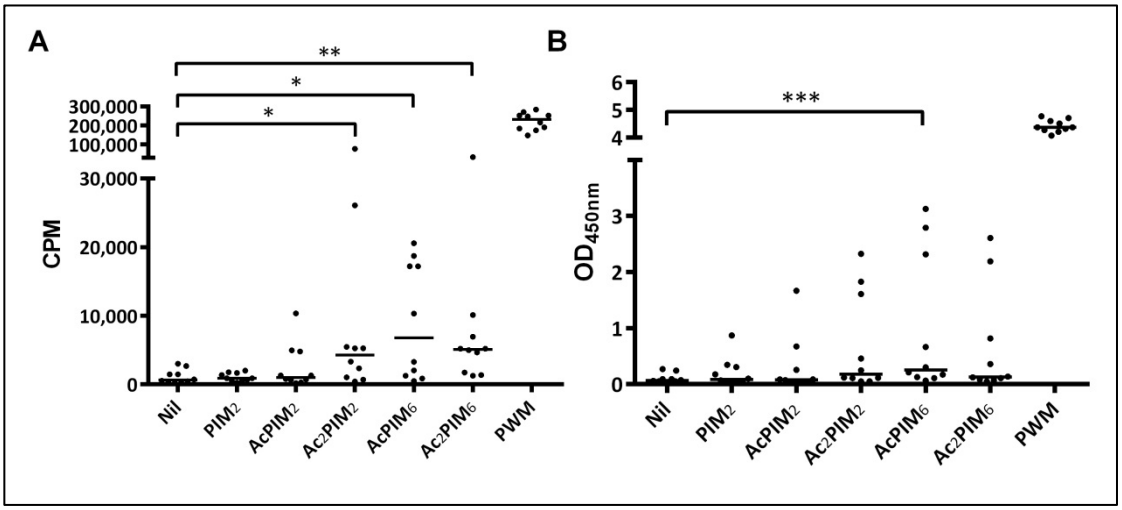

Figure 6:5 - Effect of stimulation with purified PIMs on bovine PBMC.
(A) Proliferation measured by ^{3}H - Thy incorporation; (B) IFNγ production measured by Bovigam. Points represent triplicate wells for each of 10 animals tested; lines indicate sample median; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using Friedman repeated measures ANOVA with Dunn's multiple comparisons test.

Phenotyping of $AcPIM_6$ Responsive Cells by Flow Cytometry

As $AcPIM_6$ was the only PIM molecule to generate significantly increased levels of IFN-γ (figure 6:5 A) and produced the greatest increase in the median proliferative response (figure 6:5 B), this antigen was used to stimulate PBMC from an *M. bovis* infected animal to characterise the proliferating cell populations by flow cytometry.

CellTrace Violet labelled cells were incubated for 5 days with antigen before being harvested and labelled for flow cytometric analysis. After stimulation with either PPD - B or $AcPIM_6$, 3 populations of proliferating cells were identified based on cell surface

phenotyping: (i) $CD4^+$ T cells ($CD3^+$ $CD4^+$); (ii) $CD8^+$ T cells ($CD3^+$ $CD8^+$); and (iii) NKT like cells ($CD3^+$ $CD335^+$). An example of the gating strategy for identifying proliferating NKT like cells is highlighted in figure 2:4, which demonstrates a greater level of proliferating cells in response to stimulation with $AcPIM_6$ (58.29 %) when compared to the nil antigen control (29.87 %).

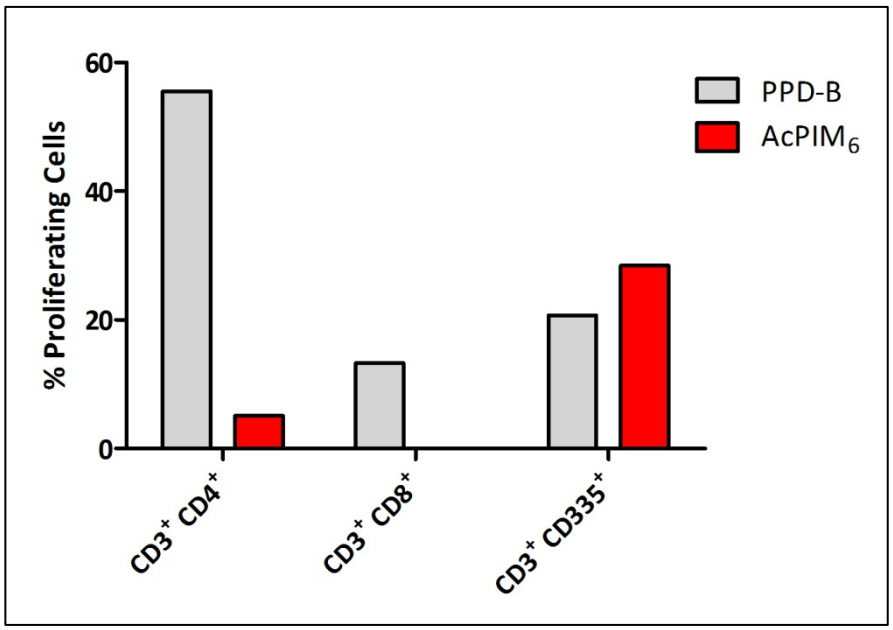

Figure 6:6 - Assessment of proliferating cell phenotype by flow cytometry
Proliferation of $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$ or $CD3^+$ $CD335^+$ cells in response to either PPD - B (grey bars) or $AcPIM_6$ (red bars). Each bar represents the percentage of cells proliferating after subtraction of the unstimulated control.

The effect of stimulation with either PPD - B or $AcPIM_6$ on the three different cell populations are summarised in figure 6:6. Stimulation with PPD - B drove antigen specific proliferation of approximately 60 % of the $CD4^+$ T cells ($CD3^+$ $CD4^+$). Similarly, an antigen specific proliferative response was seen in approximately 15 % of the $CD8^+$ T cells ($CD3^+$ $CD8^+$) to PPD - B. A slight increase in NKT cell ($CD3^+$ $CD335^+$) proliferative responses (approximately 20 %) was also seen to these antigens (figure 6:6).

Stimulation with $AcPIM_6$ induced only limited proliferation of $CD4^+$ T cells (approximately 5 %) and no proliferation of $CD8^+$ T cells above the background (figure 6:6). In contrast, approximately 30 % of the NKT cell population mounted a proliferative response after stimulation with $AcPIM_6$ (figure 6:6). Little or no proliferation above the unstimulated negative control was seen in the $CD3^-$ $CD335^+$ cells or the $CD3^+$ $\gamma\delta TCR^+$ populations (data not shown).

Discussion

Having tested the lipid subfractions on bovine MDDC and found no differences (see Chapter Five), it appeared that both lipid and lipopeptide may have an important role to play in the ability to mediate bovine immune responses. Lipopeptides have been shown to be important immune mediators, primarily in the context of adaptive immunity[257, 360, 364] and the fact that lipids can generate adaptive immune responses has been well documented[199, 202, 205, 209, 215, 216, 226, 342, 350, 352, 353]. However, before assessing the potential roles of lipopeptide and lipid on bovine adaptive immune responses, the existing lipid fractions had to be screened to see if they were capable of driving such responses.

Similarly to the effects seen when screened in MDDC, the polar lipid fraction generated significantly greater responses in PBMC than the apolar fraction (figure 6:1) and the response driven by the AN5 - derived polar lipids was comparable or stronger (figure 6:2). There is no difference in overall lipid content between the fractions when analysed by TLC (figure 3:1 and figure 3:3), nor is any difference apparent after staining with ninhydrin (figure 3:2 and figure 3:4). However, analysis of the densitometry of both fractions highlighted the largest difference between the two bacterial strains is the proportion of the polar fraction made up by PIM which may account for the stronger responses generated after exposure to the AN5 - derived lipid fraction.

To assess the role of lipopeptide in the AN5 polar fraction, proliferation and IFNγ were measured after either the addition of antibodies to block MHCII or CD1 or after treatment

ofthe lipid fractions with Proteinase K. The blocking of MHCII, but not CD1, reduced ability of the AN5 - derived polar fraction to drive proliferation (figure 6:3) as did treatment of the fraction with Proteinase K (figure 6:4). Clearly a proportion of the responses measured are restricted through MHCII and degradable by Proteinase K treatment, but these treatments do not completely abrogate the responses. It may be that the remaining responses are lipid mediated, however the possibility that some lipopeptide remains after Proteinase K treatment or that MHCII blocking is not absolute cannot be ignored. These data are in line with other work showing that Proteinase K treatment of chloroform : methanol extracts of *M. tuberculosis* reduced, but did not abrogate, the ability of the fraction to drive IFNγ production from human PBMC[(257)]. Interestingly, the authors also treated their fraction with lipase and found similar results, suggesting that, in crude extracts at least, lipopeptide plays a role in driving T cell responses but is not responsible for all the activity seen. More recent studies have highlighted specific mycobacterial lipopeptides capable of modulating the $CD4^+$ T cell response[(364)] and suggested a recognition mechanism[(360)].

Many cell populations found within PBMC have been shown to be affected by lipid antigens. Invariant NKT cells, restricted through CD1d, are particularly well characterised[(356, 357)] but conventional $CD4^+$ T cells have been shown to be reactive to lipids presented in the context of CD1b[(202, 203)] and lipid - induced, IL - 17 secreting γδ TCR^+ T cells have been shown to be important in early granuloma formation[(359)]. Unfortunately, the presence of lipopeptide in the crude fractions, as well as the subfractions, meant that they were not suitable for further dissection of the bovine adaptive immune response to lipids. To address this, a selection of highly purified PIM

molecules was obtained and the ability of these highly purified PIMs to drive immune responses was assessed.

The larger and more complex PIM molecules tested (Ac_2PIM_2, Ac_2PIM_6 and $AcPIM_6$) drove significant levels of proliferation (figure 6:5 A) while only $AcPIM_6$ was able to drive significant production of IFNγ (figure 6:5 B). Interestingly, the same antigens were used to stimulate whole blood overnight and the supernatants were assayed for IFNγ but none was seen (data not shown).

There are very few studies showing the effect of mycobacterial lipids in short incubation, whole blood assays. Cell - mediated immune responses to lipid antigens are more commonly assessed by EliSPOT with incubation times of at least 48 hours required before measurable responses become apparent[228, 300]. The discrepancy between the whole blood and PBMC IFNγ responses may be due in part to the requirement for antigen processing and presentation of PIMs[208]. Another possibility is that the frequency of lipid responsive cells is low and an extended incubation allows for expansion of these cells. This is supported by the demonstration of strong proliferative responses induced after stimulation of PBMC with PIMs (figure 6:5 A). A recent study has shown that bovine NKT cells are present only at low frequencies in peripheral blood (0.1 % - 1.7 %)[365].

Previous work using mice has shown that the ability of PIMs to generate cell - mediated responses is dependent on the acyl structures of the molecules. Early work performed using PIM_2 and PIM_6 demonstrated that the acyl chain was essential for NKT cell recruitment while the complexity of the mannose residues did not alter the response[323]

however it was subsequently shown that the second acyl chain of PIM_4 enhances binding to murine CD1d but that the polar mannose head was essential for antigen recognition, proliferation and IFNγ production[228]. As well as the number and location of acyl chains, their degree of unsaturation and *cis*, but not *trans*, stereochemistry is critical in determining antigenicity[357, 366].

As the only molecule to drive significant IFNγ responses and one of the most potent inducers of proliferation, $AcPIM_6$ was used to characterise the proliferative response. Stimulation with $AcPIM_6$ induced higher levels of proliferation in NKT cells than in $CD4^+$ or $CD8^+$ T cells (figure 6:6). However, from these data it is not possible to tell if the proliferative $CD4^+$ or $CD8^+$ also co - express CD335 as our flow cytometric labelling panels do not allow the discrimination, however this is a distinct possibility.

Although well characterised in humans and mice, the presence of NKT cells in cattle has been a controversial issue. Originally bovine *CD1D* was identified as a pseudogene[367, 368] and it was assumed that cattle lacked an NKT population as *CD1D* genes are a prerequisite for NKT cell development[369, 370]. Nevertheless, further studies have shown that despite its unusual structure the bovine *CD1D* gene is expressed and translated *in vivo*[371]. Furthermore, recent work has identified a subset of cattle lymphocytes that express both T cell (CD3) and NK cell (NKp46) markers, suggesting the presence of an NKT cell population in bovine peripheral blood[365]. In addition to CD1d restricted invariant cells, human CD1b restricted variant NKT cells have been described in transgenic mice[355] and, given the presence of functional CD1b in cattle, the existence of a CD1b restricted NKT cell population cannot be ruled out. Furthermore, bovine NKT cells have been

shown to express both αβ and γδ TCRs, have a broad TCR repertoire and have fully functional NKp46, CD16 and CD3 signalling pathways[365]. Interestingly these cells require ligation of their CD3 to produce IFNγ. While this may initially suggest a CD3 binding component may be present in the PIM preparations, it is worth noting that the cytokine producing cells have not been identified.

In conclusion, this chapter demonstrates that the crude lipid preparations are capable of generating adaptive immune responses. A significant portion of the proliferative response was shown to be restricted through MHCII and treatment of the lipid fraction with Proteinase K also reduced its proliferative activity. The use of highly purified PIM molecules enabled phenotypic identification of NKT proliferating in response to PIM stimulation. Given that NKT cells have been shown to be important in the development of protective immunity to certain intracellular pathogens including the influenza virus[372] and *Leishmania major*[373], lipid molecules such as $AcPIM_6$ may have a role to play in future vaccines either as protective antigens or biological adjuvants.

