

UNIVERSITY OF GHANA, COLLEGE OF BASIC AND APPLIED SCIENCES



**MEMBRANE VESICLES OF *MYCOBACTERIUM ULGERANS* AND THEIR ROLE
IN BURULI ULCER PATHOGENESIS**

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(10702149)

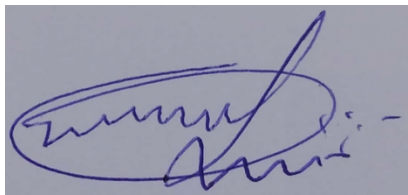
THIS THESIS/DISSERTATION IS SUBMITTED TO THE DEPARTMENT OF
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MPHIL IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE

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DECLARATION

I ERIC NTIFO OSEI, do hereby declare that with the exception of references to other people's work which have been duly acknowledged, this thesis is the outcome of a research I duly conducted at West Africa Center for Cell Biology of Infectious pathogens, Department of Biochemistry, Cell and Molecular Biology, College of Basic and Applied Science, University of Ghana, Noguchi Memorial and Medical research, University of Ghana and Laboratory of Professor Karen M. Dobos, University of Colorado, USA. The research was carried out under the direct supervision of Dr. Lydia Mosi. Also, I declare that neither all nor part of this thesis has been presented elsewhere for another degree.



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ABSTRACT

The release of bacterial extracellular membrane vesicles (EMVs) is essential for pathogen's adaptation and virulence. *Mycobacterium ulcerans*, the causative agent of Buruli ulcer, remains with queries in its pathogenic mechanism. The current study interrogated biological functions and protein content of EMVs from viable *M. ulcerans*' cells as potential medium of virulence in BU pathogenesis. Here, we demonstrate release of intact EMVs from the thick cell wall of log-phase *M. ulcerans* (Nm 209) as well as *M. marinum* (Sa 200695) in respective liquid cultures. Size distributions of isolated EMVs were similar between the two strains and did not differ from EMVs released by *M. smegmatis* used as a positive controlled strain. Mycolactone could not be detected in isolated EMVs from *M. ulcerans* (Nm 209). However, presence of *M. ulcerans* EMVs was associated with higher total intracellular reactive oxygen species which eventually compromised viability of RAW264.7 cells through oxidative stress. After 48 hours of co-incubation, native and UV-A irradiated EMVs induced 45% and 40% loss in viability of RAW264.7 cells, respectively. Moribund phagocytes exhibited apoptotic changes. Proteomic analysis on the isolated *M. ulcerans* EMVs revealed an enrichment of 32 unique proteins mostly localized in the pathogen's cell wall/membrane. A conserved hypothetical protein (MUL_2313), had the highest log₂ fold change (11.92) followed by Amidase amiC, a cell-wall remodeling hydrolase (4.19). Others included integral membrane indolylacetyltransferase EmbA/B, and many conserved hypothetical proteins. Direct contributions of these proteins to EMVs cytotoxicity could not be established. Yet, protein moon-lighting or possible cross-linking could have potentially contributed to EMV-associated toxicity on RAW264.7 cells. Our results suggest that *M. ulcerans* EMVs can elicit toxic response from host's macrophage cells through yet to be established mediators. This potentially reveals new dimension on macrophage-*M. ulcerans* interactions with possible contribution to local immunosuppression in BU and paradoxical reactions observed in its treatment.

DEDICATION

I dedicate the research outcome to God Almighty and His beloved son, Jesus Christ, for the motivation, revelations and continuous support in my entire academic life. Finally, I dedicate this thesis to the entire staff at Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon.



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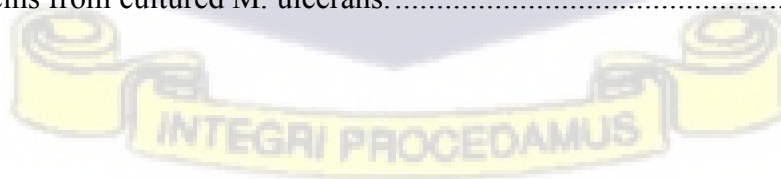
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LIST OF ABBREVIATION

EMV	Extracellular Membrane Vesicles
PLC	Phospholipase C
PLD	Phospholipase D
MVB	Multi-vesicular bodies
ILVs	Intra-Luminal Vesicles
μL	Microliter
TLC	Thin layer Chromatography
UV	Ultra-violet
CF	Culture filtrate
PBS	Phosphate Buffered Saline
MVs	Membrane vesicles
M7H9	Middlebrook 7H9
TBHP	Tert-butyl Hydrogen Peroxide
EVs	Extracellular Vesicles
ECM	Extracellular vesicles
BMVs	Bacterial Membrane Vesicles
MVs	Membrane Vesicles
WHO	World Health Organization
OMVs	Outer membrane vesicles

nM	Nanomolar
°C	Degrees celcius
BU	Buruli ulcer
ZN	Ziehl Neelsen
TEM	Transmission electron microscopy
BMVs	Bacterial membrane vesicles
DMEM	Dulbecco's Modified Eagle Medium
MABA	Microplate alarmablue assay
DCFDA	Dicholofluoreceine diacetate
OADC	Oleic acid, Albumin, Dextrose and Catalase
SDS-PAGE	Sodium Dodecyl sulfate Polyacrylamide Gel Electrophoresis
BMDM	Bone marrow-derived macrophage (Murine



CHAPTER ONE (1)

INTRODUCTION

1.1 Background

Buruli ulcer (BU), caused by infection with *Mycobacterium ulcerans*, remains a formidable Neglected Tropical Disease (NTD) with many measurable evidence gaps in disease transmission, pathogenesis and rapid diagnostics (WHO, 2019). As a result, many victims who are mostly productive individuals in endemic rural Western and Central Africa, as well as specific focal areas in Western Pacific region, suffer chronic, debilitating skin ulcers (WHO, 2006). The consequences are far-reaching. Victims can get disfigured, disabled and immobilized and have to endure considerable issues of physical deformity, psychosocial and socio-economic losses which in turn keep them in vicious cycle of poverty (Garchitorena *et al.*, 2015).

In response to this, the World Health Organization (WHO) has taken several steps to find the most cost-effective means to stem the current tide of this devastating skin disease. Efforts include the basal Yammosoukrom Declaration (WHO, 1998), the World Health Assembly (WHA) Resolution 57.1 (WHO, 2004), Global Plan to Combat Neglected Tropical Diseases, 2008-2015 (WHO, 2007), the Cotonou Declaration (WHO, 2009), The Four (4) Programmatic targets, 2013, Collaborations with Foundation for Innovative New Diagnostics (FIND) (WHO, 2018) and many more. Yet, the overall progress against Buruli ulcer is reported sub-optimal, particularly in aspects of molecular confirmation of suspected cases by PCR and search for additional *M. ulcerans*-specific biomarker (s) which could serve as potential target (s) for rapid detection of the disease for appropriate, now recommended fully oral antibiotic treatment and also help to explain the pathophysiology of *M. ulcerans* (Omansen *et al.*, 2019;Phillips *et al.*, 2020;WHO, 2018;WHO, 2019).

Bacterial pathogens have evolved several mechanisms to circumvent the host's defense systems and induce virulence. Species like *Neisseria meningitidis*, *Streptococcus pneumoniae*, and some strains of *Mycobacteria* have their antigenic surfaces covered with complex polysaccharide-rich layer called capsules. This helps pathogens to evade the host's defense surveillance and resist phagocytic attacks (Paton *et al.*, 2019). Other species including *Escherichia coli* possess appendages like flagella, fimbriae and adhesins by which they move and attach to host's epithelium to avoid the sweeping current on mucosal surfaces. Many more pathogens like *Shigella* and *Salmonella* species have evolved powerful protein secretory systems like "injectasomes" that could shuttle exotoxins from the bacterium to either proximal or distant host's targets for diseases onset (Diard *et al.*, 2017; Galán *et al.*, 2018; Green *et al.*, 2016; Moradali *et al.*, 2020). All these structural adaptations are meant to facilitate bacterial colonization fitness in host tissues and ideal conditions for infectious diseases (Diard *et al.*, 2017; Lacey *et al.*, 2020). On the contrary, traces of these Pathogen Associated Molecular Patterns (PAMP's) can provide significant source of information on the presence of such microbes in host's tissues, disease pathogenesis and development of effective control measures against such infectious organisms.

The only known biomarker of *M. ulcerans*, which also acts as the sole virulent factor of this unique pathogen, is a plasmid encoded polyketide-derived macrolide called mycolactone (Demangel *et al.*, 2018; George *et al.*, 1999). This molecule is membrane diffusible and has many cytotoxic activities (Hall *et al.*, 2014). It can perniciously engage critical actin-nucleating factors like ARP 2/3 to polarize actin polymerization and cytoskeletal remodeling in the cytosol of host's cells. Eventually, the cells die by anoikis apoptosis as they detach, round up and lose viability (Guenin-Mace *et al.*, 2013).

