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A MYCOLACTONE BASED VACCINE AGAINST BURULI ULCER DISEASE

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[redacted]

MAY 1, 2023

PHD THESIS

Supervised by Prof Rajko Reljic, Dr Mark Wansbrough-Jones and Prof Tom Harrison.

DECLARATION:

I hereby declare that this thesis and all data contained are mine. Any work/data obtained from other sources were duly acknowledged.

[redacted]

Justice Kofi Boakye-Appiah

APPRECIATION

‘For there is a God, who mediates in the affairs of men.’

When I first dreamt of obtaining a PhD, the path to achieving this was all but clear. But three men made this possible; My mentor, Professor Richard Odame Phillips who introduced me to research; His mentor, Dr Mark Wansbrough-Jones who provided the PhD opportunity through his association with St George’s; and my PhD supervisor, Professor Rajko Reljic. I will always be grateful to these individuals. Rajko became more than a supervisor. He was a teacher, a father and a friend. I could not have had a better supervisor.

I am also grateful to all members of the Ma’jic lab in St George’s for their support. Special recognition to Prof Julian Ma who helped secure additional funding to pay my tuition. I would also like to appreciate Prof Tom Harrison, Dr Matthew John Paul, Dr Peter Hart, Dr Pascal Drake and Mrs Thais Guerra for all their support.

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This thesis is dedicated to my father, Andrews Kwame Boakye who so badly looked forward to my graduation but did not live to attend. May he rest in peace and be proud of what his support has speared our family on to achieve together,

ABSTRACT

Background

Buruli ulcer (BU), a neglected necrotizing tropical skin disease caused by *Mycobacterium ulcerans* is the third most common Mycobacterial disease after TB and leprosy. Infections mostly occur in remote, rural areas of Central and West Africa, but also in Australia, Japan and Papua New Guinea. Notwithstanding the fact that more than 42,000 BU cases have been reported worldwide in 33 countries in the past decade, the actual disease burden is difficult to objectively assess considering the remoteness of affected populations and a lack of credible health systems data on the incidence of Buruli Ulcer in quite a significant number of the countries in which cases are known to occur. Most patients are children aged under 15 years and even though the disease is known to carry a low mortality risk, it bears a huge morbidity and economic burden with costs of treatment rising as high as $30,000 per patient in some cases. The condition usually presents as a painless nodule, a firm plaque, or edematous lesion, which soon ulcerates with characteristically undermined edges. Mycolactone, a toxin produced exclusively by *M. ulcerans* is the main pathological factor in the pathogenesis of Buruli ulcer. Being a neglected disease of the poor, access to surgical treatment is limited and the cost of treatment, a big disincentive. The thought of vaccine development is therefore taking centre-stage in attempts to control the disease.

This work focuses on the development of a mycolactone-based vaccine against Buruli ulcer. Previous attempts at developing a vaccine using closely related bacteria and subunit proteins have not been successful.

Methods

C57BL/6J black mice were immunized with synthetic mycolactone, mycolactone-deficient *M ulcerans* and purified enzyme proteins expressed in *E. coli* combined with selected adjuvants and delivery systems. Immunised mice were challenged with pathological *M ulcerans* and observed for footpad swelling. Various immunological assays were performed to understand the immune mechanisms underlying protection.

Results

When combined with the delivery platform YC NaMA, mycolactone was immunogenic at concentrations below 18ng/ml. The enzyme proteins were able to induce varying types and levels of immune response. Antibodies were developed to KRA and Ag85A. BURULIVAC, a composite vaccine construct comprising Mycolactone, KRA, Ag85A and Quil A conferred absolute protection against Buruli ulcer disease in mice over a 14-week period of observation. This was observed in mouse groups irrespective of whether or not mice had been primed with Delta, the mycolactone deficient strain of *M ulcerans*. Interleukin 10 emerged as a reliable correlate of protection.

Conclusion

With the right adjuvants and adequate doses, mycolactone and some of its polyketide synthase enzymes involved in its synthesis are able to produce good immune responses against Buruli ulcer and the immunosuppressive effects of mycolactone can be circumvented. BURULIVAC is a promising vaccine candidate against Buruli ulcer disease and must be explored further.

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Table of Abbreviations

Abbreviation	Abbreviation Expanded
µ	Micro
ACK	Ammonium Chloride Potassium
ACP	Acyl Carrier Protein
AFB	Acid-Fast Bacillus
AFRO	African Region (World Health Organisation)
Ag85A	Antigen-85A
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen-Presenting Cells
ATAC	Acyltransferase Acetate
ATP	Acyltransferase Propionate
BCG	Bacillus Calmette-Guerin
BSA	Bovine Serum Albumin
BU	Buruli Ulcer
BUD	Buruli Ulcer Disease
CD	Cluster of Differentiation 4/8
CRP	C-Reactive Protein
CTL	Cytotoxic T-Lymphocytes
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ER	Enoyl Reductase
FBS	Foetal Bovine Serum
FNA	Fine Needle Aspirate
gd T-Lymph	GammaDelta T-lymphocytes
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase
HSP	Heat shock Protein
IFN- γ	Interferon Gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IS2404	Insertion Sequence 2404

Kb	Kilobase
KDa	Kilodalton
KRA	Ketoreductase A
KS	Ketosynthase
LAL	Limulus Amoebocyte Lysate
LAM	Lipoarabinomannan
MEF	Murine Embryonic Fibroblasts
MHC	Major Histocompatibility Complex
Ml	Milligram
MPL A	Monophosphoryl Lipid A
MTB	Mycobacterium Tuberculosis
NF-κ B	Nuclear Factor kappa B
Ng	Nanogram
NO	Nitric Oxide
NTD	Neglected Tropical Disease
NTM	Non-Tuberculous Mycobacteria
OADC	Oleic Albumin Dextrose and Catalase
OPD	o-phenylenediamine dihydrochloride
PANTA	Polymyxin-B, Amphotericin-B, Nalidixic Acid, Trimethoprim, Azilocillin
PBS	Phosphate Buffered Solution
PCR	Polymerase Chain Reaction
PECAM	Platelet Endothelial Cell Adhesion Molecule
Pg	Picogram
PHA	Phytohaemagglutinin
PKS	Polyketide Synthase
PPD	Purified Protein Derivative
RPM	Revolution Per Minute
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
TB	Tuberculosis
TBS	Tris-Buffered Solution
TCR	T-cell Receptor
Th1/2	T-Helper Type 1/2
TLR	Toll-Like Receptor
TNF- a	Tumour Necrosis Factor Alpha
WASP	Wiskott-Aldrich syndrome protein
WHO	World Health Organisation
WPRO	Western Pacific Region (World Health Organisation)

Contents

CHAPTER 1

INTRODUCTION

1.1 History of *M. ulcerans* Disease.

Mycobacterium ulcerans disease often referred to as Buruli ulcer was first described by Sir Albert Cook when he documented his notes in the Mengo Hospital in Uganda (1). But it was not until 1948 when the first detailed description of a case was published by MacCallum et al. In his paper, MacCallum established the aetiology of the disease in a small group of 6 Australian patients in the Bairnsdale district. Hence, its other name Bairnsdale ulcer. Upon this initial publication, several attempts to culture the causative organism failed. Years later however, *M. ulcerans* was identified by coincidence when an incubator broke down. This incubator which when functional was usually set at 37ºC was observed to have had a stable temperature of between 30-33ºC. This was how the optimal growth temperature for the slow growing *M. ulcerans* was discovered (2). Later in 1950, the first report in Africa was from the Democratic Republic of the Congo but there are suggestions that the disease had been in the Democratic Republic of the Congo (Former Zaire) since 1935. A series of 170 cases had been recorded over a 10-year period (3). The name Buruli ulcer originates from the Buruli District in Uganda where the first major outbreak was recorded (4, 5). Not too far from the Buruli county and around the Nile River, an outbreak involving 220 BU (Buruli Ulcer) cases occurred between 1964 and 1969 in a Rwandan refugee camp. Investigations involving this cohort provided much of the basic epidemiology of BU that we know. Reports of Buruli ulcer disease remained predominantly a West African phenomenon with cases principally emerging from Ghana, Nigeria, Cote D'Ivoire and Benin with few case reports from Australia and Papua New Guinea until the beginning of the twenty first century (6). Following on from then, sporadic cases have been reported in some temperate and subtropical regions (7). A total of 34 countries have at present reported cases of BU disease. It must however be said that the focus had until recently been on ulcerative lesions. The emergence of the disease in highly endemic parts of Australia especially brought attention to pre-ulcerative forms of presentation (8, 12).

A major global intervention was introduced in 1998 when the World Health Organisation launched its Global BU initiative which served as a forum for disease control, research and fundraising towards efforts aimed at managing the global BU burden. Resulting from the efforts of this unit, reports in excess of 58,000 cases of BU were collated from 20 countries over the next 20 years. The WHO (World Health Organization) has also coordinated the formation of national control programs in various affected countries. Whilst this has resulted in an overall global decrease in disease burden since 2008, some countries have rather reported increases in the number of cases reported. This has been attributed to improved disease surveillance and active case search with improved diagnostics. Much of the obscurity the disease previously suffered may be attributed to the fact that it predominantly affects the poor (8). Following many years of further research, the toxin mycolactone which is responsible to much of the pathogenesis of the disease was discovered (9).

1.2 Mycobacteria Affecting Man

The genus *Mycobacterium* has over 190 well characterized species. With the most well-known ones being *M tuberculosis* which causes tuberculosis and *M leprae* which causes leprosy. The third being *M ulcerans* which causes Buruli ulcer disease (10). The prefix Myco, is from the Greek word for fungus and this was adopted to explain the fact that mycobacteria usually grow like mould on the surface of culture plates. Mycobacteria are generally slow growing and considered to be acid-fast bacteria, hence cannot be characterized through gram staining (11).

1.3 Non-Tuberculous Mycobacteria (NTM).

Non-tuberculous mycobacteria causing human disease include *M. avium, M. intracellulare, M. kansasii, M. paratuberculosis, M. scrofulaceum, M. simiae, M. habana, M. interjectum. M. xenopi, M. heckeshornense, M. szulgai, M. fortuitum, M. immunogenum, M. chelonae, M. marinum, M. genavensae, M. hemophilum, M. celatum, M. conspicuum, M. malmoensae, M. ulcerans, M. smegmatis, M. wolinskyi, M. goodii, M. thermoresistible, M. neoaurum, M. vaccae, M. palustre , M. elephantis, M. bohemicam, and M. septicum.*(13,14)

NTM are increasingly being recognised as causative agents for both pulmonary and extra-pulmonary diseases. This has been associated with the increasing number of people living with various forms of immunosuppression especially those with Acquired Immunodeficiency Syndrome (AIDS) (15, 20).

1.4 Mycobacteria Causing Cutaneous Manifestations

Among the organisms in the group of non-tuberculous mycobacteria, some have been recognised to cause cutaneous diseases. These include, *Mycobacterium ulcerans, M. szulgai* (16), *M. vaccae* (17) etc. *M. marinum* causes swimming pool granuloma or fish tank granuloma (18 -20). *M. fortuitum* and *M. chelonae* (21) cause wound abscesses and *M. immunogenum* causes wound infection with sepsis (22). *M. smegmatis* (23), *M. wolinsky, M. goodii, M. thermoresistible* (23) and *M. palustre* (24) also cause wound infections and bacteraemia. Members of the *M. terrae* family (*M. terrae, M. nonchromogenicum*, and *M. triviale*) occasionally cause acupuncture induced infections (25). *M. septicum* has also been associated with skin catheter related bacteraemia (26).

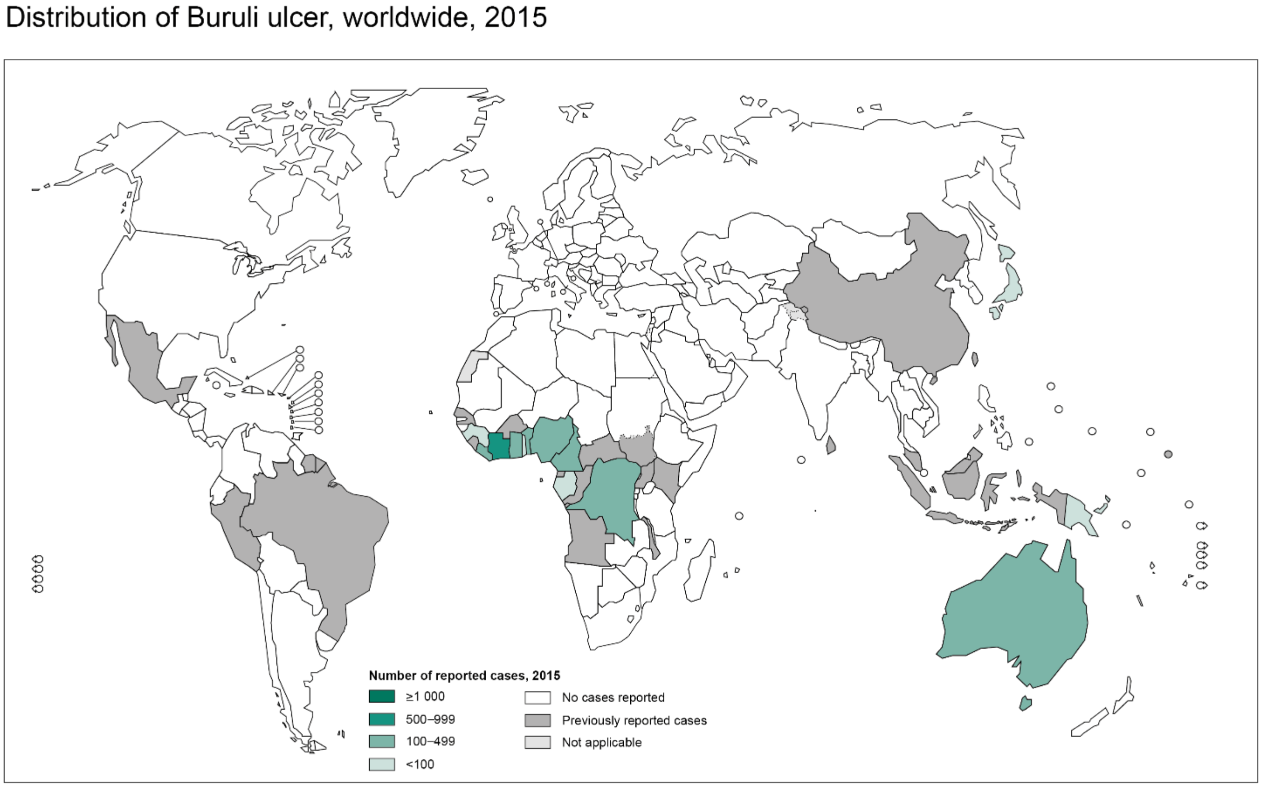
It is however noteworthy that *M. ulcerans* and *M. marinum* are very closely related with over 98% DNA sequence homology (28).

1.5 *Mycobacterium ulcerans* Disease

Mycobacterium ulcerans infection causes a panniculitis with extensive necrosis of the subcutaneous fatty tissue which leads to the formation of ulcers with undermined edges known as Buruli ulcer (BU). It is recognized as a common and serious disease in some parts of the tropics (29). In recent times, outbreaks of Buruli ulcer in various countries including Australia have brought Buruli ulcer into the limelight and is thus considered to be the third most common mycobacterial disease after Tuberculosis and leprosy.

1.6 Epidemiology

Infections mostly occur in remote, rural areas of Central and West Africa, but also in Australia, Japan and Papua New Guinea. Notwithstanding the fact that more than 42,000 BU cases have been reported worldwide in 33 countries(Angola, Australia, Benin, Brazil, Burkina Faso, Cameroon, Central African Republic, People's Republic of China, Congo, Côte d'Ivoire, Democratic Republic of the Congo, Equatorial Guinea, French Guiana, Gabon, Ghana, Guinea, Indonesia, Japan, Kenya, Republic of Kiribati, Liberia, Malawi, Malaysia, Mexico, Nigeria, Papua New Guinea , Peru, Senegal, Sierra Leone, South Sudan, Sri Lanka, Suriname, Togo, and Uganda) in the past decade, the actual disease burden is difficult to objectively assess considering the remoteness of affected populations and a lack of credible health systems data on the incidence of Buruli Ulcer in quite a significant number of the affected countries (Fig 1, 2, 3,4). Most patients are children aged under 15 years (About 70% of patients) and even though the disease is known to carry a low mortality risk, it bears a huge morbidity and economic burden with costs of treatment rising as high as $30,000 per patient in some cases (29,30). At present, the highest prevalence of *M. ulcerans* disease is in West Africa. In Ghana for instance, epidemiological surveillance data suggests that the prevalence of Buruli ulcer in some endemic areas is more than that of leprosy and equals that of tuberculosis. The situation may be worse in some endemic areas in Benin and Cote d'Ivoire (31).



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement. © WHO 2016. All rights reserved

Data Source: World Health Organization
Map Production: Control of Neglected Tropical Diseases (NTD)
World Health Organization

World Health Organization

Fig 1. Global burden and distribution of Buruli ulcer disease worldwide.

Global burden and distribution of Buruli ulcer disease. Though not reported in all countries, has been quite widely distributed globally. The tropical regions of West Africa and Australia are the most affected regions. Image adopted from World Health Organization (Last updated in 2015)

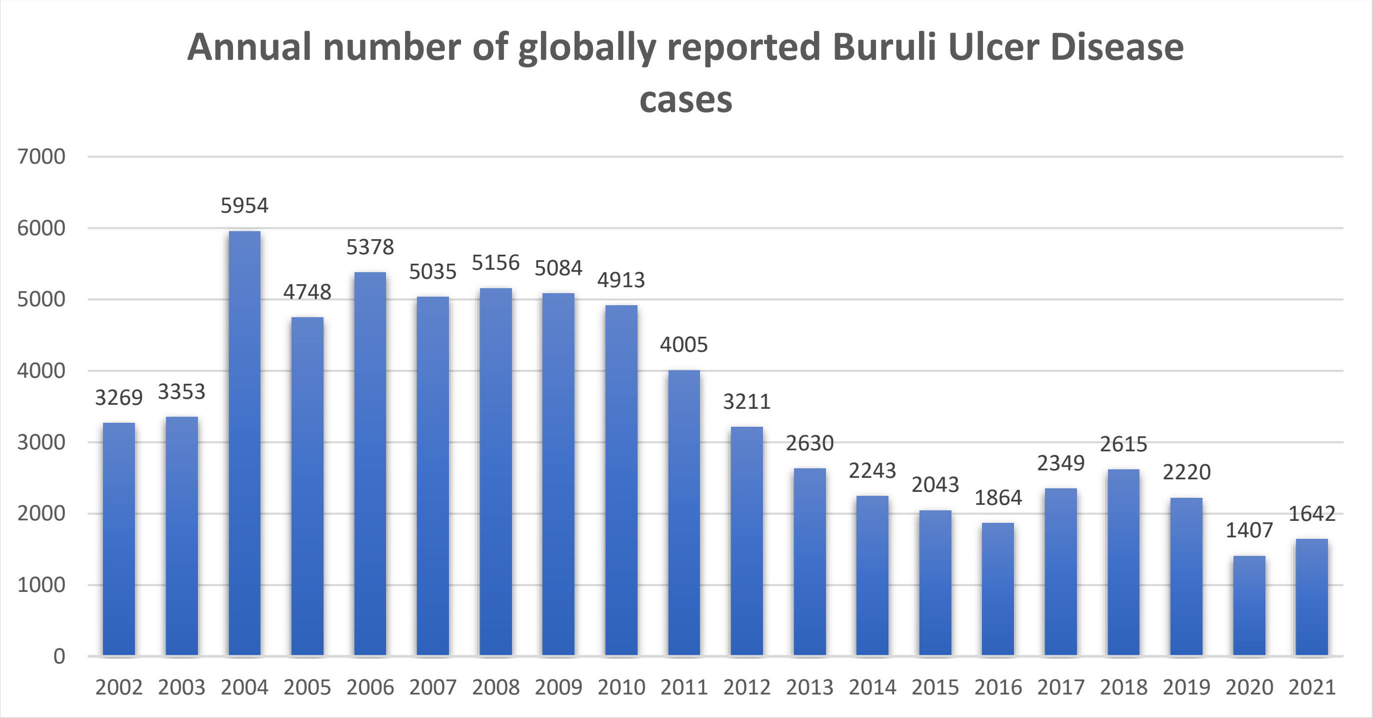
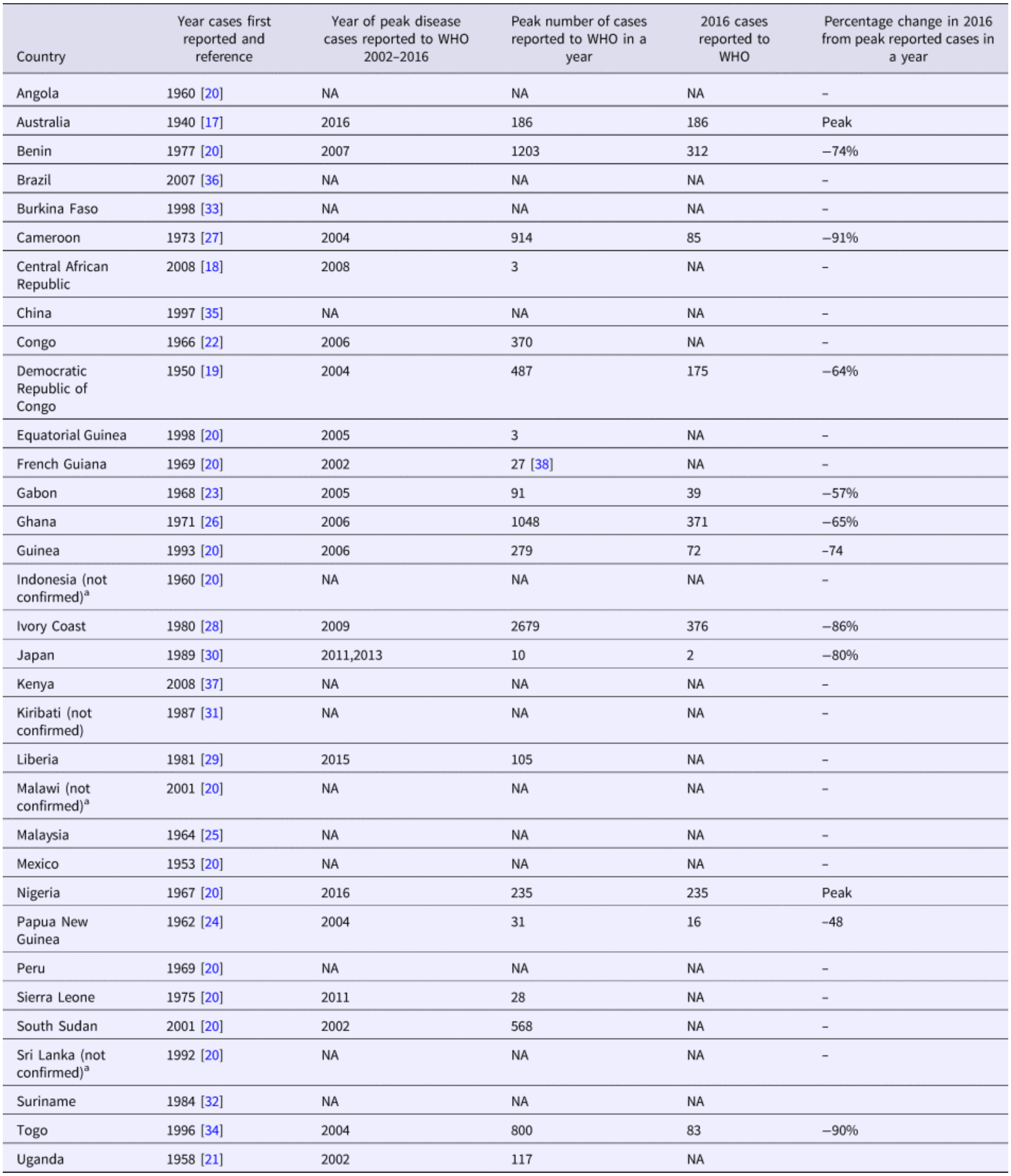


Fig 2. Annual Number of BU cases reported worldwide since 2002 according to the World Health Organisation.

There is a trending decline in the number of cases reported globally attributable to active epidemiological interventions set up by the WHO and various endemic countries. Source: WHO, July 2022, Number of new reported cases of Buruli ulcer (who.int). Accessed on 15th Nov 2022.



Country	Year cases first reported and reference	Year of peak disease cases reported to WHO 2002–2016	Peak number of cases reported to WHO in a year	2016 cases reported to WHO	Percentage change in 2016 from peak reported cases in a year
Angola	1960 [20]	NA	NA	NA	–
Australia	1940 [17]	2016	186	186	Peak
Benin	1977 [20]	2007	1203	312	−74%
Brazil	2007 [36]	NA	NA	NA	–
Burkina Faso	1998 [33]	NA	NA	NA	–
Cameroon	1973 [27]	2004	914	85	−91%
Central African Republic	2008 [18]	2008	3	NA	–
China	1997 [35]	NA	NA	NA	–
Congo	1966 [22]	2006	370	NA	–
Democratic Republic of Congo	1950 [19]	2004	487	175	−64%
Equatorial Guinea	1998 [20]	2005	3	NA	–
French Guiana	1969 [20]	2002	27 [38]	NA	–
Gabon	1968 [23]	2005	91	39	−57%
Ghana	1971 [26]	2006	1048	371	−65%
Guinea	1993 [20]	2006	279	72	-74
Indonesia (not confirmed)[a]	1960 [20]	NA	NA	NA	–
Ivory Coast	1980 [28]	2009	2679	376	−86%
Japan	1989 [30]	2011,2013	10	2	−80%
Kenya	2008 [37]	NA	NA	NA	–
Kiribati (not confirmed)	1987 [31]	NA	NA	NA	–
Liberia	1981 [29]	2015	105	NA	–
Malawi (not confirmed)[a]	2001 [20]	NA	NA	NA	–
Malaysia	1964 [25]	NA	NA	NA	–
Mexico	1953 [20]	NA	NA	NA	–
Nigeria	1967 [20]	2016	235	235	Peak
Papua New Guinea	1962 [24]	2004	31	16	-48
Peru	1969 [20]	NA	NA	NA	–
Sierra Leone	1975 [20]	2011	28	NA	–
South Sudan	2001 [20]	2002	568	NA	–
Sri Lanka (not confirmed)[a]	1992 [20]	NA	NA	NA	–
Suriname	1984 [32]	NA	NA	NA	
Togo	1996 [34]	2004	800	83	−90%
Uganda	1958 [21]	2002	117	NA	

Fig 3. Country level data of BU cases reported to the WHO as at 2016. 33 countries have reported the occurrence of Buruli ulcer disease since 2002. Source: WHO

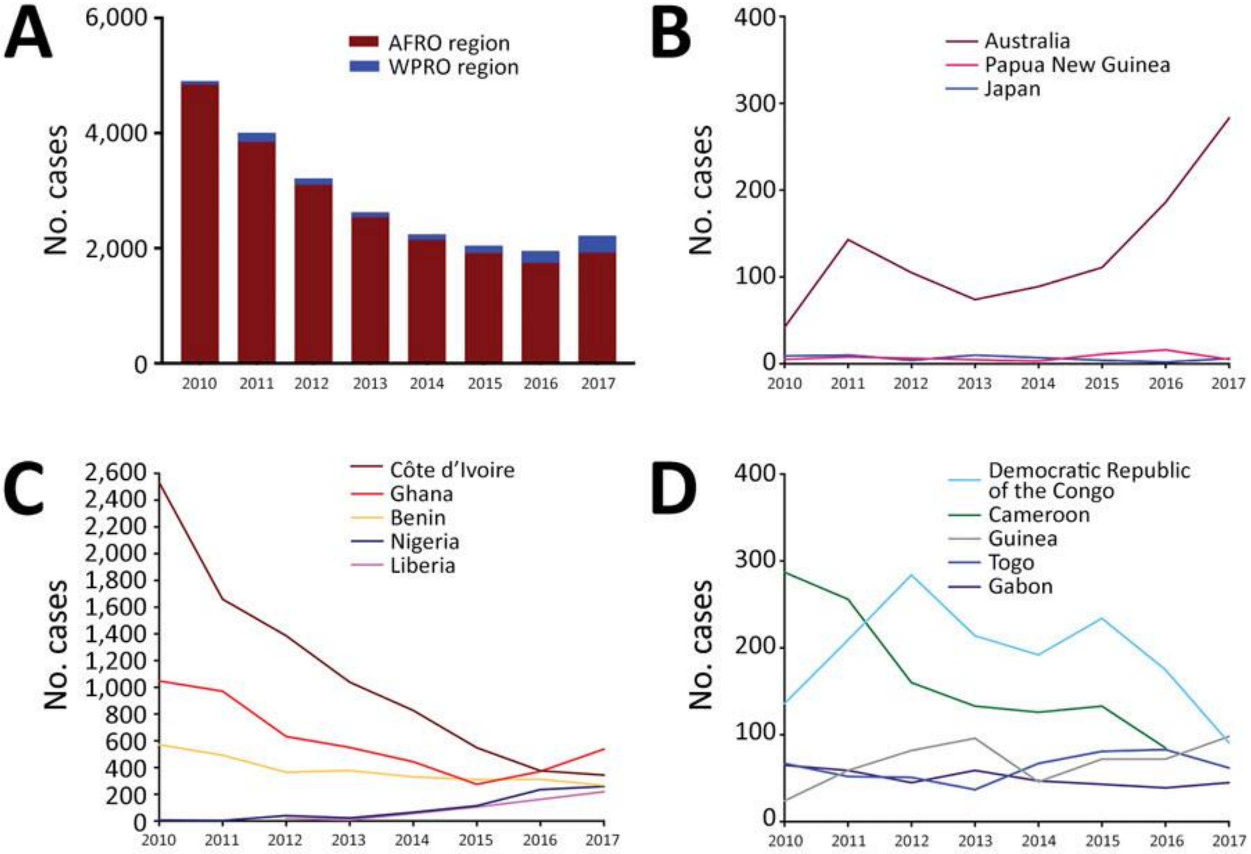


Fig 4. Disease burden and dynamics of Buruli ulcer disease reported to the WHO from 2010 to 2017 in the most endemic countries and Sub-Regions globally.

A) Globally, reported cases declined over time, but the proportion of cases reported from WPRO increased. B) WPRO data show an increase in cases in Australia. C) In AFRO, cases drastically declined in Côte d'Ivoire but recently increased in other countries such as Ghana, Nigeria, and Liberia. D) Countries in AFRO that reported fewer cases overall showed stagnant or varying numbers. AFRO, WHO African Region; WPRO, WHO Western Pacific Region. *AFRO represents the African Region whereas WPRO represents the Western Pacific Region.

1.7 Environmental Evidence of *M. ulcerans*

The disease generally occurs in wet tropical areas and is considered to be a disease of the poor, affecting inhabitants of remote rural areas with limited access to health services. Several attempts have been made in the past to investigate the presence of *M. ulcerans* in the environment. Ross et al. (32) were the first to detect DNA of *M. ulcerans* by polymerase chain reaction (PCR) from environmental samples (Fig 5). Even though attempts to culture *M. ulcerans* from environmental samples are yet to yield any positive outcomes, *M. ulcerans* has recently been cultured from salivary glands of aquatic insects captured in Cote d'Ivoire (33). Difficulties in isolation have been attributed either to low numbers of the bacterium or decontamination methods to prevent overgrowth by other organisms. The belief is that these methods may have a detrimental effect on the viability of *M. ulcerans* as well (34). It is not clear exactly where *M. ulcerans* exists in the environment but in an *M. ulcerans* PCR, aquatic plants tested positive for the organism (35). This uncertainty became even more apparent when a report demonstrated that 5% of aquatic bugs (Naucoris cimicoides) tested positive of the *M. ulcerans* insertion sequence IS2404 in endemic areas (36). An *M. ulcerans* biofilm on aquatic plants has been demonstrated by electron microscopy and snails have been shown to transiently harbour *M. ulcerans* (37).

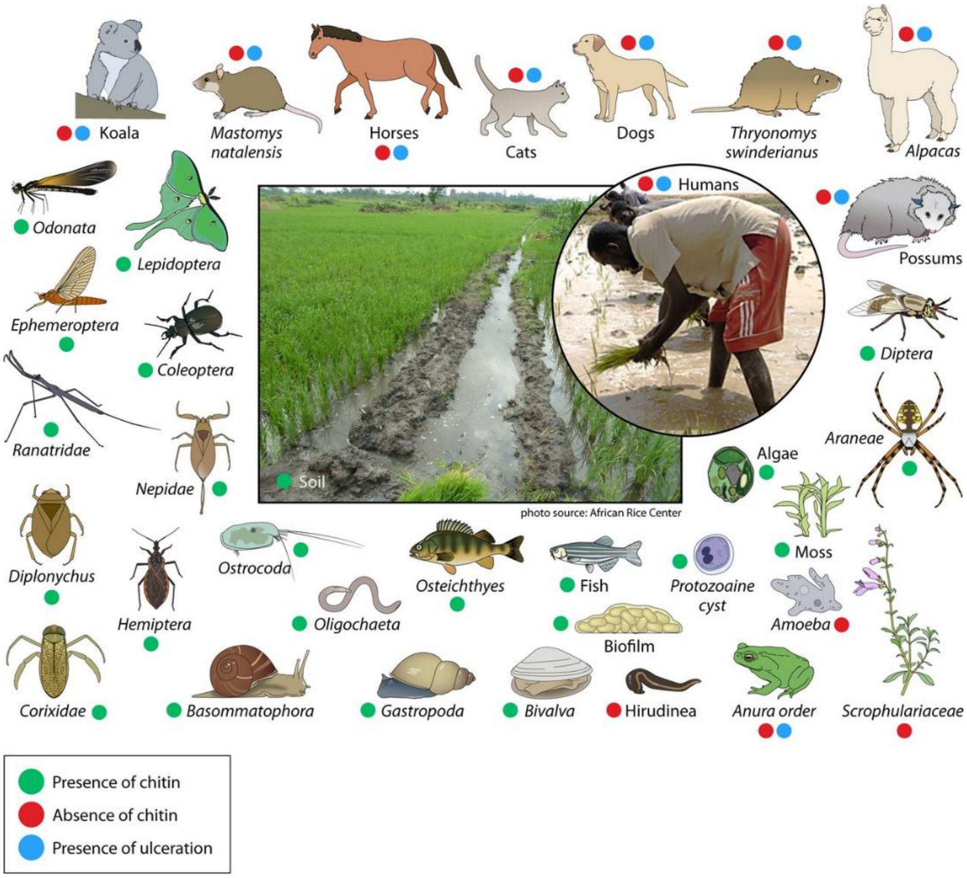


Fig 5. Environmental sources of *M. Ulcerans,* and *M. ulcerans* reservoirs.

Transmission mechanism remains unknown. Environmental sources and reservoirs have however been successfully identified. Predominantly aquatic, *M ulcerans* survives in a number of reservoirs. Image taken from (38).

1.8 Transmission

The exact mode of transmission of *M. ulcerans* is unknown. It is however thought to enter the skin through small abrasions and contact with aquatic insects (Fig 5). Minor trauma has been implicated as playing a major role in the transmission process. Hoffer et al described a case of disseminated osteomyelitis from *M ulcerans* after a snake bite (39, 40). Inoculation of *Mycobacterium ulcerans* bacteria into intact skin by aquatic insects has also been proposed as a possible mode of transmission (38, 40). To date, there has not been any published evidence of human to human transmission. However, in 2002 Marsollier et al. demonstrated in a laboratory aquarium that insects in the Naucoridae family (whose salivary glands were experimentally infected with *M. ulcerans*) were able to transmit the disease to laboratory mice by biting their tails (36). Naucoridae bites are painful and are likely to be remembered by patients. Considering the fact that patients generally do not recall or report any such insect bites, it is still unclear if this mode of transmission occurs in humans (41).

1.9 Microbiology

M. ulcerans is a slow growing environmental mycobacterium with a generation time of 20 hours. This is characteristic of other slow growing mycobacterial species. Although Middlebrook 7H10 and 7H11 are commonly used, Löwenstein Jensen (L-J) medium is the preferred solid medium for cultivation (Fig 6) (42). Cultivation has also been performed in media containing albumin: Dubos albumin medium (44) and Middlebrook 7H9 medium supplemented with oleic, albumin, dextrose and catalase (OADC) (45). The organism grows at optimal conditions of pH 5.4 to 7.4; temperature of between 30 to 33°C; microaerophilic conditions of 2.5-5.0% oxygen and finally, culture period of between 6-8 weeks or possibly more if dealing with primary cultures. Subcultures may however take 3 to 4 weeks to give good yields (46).

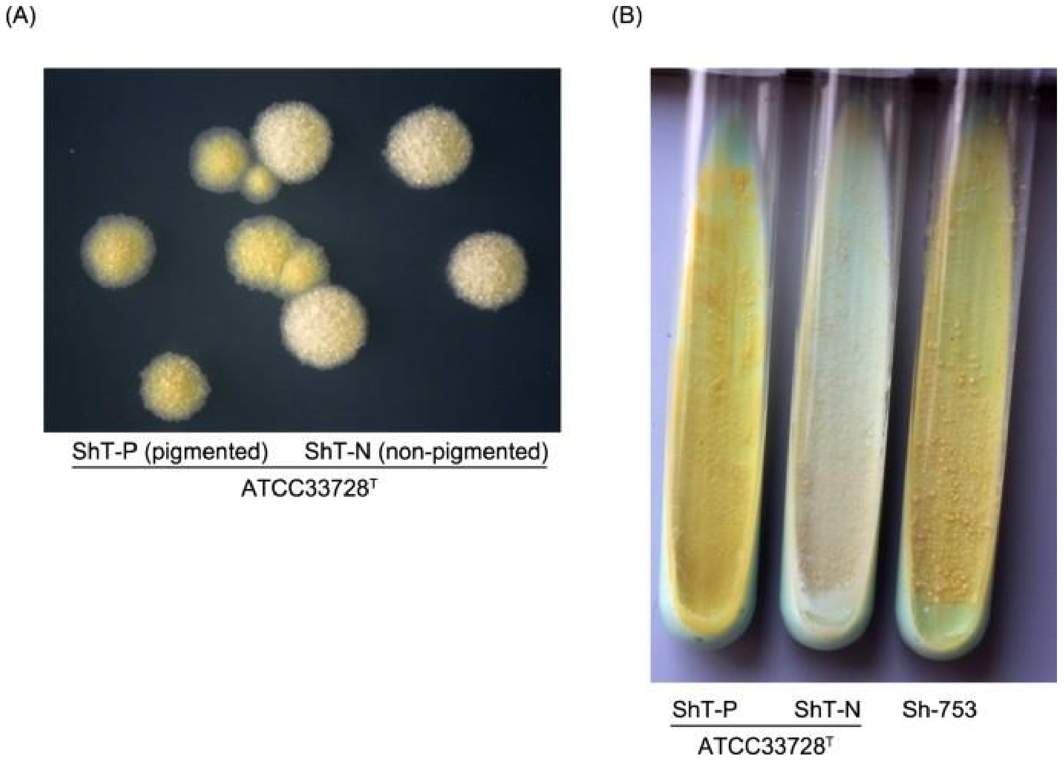


Fig 6. *M ulcerans* (sub species *M. Shinshuense)* colonies growing on 7H11Agar(A) and Lowenstein-Jensen Medium(B). It has the characteristic yellow pigmentation believed to be the colour of mycolactone. Image adopted from (43).

1.10 Clinical Presentation

Buruli ulcer disease presents as a painless skin lesion and may be of one of five forms: nodule, papule, plaque or oedematous lesion and then progressively to ulceration (159). Ulcers are the most commonly identified forms especially in places with poor healthcare. Pre-ulcerative lesions are predominantly diagnosed in places such as Australia where health seeking habits are generally much better (Fig 7 and 9). The most acute form of the disease presents as a rapidly progressive oedematous disease. This is usually managed by surgery during which a wide excision of the lesion to include all the oedematous tissue is performed (Fig 11). A recurrence rate of 16% was reported in one study but may significantly vary among patient groups, infection site, and stage of infection (46,47, 129). The natural history of the disease is a progression to an ulcer if no intervention is instituted. If treatment is applied, lesions at various stages may heal without ulcerating (48, 128). Healed lesions often leave behind a scar with often very significant deformity. Mortality from the disease is however not very common. In one very rare instance, Sarfo *et al* reported a case of pleural effusion following a Buruli ulcer lesion on the chest. The pleural aspirate tested PCR positive for *M ulcerans* (49). The more common sites where lesions occur are the extremities/limbs (Fig 8). This may be related to the variations in basal temperature across various regions in the body. The extremities tend to have lower temperatures which favour the growth of *M ulcerans* better than core parts of the body (Fig 8) (38).

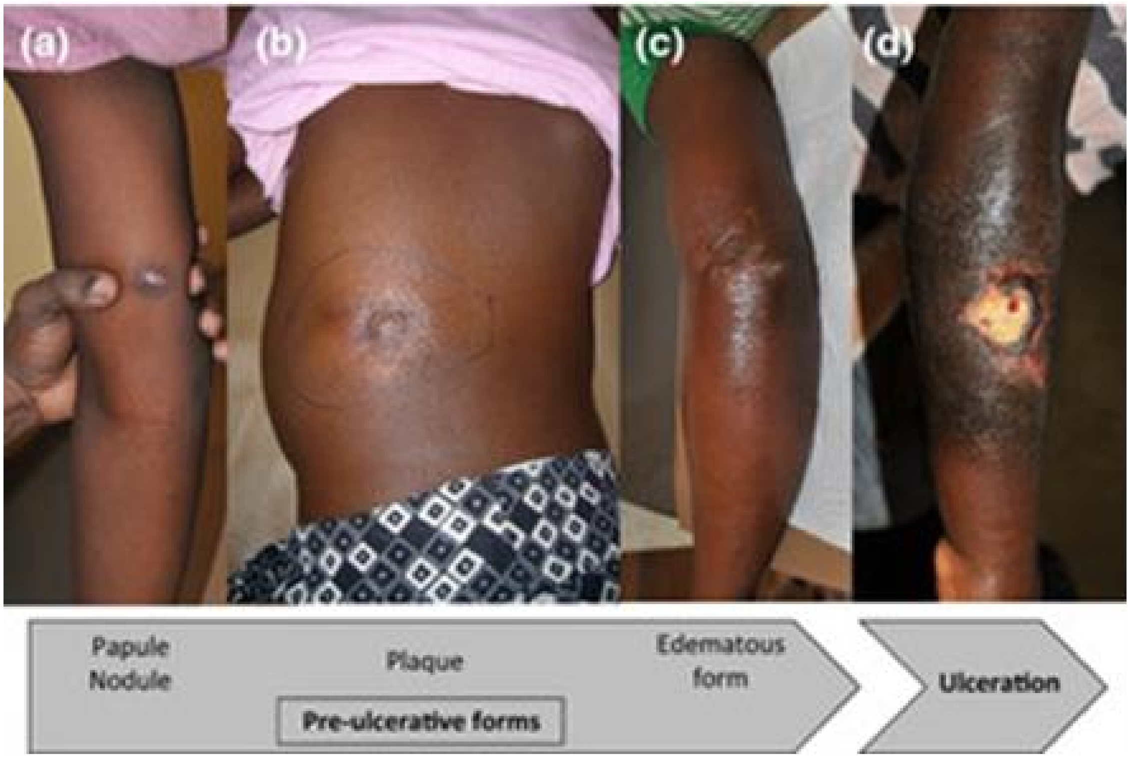


Fig 7. Clinical forms of Buruli ulcer disease. The lesions range through a spectrum from papule to nodule to plaque and then to the point of ulceration. Sometimes, patients develop an oedematous lesion before they ulcerate. Images by kind courtesy of Prof Richard Phillips.

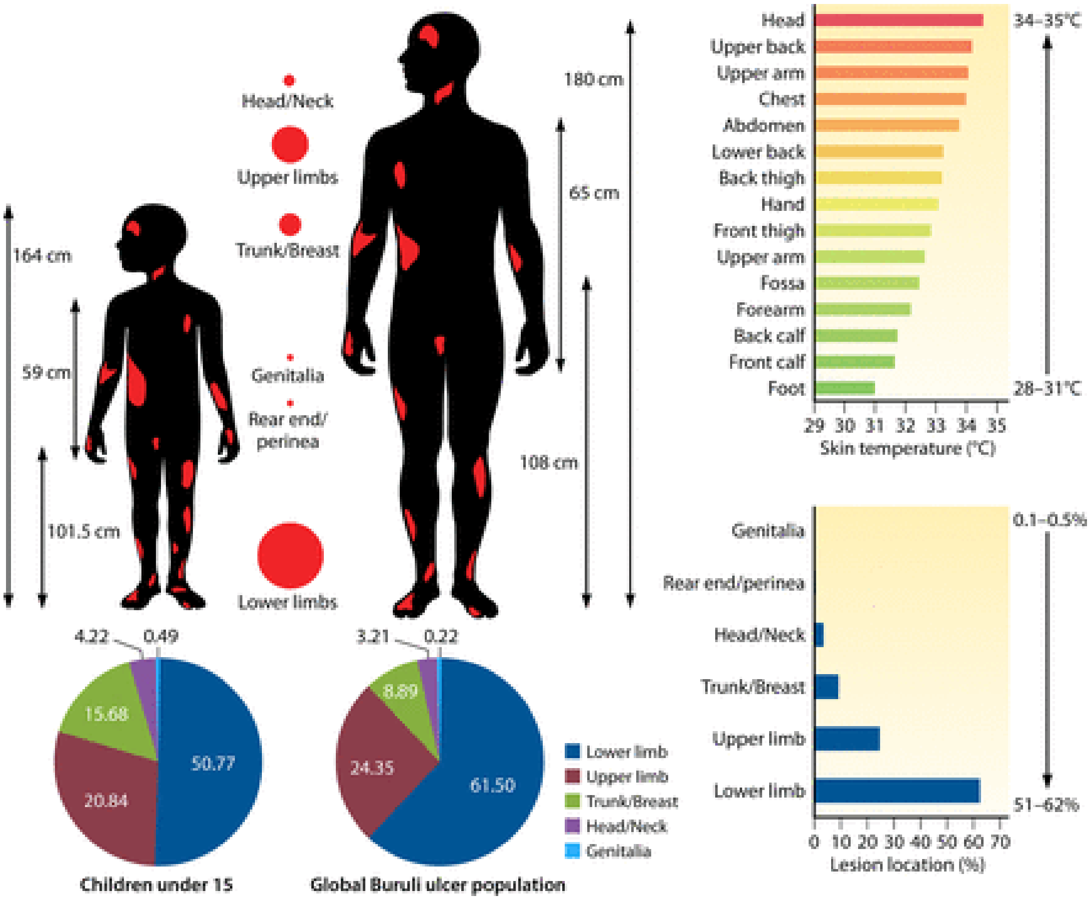


Fig 8. Pattern of distribution of Buruli ulcer lesions on the bodies of human patients in Africa. This figure is a composite of data from 10 independent studies adopted from (38). The histograms show that there is an inverse correlation between the gradient of body temperature and the location of lesions.