Chapter Summary

- **Objective**

  Assessment of the lipid fractions ability to generate adaptive immune responses and identify if the presence of lipopeptide in the fractions was responsible for any stimulation. Characterisation of responding cell populations within PBMC.

- **Results**

  Crude polar lipid fractions were capable of generating both proliferative and cytokine responses. Much of the proliferative response was shown to be restricted through MHCII and the proliferative activity of the polar fraction could also be reduced by treatment with Proteinase K. Not all proliferative response was driven through MHCII or degraded by Proteinase K activity suggesting a lipid component existed. As the most abundant lipid family in the polar fraction, highly purified PIMs were used to stimulate PBMC and NKT were cells found to be the main proliferative population.

- **Conclusions**

  The presence of lipopeptide in lipid preparations is of significance and may be responsible for much of the response seen when using crude fractions. However, lipid molecules are also capable of driving functional responses from cells of the adaptive immune system.

  Bovine NKT cells are capable of recognising, and proliferating in response to the highily mannosylated lipid $AcPIM_6$.

Chapter Seven

Concluding Remarks

The primary purpose of this study has been to address the hypothesis that lipids from *M. bovis* constitute a source of molecules that could aid in the development of control measures for BTB such as subunit vaccines (as antigens or immunomodulatory adjuvants) or diagnostic reagents. To work towards these goals it is necessary to characterise their immunological properties and to identify individual compounds with the desired properties. The data presented here describe bovine immune responses directed at lipids extracted from 2 strains of *M. bovis*.

The 3 main objectives of the work reported here were laid out at the end of Chapter One and each shall be addressed here:

- **Develop methods to isolate and characterise lipid moieties from *M. bovis*:**

  While the method used to extract polar and apolar lipid fractions in this study is not novel, a full 2D TLC analysis of lipids from *M. bovis* AF 2122/97 has not been published previously. At this macroscopic level, little difference can be seen between AF 2122/97 - derived and AN5 - derived lipid fractions. However, the application of densitometrical analysis allowed for some further refinement to the comparisons. Also shown here, and not previously published, is ninhydrin staining

of these lipid fractions which demonstrated the presence of a range of unidentified amine residues within the polar fractions. This work constitutes the first published documentation of the polar and apolar lipid fractions from *M. bovis* AF 2122/97.

Further analysis of the polar fraction was attempted by chemical subfractionation. This proved to be technically demanding, partly due the poor efficiency of column chromatography methods and subsequently due to the difficulty in removing colloidal silica from TLC preparations. Despite these challenges, lipid subfractions were successfully prepared but ninhydrin staining of 2D TLCs demonstrated lipopeptide contamination in all subfractions.

That lipopeptides can mediate immune responses is well known but, despite this, no comprehensive lipopeptide screening has been published for any members of the *M. tuberculosis* complex. The ability to extract and purify lipids using simple methods is of limited use without further work to analyse and identify lipopeptide contaminants and their role in modulating host immunity.

- **Assess the effect of *M bovis* lipids on bovine APC to identify potential immunomodulatory activity which could be exploited in the development of novel adjuvants:**

  Stimulation of bovine MDDC with the polar and apolar lipid fractions showed that components within the polar lipid fraction are capable of mediating effects on the host APC. Significant reduction in important molecules such as MHCII and CD1b was seen, as well as increased IL - 10 and IL - 12 secretion, and these responses could hamper the hosts ability to mount an appropriate immune response.

Despite the presence of lipids known to stimulate Th1 responses, the potentially deleterious effect of the polar lipids on APC may constitute a strategy employed by the bacilli to increase the chances of successful infection. Further refinement of the polar fraction was attempted to try to identify which moieties might be responsible for these effects however, despite the different lipid composition of the subfractions, no differences were found in the ability of the subfractions to mediate reduction in MHCII expression or increase the secretion of IL - 10. Despite the increased levels of IL - 12 measured after stimulation of APC with the polar lipids, the elevated levels of IL - 10 and reduction in important antigen presentation molecules mediated by the lipid fractions is precisely the opposite of an adjuvanting response.

Clearly further work is required on purification of individual lipids. More complex chemical technologies and more advanced purification methods have been applied by others to obtain individual lipid molecules free from contamination with lipopeptide and it is these approaches that must be taken to further separate and define the lipid fractions from *M. bovis*.

- **Identify the lipid targets of the bovine adaptive immune response which could be used as innovative vaccine candidates:**

  Initial experiments documented in this thesis demonstrated the ability of the crude lipid fractions to drive proliferative and cytokine responses in bovine PBMC. Interestingly, stronger responses were seen to the polar fraction, as had been the case with innate immune cells. The finding that much of the proliferative response to the polar fraction was restricted through MHCII and could be partially

degraded by treatment of the lipid fraction with Proteinase K again highlights the potential role of lipopeptide in these lipid preparations. However, neither blockade of MHCII nor enzymatic degradation of lipopeptide completely abrogated proliferative responses and the use of purified PIM molecules enabled the identification of $AcPIM_6$ as a natural ligand of bovine NKT cells. The presence of these cells in cattle has only recently been shown and the discovery of a ligand so soon is a remarkable achievement.

Whether or not $AcPIM_6$ or other lipid molecules could be used as vaccine candidates remains an open question. The non - polymorphic nature of CD1 molecules makes them good targets for a vaccine antigens and the ability to target a specific cell population with an individual antigen has been demonstrated in this thesis. However, the restriction of the proliferating NKT cells is currently unknown but warrants further investigation.

The main achievements of this thesis are the extraction and screening of the *M. bovis* - derived lipid fractions, the documentation of the effects of the lipid fractions on bovine APC and the identification of a specific lipid ligand capable of driving proliferation in bovine NKT cells. However, this thesis also highlights the lack of knowledge surrounding the lipopeptide content of the mycobacterial cell wall and underlines the requirement for cross - discipline collaboration to separate the individual components of these extracts and fully understand the interaction at the point of contact between host and pathogen.

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Appendix

Appendix

Publications Associated with this Thesis

C. Pirson, G. J. Jones, S. Steinbach, G. S. Besra & H. M. Vordermeier, (2012). "**Differential effects of *Mycobacterium bovis* - derived polar and apolar lipid fractions on bovine innate immune cells**" Veterinary Research **43**:54

C. Pirson, R. Engel, G. J. Jones, T. Holder, O. Holst & H. M. Vordermeier, (2015) "**Highly purified mycobacterial phosphatidylinositol mannosides drive cell mediated responses and activate NKT cells in cattle**" Clinical and Vaccine Immunology **22**:2

Poster presented at VIth International *M. bovis* Conference

Differential effects of *Mycobacterium bovis* - derived polar and apolar lipid fractions on bovine innate immune cells

VETERINARY RESEARCH

RESEARCH Open Access

Differential effects of *Mycobacterium bovis* - derived polar and apolar lipid fractions on bovine innate immune cells

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Abstract

Mycobacterial lipids have long been known to modulate the function of a variety of cells of the innate immune system. Here, we report the extraction and characterisation of polar and apolar free lipids from *Mycobacterium bovis* AF 2122/97 and identify the major lipids present in these fractions. Lipids found included trehalose dimycolate (TDM) and trehalose monomycolate (TMM), the apolar phthiocerol dimycocersates (PDIMs), triacyl glycerol (TAG), pentacyl trehalose (PAT), phenolic glycolipid (PGL), and mono-mycolyl glycerol (MMG). Polar lipids identified included glucose monomycolate (GMM), diphosphatidyl glycerol (DPG), phenylethanolamine (PE) and a range of mono- and di-acylated phosphatidyl inositol mannosides (PIMs). These lipid fractions are capable of altering the cytokine profile produced by fresh and cultured bovine monocytes as well as monocyte derived dendritic cells. Significant increases in the production of IL-10, IL-12, MIP-1β, TNFα and IL-6 were seen after exposure of antigen presenting cells to the polar lipid fraction. Phenotypic characterisation of the cells was performed by flow cytometry and significant decreases in the expression of MHCII, CD86 and CD1b were found after exposure to the polar lipid fraction. Polar lipids also significantly increased the levels of CD40 expressed by monocytes and cultured monocytes but no effect was seen on the constitutively high expression of CD40 on MDDC or on the levels of CD80 expressed by any of the cells. Finally, the capacity of polar fraction treated cells to stimulate alloreactive lymphocytes was assessed. Significant reduction in proliferative activity was seen after stimulation of PBMC by polar fraction treated cultured monocytes whilst no effect was seen after lipid treatment of MDDC. These data demonstrate that pathogenic mycobacterial polar lipids may significantly hamper the ability of the host APCs to induce an appropriate immune response to an invading pathogen.

Introduction

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis* (*M. bovis*), is a zoonotic disease of significant economic, animal and public health burden. Consumption of raw or unpasteurised animal products and contact with infected carcasses plays a large role in zoonotic *M. bovis* infection of humans in Africa and South America [1,2]. Yet this is not a problem solely associated with poverty and less developed countries as evidenced by a recent report that up to 45% of all TB infected children in San Diego were caused by *M. bovis* [3]. The incidence of BTB in cattle in Great Britain (GB) has undergone steady and continual increase since 1998 despite the implementation of control measures, possibly due to the presence of a wildlife reservoir [4]. Within GB, BTB has spread drastically since the Foot & Mouth disease outbreak in 2001 with the annual number of animals slaughtered rising from a mean of 7116 animals between 1998 and 2001 to an annual mean of 26 277 cases between 2002 and 2010 inclusive [5].

The first point of contact between *M. bovis* and its host is likely to be interaction between receptors of its sentinel cells of the innate immune system such as macrophages or dendritic cells (DC) and the surface expressed molecules of the bacilli, the majority of which are lipid in nature [6,7]. Recognition of mycobacterial lipids by macrophages has been demonstrated via the mannose receptor, complement receptors, scavenger receptors and CD14 [8-10]. Furthermore, various lipids

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from *M. tuberculosis* have been shown to ligate TLR2 on human macrophages [11]. DC are known to possess and utilise these same receptors as well as other, structurally related molecules such as the DC specific C - type lectin (DC - SIGN) which have also been heavily implicated in immune recognition of the bacilli [12-14].

Many lipids have been implicated in mycobacterial virulence which are not found in other bacterial genera. Lipomannan (LM), lipoarabinomannan (LAM), the phosphatidylinositol mannosides (PIMs), the cord factors trehalose mono- and dimycolate (TMM and TDM) and the phthiocerol dimycocerosates (PDIMs) are all surface bound mycobacterial lipids capable of modulating innate immunity [15-17]. Recently, newly identified lipids, such as monomycolyl glycerol (MMG), have been shown to modulate host immunity [18] and hypervirulence [19]. However, little data exist which describe the effect of *M. bovis* derived lipid on bovine innate cells. Previously published work usually relies on non - pathogenic mycobacterial species or makes use of an animal model rather than the pathogen's natural host. For example, work performed by Hope et al. uses bovine monocyte-derived DC (MDDC) but these cells are stimulated with a synthetic lipopeptide [20] rather than a *M. bovis*-specific lipid antigen. Additionally, although Reed et al. demonstrated that blockage of synthesis of the phenolic glycolipid (PGL) correlated with increased secretion of TNF-α, IL - 6 and IL - 12 by the host, and removed the "hyperlethal" phenotype displayed by the bacilli, this was only shown in the murine model [19].

Thus, to assess host immune responses to its natural pathogen's lipid constituents, lipids were extracted from virulent *M. bovis* AF 2122/97 and used to stimulate various bovine innate immune cells isolated from live, TB free, cattle. Cellular responses were evaluated by measuring cytokine production, alterations in cell surface molecules and induction of T-cell proliferation.

Materials and methods

Preparation of bacterial isolates for lipid extraction

Bacterial isolates were grown in Middlebrooks 7H9 medium as previously described [21].

Briefly, bacterial cells were grown in 100 mL volumes in rolling culture flasks inoculated with 1 mL of a starting culture. At mid - log phase, cultures were decanted into sterile tubes and pelleted before being washed twice in sterile water. Finally, pellets were resuspended in 5 mL sterile water and heat killed in a water bath at 80 °C to 90 °C for between 1 and 2 h and finally freeze dried.

Extraction of crude free mycobacterial lipid

The extraction of mycobacterial lipid has been previously described [22]. Briefly, freeze dried bacterial cells were suspended in methanolic saline before an equal volume of petroleum ether was added and the mixture stirred for 12 to 16 h. Cells were pelleted by centrifugation (7000 *g* for 10 min) and the non-aqueous phase containing the apolar lipids was removed and stored. An equal amount of petroleum ether was added to the aqueous lower phase and the mixture stirred for 2 h before being centrifuged (7000 *g* for 10 min) and the non-aqueous layer removed and pooled with the first. These non-aqueous petroleum ether extracts were dried using a rotary evaporator with cold finger condenser and the lipid transferred to a pre-weighed glass tube in 4 : 1 $CHCl_3$: CH_3OH. Evaporation of the $CHCl_3$: CH_3OH was achieved using a heating block and N_2 gas stream and the tube weighed to determine the mass of apolar lipids extracted.

Extraction of polar lipids was performed by adding $CHCl_3$, CH_3OH and 0.3% aqueous NaCl in a 9 : 10 : 3 ratio to the cell pellet. The mixture was stirred for 12 to 16 h before being passed through 2 Whatman # 91 filters. Once dried, the cells were recovered from the filter papers and re-extracted twice using a 5 : 10 : 4 mixture of $CHCl_3$, CH_3OH and 0.3% NaCl. After a final filtration to remove the cells, equal volumes of $CHCl_3$ and 0.3% NaCl were added and the mixture stirred for one hour, after which the aqueous phase containing the polar lipids was removed and dried in a rotary evaporator. Final polar lipid mass was ascertained as described for the apolar petroleum ether extracted lipid fraction.