Mycolactone can also attack and block critical functions of heterotrimeric Sec61 translocon on rough endoplasmic reticulum (ER), which serves as a conduit for transport of nascent polypeptides from ribosomes into the ER for subsequent post-translational modification and localization (Hall *et al.*, 2014). In consequence, mammalian cells and tissues get depleted of essential proteins like type-I transmembrane proteins, immune effectors, secretory and structural proteins (Hall *et al.*, 2014). Mycolactone can also interact and block the nociceptive function of sensory neurons via angiotensin-II, type 2 receptor (AT2R) and cause the perceived lack of pain in Buruli ulcers lesions (Song *et al.*, 2017). All these toxic, pleiotropic interactions of mycolactone released by *M. ulcerans* are considered necessary and sufficient for tissue necrosis, hypoalgesia, local immunosuppression and the chronic, progressive nature of Buruli ulcer lesions on the skin (Demangel *et al.*, 2018). As a result, this lipid-like macrolide is being investigated as a specific biomarker for early and rapid diagnosis of *M. ulcerans*' infections (Naranjo *et al.*, 2019).

Although feasible and laudable as it now appears, such an effort may come with many possible downsides. Firstly, the current understanding of the dynamics and kinetics of mycolactone, particularly in mammalian tissues, appears incomprehensive. Secondly, the presence of *M. ulcerans* in the host's tissues may not necessarily guarantee bioavailability of mycolactone for detection. Thirdly, mycolactone is unstable in host's tissues and rapidly degrades once released from *M. ulcerans* (Babonneau *et al.*, 2019). Fourthly, the biosynthetic pathway of this macrolide is liable to inhibition by certain sugars like glucose, maltose and maltopentaose suggesting that *M. ulcerans* can survive in environments with these sugars without the presence of mycolactone (Deshayes *et al.*, 2013). Finally, it is discussed that *M. ulcerans* can turn off its biosynthetic machinery for mycolactone production when it is challenged with unfavorable growth condition (Converse *et al.*, 2014).

All these uncertainties surrounding the utility of this unique virulent factor as a potential target for rapid Buruli ulcer diagnosis necessitate the need to re-consider a more robust, specific exogenous protein (s) from *M. ulcerans* which can serve the needed purpose as an ideal biomarker for early detection of the disease.

Empirical evidence from earlier studies supports the potential secretion of specific virulent proteins from *M. ulcerans* into its growth medium. Dobos *et al.*, (2000) used one (1) dimensional SDS-PAGE to resolve three (3) unique proteins in culture supernatant of this pathogen at mid-logarithmic growth phase. The proteins were immunogenic and could be recognized by antibodies found in sera of patients with confirmed Buruli ulcer cases. The identities of these proteins could not be established. But their sero-diagnostic potential was suggested (Dobos *et al.*, 2000). Such a seminal study propelled subsequent detection of hemolytic Phospholipase C (PLC) and Phospholipase D (PLD) in culture supernatant of viable *M. ulcerans* cultures in mid-log phase of growth (Gomez *et al.*, 2000). Functional activities of these lipases were confirmed by the authors. Bacterial phospholipases are virulent on mammalian cells and implicated in many diseases caused by mycobacterial pathogens (Kumari *et al.*, 2020; Roberts *et al.*, 2018).

Possible activities of these exogenous proteins from *M. ulcerans* were further confirmed when it was observed that 20µg of its culture filtrate (CF) can induce apoptotic changes in monolayer of human adipose cell line (SW872) including intra-cellular granules, pseudopodium formation, accumulation of vacuoles and cellular debris while purified mycolactone induced necrosis (Dobos *et al.*, 2001). This apoptotic effect of CF was significantly abrogated when the filtrate was treated with proteinase K coupled with heating to denature at 100°C suggesting that potential contribution of specific polypeptides in the CF to the apoptotic effect. Therefore, it

was not much of a surprise for the authors to isolate 38-kDa protein and high-molecular weight lipoproteins from the CF (Dobos *et al.*, 2001). These toxic proteins may have acted either independently or in synergy with mycolactone to cause the apoptosis in the SW872 cells (Dobos *et al.*, 2001;Kubicek-Sutherland *et al.*, 2019).

Additionally, 19 unique proteins from *M. ulcerans* in culture were isolated and identified in a seven (7)-week culture supernatant of this mycobacterial pathogen. These proteins included an ESAT6-like protein, ExG (MUL_1209), a chitinase (MUL_2210), three (3) members of the PE/PPE family (MUL_1207, MUL_2427, MUL_2737), eight (8) secreted antigens (Fbp A/B/C/D, MUL_2970, 4793, 4986, 4987) and six (6) conserved secreted proteins (Mtc28, MUL_0055, MUL_0607, MUL_2974, MUL_3477, MUL_4936) (Tafelmeyer *et al.*, 2008). Even though, the authors could not confirm the exact contributions of these exogenous antigens to Buruli ulcer lesions, their presence in the environment of *M. ulcerans* suggests possible secretory machinery and pathogenic mechanism (s) of this mycobacterium.

How these proteins got released into the growth medium of *M. ulcerans* is unknown. *M. ulcerans* is known to lack active putative ESX-1 secretory system common to many pathogenic strains of mycobacterium (Huber *et al.*, 2008). Yet about 20% of total proteins found in its growth medium had signal peptide sequence (Tafelmeyer *et al.*, 2008). The canonical secretory system might have contributed in this regard. However, reason for the 80% protein residue found in the extracellular milieu without secretory signal was proposed as potential bacterial cell lysis in culture or yet to be characterized protein secretion mechanism(s) (Tafelmeyer *et al.*, 2008). Accumulating evidence suggest pathogenic mycobacteria to utilize extracellular membrane vesicles (EMVs) as medium of transport of virulent proteins in host's tissues (Athman *et al.*, 2015;Maas *et al.*, 2017;Wang *et al.*, 2019).

EMVs are lipid bilayer nanoparticles which mostly originate from membranous compartments of viable bacterial cells by yet to be established mechanism and get eventually discharged into the environment. They play a critical role in bacterial cell adaptations and pathophysiology of many pathogens through various complexes of proteins, lipids, polysaccharides, nucleic acids and other small molecules they often carry (Zingl *et al.*, 2019). As a result, EMVs are reported in processes of biofilm formation, immune evasion, excretion of toxic waste molecules from parent bacterial cells, scavenge for nutrients in host's environment, horizontal gene transfer and also, act as molecular "punch bags" by which pathogens launch cytotoxic cargoes at both proximal and distant host's cells for disease onset (Chiu *et al.*, 2020; Rueter *et al.*, 2020; Schorey *et al.*, 2016; Vdovikova *et al.*, 2017). The release of MVs is an evolutionary conserved one and reported among various species of both gram negative and positive bacteria acting as key mediators in both intra-species and inter-species communications (Coelho *et al.*, 2019; Jurkoshek *et al.*, 2016; Maas *et al.*, 2017).

Strains of *Mycobacterium* are no exception to this adaptive cellular process. Fast and slow growers as well as pathogenic and non-pathogenic strains are reported to release heterogenous EMVs in both natural and artificial niches, ranging in diameter from 20 nm to 300 nm range (Prados-Rosales *et al.*, 2011). Vesicles from mycobacterium also carry various proteins many of which are either toxic to mammalian host's cells or possess immunomodulatory functions against host's defense systems (Brown *et al.*, 2015; Gupta *et al.*, 2018).

Many of these proteins have been identified in isolated membrane vesicles from patients' samples and various culture media using advanced technologies like Multiple Reaction Monitoring Mass Spectrometry (MRM-Ms) and other biochemical means. Isolated proteins included Antigen 85B, Antigen 85C, Apa, BfrB, GlcB, HspX, KatG, Mpt64, LpqH, LprG and

other toxic lipoproteins (Athman *et al.*, 2015;Kruh-Garcia *et al.*, 2014). Further studies found as much as 287 different proteins in MVs released from viable *M. tuberculosis*. Among other antigens included HbhA, TatA, Hup, Acn, FbpA, FbpB, FbpC and SodB which are critical for the pathogen's intracellular survival, growth and dissemination in host's tissues (Lee *et al.*, 2015). Additional virulent proteins like Cfp2, Mpt32, Mpt64 and BfrB have been reported as cargoes in membrane vesicles released by *M. tb* (Mehaffy *et al.*, 2017).

More recently, an exploratory study found LpqH to be enriched in isolated MVs of *M. tb* and indicated its potential utility as a differentiating biomarker to discriminate bovine diseases caused by *M. avium subsp paratuberculosis* from infections caused by either *M. tuberculosis* or *M. bovis* (Palacios *et al.*, 2019). Many more proteins are identified in vesicles released by diverse pathogens of mycobacterium as extensively summarized in a recent report (Wang *et al.*, 2019). Such membrane vesicles are stable in biological systems and maintain their physiologic activities against host tissues. They could elicit either strong pro-inflammatory response from both innate macrophage and endothelial cells (Athman *et al.*, 2015;Li *et al.*, 2018;Prados-Rosales *et al.*, 2011) or profound anti-inflammatory response with T-cell anergy (Athman *et al.*, 2017). All these reports suggest that exogenic membrane vesicles from pathogenic mycobacteria can carry specific PAMP's to play critical role in colonization fitness of mycobacteria. Also, these vesicles-associated PAMPs can provide useful sources of biomarkers for purposes of diagnostics and therapeutics, especially against "tool-deficient" disease like Buruli ulcer (Raeven *et al.*, 2018;Wang *et al.*, 2019;Ziegenbalg *et al.*, 2013).