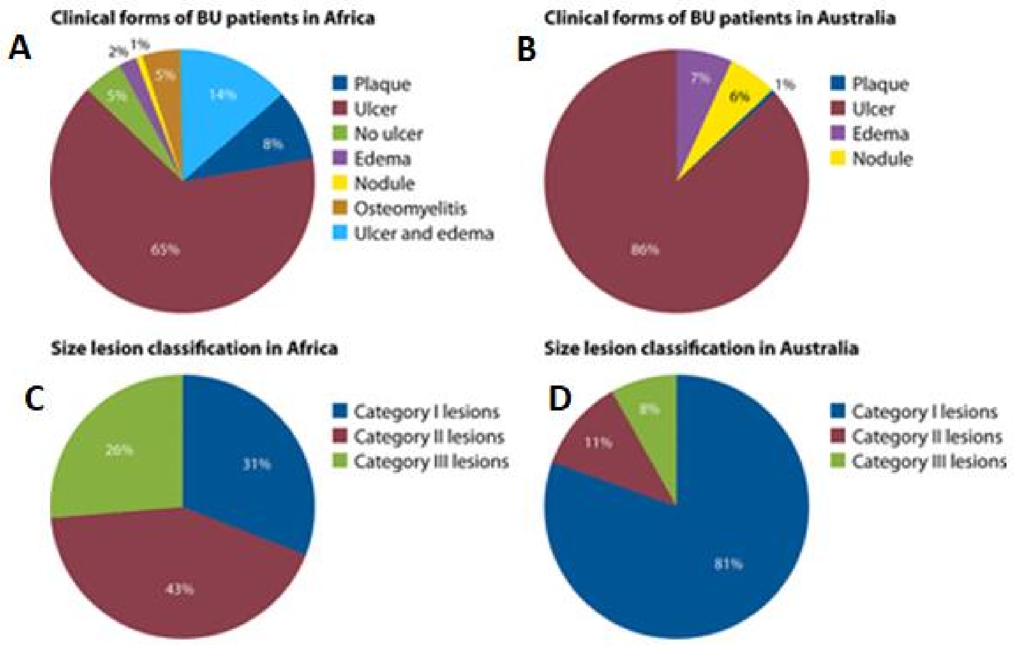


Fig 9. Comparison of the percentages of Buruli ulcer clinical forms and WHO-classified sizes of lesions between African and Australian Buruli ulcer patients. Source: WHO

1.11 Histopathology

The classical histopathological appearances of *M. ulcerans* disease are necrosis, extracellular acid-fast bacilli (AFB) and few inflammatory cells for the nodular and early ulcerative stages but few or no acid-fast bacilli, a mixture of acute and chronic inflammatory response and non-caseating granuloma formation for the healing stages (Fig 10). In particular, the oedematous form of the disease, which is the most acute form, tends to have abundant acid-fast bacilli and minimal or no inflammatory response. The necrosis occurs in the panniculus (the fatty layer below the epidermis), dermis, and sometimes fascia. Underlying muscle is usually not involved. The acute inflammatory response is characterised by neutrophilia whereas lymphocytes, plasma cells are seen in the chronic inflammatory response (51). Bacterial infections are usually characterised by neutrophilia. However, Adusumilli in 2005 (50) observed that neutrophil chemotaxis in *M. ulcerans* is poor and that neutrophil toxicity was accelerated by the presence of mycolactone. The defect in chemotaxis may however not be as a result of mycolactone since this observation was also made in mycolactone-negative mutant strain (51). Granulomas also occur in the dermis and panniculus of healing lesions. Other associated findings may include marked vasculitis resulting in thrombus formation and occlusion of small and medium sized vessels, which leads to continuing necrosis of the adjacent skin. Fat cells often die and lose their nuclei but retain their cell membrane and hence are referred to as ghost cells (52).

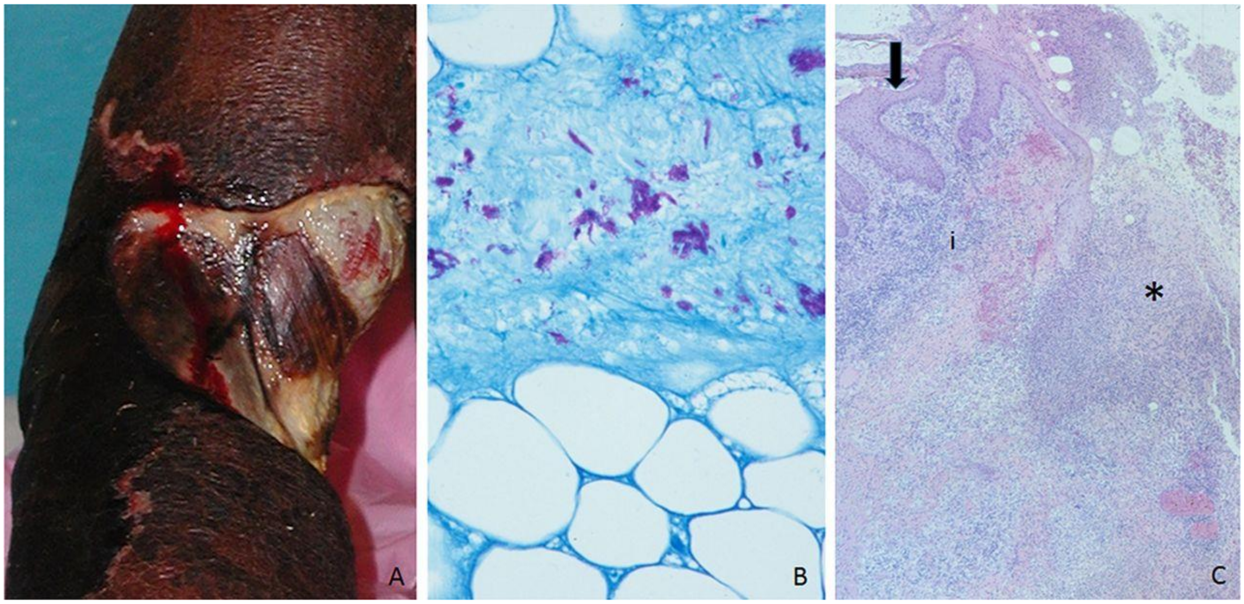


Fig 10. Histological slides of Buruli ulcer lesions

(A) Category 3 ulcer in the upper extremity, revealing the patient's tendons and muscle. (B) Ziehl-Neelsen stain demonstrating abundant clusters of acid-fast bacilli in the necrotic adipose tissue. This was a tissue sample from a nodule. The photograph was taken under oil immersion. (C) Histopathology of an ulcer, showing hyperplastic epidermis (arrow), necrosis (*), and inflammation (i). The photograph was taken at low magnification (4× objective). Photographs were obtained from the Centers for Disease Control and Prevention (52).

1.12 Treatment of *M. ulcerans* Disease

1.12.1 Antimycobacterial susceptibility

In vitro, *M ulcerans* has been shown to be susceptible to rifampicin, amikacin, streptomycin, clarithromycin, ofloxacin and dapsone but resistant to isoniazid (53, 54, 55, 58). Though clofazimine, an-anti-leprosy drug was highly effective *in vitro*, a controlled trial showed that clofazimine alone was not beneficial even in patients with pre-ulceration lesions (56, 57). Currently, antibiotics have taken center stage in the management of Buruli ulcer. A combination of streptomycin and rifampicin (for 8 treatment weeks) has been in use for a while now and has proven to be effective. In an attempt to tackle the poor compliance associated with streptomycin which is an injectable, the WHO is led a drug trial to replace streptomycin with clarithromycin in the combination. The trial which ended in 2020 proved that an 8-week oral dose of Clarithromycin and Rifampicin provides comparable cure for uncomplicated Buruli disease. It has since been recommended by the WHO as the standard treatment regimen against the disease (59). With effective antibiotic treatment, surgery will now be reserved for very complex lesions and complications such as contractures (60, 130).

1.12.2 Surgery

In the past, surgical excision of lesions used to be the standard treatment. In recent times however, antibiotic therapy has brought about a significant reduction in the number of surgeries performed. Surgery is now reserved for complex cases which require debridement, skin grafting or in complicated cases, contracture release (Fig 11). These types of surgeries require skilled personnel and lengthy hospital stay, thus increasing the cost of treatment and economic burden imposed on patients by the disease (60,61).

Fig 11. A skin grafted Buruli ulcer (Left) and a complicated contracture (Right) resulting from Buruli ulcer Disease (62)

1.13 Immunology of Buruli Ulcer Disease.

The immune system defends individuals against infections. Divided into two, its innate component is the first line of defence. It consists mainly of physical barriers present at birth which change very little with age or with experience of infection. It has no immunological memory. The adaptive component on the other hand, makes use of lymphocytes bearing highly diverse antigen specific receptors which facilitate recognition of a wide variety of pathogens. The adaptive immune system thus generates a host of memory lymphocytes which allow a more rapid and effective immune response upon re-infection in future.

Much of what is presently known regarding interactions between the host and mycobacteria has been studied in relation to *Mycobacterium tuberculosis*. In BU lesions, immunohistochemical studies of biopsy samples taken out of lesions have shown high concentrations of leukocytes. This observation is thought to support the thinking that the innate immune system may have its own mechanisms for identifying mycobacterial infections (52, 60, 63, 127). In some serological studies, control participants selected from endemic areas did not themselves experience the disease but tested positive for *M ulcerans* specific antibodies. This provided evidence of exposure to the organism and gave credence to the claim that there may be some protective immune features at play in the causation of Buruli ulcer disease. Only a small percentage of individuals who come into contact with the organism go on further to have the disease (64). In view of the fact that most of the distinctive immunological features of Buruli ulcer disease are as a result of the far-reaching effects of the *M.ulcerans* related toxin mycolactone (68), these features will be discussed in detail with mycolactone (Section 1.15).

Mycolactone, a lipidic molecule produced by *M. ulcerans* has been proven to have multiple effects on the immune system. George et al. proved it to be the main pathological factor involved in the disease process (65). Inoculation of Guinea pigs with mycolactone deficient strains of *M ulcerans* did not cause the characteristic Buruli ulcer lesions when attempted. It produced lesions similar to those produced by other non-mycolactone producing mycobacteria such as *M marinum*. However, site injections of purified mycolactone alone resulted in characteristic Buruli ulcer lesions (66,67). The immunological effects of mycolactone will be discussed further. Even though little is known about the specific immune mechansims underlying the pathology of buruli ulcer disease, preliminary projections based on the descriptions of pathological findings and lessons learned from Mtb give credence to the belief that cell mediated responses play a central role in the immunopathogenesis of the disease. And with the active involvement of the toxin mycolactone which is a lipid and non-protein based molecule, Non-classical MHC may play a significant role here. We therefore go on to discuss T-cell activation mechanisms.

1.13.1 Activation of T Lymphocytes

T cells classically fall into two major classes depending on which cell surface proteins they express. They bear the surface molecules CD8 or CD4. Mycobacterial peptides complexed with MHC class 1 molecules are presented for activation of T cells expressing CD8 receptors whereas peptides complexed with MHC class II molecules are presented for activation of T lymphocytes expressing CD4 molecules. T cells require two independent signals delivered by the same antigen presenting cells for activation. Binding of the foreign peptide/MHC II complex to the TCR:CD3 and CD4 co-receptor transmits the first signal but this can induce clonal expansion only when the co-stimulatory signal is given by binding of CD28 to B7 glycoprotein molecules like CD80 (B-7.1) or CD86 (B-7.2). On the contrary binding of B7 molecules to another protein CTLA-4 delivers inhibitory signals to the activated T cells. CD8 T lymphocytes that encounter peptide: MHC I complexes are activated in similar fashion but require stronger co-stimulatory signals for activation. This happens if activation is by mature dendritic cells that have high intrinsic co-stimulatory activity and can directly stimulate CD8 T cells to synthesize IL-2 that drives T cell proliferation and differentiation. In some cases, armed effector CD4 cells are required when there is inadequate co-stimulation of naïve T cells by the infected antigen presenting cell (APC). These armed effector CD4 cells then activate the APC by binding of CD40 ligand to CD40 on the APC to secrete high levels of co-stimulatory molecules. Activation of T cells results in secretion of IL-2 and expression of high affinity IL-2 receptors. IL-2 binds to the high affinity IL-2 receptors to promote T cell growth (69).

1.13.2 CD4 positive T lymphocyte

CD4 T lymphocytes have classically been divided into Th1 or Th2 and recognise peptides processed from the intracellular vesicles and displayed on the surface by MHC class II molecules. Activated Th1 lymphocytes express the CD4 ligand and produce cytokines such as Interferon Gamma (γ), tumour necrosis factor-a (TNF-a) and lymphotoxin-a. Together, these newly synthesized proteins activate the infected macrophage. Macrophages require two signals for activation one of which is provided by IFN-γ and the other by binding of CD40 ligand to CD40. TNF-a or TNF-b (lymphotoxin) also secreted by activated Th1 lymphocytes can substitute for CD40 ligand in macrophage activation. CD4 T lymphocytes also produce lymphotoxin-a, which participates in protection against tuberculosis (70). In tuberculosis for example IFN-γ and TNF-a act in synergy to activate macrophages. Macrophages fuse their lysozomes more effectively with phagosomes after activation and production of nitric oxide (NO) and superoxide ions (O2) which have powerful antimicrobial activity is induced. Th2 lymphocytes produce cytokines like IL-4, IL-5, IL-10 and IL-13 which act on B cells to induce antibody production (71)

1.13.3 CD8 positive T lymphocytes

CD8 T lymphocytes recognise mycobacterial peptides processed in the cytosolic compartment (72) and are referred to as cytotoxic T lymphocytes (CTL). They contribute to protective immunity in three ways. Like CD4 T lymphocytes they secrete cytokines and can be grouped into Tc1 or Tc2 depending on the cytokines they produce. Tc1 CD8 T lymphocytes are characterized by secretion of cytokines IL-2, IFN-γ and TNF-a (73, 74, 75) whereas Tc2 CD8 T cells secrete IL-4, IL-5 and IL-10 (63, 64). It is the Tc1 CD8 T lymphocytes that are associated with protective immunity in tuberculosis as they secrete IFN-γ and TNF-a (76, 77). This may account for the high frequency of IFN-γ -secreting CD8 T lymphocytes in the peripheral blood of healthy individuals who have been exposed to *Mycobacterium tuberculosis* (78). They also cause cell-mediated cytotoxicity either in a Ca2+-dependent release of cytolytic granules containing perforin and granzyme proteases leading to apoptosis of the target cell or in a receptor-mediated way which involves the engagement of Fas ligand (CD95L) on the T cell membrane with the target cell surface receptor Fas (CD95) also resulting in programmed cell death (79). Induction of apoptosis by these cells may account for the reduced viability of mycobacteria within such cells. CD8 T lymphocytes also possess a granule associated molecule granulysin (protein 519) that has direct mycobactericidal activity against intracellular mycobacteria (80).

1.13.4 Non–classical T lymphocytes

CD1-restricted T lymphocytes and the GammaDelta-T lymphocytes (gd T-Lymphocytes) all function like CD8 T lymphocytes by producing IFN-γ and killing infected cells by producing granules (81). They recognise lipoarabinomannan (LAM) or mycolic acids presented by the MHC class I–like molecules (CD1 family). They may also recognise an evolutionarily conserved family of isoprenoid glycolipids that include essential components of protein glycosylation and cell wall synthesis pathways. GammaDelta-T lymphocyte numbers have been shown to be increased during *M. tuberculosis* infection and reduced after treatment (82).

1.14 Buruli Ulcer Disease And HIV

HIV coinfection with tuberculosis, another Mycobacterium has been well studied and strongly correlated. The exact situation with HIV- Buruli ulcer disease coinfection has only been studied in very small populations and the results published seem contrasting. Very little is therefore known about treatment outcomes, mortality, incidence of paradoxical reactions secondary to antibiotic treatment (equivalent of Immune Reconstitution Syndrome as seen in HIV-TB coinfection), time to healing, rate of recurrence etc (83). In a 6-year study in Benin involving 1511 Buruli Ulcer patients of whom 34 were HIV positive, it was observed that while HIV negative patients stood a 50% chance of developing severe BU, HIV positive patients stood a 70% chance at suffering severe BU. This study however did not look at parameters such as CD4 count, viral load and treatment stage of the HIV positive patients. Also, as limitation of the study, the authors acknowledge that the study was not well controlled (84). These findings confirmed the findings of a previous retrospective study performed in Cameroon where HIV prevalence was found to be higher in the Buruli ulcer group than in the normal population and HIV positive patients were confirmed to have more severe forms of Buruli ulcer disease than HIV negative BU patients (83). In a previous study in Ghana involving 6 HIV positive out of 116 BU patients, there was no significant difference in the progression of Buruli Ulcer disease between HIV positive and HIV negative patients (85). There is also no study outlining the specific immune mechanisms which come to play in HIV-BU coinfections. With this level of uncertainty in HIV-BU coinfection, much of the discussion in this regard is based on knowledge borrowed from TB-HIV coinfection. The WHO also recommends treating HIV-BU coinfection with the same principles applied in managing HIV-TB coinfection (86).

1.15 *M. ulcerans* Toxin-Mycolactone

M. ulcerans produces a lipid-like molecule called mycolactone (Fig 12). This molecule over time has been found to play a crucial role in the pathogenesis of *M ulcerans* disease. It comes in variant structures with differences even though the central components of the structure remain the same. They are similar in structure to macrolides produced as secondary metabolites by soil bacteria, such as Streptomyces and Saccharopolyspora species in the order Actinomycetales (87). Connor and Lunn (88) suggested in 1965 that the extensive necrosis of *M. ulcerans* infection could be due to a diffusible substance. Following on from this, Read et al. (89) in 1974 reported the presence of cytopathic activity in culture supernatants of *M. ulcerans*. It was not until 1998 that a significant breakthrough was made in the purification and isolation of this substance (mycolactone) from acetone soluble fractions of lipid extracts of *M ulcerans* (9). Its chemical and immunological features are gradually being better appreciated with ongoing research.

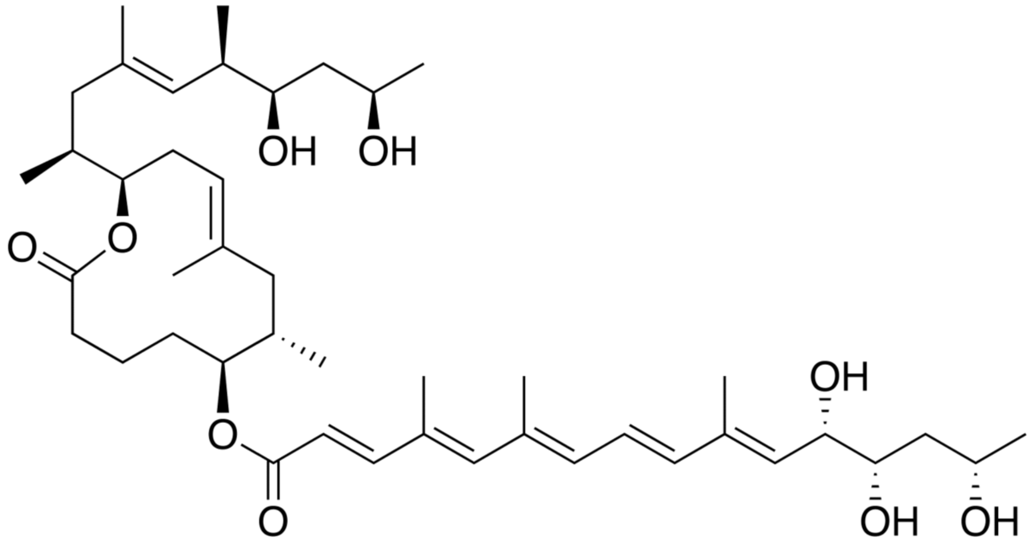


Fig 12. Molecular Structure of mycolactone. It consists of a 12-membered macrolide core with an ester-linked polyketide chain (190).

1.15.1 Biochemical Properties of Mycolactone

Mycolactone is heat-stable and present in *M. ulcerans* cytoplasmic fractions but absent from the cell wall (68). Filtered supernatants of culture media used to grow *M. ulcerans* have demonstrated mycolactone activity. Krieg RE (1974) suggested that it is composed of a "high molecular weight phospholipoprotein-polysaccharide complex" (67) and George K et al. partially purified the toxin and quantitated its cytopathic activity on mouse fibroblast L929 cells and characterized it as a relatively polar lipid (66). Mycolactone is photosensitive and its biological activity is lost upon prolonged exposure to UV light (Fig 13). The molecular degradation follows first-order kinetics with a half-life time 7.5 min (90).

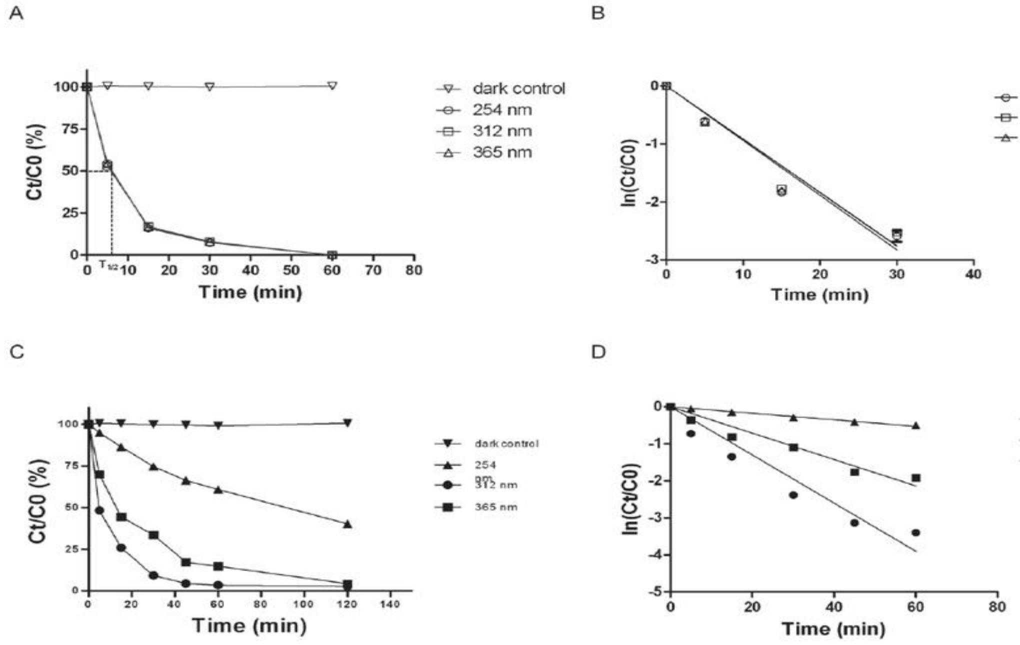


Fig 13. Variation in the quantity of native mycolactone after exposure to UV irradiation in quartz and glass tubes. (A) Remaining native mycolactone after different exposure times and UV wavelengths in quartz tubes. (B) Representation of the linear relationship between mycolactone and time irradiation to calculate half-life time in quartz tubes. (C) Remaining native mycolactone after different exposure times and UV wavelengths in glass tubes. (D) Representation of the linear relationship between mycolactone and time irradiation to calculate half-life time in glass tubes. Results in (A) and (C) represent mean values of triplicate experiments. Standard deviations represent less than 2.5% of the obtained mean values. C0 and Ct are the concentration at times 0 and t, where t is the irradiation time (90).

1.15.2 Mycolactone Gene is Carried on Plasmid pMUM001

Jenkin et al. through comparative genomic experiments between *M. ulcerans* and *M. marinum* fragments identified *Mycobacterium ulcerans*-specific polyketide synthase (PKS) genes (91). This was possible because *M. ulcerans* is known to share about 98% DNA sequence identity with *M. marinum* (28). These experiments led to the discovery of a *Mycobacterium ulcerans* virulence plasmid pMUM001 and the PKS locus it encodes. The plasmid is a 174kb plasmid bearing a cluster of genes encoding giant polyketide synthases (PKSs) and polyketide modifying enzymes, which are necessary for mycolactone synthesis (92). Following on, Tim Stinear and Porter JL have gone on to isolate and perform further experiments with these enzymes which are to be used in this project also.

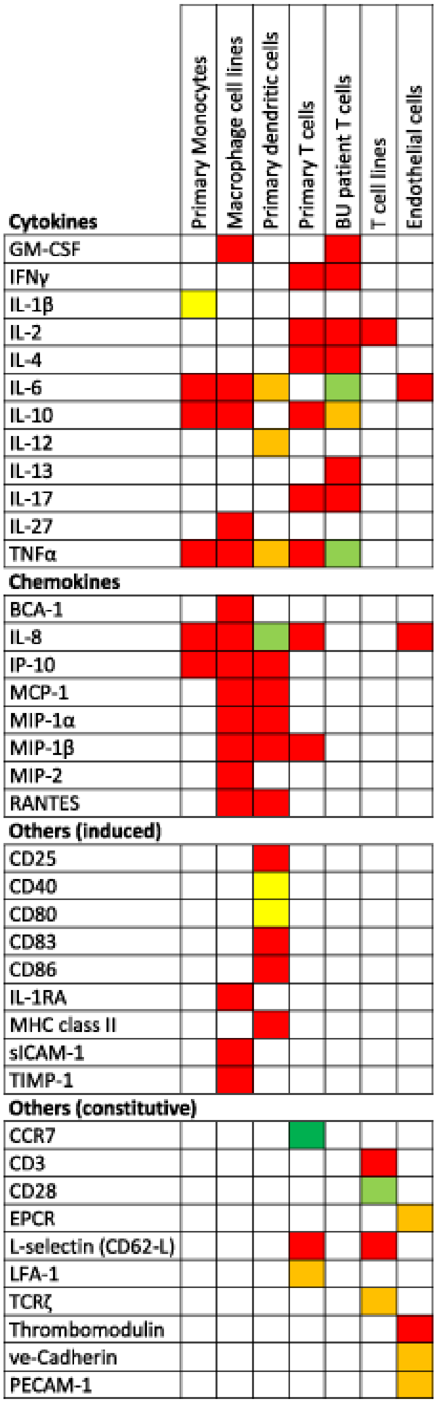
1.15.3 Biological Effects of Mycolactone

Mycolactone caused a cytopathic effect in mouse fibroblast L929 cells characterized by cytoskeletal rearrangement, subsequent rounding up and detachment from tissue culture plates within 48 hours (66). L929 and J774 mouse macrophage cells die via apoptosis after 3-5 days exposure to mycolactone and treatment with pan-caspase inhibitor inhibited mycolactone–induced apoptosis (66, 67). Mycolactone caused arrest in G0/G1 phase of the cell cycle within 48 hours (93, 9). When injected intradermally in the guinea pig it caused lesions similar to those produced by infection with the organism (66, 67).

Pahlevan A et al. showed that mycolactone causes more than 95% inhibition of lipopolysacharide-induced release of TNF and IL-10 from human monocytes and caused a loss of adherence of the cells without cell death. It also blocked the production of IL-2 from activated T lymphocytes (Table 1). It had no effect on TNF induced cytotoxicity, but abrogated downstream tumour necrosis factor-induced nuclear factor kappa B (NF-κ B) activation. Mycolactone had no effect on IL-1 or lipopolysacharide induced NF-κB activation. It also did not inhibit the degradation of IκBα induced by TNF, indicating that the target for this activity probably lies within an undefined part of the TNF signalling mechanism. These results were proposed as a possible explanation for the underlying immunological unresponsiveness and specifically the poor inflammatory response during the necrotizing stage of Buruli ulcer (94).

Most bacteria are phagocytosed by macrophages but this does not apply to *M. ulcerans*. Mycolactone secretion disrupts the cytoskeleton of macrophages by directly binding to the Wiskott-Aldrich syndrome protein (WASP). In so doing, there is cytoskeletal rearrangement which results in defective cell adhesion and directional migration. This allows the living *M. ulcerans* to escape phagocytosis and further proliferate (95). This supports the finding of multiple intracellular *M. ulcerans* in pathological samples in early Buruli ulcer but a reduction in this population with a shift to more extracellular *M. ulcerans* organisms with many dead macrophages later in the disease.

It has been possible to conjugate mycolactone to proteins in attempts to develop antibodies to it. Dangy et al conjugated truncated mycolactone to BSA and through mouse hybridoma techniques, developed monoclonal antibodies which demonstrated significant neutralising potential against synthetic mycolactone (96). These antibodies have however not been tested as candidates for passive immunity. Neither has the conjugated mycolactone molecule been tried as a vaccine candidate. In demonstrating that it is possible to induce in vivo antibody production to unconjugated mycolactone, Naranjo et al used various in vitro display techniques, comprising both phage and yeast display, to select antibodies against mycolactone. 10 antibodies were selected in all (97).



Cytokines	Primary Monocytes	Macrophage cell lines	Primary dendritic cells	Primary T cells	BU patient T cells	T cell lines	Endothelial cells
GM-CSF		🟥			🟥		
IFNγ				🟥	🟥		
IL-1β	🟨						
IL-2				🟥	🟥	🟥	
IL-4				🟥	🟥		
IL-6	🟥	🟥	🟧		🟩		🟥
IL-10	🟥	🟥		🟥	🟧		
IL-12			🟧				
IL-13					🟥		
IL-17				🟥	🟥		
IL-27		🟥					
TNFα	🟥	🟥	🟧	🟥	🟩		
Chemokines							
BCA-1		🟥					
IL-8	🟥	🟥	🟩	🟥			🟥
IP-10	🟥	🟥	🟥				
MCP-1		🟥	🟥				
MIP-1α		🟥	🟥				
MIP-1β		🟥	🟥	🟥			
MIP-2		🟥					
RANTES		🟥	🟥				
Others (induced)							
CD25			🟥				
CD40			🟨				
CD80			🟨				
CD83			🟥				
CD86			🟥				
IL-1RA		🟥					
MHC class II			🟥				
sICAM-1		🟥					
TIMP-1		🟥					
Others (constitutive)							
CCR7				🟢			
CD3						🟥	
CD28						🟩	
EPCR							🟧
L-selectin (CD62-L)				🟥		🟥	
LFA-1				🟧			
TCRζ						🟧	
Thrombomodulin							🟥
ve-Cadherin							🟧
PECAM-1							🟧

Table 1. Summary of findings of multiple publications that have reported the effect of mycolactone directly on the production of various cytokines, chemokines and other proteins by monocytes (Simmonds et al., 2009), macrophages (Hall et al., 2014), dendritic cells (Coutanceau et al., 2007), primary T-cells (Phillips et al., 2009, Guenin-Mace et al. 2011), T-cell lines (Pahlevan et al., 1999, Boulkroun et al., 2010) and endothelial cells (Ogbechi et al., 2015). Colour coding is as follows: Red, profound inhibition; orange, some inhibition; yellow, little inhibition; green, no inhibition; dark green, increased expression; white, not studied (51).

1.16 Vaccine Against Buruli Ulcer Disease

There currently exists no vaccine against Buruli. Vaccine studies against Buruli ulcer date back to 1956 when Fenner carried out immunizations with BCG, low dose *M. ulcerans*, high dose *M. ulcerans* and *M. balnei* (Marinum). He concluded that BCG protection was poor and even though *M. marinum* and *M. ulcerans* provided some limited protection against Buruli ulcer, this protection was not by antibody transfer. [98, 108]

Case control studies have proven that even though BCG confers some protection against severe forms of the disease, it does not prevent the occurrence of the disease (99,100). In two randomised controlled trials in Rwanda and Uganda, there was evidence that BCG did confer some protection even though this protection was thought to be short-lived (101, 102). However, in multiple other case control studies performed involving Ghana, Benin, Cameroon and Togo, there was no evidence that BCG does confer any protection. The protective ability of BCG against BU in humans therefore remains a matter of controversy (103, 106). In the trial of BCG in animals, it has been observed that BCG use in mice against BU only provides temporary protection as even booster doses do not seem to stop mice from developing footpad swellings on challenge. This is observed even when the dose of *M ulcerans* used in the challenge is reduced (107).

Mouse trials involving several antigenic candidates have also been tried but none has been successful so far. These span from attenuated *M ulcerans* to the use of other closely related mycobacterial organisms and in recent times, subunit vaccine candidates involving immune-active *M. ulcerans* surface proteins such as MU-Ag85A, MUL_2232 and MUL_3720. None of these have however been proven to be efficacious vaccine candidates (108, 132). Even though BCG has proven to only confer temporary protection, animal experiments have provided important lessons on immunological determinants of protection and immunological correlates involved in Buruli ulcer immunity. For example, an experiment demonstrated the interplay of cytokines during the protective phase and the time period during which mice developed footpad swellings on challenge. These principles and lessons have been adopted and applied in designing and trying various vaccine candidates against BU.

In recounting attempts at developing vaccines against BU, we would like to categorize the various vaccine candidates under two broad arms: Whole bacteria vaccines and subunit vaccines (Including mycolactone (toxin) based vaccines).

1.16.1 Whole Bacteria Vaccines

BCG based vaccines had for years been the focus of BU vaccination. In the first attempt at a vaccine, Fenner worked with BCG, *M ulcerans* (1615E) and *M. marinum* in mice (98, 109). *M marinum* is a slow-growing non-motile mycobacterium that shares 98% genetic homology with *M ulcerans* (28). In humans, it causes granulomatous hand lesions called Fishtank granuloma (18). Fenner found that protection against BU is not antibody dependent. He also found that even though BCG provided some protection against BU, it was short-lived and inferior to the protection provided by low dose *M ulcerans* and *M marinum*. Despite not being able to elucidate the underlying mechanisms, he observed that the protection conferred by low dose *M ulcerans* and *M* marinum were also short lived and waned with time thus causing the experimental mice to develop footpad swellings a little longer after challenge. Challenged mice were observed for 6 months with intermittent tissue collection for pathological and microbiological studies. Protection was ranked according to the number of colony forming units on culturing footpad extracts post vaccination and challenge and size of infected footpads measured at various intervals. *M ulcerans* (1615E) provided the best protection followed by *M marinum* and lastly by BCG. His conclusion was that all protection conferred was only temporary and will require optimisation to enhance immunity. He, however, also observed that the superior immunity conferred by *M ulcerans* (1615E) was lost when mice were vaccinated with high doses of the bacteria. Though not explained, this may have been our first hint of the immunomodulatory effect of the toxin mycolactone in high doses.

M marinum has since this experiment been retried in different forms. Hart et al in 2016 performed an experiment with recombinant *M marinum* which expressed high levels of the autologous *M ulcerans* specific antigen Ag85A. By this design, homologous antigens of the bacteria were presented in a vaccine which lacked the virulent and immunomodulatory potential of the toxin mycolactone. This construct was compared to the empty vector *M marinum* and BCG. In the end, at a cut off humane endpoint of footpad swelling up to 4.5mm, BCG vaccination temporarily raised the average lifespan by 5.5 weeks compared to a negative control. Empty vector *M marinum* increased it by 17 weeks while *M marinum* MU-Ag85A did this by 19 weeks. Although the increased expression of MU-Ag85A did not seem to have increased the lifespan of these mice, it did significantly reduce the bacterial load of the challenged footpads. Ultimately, the main parameter measured was lifespan and with respect to this, the conclusion was that standard *M marinum* (Aronson strain) conferred superior protection against *M ulcerans* when compared to the

BCG. This protection was however not absolute and a construct incorporating MU-Ag85A did not enhance this protection (110).

Hart et al also applied this same technology to BCG. BCG expressing the immunodominant Ag85A and EsxH which is the *M ulcerans* ortholog of Tuberculosis TB10.4 was constructed and used in experiments in comparison to standard BCG. Vaccination with BCG MU-Ag85B-EsxH induced better Ag85 specific CD4$^+$ T cell proliferation than both BCG and BCG MU-Ag85A. It also produced the strongest IFN-γ splenocyte responses to whole MU and recombinant antigens. Mice challenged with MU following a single subcutaneous vaccination with BCG MU-Ag85B-EsxH displayed significantly less bacterial burden at 6- and 12-weeks post-infection, reduced histopathological tissue damage, and significantly prolonged survival times compared to vaccination with either BCG or BCG MU-Ag85A. While BCG vaccination increased the mean survival time from 6.3 weeks for unprimed mice to 8 weeks, subcutaneous vaccination with BCG MU-A85gA significantly increased survival time over BCG alone to 17.4 weeks ($p<0.01$). A single subcutaneous dose of BCG MU-Ag85B-EsxH further significantly increased the survival time of MU-challenged mice over that of BCG MU-Ag85A to a mean of 29.4 weeks ($p<0.001$). Though improved, protection was still not absolute as mice reached the endpoint swelling of 4.5mm at various points in time (111). Hart et al had in a previous study compared BCG MU-Ag85A to standard BCG and the outcomes were comparable to the experiment described above (112).

Still on whole cell vaccine candidates, Fraga et al (113) in attempt to develop a vaccine devoid of the immunosuppressive toxin mycolactone, tried using a mycolactone deficient strain of *M* *ulcerans* ie *M ulcerans* strain 5114 which has become a mycolactone-negative strain due to repeated sub-culturing, leading to the spontaneous loss of MUP038 encoding some genes that are involved in the synthesis of mycolactone (114). In this experiment, the strain only managed to delay onset of swelling post-challenge but was unable to protect against it altogether. Median time for the development of footpad swelling after challenge was 129 days. A major component of this experiment was the study of the immune mechanisms underlying this partial protection. Roles were identified for sustained levels of IFN-γ and TNF with no detection of IL-4, IL-10 and IL-17 activity. It suggested that the mechanisms underlying protection against BU infection may be TH1 mediated. In discussing why this candidate only achieves limited success, the authors noted the possibility of a loss of significant immunogenic antigens during the process of repeated passaging (116). Thus, though limited in success, these experiments proved the importance of mycolactone or mycolactone based antigens in the design of vaccine candidates against Buruli ulcer disease.

With BCG still maintaining relevance in BU immunogenicity, Tanghe et al tried BCG booster on a BCG prime but this did not demonstrate any additional advantage over a single BCG vaccine (115).

1.16.2 Subunit Vaccines

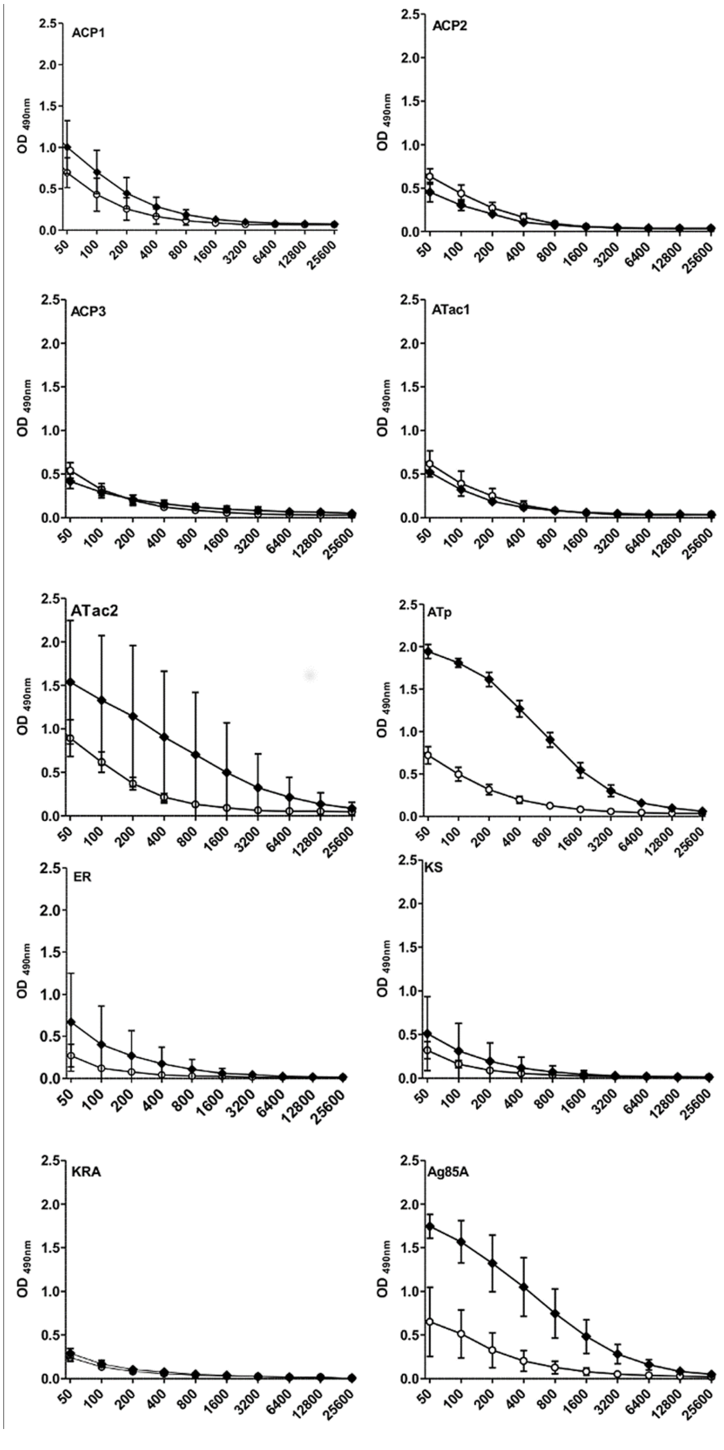
Learning lessons from TB protein immunogenicity, the first *M ulcerans* protein tested as potential vaccines was MU-Ag85A which is a 30-32kD protein with analogue forms in BCG and MTB (117). It is involved in the synthesis of cord factor and the organisation of mycolic acids in the bacterial cell wall. This it does through its enzymatic activity (118). Prior to this, all studies of cellular immune responses used whole *M ulcerans* bacteria or burulin which is a crude heat-killed lysate of the bacteria (119, 124).

Ag8A was first tried as an encoded DNA-based vaccine by Tanghe et al (120) and then as an *E. coli* expressed and purified recombinant protein by the Kris Huygen group in 2014(121). As a DNA based vaccine encoding Ag85A from *Mycobacterium bovis* BCG, it reduced *M ulcerans* bacterial load by 20-fold as compared to empty vector DNA controls in infected footpads after 8 weeks of infection. There was however no point of sterilisation. The shortcoming with this experiment is that it did not measure footpad swellings. In addition to demonstrating a reduction in bacterial load, the experiments also demonstrated that immune responses towards protection against Buruli ulcer disease was mostly local and Th1 mediated with strong roles for Il-2 and IFN-γ.

When tried as a purified protein expressed in *E coli*, Ag85A was tested in combination with 9 domains isolated from polyketide synthase enzymes involved in the synthesis of mycolactone. This experiment provided salient substrate data for the current project. Nine polyketide synthase enzyme domains derived from the three large polyketide synthases encoded by mlsA1, mlsA2 and mlsB localized on the 174 kb pMUM001 virulence plasmid of *M ulcerans* were expressed both as DNA vaccines and recombinant protein vaccines. These were acyl carrier protein 1, 2, and 3 (ACP 1,2,3) acyltransferase (acetate) 1 and 2(ATAC 1&2), acyltransferase propionate (ATP), enoylreductase (ER), ketoreductase A (KRA), and ketosynthase (KS) load module. These are the polyketide synthase domains involved in the synthesis of mycolactone which have the ability to induce antibodies in BU patients and healthy controls living in endemic regions of Buruli ulcer. Immunized mice were first primed with DNA constructs on Day 0 and 21 and boosted with *E coli* expressed and purified proteins on Day 42. Control groups received vaccinations with empty vectors on the corresponding dates. A group received log5 of *M bovis* BCG. Some of the mice were then culled at 3 weeks and others at 6 weeks after the 3^{rd} vaccination for immunogenicity studies. Remaining mice were then challenged 6 weeks after the protein boost with log 5 of *M. ulcerans* 1615. Inoculation was into the right footpads. Mice were then followed up with regular footpad size measurements and culled when swellings reached 4mm. 6 weeks after challenge, some mice were also culled for enumeration of bacterial load in footpads.

In the immunogenicity studies which looked at IgG responses, IL-2 and IFN-γ responses on antigen recall by splenocytes, some of the polyketide synthase domains did generate significant IgG antibody levels in mice.

As shown in **Fig 14**, responses were strong at week 3 post-protein boost in mice for ATac2 and ATp. Vaccination against ACP1 (acyl carrier protein 1) and ER induced weak IgG responses with only a quarter of mice responding. Disappointingly, IgG levels induced by vaccination against ACP2, ACP3, ATac1 KR A and KS were similar to levels in naïve mice. As previously determined (114), vaccination against Ag85A induced strong IgG antibody levels. Six weeks post vaccination, IgG antibodies against ATac2 and ATp were still present, but lower than at week 3. ACP1 and Ag85A specific antibody maintained their levels. However, antibodies against ER were found to be higher at week 6 than they were at week 3. IgG levels induced by vaccination against ACP2, ACP3, ATac1, KR A and KS remained negative.



Antigen-specific IgG antibodies in naïve and vaccinated mice.

Fig 14. IgG antibodies in C57BL/6 mice vaccinated twice with pDNA encoding the nine Pks synthase domains and boosted with the homologous recombinant protein. Sera were collected three weeks after the protein boost and tested by ELISA, using serial twofold dilutions, starting at 1:50 **(X-axis)**. Open circles: naive mice, closed circles vaccinated mice. Results presented as mean O.D. values ± SD of 4–6 mice tested individually (121).

On IL-2 levels in cytokine recall experiments (Fig 15), the Polyketide synthase domains (ATac2 and ATp) that induced strong antibodies, were also good inducers of IL-2. Contrastingly, vaccination against KRA which did not induce an antibody response did induce strong IL-2 responses. Vaccination against ACP1, ACP2, ACP3, ATac1 and ER however only resulted in very modest IL-2 levels. Stimulation of cells from unvaccinated mice with the recombinant proteins induced IL-2 levels that were close to the detection limit.