Analysis of lipid fractions by 2D Thin Layer Chromatography (TLC)

Aluminium backed silica gel 60 F_{254} TLC plates (Fisher Scientific, Loughborough, Leics, UK) were cut into approximately 6 cm squares and 100 μg of lipid extract was spotted onto the plates using glass micro - capillary pipettes. Plates were dried thoroughly before being placed in TLC tanks containing appropriate solvent mixtures (systems A - E, Table 1). TLC plates were dried between each run and before staining to ensure that residual solvent was removed. Staining was performed using a 5% solution of molybdophosphoric acid (MPA) (Sigma Aldrich, Poole, Dorset, UK) in 95% ethanol (Figure 1). Stains were sprayed onto TLC plates which were subsequently charred using a hot air gun before being photographed or scanned. Identification of individual lipids was performed by comparison with previously published TLC analysis [23].

Cattle

BTB free cattle between the ages of 6 and 36 months were obtained from herds within 4 - yearly testing parishes with no history of a BTB breakdown in the past 4 years. These animals were purchased when around 6 months old and transported to AHVLA. Whilst at

Table 1 Solvent systems for TLC analysis of mycobacterial lipids (adapted from [22]).

Solvent System	Run Direction	Components	Runs	Lipids Resolved
A	1	petroleum ether : ethyl acetate (98 : 2)	3	PDIM, TAG, MQ
	2	petroleum ether : acetone (98 : 2)	1	
B	1	petroleum ether : acetone (92 : 8)	3	AT, FA
	2	toluene : acetone (95 : 5)	1	
C	1	chloroform : methanol (96 : 4)	1	FA, GLY
	2	toluene : acetone (80 : 20)	1	
D	1	chloroform : methanol : water (100 : 14 : 0.8)	1	CF, SL, DAT
	2	chloroform : acetone : methanol : water (50 : 60: 2.5 : 3)	1	
E	1	chloroform : methanol : water (60 : 30 : 6)	1	DPG, PE PI, PIM
	2	chloroform : acetic acid : methanol : water (40 : 25 : 3 : 6)	1	

AHVLA they tested negative for BTB using both the Bovigam IFNγ assay (Prionics AG, Schlieren-Zurich, Switzerland) and the single intradermal comparative cervical tuberculin test (SICCT).

Isolation of bovine PBMC from whole blood

Whole blood was mixed in equal amounts with sterile HBSS containing 10 U mL^{-1} heparin. This mixture was overlaid onto Histopaque 1077 (Sigma Aldrich) and centrifuged at 800 *g* for 40 min. The PBMC interface was removed using a pastette and washed twice in HBSS containing heparin. Live cells were identified via trypan blue exclusion and enumerated using a haemocytometer.

Isolation of $CD14^+$ monocytes from bovine PBMC

PBMC were suspended in 80 μL of MACS rinsing buffer per 10^7 cells before the addition of 10 μL of MACS anti - CD14 MicroBeads (Miltenyi Biotec, Bisley, Surrey, UK) per 10^7 cells. After a 15 min incubation at +4 °C on a rotator, cells were pelleted and resuspended in 500 μL per 10^8 cells and passed through MACS LS columns as per the manufacturer's instructions.

The $CD14^+$ fraction was counted and cells diluted to 1.5×10^6 mL^{-1} in cell culture medium (RPMI 1640 containing 25 mM HEPES, 10% FCS, 1% NEAA, 5×10^{-5} mM β2 - mercapto-ethanol, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin [Gibco Life Technologies, Paisley, UK]).

Generation of bovine cultured monocytes and MDDC

$CD14^+$ monocytes were plated in 1 mL volumes at 1.5×10^6 mL^{-1} in 24 well plates (Nunc Nunclon, Roskilde, Denmark) before adding either 1000 U mL^{-1} equine GM-CSF (Kingfisher Biotech, St Paul, MN, USA) (cultured monocytes) or 1000 U mL^{-1} equine GM-CSF and 4 ng mL^{-1} bovine IL-4 (AbD-Serotec, Kidlington, Oxon, UK) (MDDC). Cells were cultured at 37 °C + 5% CO_2 for 3 days [20], following which they were harvested, re-plated at 1.5×10^6 mL^{-1} in fresh cell culture medium and the appropriate volume of lipid solution was added. Cells were cultured for a further 12 to 16 h before supernatants were collected and cells harvested for subsequent flow cytometric analysis.

Preparation of lipid antigen suspensions

Suspensions of all lipid antigens were prepared in an aqueous phase for use in cell culture experiments after first removing any $CHCl_3$: CH_3OH by evaporation using an N_2 gas stream. Cell culture medium was added to the dried lipid and the mixture subjected to 2 cycles of heating at 80 °C and then sonication for 5 min. Apolar and polar lipids were used to stimulate cells in vitro at 20 μg mL^{-1} for 12 to 16 h. These conditions were shown to be optimal in previous experiments (data not shown).

Measurement of cytokine production

Culture supernatants were assayed for cytokine levels using the MSD multiplex platform (Meso Scale Discovery, Gaithersburg, MD, USA) as previously described [24,25]. Briefly, supernatants were analysed using a custom multiplex electrochemiluminescent system which allows simultaneous detection of IL-1β, IL-6, IL-10, IL-12, MIP-1β and TNF-α (Meso Scale Discovery). Multiplex 96 well plates were supplied with target capture antibodies spotted onto 6 separate carbon electrodes in each well (anti-bovine TNF-α [Endogen, Rockford, IL, USA]; anti-bovine IL-10 and anti-bovine IL-12 [AbD-Serotec]; anti-bovine IL-1β, anti-bovine IL-6 and cross-reactive anti-human MIP-1β [Meso Scale Discovery]). Plates were blocked with MSD assay buffer for 30 min at room temperature before the addition of samples or standards for 1 h at room temperature. Recombinant standard controls (Meso Scale Discovery) were prepared by serial dilution. After incubation, plates were washed and combined biotinylated secondary detector antibodies were added for a further hour. Finally, plates were washed, loaded with MSD read buffer and analysed using an MSD Sector Imager 6000.

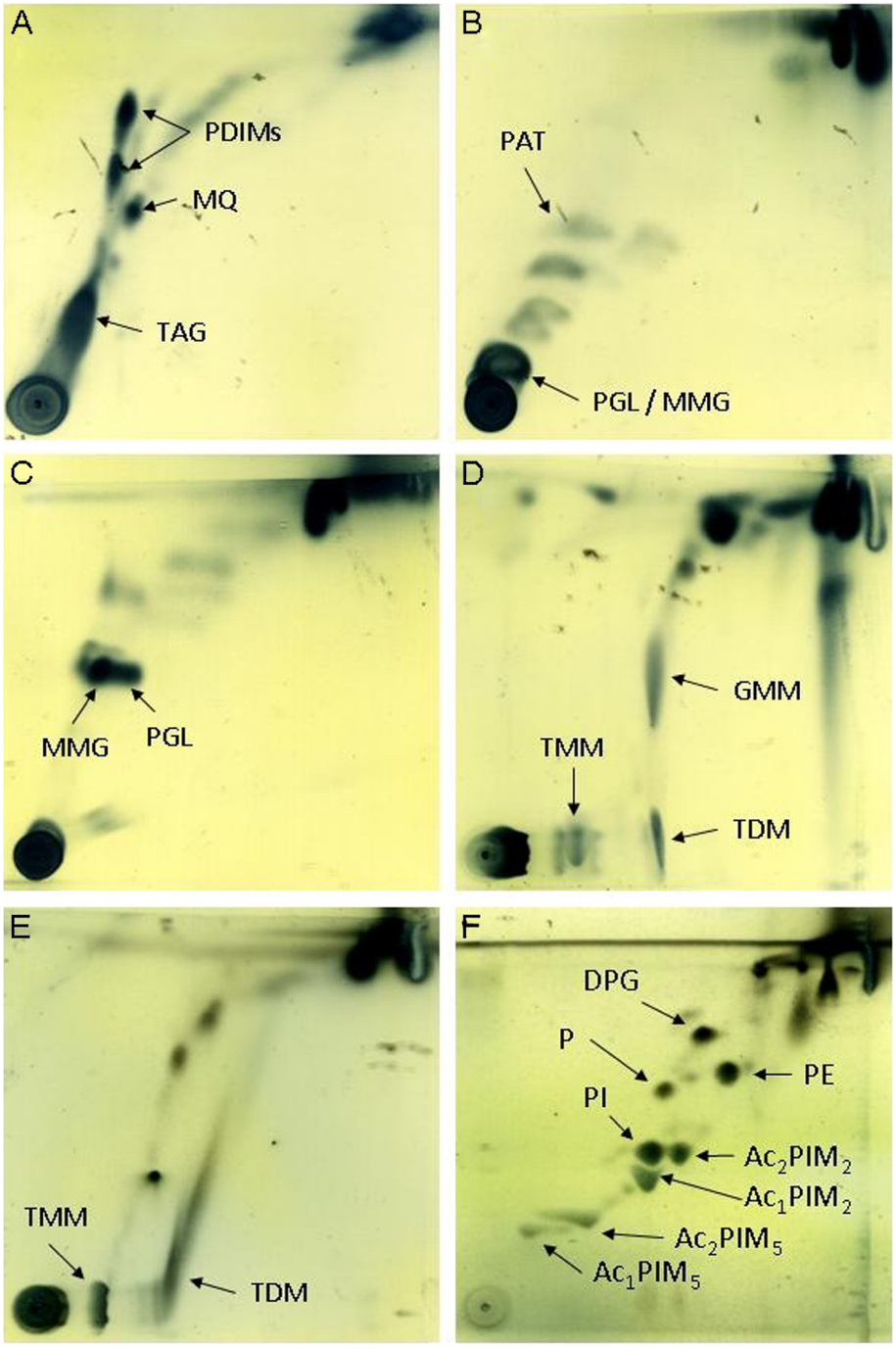

Figure 1 2D TLC analysis of crude, free lipids extracted from M. bovis AF 2122/97 and stained with MPA. A - D: Apolar fraction analysed with TLC systems A, B, C and D. **E** and **F**: Polar fraction analysed with systems D and E. PDIM - phthiocerol dimycocerosate; MQ - menaquinone; TAG - triacyl glycerol; PAT - pentacyl trehalose; PGL - phenolic glycolipid; MMG - monomycolyl glycerol; TMM - trehalose monomycolate; TDM - trehalose dimycolate; GMM - glucose monomycolate; DPG - diphosphatidyl glycerol; PE - phosphatidyl ethanolamine; PIM - phosphatidylinositol mannosides (integers denote number of mannoside or acyl groups); PI - phosphatidyl inositol; P - phospholipid.

Cell labelling and analysis by flow cytometry

Cultured cells were suspended in PBS and labelled with the live / dead indicator ViViD (Invitrogen Life Technologies, Paisley, UK) before being transferred to a 96 well plate and washed using 150 μL MACS rinse buffer. Cells were stained for 15 min using either anti-bovine CD14 (ccG33; Institute for Animal Health; 1:50 dilution), anti-equine MHCII (MCA1085; AbD Serotec; 1:50

dilution), anti-bovine CD40 (IL-A156; cell supernatant; AHVLA; 1:10 dilution), anti-bovine CD80 (IL - A159; cell supernatant; AHVLA; 1:10 dilution), anti-bovine CD86 (IL-A190; cell supernatant; AHVLA; 1:10 dilution), anti-bovine CD1b (CC14; AbD Serotec MCA831G; 1:10 dilution) or an IgG1 isotype control (Av20; Institute for Animal Health; 1:50 dilution). Labelled cells were washed using 150 μL MACS rinse buffer and secondary labelling was performed using a 1:400 dilution of anti-IgG1 conjugated to R-Phycoerythrin (R-PE) (Invitrogen; P21129) in 50 μL volumes for 10 min. After incubation, cells were washed by the addition of 150 μL PBS, pelleted and resuspended in 100 μL of 2% paraformaldehyde (Cytofix; BD Biosciences, Oxford, Oxon, UK) for at least 30 min at 4 °C before analysis on a CyAn ADP analyser. For capture and analysis, initial gating was on single, ViViD^{lo} (live) cells into a subsequent small cell/lymphocyte exclusion gate.

One way mixed lymphocyte reaction

Bovine MDDC and cultured monocytes were prepared from 1 animal as described above. Following 3 days in culture, cells were pulsed with lipid antigen overnight before being enumerated, washed to remove any cytokines and lipid from the media and incubated at 10^7 cells mL^{-1} in the presence of Mitomycin C at 100 μg mL^{-1} for 30 min at 37 °C + 5% CO_2. Lipid pulsed, Mitomycin C treated MDDC or cultured monocytes were cultured in 1 mL at 37 °C + 5% CO_2 at 2×10^5 with 1×10^5 PBMC isolated from a second, allogeneic animal. After 5 days, cells were pulsed overnight with 1 μCi well^{-1} of ^{3}H-thymidine before being harvested using a Harvester 96 Mach III (TomTec Inc, Hamden, CT, USA). Lymphocyte proliferation was assessed by the increased cellular incorporation of ^{3}H-thymidine which was measured using a MicroBeta^2 2450 (Perkin Elmer, Waltham, MA, USA).