1.2 Justification

Intriguingly, almost all the existing data on mycobacterial EMVs originate from clinically significant tuberculous strains including *M. tuberculosis* and *M. bovis* (Athman *et al.*, 2017;Prados-Rosales *et al.*, 2011;Ziegenbalg *et al.*, 2013). Data on clinically relevant non-

tuberculous strains are sparse in literature. Also, information on EMVs released by *M. ulcerans* is scarce. Only two studies, all emanating from a single Laboratory (Marsollier *et al.*, 2007 and Foulon *et al.*, 2020) report on *M. ulcerans* EMVs. Interestingly, none of these studies had EMVs release and functions as its primary objective. Their reports were secondary to the overall objective of the study. *M. ulcerans* EMVs, found in extracellular matrix, was proposed to transport mycolactone to induce virulence (Marsollier *et al.*, 2007). Such vesicles from mycolactone-producing strain demonstrated cytotoxic activities on cultured macrophage, HeLa and cos cell lines. Vesicles from mycolactone-deficient *M. ulcerans* failed to exhibit similar effect on the mammalian cells. Another study suggested *M. ulcerans* vesicles to supply the first signal required for mycolactone to induce IL-1 β maturation and secretion from host macrophage cells (Foulon *et al.*, 2020). However, we do not know how EMVs, with/without mycolactone, affect survival of interacting macrophage cells which are critical to appropriate immune response to invading mycobacterium. Also, not much is known on proteomic contributions to BU pathogenesis. Detailed proteomic analysis on discharged EMVs from *M. ulcerans* may provide additional insight on pathogenic mechanism and development of Buruli ulcer.

1.3 Hypothesis

The current study hypothesized that extracellular membrane vesicles from *M. ulcerans* modulate survival of host's macrophage cells through their protein content. This was based on three (3) key observations. First, cytotoxic lipoproteins, chitinase, antigen 85 complex, phospholipases C and D among others, existed in growth medium of growing *M. ulcerans* which lacks the putative ESX-1 secretory system common to many pathogenic mycobacteria (Dobos *et al.*, 2001; Franco-Paredes *et al.*, 2019; Gomez *et al.*, 2001; Tafelmeyer *et al.*, 2008). Secondly, other pathogenic strains of mycobacterium, which do not produce mycolactone,

actively release MVs to mediate virulence in their environment (Athman *et al.*, 2015; Prados-Rosales *et al.*, 2011; Ziegenbalg *et al.*, 2013). Finally, tissue-resident macrophage cells are key targets for manipulation by invading pathogenic mycobacteria (Weiss *et al.*, 2015).

1.4 Overall Aim

Therefore, the study interrogated biological functions and protein content of *M. ulcerans* EMVs.

Specifically, we;

1. Isolated extracellular membrane vesicles from growth medium of *M. ulcerans*.
2. Assessed the effect of *M. ulcerans*' EMVs on viability of RAW 264.7 cell lines.
3. Analyzed mycobacterial proteins associated with isolated EMVs from *M. ulcerans*.

1.5 Study's Relevance

To date mycolactone remains the only known virulence factor of *M. ulcerans*. No other factor/mechanism of virulence is known to date. The current study provides interesting new dimension on *M. ulcerans* pathophysiology providing details on how active membrane remodeling and subsequent release of EMVs from viable *M. ulcerans* cells modulate functions and survival of interacting macrophage cells. Thus contribute to Buruli ulcer pathogenesis through potentially unique form of host-*M. ulcerans* interactions. Also, proteomic evidence provides insight on other factors that may possibly play to Buruli ulcer development. The evidence further initiates a potential search for protein-based biomarkers that may serve as ideal targets for rapid detection of *M. ulcerans*' infections and also vaccine development (Wang *et al.*, 2019). Eventually, a more effective disease control measures could be developed to minimize morbidity and disability associated with Buruli ulcer (Omansen *et al.*, 2019).

CHAPTER TWO (2)

LITERATURE REVIEW

2.1.0. Buruli ulcer Disease

Buruli ulcer is a neglected tropical skin disease (skin NTD) that is often described as destructive, unsightly, debilitating and incapacitating to affected individuals. The causative agent, *Mycobacterium ulcerans*, has special preference to the dermis and subcutis where it wrecks considerable damage to cells and skin tissue integrity mainly through its plasmid encoded polyketide-derived macrolide called mycolactone. The resulting skin lesions are often chronic and progressive and can cover as much as 15% of skin surface if not treated leaving in its wake an unsightly necrotic slough of subcutaneous fat with typically undermined wound edges (Kumar *et al.*, 2015;WHO, 2013). Long-term consequences include physical incapacitation, stigmatization, and loss of productive ventures (Yotsu *et al.*, 2018).

2.1.1. Brief Historical Perspective

The disease was first described by a British missionary and a physician, Sir Albert Ruskin Cook, in 1897 (Billington, 1970). He described cases of chronic progressive skin ulcers in medical records of patients he managed in a grass-roofed and reed-walled, 12-bed capacity Mengo Hospital located in Uganda, Africa. This description was later found to be consistent with Buruli ulcer. After three (3) decades of virtually no scientific report on the disease, physicians working in the Bairnsdale region of Victoria, Australia, were challenged with “unfamiliar” skin ulcers on six (6) patients from the region. Samples from the lesions contained Acid-Fast Bacilli (AFBs) but such laboratory diagnosis was inconsistent with the then known mycobacterial diseases, tuberculosis and leprosy. Therefore, the patients were referred to Alfred’s Hospital in Melbourne, Australia, for tertiary healthcare. Skin biopsies from the lesions were also transported to University of Melbourne for scientific investigations into the

microbiology of the disease. These laudable efforts led to the first successful *in vitro* isolation of *Mycobacterium ulcerans* (Johnson, 2020).

In 1948, MacCallum *et al*, published the first scientific report on the disease, titled “*A New Mycobacterial Disease in Man*”. The report detailed histological features of Buruli ulcer lesions and showed how animal models can be used to obtain colonies of this unique mycobacterial pathogen which exhibited unique growth requirement (MacCallum *et al.*, 1948). In recognition of where the first infectious samples originated, the isolated mycobacterium was initially named Bairnsdale bacillus and the disease, Bairnsdale ulcer (BU). Several other names like Daintree ulcer, Searl ulcer and Mossman ulcer emanated afterwards depicting where and how the disease was reported for clinical intervention. However, following the first reported outbreak of this disease in Buruli county, Uganda, Africa, in 1961 and also case incidences in other parts of the world, the pathogen’s name was changed to *Mycobacterium ulcerans* and the disease, Buruli ulcer (BU) (Pluschke *et al.*, 2019). Now, BU remains a global threat to public health with current global prevalence ranging from 3.2 to 29.6 cases in every 10,000 populations (Simpson *et al.*, 2019).

2.2.0. Disease Presentation

2.2.1. Manifestations of Buruli ulcer Lesions

Cases of Buruli ulcer vary in manifestation and clinical presentations. Skin infections with *M. ulcerans* can manifest as a 1-2 cm skin induration/nodule that extends deep into the subcutaneous tissues showing discolored surroundings (**Figure 2a**). The disease can also begin as painless, slightly raised skin papule or plaque measuring < 1 cm in its widest diameter with inflamed surroundings (**Figure 2b**). Another non-ulcerative manifestation is an extensive, diffused skin oedema with ill-defined borders (WHO, 2004) (**Figure 2c**).



Figure 2.0: Clinical manifestations of Buruli ulcer (WHO, 2012, Walsh *et al.*; 2009). (a) Nodule, (b) edema, (c) plaque, (d) small ulcer (e) large ulcer and (f) disability

More characteristically, Buruli ulcer manifests as a small skin ulcer (**Figure 2d**) that can steadily spread irregularly, showing extensive slough of necrotic subcutaneous fat and typically undermined edges (**Figure 2e**). Some evidence suggests that BU progresses from non-ulcerative nodule or plaque through edema to ulcerative lesions (Röltgen *et al.*, 2020). However, there is currently no predictable pattern of Buruli ulcer manifestation (Omansen *et al.*, 2019). Any of the above manifestations can occur first and factors which determine how infection with *M. ulcerans* manifests include the immune competence of affected individuals, genetic constitution of species/lineage involved in infection, type and amount of mycolactone it can synthesize and secrete and the likely mode of transmission of infection (Mve-Obiang *et al.*, 2003; Nakanaga *et al.*, 2011; Omansen *et al.*, 2019; Takahashi *et al.*, 2020; Wallace *et al.*, 2017). Therefore, ulcerative lesions may not always occur as a direct consequence of late detection, neither does it predict late manifestation of *M. ulcerans* infection (Omansen *et al.*, 2019).