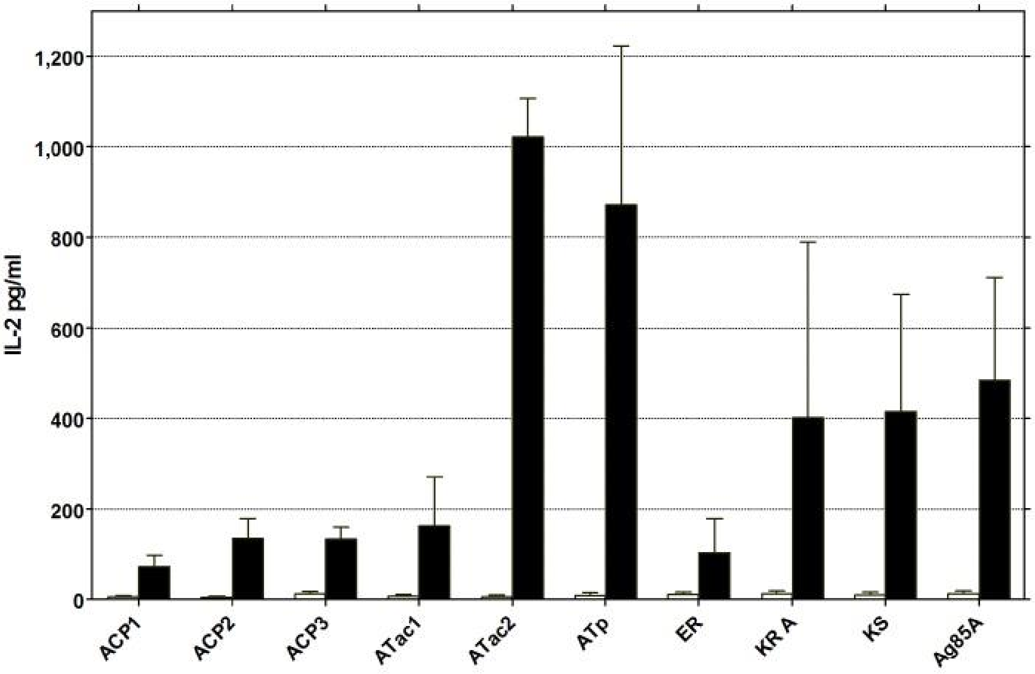


Fig 15. Antigen-specific IL-2 production in naïve and vaccinated mice, as tested by ELISA. Interleukin-2 levels in 24 hr spleen cell culture supernatants of C57BL/6 mice vaccinated against the 9 PkS domains using the pDNA prime/protein boost protocol and stimulated *in vitro* with the corresponding recombinant protein antigen (5 µg/ml). Results represent mean ± SD IL-2 values (pg/ml) of 4–6 mice tested individually. Data are representative of one of three experiments (121).

On IFN-γ, vaccination against ACP1, ACP2, ATac1, ATac2, ER and KS resulted in maximum IFN-γ levels of 2,500 pg/ml, responses against KR A and ACP3 mounted to 5,000 pg/ml and 7,500 pg/ml respectively and vaccination against ATp and Ag85A resulted in mean IFN-γ levels of more than 10,000 pg/ml (Fig 16).

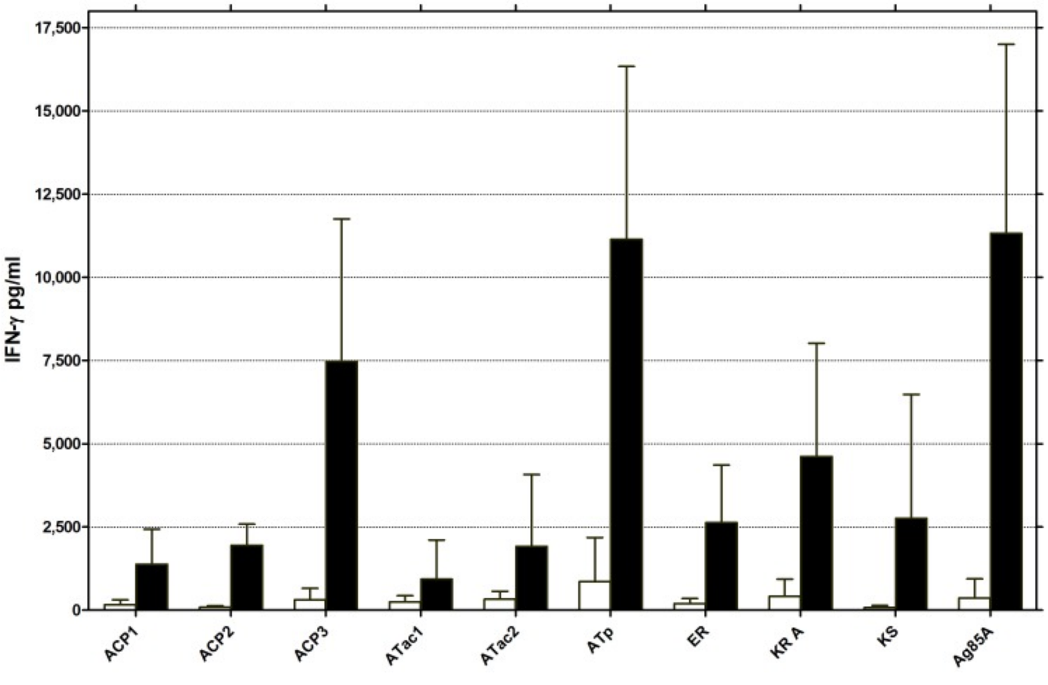


Fig 16. Antigen-specific IFN-γ production in naïve and vaccinated mice, as tested by ELISA.

IFN-γ levels in 72 hr spleen cell culture supernatants of C57BL/6 mice vaccinated against the 9 PkS domains using the pDNA prime/protein boost protocol and stimulated *in vitro* with the corresponding recombinant protein antigen (5 µg/ml). Results represent mean ± SD IFN-γ values (pg/ml) of 4–6 mice tested individually. Data are representative of one of three experiments (121).

Overall, the most important measured marker was protection as measured by footpad swelling. Mice were considered to have reached terminal point when the measured footpad swelling reached 4mm. The best protection as judged by time taken to reach endpoint was offered by BCG control followed by Ag85A. Of the polyketide synthase enzymes, only ATP followed by KRA offered moderate protection (Fig 17).

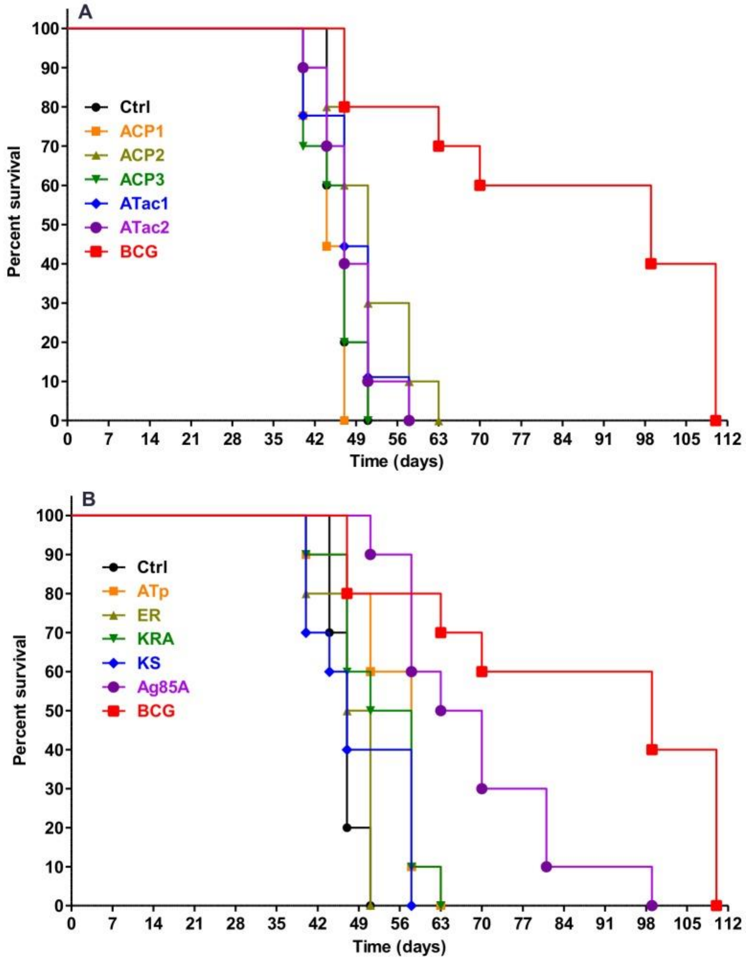


Fig 17. Survival of C57BL/6 mice vaccinated against Pks domains and challenged with *M. ulcerans* 1615. Ten mice/group were challenged 6 weeks after the protein boost with 10^5 Acid Fast Bacillus of *M. ulcerans* 1615 and subsequently monitored for 112 days for footpad swelling after *M. ulcerans* 1615 infection. Animals were euthanized when footpad swelling exceeded 4 mm. Fig shows the percentage of surviving mice during the 112 day follow-up. A: Survival curves of unvaccinated (ctrl) mice or mice vaccinated with ACP1, ACP2, ACP3, ATac1, ATac2 or *M. bovis* BCG. B: Survival curves of unvaccinated (ctrl) mice or mice vaccinated with ATp, ER, KR A, KS, Ag85A or *M. bovis* BCG (121).

In this project, four of these enzymatic modules namely acyltransferase (propionate) also called ATP (Acyltransferase propionate), ketoreductase A also called KRA, enoylreductase also called ER and acyltransferase (acetate) 2 also called ATP have been selected for further experimentation. These were selected based on previous immunogenicity work done by Kris Huygen to demonstrate that these were the most immunologically active of the nine polyketide synthases worked with and work done in Richard Phillips' lab at KCCR (Kumasi Centre for Collaborative Research) in Kumasi, Ghana to determine which of these synthase enzymes presented the greatest immunological distinctions between patients and controls (122). The immunodominant Ag85A was also included.

With limited success so far on both whole bacteria and subunit protein candidates including DNA based candidates, we postulate that a vaccine design based on the pathologically important toxin mycolactone could provide the much sought-after protection against Buruli ulcer disease. This project seeks to explore this with lessons from previous use of toxoids in vaccines against Diphtheria and Tetanus (123). Even though not a polypeptide like the toxoids mentioned, mycolactone when conjugated to Bovine Serum Albumin (BSA) via the amino group on its diethylene glycol-based linker and the carrier protein conjugate (Fig 18) was used for immunization of mice, elicited protein based immune responses with the production of neutralising antibodies as determined by ELISA and various neutralisation assays as well as protein targeting T-cell responses (96). Also, Naranjo et al demonstrated the immunogenicity/ vaccine potential of mycolactone by eliciting antibody responses to it via in vitro display methods comprising both phage and yeast. Thus, they demonstrated that if appropriately explored, mycolactone does have immunogenic/vaccine potential (97).

Against this background, we seek to explore the actions of mycolactone further when combined with selected polyketide synthase enzyme proteins.

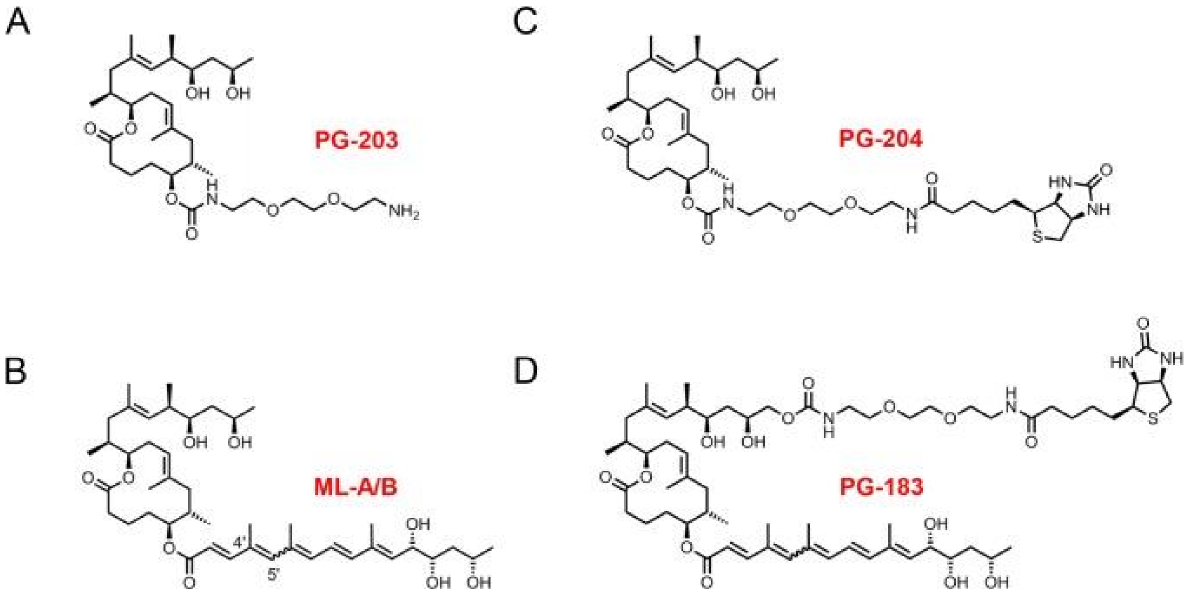
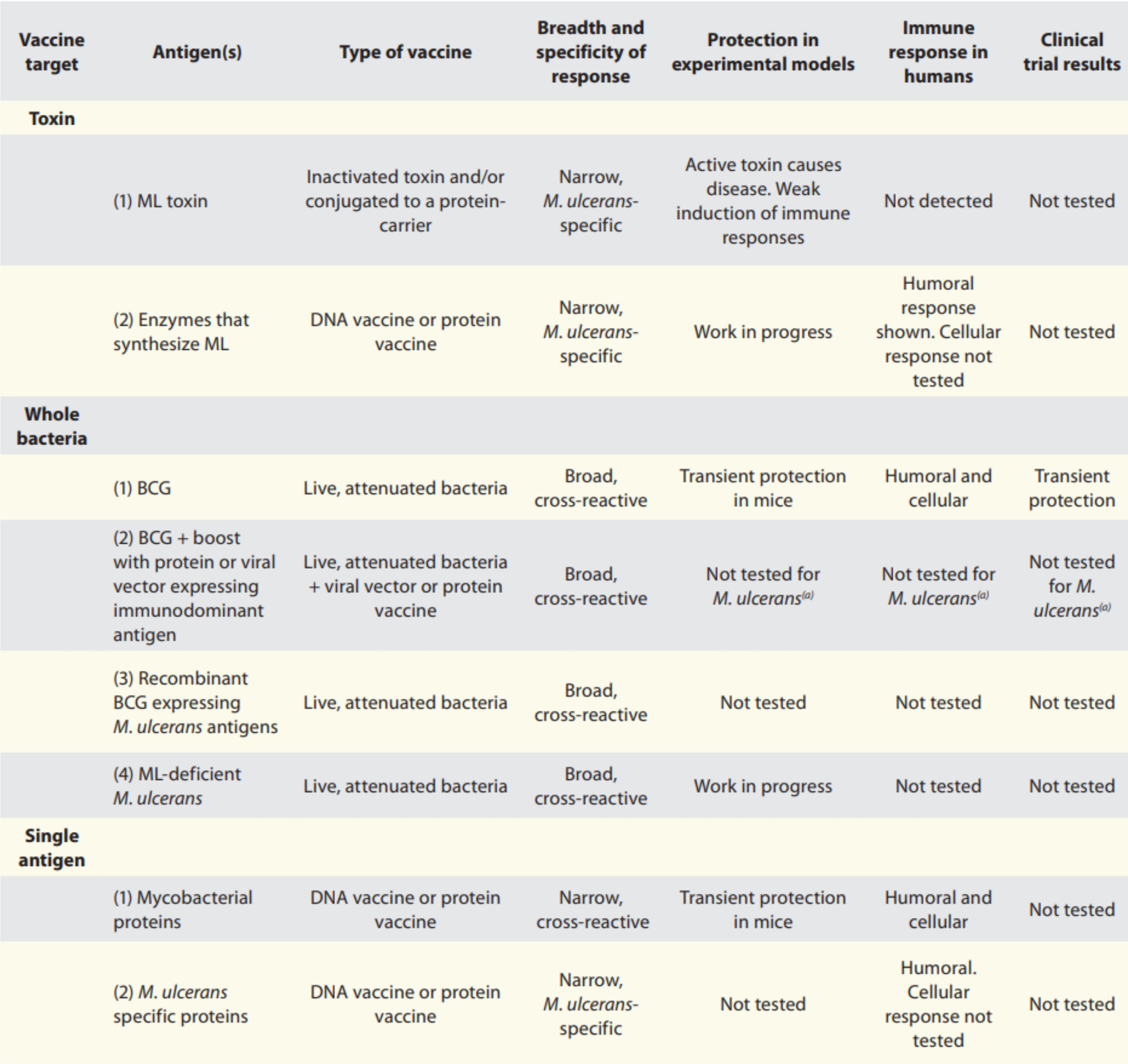


Fig 18. Synthetic mycolactone derivatives used for the generation and detection of mycolactone specific antibody responses.

PG-203 (A) was conjugated to BSA via the amino group on its diethylene glycol-based linker and the carrier protein conjugate was used for the immunization of mice. PG-204 (C) and PG-183 (D) are biotinylated mycolactone derivatives used for analytical purposes. Unmodified synthetic mycolactone A/B (B) was used as effector molecule in cytotoxicity assays (96).

It is also important to mention that since the publication from Tanghe et al, *Mycobacterium ulcerans* surface proteins MUL_2232 and MUL_3720 following their strong induction of IgG antibodies have been tried as vaccine candidates but failed to provide any protection (125). Prior to this, Mangas et al had tried vaccine candidates designed to contain HSP 18, a protein associated with biofilm formation in *M ulcerans infection (*126, 132). In this experiment, HSP 18 did not confer any protection despite inducing strong IgG antibody responses. It in effect gave credence to the thinking that T-Cell responses may have a significant role in *M ulcerans* immunity.

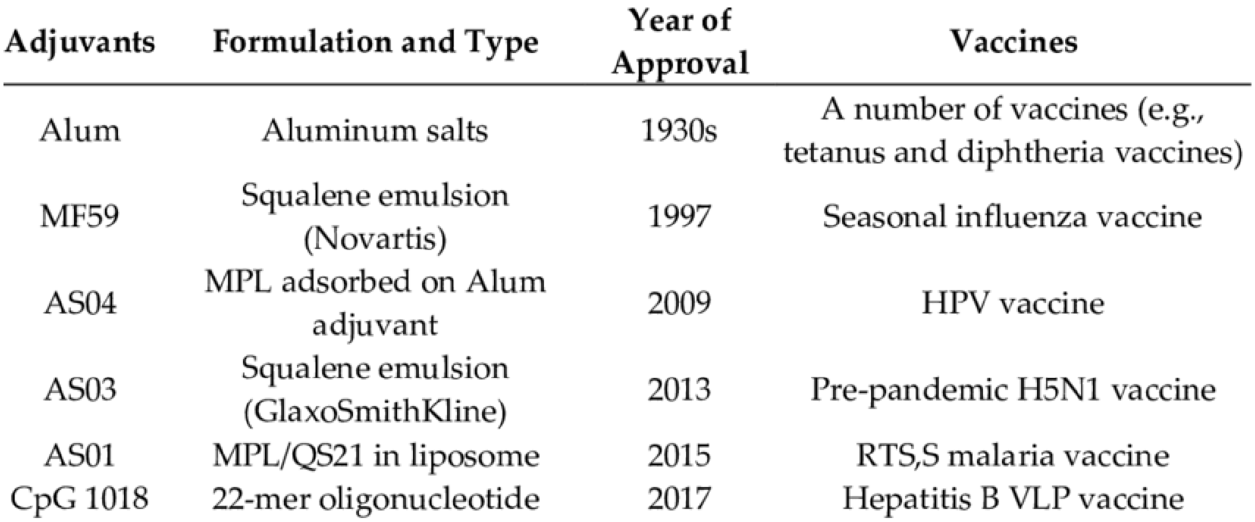


Vaccine target	Antigen(s)	Type of vaccine	Breadth and specificity of response	Protection in experimental models	Immune response in humans	Clinical trial results
Toxin						
	(1) ML toxin	Inactivated toxin and/or conjugated to a protein-carrier	Narrow, *M. ulcerans*-specific	Active toxin causes disease. Weak induction of immune responses	Not detected	Not tested
	(2) Enzymes that synthesize ML	DNA vaccine or protein vaccine	Narrow, *M. ulcerans*-specific	Work in progress	Humoral response shown. Cellular response not tested	Not tested
Whole bacteria						
	(1) BCG	Live, attenuated bacteria	Broad, cross-reactive	Transient protection in mice	Humoral and cellular	Transient protection
	(2) BCG + boost with protein or viral vector expressing immunodominant antigen	Live, attenuated bacteria + viral vector or protein vaccine	Broad, cross-reactive	Not tested for *M. ulcerans*$^{(a)}$	Not tested for *M. ulcerans*$^{(a)}$	Not tested for *M. ulcerans*$^{(a)}$
	(3) Recombinant BCG expressing *M. ulcerans* antigens	Live, attenuated bacteria	Broad, cross-reactive	Not tested	Not tested	Not tested
	(4) ML-deficient *M. ulcerans*	Live, attenuated bacteria	Broad, cross-reactive	Work in progress	Not tested	Not tested
Single antigen						
	(1) Mycobacterial proteins	DNA vaccine or protein vaccine	Narrow, cross-reactive	Transient protection in mice	Humoral and cellular	Not tested
	(2) *M. ulcerans* specific proteins	DNA vaccine or protein vaccine	Narrow, *M. ulcerans*-specific	Not tested	Humoral. Cellular response not tested	Not tested

Table 2. **Listed are the vaccine targets (toxin, bacteria or specific protein) and type, whether the vaccines are specific for *M. ulcerans* or cross-react with other mycobacteria**. Results from experimental models and published immune responses in humans are listed, as well as whether the vaccine has been tested in clinical trials for protection against BU disease (131).

1.16.3 Vaccine Adjuvants

Derived from the latin word ‘adjuvare’ which means to help, adjuvants are defined as chemical compounds which enhance immune responses to vaccine antigens (133). They are so called because of their ability to modulate (upwards) the humoral and/or cellular responses to an antigen. With the advent of subunit vaccines, vaccine candidates needed immunogenicity boosts in view of the narrower antigen spectrum and weaker immune response they induce as compared to previously popular whole bacteria/viral vaccine candidates (133, 134) (Table 2). Though still a subject of scientific controversy, adjuvants are gradually gaining prominence in their use. Alum for a long time was the only adjuvant approved for use in humans (138, 139). Concerns of safety have over time been resolved and there are currently many other approved adjuvants which have been used for vaccines in current use. These include Monophosphoryl Lipid A (MPL) used in the cervarix vaccine; MF59 an oil-in-water emulsion composed of squalene which is a naturally occurring oil found in many plant and animal cells, as well as in humans is the adjuvant contained in Fluad (an influenza vaccine licensed for adults aged 65 or older) ; AS01$_B$ an adjuvant suspension used with the antigen component of Shingrix zoster vaccine; CpG 1018 a recently developed adjuvant used in Heplisav-B vaccine against hepatitis B and a few other examples of adjuvants(135, 136) (Table 3). There are however some other adjuvants which though not approved for human use, have shown promise in research and are still under development even. Examples are Quil A (A saponin derived detergent), PolyI:C (A TLR3 agonist), Flagellin (A TLR5 Agonist) etc (137). Adjuvants are not approved in isolation but as components of vaccines which demonstrate safety and efficacy. These adjuvants will therefore have to be used as such for eventual approval. Recently, toll-like receptors are taking center stage in adjuvant science. Studies have shown that antigens using toll-like receptor ligands (TLRs) induce exceptionally high humoral and cellular response levels. Adjuvants targeting these receptor ligands are now established to be of good use in vaccines (140, 141).



Adjuvants	**Formulation and Type**	**Year of Approval**	**Vaccines**
Alum	Aluminum salts	1930s	A number of vaccines (e.g., tetanus and diphtheria vaccines)
MF59	Squalene emulsion (Novartis)	1997	Seasonal influenza vaccine
AS04	MPL adsorbed on Alum adjuvant	2009	HPV vaccine
AS03	Squalene emulsion (GlaxoSmithKline)	2013	Pre-pandemic H5N1 vaccine
AS01	MPL/QS21 in liposome	2015	RTS,S malaria vaccine
CpG 1018	22-mer oligonucleotide	2017	Hepatitis B VLP vaccine

Table 3. List of some licensed adjuvants for human use. Adjuvants are approved as components of vaccines. Thus, all listed adjuvants are components of vaccines determined to be safe and effective for humans. (142)

Beyond these specific examples, adjuvants in general are known to be able to stabilize otherwise unstable antigens and to regulate prolonged release for optimal responses. Chitosan containing nanoparticles for example do this very well. Based on their modes of actions, adjuvants may be classified into five groups (133).

i) Presentation adjuvants

ii) Immunomodulating Adjuvants (These modify cytokine networks)

iii) Cell specific targeting adjuvants (Uptake and intracellular distribution of antigen in APCs (Antigen Presenting Cell))

iv) Cytotoxic T-Lymphocyte inducing adjuvants

v) Depot generating adjuvants (The most notable being Aluminium hydroxide gels, they)

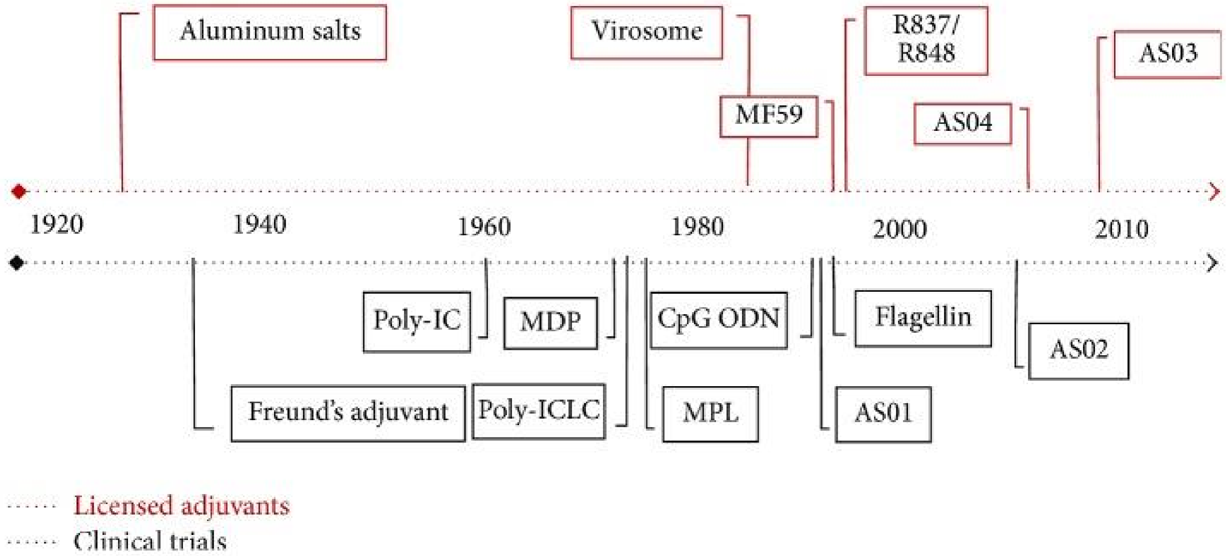


Fig 19. Timeline of Vaccine Adjuvants discovery. Adjuvants discovered from 1920 to 2010 (143)

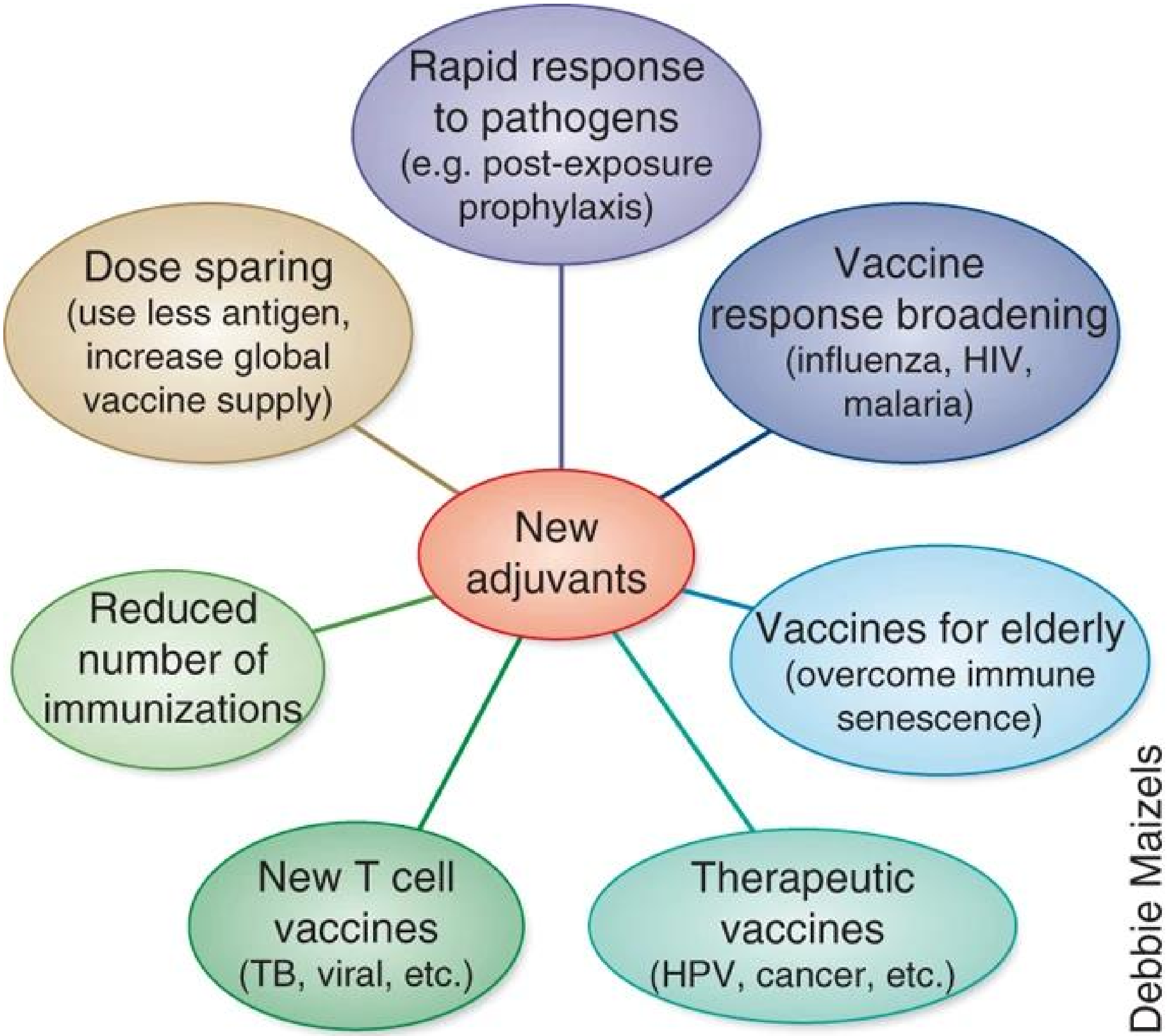


Fig 20. **Benefits of Adjuvants** (144)

In this project, adjuvants will be used in an attempt to enhance mycolactone immunogenicity and presentation. Knowing that properties such as size, charge and hydrophobicity influence the suitability of adjuvants for specific antigens, we will be testing different adjuvants and delivery systems to select the most ideal candidates. Specifically, selected adjuvants will be discussed in the appropriate chapters.

1.16.4 Selected Adjuvants

In this project, adjuvants have been selected based on experience working with various adjuvants in the Reljic lab and their ability to induce both humoral and cellular immune responses.

i) QUIL A

Quil A is a saponin adjuvant derived from the South American tree *Quillaja Saponaria* Molina. They induce strong T-dependent and T-independent antigens and also induce strong cytotoxic CD8+ lymphocyte responses and potentiate the response to mucosal antigens. When combined with cholesterol and phospholipids, it has the ability to activate both T-cell and B-cell immune responses to a broad range of antigens. (193)

ii) YC NaMA

YC NaMA is produced via the emulsification of Yellow Carnauba (YC) wax with sodium myristate (NAMA). They have an average diameter 400nm and are anionic with a zeta potential of approximately -75mV, thereby giving it high colloidal stability on YC-NaMA in suspension. Its mechanism of action of poorly understood and remains undefined but has been observed to strongly drive T-cell immune responses. (151)

iii) SPORES (*Bacillus subtilis*)

Bacillus subtilis is a non-pathogenic, endospore-forming gram-positive bacterium which is has the ability to induce both T-cell and B-cell directed immune responses. When administered as component of the inactivated avian influenza virus H9N2, its adjuvanticity was observed through the enhancement of H9N2 virus-specific IgG responses as well increased expression of the pro-inflammatory cytokines IL-1B and IL6 as compared to H9N2 only. It is also known to broaden the

T-cell responses to include both antigen-specific CD4+ and CD8+ cell responses to specific antigens. (194)

iv) LIPOSOMES (Monophosphoryl Lipid A)

Liposome containing monophosphoryl lipid A (MPL) is a cationic molecule with inherent cytotoxicity properties. By inducing cell death, it stimulates immune cell infiltration and thus enhances antigen presentation. It has been used an adjuvant in vaccines against infectious diseases and also in cancer immunotherapy. By its mechanism of action, MPL induces both humoral and cell-mediated immunity. (195)

CHAPTER 2

PROJECT OVERVIEW

2.1 Research Questions

This research project seeks to answer three main questions:

1. Does immunization with mycolactone alone or in combination with novel *M. ulcerans* candidate antigens prevent the establishment of Buruli ulcer disease?

Even though mycolactone alone has not been used in the literature as a vaccine candidate, we are of the view that mycolactone in addition to established adjuvants can induce an immune response with capacity to protect against infection. In the past, equally small molecules like azaspiracid have been modified into immunogenic compounds for the production of protective monoclonal antibodies against diseases (64). The dogma that mycolactone is not immunogenic has been based on analysis of the structure of the molecule. It is not of the form of characteristic protein vaccine candidates. It is a lipidic polyketide molecule with cytotoxic and anti-proliferative properties. We intend to enhance its presentation and achieve protection following *M ulcerans* challenge. We also want to investigate the potential of a mycolactone based vaccine's ability to protect against the classic debilitating form of BU by efficiently neutralising mycolactone and possibly reducing the severity of infections if they do occur post-immunization.

2. What are the effects of Mycolactone on the cells of the immune system?

Even though *in vitro* effects of mycolactone on cells of the immune system have been partially studied over the period, there still remain gaps in the knowledge available. Not much is known on the *in vivo* effects of mycolactone on cells of the immune system. We wish to characterise these effects and seek further insights into the *in vivo* physiology and mechanisms of action of mycolactone using our mouse model of immunization and infection.

3. Do adjuvants improve the immunogenicity of Mycolactone and its polyketide synthase domains?

Upon the identification and description of the pMUM giant plasmid and its associate enzymes involved in mycolactone synthesis, the Huygen laboratory attempted to develop a DNA based vaccine against BU by immunizing mice with the constructs (65). Though some candidates conferred some level of protection, none was better than BCG. We want to see if recombinant protein-based forms of these enzymes will confer better protection and if adjuvants will enhance this protection. Selection of these adjuvants will be influenced by their known abilities to enhance T-cell or B-cell responses.

4. Will protection against BU be mediated by B-cell or T-cell induced mechanisms?

With evidence gathered so far indicating places for both T-cell and B-cell effects in the immunology of Buruli ulcer disease, we seek to identify which of these mechanisms will induce protection against the disease. We will generate both B and T-cell responses and attempt to identify correlates of protection.

To address these research questions, this study has set the following specific aims and objectives:

2.2 Aims

- To investigate and establish the immunogenicity of Mycolactone
- To determine the protective ability of the Polyketide synthase enzymes involved in the synthesis of Mycolactone.
- To design and investigate the protective ability of a mycolactone based vaccine against Buruli ulcer disease.

2.3 Objectives

- To express and purify and *M ulcerans* antigenic proteins.
- To design and develop multiple mycolactone based vaccine candidates with adjuvants and delivery systems.
- To set up footpad infection model of Buruli ulcer disease in mice.
- To immunize and challenge mice with vaccine candidates and determine protection and immunogenicity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Regulatory Declaration

In this project, activities involving genetically modified organisms were conducted according to the genetically modified organisms regulations 2014, GM activity number GM254. All work with mice was carried out by trained and Home office licensed individuals in the biological research facility at St George's University of London, which at the time of performing experiments, was a licensed institution. This project was also licensed by the Home office and all work was carried out under the Animals Scientific Act 1986 and following the principles of '3Rs'.

3.2 Mycolactone

Purified mycolactone was obtained in 0.5ml glass vials from Prof Yoshito Kishi's laboratory at Harvard University. Samples came in ethyl acetate or ethanol carrier media at concentrations of 200µg/ml. This mycolactone is WHO verified and has been used extensively by researchers in other labs for Buruli ulcer related work.

3.3 Polyketide Synthase Enzymes:

3.3.1 Transformation and Expression

The plasmid constructs were obtained from Prof Tim Stinear's laboratory in the University of Melbourne. These were expressed from transformed BL21 Plys s E coli strains (Invitrogen) for Ag85A and KRA; Rosetta strains (Novagen) for ATP, ER and ATAC2. The E coli was first transformed by incubating the bacteria with corresponding DNA for 20 minutes on ice and heat shocking the bacteria at 42ºC for 45 seconds. 300µl of SOC medium (Invitrogen) was then added and incubated for an hour in a shaking incubator. This was then transferred onto an LB agar plate with selected antibiotics (Carbenicillin only for BL21 cells and Carbenillin + chloramphenicol for Roseta cells) for overnight incubation at 37ºC. Transformed colonies were then transferred into 5ml liquid cultures with appropriate antibiotics for overnight incubation at 37ºC. Larger volume cultures of 200mls were then made by transferring 1ml of the mini culture sample into 200mls of LB liquid media in conical flasks of at least 1000ml volume. This was then incubated at 37ºC to obtain an OD of 0.6. The media was then allowed to cool at room temperature and Isopropyl β-D-1-thiogalactopyranoside (IPTG) added to induce protein expression. IPTG was added to a final concentration of 1mM and incubated for four hours with shaking at 37^{O}C. The cells were then collected after 20 minutes of centrifuging at 3500g. The pellet was either frozen for later use or immediately lysed for protein purification.

3.3.2 Protein Purification

Solution	DIMAC 5	DIMAC 20	DIMAC 100	DIMAC 200	DIMAC 1000	STRIP Buffer
2M Imidazole	1.25ml	5ml	25ml	50ml	250ml	
Urea	240.24g	240.24g	240.24g	240.24g	240.24g	
2M Tris-HCl pH 7.9	5ml	5ml	5ml	5ml	5ml	1ml
5M NaCl	50ml	50ml	50ml	50ml	50ml	10ml
0.5M Na2EDTA (pH 8.2)						20ml
dH2O	To 500ml	To 500ml	To 500ml	To 500ml	To 500ml	To 100ml

Table 4. Composition of Reagents for Purification

Protein purification was done using a sepharose-Nickel column. The pellet obtained after inducing protein expression was sonicated in DIMAC5 solution. The supernatant after centrifuging was then filter sterilized and passed through the sepharose-nickel column (GE 17-0575-01 Chelating Sepharose Fast Flow) which had been prewashed with water and DIMAC 5. The desired proteins were then eluted with DIMAC 100 and dialyzed in PBS or $NaHCO_3$ depending on the protein's solubility in PBS. Samples were then aliquoted and stored at 4°C.

3.3.3 Coomassie and Western blots

Coomassie and Western blots were performed to further characterize and confirm the presence of purified proteins. This also aided in estimating the percentage purity of the proteins post-purification. The protein samples were incubated with LDS loading buffer and β-mecaptoethanol (sigma) at 85 ^{o}C for 5 minutes and immediately cooled on ice for 2 minutes. Loading was then done onto 4-12% w/v BIS-TRIS SDS-PAGE gels (Invitrogen) alongside a reference ladder (Biorad Precision Plus Protein, All Blue Standards) and run at 140V for 1 hour in MES buffer (Life Technologies, Novex). The proteins in the gel were then either stained with Coomassie dye for 2 hours, de-stained with distilled water and captured using a gel reader (Syngene G) (Fig 21) or transferred onto a nitrocellulose membrane for a Western blot using the semi-dry transfer method (0.04A per gel for 60 minutes).

In the semi-dry transfer method, the gel is first equilibrated in semi dry transfer buffer for 15 minutes before being sandwiched between equilibrated nitrocellulose membrane and blot paper soaked in the transfer buffer. Additional blot paper is used to pad the gel following which a pipette is rolled over the topmost blot paper to remove all bubbles. The blot paper, nitrocellulose membrane and gel are kept the same size. The cathode plate is placed on the stack and pressed down to engage the latchets and to ensure proper contact for efficient transfer. The transfer is then run at a current 0.04A for one hour.

On completion of the run, nitrocellulose membranes were blocked with 5% w/v skimmed milk protein (Marvel original) in PBS overnight at 4^{o}C (192). In order to make it possible to stain proteins, membranes were incubated with primary antibodies against the heavy and light chains of IgG (Sigma A7058 Monoclonal Anti-polyhistidine−Peroxidase antibody produced in mouse) for 2 hours at 1:1000 dilution in PBS 5% w/v milk. The membrane was then washed between staining steps 3 times with TBS 0.1% w/v Tween-20. To visualize the membrane ECL prime substrate (Amersham) was added and the membrane captured on a gel reader (Syngene G box).

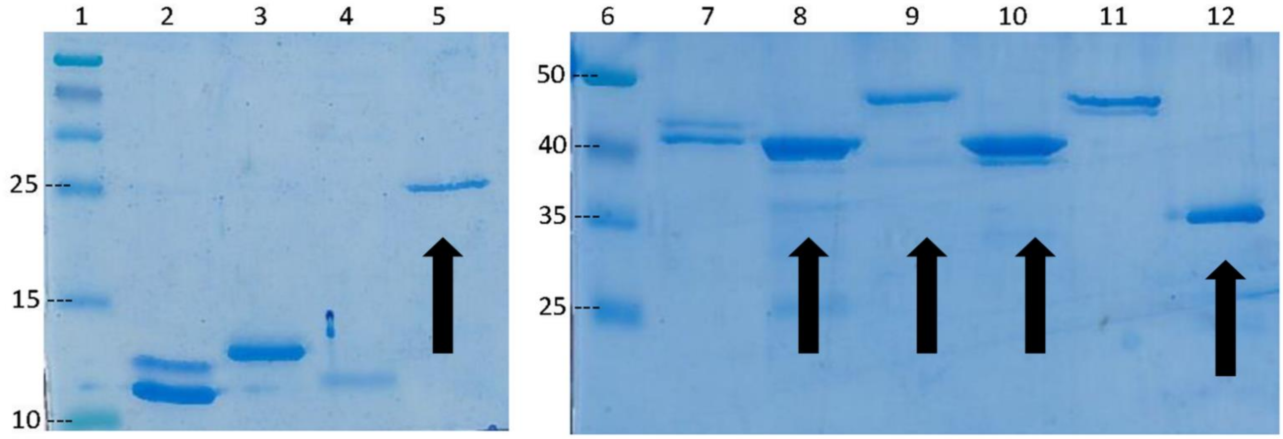


Fig 21. Purified Recombinant Proteins (2.5 µg each) were separated by 15% (left) or 12% (right) SDS-PAGE and stained with PageBlue™. Lane 1, molecular weight markers (kD); 2, acyl carrier protein 1 (ACP 1); 3, acyl carrier protein 2 (ACP 2); 4, acyl carrier protein 3 (ACP 3); 5, ketoreductase A (KRA); 6, molecular weight markers (kD); 7, acyltransferase (acetate) 1 (ATac1); 8, acyltransferase (acetate) 2 (Atac2); 9, acyltransferase (propionate) ATp; 10, enoylreductase (ER); 11, ketosynthase (KS); 12, Ag85A (MUL4987). Antigens selected for immunization highlighted in green and indicated by arrows on the gel.

3.3.4 Protein Dialysis, Lipopolysacharide Removal and Protein Concentration

Using Thermofischer dialysis cassettes, the membranes were Pre-wet with dialysis buffer. The buffer of choice for was either PBS or Ammonium sulphate depending on the extent of protein solubilisation. The most ideal buffer was one which completely solubilised the purified protein. The protein sample was then loaded into the cassette and placed in a flask containing at least 200-fold volume of dialysis buffer solution and dialysed for an hour at room temperature. The buffer was changed after an hour and dialysis continued overnight at a temperature of 4°C. The dialysis was carried out amidst magnetic stirring to augment the process. On completion of dialysis, lipopolysaccharides were removed from the proteins by running the protein solution through Sigma Polymyxin B columns at a slow rate of 1 drop/minute. The run through was performed 8-10 times to ensure complete LPS removal. This was confirmed by performing a LAL test on the eluted sample to ensure the proteins were endotoxin free. Amicon centrifugation filter concentration tubes with molecular weight cut-off of 5KDa were then used to concentrate the solubilised proteins through repeated centrifugation. The resulting purified, dialysed and endotoxin free protein solution was then filter sterilised and stored in tubes at 4°C.

3.3.5 Formulation of Vaccines

All vaccine candidates were formulations of the active ingredients outlined in Tables 5 and 6 solubilised in distilled water. Sterile stocks were prepared for use during entire project for uniformity.

3.4 Immunisation Protocol

Two sets of immunizations were performed. The first set was with various formulations of mycolactone with adjuvants and delivery systems (Table 5) while the second was with the purified polyketide synthase proteins combined with the adjuvant Quil A (Table 6). Each vaccine group was comprised of three C57BL/6J black mice which were immunized subcutaneously with the vaccine of interest. Each mouse was immunized four times in total and at two-week intervals with the maximum vaccine volume administered being 100µl. The mice were eventually culled at week 8 i.e. two weeks after final immunization and tissues (blood and spleen) were harvested for various immunological assays (Fig 22).

GROUP	ANTIGEN	ADJUVANT	ADJUVANT	Number of mice
1		PBS		3
2	0.5µg Mycolactone	100µg YC NaMA	10µg Poly IC	3
3	0.5µg Mycolactone	10µg Poly IC	1*109 Spores	3
4	0.5µg Mycolactone	12.5µg MPL	-	3
5	0.5µg Mycolactone	1.5µg Quil A	-	3
6	0.5µg Mycolactone	-	-	3

Table 5. Mycolactone Immunization Groups and composition.

GROUP	ANTIGEN	ADJUVANT	Number of mice
1	PBS	15µg Quil A	3
2	10µg Ag85A	15µg Quil A	3
3	10µg KRA	15µg Quil A	3
4	10µg ATP	15µg Quil A	3
5	10µg ER	15µg Quil A	3
5	10µg ATAC2	15µg Quil A	3

Table 6. Polyketide Synthase Immunization Groups and composition.

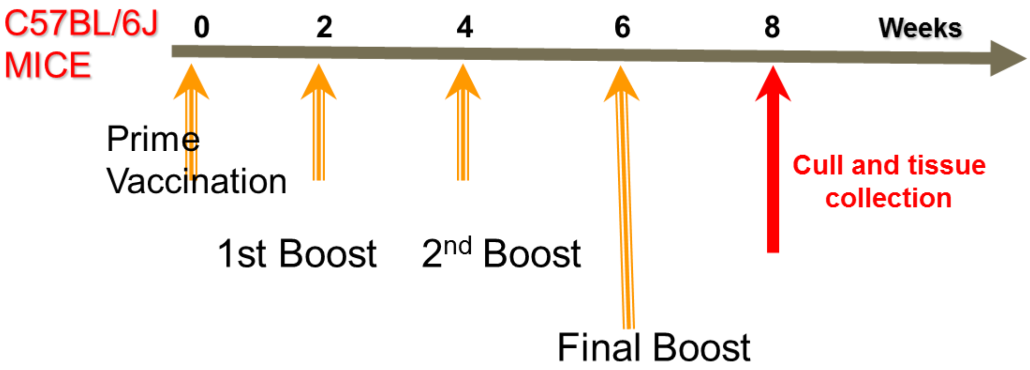


Fig 22. Immunization Schedule. Mice were primed with the vaccine at week 0 and given 3 repeated boosts at two-week interval. They were then culled two weeks after the last boost for tissue/organ harvesting and analysis. Route of immunisation was subcutaneous. There were three mice per group.