Data and statistical analysis

All data representation and statistical analysis was performed using GraphPad Prism version 5.04 and GraphPad InStat version 3.06 (GraphPad Software, La Jolla, CA, USA). Flow cytometric data was analysed using repeated measures ANOVA with a Bonferroni multiple comparisons post test. Cytokine profile analysis was performed using a Friedman non-parametric repeated measures ANOVA with Dunns multiple comparisons test.

Results

Extraction and analysis of lipid from AF 2122/97

In order to identify individual lipid components within the polar and apolar fractions, lipids extracted from the *M. bovis* reference strain (AF 2122/97) were subjected to 2D TLC analysis using solvent systems of increasing polarity and subsequently stained with MPA. Analysis of the apolar fraction using the least polar TLC system (Figure 1a) identified the presence of phthiocerol dimycocerosates (PDIMs), menaquinone (MQ) and triacyl glycerol (TAG). System B (Figure 1b) revealed that the apolar fraction also contained pentacyl trehalose (PAT), phenolic glycolipids (PGL) and monomycolyl glycerol (MMG). System C (Figure 1c) allowed further resolution of both MMG and PGL. The most polar system used to analyse the apolar fraction (system D, Figure 1d) identified both trehalose monomycolate (TMM) and dimycolate (TDM; cord factor) as well as glucose monomycolate (GMM).

TLC analysis of the polar lipid fraction using system D showed the presence of TMM and TDM (Figure 1e) while the most polar solvent system (E, Figure 1f) enabled identification of the most polar lipids, which included diphosphatidyl glycerol (DPG), phosphatidyl ethanolamine (PE), phosphatidylinositol mannosides (PIMs, integers denote number of mannoside or acyl groups), phosphatidyl inositol (PI) and an unknown phospholipid (P).

Cytokine responses to *M. bovis* - derived lipids

Innate immune cells are known to produce cytokines in response to appropriate antigenic stimuli. In order to assess the effect of *M. bovis* - derived lipids on 3 types of bovine innate cells (freshly isolated monocytes, cultured monocytes and MDDC), cytokine production was measured after stimulation with the lipid fractions (Figure 2). The cytokines investigated included IL-10, IL-12, TNF-α, MIP-1β, and IL-6.

Significantly increased IL-10 secretion was seen from all 3 cell types following stimulation with the polar lipid fraction (Figure 2a). Strong IL-10 responses were seen for 3 animals, while only modest increases were noted for the remaining cattle (Figure 2a). In contrast, little or no significant increase in IL-10 production was seen following stimulation with the apolar lipid fraction, although apolar lipids induced some IL-10 production by cultured monocytes and MDDC from 3 animals. All responses to apolar lipids were by far lower than those induced by the polar lipid fraction.

IL-12 levels in the culture supernatants were measured simultaneously and the results are shown in Figure 2b. Stimulation with the polar lipid fraction induced a large increase in IL-12 production by MDDC, while lower responses were also observed from monocyte and cultured monocyte populations. In contrast to the polar lipids, stimulation with the apolar lipid fraction resulted in minimal increases in IL-12 production by monocytes, cultured monocytes or MDDC.

Levels of MIP-1β were also found to be significantly increased after exposure to the polar lipid fraction with

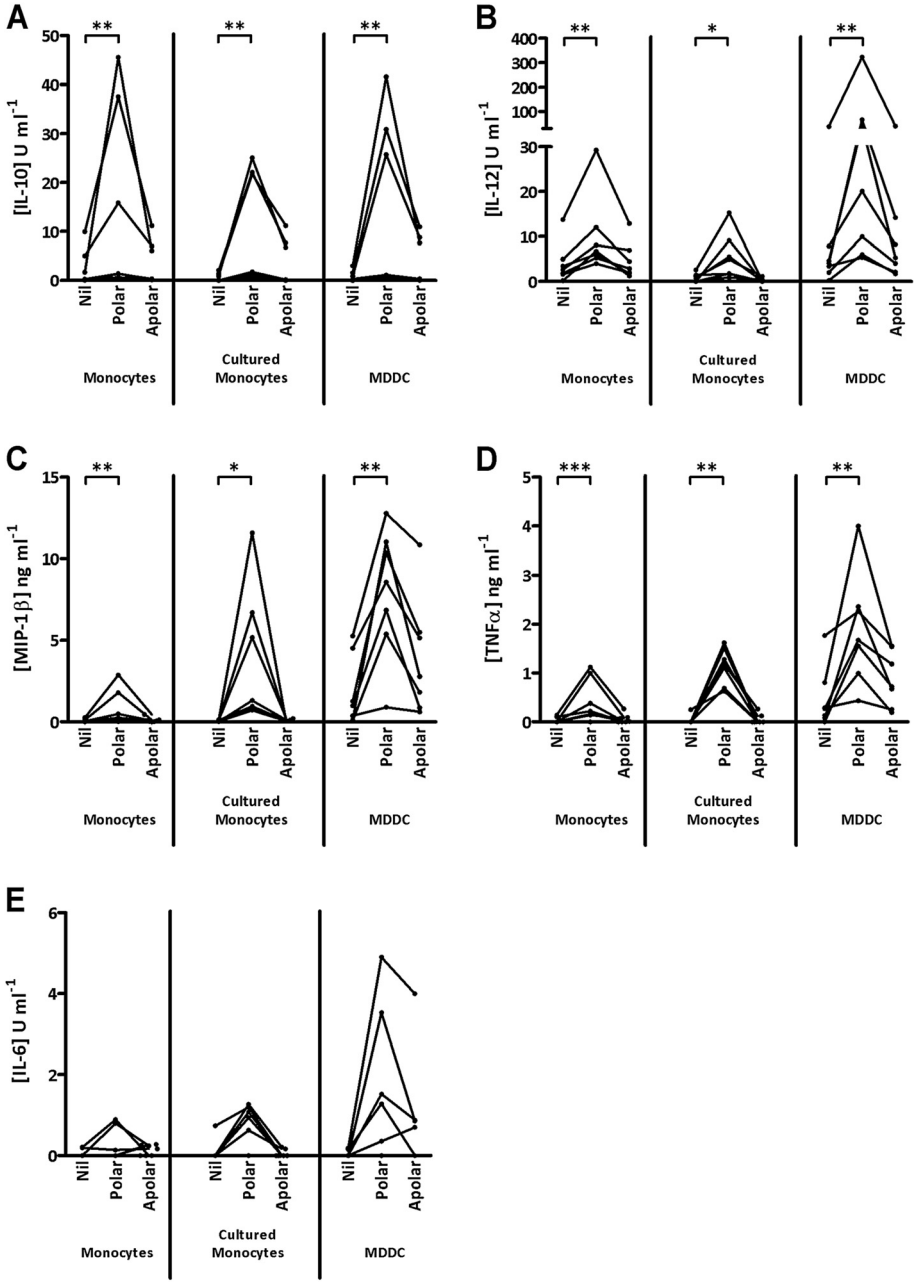

Figure 2 IL - 10 (A), IL - 12 (B), MIP - 1β (C), TNFα (D) & IL - 6 (E) production by monocytes and cultured cells in response to stimulation with crude lipid fractions. Points represent mean responses from duplicate wells for each of 7 animals tested. Lines indicate that cells were derived from the same animal; * $0 < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

no significant increase seen after apolar lipid stimulation (Figure 2c). Both cultured monocytes and MDDC produced noticeably more MIP-1β than fresh monocytes, with MDDC from 6, and cultured monocytes from 3, of the 7 animals responding strongly (Figure 2c).

Significant increases in TNFα production were also seen, again in response to the polar lipid fraction (Figure 2d). While polar lipid treated cultured monocytes from all 7 cattle produced significant levels of TNFα, considerably more TNFα was produced by MDDC (Figure 2d). Further, the level of TNFα production was similar between fresh and cultured monocytes (Figure 2d).

The production of IL-6 (Figure 2e) followed a broadly similar pattern to that of TNFα (Figure 2d) although statistical significance was not achieved. Fresh monocytes from 2 cattle produced more IL-6 after exposure to the polar lipids, which also drove increased IL-6

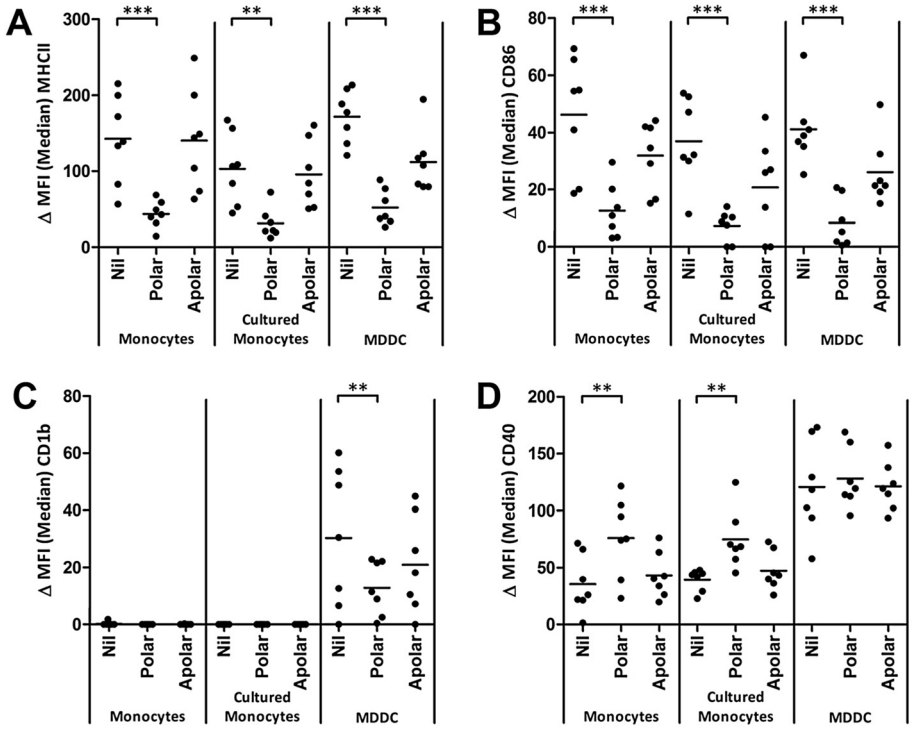

Figure 3 Surface expression of MHCII (A), CD86 (B), CD1b (C) and CD40 (D) on cultured cells and fresh monocytes after exposure to crude polar and apolar lipid fractions. A single point represents the median fluorescence intensity of the specific stain after subtraction of an isotype control (ΔMFI) for each of 7 animals tested; ** $p < 0.01$; *** $p < 0.001$.

production in cultured monocytes from 6 cattle (Figure 2e). Polar lipid driven IL-6 production by MDDC was noted in 5 of the 7 animals screened, with one of these animals producing more IL-6 to the apolar lipid fraction than the polar. While statistical significance was not achieved, the levels of IL-6 produced by MDDC are notably higher than from fresh or cultured monocytes (Figure 2e).

These data clearly demonstrate that the polar lipid fraction drives the production of significant amounts of IL-10, IL-12, MIP-1β and TNFα from all cell types. Furthermore, it is clear that MDDC produced more IL-12, TNFα and IL-6 than fresh or cultured monocytes and MIP-1β production is greater from both cultured monocytes and MDDC than in fresh monocytes.

Phenotypic responses to *M. bovis* - derived lipids

Exposure of bovine antigen presenting cells to the polar lipid fraction lead to significant increases in the production of a variety of cytokines (Figure 2a-e), all of which can play important roles in directing the subsequent cell mediated response. In order to further assess the effect of *M. bovis* - derived lipids and the local cytokine milieu on these cells, analysis of the expression of key antigen presentation related molecules was assessed by flow cytometry (Figure 3).

Stimulation with the polar lipid fraction resulted in a significant reduction in the cell surface expression of MHCII on all three cell types (Figure 3a). Furthermore, MHCII expression was also lower on MDDC following stimulation with apolar lipids, although this did not achieve statistical significance (Figure 3a). CD86 expression was also significantly reduced on all three cell types following stimulation with the polar lipid fraction (Figure 3b). While there was a trend for lower CD86 expression on all three cell types following stimulation with the apolar lipid fraction, this again did not achieve statistical significance (Figure 3b). Interestingly, the lipid-specific antigen presentation molecule CD1b, which was constitutively expressed on MDDC, was not present on monocytes or cultured monocytes nor could it's expression be modulated in these cell types by mycobacterial lipids (Figure 3c). By contrast, MDDC expressed CD1b constitutively and incubation with the polar lipid fraction resulted in a significant reduction in CD1b surface expression (Figure 3c).

Not all cell surface molecules were down-regulated following treatment with lipids. CD40 expression on both monocytes and cultured monocytes increased significantly following stimulation with the polar lipid fraction (Figure 3d), although no effect was seen on MDDC. Finally, no significant difference was seen in CD80 levels

following stimulation with either the polar or apolar lipid fractions (data not shown).

In summary, these data demonstrate that *M. bovis* - derived lipids, and in particular the polar fraction, downregulate the expression of several key cell surface molecules involved in antigen presentation.

Consequence of exposure to *M. bovis* - derived lipids

To identify and assess any functional consequence of the lipid induced reduction in molecules related to antigen presentation the ability of lipid treated innate cell types to stimulate an alloreactive response was assessed. Co-culture of the responder PBMC population with either untreated MDDC or cultured monocytes resulted in a 5 - fold increase in their proliferation (Figure 4). No proliferation was noted for either MDDC or cultured monocytes in the absence of responder cells (data not shown). Polar lipid treated MDDC retained their ability to induce proliferation in the responder population despite the downregulation of important costimulatory molecules. In contrast, allo - stimulation of the responder population by polar lipid treated cultured monocytes resulted in significantly reduced proliferative responses to levels comparable with the unstimulated responder control cells (Figure 4).