2.2.2. Clinical Categories of Buruli ulcer Lesions By WHO

To enhance better understanding of BU for appropriate clinical recognition and intervention, lesions of Buruli ulcer group into three (3) categories by WHO. This is based on lesion's widest diameter and level of tissue involvement (WHO, 2012). According to the scheme, Category I lesions are defined as a single non-ulcerative nodule, papule or necrotic ulcers that measure < 5 cm in its widest diameter. Category II lesions are considered as plaque, edema or necrotic ulcers measuring within 5 - 15 cm. Category III lesions occur as diffused edema, plaque or necrotic ulcers that measure > 15 cm in its widest diameter with or without complications like osteomyelitis, osteitis and joint involvement (WHO, 2004; WHO, 2013). In many endemic settings in Africa, cases of Buruli ulcer are usually reported for management in stages of categories II and III (Omansen *et al.*, 2019; Sakyi *et al.*, 2016). This is different from endemic developed countries like Australia and Japan where most cases of Buruli ulcer are detected for management at Category I or II (Pluschke *et al.*, 2019).

Such geographic difference in BU detection reflected in a recent review on Buruli ulcer surveillance data in Ga West municipality in Ghana. A total of 267 cases were recorded, 85.9% were ulcerative lesions, 1.5% as oedema, 2.7% as nodule, 2.9% as plaque and 7% as mixed forms (Rufai *et al.*, 2019). This translated into 31.4% Category I, 18.4% Category II and as much as 50.6% Category III lesions. A similar surveillance report in Liberia did not differ from what was reported Ghana; Among the total BU cases that were recorded in the country around 2012-2013, 52% were WHO's Categories II and 48% were and Categories III out of which three (3) cases were complicated with osteomyelitis (Kollie *et al.*, 2014). A case report in Nigeria corroborated the preponderance of ulcerative lesions in Africa, with report of a large multifocal ulcers on skin of a patient who was also being managed with antiretroviral therapy (Oke *et al.*, 2019).

On the contrary, a case series in Honshu Island, Japan, indicated that only 1 case out of a total of 19 Buruli ulcer cases had lesion that measured >10 cm in its widest diameter (Nakanaga *et al.*, 2011). Similar evidence was recently reviewed in Victoria, Australia, with a report that despite average clinical presentation delay of 30 days many of clinically presented BU among affected children (< 15 years) and older generation (> 65 years) were nodules, papules and plaques that measured less than 5 cm in diameter (Coutts *et al.*, 2019). Reasons for this early detection rate could be ascribed to better knowledge and understanding of the disease among the citizenry and healthcare workers in the developed world compared to stakeholders in the developing world (Yotsu *et al.*, 2018).

2.2.3. Lesion Topography

Although Buruli ulcer can occur on any part of the body, it usually manifests in subcutaneous tissues of the legs and hands where average tissue temperature falls below the average body temperature of 37°C (Figure 2). This reflects and confirms the optimal growth condition of heat-sensitive *M. ulcerans* around 33°C (Sexton-Oates *et al.*, 2020).

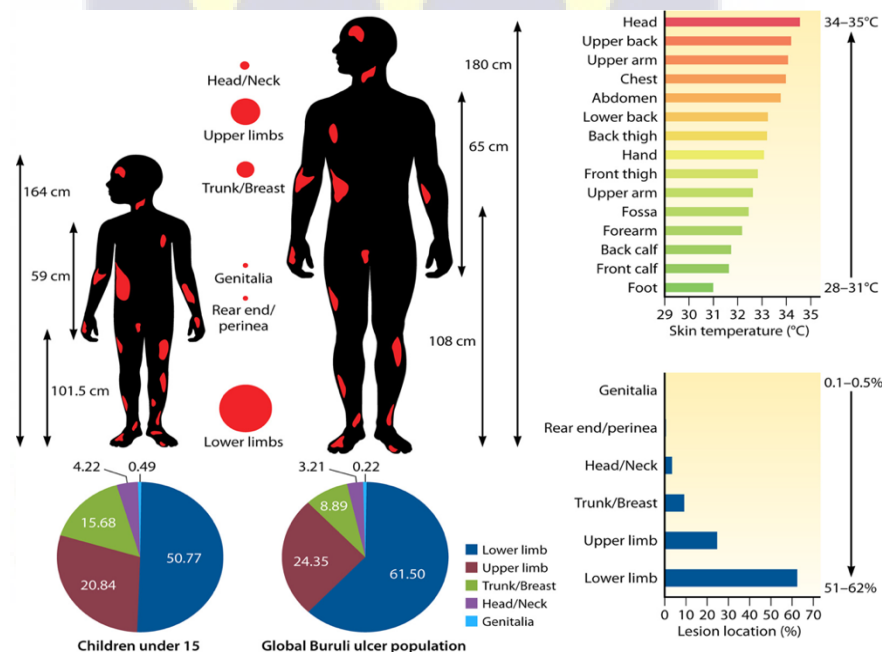


Figure 2.2: Distribution pattern of Buruli ulcer lesions on skin in relation to gradient body temperature (Zingue *et al.*, 2018).

Such unique tissue tropism reflected well in a surveillance data on Buruli ulcer in Liberia. Out of a total of 21 cases, 17 occurred on the legs and 3 on the hands (Kollie *et al.*, 2014). Similar observation was made in subsequent systematic review of a 17-year data (1998-2015) on Buruli ulcer in Australia; 70% of total 649 lesions were found on the leg (lower legs, ankles, calves and thighs), 27.1% on the hands (arm, axilla, elbow and the shoulder) and 2.9% on other parts of the body including the trunk and the face (Yerramilli *et al.*, 2017).

These empirical shreds of evidence on *M. ulcerans* preference to the body's extremities were summarized by Zingue *et al.*; (2018) in a recent systematic review in which data from 10 peer-reviewed articles in Africa estimated 30% of BU lesions occurred on the hands, 60% on legs and 10% on the trunk and the head. Cases were mostly found on the feet, calf, thigh, forearm and the arm with tissue temperature range below 34°C (Figure 2). All these observations suggests that the unclothed or less protected body parts (feet and the hands) which are often in direct contact with the environment may be more susceptible to *M. ulcerans* infections and Buruli ulcer manifestations than other parts of the body where tissue temperature is around the pathogen's growth-limiting temperature of 37°C (Zingue *et al.*, 2018).

2.3.0 Epidemiology of Buruli ulcer

2.3.1. World-wide Distribution

Overall, about 34 countries have, at a point in time, reported cases of Buruli ulcer to the WHO. These countries are localized within the range of Tropic of Capricorn (23.5 ° south) and Tropic of Cancer (23.5 ° north) where dry and wet seasons generally alternate (Figure 3). Even though the disease has a global picture of scattered but focal spread, Buruli ulcer is common in African disease with high recorded cases in Western and Central Africa where tropical climate prevails (Omansen *et al.*, 2019)..

Ghana, Nigeria, Liberia, Cote d'Ivoire, Benin, Cameroon, Guinea, Democratic republic of Congo, Gabon and Togo are current global hotspots for Buruli ulcer. Each of these endemic African countries reports more than 200 new cases of human *M. ulcerans*' infections every year (WHO, 2019). Australia, Japan and Papua New Guinea are the other non-African hot spots for Buruli ulcer across the globe.

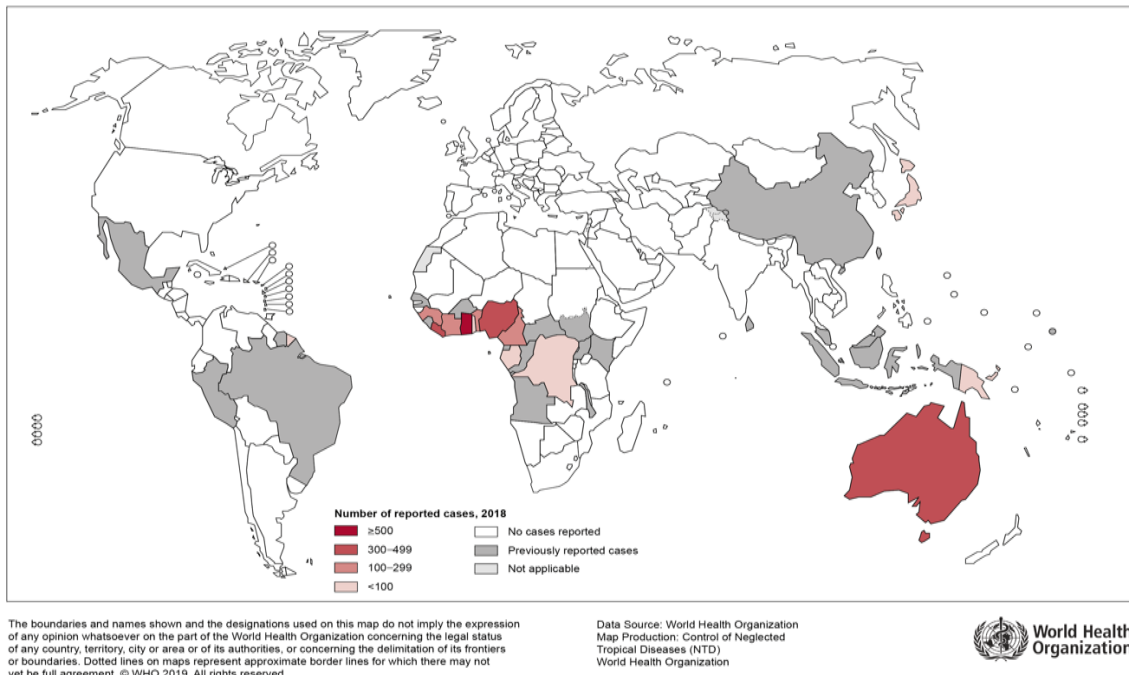


Figure 2.3: Sparse and focal distribution of Buruli ulcer cases world-wide (WHO, 2019)