3.5 Antibody Detection

ELISA plates (Maxisorb, Thermo Fisher Scientific) were coated with 5µg of corresponding proteins in 100µl of PBS per well overnight at 4°C. The wells were then washed thrice with 200µl of 0.5% PBS Tween-20 per well. The wells were then blocked with 200µl 5%w/v PBS skimmed milk for 2 hours at room temperature. Washing was again performed (3x). Corresponding supernatant samples dissolved in the blocking buffer were then added to the topmost wells with all other wells receiving just the blocking buffer. The topmost wells were then serially diluted downwards at a predetermined dilution factor. The plate was sealed and incubated overnight at 4°C. After repeating the washing step, wells were incubated with a secondary conjugated antibody at 1:1000 dilution in 5% W/V PBS skim milk for 1 hour at room temperature and then washed again (5x). Colour signal was then developed using sigmaFast OPD substrate. The developing signal was read at 450nm by a plate reader (Tecan Infinite 200 Pro) measuring absorbance.

3.6 Mouse Immunisation

All immunisations were carried out via the subcutaneous route. With mycolactone being a fat soluble molecule, the subcutaneous route presents an opportunity to inject it into the fatty layer of the skin, mimicking what happens in human infections. The subcutaneous route is also known to reduce the risk of complications resulting from injecting nerves and blood vessels as there are few within this layer. Using 32G needles, mice immobilised in a rodent restrainer were immunised subcutaneously at the base of the tail. Each mouse received 100µl of various vaccine formulations as detailed in Table 5. Mice were then monitored for at least 30 minutes to ensure they did not develop any acute adverse reactions such as skin irritations, swellings, bleeding, paralysis etc.

3.7 Mouse Infection/Challenge

Local infection challenge with wild type S141 strain of *M. ulcerans* was done by directly inoculating the right footpads of mice. First, the mice were anaesthesized using isofluorine in gas chambers supplied with oxygen. Once fully anaesthesized, animal is placed on tissue towel on the back. Using flat-end clean forceps to stabilise the right foot, a preloaded syringe containing 10^3-10^6 organisms in 0.05 ml PBS is then used to inject inoculum into the middle of the mouse footpads carefully aiming for this at angle of 45 degrees. Injections were slowly carried out to prevent backflow. When properly angulated and well timed, there was no resistance to flow. Success of the inoculation was confirmed visually: the footpad become visibly inflated and there was no backflow. The uninoculated left footpad was used as a control. Each animal was then observed in the cage until it regained conciseness and normal mobility.

3.8 Footpad Measurements

Prior to culling mice, footpads were measured in experiments which involved footpad infections. On the presumption that mouse footpads are of the same height, dimensions of footpad breadth and thickness are taken. These measurements were taken with an electronic Digital Vernier caliper from Visenta. In such experiments, these measurements were taken at various specified stages of the experiment. The trend was to take weekly measurements. To keep mice still during measurements, they were anaesthetised with inhaled isoflurane in a gas chamber.

3.9 Post-Cull Mouse Handling

At the termination of experiments, mice were culled under schedule 1 by the cervical dislocation method. Death was confirmed by femoral artery dissection and severance. Various samples were obtained by various methods described in detail below.

3.9.1 Preparation of footpads for supernatant and culture

After culling mice, footpads were obtained by amputating the foot at the ankle joint. Amputated foot pieces were immediately placed in 4ml homogeniser tubes containing 1.5mls of Phosphate Buffer Saline solution and ceramic homogeniser beads (Part of the Precellys Evolution homogenizer lysing kit). The tubes were placed on ice and moved to the Category three lab for processing. Amputated footpads were mashed up using a homogenizer (Precellys Evolution from bertin Technologies) at a speed of 7500 Rounds per Minute and in three cycles lasting 25 seconds with 90 second breaks between cycles. Using cut tip pipettes, Liquid components of the resulting mash up is extracted. 50µl of this is directly transferred for plating at various dilutions while the rest is filtered through 0.45µm filters. The resulting filter supernatant is stored in liquid nitrogen for later use in various assays. In some instances where experiments were already ongoing, the supernatants are immediately plated for various experimental measurements. These were mostly cytokine ELISA assays. The supernatants were also used in C-reactive Protein assays (ThermoFisher Mouse CRP ELISA Kit).

3.9.2 Serum Collection

After culling the mice, abdominal dissections were performed, and blood samples obtained from the intraperitoneal space by cutting into the abdominal aorta and collecting free flowing blood. Averagely, 2mls of blood is obtained per mouse. Vials containing collected blood are then left overnight on the bench for the blood to clot and for serum to separate. Clotted blood is then centrifuged at a speed of 15000g for 30 minutes and separated serum later is collected for antibody ELISA assays.

3.9.3 Preparation of Spleen Cells

Spleens harvested from immunised mice were transported in RPMI media (supplemented with 100µg/ml Penicillin, 100µg/ml streptomycin, 10% bovine serum albumin and 0.3g/L of L-Glutamine) on ice (Sigma-Aldrich). The spleens were passed through cell strainers to obtain cells which were further incubated for 5 minutes in ACK lysis buffer (Sigma-Aldrich) to lyse the red blood cells in the mixture. The spun down splenocytes were washed twice with the RPMI media remove debris. They were then placed in round bottom 96-well tissue culture plate wells at concentrations of 500,000 cells per well for various assays to be described. Counting was by trypan blue (ThermoFisher) staining.

3.4 *M. ulcerans* Culture Assay

3.4.1 Decontamination of samples before culturing

Prior to culture of *M. ulcerans*, the modified Petroff homogenization and decontamination method is performed to remove other bacteria and fungi. As *Mycobacterium ulcerans* are slow growing bacteria, other microorganisms would be overgrown and inhibits the growth of *M. ulcerans* if no decontamination step is performed. Before handling of diagnostic material the Biosafety hood is allowed to run for 15 minutes. All steps of homogenization and decontamination are performed under sterile conditions under the laminar flow. It is important to strictly follow the incubation periods given in this protocol.

3.4.2 Decontamination

50 ml Falcon tubes are labelled with the mouse ID. Swab and Fine Needle Aspirate (FNA) specimens (still placed in the transport container with PANTA containing medium) are shaken for 5 minutes and the fluid is poured into the labelled 50ml falcon tubes. An equal volume of 4% NaOH is added into the falcon tubes (equal to the volume of specimen/fluid already collected in the falcon tubes). The falcon tubes are shaken for 15 minutes on a shaker, then centrifugation is carried out at 3000rpm for 15 minutes. The falcon tubes must be tightly closed. The supernatant is gently discarded into the waste bottle under the laminar flow. 15 ml of sterile 0.9 % NaCl is added to the falcon tube and subjected again to centrifugation at 3000 rpm for 15 minutes. The falcon tube must be tightly closed! Afterwards the supernatant is gently discarded. 0.5 ml 0.9 % NaCl is added to dissolve the pellets. All reagents were from Sigma-Aldrich.

3.4.3 Culturing *M ulcerans*

One vial of Middlebrook 7H9 Middlebrook media supplemented with OADC (Oleic Albumin Dextrose Catalase) Growth Supplement was used as the medium of choice. Contamination was reduced by supplementing the medium with a mixture of the antimicrobial, PANTA prior to inoculation. PANTA contains polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin, and was available in a lyophilized form. It was prepared with 5 ml of reconstituting fluid. All reagents were from Sigma-Aldrich.

3.4.3.1 Inoculation of liquid culture media

500ml conical tissue culture flasks (with filtered screw cap to allow exchange of gases) containing 100ml of media were then inoculated with stock culture. Flasks were securely placed in plastic containers which were on only partially covered and placed in a 30^{O}C incubator with 5% CO2 atmosphere. As *M ulcerans* is a slow growing microorganism, growth was checked weekly.

3.4.3.2 Culturing on solid medium

0.1ml of culture was transferred to a Middlebrook 7H11 or Loewenstein Jensen medium plate and dispersed using L-shaped disposable spreader. The inoculated plates were then double bagged and incubated at 30°C. Plates were checked weekly for growth of *M. ulcerans*. If no colonies were detected after 6 months, the culture will be reported as negative.

3.5 *M ulcerans* Lysate Processing

To obtain antigen rich solutions for recall assays, Purified Protein Derivative of *M ulcerans* was prepared by lysing live *M ulcerans* bacteria through sonication (Sigma-Aldrich Ultrasonic Processor 500W) and filter sterilising the lysate to ensure no whole bacteria was contained in the lysate. A uniform single stock solution which was used throughout the project was prepared at the onset by sonicating 5 x 109 live *M ulcerans* bacteria in 50mls of Phosphate Buffer Saline. This sample was used throughout the project for uniformity.

3.6 Antigen Recall Assays

Spleens taken out of culled immunized mice were homogenized and placed in wells (In triplicates of 500,000 splenocytes per well). The wells contained 200µl of RPMI media each. 5µg of corresponding antigens were added to the wells and appropriate controls were set up. After 3/5 days of incubation, the cells were spun down and supernatant harvested for various cytokine ELISAs while the pelleted cells were washed for onward flow cytometric analysis. To enhance antigen presentation, DC2.4 Mouse Dendritic cells (20% of splenocyte population per well) from Sigma-Aldrich were added to some splenocyte containing wells.

3.7 Cytotoxicity Assay

In order to demonstrate the cytotoxic effects of mycolactone on various cell lines, cells (500,000 Splenocytes per well, 50,000 J774 cells per well, 50,000 murine embryonic fibroblasts (MEFs) per well and 50,000 L929 fibroblasts per well) were incubated in cell culture plate wells with reducing concentrations of mycolactone (serial dilutions of 1 in 2) in duplicates for 72hrs. Previous pilot experiments had been performed to determine the appropriate cell concentration per well as well as the most appropriate incubation time period. The Splenocytes were incubated in complete RPMI media while J774, MEFs, and L929 cells were incubated in complete DMEM media (Sigma Aldrich). After 72hrs of incubation, cytotoxicity was determined by adding resazurin (1 in 10) to all the wells and measuring fluorescence at excitation wavelength of 540nm and emission wavelength of 580nm after 4-6 hours of incubation with resazurin. Viable cells are able to reduce resazurin which is itself very weakly fluorescent to the pink coloured and highly fluorescent resorufin.

3.7.1 Resazurin Assay (Alamar Blue)

Cells were set up in 96 well flat-bottom plates at a concentration of 1-10 x 10^4/ml in 100µl of complete medium (High glucose DMEM/RPMI + 10% FBS + Antibiotics and L- Glutamine. All from Sigma-Aldrich). The plates were then incubated overnight at 37ºC/5% CO_2. At the end of the incubation period, dilutions of mycolactone in complete medium were added to each well. Stock mycolactone dilutions were twice the desired concentrations such that on adding 100µl per well, the final desired concentration was attained. Control wells without mycolactone were also included. The plates were then incubated over various time periods. Incubation times varied from cell line to cell line and were determined through optimisation experiments performed.

To measure growth/cell death, 20µl of resazurin solution (0.125mg/ml in PBS, filter sterilised, store at 4°C) was added at the end of the incubation period and plates incubated again for 6-24 hours depending on cell type and density. Fluorescence was then measured on a fluorescent plate reader: excitation, 530 nm; emission, 585 nm; filter cut-off, 570 nm. Alternatively, measure of absorbance was also sometimes taken at 600nm for comparison.

3.8 Cytokine Detection Assays

In another arm of this set of experiments, a potent cell proliferation stimulators Lipopolysaccharide (LPS) or phytohemagglutinin was added to the wells already containing splenocytes and serially diluted concentrations of mycolactone. As previously highlighted (Table 1), Mycolactone is believed to suppress the expression of cytokines *in vitro*. This arm of the experiment was aimed at confirming this fact and determining the exact concentrations of mycolactone at which this phenomenon is observed. Following three days of incubation, the plates were centrifuged at 600 RPM and supernatant harvested for various cytokine measuring ELISAs (ThermoFisher scientific kits). With little variations for specific cytokines according to the invitrogen protocol (See appendix) for cytokine ELISAs, ELISAs were performed for various cytokines of interest. Cytokines measured were Interferon gamma, Tumour necrosis factor alpha, Interleukin 10 and Interleukin 17A.

CYTOKINE ELISA ASSAY (ThermoFisher Protocol)

1. Prepare Coating Solution by diluting the **Capture antibody** in **Coating buffer**. Refer to manufacturer for dilution recommendations.

2. Coat plates with 100 µL per well of Coating Solution. Cover plates, and incubate overnight (12–18 hours) at 2–8 °C.

3. Aspirate wells and wash 1 time with >200 µL of **Wash buffer** per well. Following wash, invert and tap on absorbent paper to remove excess liquid.

4. Block plate with 200 µL per well with **Blocking buffer** for 1 hour at room temperature.

5. Aspirate, invert, and tap on absorbent paper to remove excess liquid.

6. Prepare standards and sample dilutions in **Blocking buffer**.
7. Pipette 100 µL of standards (in duplicate) and samples into designated wells. Incubate for 1 hour at room temperature with gentle continual shaking (~500 rpm).
8. Aspirate and wash 5 times with >200 µL of **Wash buffer** per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
9. Prepare detection antibody solution by diluting the **Detection antibody** in **Blocking buffer**. For recommended antibody dilution, refer to manufacturer's instruction.
10. Add 100 µL of the detection antibody solution into each well. Incubate for 2 hours at room temperature with gentle continual shaking (~500 rpm).
11. Aspirate and wash 5 times with >200 µL of **Wash buffer** per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
12. Make working solution of **Streptavidin-HRP** with **Blocking buffer** by diluting 1:5,000. For example, to make enough for 1 plate, add 2 µL of streptavidin-HRP to 9.998 mL of Blocking buffer.
13. Add 100 µL of working streptavidin-HRP solution into each well. Incubate for 30 minutes at room temperature with gentle continual shaking (~500 rpm).
14. Aspirate and wash 5 times with >200 µL of **Wash buffer** per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
15. Add 100 µL of **TMB substrate solution** to each well. Incubate plate for 30 minutes at room temperature.
16. Add 100 µL of **Stop solution** to each well.

17. Measure absorbance at 450 nm within 30 minutes of adding **Stop solution**.

18. Calculate results using a log-log or 4-parameter curve fit.

3.9 Flowcytometry

Mouse splenocytes which had been incubated in round bottom tissue culture plates with corresponding recall antigens were washed in 200ul of PBS three times and re-suspended in 25ul per well of viability dye. The plate was then incubated at 4oC for 20 minutes. Following this, 200ul of FACS buffer (consisting of PBS, 0.5% Bovine Serum Albumin and 0.1% Sodium Azide) was added to each well. The plate was then spun at 400rcf for 5 minutes and supernatant flicked, leaving cells at the bottom of the plate. A mastermix of antibodies was prepared and 25ul of this was added to each well. Control wells with a master mix minus one antibody were also setup for each antibody used. The plate was not incubated at 4ºC for another hour and cells washed with 200ul per well afterwards. Cells were prepared for flowcytometry according to the Biolegend kit procedure for staining cells in u-bottom plate wells. The following antibody-colour panel was used.

Viability Dye: Efluor 780/APC-Cy7
CD3+: AF488
CD4+: Percpcy 5.5
CD8+: BV510
CD45+: RA PE/Cy7
CD69+: PE

3.10 Statistics

Statistical analysis were performed using graphPad Prism 7 and Microsoft Excel 2013. Experiments were carried out in duplicates or triplicates (Stated in describing those experiments). Bars represent averages of repeat experiments and error bars represent standard deviations of the mean. Specific circumstances are described in detail in the results and discussion sections.

CHAPTER 4.

UNDERSTANDING THE IMMUNOLOGY OF MYCOLACTONE AND MYCOLACTONE SYNTHESIZING ENZYMES

4.1 Overview

Mycolatone is the toxic immunopathological moderator molecule secreted by *M ulcerans* (9, 51). Its secretion makes *M ulcerans* unique in that, no other mycobacterial pathogen secretes such (50,66). In this project, we intend to design and test mycolactone based vaccines against Buruli ulcer disease, the disease caused by *M ulcerans*. To make this possible, we first need to understand the activity of mycolactone and determine means to mitigate its immunosuppressive ability in order to design a potent vaccine with it. We therefore went ahead to determine its toxic dose range, its interaction with adjuvants and delivery systems as well as the immune potentials of the polyketide synthase enzymes involved in its synthesis.

4.2 Results

4.2.1 Mycolactone Spectroscopy

To confirm the presence of Mycolactone in the sample from the supplier, a spectroscopic determination of mycolactone was performed. Maximal absorbance peaks were obtained at wavelength of 362nm (Fig 23) in accordance with published values in the literature (90). Ethyl acetate, the solvent of choice for synthetic mycolactone was used as the blank.

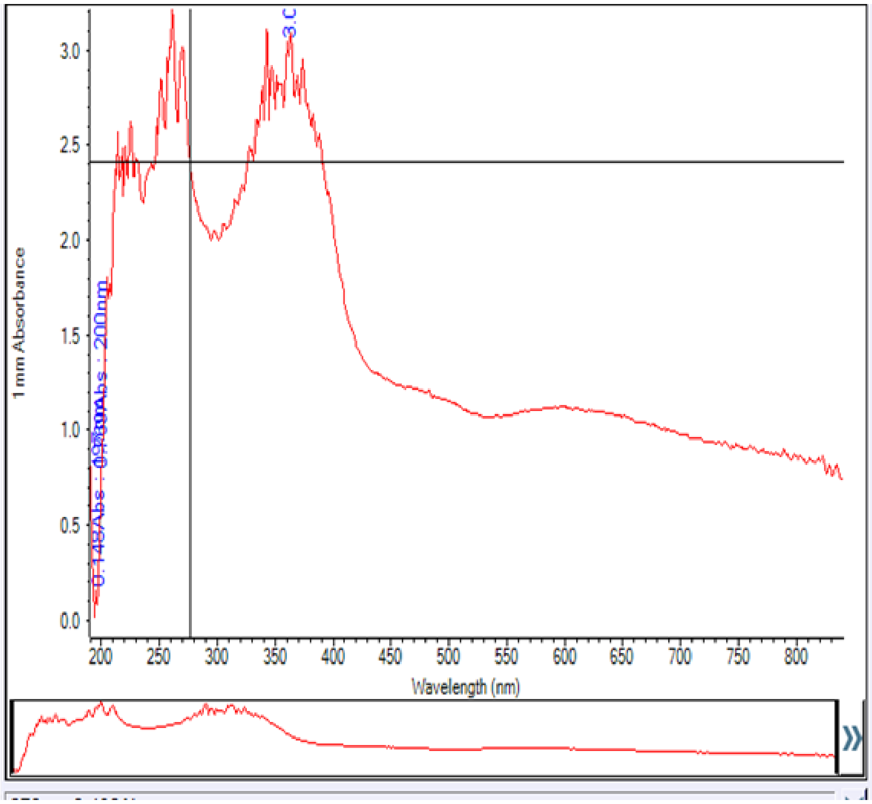


Fig 23. Mycolactone Spectrophotometry Graph. Peak absorbance was at 362nm just as is indicated in literature (90).

4.2.2 Mycolactone Recall Assays

Mycolactone as a toxin is known to be immunosuppressive (51). A key aim of this project is to elicit immunogenic responses to it and to curb its immunosuppressive abilities. If this is achieved, the ultimate goal will be to use it as a vaccine candidate or a component of a conjugate vaccine candidate against BU disease.

To determine this, C57/BL6 mice were immunised with mycolactone delivered in combination with various adjuvants and delivery systems. Spleen cells from immunized mice were harvested and incubated *in vitro* with 5µg of mycolactone in 200µl of media (500,000 cells per well) for three days in one plate and six days in another. To enhance antigen presentation, antigen presenting cells (DC2.4 Mouse dendritic cells from Sigma-Aldrich) were added in combination with mycolactone to a set of wells. This was to enhance mycolactone presentation to specific T cells via well studied CD4+/CD8+ activation mechanisms (187). Harvested media supernatant was then tested for interferon gamma concentrations and pelleted cells were analysed by flow cytometry to measure T-cell proliferation in response to antigenic (mycolactone) recall. The results obtained showed no detectable recall responses. There was no interferon gamma secretion in any of the recall wells even though in the positive control wells, significant levels of the cytokine were detected (Fig 24). This ordinarily means that recall responses to mycolactone were potentially not inducible. We however had to go further to ascertain this with certainty.

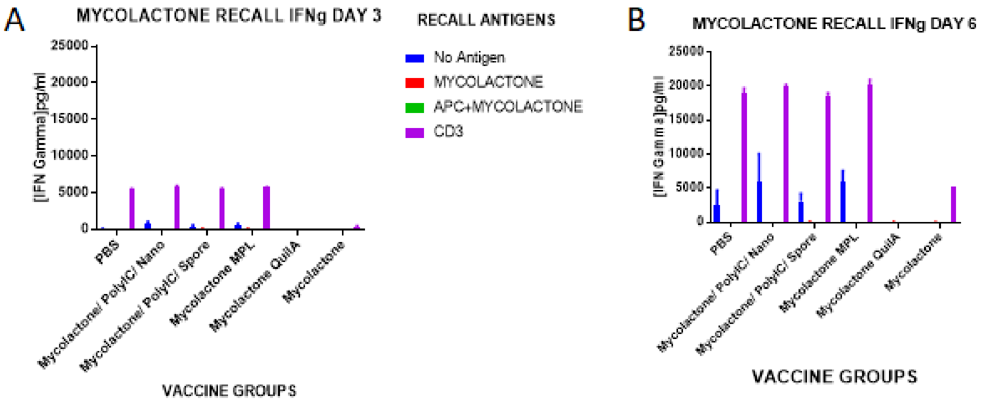


Fig 24. Interferon Gamma secretion by splenocytes of mycolactone immunized mice upon three and six day *in vitro* recall with mycolactone. The colours indicate what the recall material was.i.e. Blank media (PBS), Antigen of interest/immunization (Mycolactone), Mycolactone pulsed with DC2.4 mouse dendritic cells to enhance antigen presentation (APC + Mycolactone) and anti-CD3 which was used as a positive control (CD3). Secreted IFN-γ was measured using a commercial ELISA kit and quantified by interpolating a standard curve. N = 5, error bars indicate Standard Deviation.

However, looking at the various cell counts on flow cytometry (Fig 25), it becomes quite clear that the quality and quantity of spleen cells in the various wells following *in –vitro* –mycolactone recall had been affected by an additive. The percentage of surviving lymphocytes in the wells incubated with mycolactone ranged between 2-30% whereas control wells which had not been incubated with mycolactone recorded up to 80% lymphocyte viability. A further breakdown looking into the details of cell types constituting the already poorly populated live lymphocytes showed that the wells contained relatively fewer live T-cells as compared to corresponding control wells to which mycolactone had not been added. The data showed that even though as a percentage of the few T-cells in the wells, the level of proliferation of both conventional and unconventional T-cells may be markedly different (Fig 25), the absolute cell count in numbers tells a different story. The cell population was too small to determine any significant proliferation. It is therefore not surprising that there was no cytokine secreted in response to the recalls. There were not enough viable cells to secrete the cytokine. This however did not apply in the non-mycolactone containing controls.

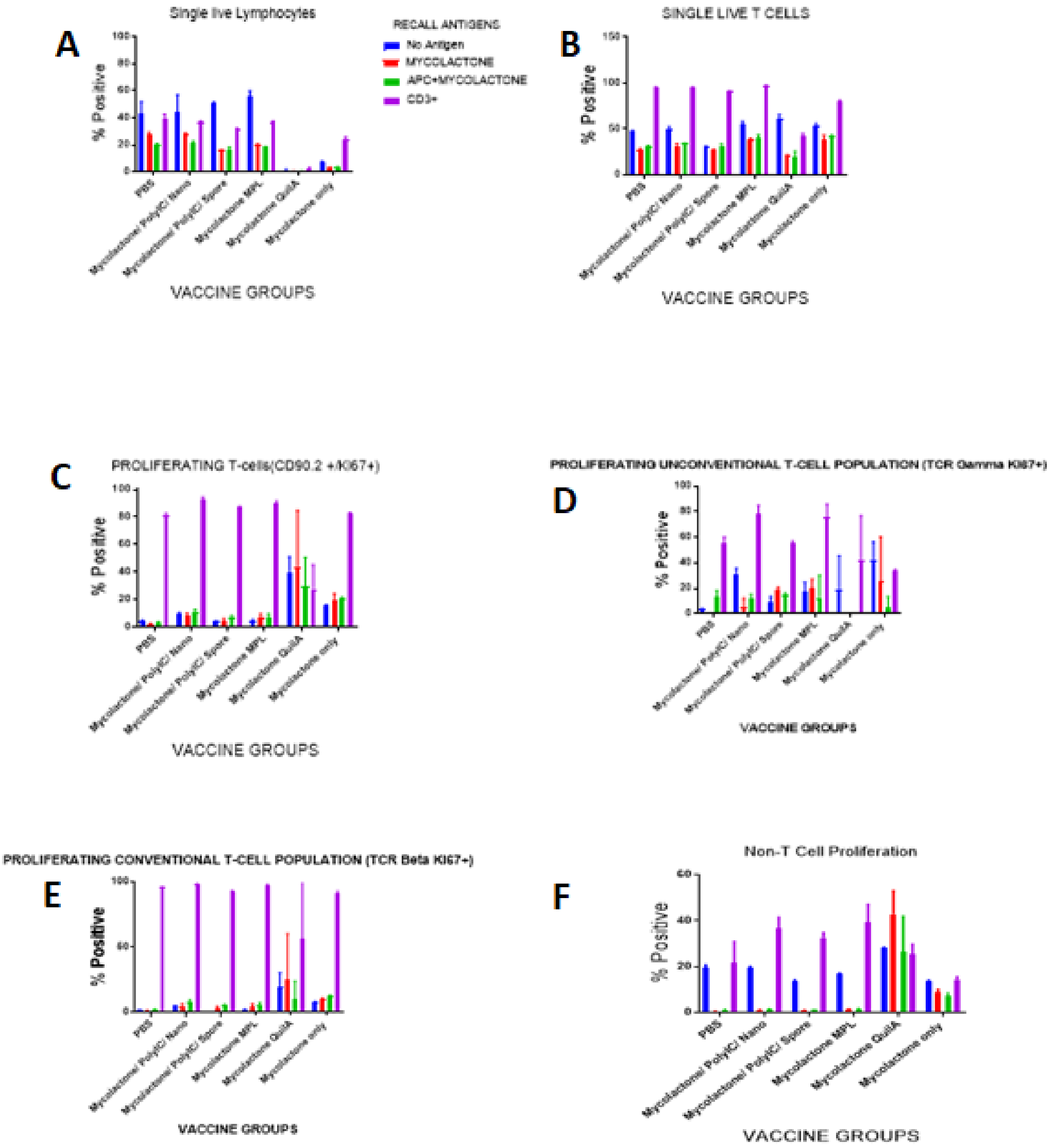


Fig 25. Flow cytometric determination of cell type populations of mycolactone-Immunised mice upon recall with mycolactone. Bars are colour-coded by recall antigen. Efluor 780 was used to monitor cell viability, while-Ki67 was used as a marker for proliferation. Error bars indicate Standard Deviation.

Following on from there, the question of exactly what had accounted for these poor responses had to be answered. Series of experiments were thus carried to confirm the following:

4.2.3 Mycolactone Was Cytotoxic at The Concentrations Used For The *In Vitro* Recall Assays.

In establishing this, various cell types were incubated with increasing concentrations of mycolactone (0-500 ng/ml) for 72 hours and then incubated with resazurin for 4-6 hours to determine cell viability. The selected cell types as mentioned in the methods section were Murine embryonic fibroblasts (MEF), L929 fibroblasts, J774 macrophages and splenocytes from unimmunized C57BL/6J mice. It was observed (Fig 26) that mycolactone was highly toxic to the Murine embryonic fibroblasts even at concentrations as low as 100pg/ml. The cytotoxic potency however varied between cell types as it was found to be significantly cytotoxic to L929 at higher concentrations only. The effect could only be noticed at concentrations of 62.5ng/ml and above. J774 cells were however robust. The cell survival rates were not significantly different from the mycolactone vehicle and PBS controls. The observation was same for spleen cells. It is however worth noting that the highest concentration of mycolactone used in these cytotoxicity assays was 500ng/ml whereas in the mycolactone recall assays, the concentration of mycolactone used was 5μg/ml. This set of experiments therefore confirmed that mycolactone is cytotoxic to various cell types and that this effect is also dose dependent. And that even for the remaining viable cells, their ability to proliferate may have been inhibited by mycolactone.

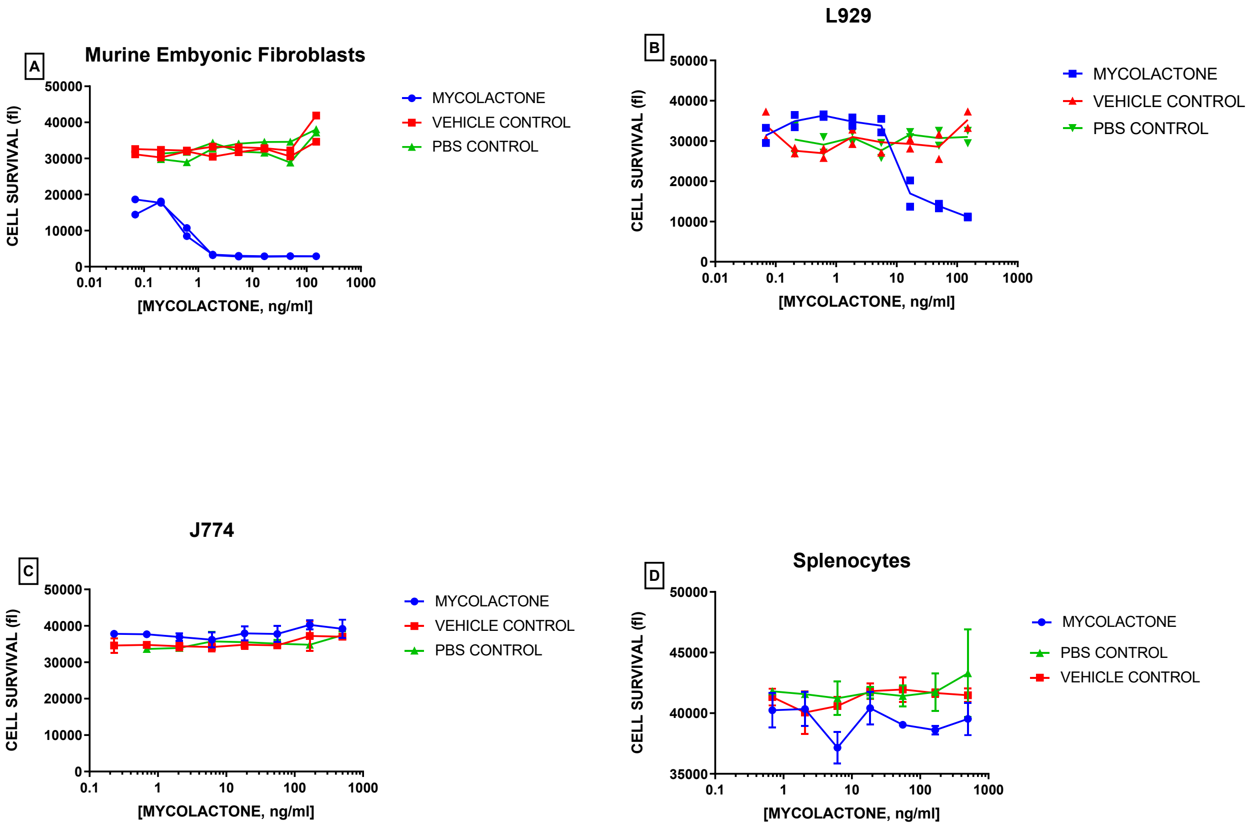


Fig 26 Cytotoxic effect of Mycolactone on cells. Cells after 72hrs of incubation with different concentrations of mycolactone were incubated with Resazurin for 4-6 hours to determine survival. This was measured by fluorescence. There is a direct relation between cell viability and fluorescence (Excitation wavelength of 540nm and Emission wavelength of 580nm). Murine Embryonic Fibroblasts (MEF) cells were the most affected, followed by the L929 fibroblasts. J774 and mouse spleen cells maintained high survival rates. Error bars represent Standard Deviation.

4.2.4 The Observed Cytotoxicity Is Due To Mycolactone, Whether Synthetic Or Natural.

To determine this, cytotoxicity assays as described above were performed using mycolactone obtained from diverse sources. In Fig 27, Mycolactone obtained from Dr Nicholas Blanchard's lab was compared to the synthetic mycolactone we had used in all our experiments. Dr Blanchard prepares synthetic mycolactone by a different enzymatic process (190, 191). From the graphs, it is observed that the cytotoxic activity of both mycolactones were comparable and similar. There was no significant difference even at the various concentrations tests were performed.

In Fig 28, footpad supernatants obtained from homogenised footpads of infected mice were used. This was on the assumption that based on the pathophysiology of buruli ulcer disease and as demonstrated by Sarfo et al (146), footpads of mice suffering buruli ulcer disease would contain mycolactone. The first group of supernatants was obtained from infected footpads of mice. The second group was from the contralateral foot of the directly infected footpad of the mouse herein labelled uninfected footpad. The third group was obtained from control mice which suffered no infection (Untreated). The graph demonstrates that supernatant from the directly infected footpads exerted the strongest cytotoxic ability. This was followed by supernatant from the contralateral footpad which was more cytotoxic than supernatant from untreated footpads. Again, the most significant difference here was the presence or absence of mycolactone and the quantities thereof.

Fig 29 demonstrates the cytotoxic activity of mycolactone obtained from culture filtrate supernatants of wild type (S117) *M ulcerans* in comparison with supernatant obtained from a mycolactone deficient strain of *M ulcerans* a negative control sample. The trend is demonstrated once again in the graph that the mycolactone containing culture supernatant from the wild type *M ulcerans* is found to be more cytotoxic than that obtained from the mycolactone deficient strain which showed indistinguishable cytotoxity to the negative control sample. Measurable cytotoxicity for the wild-type sample is observed beginning at 1% concentration t and increases as the concentration increases.

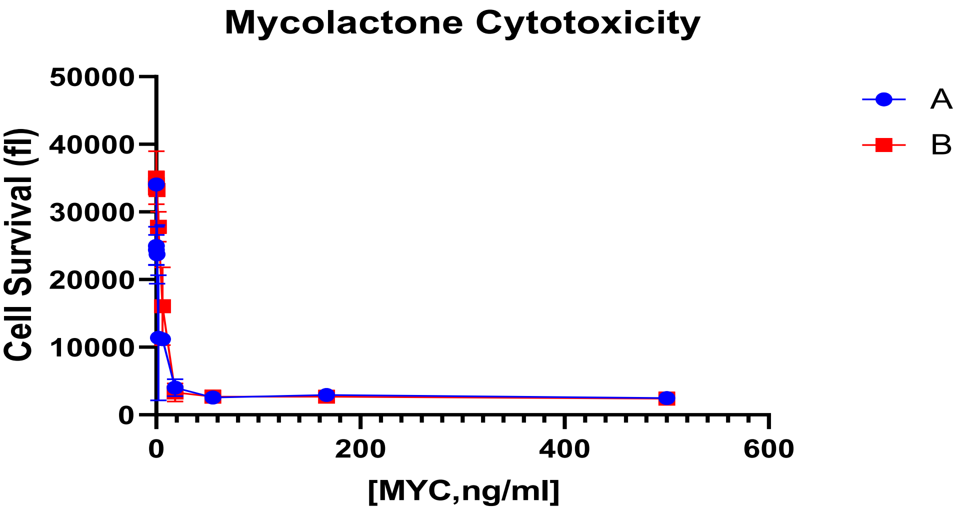


Fig 27. A graph comparing the cytotoxic actions of two synthetic mycolactone samples obtained from different sources. Sample A is the more widely used Mycolactone A/B produced in the Kishi Lab in Harvard University. Sample B is synthetic Mycolactone A/B produced in the Blanchard lab at the University of Strasbourg, France. Both samples exerted comparable cytotoxic effects on Murine Embryonic Fibroblast cells.

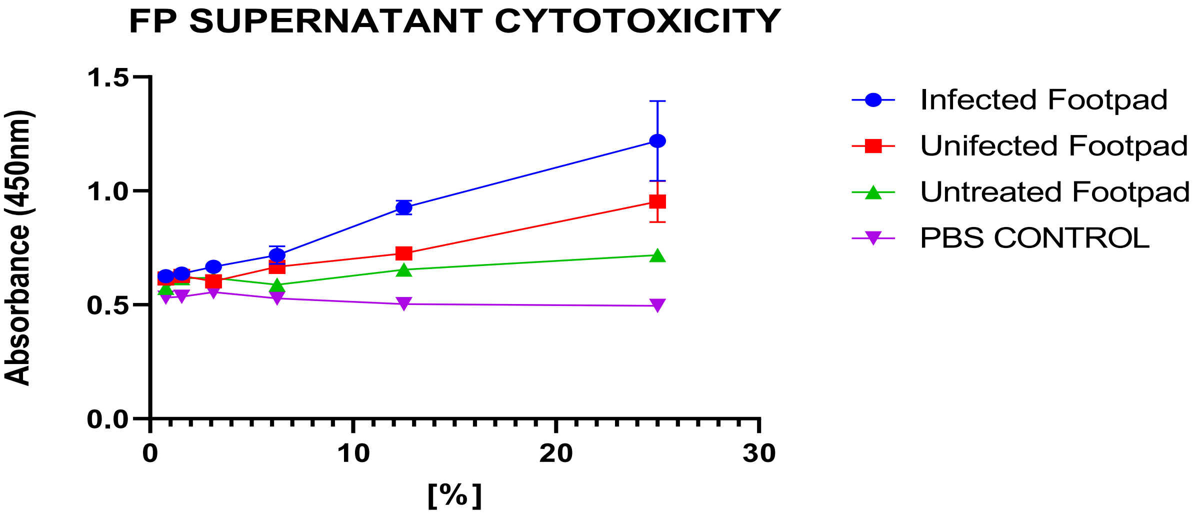


Fig 28 Graph demonstrating the cytotoxicity of mycolactone secreted in natural infection. Samples consist of footpad supernatant obtained from infected footpads, contralateral uninfected footpads from the same mice and footpad supernatant from uninfected mice. PBS was used as a control. Supernatant from infected footpads presumed to contain significant levels of mycolactone demonstrated highest cytotoxic ability. This was followed by supernatant from the contralateral footpads. Sarfo et al in previous work

showed the presence of mycolactone in contralateral footpads of infected mice albeit in reduced quantities (146).

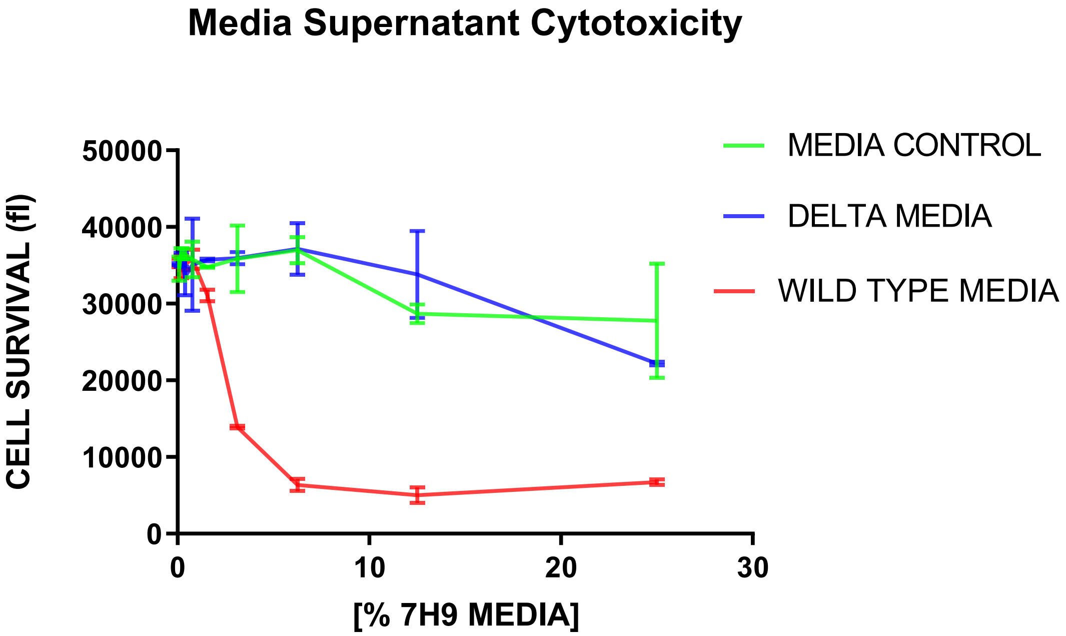


Fig 29. Graph comparing the cytotoxic potential of culture media filtrate obtained from growing wild type *M. ulcerans* (S114) which is known to secrete natural mycolactone A/B in media; Mycolactone-deficient *M ulcerans* which has lost the giant plasmid pMUM001 responsible for the synthesis of mycolactone and so is unable to secrete mycolactone; and a freshly prepared Negative media control. While filtered media from wild type *M ulcerans* demonstrated clearly cytotoxic ability comparable to synthetic mycolactone whereas media from the mycolactone-deficient *M ulcerans* and Negative control showed no difference.

4.2.5 Mycolactone Suppresses Cytokine Secretion In Murine Splenocytes by a Dose Dependent Mechanism.

Upon observing extremely low levels of interferon gamma secretion by the splenocytes of the immunized mice in response to *in vitro* mycolactone recall, it became necessary to establish if this were the case of a true absence of recall memory to mycolactone or a direct dampening/suppressive effect of the mycolactone used as the *in vitro* recall antigen. A setup as described in the methods section (Cytokine detection assays) was used. The data (Fig 30) demonstrated that at mycolactone concentrations 2ng/ml and above, interferon gamma secretion was completely abolished in splenocytes. At a fixed concentration of PHA (10µg/ml), increasing concentrations of mycolactone consistently reduced the amounts of interferon gamma secreted until it was totally abolished from concentrations of 2ng/ml and above. A similar trend is observed in the TNF α arm. However, the minimum concentration at which TNF α secretion was abolished was relatively higher i.e., 18ng/ml.

This confirmed that indeed, mycolactone does have the ability to suppress cytokine secretion and that this effect was also dose dependent.

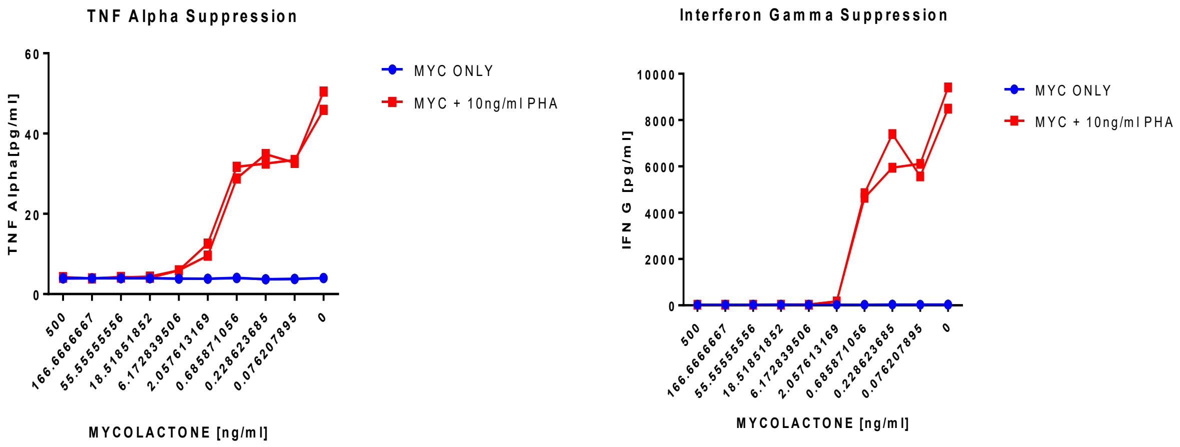


Fig 30. Dose dependent Mycolactone Suppression of cytokine secretion in splenocytes. Phytohemagglutinin (PHA) was used as a positive control. And its ability to stimulate splenocytes to secrete IF- ɣ and TNF α was abolished by mycolactone at specific concentrations i.e., Concentrations of 2ng/ml and above for IFN-ɣ and 18ng/ml and above for TNF α.

4.2.6 Effects of Mycolactone May Be Influenced By Adjuvants

As stated in the introduction, a major theme of this project is to enhance the delivery and recognition interactions between mycolactone as a vaccine and the immune system. Specific adjuvants and delivery systems have thus been selected for this purpose. It was therefore important to evaluate the *in vitro* effects of these adjuvants on cells of the immune system. Spleen cells from naïve mice were therefore incubated with fixed concentrations of lipopolysaccharide (a potent endotoxin for immune cell stimulation) (188), increasing concentrations of mycolactone and various adjuvants in their working concentrations. As already known from previous experiments, mycolactone has a dose-dependent ability to suppress secretion of cytokines. The data (Fig 31) showed that this effect was not reversed by any of the adjuvants apart from YC-NAMA. It enabled cells to secrete significantly elevated levels of interferon even in the presence of mycolactone at concentrations which had abolished interferon gamma secretion in control wells. This will presumably be an advantage *in vitro*. In the Quil A wells however, the observation was that the working concentration of Quil A abolished interferon gamma secretion altogether giving rise to the question of what the exact interaction between Quil A, splenocytes and interferon gamma is. It is known from literature that Quil A is toxic to cells vitro as it lyses them as its main mode of action (189). In vivo, local lysis of cells enhances inflammatory response and contributes to adjuvantic effect. Interferon was selected as a cytokine to measure because of its relative stability in solution over longer periods and its already discussed role in mycobacterial infections (144, 125).