Discussion

Mycobacterial lipids have long been implicated in the interaction between the pathogen and its host. Here we describe the consequence of exposure to lipids derived from a virulent *M. bovis* on the innate immune cells of cattle.

Extraction of mycobacterial lipids and their subsequent analysis by 2D TLC has been previously described [22], but little data exists on total lipid profiling of *M. bovis.* Previous work by Dandapat et al. [26] attempted to characterise *M. bovis* based on the expression of PGL and PDIMs, but only as a tool for identification of the organism. In Figure 1, we have applied a complete range of TLC analyses to polar and apolar lipid extracts which has allowed the identification of a broad range of characteristic mycobacterial lipids including PDIMS (Figure 1a), the *M. bovis* characteristic PGL [27] (figure 1 B-C), TDM (Figure 1d-e) and PIMs (Figure 1f). As expected, no sulphoglycolipid was found (Figure 1d). Interestingly, TDM was found in both the polar and apolar extracts (Figure 1d-e). This may be related to it's particularly amphipathic nature [28] and variable acylation states which are known to alter both immunogenicity and hydrophobicity [29,30] and may cause the molecule to split differentially across the biphase interface during lipid extraction.

To discover if the lipid fractions were capable of mediating responses of bovine innate immune cells, stimulation experiments were performed and the level of a range of cytokines was analysed. Significant increases in the production of various cytokines were measured only after cells were exposed to the polar lipid fraction. Perhaps most striking is the significant increase in the production of the Th-1 polarising IL-12 and the anti-inflammatory cytokine IL-10 (Figure 2a-b). While this may seem contradictory, it is important to note that the fractions used are complex mixtures of a variety of lipids some of which are known to induce potent

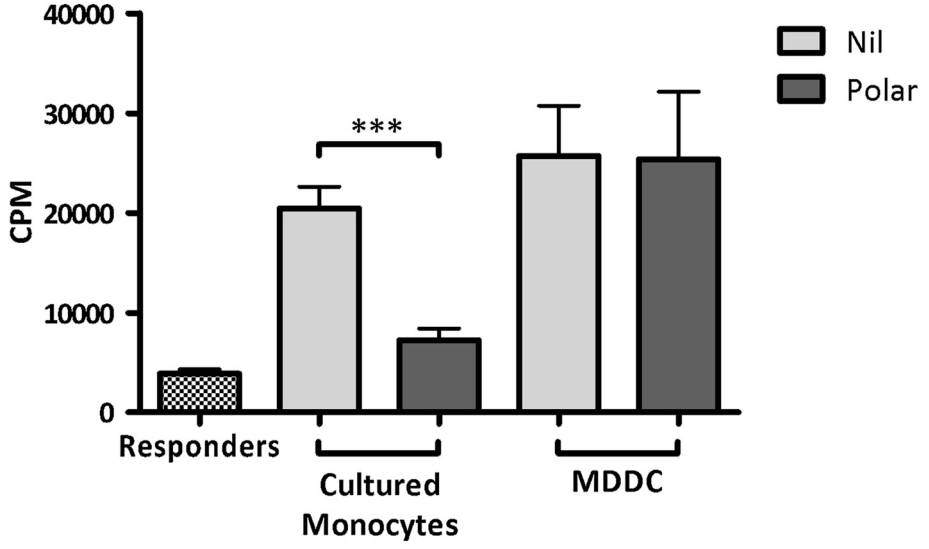

Figure 4 Proliferative responses (in counts per minute [CPM]) of 1×10^5 PBMC after allotypic stimulation with either untreated (grey bars) or polar lipid treated (red bars) MDDC or cultured monocytes. Bars represent the mean of triplicate wells ± standard error of the mean; *** $p < 0.001$.

immunostimulatory cytokine profiles, such as MMG [18] and others, such as glycerol monomycolate (GroMM) are known Th2 polarisers [31].

Little evidence exists on cytokine production by antigen presenting cells after treatment with lipids, however many lipids have been assessed in the context of both $CD4^+$ and $CD8^+$ T cells. For example, TDM, which is present in the polar and apolar fractions (Figure 1d-e), has been shown to induce both Th1 and Th2 cytokines. The induction of IFNγ and IL-12 and the depletion of IL-4 producing NK cells has been attributed to TDM [32,33] as well as a role, along with IL-6 and TNFα, in stable granuloma formation [34]. Yet TDM is also implicated in the production of IL-5 and IL-10 in a CD1 dependent manner [35]. Furthermore, GroMM has been implicated in the induction of Th2 polarising responses [31] where as the closely related GMM has been shown to induce Th1 cytokine responses in T cells [36]. Anti-inflammatory effects have also been attributed to PIM_2 and PIM_6 where, upon lipid treatment of LPS activated macrophages, Doz et al. measured downregulation of TLR4, TNFα, IL-12p40, IL-6, KC and IL-10 as well as MyD88 mediated NO release [37].

Given the significant increase in IL-10 production by all innate cell types assessed, and the important role these cells play in generating and directing the immune response, we analysed the expression of antigen presentation associated cell surface molecules after lipid exposure. Lipid treatment of APCs leads to a significant decrease in the levels of costimulatory molecules associated with antigen presentation including MHCII and CD86 on all cell types studied and CD1b on MDDC (Figure 3a-c). Negative regulation of these molecules by a variety of lipid components has been noted previously, especially MHCII in human and murine systems. Similar to the data presented here, the 19-kDa lipoprotein is capable of downregulating MHCII expression on human THP-1 macrophages by inhibiting activation of the IFNγ - induced CIITA [38,39]. Downregulation of MHCII, as well as TLR2 and TLR4, has also been reported on human MDDC after lipid exposure [40] and a further study also found impaired expression of CD1a, MHCII, CD80 and CD83 on human MDDC [41].

Downregulation of CD1 molecules has also been shown through the discovery that MDDC generated from BCG treated monocytes did not express CD1 and showed reduced MHCII, CD40 and CD80 [42] and this has since been shown to be due to cell wall associated carbohydrate α-glucan [43] and mediated through the p38 MAPK pathway [44]. However these experiments have all been performed in human or murine systems and with specific lipids, often from avirulent bacterial isolates.

Interestingly, treatment of fresh and cultured monocytes with the polar lipid fraction significantly increased the level of CD40 expression (Figure 3d) and this effect is not seen on MDDC. This finding seems contradictory to the published literature [42,45] however these studies used BCG or TDM alone, rather than the complex and more biologically representative lipid preparations derived from virulent mycobacteria used here, as well as being performed in human or murine macrophage models. Bovine MDDC expression of CD40 does not alter after stimulation with either polar or apolar lipids which may be due to its constitutively higher levels of expression than on fresh or cultured monocytes.

Finally, no difference was seen in the expression of CD80 after lipid treatment, although this has also been reported in other systems using virulent *M. tuberculosis* or avirulent BCG derived lipids [41,42,45].

The significant loss of MHCII, CD86 and CD1b is consistent with the phenotype of an impaired antigen presenting cell [46]. Given the effect of the polar lipids on the expression of these molecules and the concurrent increase in IL-10 production, we hypothesised that the polar lipid fraction, or one of its components, hampers the ability of the cells to successfully present antigen to T cells and may be able to suppress the induction of a Th1 response during infection. To assess any functional deficit in these cells, especially due to the loss in MHCII, lipid treated and untreated cells were used to drive allotypic proliferative responses.

Cultured monocytes drove proliferation of allogeneic PBMC (Figure 4) and treatment of cultured monocytes with the polar lipid fraction significantly abrogated these responses as suggested by the downregulation of MHCII and other costimulatory molecules. Proliferative responses were also seen when allogeneic PBMC were combined with untreated MDDC (Figure 4) however no difference in proliferation was seen using lipid treated MDDC despite flow cytometric analysis revealing characteristic reduction in the level of MHCII on the MDDC (data not shown). While these results seem at odds with each other, it is possible that the loss of MHCII may be overcome by the high level of CD40 expressed by MDDC (Figure 3d) or the constitutively higher levels of IL-12 produced by these cells which further increases significantly after lipid stimulation (Figure 2b). Also, some evidence exists that the presence of CD80 is enough to stimulate allogeneic T cells in the absence of CD86 signalling [47]. Given the significant reduction in CD86 expression on MDDC, the maintenance of CD80 may play a role. Finally, it is possible that, due to constitutively higher levels of MHCII and CD40 present on MDDC, as well as their expression of CD1b, the levels of MHCII and CD86 on these cells remains sufficient to drive an allotypic reaction.

These data demonstrate that *M. bovis* derived lipid fractions are capable of stimulating responses in bovine innate cells and that these different cell types respond in distinct ways.

Interestingly, the alteration in cell surface phenotype of both cultured monocytes and MDDC seen after polar lipid stimulation is also evident after exposure to the apolar lipid fraction, albeit to a lesser, not statistically significant, extent. This may be due to specific lipid components present in both the polar fraction and the apolar preparation, such as TDM. However, it may also be due to the insolubility of less polar lipids in the aqueous environment an in vitro culture system which may limit lipid bioavailability.

In conclusion, we present here the first data to demonstrate the regulatory effects of *M. bovis* - derived lipids on bovine innate cells. These lipids, especially those contained within the polar fraction are capable of interacting with the host's innate immune cell's such that the cells ability to initiate an adequate T cell response may be compromised, although this effect could only be demonstrated for cultured monocytes and not MDDC. The lipid fractions used in this study contain the total free extractable lipid from *M. bovis* AF 2122/97, hence we were not able to attribute these effects to any specific lipid entities. However work is currently being undertaken in our laboratory to further define these responses and identity the lipids which are responsible for mediating the effects we have shown. Nevertheless, the effects mediated by these lipids may play a pivotal role in the outcome of infection and aid further identification of individual lipid components responsible for the immunomodulatory effects as well as new targets for attenuation and novel vaccine candidates and adjuvant preparations.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
CP - Carried out the studies and prepared the manuscript. SS - Assisted with dendritic cell culture methods. GJ - Participated in the study design and proofing of the manuscript. GB - conceived of the study and participated in its design and coordination. MV - conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Highly Purified Mycobacterial Phosphatidylinositol Mannosides Drive Cell-Mediated Responses and Activate NKT Cells in Cattle

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Mycobacterial lipids play an important role in the modulation of the immune response upon contact with the host. Using novel methods, we have isolated highly purified phosphatidylinositol mannoside (PIM) molecules (phosphatidylinositol dimannoside [PIM_2], acylphosphatidylinositol dimannoside [$AcPIM_2$], diacyl-phosphatidylinositol dimannoside [Ac_2PIM_2], acylphosphatidylinositol hexamannoside [$AcPIM_6$], and diacylphosphatidylinositol hexamannoside [Ac_2PIM_6]) from virulent *Mycobacterium tuberculosis* to assess their potential to stimulate peripheral blood mononuclear cell (PBMC) responses in *Mycobacterium bovis*-infected cattle. Of these molecules, one ($AcPIM_6$) induced significant levels of gamma interferon (IFN-γ) in bovine PBMCs. Three PIM molecules ($AcPIM_6$, Ac_2PIM_2, and Ac_2PIM_6) were shown to drive significant proliferation in bovine PBMCs. $AcPIM_6$ was subsequently used to phenotype the proliferating cells by flow cytometry. This analysis demonstrated that $AcPIM_6$ was predominantly recognized by $CD3^+$ $CD335^+$ NKT cells. In conclusion, we have identified PIM lipid molecules that interact with bovine lymphocyte populations, and these lipids may be useful as future subunit vaccines or diagnostic reagents. Further, these data demonstrate, for the first time, lipid-specific NKT activation in cattle.

Members of the mycobacterial genus are renowned for their waxy, lipid-rich outer envelope. Under physiological conditions, this outer layer is likely to be the first point of contact between the bacterial cell and the host's immune system, and the outcome of this interaction is pivotal in the establishment of infection. One of the most important groups of membrane bound lipids consists of the phosphatidylinositol mannosides (PIMs). Interest in PIMs was stimulated since it was shown that phosphatidylinositol dimannoside (PIM_2) forms the phosphoglycolipid anchor which tethers a large array of glycolipids and lipoglycans, including lipomannan (LM) and lipoarabinomannan (LAM), to the cellular membrane (1). PIMs have been shown to interact with a variety of immune components and mediate significant effects on the host. Ever since the realization that NKT cells could respond to lipid antigens (2–4) and the subsequent discovery of the CD1d-restricted lipid antigen α-galactosylceramide (α-GalCer) (5, 6), much research effort has been concentrated on understanding lipid antigens. Although work was initially focused on invariant NKT cells, it has since been shown that great diversity exists in the lipid-responsive T cell receptor (TCR) repertoire (7–9) and that these diverse NKT cells contribute to the Th1/Th2 balance (7, 10). It has been shown that CD1d-restricted NKT cells are capable of recognizing a variety of lipid antigens, including phospholipids (11). More recently, a CD1b-restricted subset of T cells has been found (12). Similarly to CD1d-restricted invariant NKT (iNKT) cells, the CD1b-restricted variant cells require CD1B for their development and produce proinflammatory cytokines in response to CD1b-expressing dendritic cells (DCs) (12). It is clear that NKT-like cells have a significant role to play in lipid-mediated responses, and the hunt for their antigens has continued (13–15).