A recent systematic review on global BU data over a 65-year period (1952-2017) indicated that 94.9% of global BU burden is on Africa, 4.9% on Western Pacific region and <1% on Eastern Mediterranean region (Simpson *et al.*, 2019). This was confirmed by the WHO based on global incidence data in 2018. A total of 2,713 cases were reported to WHO out of which 86% emanated from Africa, 13% from Western Pacific and < 1% Southern America (WHO, 2019). Such a disproportionate disease burden between the developed and the developing countries reflected in recent prevalent data which indicated Buruli ulcer prevalence of 0.12 cases / 10,000 population in Australia, 0.007 cases / 10,000 population in Papua New Guinea and 0.0048 cases/10,000 population in Japan (Omansen *et al.*, 2019).

These figures are in sharp contrast to the average African prevalence rate 29.6 cases / 10,000 population in Benin, 21.0 cases / 10,000 population Cameroon, 11.3 cases / 10,000 population in Cote d'Ivoire, 10.9 cases / 10,000 population in Congo and 6.9 cases / 10,000 population in Ghana (Simpson *et al.*, 2019). Even at the sub-national level, Buruli ulcer distribution remains focal dispersed and focal with a possible reason that *M. ulcerans* is heat-sensitive and may fail to propagate in environments where climatic conditions are predominantly dry and hot or extremely cold (Drancourt *et al.*, 2019). Endemic African countries like Ghana (Figure 4A) and Cameroon (Figure 4B), Benin, Nigeria, among others have high prevalence of Buruli cases mostly in southern belt where tropical rains alternate with dry seasons. The northern belt of such countries where climatic conditions are continuously dry and hot rarely reports cases of Buruli ulcer.

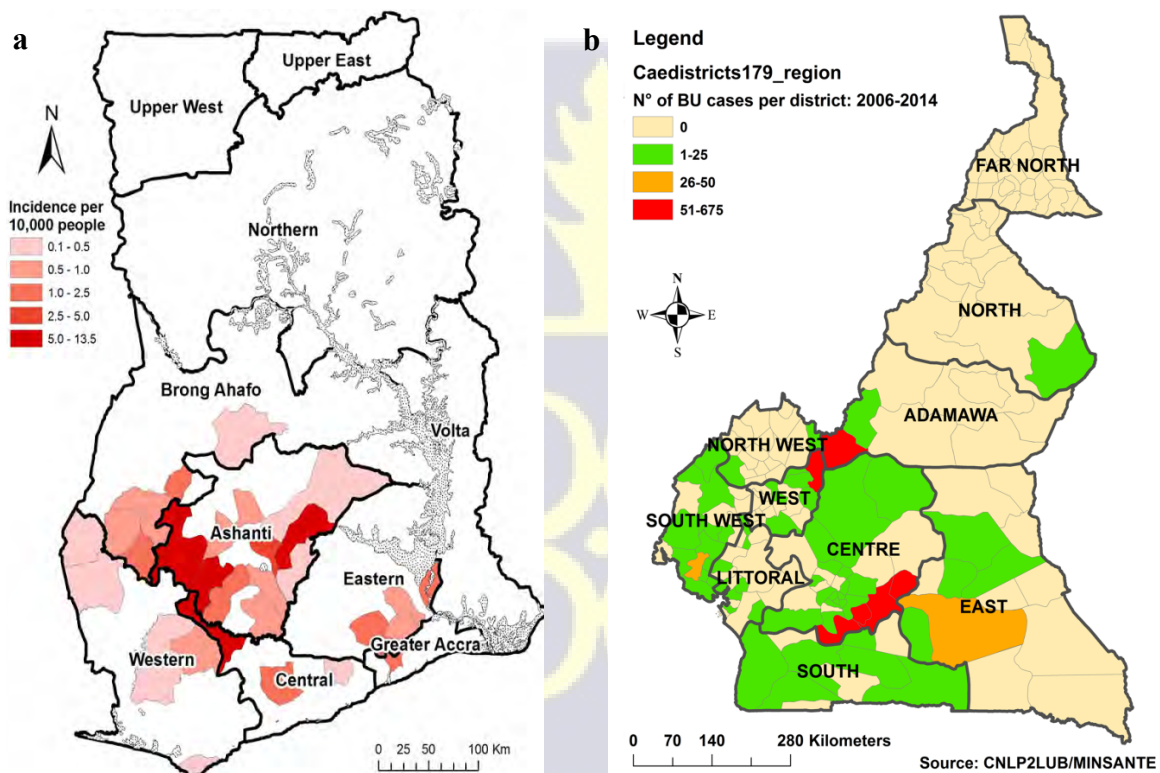


Figure 2.3.1: Foci distribution of Buruli ulcer cases in tropical Southern belts of (a) Ghana (Wansbrough-Jones, 2015) and (b) Cameroon (Tabah *et al.*; 2016)) compared to hot Northern belts: The potential influence of climate change on incidence and sub-national distribution of Buruli ulcer (Drancourt & Zingue, 2019).

All these data on Buruli ulcer distribution may suggest that environmental conditions can influence the niche of *M. ulcerans* and distribution pattern of cases and that climate change may increase incidence of Buruli ulcer in the already established regions and further contribute to spread of the disease to regions of no case report (Coates *et al.*, 2020; Drancourt *et al.*, 2019). It may be worthwhile to investigate this concept in an extensive ecological survey in bordering endemic and non-endemic territories.

2.3.2. Environmental Reservoirs and Transmission

Currently, there is no evidence of human transmission of *M. ulcerans* nor is there any indication as to where exactly in the environment this pathogen resides. However, the pathogen's DNA has been detected in diverse environmental samples. These included the soil, detritus, biofilms, water filtrates, and some aquatic vegetation (Zingue *et al.*, 2018). Biomarkers of the pathogen have also been amplified in samples from aquatic fauna like the Naucoridae and Belostomatidae and an eventual successful *in vitro* isolation of *M. ulcerans* from a Gerridae in Benin is reported (Portaels *et al.*, 2008). Also, the relation between the presence of the pathogen's DNA in the environment and human incidence of Buruli ulcer has been positively predicted suggesting an environmental niche of *M. ulcerans* (Garchitorena *et al.*, 2015). Yet, there is no specific evidence to confirm where exactly *M. ulcerans* resides in the environment or how it is transmitted to humans (Djouaka *et al.*, 2017; Merritt *et al.*, 2010).

2.4.0. Pathogenesis of Buruli ulcer

2.4.1. Mycolactone as the major virulence factor in Buruli ulcer

Only one (1) virulence factor is known to account for the numerous manifestations of *M. ulcerans*' infection. This molecule is a lipid-like macrolide, called mycolactone (George *et al.*, 1999; George *et al.*, 2000). Mycolactone appears light-yellowish, UV sensitive, acetone soluble and has a retention value of 0.23 on Thin Layer Chromatographic (TLC) plate and a mass-to-

charge (m/s) ratio of around 765 (Kubicek-Sutherland *et al.*, 2019). The native structure of mycolactone is synthesized by three (3) giant, Type I Polyketide synthases (mlsA1, mlsA2 and mlsB) which make the core lactone ring and the southern and northern fatty-acyl side chains. The three (3) synthases act in synergy with additional three (3) accessory enzymes, encoded by *pmu037*, *pmu045* and *pmu053*, to add various chemical groups to the southern unsaturated fatty-acyl side chain which potentiates mycolactone cytotoxicity (Kishi, 2011).

All these six (6) enzymes are encoded on a giant 174 Kb circular DNA plasmid (pMU001) which is believed to have been acquired by the evolving non-pathogenic strain of *M. ulcerans* (Stinear *et al.*, 2004). Figure 5 demonstrates how activities of these synthetic enzymes synergize in different mycolactone producing mycobacterial (MPMs) to create five (5) structurally different variants of mycolactone (A/B, C, D, E and F). These congeners of mycolactone differ in natural sources and also level of virulence. Mycolactones (A/B, C and D) are found in lineages of *M. ulcerans*, variant E mostly occurs in frog pathogen, *M. liflandii*, while mycolactone F exists in *M. marinum*- a fish pathogen (Matthias *et al.*, 2017; Mve-Obiang *et al.*, 2003).