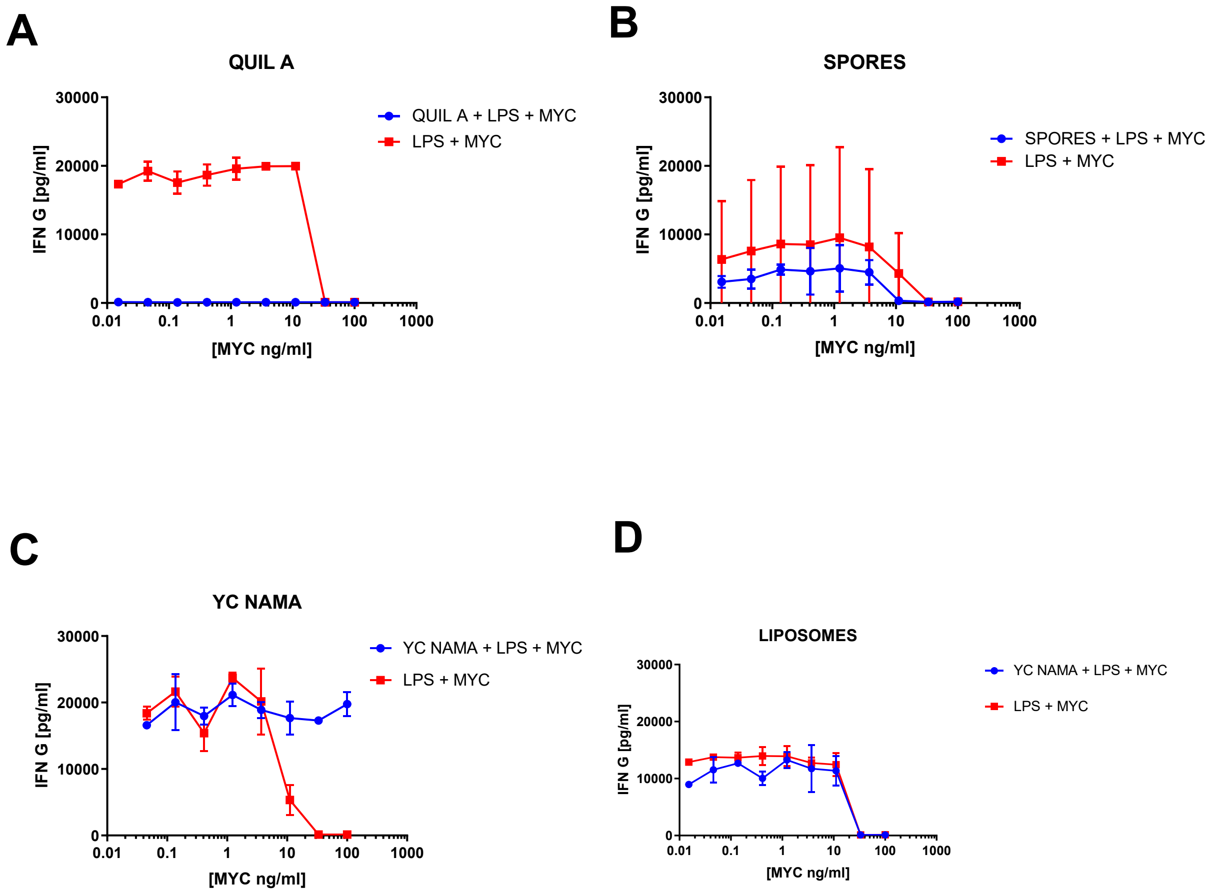


Fig 31. Effect of adjuvants on mycolactone's Interferon gamma suppressive property. Using a setup involving splenocytes, varying concentrations of mycolactone, LPS as a potent stimulator of interferon gamma secretion and the four adjuvants used, it was determined that YC NaMA had the ability to stop mycolactone suppression of IFN-ɣ.

4.2.7 Polyketide Synthase Enzyme Assay Results

Mycolactone synthesis is modulated by polyketide synthase enzymes as discussed in the introduction chapter. As a way of circumventing the toxic and immunosuppressive effects of constituted mycolactone, an aim of this project is experiment with these polyketide synthase enzymes to determine their immunogenicity and eventual vaccine potential.

Going by the protein purification process described in the methods section, protein constructs of some of these synthase enzymes and the recombinant *M ulcerans* specific immunodominant protein Ag85A were made. Of the 12 mycolactone synthase enzymes in its synthesis, 4 were selected in accordance with previous findings. The selected ones were Ketoreductase A(KRA), Acyltransferase propionate (ATP), Acyltransferase acetate 2(ATAC 2) and Enoyl Reductase (ER). Fig 32 shows the Coomasie stain product of the purified proteins and Fig 33 shows the Western blot images of the purified proteins. The proteins corresponded with the known Molecular weights as demonstrated in previous works by Kris Huygen et al (These purified proteins were taken into mouse immunisation experiments and the results are as below).

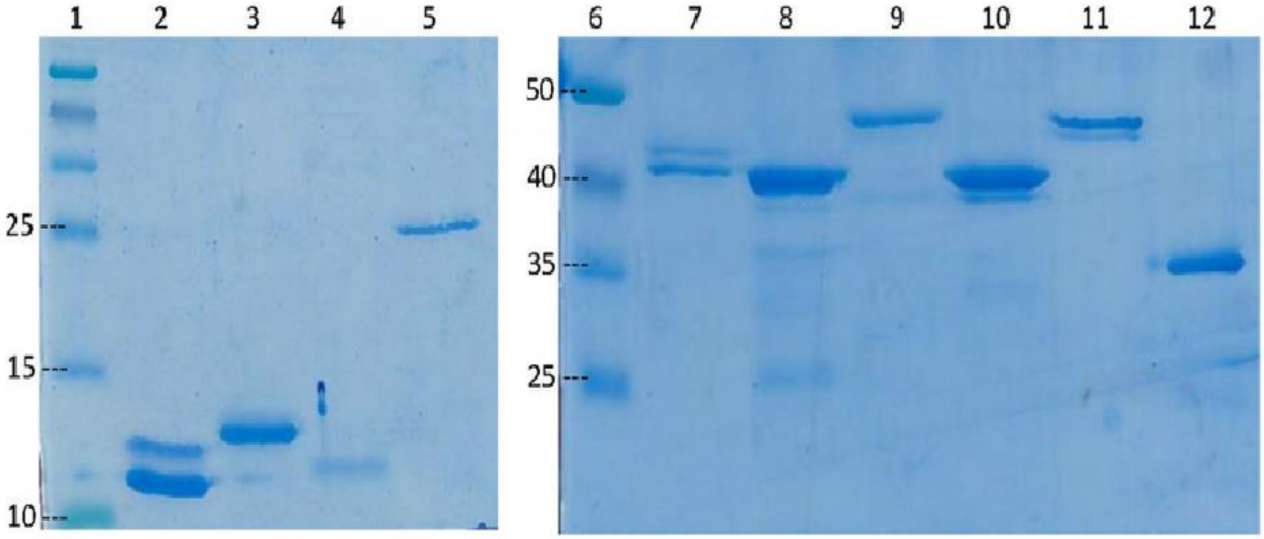


Fig 32 Purified Recombinant Proteins (2.5 µg each) were separated by 15% (left) or 12% (right) SDS-PAGE and stained with PageBlue™. Lane 1, molecular weight markers (kD); 2, acyl carrier protein 1 (ACP 1); 3, acyl carrier protein 2 (ACP 2); 4, acyl carrier protein 3 (ACP 3); 5, ketoreductase A (KRA); 6, molecular weight markers (kD); 7, acyltransferase (acetate) 1 (ATac1); 8, acyltransferase (acetate) 2 (Atac2); 9, acyltransferase (propionate) ATp; 10, enoylreductase (ER); 11, ketosynthase (KS); 12, Ag85A (MUL4987). Antigens selected for immunization highlighted in green.

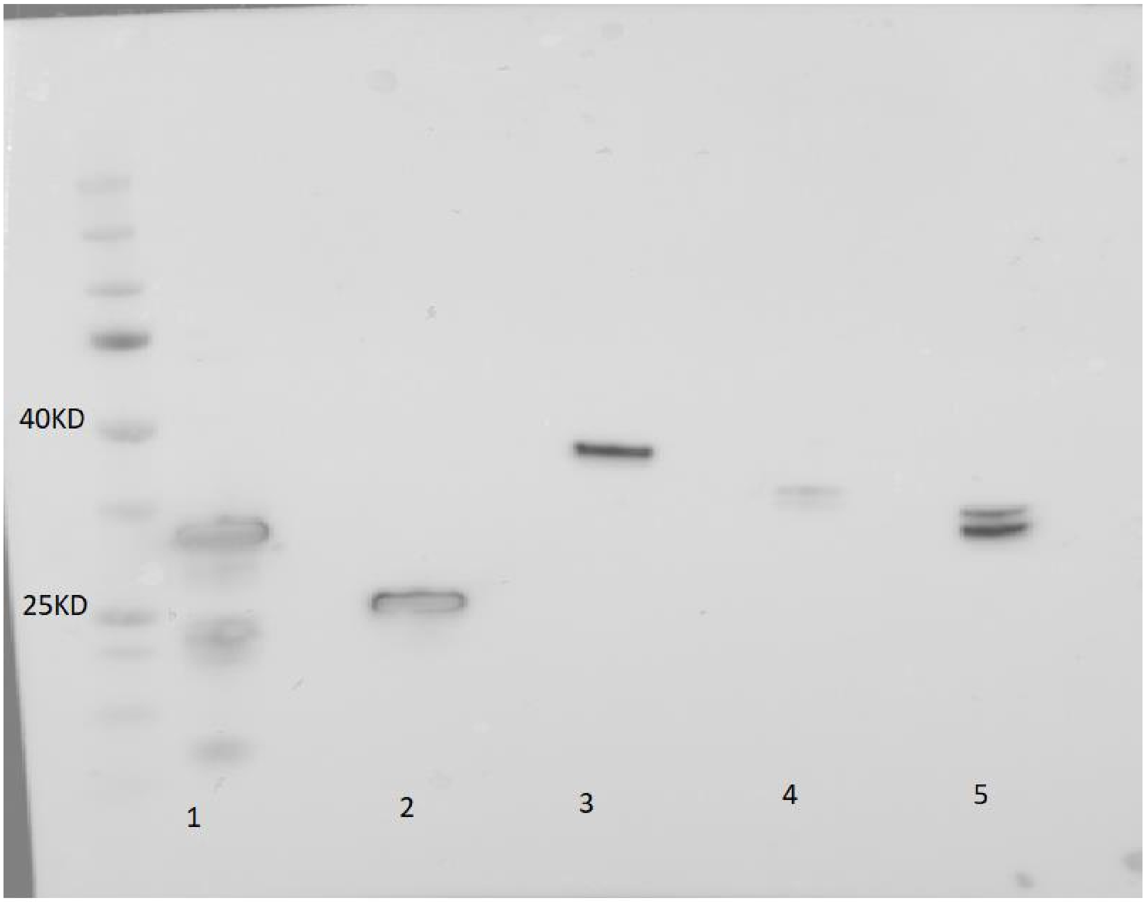


Fig 33. Western blot image of purified Ag85A(35KDa) in Lane 1, Ketoreductase A (25KDa) in Lane 2, Acyltransferase Acetate 2 (40KDa) in lane 3 and Enoyl Reductase (38KDa) in Lane 5. Detection was done with Anti-His antibody tagging. These were the proteins selected for the final set of experiments aimed at vaccine design.

4.2.8 Antibody Responses

As mentioned earlier, mice were immunized subcutaneously at two-week intervals with 10µg of corresponding antigens and adjuvants constituted into a 100µl mixture of various antigens and pre-determined adjuvants for subcutaneous administration. They were immunized with 4 doses each. Serum from immunized mice was used in antibody ELISAs to determine the presence of IgG antibodies in mice. The starting dilution was 1 in 20 and the titrations were done in 1 in 2. Five dilutions were made for the various antigen groups.

Even though the exact correlates of protection against Buruli ulcer disease are yet to be identified, antibodies present an option. In this assay, antibodies specific to the active Mycolactone related antigens were tested for and measured. Mice immunized with Ag85A, KRA and ATP produced antigen-specific antibodies. ER and ATAC2 groups did not show any evidence of the presence of IgG antibodies (Fig 34). The strongest antibody response was detected to Ag85A. In previous work done involving DNA vaccines, antibodies were not produced in response to KRA (65). Therefore, the detection of antibodies to KRA in this assay is novel.

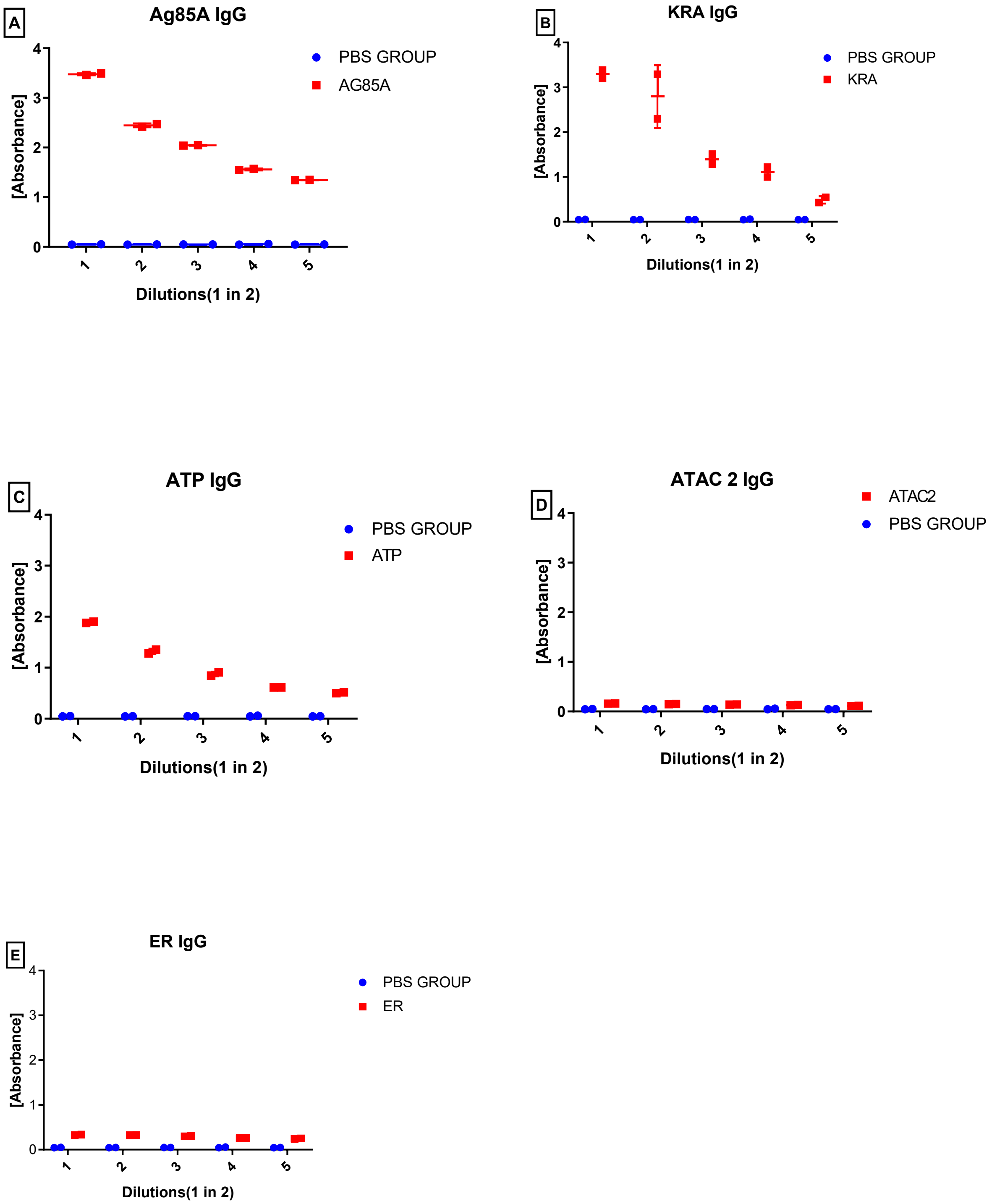


Fig 34. IgG Antibody ELISA responses in sera of mice immunised with various *M ulcerans* antigens of interest. N=5. The best titrating responses was in the Ag85A. KRA produced antibodies too: $ATAC_2$ however gave the poorest response.

4.2.9 Cytokine Responses

As indicated previously, supernatants from wells containing splenocytes (obtained from immunized mice) which had been incubated for three days with the antigen with which the mice had been immunized with, were collected for measurement of cytokines. This was to determine if the splenocytes had recall memory of the antigens and if the recall had stimulated adequate proliferative responses. The cytokines measured were Interferon gamma (IFN-γ), Interleukin 10 and Interleukin 17A. IL-10 was selected for its anti-inflammatory character while the others were selected for their pro-inflammatory nature. Together, they have also been implicated in the immunology of mycobacteria. In all, IFN-γ responses were strongest. This may be due to its chemical stability and a sustained production by cells over days. Even though phytohemagglutinin (PHA) was used as a positive control in this experiment, it proved to be a relatively weak inducer of interferon gamma secretion (66). Ag85A and KRA groups induced higher concentrations of IFN-γ than the PHA controls. The control group antigen was a recombinant protein expressed in *E. coli* and purified by the same system and methods by which the antigens of interest had been produced. This was to allow for subtraction of background *E. coli* (system of expression) effects.

In comparison, (Fig 35) the Ag85A mice splenocytes secreted the highest levels of IFN-γ in response to the recall antigen. This was followed by the KRA and ATP group splenocytes. The levels in the ER and ATAC2 wells were not significantly different from the controls. Interferon gamma secretion in response to recall antigens was therefore poor in the ER and ATAC2 groups. This trend seems to generally have followed through for the other cytokines.

Again, the Ag85A mice splenocytes secreted the highest levels of cytokines in the measurement of IL-10 (Fig 36) and IL-17A (Fig 37). This was closely followed by KRA. $ATAC_2$ wells however had IL-10 levels significantly higher than the controls, in contrast to the results for Interferon gamma. This was however still less than the levels secreted in the KRA and ATP wells. Again, splenocytes of the ER immunized mice did not secrete significant levels of IL-10. The trend follows for IL-17A as well where the levels in the ER labelled wells were not significantly higher than the controls.

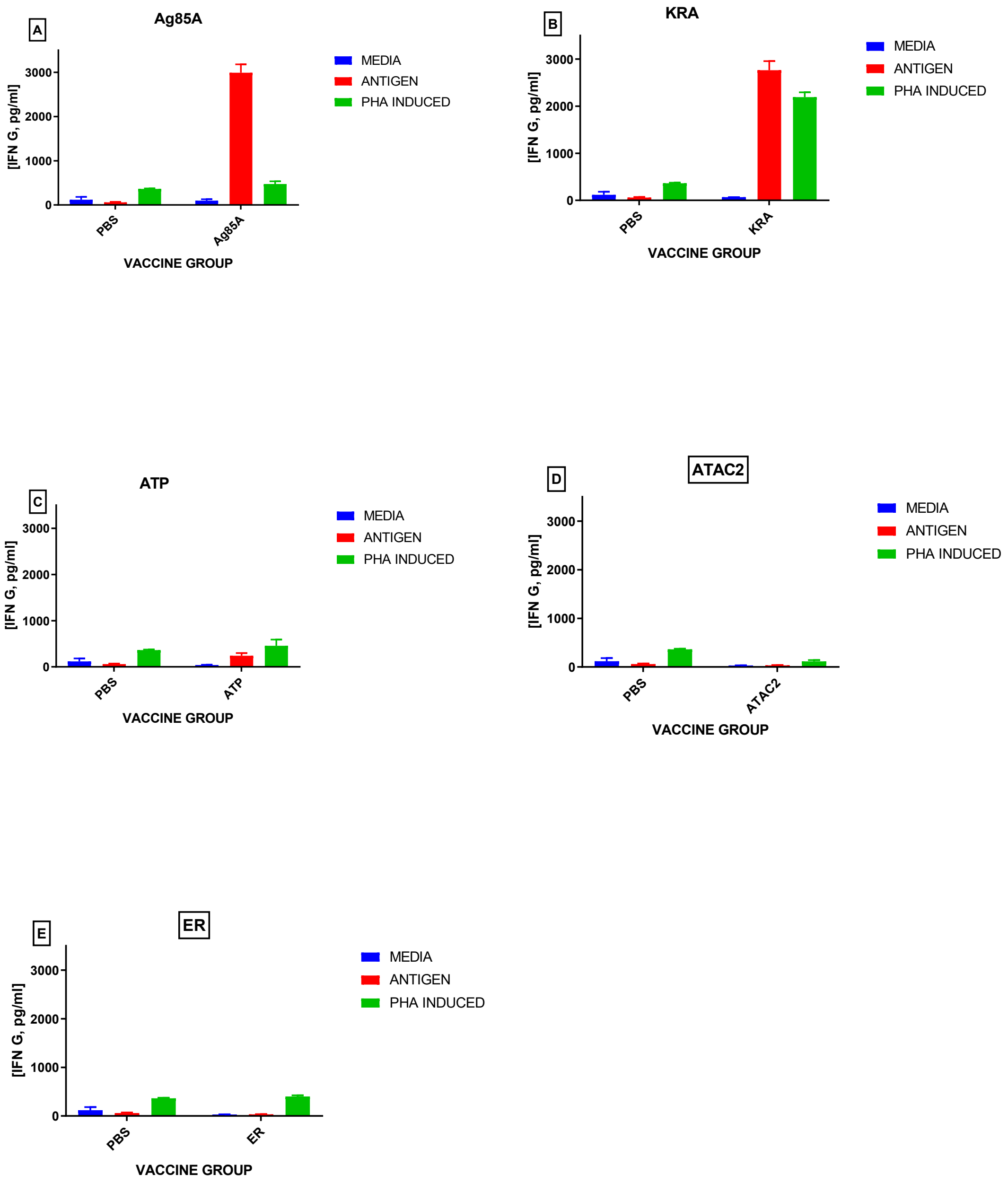


Fig 35. *In vitro* Interferon Gamma secretion by splenocytes of various antigenic mouse groups upon recall with corresponding antigens. The colours indicate what the recall material was.ie Blank media (Negative control), Antigen of interest/immunization and the Phytohemagglutinin (PHA) which was used as a positive control. Error bars represent standard deviation of the mean and bars are means of triplicate wells.

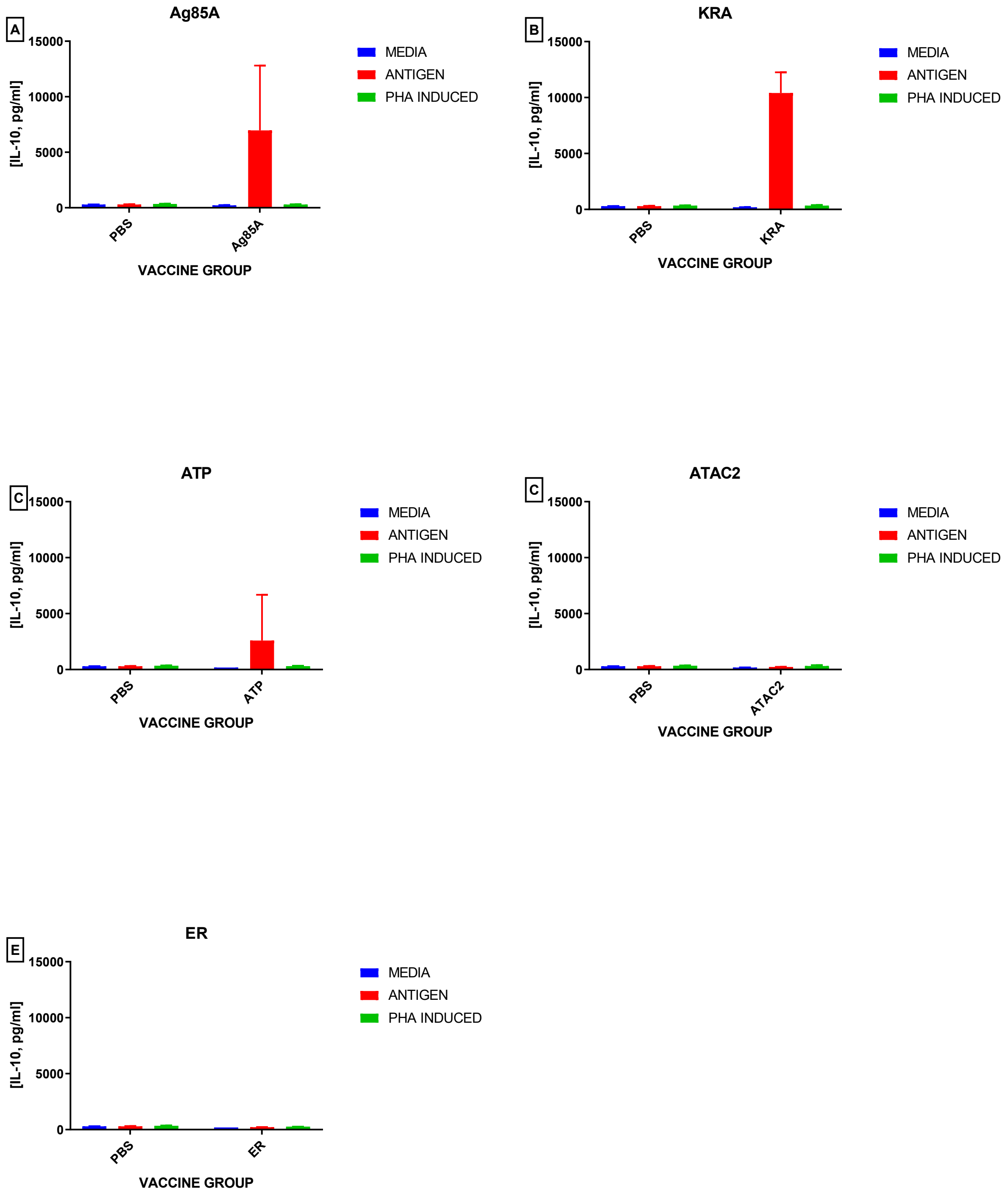


Fig 36. ***In vitro* Interleukin 10 secretion by splenocytes of various antigenic mouse groups upon recall with corresponding antigens.** The colours indicate what the recall material was.ie Blank media (Negative control), Antigen of interest/immunization and the Phytohemagglutinin (PHA) which was used as a positive control. Error bars represent standard deviation of the mean and bars are means of triplicate wells.

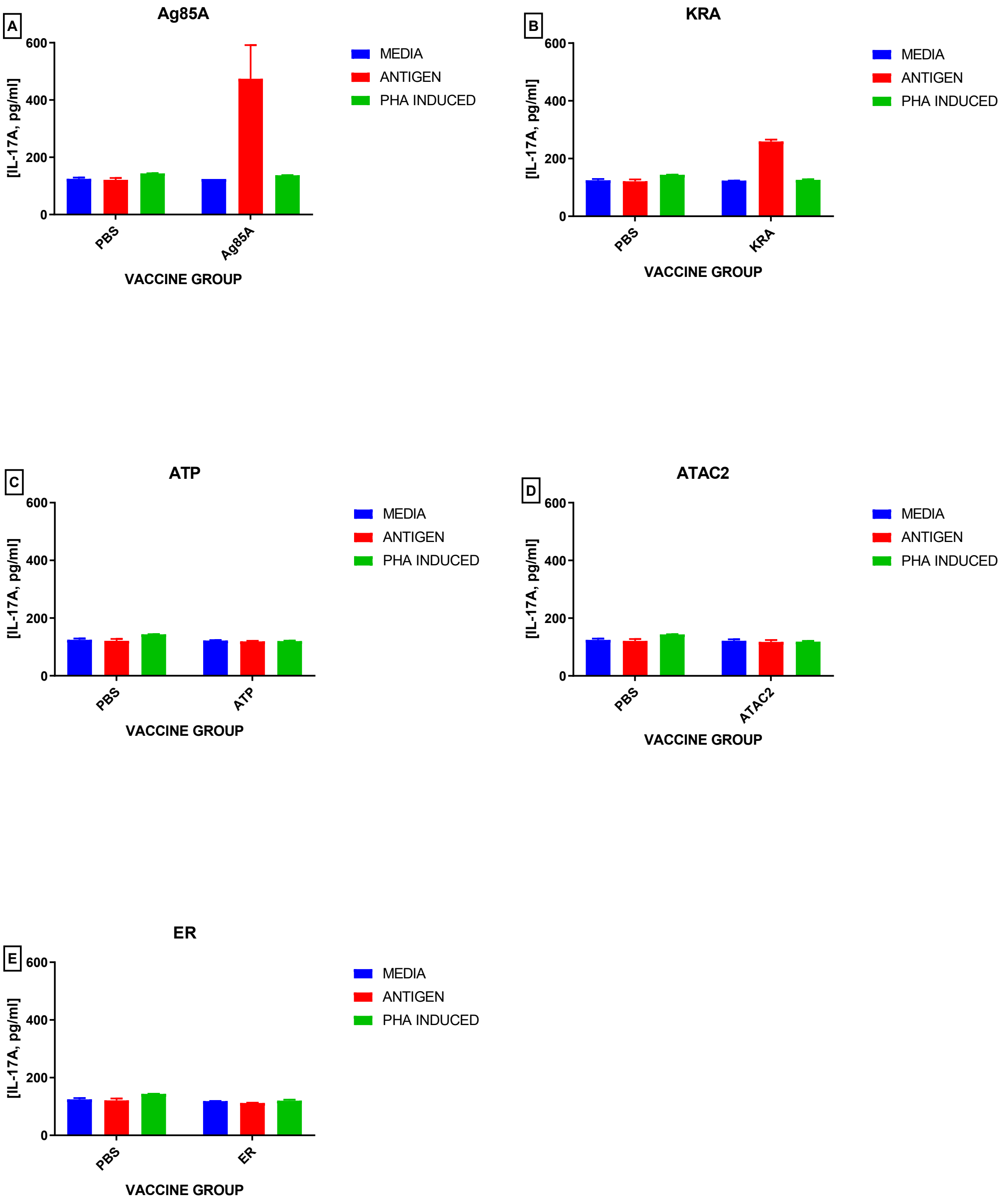


Fig 37. ***In vitro*** **Interleukin-17 Interferon gamma secretion by splenocytes of various antigenic mouse groups upon recall with corresponding antigens.** The colours indicate what the recall material was.ie Blank media (Negative control), Antigen of interest/immunization and the phytohemagglutinin (PHA) which was used as a positive control. Error bars represent standard deviation of the mean and bars are means of triplicate wells.

4.2.10 T- Cell Proliferation in Response to Recall Antigens

To measure CD4+ and CD8+ T-cell proliferation in response to recall with appropriate antigens, the spleen cells which had been incubated for three days with their corresponding antigen were evaluated by flow cytometry. CD4+ T-cell responses were either extremely low or non-existent in the KRA and ER vaccine groups (Fig 38). There however was significant CD4+ T-cell proliferation in the Ag85A, ATP and ATAC2 groups with the highest being for the Ag85A group response, followed by the $ATAC_2$ group. KRA was not a potent inducer of proliferation in CD4+ T-cells. Phytohemagglutinin was used as positive control due to its intrinsic ability to induce cell proliferation and Ki67 was the tissue proliferation marker used.

In measuring CD8+ T-cell proliferation, it was observed that the trend varied and was not the same as occurred with CD4+ cells. CD8+ T-cell proliferation was either very low or non-existent in the KRA, ATP, ER and ATAC2 vaccine groups (Fig 39). Only the Ag85A group splenocytes demonstrated significant CD8+ T-cell proliferation. At this point, it is obvious Ag85A has a potent T-cell stimulating ability. This ability is however non-existent in KRA and ER while ATP and ATAC2 retain partial potency.

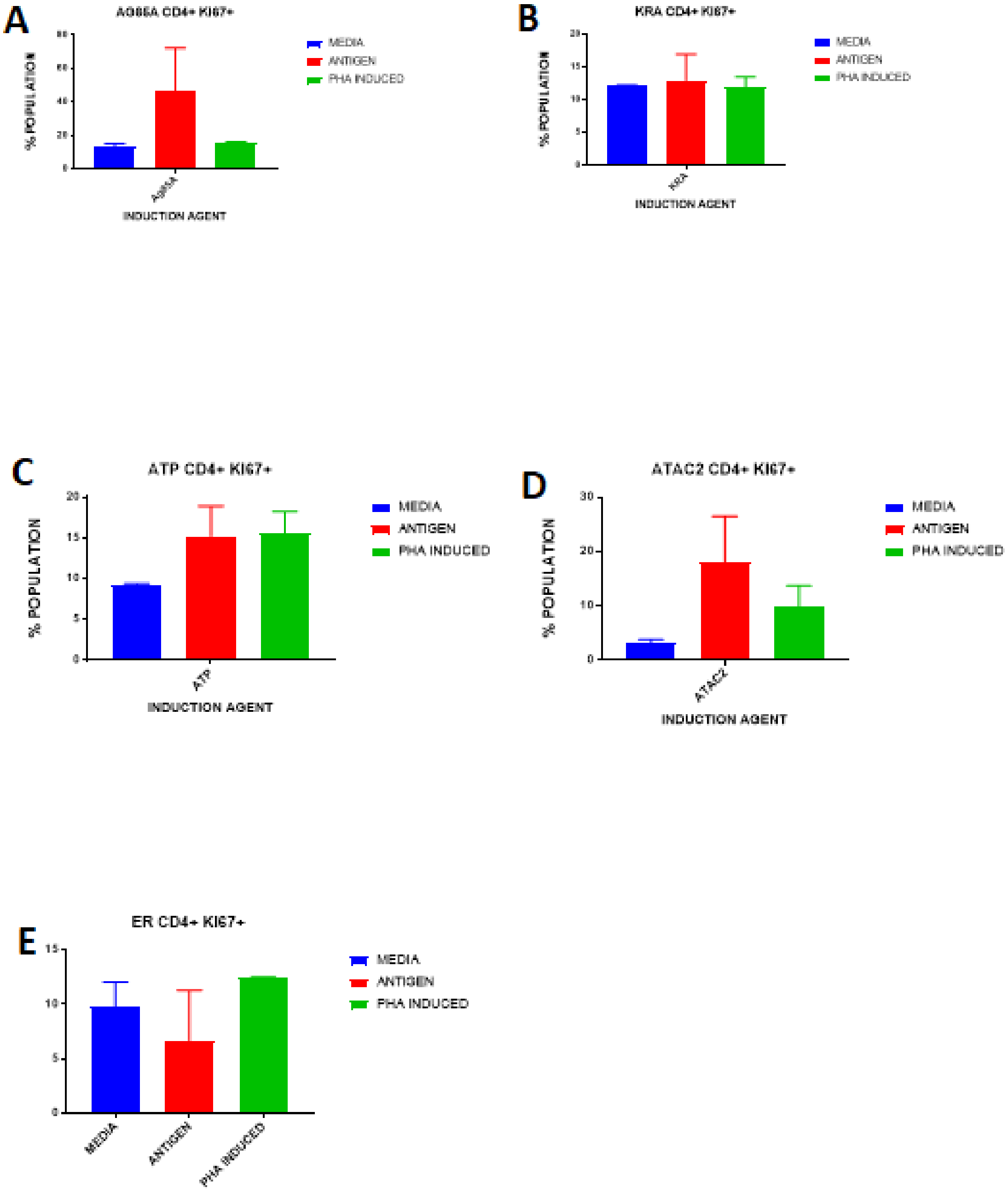


Fig 38. Flow cytometric determination of CD4+ T-cell proliferation in splenocytes of antigenic mouse groups in response to recall antigens *in vitro*. The induction agent represents the antigenic group. The colours represent the recall the recall material i.e. Plain media for blue, the antigen of immunization for red and the PHA positive control for green. Columns indicate percentage of CD4+ T-cells expressing Ki67 which was the tissue proliferation marker used. Splenocytes were obtained from the pooling of spleens from 5 mice in each group. Error bars represent standard deviation of the mean and bars are means of triplicate wells.

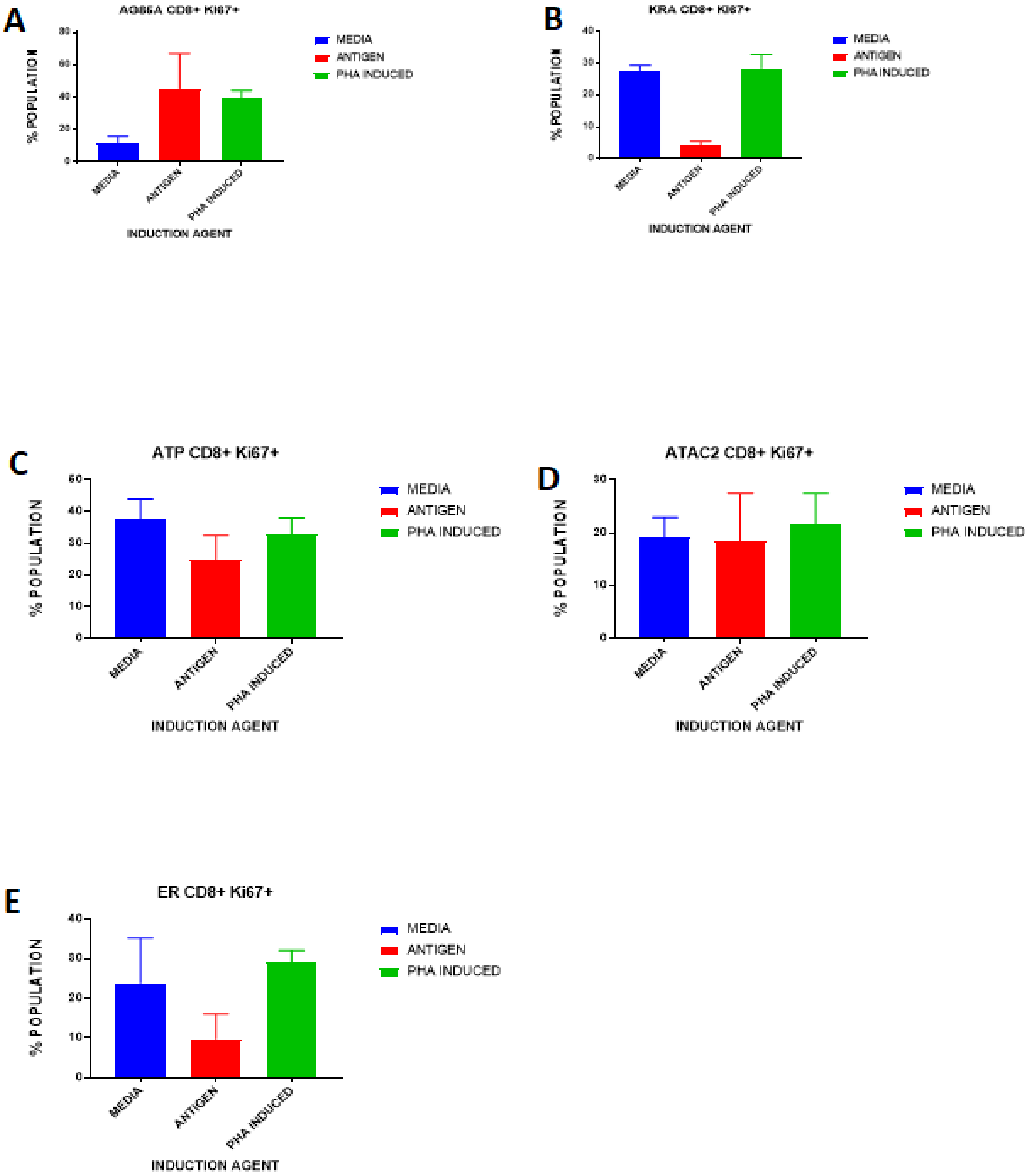


Fig 39. Flow cytometric determination of CD8+ T-cell proliferation in splenocytes of antigenic mouse groups in response to recall antigens *in vitro*. The induction agent represents the antigenic group. The colours represent the recall the recall material i.e. Plain media for blue, the antigen of immunization for red and the PHA positive control for green. Columns indicate percentage of CD8+ T-cells expressing Ki67 which was the tissue proliferation marker used. Splenocytes were obtained from the pooling of spleens from 5 mice in each group. Error bars represent standard deviation of the mean and bars are means of triplicate wells.

4.3 Discussion

This chapter focused on elucidating the immunogenicity of synthetic mycolactone, the toxin produced by *M ulcerans* and its synthase enzyme proteins. Its effects on cell lines have been comprehensively studied (51) in the past but we sought to specifically determine if these effects could be circumvented for the purposes of making mycolactone immunogenic and designing a vaccine with it. Specific assays to determine the exact extent and mechanism of its cytotoxic and immunosuppressive potential were designed to demonstrate this.

So, firstly, mice were immunized with mycolactone combined with various adjuvants and delivery systems designed to enhance antigen presentation to the immune system. Knowing the established role of interferon gamma in the pathogenesis of mycobacteria (124, 145), we sought to measure the levels produced in response to the recall of mycolactone following exposure of splenocytes of mice immunized to it. From the initial results, there was no interferon gamma secretion after incubation for 3 and 5 days. On flow cytometric analysis however, the viable cell counts had significantly gone down (Fig 25). There had been significant cytotoxicity with some wells having close to no viable cells at all. The range was between 0-25% viable cells. So even though the low levels of interferon gamma could be explained by the absence of viable cells in those wells, there was no explanation for the wells which had up to 20% viable cells as these cells ought to have been able to secrete IFN-γ. High cytotoxicity outcome could imply that even surviving cells may be moribund and thus dysfunctional. Thus, the key outcome is the demonstration of the effect of mycolactone on cell viability.

A potential explanation could however be that mycolactone at the used concentration was also suppressing cytokine secretion in the viable cells. This was proven in the experiment to determine the exact minimum concentration of mycolactone required for this suppression. From the experiment, we find in Fig 30 that interferon gamma secretion is inhibited at concentrations above 2ng/ml. Suppression of TNF-α started at concentrations of 18ng/ml. Mycolactone at these concentrations did not kill the cells, but significantly inhibited the secretion of various cytokines. From this, it was clear that at the 5µg of mycolactone added to 200µl of media, mycolactone had induced significant cytotoxicity and even the surviving viable cells could not have secreted any cytokines due to the very potent suppressive effect of mycolactone at such high concentrations. It is also noteworthy that the cytokine suppressive effect of mycolactone occurs at different concentrations for different cytokines. There is therefore not a one-for all minimal concentration of inhibition.

In order to determine the exact concentration we ought to have used to avoid killing the spleen cells, we set up cytotoxicity assays using rising concentrations of mycolactone with the highest concentration being 500ng/ml. This was chosen bearing in mind the fact that average mycolactone levels measured from tissue samples taken from infected lesions do not exceed 500ng/ml (51, 146). From the experiments, it was found that mycolactone was very toxic to Murine embryonic fibroblasts (MEFS) and L929 cell lines even at low doses. J774 macrophage cells and spleen cells were however resistant to mycolactone toxicity even at concentrations of 500ng/ml for 3 days (Fig 26). We potentially exposed the spleen cells in the mycolactone recall to exceedingly high concentrations: hence the cytotoxicity. However, knowing now that we do not have to exceed 10ng/ml if we are to measure interferon gamma secretions and also that mycolactone concentrations do not exceed 500ng/ml in real patients, it is a progressive step to know that spleen cells are capable of resisting mycolactone induced cytotoxicity at such concentrations for at least three days.

It is worthy of note that even though we use synthetic mycolactone in these experiments, the uniformity in the immunosuppressive potential of mycolactone obtained from various sources (both natural and synthetic) has been demonstrated in Figures 27, 28 and 29. Synthetic mycolactone obtained from two different labs, which use different methods of synthesis, demonstrated comparable cytotoxic abilities. In this project, we use mycolactone produced by Prof Kishi's lab. Natural mycolactone contained in footpad supernatant obtained from infected footpads demonstrated higher cytotoxic ability than that obtained from uninfected contralateral footpads. Supernatant from the contralateral footpad also demonstrated higher cytotoxicity than supernatant obtained from footpads of naïve mice and PBS. The inference being that mycolactone concentrations are significantly higher in the local region of infection but may seep in lower doses to other regions of the body such as the contralateral uninfected foot. Therefore, though visibly local as an infection, the immunosuppressive consequence of Buruli ulcer disease as mediated by the toxin mycolactone is potentially systemic. We also went further to demonstrate the absence of mycolactone in secretions from the mycolactone deficient delta strain of *M ulcerans*. Media filtrate obtained from cultures of Delta did not show any significantly different cytotoxic potential from negative control media whereas media from wild type S114 demonstrated high cytotoxic potential comparable to that of synthetic mycolactone (Fig 29). We have considered the use of Delta as a prime whole bacteria vaccine against Buruli ulcer disease to be boosted with mycolactone based candidates. This therefore paves way for its use in this regard as we will not be causing any active infection in mice with the loss of its mycolactone secreting ability, which according to literature is key in the pathogenesis of Buruli ulcer disease (9, 147, 148).

Following successful purification of the synthase enzymes, mice were immunized with these proteins in combination with adjuvants and recall experiments were conducted to determine the immunogenicity of these proteins. Selected readouts were for IgG antibody responses, TNF alpha, IFN-γ, Interleukin 17, and Interleukin 10.

Of the five proteins selected, interferon gamma responses were significantly high for three: Ag85A, KRA and ATP. Appreciably significant levels of TNF α were also secreted by the spleen cells on recall with corresponding antigens. In the measurement of IL-17 however, recall response in the ATP group was poor. Ag85A and KRA maintained elevated levels of IL-17 secretion.
IgG antibody ELISAs were significantly positive for Ag85A, KRA and ATP. This was performed *in vitro* with serum obtained from immunized mice and incubated with the corresponding antigen of interest. There were no IgG antibodies to ATAC2 and ER. We report that IgG antibodies to KRA are novel since in previous works where DNA vaccines were used, no antibodies were produced. (86)

As with other mycobacterial diseases, it is still unclear what the immune correlates of protection against Buruli ulcer disease are. However, taking a cue from *M tuberculosis* which is a closely related organism, one would expect interferon gamma to play a significant role in protection. Unfortunately, the role of interferon gamma in TB infection and disease is still far from well understood (141). We are therefore unable to tell exactly which of our measured parameters is most significant and indicative of protection at this stage. Following the results of the *M. ulcerans* challenge study in mice to be discussed in another chapter, we will be able to take a retrospective look at the data to determine what our best correlates of protection are and their extent of significance.

Domain	T-Cell Proliferation (CD4+/CD8+)		IFN-γ	IL-10	IL-17A	IgG	Protection
Ag85A	+	+	+	+	+	+	?
ATac2	+	-	+	-	-	-	?
Atp	+	-	+	+	-	+	?
ER	-	-	+	-	-	-	?
KR A	-	-	+	+	+	+	?

Table 7. Summary of results from the polyketide synthase enzyme recombinant protein vaccine experiments.

The main difficulty we face in eliciting mycolactone-directed immunity stems from the fact that it is immunosuppressive and by virtue of its mode of action, cells of the immune system which are expected to play a role in the immune response are rendered either dysfunctional or ineffective (87). Thus, a way by which mycolactone can be presented to the immune system while rendering it non-immunosuppressive will enhance its vaccine potential. This is what we sought to achieve with adjuvants and delivery systems and optimisation of mycolactone doses. The adjuvants were to enhance mycolactone delivery and presentation and reverse its immunosuppressive and cytotoxic activities.