Given the ability of lipid molecules to generate responses in peripheral blood mononuclear cells (PBMC), we decided to assess the ability of individual, highly purified natural PIMs to activate bovine lymphocytes. In this study, we developed a novel extraction method which allowed us to extract and highly purify a variety of PIM molecules from virulent *Mycobacterium tuberculosis* H37Rv. The ability of these molecules to induce lymphocyte responses in *Mycobacterium bovis*-infected cattle was investigated by measuring lymphocyte proliferation and gamma interferon (IFN-γ) production. Furthermore, flow cytometry techniques were utilized to characterize responding cell populations.

MATERIALS AND METHODS

Extraction of PIMs. Using the novel methodology outlined below, highly pure phosphatidylinositol dimannoside (PIM_2), acylphosphatidylinositol dimannoside ($AcPIM_2$), diacyl-phosphatidylinositol dimannoside (Ac_2PIM_2), acylphosphatidylinositol hexamannoside ($AcPIM_6$), and diacylphosphatidylinositol hexamannoside (Ac_2PIM_6) were successfully isolated. Individual PIM molecules were analyzed by electrospray ionization mass spectrometry (ESI-MS) to confirm identity and purity as shown in Fig. 1. Up to 1 g of dry bacterial mass of *Mycobacterium tuberculosis* H37Rv was suspended in 20 to 30 ml of H_2O and ruptured utilizing a French press at a minimum of 20,000 kPa. This procedure was performed five times, and the combined sample was lyophilized.

Up to 0.5 g of this lyophilized material was extracted three times according to the method of Bligh and Dyer (16). The dry mass was suspended in 4 ml of H_2O and washed twice in an additional 2 ml of H_2O

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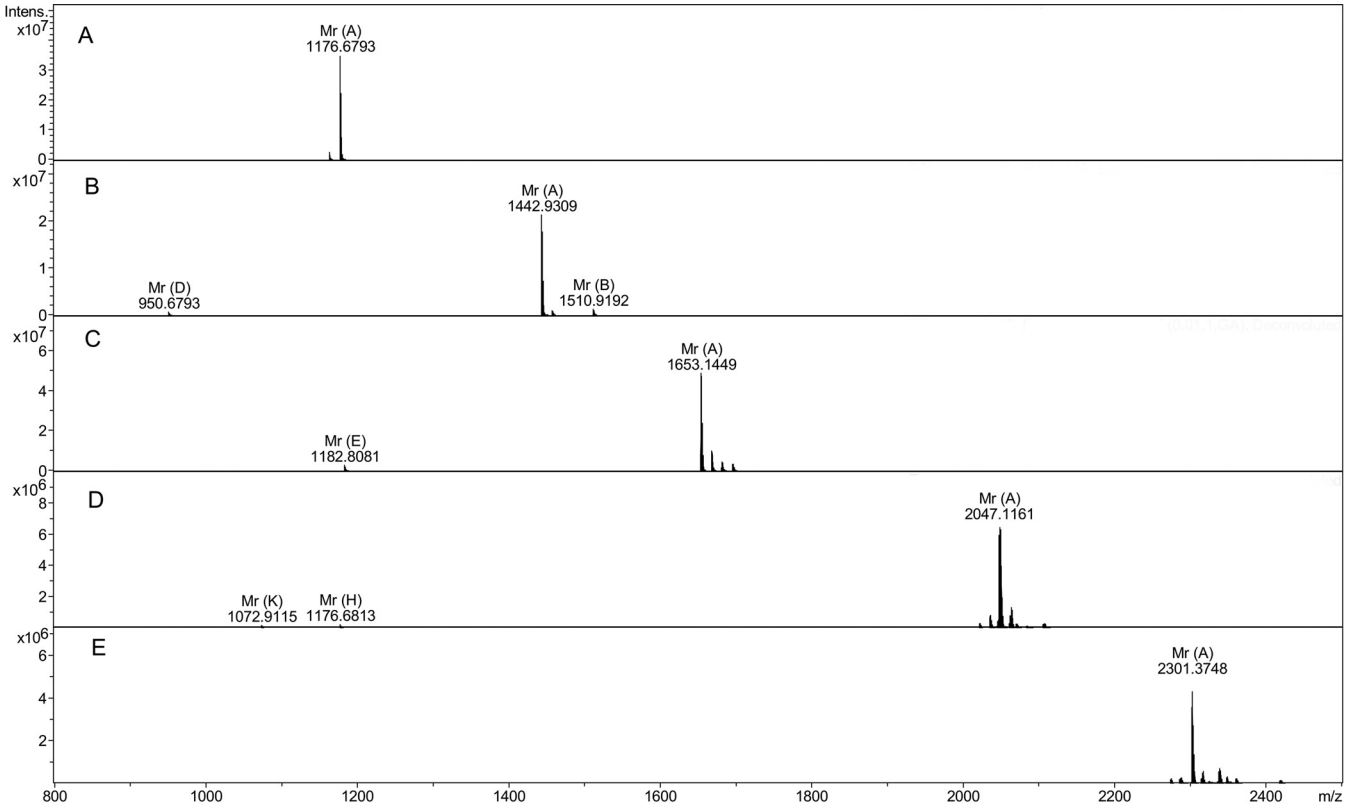

FIG 1 Mass spectrometric proof of purity of isolated PIMs. Identified molecular masses of PIM_2 (calculated molecular mass, 1176.6784 u) (A), $AcPIM_2$ (calculated molecular mass, 1442.9394 u) (B), Ac_2PIM_2 (calculated molecular mass, 1653.1378 u) (C), $AcPIM_6$ (calculated molecular mass, 20147.0881 u) (D), and Ac_2PIM_6 (calculated molecular mass, 2301.3491 u) (E). Letters in parentheses identify the peaks.

before being transferred into a 100-ml Erlenmeyer flask. To this sample was added 30 ml of $CHCl_3$·CH_3OH (1:2, vol/vol), and the sample was sonicated (Branson Sonifier 250; output 2, 40% duty cycle, 20 min). Then, 10 ml of $CHCl_3$ was added, and the sample was sonicated for a further 5 min. Subsequently, an additional 10 ml of H_2O was added, and the sample was sonicated for a final 5 min. The sample was decanted evenly into between two and four 50-ml Nalgene Teflon tubes and centrifuged for 30 min at 10,000 × *g* to generate three phases (a water phase, a $CHCl_3$ phase, and an interphase). The water phases (containing LAM and LM) were removed, combined, and lyophilized, while the $CHCl_3$ phases were transferred into a single 100-ml pear-shaped flask. The total yield of material after three extractions was about 11% of the bacterial dry mass.

Since the remaining interphases contained a lot of $AcPIM_6$, they were suspended in 8 ml of H_2O and combined into 30-ml Kimble high-speed glass tubes before being lyophilized. These phases were then extracted overnight with 30 ml of $CHCl_3$·MeOH (1:2, vol/vol) using a shaker. The sample was then centrifuged for 30 min at 10,000 × *g*, and the $CHCl_3$·MeOH phase was removed. This phase was dried under an N_2 gas stream before being resuspended in equal parts of $CHCl_3$·MeOH, filtered through a 0.2-μm-pore-size polytetrafluoroethylene (PTFE) filter, and dried under N_2. This extraction was repeated two times until no PIMs could be identified (total yield of about 2% of the bacterial dry mass). The obtained PIMs were further purified with silica gel 60 chromatography (see below), as were the $CHCl_3$ phases of the Bligh and Dyer extraction.

Purification of PIMs. All samples were separated on a column (7 by 1 cm) of silica gel 60 (0.04 to 0.063 mm) which was successively eluted with (i) 80 ml of $CHCl_3$·MeOH (8:2, vol/vol), (ii) 60 ml of $CHCl_3$·MeOH (1:1, vol/vol), and finally (iii) 40 ml of $CHCl_3$·MeOH·H_2O (10:10:3, vol/vol/vol). Coextracted cardiolipin and other lipids eluted in the first mobile phase, most PIMs eluted in the second, and the rest of the PIMs eluted in the third. Fractions ii and iii were dried under N_2, resuspended in 10 ml of $CHCl_3$·MeOH (8:2, vol/vol), and passed through a 0.2-μm-pore-size PTFE filter before being analyzed by high-performance thin-layer chromatography (HPTLC). HPTLC was performed using glass-backed 10- by 10-cm silica gel 60 plates (Merck KGAA, Darmstadt, Germany) run in $CHCl_3$·MeOH·H_2O (10:8:2, vol/vol/vol) and stained with Hanessian's stain (0.5 g of ceric sulfate and 25 g of ammonium molybdate in 470 ml of water supplemented with 30 ml of sulfuric acid with stirring) and visualized at 150°C.

Fractions ii and iii from both of the $CHCl_3$ phases of the Bligh and Dyer extraction and the PIMs extracted from the interphase were then further separated by high-performance liquid chromatography (HPLC) using 5-μm Kromasil 100 C_{18} columns (250 by 20 mm) eluted with eluent A ($CHCl_3$·MeOH·H_2O [240:1,140:620, vol/vol/vol] containing 10 mM $NH_4CH_3CO_2$) and eluent B ($CHCl_3$·MeOH [1,400:600, vol/vol] containing 50 mM $NH_4CH_3CO_2$). The initial eluent B gradient was 15% for 60 min, followed by 20% for 140 min, 40% for 80 min, and finally 100% for 60 min at 4 ml min^{-1}. Samples were detected by a light-scattering detector (Sedex; nitrogen pressure, 2 × 10^5 Pa; temperature, 50°C; split, 1:70). Samples were applied in 200 μl of $CHCl_3$·MeOH (8:2, vol/vol). For analytical runs, 10 μg of sample was injected while 10 mg was injected for preparative separations.

Since PIM_2 and $AcPIM_6$ coeluted on the C_{18} column, they were separated by HPLC on 5-μm Prontosil 200-5-C_{30} reverse-phase columns (250 by 4.6 mm) using the same elution reagents. The initial eluent B gradient was 5% for 5 min, followed by 10% for 15 min, 15% for 50 min, and finally 100% for 10 min at 0.8 ml min^{-1}. Samples were injected as a mixture of 0.6 mg in 80 μl of $CHCl_3$·CH_3OH·H_2O (10:10:3, vol/vol/vol) and detected by the light-scattering detector described above.

Cattle. Blood samples were obtained from 10 naturally infected, single intradermal comparative cervical tuberculin test-positive reactors (between 6 and 36 months of age). Animals were sourced from herds with confirmed bovine tuberculosis breakdowns in Devon, Herefordshire, or Worcestershire and were housed at the Animal Health and Veterinary Laboratories Agency (AHVLA) at the time of blood sampling. Infection was confirmed by necropsy and *M. bovis* culture in all animals. All procedures involving animals were carried out under a project license granted by the Home Office of Great Britain under the Animals (Scientific Procedures) Act 1986. This project was approved by the local VLA Animal Ethics Committee prior to submission to the Home Office.

Isolation of bovine PBMC from whole blood. Whole blood was mixed in equal parts with sterile Hanks balanced salt solution (HBSS) containing 10 U ml^{-1} heparin. This mixture was overlaid onto Histopaque 1077 (Sigma-Aldrich) and centrifuged at 800 × *g* for 40 min. The PBMC interface was removed using a pastette and washed twice in HBSS containing heparin. Live cells were identified via trypan blue exclusion and enumerated using a hemocytometer.

Preparation of lipid antigen suspensions. Briefly, lipids were suspended in an aqueous phase for use in cell culture experiments after removal of $CHCl_3$·CH_3OH by evaporation using an N_2 gas stream. Cell culture medium was added to the dried lipid, and the mixture was subjected to two cycles of heating at 80°C and then sonication for 5 min. Lipids were used to stimulate cells *in vitro* at 20 μg ml^{-1} in all assays.

Lymphocyte proliferation assay. Bovine PBMC were prepared from all 10 animals as described above and were cultured in complete cell culture medium (RPMI 1640 medium containing 25 mM HEPES, 10% fetal calf serum [FCS], 1% nonessential amino acids [NEAA], 5 × 10^{-5} mM β2-mercaptoethanol, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin [Gibco Life Technologies, Paisley, United Kingdom]) at 37°C in 5% CO_2 for 5 days in the presence of antigen at 2 × 10^5 cells $well^{-1}$. After 5 days, cells were pulsed with 1 μCi $well^{-1}$ of [^{3}H]thymidine overnight, before being harvested using a Harvester 96 Mach III (TomTec, Inc., Hamden, CT, USA). Lymphocyte proliferation was assessed by the increased cellular incorporation of [^{3}H]thymidine (cpm), which was measured using a MicroBeta^2 2450 plate counter (PerkinElmer, Waltham, MA, USA). Responses to individual PIMs were considered positive if the cpm exceeded the mean plus 2 times standard deviation of cpm for non-antigen-stimulated cultures from all 10 animals.

Measurement of IFN-γ by Bovigam ELISA. Levels of IFN-γ in 5-day supernatants from the proliferation assay were determined using a Bovigam enzyme-linked immunosorbent assay (ELISA) kit (Prionics AG, Switzerland). Responses to individual PIMs were considered positive if the optical density at 450 nm (OD_{450}) exceeded the mean plus 2 times the standard deviation of the OD_{450} for non-antigen-stimulated cultures from all 10 animals.

Measurement of proliferation and phenotyping by flow cytometry. Bovine PBMC were isolated as described above and labeled with CellTrace violet (Invitrogen Molecular Probes, Paisley, United Kingdom) in accordance with the manufacturer's instructions. Briefly, PBMC were suspended at 1 × 10^7 cells ml^{-1} in prewarmed phosphate-buffered saline (PBS), and 5 mM CellTrace violet was added to a final working concentration of 1 μM. Cells were incubated at 37°C for 20 min before unbound dye was quenched with five times the labeling volume of complete cell culture medium at 37°C for 5 min. Finally, cells were pelleted and washed in prewarmed complete cell culture medium, plated at 2 × 10^5 cells $well^{-1}$, and incubated at 37°C in 5% CO_2 for 5 days in the presence of antigen.