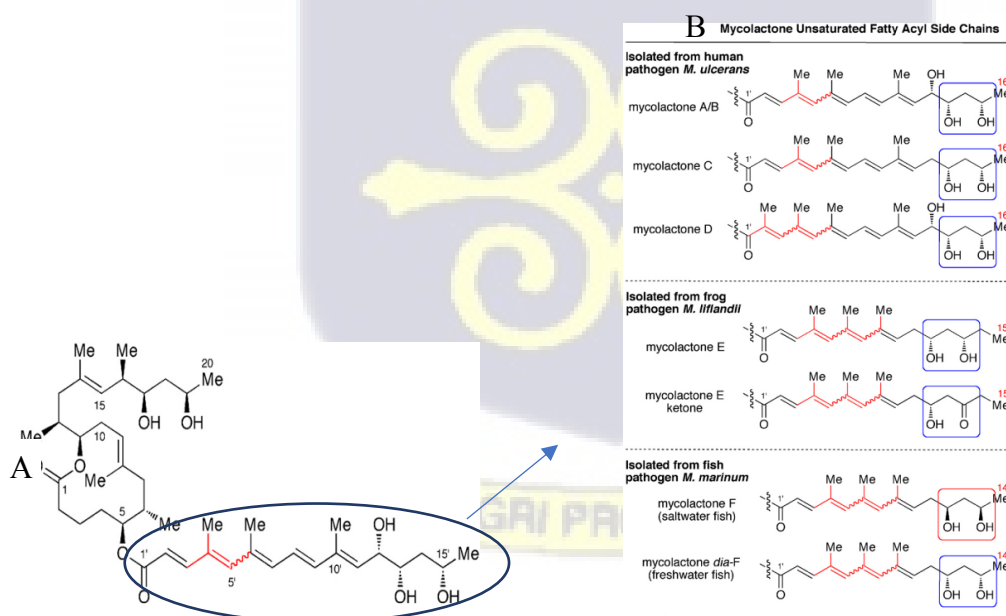


Figure 2.4: Natural Sources and structural variants of mycolactones (Kishi, 2011;Matthias et al., 2017)

Mycolactone A/B are stereo-isomers mostly in ratio mixture of 3:2. These variants are most cytotoxic and occur predominantly in classical lineage of *M. ulcerans* which are common in Africa and account for most cases of Category III lesions in Africa (Mve-Obiang *et al.*, 2003). Their lower side chains have 16 carbon chains, three (3) hydroxyl (-OH) groups and four (4) methyl groups (Figure 5). Mycolactone C lacks -OH at carbon 12 (C-12) whilst mycolactone D has an additional methyl group on C-2 (Matthias *et al.*, 2017). These structural modifications are proposed to attenuate cytotoxic potency of mycolactones C and D which are mostly responsible for Category I and II lesions reported in developed countries like Australia and Japan which are endemic in Buruli ulcer (Matthias *et al.*, 2017; Mve-Obiang *et al.*, 2003). Uniquely, mycolactone E has 15 carbon chains whilst F occurs with 14 on their respective lower side chains.

Additionally, the chains lack OH groups at C-10 and C-12, a reduced number of conjugated double bonds, an additional ethyl group at C-14 and a reversed configuration of OH groups at C-11 and C-13 (Kishi, 2011). All these structural modifications can render mycolactones E and F less cytotoxic in human tissues. However, not much is known about the contributions of other MPMs to Buruli ulcer incidences and how variants like mycolactones E and F can cause lesions in human skins. Nonetheless, any of these congeners have the potential to target key host's cell compartments to induce either apoptosis or necrosis.

2.4.2. Cellular Targets of Mycolactone Toxicity

2.4.2.1 Angiotensin-II, type 2 receptor (AT2R) and Neural degeneration

The perceived lack of pain associated with uncomplicated cases of Buruli ulcer was initially believed to result from nociceptive dysfunction of host's neurons mediated by mycolactone. Mycolactone was found to induce axonal degeneration via accumulation of apoptotic bodies in

nerve bundles (Zavattaro *et al.*, 2012). But subsequent *in vivo* models refuted this concept of neurodegeneration and suggested that mycolactone rather interacts with AT₂R to initiate signaling cascade through phospholipaseA2 - COX1 pathway to cause potassium-dependent hyperpolarization in sensory neurons (Marion, E. *et al.*, 2014). Interestingly, a follow-up study confirmed the initial idea of neurodegeneration in cases of Buruli ulcer and summarized how mycolactone alone can induce neural cell death. The authors indicated that mycolactone blocks TRPV1 or capsaicin receptor (for pain sensation) and also Sec61 to reduce levels of Gap43 (marker of neural integrity), α -tubulin while the bidirectional trafficking of mitochondria in neural axons also gets impaired (Anand *et al.*, 2016). This leads to neurite degeneration, cell death and nociceptor dysfunction. The authors could not confirm the concept of neurite hyperpolarization on dorsal root ganglia (DRG) partly due to probable lack of AT₂R on DRG in human and mice (shepherd, 2018).

However, evidence from this study suffices to suggest that cytotoxic mycolactone interacts with Sec61 as well as AT₂R in DRG, hippocampal neurons and Schwann cells to induce neurite dysfunction that may account for the general lack of pain with Buruli ulcer (Babonneau *et al.*, 2019; Demangel *et al.*, 2018; Reynaert *et al.*, 2019).

2.4.2.2 Wiscott-Aldrich Syndrome Proteins (WASP) proteins

The architecture of eukaryotic cells is critical to cell survival, function, cell-cell and cell-extracellular matrix interactions leading to ultimate tissue integrity. This depends on cytoskeletal proteins and their regulated rearrangements under the influence of WASP proteins (Smyrek *et al.*, 2019). Again, mycolactone targets these structural proteins with reported that it disrupts stable functions of N-WASP/WASP as it interacts with their N-terminal GTPase-Binding Domain (GBD) to block auto-inhibition and further induces conformational changes which expose the proteins C-terminal verprolin-coffilin-acidic (VCA) domain to constitutive

interactions with actin-nucleating complexes (ARP2/3). Such persistent interactions lead to ectopic polymerization of actin in the cytosol instead of the peripheral membranes. Eventually, cells lose their scaffold proteins, detach, shrink, and die by anoikis (Guenin-Macé *et al.*, 2019; Guenin-Mace *et al.*, 2013; Sarfo *et al.*, 2016).

2.4.2.3 Sec61 Translocon

The same mycolactone is reported to target heterotrimeric conduit, Sec61, on rough endoplasmic reticulum (ER) and deprive host cells of secreted and structural proteins which are essential to cell survival and tissue integrity. It achieves this as it blocks translocation of nascent polypeptide into the ER, during or after translation, for post-translational modification and sub-cellular localizations (Demangel *et al.*, 2018; Ogbechi *et al.*, 2018). Mechanistically, diffusible mycolactone enters cells where it interacts with the cytosolic side of non-engaged Sec61 complex through the lateral gate and wedges open the gate to keep the α helices of the translocon partially active but in a non-functional conformation. This molecular orientation masks the docking site for signal peptides of translocating proteins and prevents their subsequent passage through the translocon into ER (Gérard *et al.*, 2020). Consequently, precursor proteins accumulate in the cytosol where they get ubiquitinated for eventual degradation in 26S proteasome (Hall *et al.*, 2014). By this interaction, mycolactone denies host's cells of Type I and II transmembrane proteins, inflammatory mediators, immune effectors and other structural and humoral proteins which are critical to cell viability and tissue integrity (Hall *et al.*, 2014; McKenna *et al.*, 2016). Also, interaction between mycolactone and Sec61 is proposed to activate the suicidal arm of Integrated Stress Response (ISR) pathway as PERK (an ER kinase) gets activated to selectively phosphorylate and inhibit eukaryotic Initiation Factor-2 (eIF-2a). This leads to global translational shutdown and eventual cell death by apoptosis through Bim - Bax/Bak pathway (Hu *et al.*, 2018; Ogbechi *et al.*, 2018).

Alternatively, mycolactone can induce apoptosis in mammalian cells through the mechanistic Target of Rapamycin (mTOR) (Bieri *et al.*, 2017).

2.4.2.4 Mammalian Uncoordinated-18, isoform b (Munc-18b)

Although not proven in a wet experimental model, mycolactone again is proposed to bind strongly to Munc-18b protein through Arg405. This interaction induces a conformational change that destabilizes the protein's biologic activity and has the potential to block the exocytic functions of platelets and mast cell exocytosis which are critical for homeostasis (Kwofie *et al.*, 2019). This cell dysfunction may slow wound healing and prevent tissue remodeling partly accounting for the chronic nature of untreated Buruli ulcer lesions (Kim *et al.*, 2013; Opneja *et al.*, 2019).

In summary, all these pleiotropic interactions of mycolactone with critical cellular targets are considered necessary and sufficient for Buruli ulcer pathogenesis (George *et al.*, 1999). No other virulent factors from *M. ulcerans* are known to date. However, there have been earlier suggestions that “other virulent factors” may contribute to Buruli ulcer pathogenesis (Röltgen *et al.*, 2020). These suggestions were based on pieces of evidence that toxic lipoproteins (Dobos *et al.*, 2001), phospholipases C and D (Gomez *et al.*, 2001), antigenic ESAT6-like ExG, FbpA-D, chitinase among others, have all been isolated from growth medium of viable *M. ulcerans* in culture (Tafelmeyer *et al.*, 2008). Also, the pathogen possesses a unique extracellular matrix (ECM) found to harbor many proteins originating from the mycobacterium (Marsollier *et al.*, 2007). How these extracellular proteins got secreted from *M. ulcerans* could have resulted from other signals yet to be determined or extracellular MVs may play a role.