When mouse spleen cells were incubated with increasing concentrations of mycolactone combined with the various adjuvants and delivery systems used in this work i.e. Quil A, Liposomes, YC-NaMA and Spores for 72 hours in the presence of fixed concentrations of lipopolysaccharides (potent endotoxin for immune cell stimulation), the levels of interferon gamma secreted as mycolactone concentrations rose were either too low to be identified or possibly zero. This was no different from the control wells which had just mycolactone and LPS. Considering that mycolactone is already known to suppress LPS activity, this was expected. It is significant to note however that in the wells containing mycolactone, YC-NaMA and LPS, this inhibitory/suppressive effect of mycolactone was abolished. The levels of interferon gamma secreted remained appreciably high even at otherwise intolerably high concentrations of mycolactone. This is a novelty. By this result, we now know that YC-NaMA has the ability to reverse some aspects of the *in vitro* immunosuppressive abilities of mycolactone; a feature this project seeks to overcome. The exact mechanism by which this is achieved is still yet to be understood. It may be due to a chelating effect of YC-NaMA on mycolactone. Its surface potentially binds mycolactone thereby removing it from circulation and in effect, reducing its activity. Another possible explanation could be the role of myristic acid. YC-NaMA is known to contain myristic acid which is known to have the ability to bind to C-abl and C-src: two molecules implicated in the mechanism of action of mycolactone (50, 95, 149, 150). Future work in this project will seek to explore this in much more detail.

YC NaMA is an adjuvant of Nanoparticles Sciences, made of yellow carnauba wax. It has high colloidal stability and has been used in vaccine work involving Tuberculosis (151) and Human Immunodeficiency Virus (HIV) (152). It has a wide surface area and is capable of attracting molecule unto its surface for enhanced antigenic presentation. Even though we did not investigate the exact mechanism by which YC-NAMA reverses the immunosuppressive activity of mycolactone, it is encouraging to note that mycolactone can be presented to the immune system without altering its structure yet being able to overcome its immunosuppressive, cytopathic and cytotoxic effects on cells of the immune system. This opens doors for further experimentation with other adjuvants and delivery systems acknowledging that their in-vitro and in-vivo effects may vary.

4.4 Conclusion

In concluding this chapter, we demonstrated that mycolactone is immunosuppressive and toxic but that this can be reversed under certain circumstances. We now know that its presentation can be enhanced with adequate abolishing of its undesired immunosuppressive effects for the purpose of vaccine design and subsequent applications. Furthermore, we demonstrated that mycolactone-synthesizing enzymes vary in their own immunogenicity, inducing diverse antibody and cytokine profile. Going forward, we therefore seek to exploit these findings in designing and testing our vaccine formulations.

CHAPTER 5.

ESTABLISHING AND UNDERSTANDING THE MOUSE FOOTPAD MODEL OF *M ulcerans* INFECTION.

5.1 Introduction

Mycobacterium ulcerans occurs in nature as a human pathogen. Though infections in animals have been reported, these have not been linked to any significant morbidity/mortality (165). In a bid to unearth the mode of transmission, many animals especially aquatic ones have been tested by PCR methods for the presence of *M ulcerans*. It has thus been confirmed in literature that a number of these animals do serve as reservoirs for the pathogen (Fig 5) and that in testing positive for *M ulcerans*, these animals did not necessarily bear Buruli ulcer lesions (166). Naturally occurring lesions of buruli ulcer disease have so far only been reported in Koalas (*Phascolarctos cinereus*), Australian Possums (*Pseudocheirus peregrinus* and *Trichosurus vulpecula*) and in two domestic animals (goat and dog) in Benin, West-Africa. An active case search and testing in Cameroon found no Buruli ulcer lesions in tested domestic animals. To confirm this phenomenon of the presence of Buruli ulcer lesions in domestic animals, tests conducted in Australia on animals found to have skin lesions came positive in dogs, cats, alpacas and horses. Just as is most common in humans, these lesions also occurred especially on the extremities of the animals involved. There is however no evidence of animal to human transmission or vice versa (153 -157).

Historically, animal models have been used as a proxy to study and understand human diseases. Buruli ulcer disease is no exception. The first ever publication on Buruli ulcer disease included chapters on animal experimentation following identification and description of lesions in humans

(1). In that paper, rats, mice and guinea pigs were involved. As the bacteria causing the infection had at the time not been isolated, these experimental animals were injected with mashed-up tissues and exudates from patient lesions with the hope of inoculating the animals with the causative organisms. For the short duration of the study, none of the animals showed any evidence of disease. One of the marked experimental rats which was abandoned and not culled after the study developed swelling in its limbs and tail. Further examination of tissue from infected animal parts revealed abundant Acid-Fast Bacilli under microscopy. This was the first documented case of Buruli ulcer disease in animals. Following on from this occurrence, rats, mice, Guinea pigs, lizards and a fowl have been used as experimental animals in Buruli ulcer disease research (10,66). Mice have been the most frequently used model as they are the most susceptible to *M ulcerans* infections. The first animal studies in experimenting on immunity against *M ulcerans* infections were carried out by Fenner in mice (98, 109). Repeated by other experimenters, his findings have subsequently been confirmed in humans. A significant amount of what is known about pathology, immunology, and treatment of *M ulcerans* infection is from observations made in animal models (especially mice) some of which have subsequently been confirmed in humans (158).

As a proxy to understanding the vaccine dynamics, underlying pathology and trend of disease progression in *M ulcerans* infection, we set up mouse footpad infection models of Buruli ulcer disease in this this project. This was to answer specific questions aimed at ultimately designing experiments with our vaccine candidates.

Therefore, the specific aims in this chapter are:

1. To establish the mouse footpad model of *M ulcerans* infection.
2. To determine the inoculation dose dependent variations in the footpad infection model.

3. To elicit and understand the immunological features of the mouse footpad infection model.

5.2 Experimental Approach in Brief

C57BLJ/6J mice purchased at week 8 were infected with varying doses of *M ulcerans.* Two strains of *M ulcerans* were used: Wildtype S114 and Mycolactone deficient (Also called Delta) strain 5143. Infections were carried out by inoculating mouse footpads with the bacteria. Four mouse groups each containing 5 mice were inoculated with 10^3, 10^4, 10^5, and 10^6 bacteria of wild type *M ulcerans* S114. One group of 5 mice was infected with the mycolactone deficient strain for observation. Inoculations were to the right footpad with the left footpads used as controls. Inoculation/Challenge was carried out on mice after administering anaesthesia.

Observations of swelling were conducted both visually and by measurement of footpad size using electronic digital Vernier Callipers. Footpad measurements were taken weekly. Humane end point for mice was set at footpad swelling of 4.5mm or development of ulceration in infected mice in accordance with Biological Research Facility and Home Animal Welfare Guidelines. Animals on reaching humane end-point were culled by Schedule 1 methods (Dislocation of neck). Mice from different groups were culled at different time points throughout the 7-week period when they reached the humane end-point. The last batch of surviving mice were culled by week 7. All infections studies were carried out in Category 3 laboratories and animal housing.

Tissues were harvested from some culled mice for further experimentation. These included amputated footpads, blood for serum and spleens. Amputated footpads were processed by running them through a tissue homogenizer (as described in the methods chapter) to obtain homogenised samples for culturing and cytokine measurements. Collected blood samples were processed to obtain serum for antibody assays. Splenocytes were extracted from collected spleens for recall assays and flowcytometric analysis of proliferation. For recall assays and antibody ELISAs, a rich protein derivative of *M ulcerans* lysate was prepared by sonicating whole bacteria and filter sterilising the derivative. This provided a rich and broad-spectrum source of *M ulcerans* antigens.

5.3 Results

5.3.1 Physical Observations in Mice Infected with *M ulcerans*

On weekly mouse observation visits, swellings in challenged footpads were visible in some groups by Day 7 (Fig 40). Though physically visible, animals walked with a normal gait and significant morbidity had been observed in the affected animals. Footpad swelling was observed in mice infected with 10^6 bacteria. The footpad looked reddened but without any ulceration. By week two, mice in this group had reached the humane endpoint by either developing visible ulcers or reaching swelling 4.5mm in any of the measured dimensions described in detail in the Materials and Methods chapter. There was no swelling in any of the other groups. Weekly follow-up of mice continued unabated. However, mice infected with 10^6 of Delta (Mycolactone deficient *M ulcerans*) did not develop any visible or measurable swelling all through the 7-week follow up period. There was no physically observed difference between the inoculated right footpads and their contralateral left control footpads. This group was included to confirm the previously acknowledged non-virulence of Delta. With the intention of using Delta as a priming vaccine candidate, its non-virulence needed to be demonstrated in our animal model.

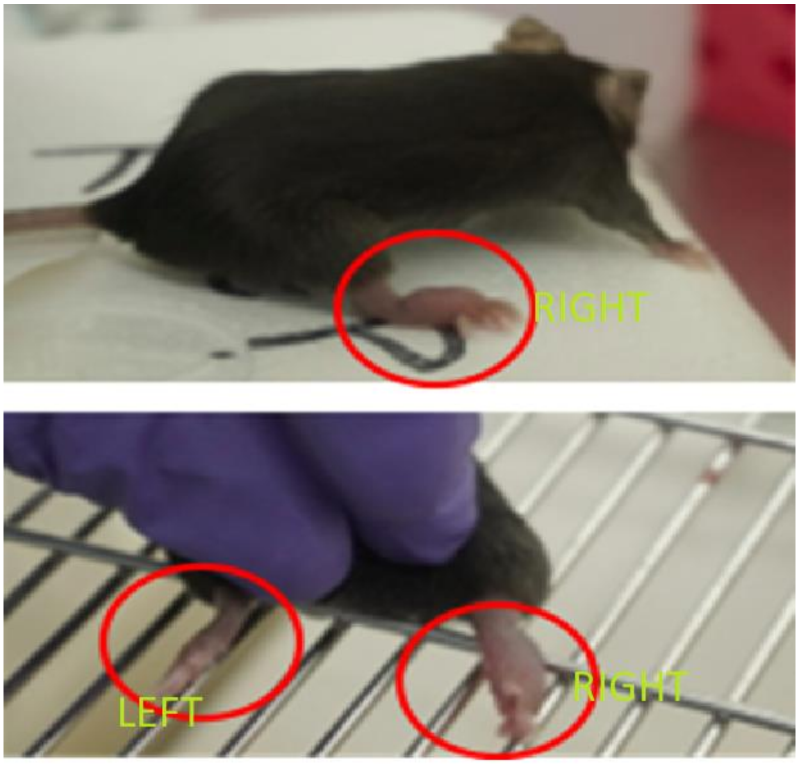


Fig 40. Swelling in right footpad of a mouse in the 10^6 group at week 1. Left footpad used as a control showed no change. Mice walked without a limp and exhibited no physical signs of unwellness.

Over the 7 week follow up period as demonstrated by the graphs in Fig 41, all mouse groups infected with wild type S114 strain *M ulcerans* developed swelling in the corresponding footpads. No contralateral control footpad developed any swelling or exhibited any change in physical appearance. Increase in infected footpad size was first observed in mice in the 10^6 group. With a mean percentage change in footpad size of 68% by week one, mice in this group were the only ones to have developed swelling this early. No other group had developed swelling. By week two, the mean change in footpad size had risen to 75% with all animals in this group reaching the humane endpoint. Measurements could therefore not be taken beyond week 2 as mice had to be culled after measurements. Except for mice in the 10^5 group which developed swelling the size close to that measured in the 10^6 group albeit at a later time point, no other animals reached humane endpoints throughout the 7-week study period.

By week 2, swelling was observed in mice in the 10^5 and 10^4 groups. Though all individual mice in the 10^5 group had developed measurable swelling and averaged about 40% in percentage swelling, swelling could only be seen in some mice footpads in the 10^4 group and average percentage swelling was only about 5% in that group. Mice in the 10^3 group had still not developed any swelling.

By week 3, all mice in the 10^3 group had developed footpad swelling and even though all mice in the 10^4 group had by this time developed swelling too, the average percentage swelling for mice in the 10^3 group was higher (40%) than mice in the 10^4 group (15%). Average swelling in the 10^5 group also increased from 40% the previous week to about 75%. Groups started to lose individual mice from this week onwards as some reached the humane endpoint by either developing ulcers or footpad swellings exceeding 4.5mm in any of the measured dimension.

Throughout the 7-week period, the peak average percentage swelling was seen at week 3 in the 10^5 (78%) group followed by the 75% seen at week 2 in the 10^6 group.

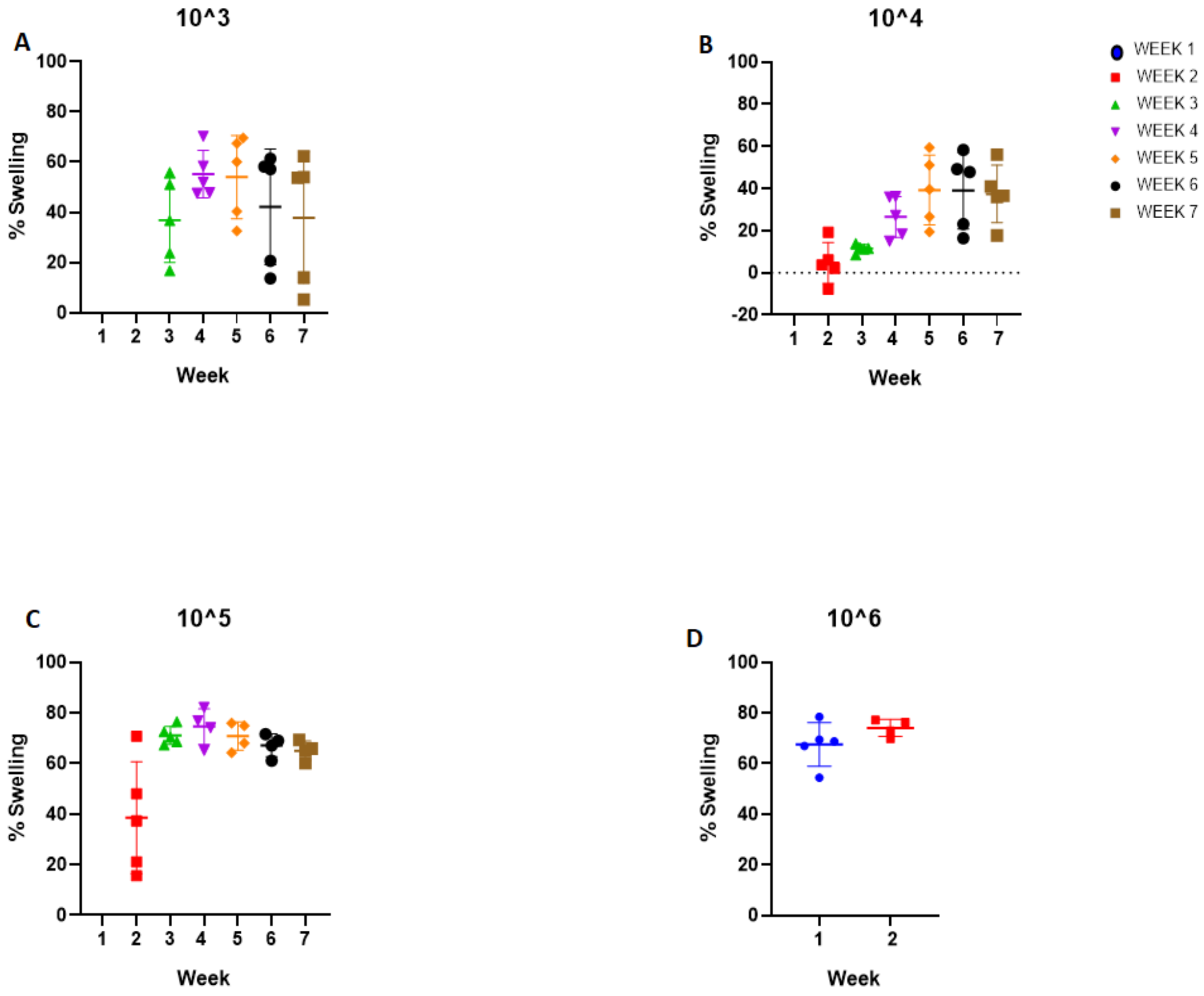


Fig. 41 Graphs of percentage swelling in infected footpads of mice inoculated with 10^3, 10^4, 10^5 and 10^6 *M ulcerans* bacteria and measured weekly over a period of 7 weeks. N=5. Error bars represent SD

Fig 42 describes the same data set reordered to show groups on the same charts. Even though the onset of swelling is in the order of increasing inoculated bacterial dose with the earliest onset seen after one week in the 10^6 group and the most delayed seen in the 10^3 group at week 3, the extent of rise in footpad size is independent of this. Mice in group 10^6 had developed swelling by the end of week 1 and even though mice in both groups 10^5 (40%) and 10^4 (5%) had developed swelling by week 2, the average right footpad size was much bigger in the 10^5 group. And despite the late onset of swelling in mice in the 10^3 group, percentage change in footpad size as first measured at week 3 was more than double that of the 10^4 group. ie. 15% for the 10^4 group and 39% for the 10^3 group.

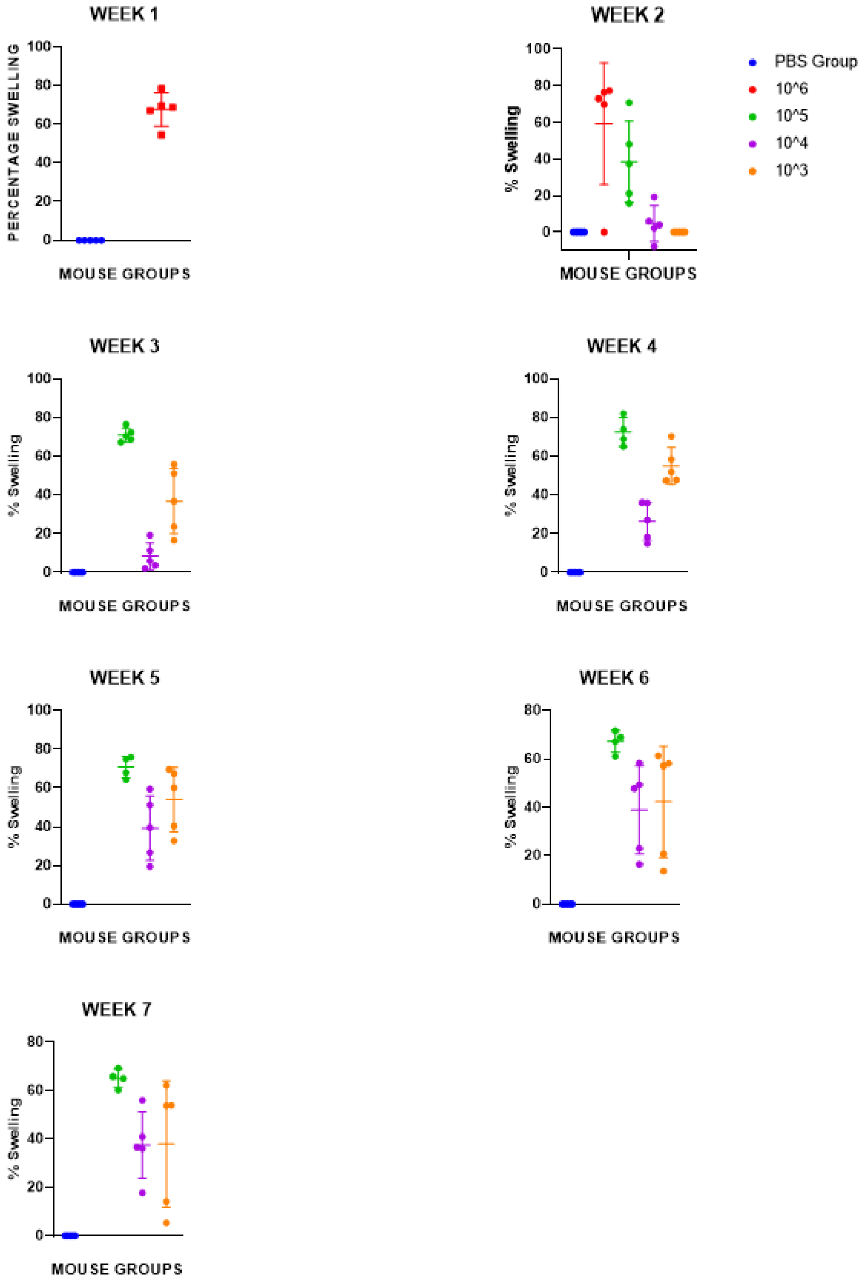


Fig 42. Weekly measurement of Percentage change in infected footpad size in mice inoculated with PBS (Control), 10^3, 10^4, 10^5 and 10^6 *M ulcerans* over a 7-week period. Measurements were taken from the point of physical swelling as observed visually until animals reached the stipulated humane endpoint.

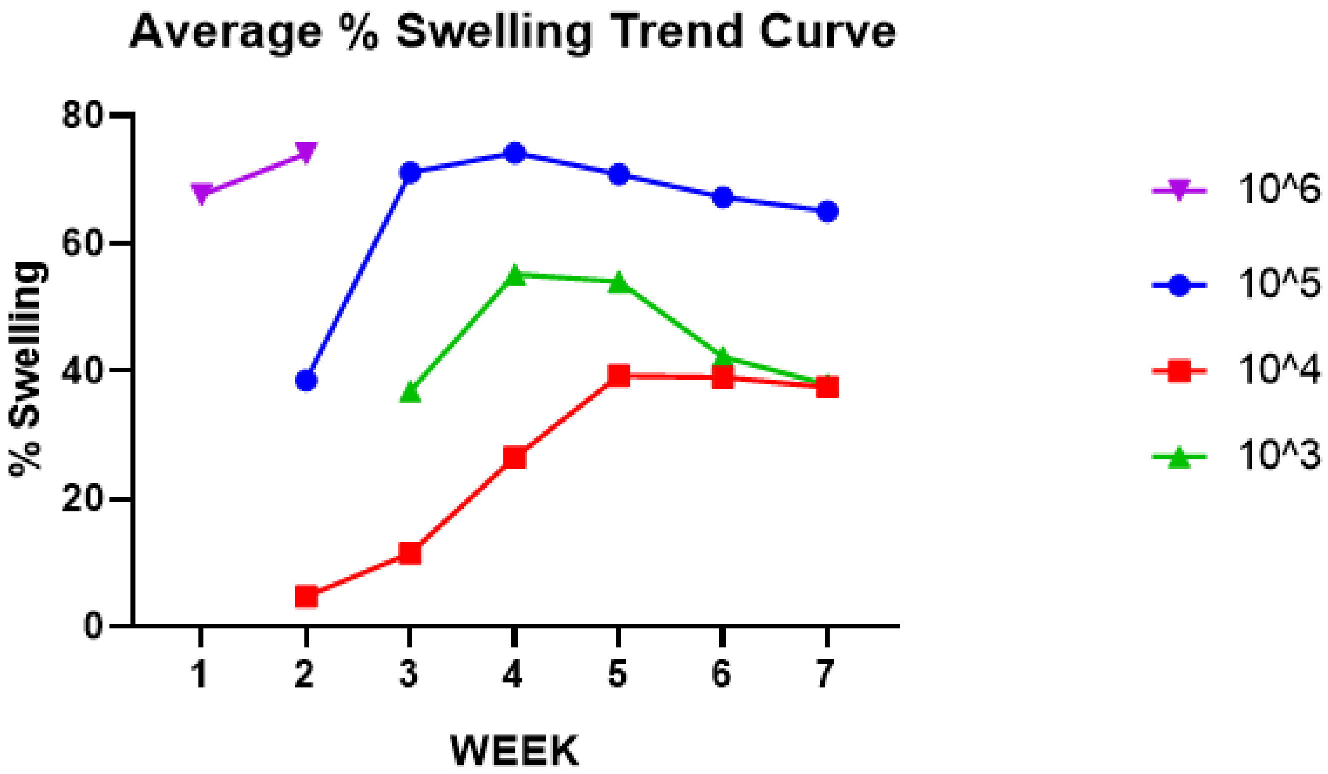


Fig 43. Trend curves of average percentage change in right footpad size measured weekly from point of infection for 7 successive weeks. Values are means from 5 mice.

Observing the trend in Fig 43, percentage change in footpad size in the 10^5 group had stabilised and was a gradual decline from week 4 despite the early onset of swelling and steep rise in infected footpad size. A similar trend was seen in group 10^3 as there was a significant decline in the footpad swelling from week 4. Even though swelling did not increase in the 10^4 group from week 5, there was also no decline in footpad size as the recordings remained averagely constant at 40%. This trend persisted until post-challenge week 7 when the experiment was terminated. The early attainment of the humane endpoint for mice in the 10^6 group meant the trend of percentage footpad change in footpad size could not be observed beyond the two-week period. It however demonstrated early onset of footpad swelling as percentage footpad swelling was 70% even as early as the first week. A steep rise to 80% was observed in week 2. This followed the steep change in footpad size of 70% in the first week.

The experiment was to determine if the dose of bacterial inoculum (for infection) influenced footpad swelling dynamics and if so, how it did this. The curves thus give a vivid demonstration of the progress and patterns of footpad swelling in mice following infection with varying inoculum load.

5.3.2 Footpad Extracts Display Cytotoxicity Linked to Infection Status; Effect of Mycolactone.

The immunopathology of Buruli ulcer disease is known to be heavily mediated by the toxin mycolactone. To confirm the presence of mycolactone and to determine whether or not it is only locally restricted to the site of infection, cytotoxicity assays as previously described in the mycolactone chapter were set up with Murine Embryonic Fibroblast cells. Supernatants were obtained from pooled homogenate of footpads of mice in the 10^6 group at the point of termination and from footpads of naïve control mice. PBS was also used as a control. Samples were taken from both the infected footpads and the contralateral control footpads in the same mice.

From the results in Fig 44, cytotoxic activity was observed to be highest in the sample taken from the infected footpads. Cytotoxicity increased as the concentration of the sample in the wells increased. At peak concentration of 25%, cytotoxic activity was highest in the wells containing sample from infected (Right) footpads. This was followed in ordinance by wells containing samples obtained from the contralateral (Left) footpads of the infected mice. Supernatant from untreated mice and PBS followed in activity. As concentration decreased, the cytotoxic activity levelled off at 5% concentrations for all samples tested.

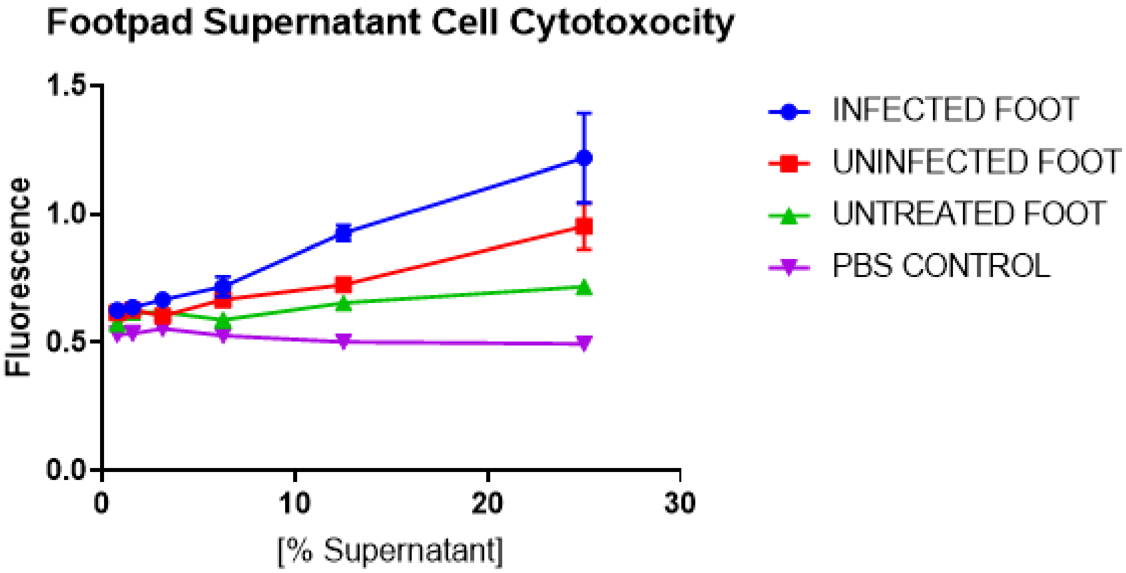


Fig 44. Cytotoxic activity of Homogenised Footpad supernatants obtained from *M ulcerans* infected mice and controls on Murine Embryonic Fibroblast (MEF) cells. This was measured by Alamar blue assays described in detail in the materials and methods chapter. Fluorescence is directly proportional to the extent of cell death. Error bars represent Standard Deviation. Experiments were in triplicates.

5.3.3 The Immunological Dynamics In *M ulcerans* Mouse Footpad Infection.

5.3.3.1 Antibody responses

Blood samples obtained from culled mice were processed and serum obtained stored for ELISA to determine titres for IgM and IgG antibodies. In order to get the full complement of all 5 mice in a group, samples taken from all 5 mice in the 10^6 group which were culled at week 2 were used in the ELISA tests to measure both IgM and IgG levels. The two-week timeline was chosen to ensure uniformity in results. Though not at a peak titre time for both antibody types, sample collection on day 14 of infection was done in order to improve chances of finding these antibodies as they are known to be absent at certain points in the timeline of infection but present in significant quantities at this point. Three sources of antigens were used: The protein derivative (MU- PPD) obtained from sonicating wild type *M ulcerans* S114, antigens from liquid culture media supernatant used to culture the wild type *M ulcerans* and that used to culture the mycolactone deficient *M ulcerans* (Delta).

Compared to control serum, serum from the five mice in the 10^6 group demonstrated significantly high titres of IgM antibodies (Fig 45). With a calculated mean titre of 1:400, mice developed antibodies very early in infection. Though to varying degrees in individual mice, all mice developed IgM antibodies to *M ulcerans* following infection. Mice were ear tagged for identification and so it was possible to correspond all outcomes to the respective mice. There was no correlation between size of swelling developed and IgM levels measured in corresponding sera.

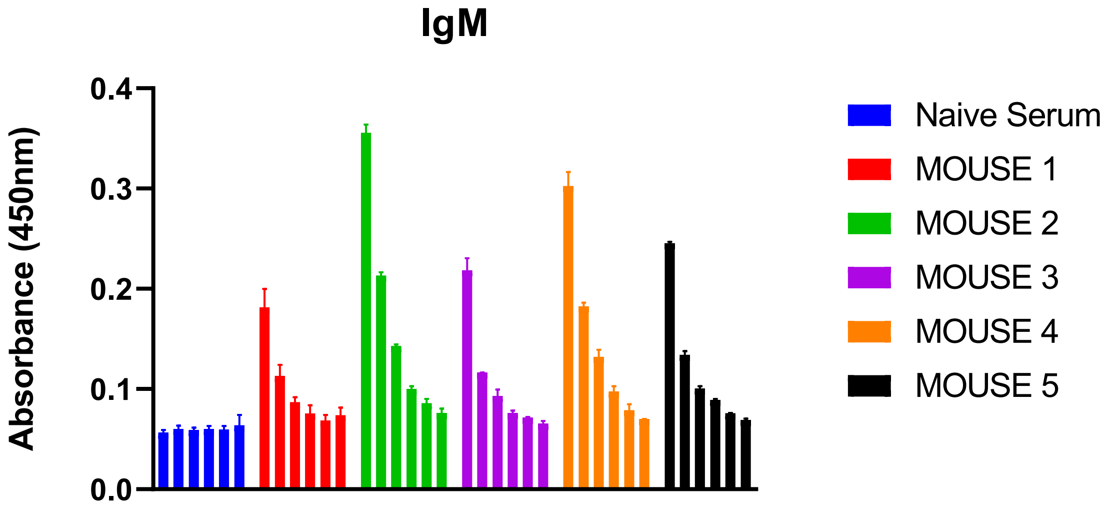


Fig 45. Antibody ELISA measuring IgM titre serum obtained from mice in the 10^6 group culled at week 2 using MU-PPD for antigenic coating. Starting concentration was 1/ 20 titred down by a dilution factor of 10. Experimental wells were in triplicates.

In comparison to control serum, IgG titres in mice from the 10^6 group were generally low. With a mean titre of 1:20, serum IgG levels were almost at baseline in some mice (Fig 46). Again, there was no correlation between serum IgG levels and extent of right footpad swelling.

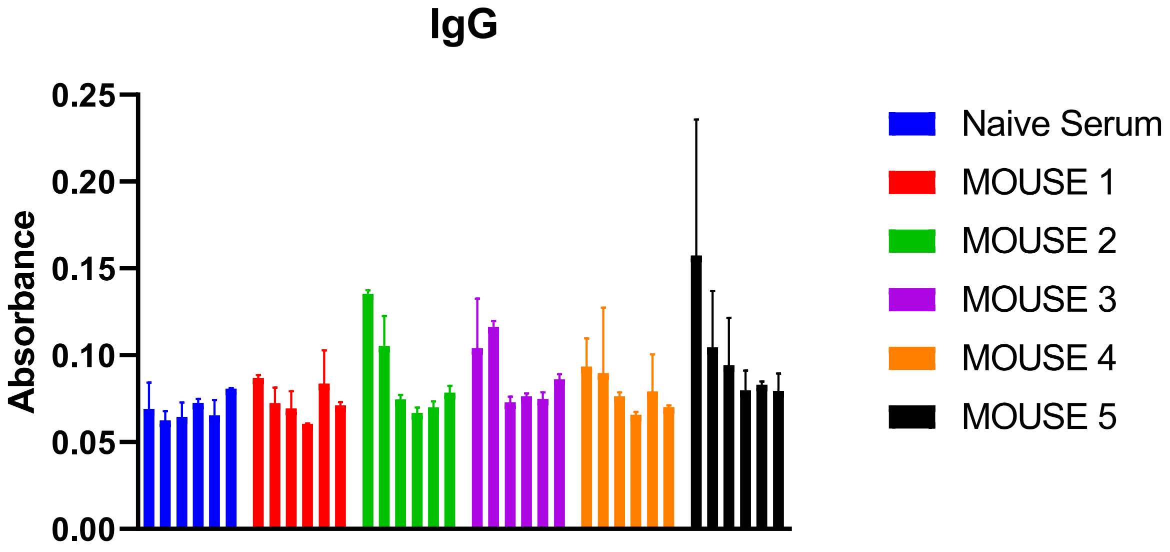


Fig 46. Antibody ELISA measuring IgG titre serum obtained from mice in the 10^6 group culled at week 2 using MU-PPD for antigenic coating. Starting concentration was 1/ 20 titred down by a dilution factor of 10. Experimental wells were in triplicates.

In order to obtain an indirect measure of possible IgM antibodies to mycolactone as secreted in the medium during liquid culturing of wild type *M ulcerans*, we used serum from 3 mice in the 10^6 group and pooled serum obtained from mice previously immunized with synthetic mycolactone and culled for immunogenicity as described in detail in the Mycolactone chapter, IgM antibody levels were measured against antigens in the culture media supernatants for both Delta and Wild type and *M ulcerans.* This was to be an indirect measure.

The results show in Fig 47 that while IgM antibodies against antigens contained in the culture media supernatant from both sources were detected in serum obtained from the mice infected with type *M ulcerans,* there were no detectable antibodies in the serum obtained from mice immunised with mycolactone. There was also no significant difference between antibody titres measured in sera from the infected mice against antigens contained in the media for both wild type and Delta *M ulcerans*.

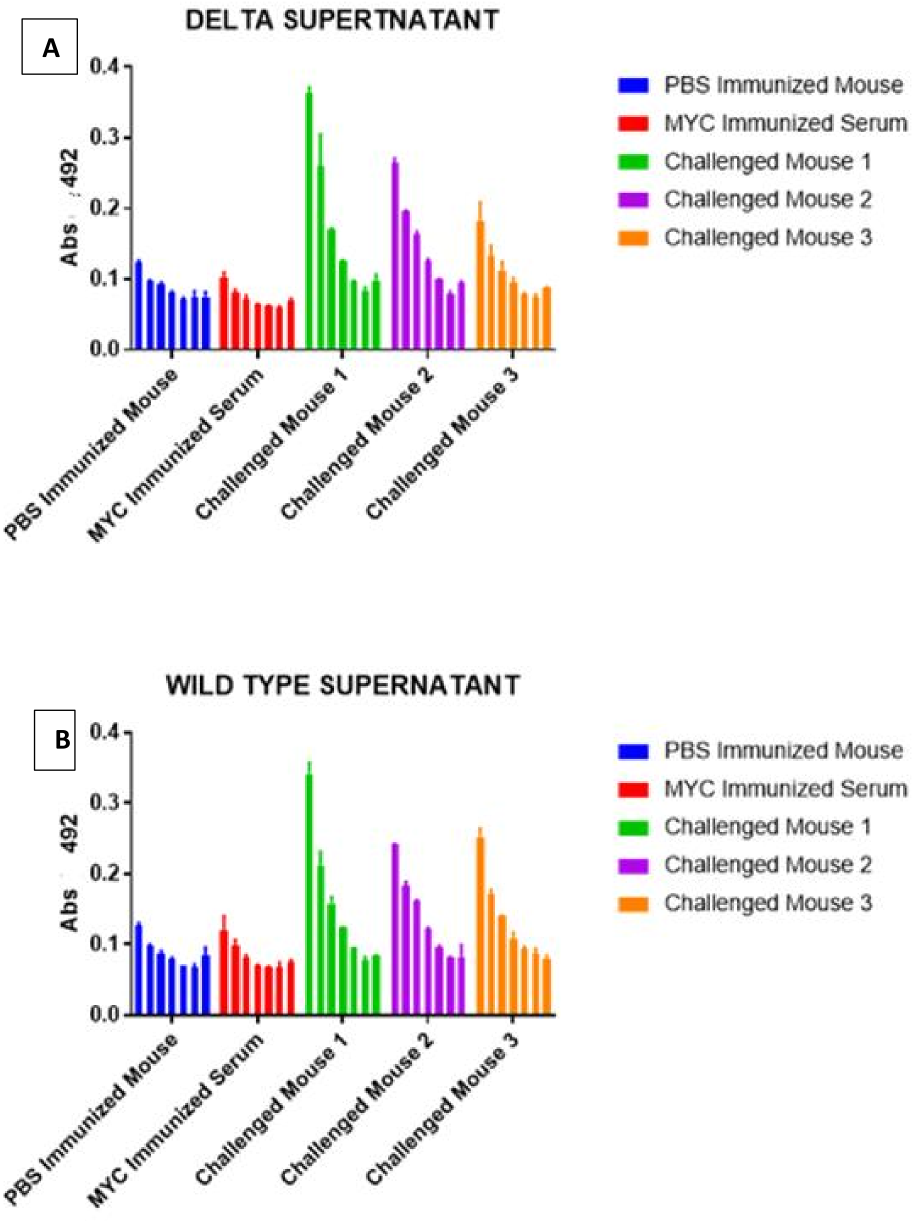


Fig 47. IgM antibody ELISA measured in pooled sera obtained from mice immunised with synthetic mycolactone but not challenged and serum obtained from 3 mice in the 10^6 group. Graph A represents results from Delta (Mycolactone Deficient *M ulcerans*) medium supernatant as the source of antigen while graph B represents results from when culture medium supernatant of wild type *M ulcerans* S114 was used for antigenic coating of ELISA Plates. Experiments were in triplicates. Error bars represent Standard Deviation.

5.3.3.2 Cell Mediated Responses

To understand the cytokine interplay at the point of infection as a proxy for the role of T-cell mediation, supernatants from homogenised infected footpads were collected to identify and quantify cytokines locally present at the time of being culled.

The highest concentration of IFN-γ was seen in the footpads of mice belonging to the 10^6 group as demonstrated in Fig 48. Mean concentration in the group was 900 pg/ml. Mean IFN-γ concentration was however similar in all the other mouse groups though significantly higher than in the control group. It ranged from 200 pg/ml for both the 10^5 and 10^4 groups and 180 pg/ml for the 10^3 group. PBS control group measured only up to 50 pg/ml of IFN-γ.

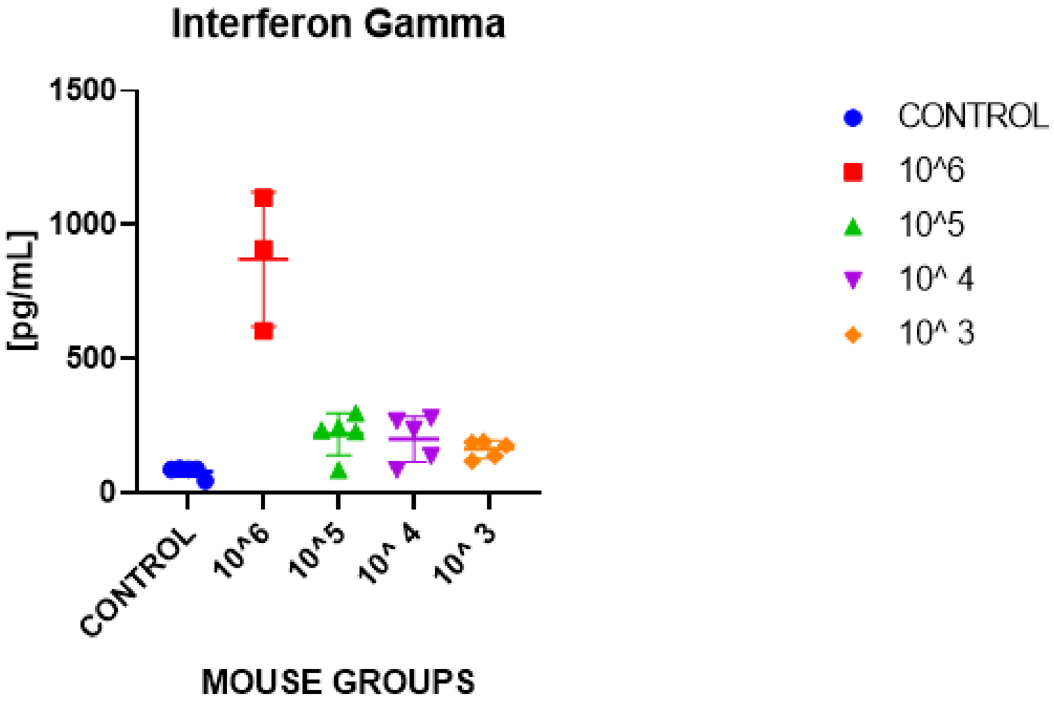


Fig. 48 Interferon Gamma concentrations in *M ulcerans* infected footpads of mice at the point of reaching humane endpoint as measured by Cytokine ELISA. N=5. Experiments were in triplicates. Error bars represent Standard Deviation.

Concentrations of Interleukin 2 and 4 in the footpads (Fig 49) show different profiles as mouse groups with lower levels of IL-2 tended to have higher levels of IL-4. Only the 10^6 group recorded IL-2 concentrations above the control group. IL-2 concentration was 100pg/ml in the 10^6 group but lingered around 25 pg/ml for the negative control group and all other experimental groups. IL-4 concentrations were lower in the experimental groups as compared to the Control group. While the control group recorded a mean concentration of 70pg/ml, the highest measure in an experimental group was in the 10^3 group (60pg/ml) followed by the 10^4 group (58pg/ml) and then the 10^5 group (55pg/ml). The lowest concentration was encountered in the 10^6 group which measured 51pg/ml. There was however no statistical difference between the 10^3 and the 10^4 groups.

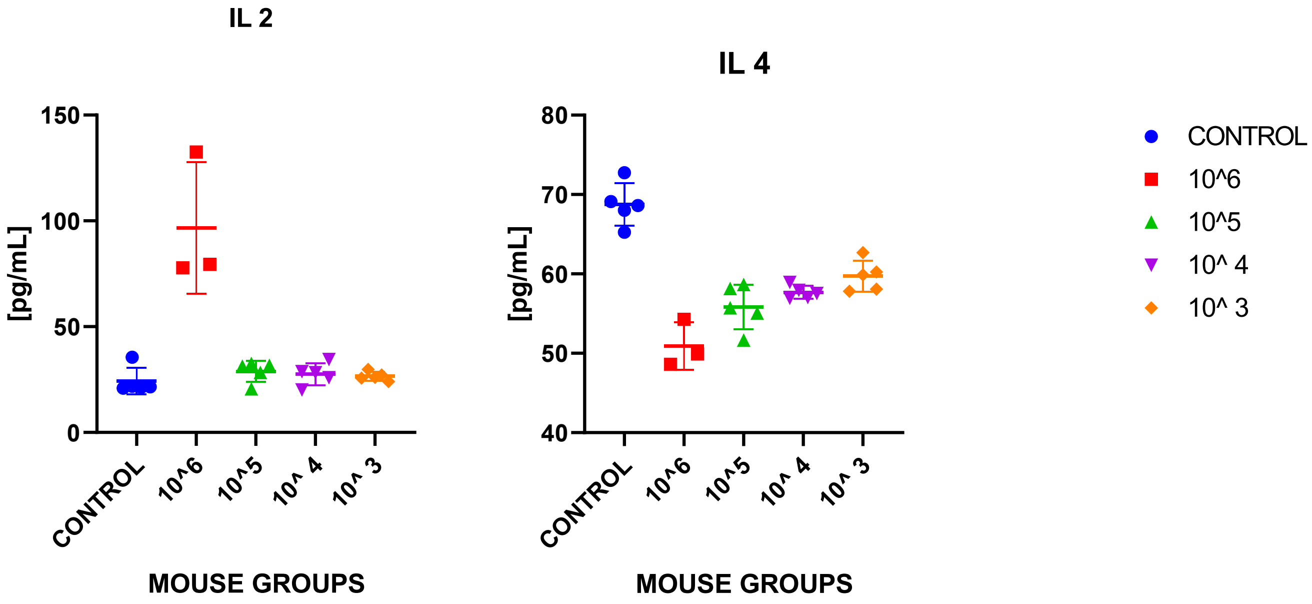


Fig 49. Interleukin 2 and Interleukin 4 concentrations in *M ulcerans* infected footpads of mice at the point of reaching humane endpoint as measured by Cytokine ELISA. N=5. Experiments were in triplicates. Error bars represent Standard Deviation.

Finally, the mean concentration of IL-10 was as at baseline for all experimental groups. Ranging between 160pg/ml and 170pg/ml, there was no statistical difference between any of the groups including the control group. For IL-17, only the 10^6 group recorded levels significantly higher than the control group. Measured concentration was 148pg/ml in the 10^6 group but levelled off between 90-95pg/ml in all the other groups including control (Fig 50).