Cultured cells were harvested and resuspended in flow cytometry buffer (PBS containing 2% FCS and 0.05% NaN_3) and labeled for 15 min with the Near-IR live/dead indicator NIRViD (Invitrogen Life Technologies, Paisley, United Kingdom) and mouse anti-bovine CD335, also known as NKp46 (AKS1; AbD Serotec, Oxfordshire, United Kingdom). Cells were washed in flow cytometry buffer, and secondary labeling of anti-CD335 was performed using a 1:400 dilution of rat anti-mouse IgG2a conjugated to allophycocyanin for a further 15 min. After a subsequent wash, cells were further labeled with combinations of *R*-phycoerythrin (*R*-PE)-Zenon-labeled (Invitrogen Life Technologies, Paisley, United Kingdom) mouse anti-bovine CD3 (MM1A; WSU Monoclonal Antibody Centre, Pullman, Washington, USA), mouse anti-bovine CD4 conjugated to Alexa Fluor 647 (CC30; AbD Serotec, Oxfordshire, United Kingdom), mouse anti-bovine CD8 conjugated to Alexa Fluor 647 (CC63; AbD Serotec, Oxfordshire, United Kingdom), and Alexa Fluor 488-Zenon-labeled (Invitrogen Life Technologies, Paisley, United Kingdom) mouse anti-bovine γδ-TCR1 (GB21a; WSU Monoclonal Antibody Centre, Pullman, Washington, USA). Finally, labeled cells were washed in flow cytometry buffer and resuspended in 150 μl of 2% paraformaldehyde (Cytofix; BD Biosciences, Oxfordshire, United Kingdom) for at least 30 min at 4°C before analysis on a CyAn ADP analyzer. For capture and analysis, initial gating was on single, NIRViD^{lo} (live) cells into a subsequent lymphocyte gate before gating on CellTrace violet^{lo} cells.

Data and statistical analysis. All data representation and statistical analysis were performed using GraphPad Prism, version 5.04, and GraphPad InStat, version 3.06 (GraphPad Software, La Jolla, CA, USA). Statistical analysis of IFN-γ and lymphocyte proliferation data was performed using a nonparametric repeated-measures analysis of variance (ANOVA; Friedman test) with a Dunn's multiple comparisons posttest.

RESULTS

Highly purified PIM molecules can be isolated from virulent mycobacteria. PIM molecules differing in the number of acyl and mannose residues were highly purified from *M. tuberculosis*. Five distinct PIM molecules were isolated: PIM_2, $AcPIM_2$, Ac_2PIM_2, $AcPIM_6$, and Ac_2PIM_6. In total, these lipids constituted 3% of the bacterial dry mass after silica gel 60 separation. The ability of the purification method to isolate highly pure PIMs is shown in Fig. 1, in which the structures and purity of the different PIM molecules were confirmed by ESI-MS. In addition, bands corresponding to $AcPIM_2$ molecules (that differed only in the number of carbon atoms in the acyl chains) were clearly resolved by thin-layer chromatography (TLC) analysis (see Fig. S1 in the supplemental material).

Purified PIM molecules activate lymphocytes from *M. bovis*-infected cattle. In order to assess the ability of purified PIMs to induce *in vitro* immune responses in cattle, PBMC from 10 naturally *M. bovis*-infected cattle were cultured for 5 days in the presence of each PIM molecule, and the level of IFN-γ was measured by ELISA (Fig. 2A). Both the frequency and strength of IFN-γ responses differed depending on the nature of the PIM molecule. $AcPIM_2$ was least recognized, inducing responses in only 2 of the 10 animals. PIM_2, Ac_2PIM_2, and Ac_2PIM_6 were more frequently recognized, with responses detected in 3 (PIM_2) and 4 (Ac_2PIM_2 and Ac_2PIM_6) out of 10 animals. $AcPIM_6$ was most frequently recognized, inducing responses in half of the animals studied. Furthermore, $AcPIM_6$ was the only PIM molecule to induce significantly greater levels ($P < 0.01$) of IFN-γ overall than nonstimulated controls.

In addition to measuring IFN-γ production, we also investigated the ability of the PIM molecules to induce PBMC proliferative responses in the same animals. Again, the frequency of responding animals differed depending upon the nature of the PIM molecule (Fig. 2B). PIM_2 failed to induce a proliferative response in any of the animals studied, while $AcPIM_2$ induced responses in only 3 out of 10 animals. In contrast, Ac_2PIM_2, $AcPIM_6$, and Ac_2PIM_6 were more frequently recognized, inducing proliferative responses in 6 (Ac_2PIM_2 and $AcPIM_6$) and 7 (Ac_2PIM_6) out of 10 animals. Overall, significantly greater PBMC proliferation was de-

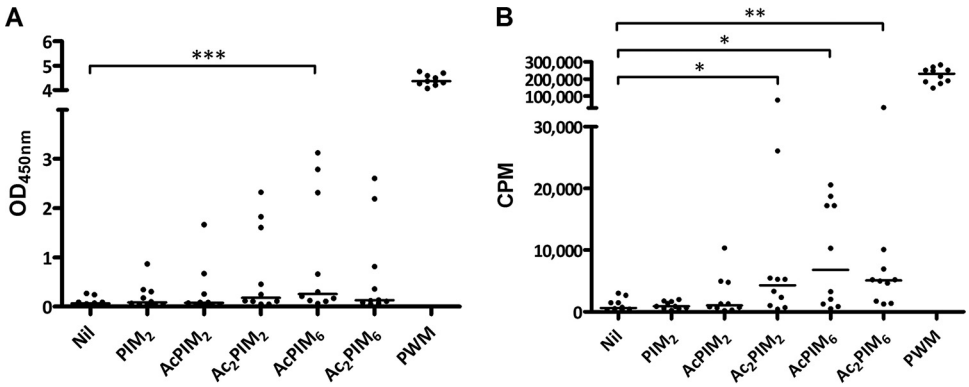

FIG 2 (A) PIM-driven IFN-γ production as measured by Bovigam ELISA. PBMC were incubated with PIMs at 20 μg ml^{-1} for 5 days. Points represent mean responses from triplicate wells for each of the 10 animals tested; lines represent the sample median. ***, $P < 0.001$. (B) PIM-driven proliferation of bovine PBMC as measured by [^{3}H]Thy incorporation. Bovine PBMC were stimulated for 5 days with individual PIMs at 20 μg ml^{-1}. Points represent mean responses from triplicate wells for each of the 10 animals tested; lines represent the sample median. *, $P < 0.05$; **, $P < 0.01$. Nil, nonstimulated controls.

tected in the Ac_2PIM_2, $AcPIM_6$, and Ac_2PIM_6 treatment groups than in nonstimulated controls, with median values tending to be greater following $AcPIM_6$ stimulation.

Phenotyping of $AcPIM_6$-responsive proliferating cells by flow cytometry. As $AcPIM_6$ was the only PIM molecule to generate significantly increased levels of IFN-γ (Fig. 2A) and produced the greatest increase in the median proliferative response (Fig. 2B), we used this antigen to stimulate PBMC from an *M. bovis*-infected animal to characterize the proliferating cell populations by flow cytometry. Purified protein derivative from *M. bovis* (PPD-B) was used as a control antigen. CellTrace violet-labeled cells were incubated for 5 days with antigen before being harvested and labeled for flow cytometric analysis. After stimulation with either PPD-B or $AcPIM_6$, three populations of proliferating cells were identified based on cell surface phenotyping: (i) $CD4^+$ T cells ($CD3^+$ $CD4^+$), (ii) $CD8^+$ T cells ($CD3^+$ $CD8^+$), and (iii) NKT-like cells ($CD3^+$ $CD335^+$). An example of the gating strategy for identifying proliferating NKT cells is highlighted in Fig. 3A, which demonstrates a greater level of proliferating cells in response to stimulation with $AcPIM_6$ (58.29%) than in the non-antigen-stimulated control (29.87%).

The effects of stimulation with either PPD-B or $AcPIM_6$ on the three different cell populations are summarized in Fig. 3B. Stimulation with PPD-B drove antigen-specific proliferation of approximately 60% of the $CD4^+$ T cells ($CD3^+$ $CD4^+$). Similarly, an antigen-specific proliferative response was seen in approximately 15% of the $CD8^+$ T cells ($CD3^+$ $CD8^+$) to PPD-B. A slight increase in NKT cell ($CD3^+$ $CD335^+$) proliferative responses (approximately 20%) was also seen to these antigens (Fig. 3B).

Stimulation with $AcPIM_6$ induced only limited proliferation of $CD4^+$ T cells (approximately 5%) and no proliferation of $CD8^+$ T cells above the background (Fig. 3B). In contrast, approximately 30% of the NKT cell population mounted a proliferative response after stimulation with $AcPIM_6$ (Fig. 3B). Little or no proliferation above the unstimulated negative control was seen in the $CD3^-$ $CD335^+$ cells or in the $CD3^+$ γδ-TCR^+ populations (data not shown).

DISCUSSION

Mycobacterial lipids have long been implicated in the induction of responses in both the innate and adaptive cell-mediated immune responses (17–21). Although one strategy has been published which allows the isolation of PIMs from the avirulent *M. bovis* BCG strain (18, 22–24) and *M. tuberculosis* H37Rv (22) and certain synthetic molecules (17, 19, 21), in this study we successfully developed a novel method for extracting and subsequently highly purifying and characterizing individual PIM species from the polar fraction of virulent *M. tuberculosis* H37Rv. The method developed here improves upon the previously published protocols primarily by using a French press to disrupt the bacterial cells, thereby increasing PIM yield. Other refinements include the removal of the hot acetone incubation and the use of different reverse-phase conditions for PIM purification. Our strategy allowed us to isolate a greater yield of more highly purified PIMs, as confirmed by ESI-MS, that could be subsequently assayed for their ability to generate responses in lymphocytes.

To assess the ability of these highly purified PIMs to drive immune responses, the individual molecules were used to stimulate peripheral lymphocytes isolated from *M. bovis*-infected cattle. Only $AcPIM_6$ drove significant levels of IFN-γ from PBMC (Fig. 2A). Interestingly, when whole blood taken from the same animals was stimulated overnight with the PIM molecules as previously described (25-27), no IFN-γ could be measured, and this was not due to a lack of viability as stimulation with pokeweed mitogen (PWM) generated high levels of IFN-γ (data not shown). Although no IFN-γ production was seen from the whole-blood assay, $AcPIM_6$ was able to drive significant production of IFN-γ from PBMC incubated for 5 days (Fig. 2A).

There are very few studies showing the effect of mycobacterial lipids in short-incubation, whole-blood assays. Cell-mediated immune responses to lipid antigens are more commonly assessed by enzyme-linked immunosorbent spot (ELISpot) assay, with incubation times of at least 48 h required before measurable responses become apparent (24, 28). Further, the requirement for antigen processing and presentation of specific PIMs has been demonstrated previously (20); perhaps the most likely explanation for the discrepancy between whole-blood and PBMC IFN-γ responses is that the frequencies of lipid-responsive cells are low and that an extended incubation allows for expansion of these cells. This is supported by our demonstration of strong proliferative responses induced after stimulation of PBMC with PIMs (Fig. 2B).