Almost all the pathogenic strains of mycobacterium have a putative ESX-1 gene cluster at Region of Difference 1 (RD1) in their genomes. This system utilizes Type VII protein secretory system (T7SS) to discharge immunoactive 6 kDa Early Secretory Antigenic Target (ESAT-6), 10 kDa Culture Filtrate Protein (CFP-10) and Heat Shock Protein X (HspX) to aid in the pathogen's intracellular survival and growth (Damen *et al.*, 2020;Houben *et al.*, 2012). Uniquely, *M. ulcerans* has sequences of this system either completely deleted or functionally disrupted through evolutionary events of genome reduction and pseudogenes accumulation (Huber *et al.*, 2008). Besides the canonical secretion system, no active ESX secretory system is known. However, *M. ulcerans* remains one of the most virulent strains of mycobacterium. Therefore, it is suggested that other virulent factors may act, either in synergy with mycolactone or independently, to mediate virulence (Dobos *et al.*, 2001) and that the release of extracellular vesicles from *M. ulcerans* is likely to mediate pathogenicity (Foulon, M *et al.*, 2020;Marsollier *et al.*, 2007) just as reported of *M. tuberculosis* and *M. bovis* (Athman *et al.*, 2017;Prados-Rosales *et al.*, 2011).

2.5. Extra-cellular Vesicles and Their Antigenic Content

Viable cells have evolved several means by which they adapt to spatio-temporal changes in their environment. Example of such adaptive mechanism is the active release of Extracellular Vesicles (EVs) from their surfaces into the environment (Coelho *et al.*, 2019). These are spherical, lipid-bilayer, nanovesicles which originate from membranous compartments of viable growing cells and often act as key mediators in both intra-species and inter-species communication carrying diverse molecules from parent cells to targeted recipient cells where they modulate biologic functions (Woith *et al.*, 2019). Major molecules in discharged extracellular vesicles include complexes of proteins, polysaccharides, lipids, nucleic acids, small molecules, and other mediators of metabolisms (Chen *et al.*, 2019;Maas *et al.*, 2017). EVs exist with several names and among others may be released as exosomes, microvesicles,

outer membrane vesicles (OMVs), membrane vesicles (MVs) depending on the source cell and cellular compartment they originate from (Witwer *et al.*, 2019).

Eukaryotic cells release EVs as exosomes through the endocytic-exocytic pathway. This occurs when early endosomes mature to late endosomes in which proteins I and II of endosomal sorting complexes required for transport (ESCRTs) induce invaginations in the membrane to form intraluminal vesicles (ILVs) (Meldolesi, 2019). Consequently, the late endosomes transform into multi-vesicular bodies (MVBs) in the cytosol. Eventually, this may fuse with the plasma membrane to empty their content into the environment. Also existing are micro-vesicles which bulge out of plasma membrane through the activities of ESCRT III and eventually pinch-off from the cell into the environment (Meldolesi, 2019). These two types of eukaryotes vesicles can convey both host's and pathogen-specific proteins in cases of infection (Chen *et al.*, 2019; Meldolesi, 2019; Schorey *et al.*, 2016).

The roles of exosomes and micro-vesicles are exemplified in events like immune “cross-dressing” or “cross-presentation” in which immature Dendritic cells (DCs) package antigens on MHC-II complex in EVs and pass on to matured DCs which are more primed to present antigen and activate naïve as well as memory CD4⁺ and CD8⁺ lymphocytes for an appropriate immune response to the invading hazard (Chen *et al.*, 2019). Also, macrophage cells can process intracellular peptides of an engulfed microbe and release their antigens through the ILVs into the host's environment where they could be recognized by sensitized T-cells (Schorey *et al.*, 2016). Moreover, mast cells and neutrophils can all let out outer membrane vesicles with effector cargoes (Chen *et al.*, 2019). Therefore, EVs from immune cells can provide an ideal source of Pathogen Associated Molecular Pattern (PAMPs) which could be harnessed for better disease understanding and control strategies, most especially in areas of

diagnostics and vaccine development (Cai *et al.*, 2018;Nagakubo *et al.*, 2019;Rodrigues *et al.*, 2018;Wang *et al.*, 2019).

2.6. Virulence of Bacterial Membrane Vesicles and Their Proteins Cargoes

Prokaryotes also rely on the release of extracellular vesicles to adapt to a new environment. Considering the “courier” services bacterial membrane vesicles (BMVs) play in cellular communications, many pathogens of bacteria have resorted to EVs as major armory against host defence systems (Rueter *et al.*, 2020). One area of such concern is the pathogen’s potential to enter the host’s tissues through functional BMVs. A more recent study indicated an enrichment of High temperature requirement enzyme (Htr A) in membrane vesicles (MVs) released by *Helicobacter pylori* in gastric tissues (Ansari *et al.*, 2019). This enzyme is serine protease and can degrade architectural proteins like E-cadherin, claudin-8 and occludin in mucosal tight junctions. This allows the pathogens to breach gastric epithelial barrier for tissue invasion (Ansari *et al.*, 2019). Additionally, species of *Vibrio cholera* release MVs with a high content of active Vibrio Cholera Cytotoxin (VCC) which can breach the epithelial layer of the intestine for the pathogen’s entry (Elluri *et al.*, 2014). Moreover, strains of Enterohaemorrhagic *Escherichia coli* (EHEC O 157:H7 and EHEC O 104:H4) are known to release membrane vesicles with high content of active ribotoxic shiga toxin 2a (stx2a). These molecules can arrest global translation and induce host’s cell death through apoptosis (Bauwens *et al.*, 2017). Moreover, MVs of *Streptococcus suis*, were characterized with increased content of active subtilisin-like protease (SspA) and DNase. These molecules could induce host’ cell death, destroy tissues and further arm the pathogen to resist phagocytic attacks in host’s environment (Haas *et al.*, 2015). All these pieces of evidence affirm the critical role exogenic vesicles of pathogens play in successful colonization of host’s tissues and virulence.

Another aspect of MVs virulence is modulation of host's immune systems required for positive selection of pathogens. VacA, is an exotoxin known to suppress T-cell activation and induce apoptosis in immune cells. This protein was found to be enriched in MVs released by *H. pylori* and confirms how this pathogen can stifle activation of adaptive immune response against its entry (Winter *et al.*, 2014). Another immune-modulatory found BMVs is ompT protease. An increased amount of this tissue degrading enzyme was found in exogenous vesicles released from surfaces of EHEC species (Urashima *et al.*, 2017). This pathogenic enzyme could degrade the host's anti-microbial peptide, cathelicidin LL-37, and protect the bacterium against onslaught from innate immune effectors (Urashima *et al.*, 2017). *V. cholera* is not an exception to vesicle-mediated virulence in host's tissues. Once in host's intestinal tissues, this pathogen can shut down its transport system for membrane phospholipids and increase the rate of release of MVs from its surface. This act of desquamation allows the pathogen to modify its antigenic surfaces immune evasion and an enhanced colonization fitness (Zingl *et al.*, 2019).

Moreover, there is evidence of efficient transport of active Lipopolysaccharides (LPS) into the cytosol of both phagocytic and non-phagocytic cells by outer membrane vesicles (OMVs) released by *E. coli*, *N. meningitidis* and *Pseudomonas aeruginosa*. In the cytosol, LPS activates caspase-11 signaling cascade responsible for inflammatory-related cell death in murine bone marrow-derived macrophage (Vanaja *et al.*, 2016). More recently, it is reported that sub-lethal dose of cephalosporins on *Acinetobacter baumannii* can stimulate high number of cytotoxic vesicles that could carry toxic LPS and other virulent proteins to distant targets for disease pathogenesis (Chiu *et al.*, 2020). MVs of *Streptococcus mutans* were found with many virulent proteins that promote cariogenesis (Cao *et al.*, 2020). In brief, exogenous vesicles of many gram negative and gram positive pathogens play critical roles in their pathophysiology and could elicit considerable influence on pathogenesis of many infectious diseases.

2.7. The Release of Membrane Vesicles by Strains of Mycobacteria

The phenomenon of MVs release is as well conserved among strains mycobacteria. This was first established when Prados-Rosales *et al.*;(2011) utilized differential ultra-centrifugations, density gradient centrifugations and Transmission Electron Microscopy (TEM) to observe intact, lipid-bilayer vesicles (measuring 60 nm to 300 nm in size range) in growth media of *M. tuberculosis*, *M. bovis*, *M. avium*, *M. kansasii*, *M. bovis* (BCG), *M. smegmatis* and *M. phlei* (Prados-Rosales *et al.*, 2011). This seminal study might have predicated on the previous report that certain vesicular bodies with virulent features existed in unique extracellular matrix of *M. ulcerans* (Marsollier *et al.*, 2007). The foundation for future studies was therefore laid.