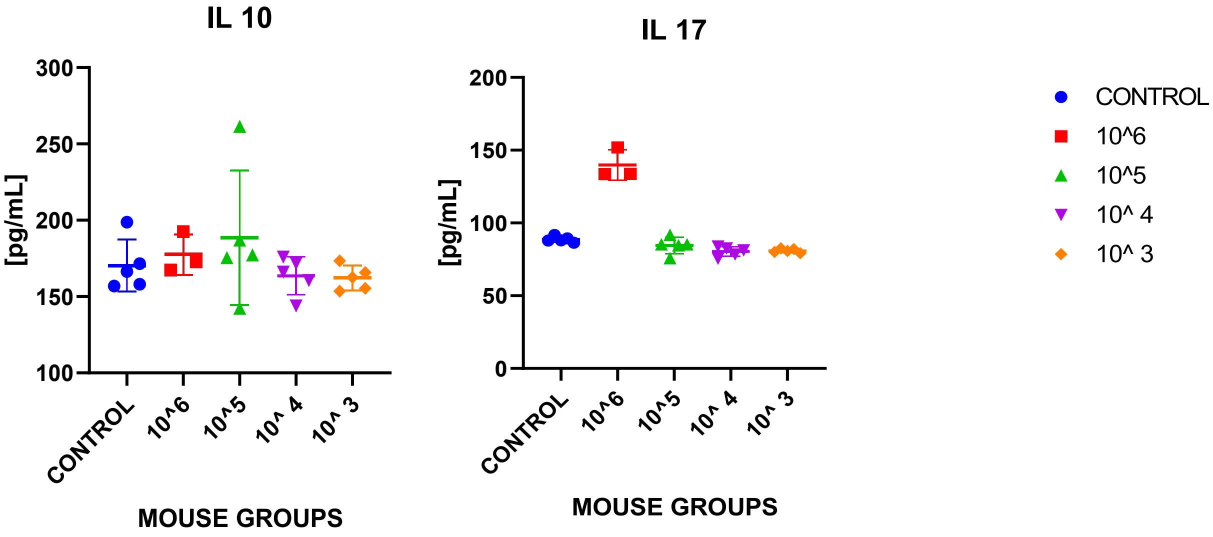


Fig 50. Interleukin 10 and Interleukin 17 concentrations in *M ulcerans* infected footpads of mice at the point of reaching humane endpoint as measured by Cytokine ELISA. N=5. Experiments were in triplicates. Error bars represent Standard Deviation.

5.4 Discussion

Buruli ulcer disease has been studied in humans but with limitations. As with other infections, knowledge on the underlying microbiology and immunology is obtained through animal models and experiments. Here, we sought to understand observe exactly what happened when mice were infected with *M ulcerans*. The ultimate aim was to apply the knowledge and principles to design and experiment with vaccine candidates against the disease in mouse models. These mice would be immunized and challenged with the bacteria.

While Buruli ulcer disease manifests in humans in 5 identified lesion forms (159), we only saw two forms in the mouse model. Footpads were either swollen or ulcerated with swelling being the only pre-ulceration lesion type observed. With ulceration being a humane endpoint, the only observational parameter we could establish as a measurable sign of disease was the presence of swelling at the site of inoculation.

With that established, the results from measuring footpad sizes showed that the onset of footpad the time to onset of disease was very much a direct consequence of the bacterial load/dose of inoculum. The higher the dose of inoculation, the earlier the onset of swelling. Accordingly, onset of swelling was delayed in the group of mice inoculated with 10^3 *M ulcerans* (Fig 41). This is very much in accordance with observations made by Ortiz et al (160) when they observed the differences in virulence and immune responses to different strains of *M ulcerans* first isolated in different regions. With an estimated generation time of 3-4 days in mouse foot pads as determined by Fenner (98, 109), it will be possible to in future determine the exact time period to expect the onset of swelling after infection challenge in vaccine testing. In humans, there have been controversies over the exact incubation period of the bacteria. While studies in Australian adults calculated the mean incubation period to be 135 days, Amoako et al in identifying and cataloguing two cases of *M ulcerans* infections in neonates 4 days old and 2 weeks old threw in limbo the certainty of the incubation period as previously found in the Australian cohort (161, 162). Following the findings in these mouse models, we submit that the incubation period could significantly vary according to dose of inoculum and is a direct consequence of the bacterial load at the time of inoculation.

Looking closely at the trend graphs in Fig 43, we observe from the results that even though the time to onset of swelling was a direct consequence of the dose of inoculum, the extent of disease measured by size of swelling did not follow any particular pattern. Despite the delayed onset of swelling in the 10^3 group, percentage change in footpad swelling was about twice that of the 10^4 group despite swelling having been observed in the latter group a week earlier. This trend continued till week 7 when a gradual decline in the swollen footpad sizes in the 10^3 group caused the average percentage change in footpad sizes to become equivalent. In effect, even though dose of inoculum had a direct effect on the time to onset of swelling, the rate of change in footpad size and the progression of the infection was not a direct consequence of this. So far, no direct relation has been observed between the disease progression and type of lesions in humans to the co-existence of any underlying medical conditions (29). Beyond the fact that some of these studies have given contradictory results, our findings provide strong reason for such studies looking to identify intrinsic factors which mediate and modulate the severity of disease experienced and its progression.

Another very important observation is the trend of reduction in size of swollen footpads with time. At the onset (Fig 43), percentage change in footpad size was on a perpetual rise in all mouse groups but with the exception of mice in the 10^6 group which could not be followed up beyond two weeks, all other mouse groups recorded either a continuous decline in footpad swelling or stabilising of the swelling size following the initial upward trend in footpad swelling. It is important to note that most mice reached humane endpoint by developing ulcers even though size of swelling was on the decline. There are two likely explanations to this phenomenon: It is either as a result of spontaneous resolution of the infection by the intervention of innate immune mechanisms or that a progression of the disease to an ulcerative stage was independent of inflammation and would happen anyway even if the extent of inflammation waned. In humans, a phenomenon where serological testing demonstrated previous exposure to *M ulcerans* but no history of clinical disease gave rise to suspicions that innate immune mechanisms could possibly have the ability to mitigate in infections and prevent the establishment of disease. Some neutralising skin specific mycolactone antibodies have been found to promote spontaneous healing of *M ulcerans* infection (163). In effect, these mechanisms could also cause a spontaneous resolution of infection without exogenous interventions. Considering that the decline in size of footpad swelling was most remarkable in the 10^3 group, this intervention of innate immune mechanisms may potentially be influenced by the dose of the inoculum. As is documented for many other pathogens, humans and animals have the innate ability to spontaneously prevent the establishment of disease especially when the infecting dose of pathogen is low. The implication of this for vaccine work is that apart from looking out for delayed or total prevention of onset of disease as a measure of vaccine efficacy, it will also be important to monitor the progress of disease after onset of swelling as vaccines may augment innate immune mechanisms to reduce the severity of disease as measured by the extent of footpad swelling and/ or prevention progress to ulceration. It will also be important to pre-determine the optimal inoculum dose for challenge as a very high dose will have the potential to overwhelm immune responses and in effect mask vaccine induced protection.

To establish the presence of mycolactone in the infected footpads, cytotoxicity assays were set up as a proxy measure to determine this. Having homogenised footpads from different mice with the same categorisation, the only significant difference between the footpad groups was the presence or absence of *M ulcerans* and its attendant toxin mycolactone. Since filtered supernatants were used in the actual assays, no bacterial cells entered the wells, save soluble moieties including mycolactone. Evidently, infected footpads exhibited the highest cytotoxic potential against MEF cells. This effect tapered down as supernatant concentration decreased in the assays. This was not surprising as infected footpads were expected to have high concentrations of mycolactone mediating the pathology of disease. Mycolactone as demonstrated in the mycolactone chapter is highly cytotoxic and by proving its presence and activity, it would follow that the mouse footpad model when used in trial of vaccine candidates against *M ulcerans* with properly simulate all the immunomodulatory effects of mycolactone. Interestingly, some significant cytotoxic activity was observed in wells containing supernatants from uninfected contralateral footpads of infected mice. These were left footpads which had no swelling and exhibited no changes or sign of disease. The right contralateral ones were however infected and visibly swollen. Not swollen or obviously diseased, it was quite surprising to have noticed the potential presence of mycolactone distantly. This is however not too surprising when looked at in light of the fact that systemic effects of mycolactone have in the past been described Hong et al (164) and Sarfo et al (146). In effect, mycolactone distribution is not limited to the site of the lesion even though concentration is likely to be much higher locally.

In understanding the immune responses generated to *M ulcerans* infection, we looked into the antibody responses and the local cytokine interplay at the site of the lesion. For the antibody responses, serum taken at two weeks post-infection was tested. Even though the exact point of appearance of IgM and IgG in *M ulcerans* infection is unknown, serological tests carried out in humans showed that IgM persisted for long after infections and is a good serological marker for distinguishing between previously infected patients, exposed household contacts and non-endemic controls (165). It was therefore not surprising to have observed significantly high titres of IgM in tested serum. IgG levels were very low. A possible explanation could be that IgG is generally known to appear late in infection and may not have been present 14 days post-infection. The presence of these antibodies was however not protective as mice still went ahead to develop swelling the size of which was in no way correlated to the antibody titres measured in individual mouse sera. As an indirect determination of the presence of antibodies to mycolactone, ELISAs performed with serum from mice in the 10^6 against culture filtrate from both mycolactone producing *M ulcerans* S114 and the mycolactone deficient Delta showed no IgM antibodies against mycolactone. There was no difference in the antibody titres irrespective of the culture filtrate used and serum from the mycolactone immunised mice gave baseline titres equal to negative serum titres. This confirms the dogma that there are no antibodies to naturally occurring mycolactone in *M ulcerans* infection. Our attempts to immobilise synthetic mycolactone onto ELISA plates for determination of the presence or absence of antibodies against synthetic mycolactone had been unsuccessful.

Finally, with the establishment of the fact that IgM presence did not necessarily correlate with protection and that there were no antibodies against mycolactone which is known to be the critical mediating factor in the pathogenesis of the infection, local footpad cytokine interplay was looked at for all mice involved in the various groups. This was to study and understand the T-cell responses at the site of infection. Pro-inflammatory cytokines Interferon Gamma, Interleukin 2 and Interleukin 17 were measured in footpad supernatants. Interleukins 10 and 4 were the selected Anti-inflammatory cytokines principally because of their documented role in mycobacterial infections. In vitro studies have already shown that with the exception of Interleukin 10 which is minimally inhibited, all the other mentioned cytokines are very heavily inhibited by mycolactone (150). Also, in a mouse study by Kiszewski et al (166) which looked at local lesion T-cell responses and pathology, Interferon Gamma concentration was found to be a good marker for the formation of granuloma which has the significance of being evidence of the strength of the cellular immune response locally generated against the infection. Increased IFN-γ levels correspond to granuloma formation whereas increased IL-10 concentration corresponds to poor or no granuloma formation.

High concentrations of pro-inflammatory cytokines IFN-γ, IL-2 and 1L-17 were measured in the footpads of mice in the 10^6 group. They however had low levels of IL-10 and 1L4. In fact, the lowest level of IL-4 measured was found in this group. Beyond evidently demonstrating the generation of a strong pro-inflammatory cellular response, the elevated levels of IFN-γ also indicate granuloma formation. Though higher than in control mice, reduced levels of IFN-γ were measured in all the other groups. Taking into cognisance the fact that samples obtained for these

ELISAs were taken at the point of cull, samples from the 10^6 group represent an early time point lesion while those from the lower dose inoculums represent later point infection. Knowing that mycolactone very significantly suppresses the actions of these pro-inflammatory cytokines, it stands that the possible explanation for the high pro-inflammatory activity which wanes with time could be that mycolactone production and secretion is a time-controlled process and that a build of mycolactone concentrations over time causes further immunosuppression and inhibition of innate immune mechanisms as infection progresses with time. To corroborate this possible explanation is the well documented pathological observation of *M ulcerans* being intracellular in the early stages of infection but becoming predominantly extracellular as infection progresses and mycolactone concentrations build up to destroy the cytoskeleton of macrophage cell walls through its WASP inhibition activity (95, 167).

The anti-inflammatory cytokine responses were opposite to what was observed in IFN-γ levels. IL-4 concentration was highest in the control group and decreased as the dose of inoculum increased. Therefore, the higher the infective dose, the higher the initial pro-inflammatory response and the lower the anti-inflammatory response. This situation reversed as infection progressed with time and is attributable to the strength of the initial innate immune response which correlates with infective dose and the gradually increasing concentration and effect of mycolactone which sets in with time.

The implication of these observations on a potential vaccine against BU will be that a vaccine candidate targeting and adequately inhibiting the production and action of mycolactone will go a long way to either prevent the establishment of disease or mitigate the severity of disease suffered.

In summary to this part of the study, we have established a mouse footpad model of MU infection which is suitable for vaccine studies and we have characterised the immunopathological features of infection having elucidated the B and T-cell profile of infection in mice. By this, measurable parameters have been understood for future vaccine work. We will therefore be able to evaluate the efficiency and efficacy of vaccine candidates we will design and test in our mouse model.

CHAPTER 6.

A MYCOLACTONE BASED VACCINE AGAINST *M ulcerans* INFECTION

6.1 Introduction

There exists no vaccine against Buruli ulcer disease. Although a number of candidates have been tried since 1956 when the first attempt was made, none has so far been successful. The pathogenesis of the disease is such that its toxin mycolactone plays a central role (9, 156). We postulate that a vaccine candidate designed to interfere with mycolactone action will stand a good chance of preventing disease. In the past, mycolactone had been thought to be immunosuppressive and without any potential to induce immune responses (51). Attempts to bypass this effect led to the use of the polyketide synthase enzymes involved in the synthesis of Buruli ulcer disease (91, 92, 114, 121). This gave some promise as partial protection was achieved. The enzymes were tried as DNA vaccines and boosted with their recombinant protein forms. Recombinant protein-based vaccines are known to induce stronger immune responses than DNA vaccines. They also have the advantages of having room for boost doses (though not exclusive to them) and the addition of adjuvants and delivery systems if need be. Additionally, multiple protein constructs may be combined in one vaccine for better synergistic effects (153, 154).

In the preceding chapters, we have explored the vaccine potential of mycolactone by looking at its dose dependent effects and determined the minimum dose at which it exerts its actions. Additionally, we investigated its interaction with adjuvants and delivery systems and sought to understand how that would affect its vaccine potential. We also looked at the polyketide synthase enzymes and their individual immunogenicity and have selected the best going forward. In this chapter, we immunised mice with various vaccine formulations consisting of modified *M ulcerans* strains which lack the ability to secrete mycolactone; mycolactone with adjuvants and delivery systems; and the selected polyketide synthase enzymes expressed as recombinant proteins and combined into single formulations. Mycolactone deficient *M ulcerans* strain 5134 herein referred to as Delta (Δ) is a wild type *M ulcerans* strain that has lost the giant plasmid responsible for mycolactone production as a result of repeated passaging. It is thus not able to secrete any mycolactone.

6.2 Results

6.2.1 Mycolactone Deficient *M ulcerans* Strain 5134 (Delta, Δ) Does Not Cause Buruli Ulcer Disease and is Partially Protective.

To determine this, mice were immunized subcutaneously at the tail with 10^6, 10^5 and 10^4 of *M ulcerans* 5134 at week 0 and challenged in the right footpad with 10^5 wild type *M ulcerans* S114 at week 5. Mice were observed daily for the development of any lesions at the base of the tail where the vaccine candidate had been injected. They were also observed daily for any footpad swelling. At the onset of swelling in the right footpad, weekly footpad size readings were taken with the electronic caliper and recorded as has been described in detail in the Materials and Methods chapter.

By week 6, no lesion was found at the base of mouse tails. The tails remained intact for the entire 14-week period of the experiment.

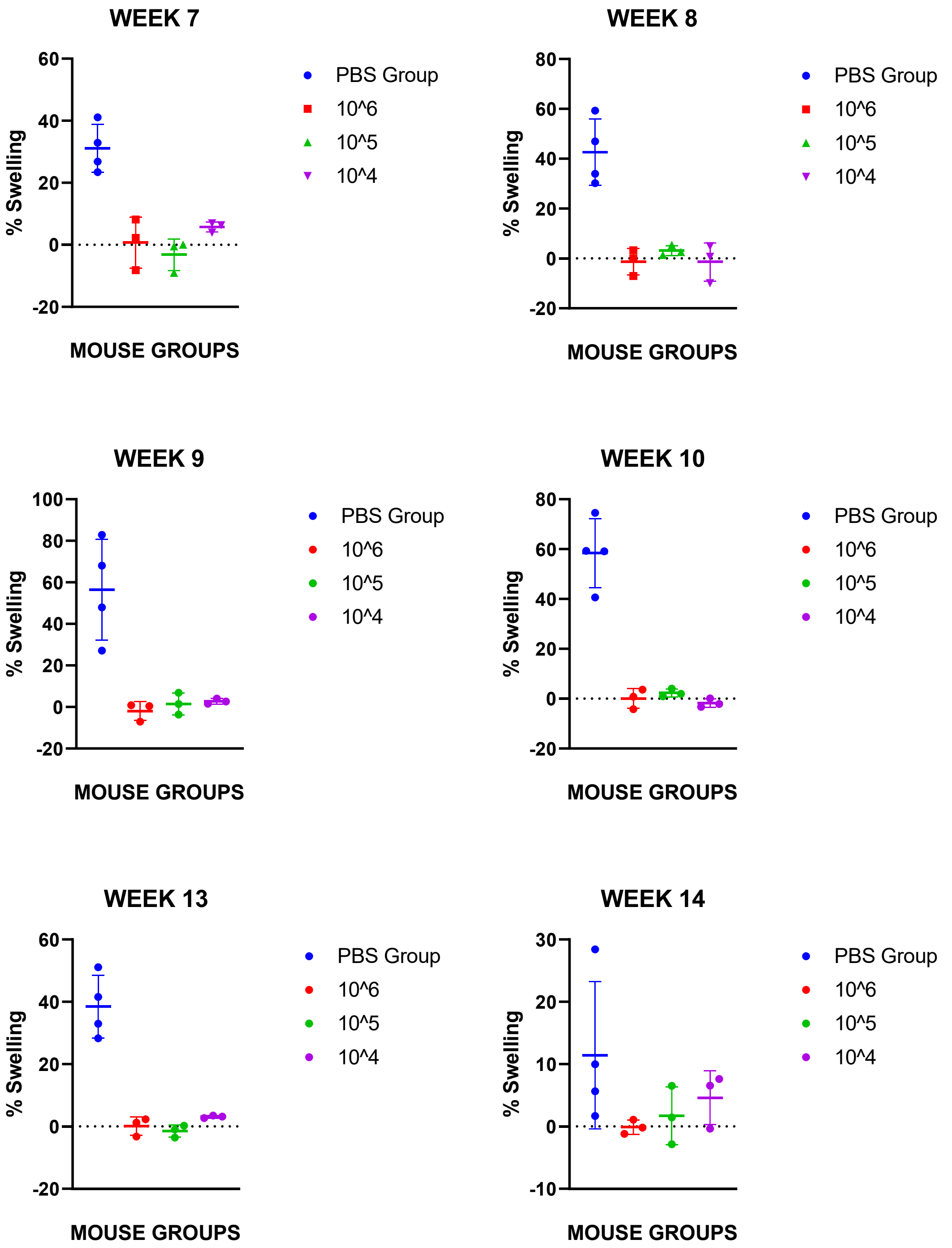


Fig 51. Percentage change in infected footpad size as measured with an electronic caliper in mice which had been immunised subcutaneously in the tail at week 0 with 10^6, 10^5 and 10^4 with *M ulcerans* 5143 (Delta) and PBS as a control and challenged in the right footpad at week 5 with 10^5 wild type *M ulcerans* (S114). Prior to week 7, there had been no swelling. N= 5. Experiments were in triplicates. Error bars represent Standard Deviation.

It was established that up to 14 weeks post-inoculation, mice infected/immunised with different doses of Delta did not develop any visible lesions. When challenged in the right footpad with the inoculation of 10^5 wild type *M ulcerans* at week 5, the mice immunised in the tail with PBS developed swelling in the right footpads from week 7 (Fig 51). It was not until week 14 that mice immunised with 10^4 and 10^5 Delta started developing Footpad swelling. Until then, infection was delayed and even at week 14, mice immunised with 10^6 Delta were still protected. Mice immunised with 10^4 had an average percentage change in footpad size of 6% at week 14 while those in the 10^5 had a change of just about 2%. Thus, the protection conferred by Delta appeared to be dose dependent with the highest immunisation dose developing no swelling at all whilst lower doses had started to develop swelling. The experiment was terminated at this point and 10^6 was selected as the dose of choice in using Delta as a vaccine candidate.

GROUP	**PRIMING ANTIGEN**	**BOOST ANTIGEN**	**ADJUVANT**	**DELIVERY SYSTEM**	**NUMBER OF MICE IN GROUP**
1	PBS	PBS	-	-	8
2	BCG	PBS	-	-	8
3	Δ *M ulcerans*	Ag85A	Quil A	-	8
4	Δ *M ulcerans*	KRA	Quil A	-	8
5	Δ *M ulcerans*	MYCOLACTONE	-	YC NaMA	8
6	Δ *M ulcerans*	MYCOLACTONE	SPORES	-	8
7	Δ *M ulcerans*	MYCOLACTONE+ Ag85A+ KRA	Quil A	-	8
8	Δ *M ulcerans*	-	-	-	8
9	PBS	MYCOLACTONE+ Ag85A+ KRA	Quil A	-	8

Table 8. Vaccine groups for Immunisation. Each group represents mice immunised with a composite vaccine candidate the components of which are described in the table. *DELTA (Δ) is a mycolactone deficient strain of *M ulcerans* *Ag85A and KRA are polyketide synthase enzymes *BURULIVAC (Myc + Ag85A+ KRA+ Quil A). *YcNAMA is a nanoparticle. *Quil A is a saponin based adjuvant *SPORES refers to heat killed Bacillus s*ubtillis* spores used as an adjuvant

Having established the potential to use Delta as a vaccine candidate without causing an infection, we formulated 5 novel vaccine candidates in addition to BCG and PBS to be used as controls (Table 8). 8 mice per group were vaccinated with each candidate according to the schedule described in the materials and methods chapter (Fig 22). Three were culled for immunogenicity studies at week 8 and the remaining five mice were challenged with 10^5 wild type *M ulcerans* and monitored for infection.

6.2.2 Immune Responses to Mycolactone and Other Antigens.

Three mice from each group were culled and blood and spleens harvested for immunogenicity studies. The studies included cytokine responses in recall assays and antibody ELISAs to various antigen preparations.

To determine antibody responses, sera prepared from blood obtained from immunised mice according to methods described in chapter 3 were used in ELISAs. Plates were coated with wild type *M ulcerans* lysate to determine IgG and IgM titres.

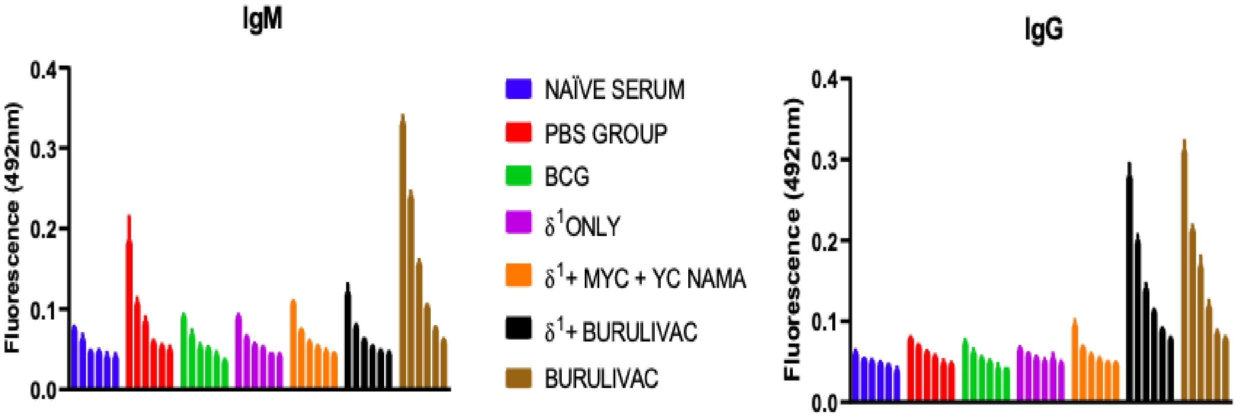


Fig 52. Wild type *M ulcerans* S114 specific IgG and IgM ELISA responses in naïve and vaccinated mice. Mice were vaccinated with various candidates containing *M ulcerans* antigens, mycolactone, Polyketide synthase enzymes and BCG. Data is presented as means and N=3. Error bars represent SD.

In Fig 52, IgM antibodies were only found in sera of mice immunised with Burulivac without a Delta prime. Serum from mice in that group also contained significant IgG antibodies. Sera from all the other vaccine groups contained no IgM beyond background. However, Delta Prime + Burulivac vaccine produced IgG antibodies. So, with the exception of mice in the Burulivac only group, no other group secreted both IgG and IgM. And even though the Delta + Burulivac vaccine contained Burulivac too, the secreted antibody profile was different, in that mice in that group did not secrete any significant levels of IgM.

On culling the mice after immunisation, splenocytes were incubated in antigen recall assays with mycolactone, KRA and *M ulcerans* lysate as described in detail in the Methods section. Supernatant was then collected after 48 hours of incubation. Supernatant was then tested by ELISA for various cytokines.

On recall with mycolactone (Fig 53), there was no IL-6 secretion by splenocytes from any of the vaccine groups. The mycolactone + spores group secreted the highest levels of IL-10, TNF α and IFN-γ. In fact, the with spores group was the only group to have generated statistically significant levels of IFN-γ. It was also the group to have secreted statistically significant levels of TNF α. Mycolactone + YC NaMA group secreted the highest level of IL-2 but did not secrete statistically significant levels of any other cytokine. Splenocytes from the Burulivac only group did not secrete any cytokines in response to mycolactone stimulation. However, the Burulivac group primed with Delta secreted significant levels of IL-2 and IL-10.

Cytokine Responses to Mycolactone Recall

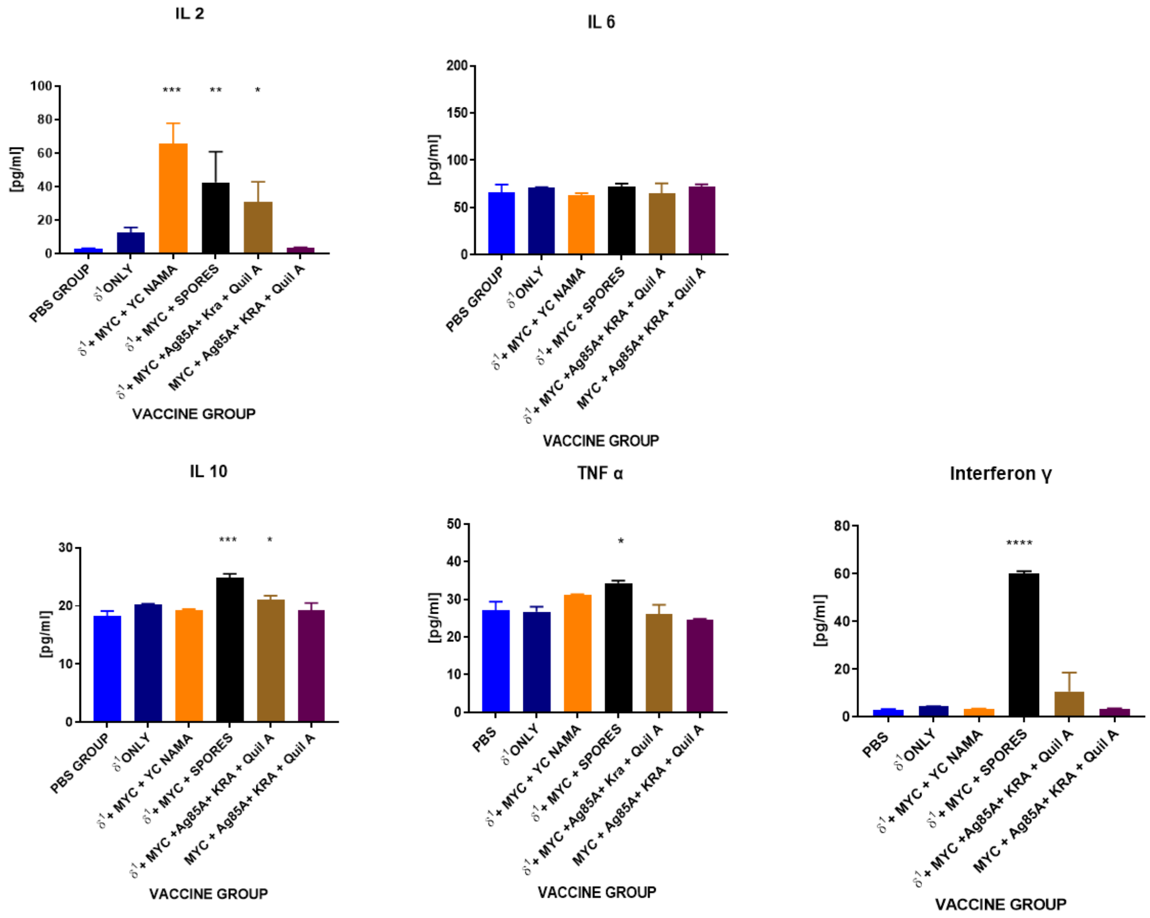


Fig 53. Cytokine profile of splenocytes obtained from immunised mice on recall with mycolactone. Measured cytokines were Interleukins 2, 6, 10, Interferon Gamma and TNF Alpha. N= 3. Error bars represent Standard Deviation. Experiments were carried out in Quintuplicates.

On recall with Ag85A, all groups apart from the PBS group secreted very high levels of IFN-γ, even beyond the upper detection limit of the test kit used. BCG responses were directed to IL-2 and TNF α secretions. For IL-2, the quantity secreted was equivalent to that secreted by Delta+ KRA + Quil A, Delta + Mycolactone + Spores and the Delta only group. For TNF α, even though a significantly higher amount than PBS control was secreted, both Burulivac groups and both protein subunit groups (Ag85A and KRA) secreted higher levels than splenocytes from the BCG group. IL-6 secretion was observed to be highest in the Ag85A group but was also significantly high in both mycolactone groups and the KRA group. The two Burulivac groups again gave the highest IL-10 responses followed by the two subunit protein vaccine groups. Responses from all the other groups were insignificant. The two subunit protein groups consistently produced significant levels of all tested cytokines in response to Ag85A. No other vaccine group did this.

Cytokine Responses to Ag85A Recall

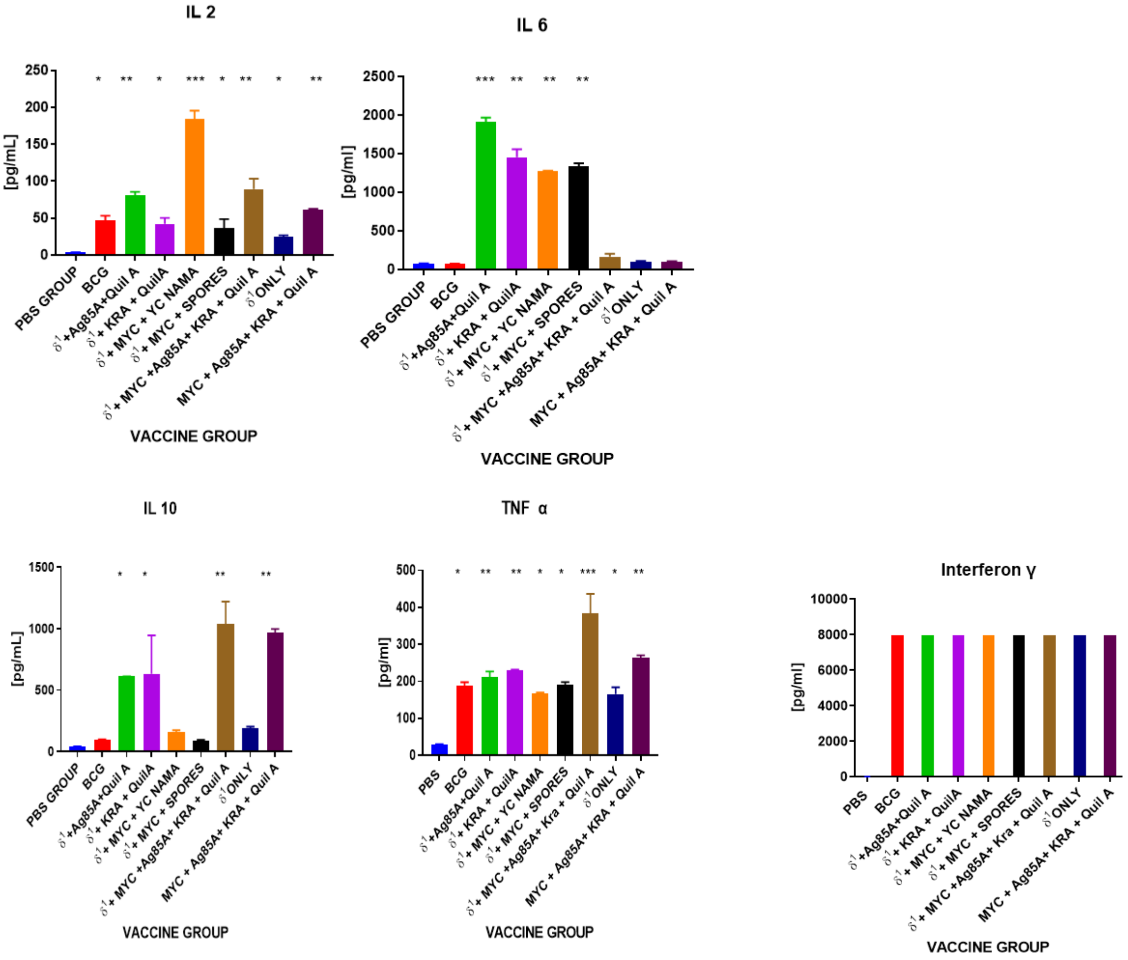


Fig 54. Cytokine profile of splenocytes obtained from immunised mice on recall with Ag85A. Measured cytokines were Interleukins 2, 6, 10, Interferon Gamma and TNF Alpha. N= 3. Error bars represent Standard Deviation. Experiments were carried out in Quintuplicates.

Ketoreductase A, the other enzymatic mycolactone-based subunit protein used in designing some of the vaccine candidates was also used as a recall antigen according to the same recall assay protocol previously described. For all cytokines measured (Fig 55), the Delta+ KRA + Quil A group gave the strongest responses. This was consistent for all cytokines. The Delta + Burulivac group followed with consistently high responses of all cytokines measured. Its IL-2, IL-6 and IFN-γ responses were comparable to the Delta+ KRA + Quil A group. Burulivac only group gave responses which were different from the Delta + Burulivac group. While it produced strong IFN-γ and TNF α responses comparable to the Delta + Burulivac group, it produced less strong IL-2 and IL-10 responses and no IL-6 response at all.

While neither mycolactone groups (ie Delta + Mycolactone + YC NaMA and Delta + Mycolactone + Spores) gave any IL-2 responses, they gave strong IL-6 responses equivalent to Delta + KRA + Quil A. Delta + Mycolactone + YC NaMA also gave no IL-10 response even though Delta + Mycolactone + Spores gave equal responses to the Delta + KRA + Quil A group. A similar trend is observed in the TNF α responses. While Delta + Mycolactone + Spores gave a very strong response, the Delta + KRA + Quil A was much less even though significantly higher than negative control. Responses from the Delta group splenocytes were generally poor. Apart from its strong Interferon gamma response it produced no IL-2, IL-6, or IL-10 response. The corresponding TNF α response though significantly higher than the negative control was the least response from any of the reactive vaccine groups.

Cytokine Responses to KRA Recall

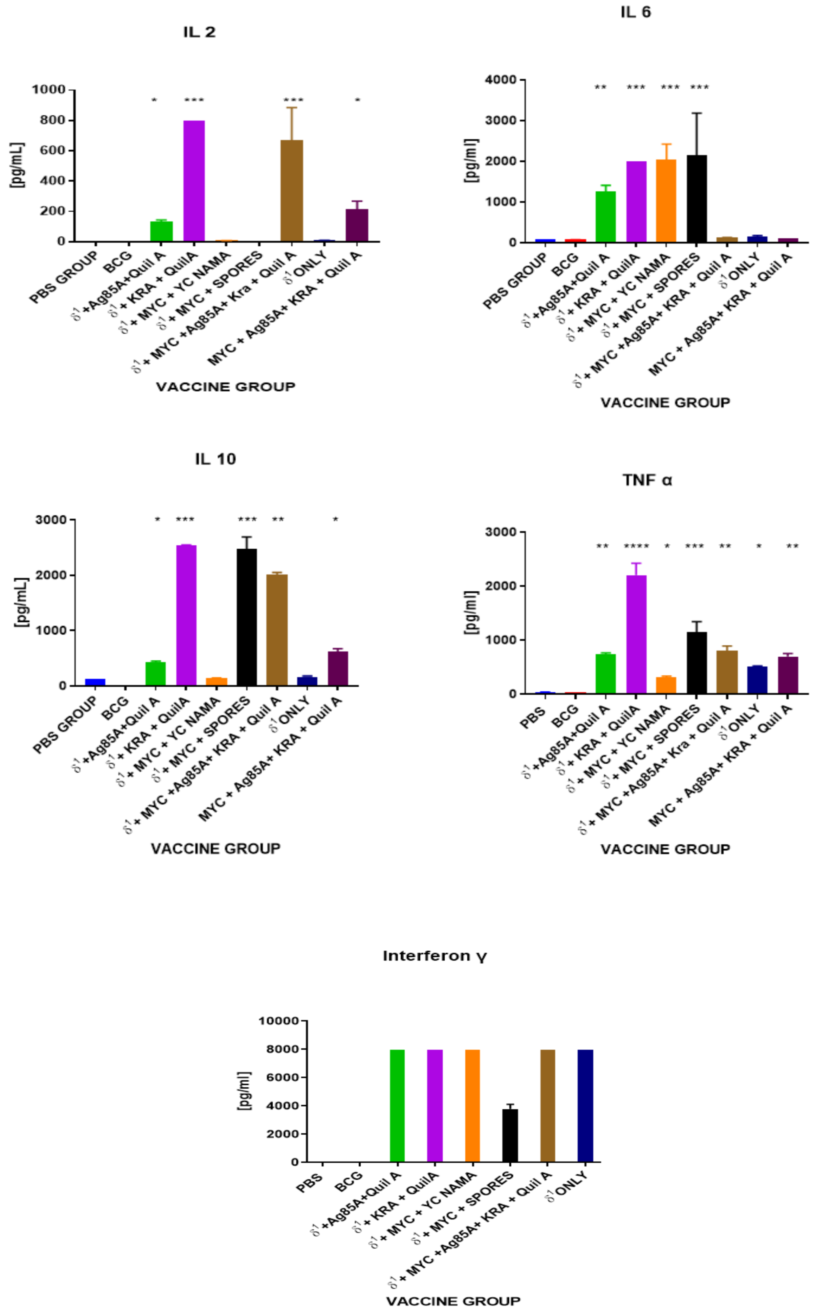


Fig 55. Cytokine profile of splenocytes obtained from immunised mice on recall with KRA. Measured cytokines were Interleukins 2, 6, 10, Interferon Gamma and TNF Alpha. N= 3. Error bars represent Standard Deviation. Experiments were carried out in Quintuplicates.

Finally, a lysate made of sonicated and filtered wild type *M ulcerans* bacteria suspended in PBS was used as a recall antigen to elicit various cytokine responses in an assay involving splenocytes obtained from mice of the various vaccine groups and as described in the methods section. As a whole, cytokine responses were poor across all measured cytokines (Fig 56). Only BCG and Delta + Burulivac gave positive IL-2 responses with BCG giving the lower response. Burulivac only and Delta + Ag85A + Quil A gave slightly higher than baseline IL-6 responses. No other group produced any signiificant IL-6 secretions. No group gave any IL-10 responses. While Delta + Burulivac produced strong TNF α and IFN-γ responses and Delta + Mycolactone + Spores gave a significant TNF α response, all other groups gave no response to either cytokine. In particular, Delta + Burulivac stood out in its IFN-γ response in that it was the only group to have produced any response at all.

Cytokine Responses to *M. Ulcerans* Recall

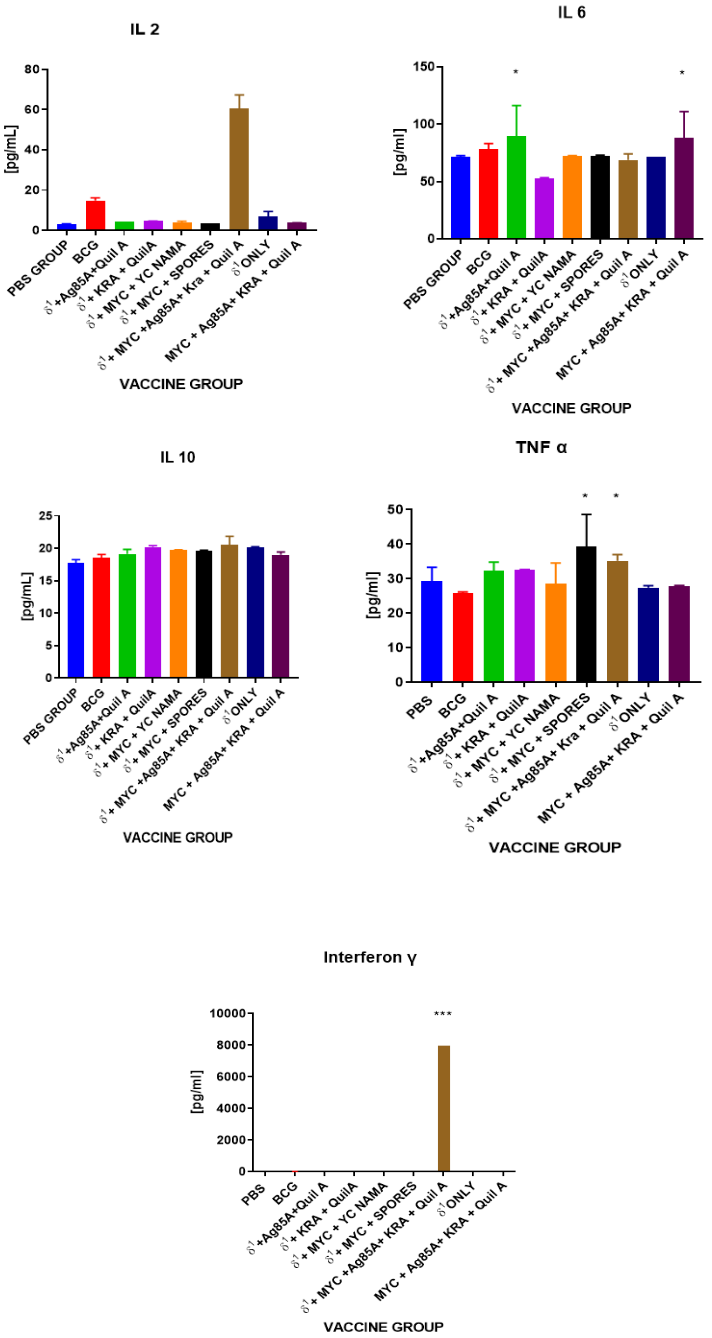


Fig 56. Cytokine profile of splenocytes obtained from immunised mice on recall with wild type *M ulcerans* lysate Measured cytokines were Interleukins 2, 6, 10, Interferon Gamma and TNF Alpha. N= 3. Error bars represent Standard Deviation. Experiments were carried out in Quintuplicates.

6.2.3 Footpad Cytokine Profile

To investigate the T-cell interplay at the peak of footpad swelling when mice were culled, infected footpads were amputated during cull and passed through a tissue homogeniser in order to homogenise the footpads in PBS solution as described in the materials and methods chapter. Resultant supernatant for each footpad was then tested by ELISA for IL-2, IL-4, IL-6, IL-10, IL-17A, TNF α, IFN-γ concentrations. As shown in Fig 57, PBS control footpads contained the highest concentrations of IL-2 (28pg/ml), IL-6 (38pg/ml), TNF α (70pg/ml), and IFN-γ (200pg/ml). That group recorded the least or baseline concentrations of IL-4 (55pg/ml) and IL-10 (520pg/ml). For IL-17A concentrations, footpads from the PBS group together with the Delta + Mycolactone + YC NaMA group contained the highest concentrations. Footpads in the BCG averagely contained about 25pg/ml of IL-2. All other groups contained averagely 20pg/ml. For IL-4, the concentration in all other groups with the exception of Delta + Mycolactone + Spores was equal to or marginally less than the BCG induced concentration of 54pg/ml. The range was 52-54pg/ml with the Burulivac only group recording the lowest concentration. Delta + Mycolactone + Spores stood out with IL-4 concentration of 61pg/ml.

PBS group and Delta + Mycolactone + Spores recorded the highest concentrations of IL-6 with levels of 38pg/ml followed by the BCG group with a concentration of 32pg/ml while all other groups followed with average concentrations of 28pg/ml. In tandem with BCG, the PBS group concentration of IL-10 was 520pg/ml. Delta + Ag85A + Quil A group recorded a slightly lower IL-10 concentration at 510pg/ml. Delta + KRA + Quil A recorded the lowest IL-10 concentration of 490pg/ml. The highest IL-10 concentration of 545pg/ml was measured in the Burulivac only group. As observed with the other cytokines, Delta + Mycolactone + Spores recorded a significantly high concentration of IL-10 at 540pg/ml. The concentration in the Delta only group was also 540pg/ml. Delta + Burulivac followed with a concentration of 538pg/ml.

With respect to IL-17A and TNF α, the Delta + Mycolactone + YC NaMA group, concentrations were on level pegging with the PBS group. The lowest concentrations of both cytokines were observed in the Burulivac and Delta only groups with the two protein subunit vaccines following closely behind in concentrations of IL-17A and TNF α. PBS group with its IFN-γ concentration of 200pg/ml was the highest. IFN-γ concentrations in the BCG, Delta + KRA + Quil A, Delta + Mycolactone + Quil A and Delta + Mycolactone + Spores were 50pg/ml, with all other groups hovering around the minimum detectable concentrations.