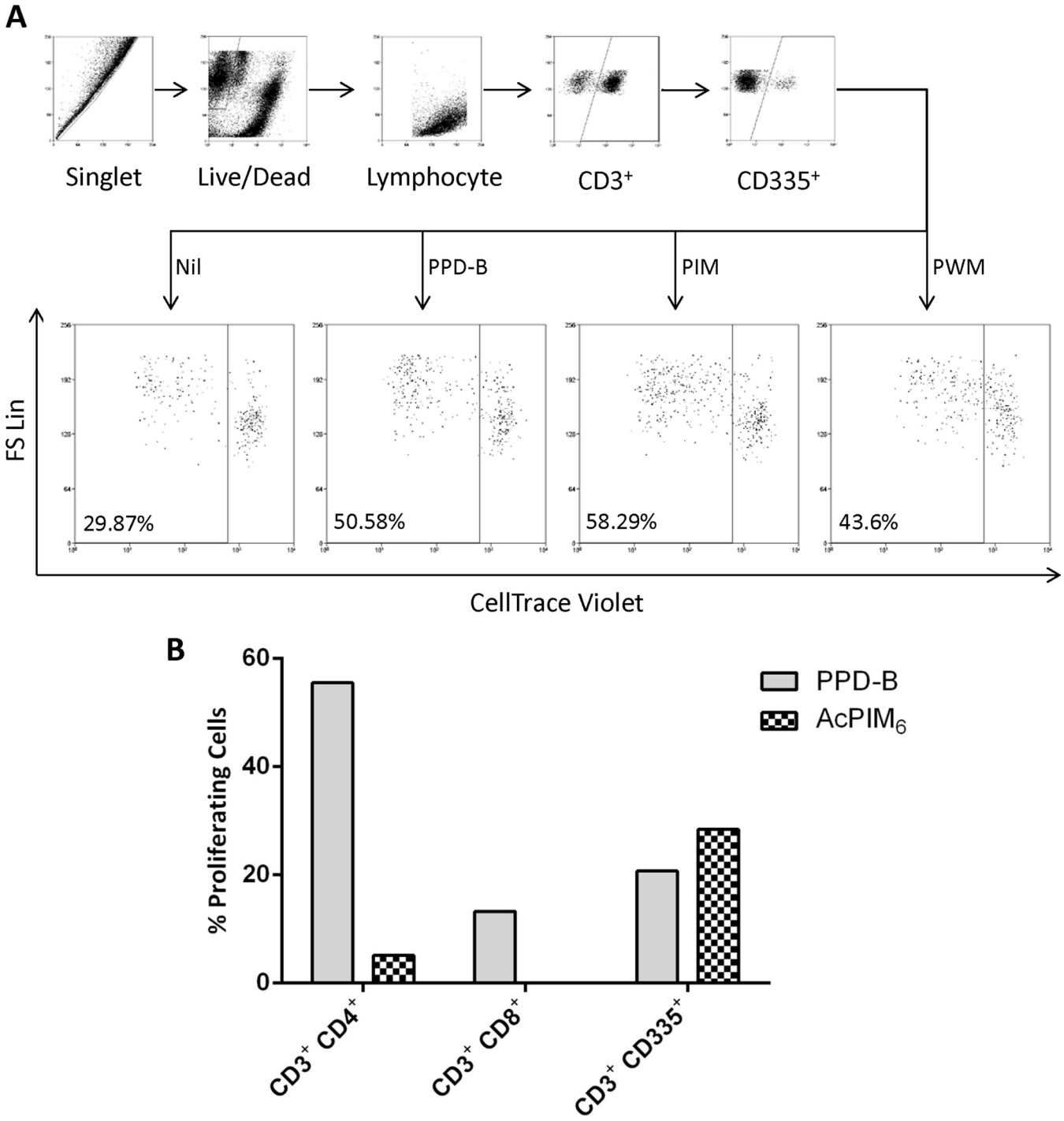

FIG 3 Assessment of proliferating cell phenotype by flow cytometry. (A) Flow cytometric gating strategy. Single, live $CD3^+$ $CD335^+$ lymphocytes were assessed for CellTrace violet labeling, and cells expressing low levels of CellTrace violet were gated for phenotyping. Numbers represent the percentages of proliferating cells in response to each antigen. FS Lin, forward scatter (linear). (B) Proliferation of $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$, or $CD3^+$ $CD335^+$ cells in response to either PPD-B or $AcPIM_6$. Each bar represents the percentage of cells proliferating after subtraction of the unstimulated control.

A recent study has shown that bovine NKT cells are present only at low frequencies (0.1% to 1.7%) (29).

Previous work using mice has shown that the ability of PIMs to generate cell-mediated responses is dependent on the acyl structures of the molecules. Early work performed using PIM_2 and PIM_6 demonstrated that the acyl chain was essential for NKT cell recruitment while the complexity of the mannose residues did not alter the response (23); however, it was subsequently shown that the second acyl chain of PIM_4 enhances binding to murine CD1d but that the polar mannose head was essential for antigen recognition, proliferation, and IFN-γ production (28). As well as the number and location of acyl chains, their degree of unsaturation

and *cis*, but not *trans*, stereochemistry are critical in determining antigenicity (14, 30).

The larger and more complex PIM molecules tested here (Ac_2PIM_2, Ac_2PIM_6, and $AcPIM_6$) drove significant levels of proliferation. $AcPIM_2$ also drove proliferation in 3 animals while PIM_2 generated no positive responses.

As $AcPIM_6$ was the only molecule to drive significant IFN-γ responses in our study (Fig. 2A) and one of the most potent inducers of proliferation (Fig. 2B), we decided to use $AcPIM_6$ to characterize the proliferative response. Stimulation with $AcPIM_6$ induced higher levels of proliferation in NKT cells than in $CD4^+$ or $CD8^+$ T cells (Fig. 3). However, from these data it is not possible to tell if the proliferative $CD4^+$ or $CD8^+$ cells also coexpress CD335 as our flow cytometric labeling panels do not allow the discrimination; however, this is a distinct possibility.

Although well characterized in humans and mice, the presence of NKT cells in cattle has been a controversial issue (31–34). Nevertheless, studies have shown that the bovine CD1D gene is expressed and translated *in vivo* (35), and recent work has identified a subset of cattle lymphocytes that express both T cell (CD3) and NK cell (NKp46) markers, suggesting the presence of an NKT cell population in bovine peripheral blood (29). Furthermore, bovine NKT cells have been shown to express both αβ- and γδ-TCRs, to have a broad TCR repertoire, and to have fully functional NKp46, CD16, and CD3 signaling pathways (29). Interestingly, these cells require ligation of their CD3 molecules to produce IFN-γ. While this may initially suggest that a CD3 binding component may be present in our PIM preparations, it is worth noting that we have not identified the cytokine-producing cells.

The identification of $AcPIM_6$ as a potent immunostimulatory molecule is of great interest both as a potential vaccine candidate (21), an adjuvant formulation (36), or a target for attenuation in the development of novel live vaccines (37). PIMs have also been used previously as diagnostic reagents for both tuberculosis and leprosy, although with limited success (38).

In conclusion, we present here the ability to extract and selectively purify PIMs to a high level of purity. These molecules could be used to stimulate significant IFN-γ production and drive significant proliferation in PBMC from cattle. We have also been able to identify the proliferative population and, for the first time, we have shown antigen-specific NKT activation in cattle.

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We have no competing interests.

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Differential effects of *Mycobacterium bovis* - derived polar and apolar lipid fractions on bovine innate immune cells

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Introduction

The first point of contact between *M. bovis* and its host is likely to be an interaction between the innate immune system and surface expressed molecules of the bacilli. The mycobacterial cell surface contains large numbers of diverse and complex lipids which are known to interact with innate cell receptors.

Little data exist describing the effect of pathogen derived lipids on innate immune cells from the appropriate host. Thus, to assess the immune response of the host to the lipids of its natural pathogen, lipids were extracted from virulent *M. bovis* AF 2122/97 and used to stimulate various bovine innate immune cells isolated from live, TB free, cattle.

M. bovis AF 2122/97 derived lipids

Lipids were extracted from freeze dried *M. bovis* AF 2122/97 in 2 fractions; apolar lipids were extracted in petroleum ether and polar lipids in chloroform and methanol. Lipids were analysed using standard 2D TLC systems and individual lipid species were identified (figure 1).

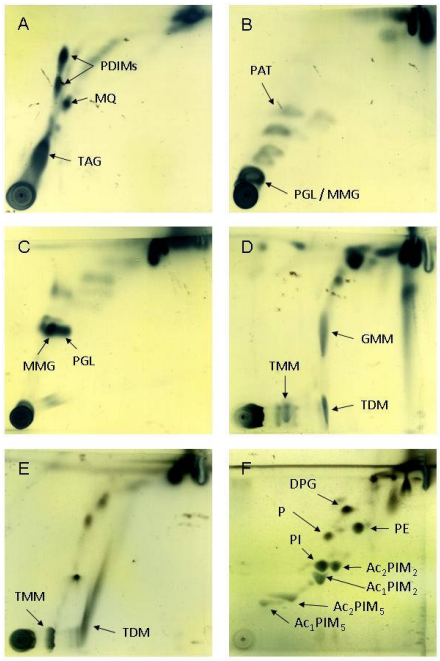

Figure 1:

A - D: Apolar fraction analysed With standard TLC systems A, B, C and D

E and F: Polar fraction analysed with stadard TLC systems D and E

PDIM - phthiocerol dimycocerosate
MQ - menaquinone
TAG - triacyl glycerol
PAT - pentacyl trehalose
PGL - phenolic glycolipid
MMG - monomycolyl glycerol
TMM - trehalose monomycolate
TDM - trehalose dimycolate
GMM - glucose monomycolate
DPG - diphosphatidyl glycerol
PE - phosphatidyl ethanolamine
PI - phosphatidyl inositol
P - phospholipid
PIM - phosphatidyl inositol mannosides

Cytokine responses to *M. bovis* lipids

Monocytes were extracted from bovine whole blood and cultured with GM-CSF and IL-4 (MDDC) or GM-CSF alone (CM) for 3 days before being washed and stimulated overnight with lipid at 20 µg ml^{-1}.

Supernatants were analysed by MSD multiplex chemiluminescent ELISA for the levels of IL-10, IL-12, MIP-1β, TNFα and IL-6 (figure 2).

Polar, but not apolar, lipids caused significant increases in production of IL-10, IL-12, MIP-1β and TNFα in all cells. DC produced more IL-12 and TNFα than other cells, while DC and CM produced more MIP-1β than monocytes.

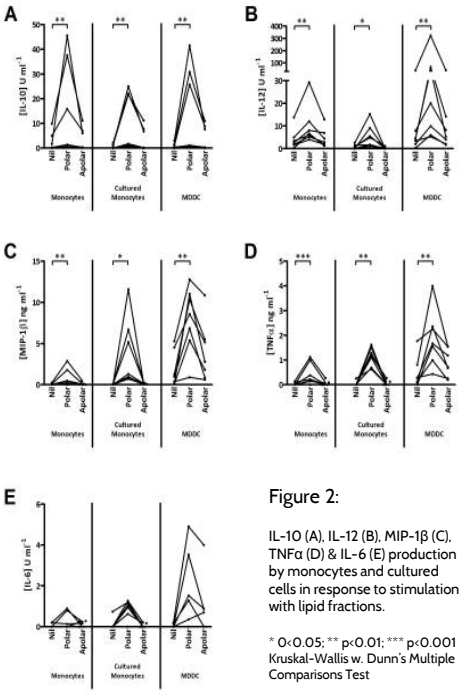

Figure 2:

IL-10 (A), IL-12 (B), MIP-1β (C), TNFα (D) & IL-6 (E) production by monocytes and cultured cells in response to stimulation with lipid fractions.

* 0<0.05; ** p<0.01; *** p<0.001 Kruskal-Wallis w. Dunn's Multiple Comparisons Test

Phenotypic responses to *M. bovis* lipids

Analysis of antigen presentation associated molecules was performed by flow cytometry (figure 3).

The polar lipid fraction caused a significant reduction in expression of MHCII and CD86 on all cell types, as well as significant reduction in CD1b expression on MDDC. CD40 levels increased significantly after polar lipid stimulation of cells where expression was not constitutively high.

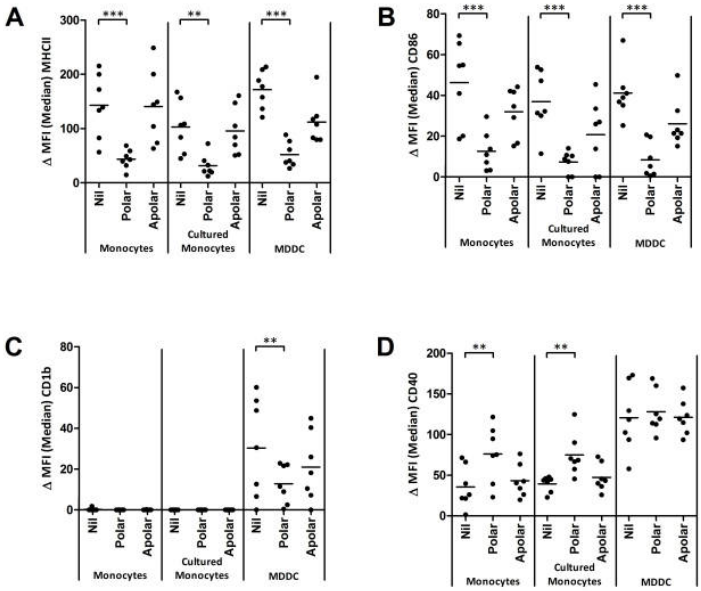

Figure 3:

Surface expression of MHCII (A), CD86 (B), CD1b (C) and CD40 (D) on cultured cells and fresh monocytes after exposure to lipid fractions. ** p<0.01; *** p<0.001; Repeated Measures ANOVA w. Bonferroni Multiple Comparisons Test

Functional consequence of lipid exposure

An MLR was performed to assess the consequence of reduction in expression of MHCII. Cultured cells were Mitomycin C treated before being mixed with pre-screened alloreactive PBMC from a different animal. Polar lipid treatment of CM led to statistically significant abrogation of proliferative responses. However, exposure of MDDC to lipids had no effect upon their ability to drive alloreactive proliferation. This may be due to constitutively high levels of CD40 found on MDDC or the significantly higher levels of IL-12 produced by these cells (figure 4).

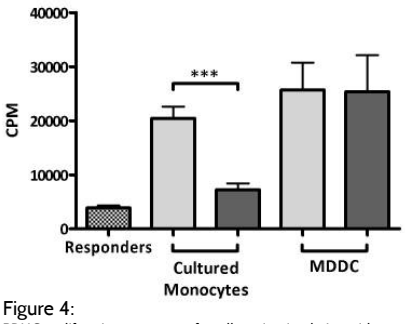

Figure 4:

PBMC proliferative responses after allotypic stimulation with untreated (light grey bars) or polar lipid treated (dark grey bars) cultured cells. Bars represent the mean of triplicate wells ± standard error of the mean.
*** p<0.001; One Way ANOVA w. Bonferroni Multiple Comparison Test

Conclusions

We present here the first data to fully categorise the lipids of *M. bovis* and demonstrate their regulatory effects of on bovine innate immune cells. Polar lipids induced significant levels of IL-10, IL-12, MIP-1β and TNFα production by innate immune cells. Polar lipids also caused a significant reduction in expression of MHCII, CD86 and CD1b. Finally, polar lipid treated cultured monocytes were shown to be compromised in the induction of T cell proliferation. Hence, polar lipids may play a pivotal role in the outcome of infection.

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