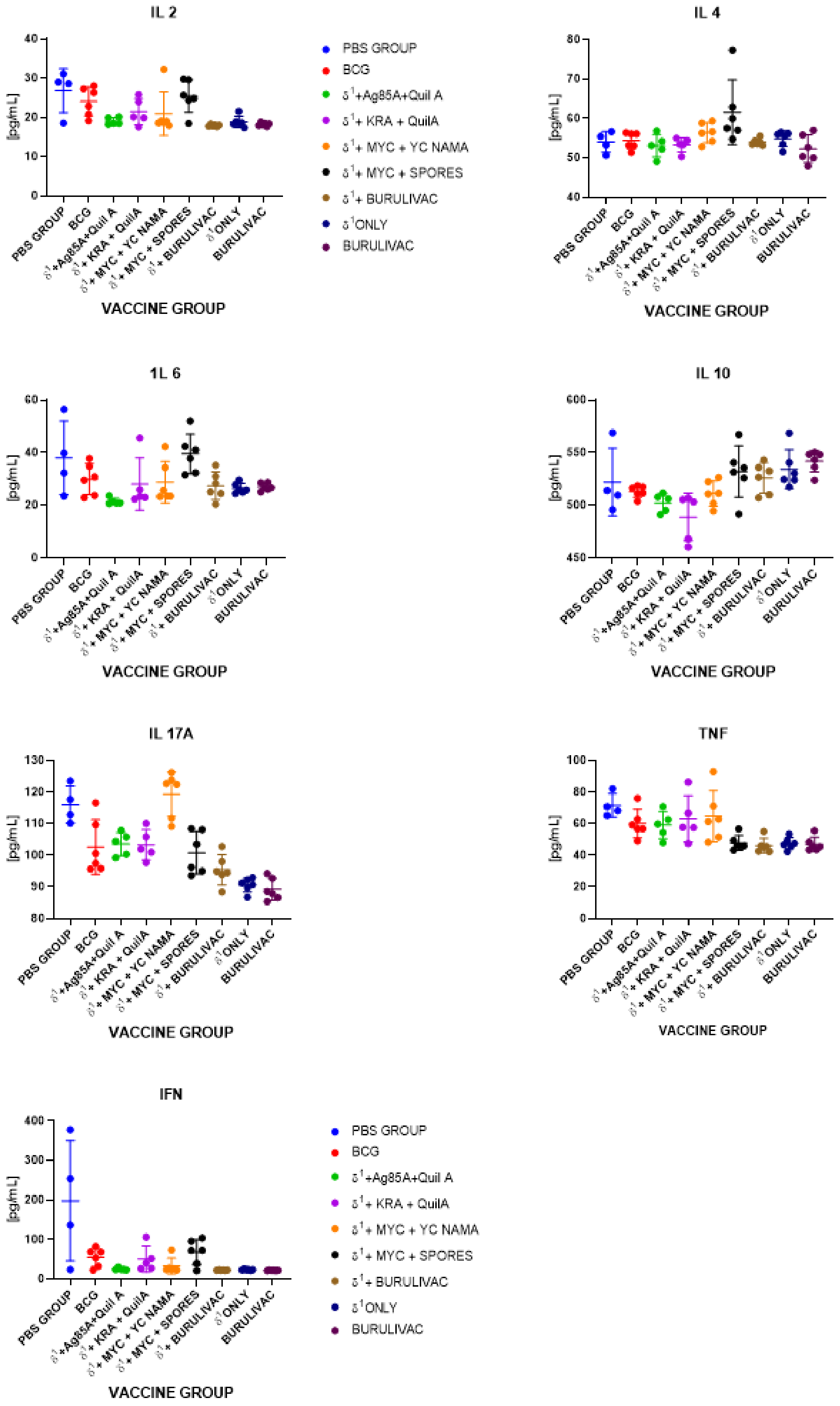


Fig 57. Cytokine concentrations in infected footpads at the point of cull. Right footpads of mice were infected with type *M ulcerans* following a complete cycle of immunization with various vaccine candidates as named in the graphs. N=5. Experiment is in triplicates. Error bars represent Standard Deviation.

6.2.4 C-Reactive Protein Concentration:

C- Reactive protein is an acute phase reactant produced by the liver in response to inflammatory injury. It has both Pro and anti-inflammatory properties but is basically used as a marker of inflammation (155). To determine the extent of inflammation ongoing in the infected pads, CRP concentration in the supernatant of each homogenised amputated foot was determined by ELISA method as described in detail in the methods section.

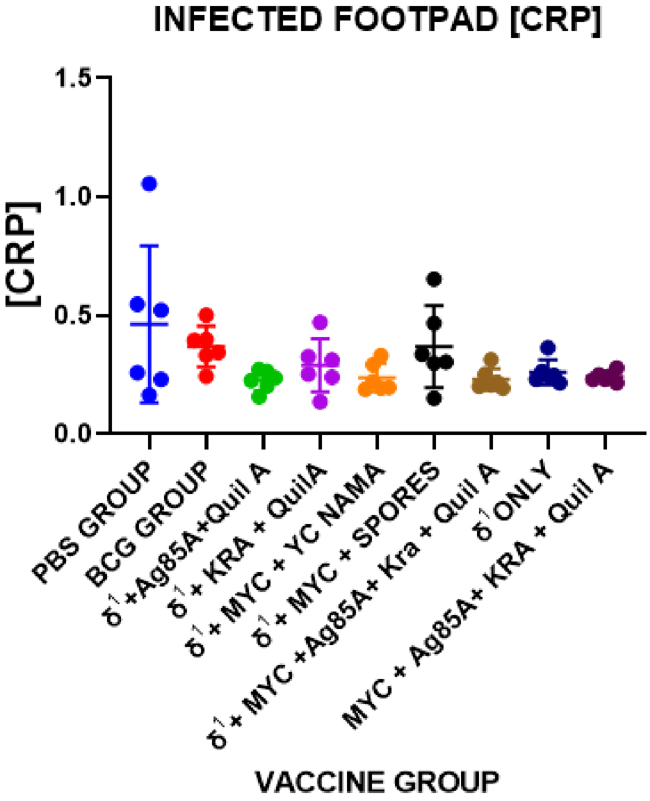


Fig 58. C-Reactive Protein concentration in infected footpads as determined by ELISA method using the ThermoFischer CRP ELISA kit. Optical density was measured at 492mm. N= 5. Error bars represent standard deviation. Unit of measurement was pg/ml.

6.2.5 Footpad Swelling and Measures of Vaccine Protection.

Measurement of Footpad thickness was carried out using electronic calipers by methods described in detail in the methods section. Percentage change in swelling of the infected right footpad was then calculated relative to the left control foot according to the formula below.

$$\frac{\text{Right(Thickness*span)} - \text{Left (Thickness*span)}}{\text{Right(Thickness*span)}} \quad \text{X 100}$$

Using percentage change in footpad as a proxy to determine severity of infection and degree of protection conferred by the respective vaccines, graphs of percentage change in footpad size were plotted for comparison. The largest swelling was observed in the PBS group with a percentage change in infected footpad of 46%. Next was BCG with a percentage change in swelling of 38%. Varied degrees of swelling were observed in the other groups with the Burulivac groups both recording no swelling at all. The Delta only, Delta + Ag85A + Quil A and Delta + Mycolactone + YC NaMA groups all recorded average percentage change in footpad size of 10% with Delta + KRA + Quil A and Delta + Mycolactone + Spores recording changes of about 20% (Fig 59).

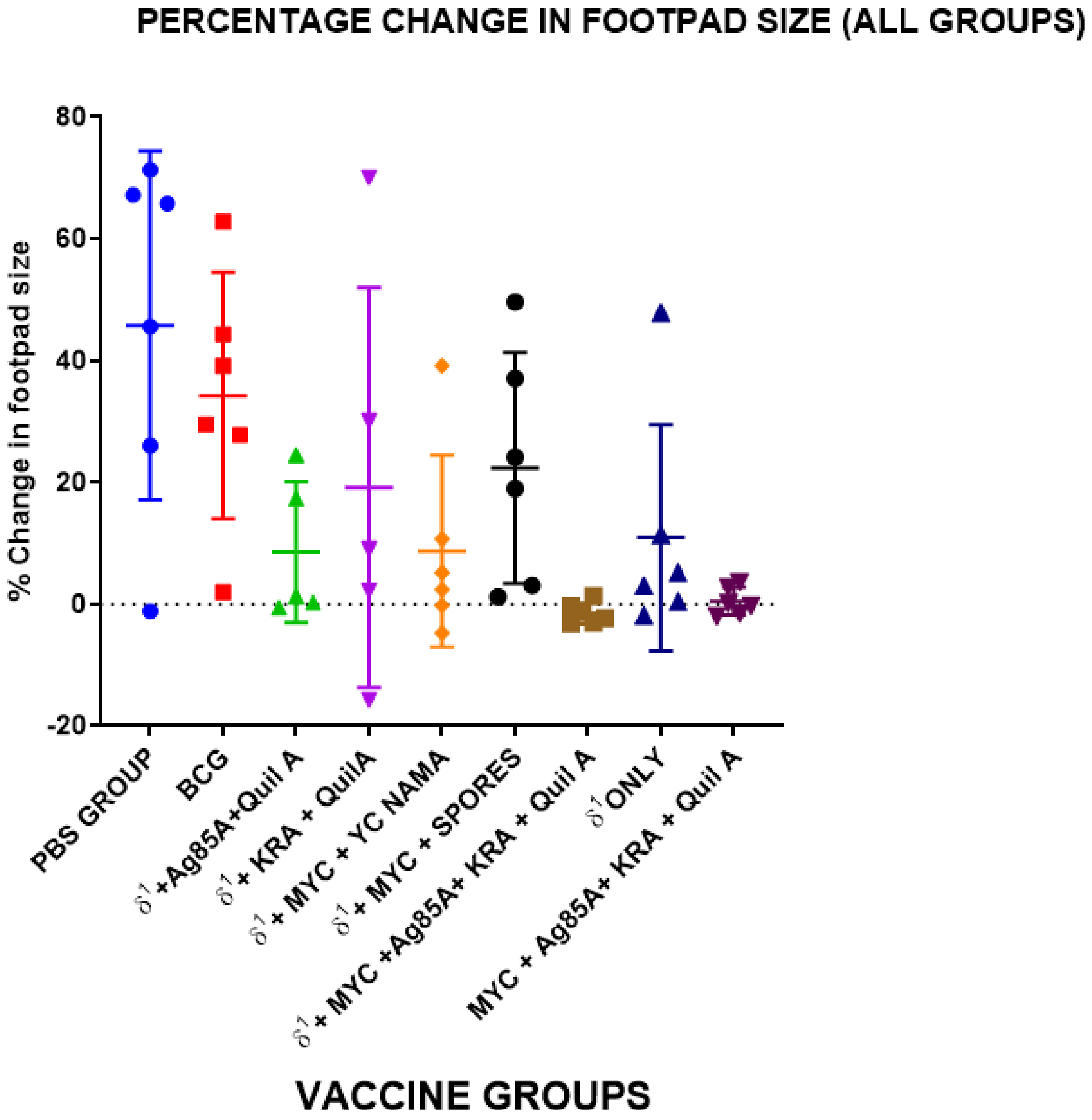


Fig 59. Percentage change in footpad size of infected mice. Right mouse footpads were infected with 10^5 *M ulcerans* bacteria while the left footpad was used as a control. Percentage change in swelling was calculated as a percentage of the control footpad size. N= 5. Error bars represent standard deviation.

In figures 60 - 63, the data is presented in smaller groups comparing percentage change in swelling among groups with similar components. Fig 60 compares groups of mice primed with Delta against control groups and the Burulivac only group. Fig 61 compares vaccine groups immunised with a mycolactone containing vaccine while sub-unit vaccine groups are compared in Fig 62. Among the Delta primed vaccine groups, the best performing candidate was the Delta prime + Burulivac boost group. There was no swelling in any of the mice in that group. In comparing groups immunized with mycolactone containing vaccines, the Burulivac groups were still the best performing. They were followed by the Delta prime with Mycolactone + YC NaMA boost with an average change in footpad size of 10%. The Ag85A + Quil A group with its 10% change in footpad size gave the best protection among the subunit vaccine groups. These results are discussed in detail in the discussion section.

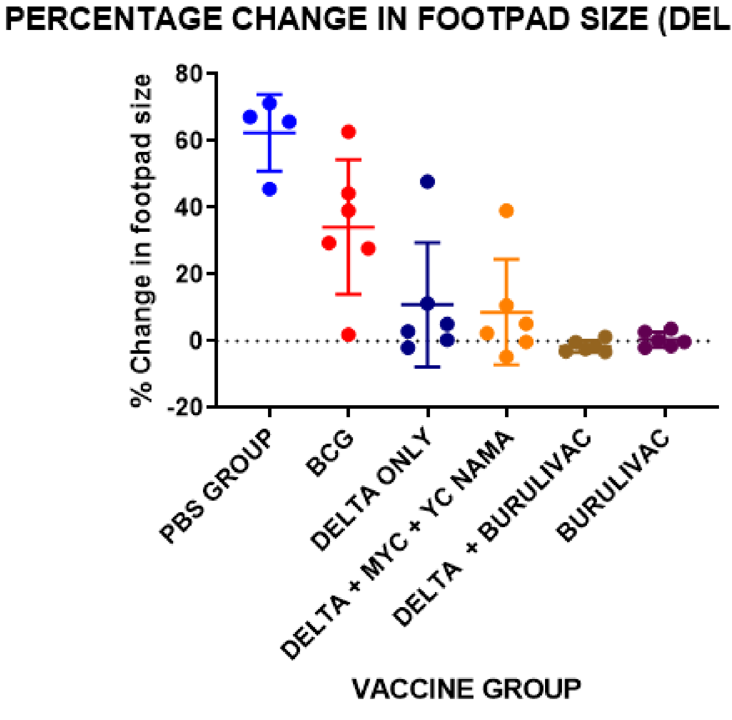


Fig 60. Comparing percentage change in footpad size of infected mice immunised with Delta as part of the vaccine regimen to control vaccine groups and the Burulivac group. Right mouse footpads were infected with 10^5 of *M ulcerans* bacteria while the left footpad was used as a control. Percentage change in swelling was calculated as a percentage of the control footpad. N= 5. Error bars represent standard deviation.

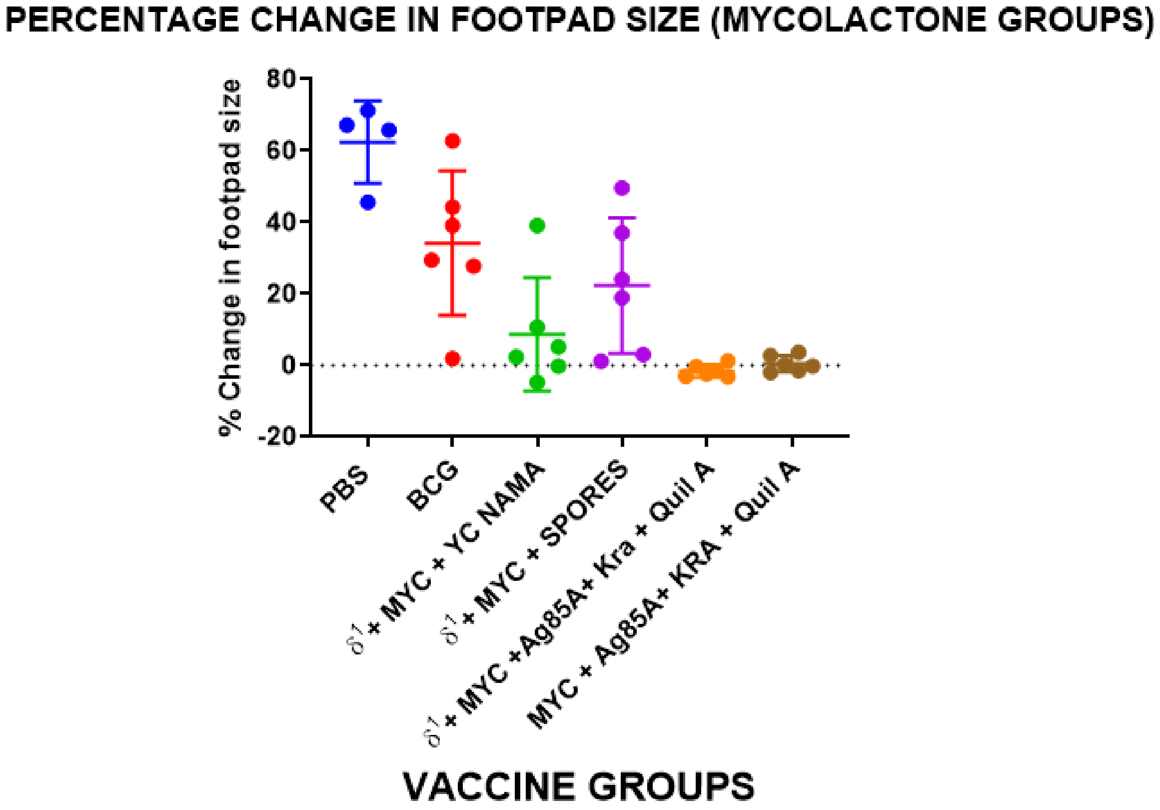


Fig 61. Comparing percentage change in footpad size of infected mice immunised with Mycolactone based vaccines to control vaccine groups and the Burulivac group. Right mouse footpads were infected with 10^5 *M ulcerans* bacteria while the left footpad was used as a control. Percentage change in swelling was calculated as a percentage of the control footpad. N= 5. Error bars represent standard deviation.

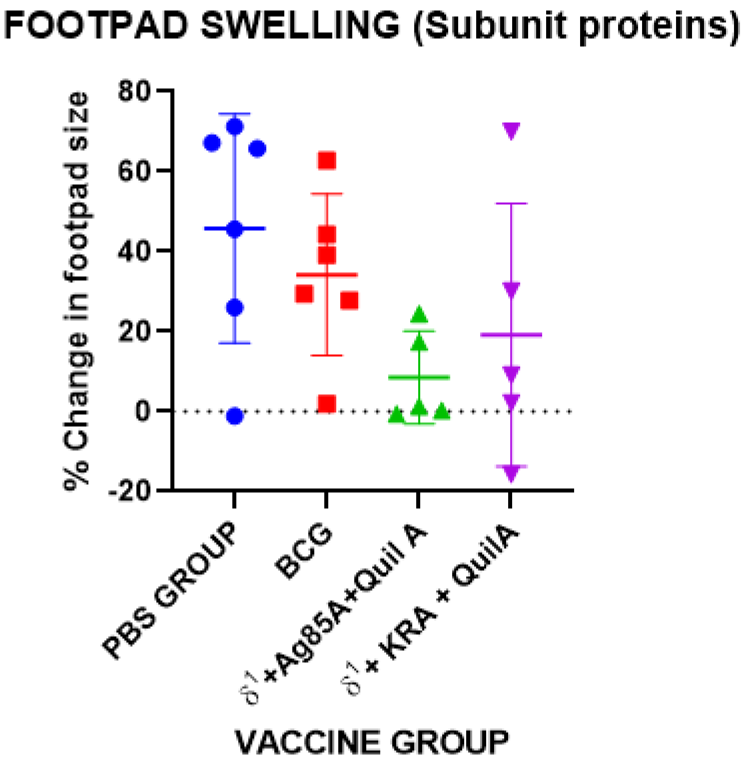


Fig 62. Comparing percentage change in footpad size of infected mice immunised with Protein-based Subunit vaccines to control vaccine groups. Right mouse footpads were infected with 10^5 *M ulcerans* bacteria while the left footpad was used as a control. Percentage change in swelling was calculated as a percentage of the control footpad. N= 5. Error bars represent standard deviation.

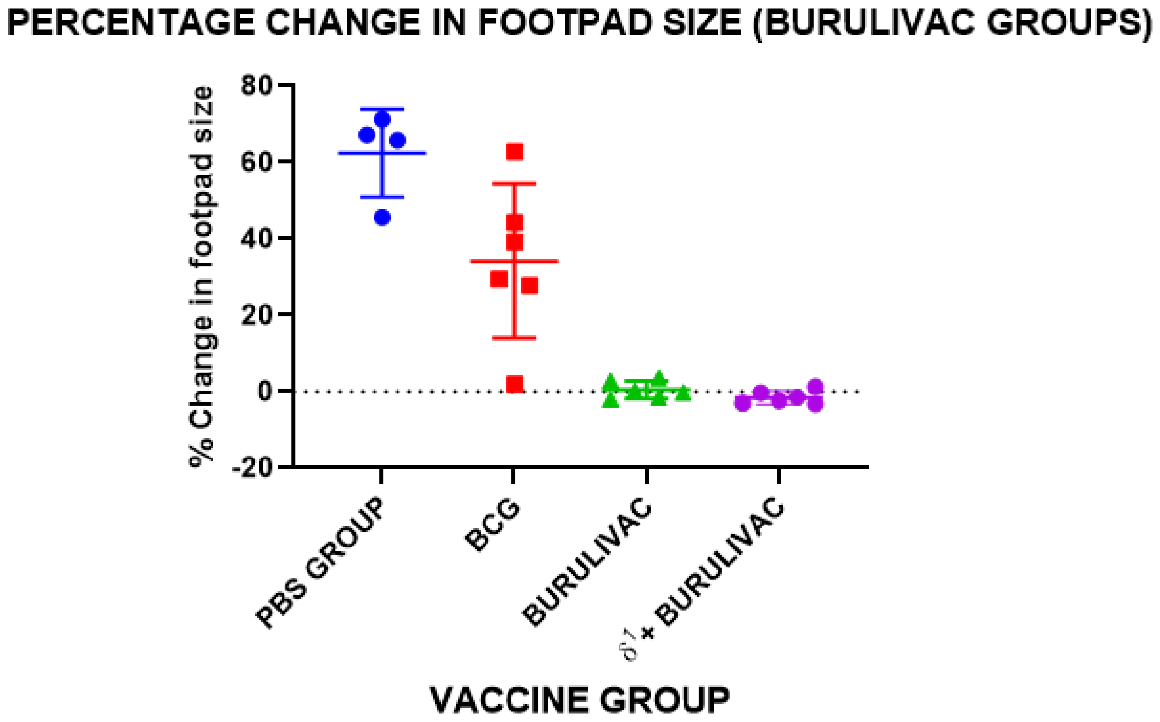


Fig 63. Comparing percentage change in footpad size of infected mice immunised with Protein-based Subunit vaccines to control vaccine groups. Right mouse footpads were infected with 10^5 *M ulcerans* bacteria while the left footpad was used as a control. Percentage change in swelling was calculated as a percentage of the control footpad. N= 5. Error bars represent standard deviation.

In determining the quality of protection offered by vaccine candidates, another way of analysing the results was to determine absolute protection. Mice were evaluated on the question of whether or not they developed swelling. Mice which developed footpad swellings were classified as unprotected (Irrespective of the average percentage change in footpad size). The extent of protection was then represented as the percentage of the mouse population in that group that did not develop any swelling at all. Results are shown in Fig 64 where absolute protection of 100% was recorded in the two Burulivac groups and a 0% protection recorded in the negative control PBS group. 15% of mice in the BCG group received absolute protection while the Ag8A group outperformed the KRA group with 60% of its mouse population receiving full protection. The

Delta only group recorded 70% absolute protection and was a rank lower than the Delta prime, Mycolactone + YC NaMA boost group which had 80% of its population being protected from developing swelling. Mycolactone delivered with spores offered a split percentage protection with 50% of the mice in that group developing protection while the remaining 50% developed swellings of varied sizes. Overall, the graph ranks the various groups in order of the protected population percentage.

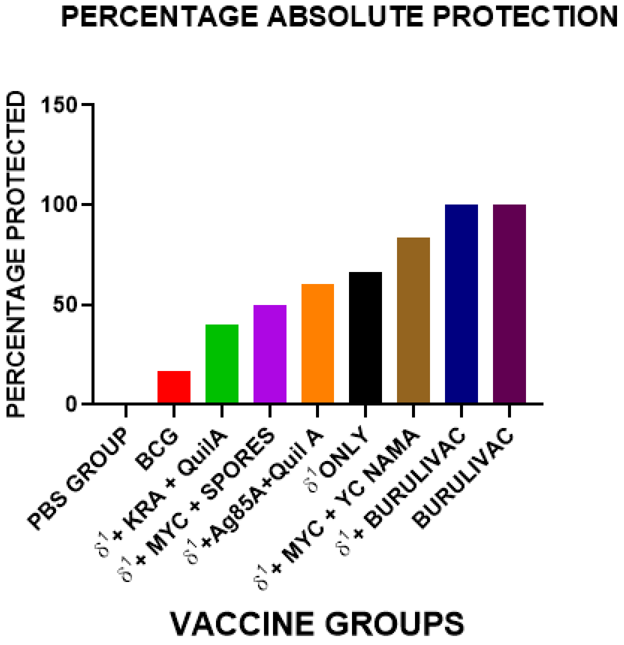


Fig 64. Percentage protection of mice within specific vaccine groups ie, mice which did not develop any swelling at all. Swelling was determined both by physical observation and measurement with callipers. Animals with any observed swelling were classified as ''Not protected''. N=5. Error bars represent Standard Deviation.

6.2.6 Residual *M ulcerans* DNA Copies In Infected Footpads

Combined 16S rRNA RT/IS2404 specific for *M ulcerans* assays were performed on supernatants taken from infected footpads. This was to determine the remnant DNA count in the footpads at the time of cull. There was a wide variation in the population in each group. Expectedly, the number of DNA copies was highest in the control PBS group. Both Burulivac groups and the Ag85A contained no DNA material. Even though an outlying mouse in the KRA group recorded a copy count of 13000, there was no overall statistical difference when compared to either of the BURULIVAC groups. DNA counts in the other groups did not statistically vary from one another. It is however remarkable to note that the DNA counts were very significantly higher in the PBS group.

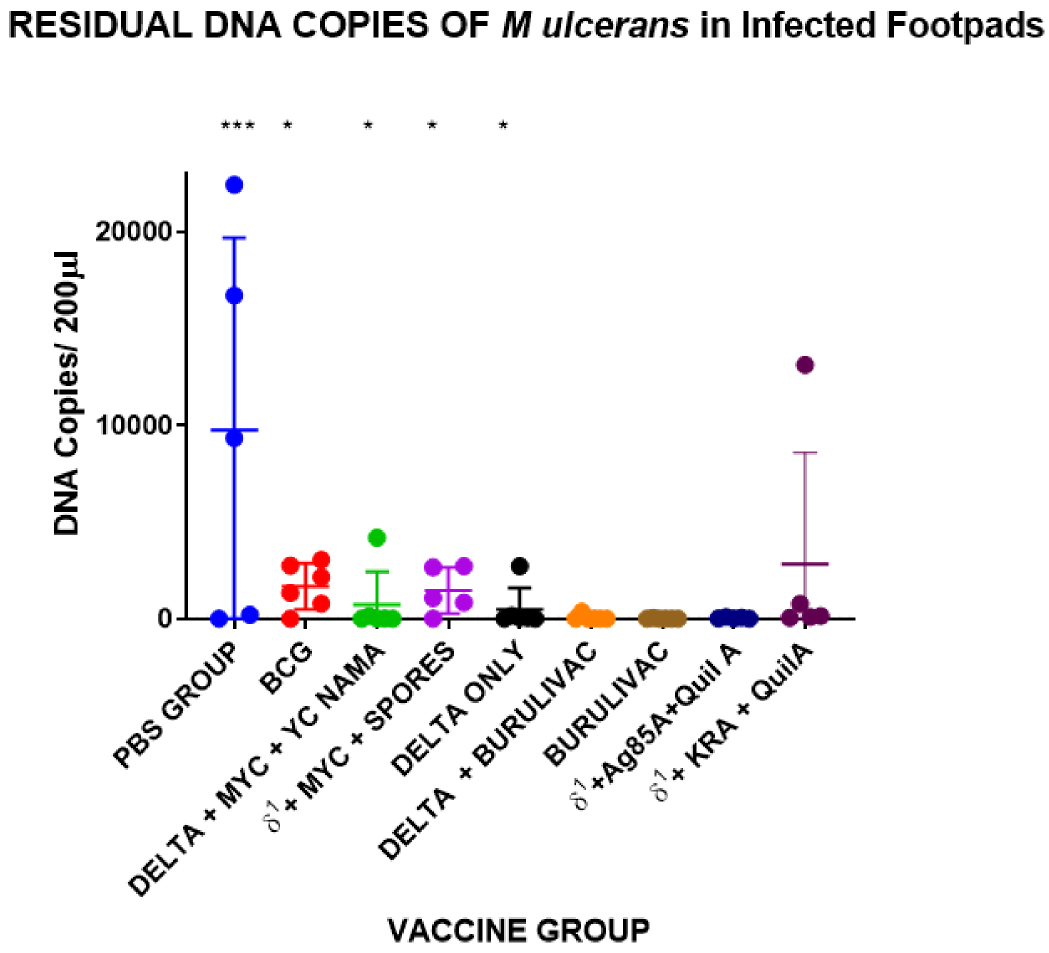


Fig 65. Combined 16S rRNA RT/IS2404 DNA copy count in infected mouse footpads at the point of cull. Vaccinated mice were challenged with Log 5 *M ulcerans* bacteria and monitored for weeks until the pre-determined experimental endpoints for each mouse. DNA copy counts were taken at the endpoint. N=5. Error Bars represent Standard Deviation.

6.3 Discussion:

This chapter being the final experimental chapter focuses on eliciting the protective potential of the designed vaccine candidates. Protection is evaluated through different measurements and experimental designs. In an attempt to unearth the immune mechanisms and correlates underlying the observed protection or otherwise, experiments looking at antibody and cytokine responses were performed on infected footpad supernatants and sera taken from challenged mice.

With the intent of using Mycolactone deficient *M ulcerans* strain 5134 (Delta), preliminary immunisation and challenge showed that compared to a PBS control, Delta from the graphs in Fig 51 was found to be protective. This experiment lasted only 14 weeks during which high dose delta used as a vaccine candidate fully protected mice challenged with 10^5 wild type *M ulcerans* S114. Lower doses of delta were able to delay onset of swelling until week 14. Evidently, Delta does have potential as a vaccine candidate and when adjuvanted or combined with other antigens, may provide the needed long-lasting protection required. It was therefore adopted as a vaccine candidate to be used as a priming vaccine to be boosted with other antigens and adjuvants. As a candidate close in homology (98%) to wild type *M ulcerans*, Delta retains most of the antigens of the wild type bacteria and without the secretion of the immunosuppressive mycolactone (43), is expected to present many common antigens without negative immunomodulation. And when enhanced by adjuvants, antigen presentation is expected to be improved.

According to Fig 52, the Burulivac group without a Delta Prime produced strong IgG and IgM responses to wild type *M ulcerans* lysate in ELISA experiments. The IgM response was however absent without the Delta prime as the Burlivac only group demonstrated good responses to IgG antibodies only. Interestingly, the BCG group responded with moderate IgM secretion while no other vaccine group generated any IgG or IgM responses. With the Burulivac only group producing both IgG and IgM responses but the addition of Delta prime ablating the IgM response, there is an indication that Delta could potentially have anti-IgM abilities by either shifting the immune responses to other pathways or by a mechanism yet to be established, ablates or dampens B-Cell responses. This is further strengthened by the lack of IgM responses in all the vaccine groups initially primed with Delta. It is also instructive to add that none of the other Delta groups generated any IgG responses either. Potentially, Delta either directs immune responses away from humoral pathways or acts by inhibiting B-Cell receptors. This concept of B-Cell inhibition has been known in science and adopted in drug design for the treatment of certain autoimmune conditions such as Rheumathoid arthritis and Multiple Sclerosis (158, 159). The widely used drug Rituximab is an example of an agent that works by this mechanism (160). An alternative explanation is that Delta like all mycobacteria skews the immune response towards cellular immunity with comparatively poor antibody responses. This mechanism has been the basis for the focus of attention on cellular immunity in developing and targeting treatments against Tuberculosis. In these experiments, ablation or inhibition of cellular responses either increased TB infectivity or worsened existent infection. Even in the specific case of *M ulcerans* infections, the effect of the immunosuppressive toxin mycolactone is found to be predominantly on cellular immunity (70, 161, 162, 163, 168). All in all, though still poorly understood, these results also confirm the previously suspected role of neutralising antibodies in the cure of Buruli ulcerans disease as described by Foulon et al (157) in their description of spontaneous healing of *M ulcerans* infection.

In previous publications and studies, attempts to elicit any form of immune responses to mycolactone have produced limited success. Mycolactone in its unaltered state has thus generally been considered non-immunogenic. The mechanisms underlying its cytotoxic and cytopathic properties have been properly studied (51, 96, 149, 150, 162, 164, 165, 166, 167). Beyond the cytotoxic and cytopathic activity of mycolactone which are believed to make it non-immunogenic, there is also the hurdle of experimental designs aimed at eliciting B-Cell responses to mycolactone. Mycolactone, as has been discussed in the introduction, is a lipidic molecule and thus cannot be immobilised onto standard ELISA plates which are designed for soluble hydrophilic molecules like protein (169). Without the ability to venture into any of the advanced experimental methods to identify and measure antibodies, the focus of this project remained on eliciting T-cell memory responses to mycolactone. Hence, splenocytes taken from immunised mice were thus incubated with mycolactone and secreted cytokines then measured by standard cytokine ELISAs. The exact concentration of mycolactone used was based on findings from chapter 4 where the dose dependent activity of mycolactone was investigated. With recall concentrations of 2ng/ml of mycolactone, splenocytes from the Delta + Mycolactone + YC NaMA group, Delta + Mycolactone+ Spores and the Delta + Burulivac groups all secreted IL-2 in response to recall with mycolactone with the highest levels being in the Delta+ Mycolactone + YC NaMA group. While there was no IL-6 response in any of the groups, Delta + Mycolactone + Spores gave IL-10, TNF α and IFN-γ responses with Delta + Burulivac also giving IL-10 responses. These T-cell recall responses to mycolactone are first to be documented in literature, as the dogma has as until now been that it is impossible to elicit and measure any immune responses to the unmodified mycolactone molecule. Also remarkable is the role of spores as an adjuvant. Delta + Mycolactone was presented with two different adjuvants i.e. YC NaMA and Spores. While the YC-NAMA vaccine only elicited IL-2 responses, the spores containing vaccine elicited IL-2, IL-10, TNF α and IFN-γ responses. With the adjuvant type being the only difference between the two candidates, this difference in cytokine profile can be attributed the role of spores. *Bacillus sibtilis* spores are known to activate both TH1 and TH2 mediated pathways by actively inducing cytokine secretion (170, 171). It is thus not surprising that when adjuvanted with spores, a Delta prime immunisation boosted with a Mycolactone based vaccine regime induced broad TH1 and TH2 cytokine responses.

M. ulcerans specific Ag85A is contained as an antigen in *M ulcerans* strain S114 and also in Delta (112, 120). In view of this, recall responses were expected in all vaccine groups as they were either primed with Delta or boosted with an Ag85A containing vaccine formulation. With the exception of the PBS control, all other vaccine groups gave very strong Interferon gamma responses beyond the upper detection limit of the test kit used. Pro-Inflammatory responses (IL2 and TNF α) were detected in all vaccine groups except PBS. With some groups showing no IL6 and IL-10 responses while others only gave weak responses, the observation is that Ag85A is a dominant antigen and drives more of a pro-inflammatory response pathway as opposed to a weakened anti-inflammatory response. It is also observed that even though the Delta only group produced positive pro-inflammatory responses, its responses were the weakest of the vaccine candidates. Delta is known to have 98% homology with wild type *M ulcerans* but without specifying all the exact antigenic differences and the effects these losses have over the immunogenicity of the bacteria, these results suggest a possible attenuation and reduction in the antigenic potency of Delta.

Ketoreductase A is a cytosolic enzyme involved in the synthesis of mycolactone (121, 173). Its immune interactions are therefore limited especially when these responses are extracellular. This is evident in the cytokine responses to various vaccine candidates which did not contain KRA as a constituent antigen. Pro-inflammatory recall responses were strongest in the KRA containing vaccine groups and in the in the mycolactone containing groups with Mycolactone + Spores giving strong responses across board. Presumptively, mycolactone as an antigen maintains antigenic sites with homologous responses to KRA. In chapter 4, we presented novel evidence of antibodies to KRA and in this chapter, we have demonstrated strong cytokine (T-cell) responses to it as well. KRA is therefore established as a potent immunogenic antigen which when used in formulations, may induce good protection against Buruli ulcer disease.

Finally, on the recall assays, cytokine responses to an *M ulcerans* lysate prepared and used as a recall antigen were generally weaker than responses to specific protein antigens. Only Delta + Burulivac group produced IL-2 and IFN-γ responses to this antigen cocktail. Without measuring the quantity of specific antigens presented, a possible explanation to this could be the low concentration of immunogenic antigens in the lysate. Also, antigenic competition in this polyvalent mixture may have resulted in these poor responses. In polyvalence where multiple antigens are pooled together, antigenic competition for limited epitopes have the potential to cause poor immunogenicity responses. Sedegah et al described this phenomenon in detail in 2004 when they tested a polyvalent DNA vaccine against individual antigenic vaccines (174). This potentially explains the poor responses noted in response to the *M ulcerans* lysate used as a recall material.

Ultimately, the extent of protection against Buruli ulcer disease conferred by these vaccine candidates was measured. This was done by looking at two parameters. The first was to determine if the vaccine candidates played any role in the reduction of footpad swelling and thus severity of disease suffered. This was by measuring the percentage footpad swelling by methods described in detail in chapter 3. With the PBS group setting the baseline average percentage change in footpad size at 45% (Fig 59), any change is footpad size below this was considered protection. In effect, all vaccine candidates including BCG conferred some protection albeit not absolute. All vaccine candidates thus mitigated in the severity of disease suffered by the mice. Taking all vaccines which conferred any form of protection into consideration, BCG made the least difference of all the candidates with an average change in footpad size of 38%. The observed effect of BCG confirms previous work done to demonstrate that even though BCG is capable of conferring protection, this is only moderate and transient (99, 102, 107, 175). In comparison, Delta which is also a whole cell vaccine conferred better protection when administered alone. Average percentage swelling was significantly reduced to about half. Delta was used as a prime vaccine and when boosted with either mycolactone or subunit proteins, protection did not significantly improve except when the boost was with the combination vaccine Burulivac. Effectively, despite retaining many of the antigens of *M ulcerans*, this was not enough to confer strong, long-lasting protection. In future, it will be beneficial to evaluate the lost antigens to ascertain if these antigens will provide better protection. Alternatively, it could also hold that, whole cell antigens may not be appropriate for this purpose as the main immunogenic antigens may not be fully expressed in terms of antigenic presentation and quantities. This limitation in the potency of whole cell vaccines can also be attributed to the activation of multiple and diverse immune mechanisms which are not specific to the pathogen of concern. Understandably, this is the result of having multiple antigens presented by whole cells. As observed in pertussis with whole cells vaccines for instance, there is an induced Th1/Th17 pathway response whereas studies have shown that optimal protection to pertussis via through Th2 response pathways. This skewed response emanates from the net effect of competing antigens and epitopes (177,176,178).

For the mycolactone containing vaccine candidates, mycolactone with YC NaMA gave the best mitigation in disease severity with mice in that group suffering just about 10% change in footpad size. This was double in the mycolactone with Spores group. Considering the fact that both vaccine regimes involved a Delta prime with boosts containing mycolactone, the difference in potency of the candidates can be attributed to the effect of the adjuvants and delivery systems used. In this regard, YC NaMA enhanced the vaccine potential of mycolactone better. We therefore establish YC-NAMA as a potent adjuvant in the induction of protective immune responses to mycolactone.

Ag85A and KRA are both subunit vaccine candidates. Combined with Quil A which is a saponin based adjuvant and used as boost vaccines to Delta primed mice, Ag85A proved to be superior. It is also important to mention that even though Ag85A as a boost vaccine did not significantly improve upon the protection conferred by the Delta prime only, it did not worsen disease severity as seen in the Delta prime, KRA boost group. Change in footpad size was almost doubled when KRA + Quil A was added on as a boost vaccine. This may be difficult to explain but could potentially exemplify antigenic competition. Antigenic competition is a phenomenon in which there is inhibition or diminution of the immune response to one antigen or determinant occurs following its administration in the presence of another antigen or determinant (179, 180). In this situation, KRA potentially displaced Delta epitopes and elicited the immune responses hitherto directed at Delta. These responses were however qualitatively not comparable to those elicited by

Delta. The augmentation of the quality of protection observed with the Ag85A boost is an example of antigenic synergy: A phenomenon in which the response observed to one antigen is augmented by the presence of another (181, 182).

Burulivac, the vaccine candidate comprising Ag85A, KRA and Mycolactone combined with Quil A turned out to give the best protection. There was no footpad swelling in any of the mice vaccinated with this candidate. One group was primed with Delta while the other wasn't. There was however no difference in the level of protection observed in the two groups, with both showing high level of protection. Even though pro-inflammatory cytokine responses were generally poor in the Burulivac groups, the response seen to IL-10 was the highest among all the vaccine groups. IL-10 as previously discussed is anti-inflammatory in action and is in this case seen to correlate with protection. The role of IL-10 has been studied in viral infections. It is known to promote and modulate viral persistence. Even in TB, this effect is observed. IL-10 blockade has thus been considered as an immune regulatory mechanism to enhance vaccine potency. Already, experiments combining BCG with IL-10 blockade demonstrated enhanced BCG effect (183, 184, 185). Our observation in this project therefore goes to reinforce the ambivalent interplay of pro and anti-inflammatory factors involved in protection against *M ulcerans* disease. It also affirms the observation of Mangas et al (186). In their paper, they concluded following mouse model experiments that a potent vaccine against Buruli ulcer disease must induce tissue-specific immune profiles with controlled inflammatory responses at the site of infection. These responses should in include high titres of cytokines such as IL-10. Further credence is given to this by the observation of low titres of pro-inflammatory cytokines such as IFN-ϒ, TNF **α** and CRP as opposed to the higher titres of IL-10 in footpads of mice with the least footpad swelling.

In Fig 64, protection is looked at as an absolute measure. Measurement of footpad swelling using percentage swelling provided discrete measures of protection. However, it is important to also know in absolute terms whether individual mice developed swelling. To do this, mice within each vaccine were categorized based on footpad size as whether protected or not protected. Any form of swelling was considered not protected. The number of protected mice were then represented as percentage of the total. All mice in the PBS group developed swelling. There was therefore 0% protection. The BCG group recorded 20% protection with 80% of mice in the group developing swellings of varying degrees. Without any boost, Delta prime conferred absolute to 70% of the mice vaccinated. When boosted with Ag85A, absolute protection waned to 57%. When boosted with KRA, it went further down to 48%. When boosted with Mycolactone, the addition of YC NaMA as an adjuvant improved protection. That group recorded absolute protection in 80% of the mice. A worsening effect was however noted when the mycolactone was adjuvanted with spores (50%). Both Burulivac groups whether primed with Delta or not conferred absolute protection in all mice (100%). An interesting finding is made with respect to three groups which recorded approximately equal average percentage swellings ie Delta only, Delta Prime wit Mycolactone + YC NaMA boost and Delta prime with Ag85A + Quila A boost. There were significant differences when absolute protection was measured. Many mice in the Delta prime, Mycolactone + YC NaMA boost group were without swelling (80%). Delta prime with Ag85A + Quil A boost however recorded a lower rate of absolute protection (57%). It was even lower than that observed in the Delta only group. So, even though these groups recorded similar average swellings, this resulted from fewer mice in the Mycolactone + YC NaMA boost group. Swellings in unprotected mice in that group were thus bigger in size as compared to the Delta prime with Ag85A + Quil A boost which recorded smaller swellings in many more mice. It is therefore evident that while Delta prime with Mycolactone + YC NaMA boost as a vaccine provided absolute protection for majority of the mice in that group, mice within that same group which did not benefit from the absolute protection recorded big swellings as well. There must be intrinsic factors within mice in these groups accounting for this intra-group variation.

Finally, it was important to identify the bacterial load in the injected footpads at the time of terminating the experiment. DNA residual copies (IS2404) were thus measured. Very significantly, the load was much higher in the PBS group. Both Burulivac groups had no bacteria. The Delta + Ag85A+Quil A group also had no bacteria in the footpads. Since there was significant footpad swelling in this group, the inference is that the swelling was potentially the result of a persisting inflammation and not the presence of bacteria. Relating it to the relatively high levels of IL-10 measured in the footpads of mice in the Burulivac groups, it supposes that protection is a factor of the extent of anti-inflammatory activity occurring at the site of infection (186). Conclusively, a good vaccine against Buruli ulcer disease should be able to induce good IgG and IgM antibodies while promoting strong anti-inflammatory or inflammation suppression at the site of infection to prevent disease establishment or mediate in the severity of disease established. This will in future guide design and selection of vaccine candidates against Buruli ulcer disease.

CHAPTER 7

CONCLUDING SUMMARY

In this study, three broad aims were set. Summarising findings under each aim:

♦ To investigate and establish the immunogenicity of Mycolactone

We have established the cytotoxic effect of mycolactone and demonstrated that this is dose dependent. We also showed that this effect is not universal as various cell types are affected to varying extents even when exposed to similar concentrations of mycolactone. This trend is also seen with regards to the cytopathic effect of mycolactone, ie its ability to suppress cytokine secretion by immune cells. Through proxy measures of mycolactone concentrations in infected mice footpads, we observed that its effect in infected mice is systemic and not only limited to the site of infection. Its concentration is however higher at the site of infection. We determined that at specific concentrations, the immunosuppressive effect of mycolactone is ablated despite its presentation and recognition by immune cells. In mouse splenocytes, we determined this concentration to be below 18ng/ml. Additionally, we have established that YC NaMA as a delivery platform is able to ablate the cytopathic effects of mycolactone even at high doses of the toxin, presumably by immobilising it on their surface and thus reducing bioavalability.

Combining these learnings, we were able to induce appropriate cytokine recall responses to mycolactone in mice immunised with the right concentrations of mycolactone presented with varying delivery systems and adjuvants. Antigen recall assays were performed with splenocytes obtained from the immunised mice and incubated with the right concentrations of mycolactone as previously determined. Through this, we established the significant role of Interferon gamma in mycolactone immunity.

Finally, in attempting to circumvent the cytotoxic and cytopathic effects of mycolactone, we experimented with the polyketide synthase enzymes involved in the synthesis of mycolactone in *M ulcerans*. We determined that these were not immunosuppressive and for the first time, induced antibody responses to KetoReductase A, one of the enzymes. All enzymes tested demonstrated immunogenic potential to varying degrees.

- To determine the protective ability of the Polyketide synthase enzymes involved in the synthesis of Mycolactone.

We have demonstrated that in combination with Quil A, the polyketide synthase enzyme KRA is able to protect against *M ulcerans* disease. This protection is however partial and short-lived. When used as a component of a composite vaccine consisting of KRA, Mycolactone, Ag85A and Quil A (BURULIVAC), pathogen-challenged mice were conferred with absolute protection over the 14-week period. KRA was selected from a pool of tested polyketide synthase enzymes after evaluating T-cell and B-cell responses elicited in immunised mice.

- To design and investigate the protective ability of a mycolactone based vaccine against Buruli ulcer disease

From understanding obtained from our mycolactone and mouse infection models, 7 vaccine candidates developed from various combinations of mycolactone, KRA, Ag85A, Delta and adjuvants/delivery systems were tested. Our lead candidate, BURULIVAC conferred absolute protection against challenged mice. This was seen irrespective of whether or not mice were primed with Delta. Delta, which is mycolactone deficient *M ulcerans* also conferred limited protection, albeit stronger than BCG. IL-10 emerged as a reliable correlate of protection. Interpreting the interplay of immune mechanisms observed, we conclude that the composite vaccine BURULIVAC, confers potent protection against Buruli ulcer disease and this is done by inducing strong antibody responses while also promoting strong anti-inflammatory mechanism at the site of infection.

Guided by these findings, we intend to progress with BURULIVAC in future studies to determine the longevity of protection offered while delving deeper into its mechanisms of action.

CHAPTER 8

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