Subsequently, growth media of *M. bovis* (NEIKER 1403), *M. avium* subsp. *paratuberculosis* (MAP K10) and *M. tuberculosis*, cultured within 2 to 4 weeks, were subjected to membrane vesicle exploration (Palacios *et al.*, 2019). Both bi-layered and uni-lamellar vesicles, ranging in size from 68 nm to 124 nm were observed by electron microscopy in pellets that were isolated from culture medium of respective test strains (Palacios *et al.*, 2019). This expanded the strains of mycobacterium involved in active release MVs. More recently, an exploratory study found intact vesicles, ranging in diameter from 100 nm to 250 nm, in growth medium of *M. kansasii* cultured for four (4) weeks (Tavassol *et al.*, 2020). Considering that, slow and fast growers as well as pathogenic and non-pathogenic strains release membrane vesicles release, it suffices to suggest the essence of MVs in pathophysiology of many pathogenic mycobacterial and that such an adaptive process could contribute to bacterial colonization and virulence in host's tissues (Prados-Rosales *et al.*, 2011;Rodriguez *et al.*, 2016). Notwithstanding, not much is known about how proteins in vesicles separated from mycobacterial pathogens could contribute to diseases pathogenesis and progression.

2.7.1 Release of Membrane Vesicles from Mycobacterium Associates with Cell Viability

Vesiculogenesis, the process of extracellular vesicles release, is an active process. Dead bacterial cells do not initiate such an adaptive process. This was confirmed in many instances where only live and growing cells of bacteria remained the only source of intact MVs in growth medium (Coelho *et al.*, 2019). A previous report failed to recover intact vesicles from growth medium when it was spiked with dead *M. tuberculosis* heat-killed at 80°C for 5 minutes (Prados-Rosales *et al.*, 2011). On the contrary, the authors recovered 135 ug/ml EMVs from similar growth medium in which live colonies of *M. tuberculosis* with 99% viability count of 99% was inoculated. This suggested a correlation of bacterial cell viability and active release of membrane vesicles packed with diverse molecules.

A subsequent report by Athman *et al.*, (2015) confirmed this evidence with an indication of high content of lipoarabinomannan (LAM) and lipomannan (LM) in extracellular vesicles released by macrophage cells infected with viable cells of growing *M. tuberculosis*. Undoubtedly, these antigenic glycolipids could not be detected when the macrophage monolayer was co-cultured with either UV irradiated or heat-killed cells of *M. tuberculosis* (Athman *et al.*, 2015) further confirming the requirement of cell viability for release of MVs by mycobacteria. A more recent report indicates a strong positive correlation between the growth rate of *M. bovis* as well as *M. avium* and quantity of membrane vesicles recovered in media of these pathogenic strains of Mycobacterium (Palacios *et al.*, 2019). Per the authors, the relatively faster growth rate of *M. bovis* is associated with increased amounts of vesicles with higher diameter compared to vesicles released by slow-growing *M. avium* (Palacios *et al.*, 2019). The more cells enter the exponential phase of growth, the higher the number of vesicles released. This underscores viability and growth of mycobacterial cells as key determinants of membrane vesicles release and a potential pre-requisite for subsequent studies (Gupta *et al.*, 2020).

2.7.2. Heterogeneity of Membrane Vesicles from Mycobacterium-Infected Macrophages

Pathogenic mycobacteria are primarily intracellular and can adapt to the harsh intracellular environment of phagocytes through the release of membrane vesicles (Prados-Rosales *et al.*, 2011). Concurrently, host phagocytes can also release membrane vesicles as part of their physiology or in response to intracellular pathogens (Wang *et al.*, 2019). Therefore, in a typical model of mycobacterial infection, it is more likely to have a heterogeneous mixture of MVs from the pathogen and vesicles from host cells all carrying diverse molecules (Wang *et al.*, 2019). This concept was proposed when molecules from mycobacterial cells were identified in EVs released by *M. tuberculosis* infected J774 macrophage cells (Giri *et al.*, 2010). Similar evidence was generated in a clinical setting with a more advanced Multiplex Multiple Reaction Monitoring-Mass spectrometry (MRM-Ms). A total of 33 *M. tuberculosis*-specific proteins co-precipitated with host's proteins found in serum extracellular vesicles of patients with active tuberculosis (Kruh-Garcia *et al.*, 2014). Interestingly, none of these studies could explain whether these antigenic proteins were being carried by either pathogen-specific vesicles or vesicles from host cells.

Insight on this gap was provided when Athman *et al.*, (2015) proposed macrophage-specific exosomes carrying pathogen-specific peptides after intracellular antigen processing (**Figure 2.7A**). Also, the authors separated and confirmed a unique mixture of *M. tuberculosis*-specific vesicles and host cell-specific vesicles in an infection medium consisting of BMDM cells and viable cells of *M. tuberculosis* (**Figure 2.7B**). These two vesicle sub-populations were different in densities based on their molecular compositions. Differences in their protein compositions further provided clear information on the origin of these extracellular vesicles (Athman *et al.*, 2015). Therefore, it is possible to harness molecular compositions of mycobacterial vesicles

from an infection milieu for useful data on biomarkers search and disease pathogenesis (Gupta *et al.*, 2018; Mehaffy *et al.*, 2017; Wang *et al.*, 2019).

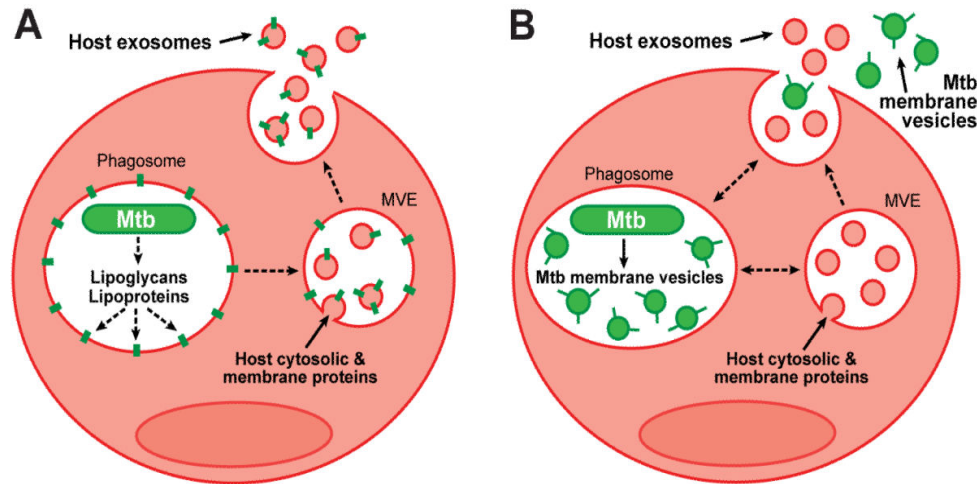


Figure 2.7: Proposed models of membrane vesicles trafficking and heterogeneity of extracellular vesicles by *M. tuberculosis*-infected macrophage cells (Athman *et al.*, 2015)

2.7.3. Factors that Influence Membrane Vesicles Formation Among Mycobacteria

Currently, it is unknown how vesiculogenesis in mycobacteria are regulated (Brown *et al.*, 2015). However, recent evidence suggests that limited iron supply in growth medium minimizes the activities of Vesiculogenesis and immune response regulator (Vir) protein. This in turn increases the rate of formation and release of MVs by *M. tuberculosis* (Rath *et al.*, 2013). It is believed that Vir forms regulatory complex with membrane-associated proteins like LpqH, Rv1488 and Rv0383c to control the number, size and molecular compositions of extracellular membrane vesicles from mycobacteria (Mohammadzadeh *et al.*, 2020). Other genetic factors also come to play. It is recently reported that increased expression of Dynamin-like proteins encoded by *iniBAC* operon facilitates membrane vesicle formation in many pathogens of mycobacteria (Gupta *et al.*, 2020).

Additionally, mutations in phosphate specific transporter (pst) increase the rate of MVs formation through the two (2) component SenX3-RegX3 transport system (White *et al.*, 2018).

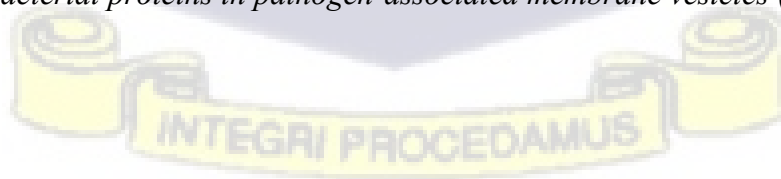
There are also reports on the influence of turgor pressure, cell wall modifying enzymes playing

various roles to incite passive packaging of proximal molecules into MVs of mycobacteria (Dean *et al.*, 2019). Yet, the actual mechanism underlying this cellular process is yet to be elucidated (Mohammadzadeh *et al.*, 2020).

2.7.4. Proteomic Composition of Membrane Vesicles by Mycobacteria Pathogens

More often, molecular composition of BMVs reflects the identity of parent cell, its physiologic or pathologic state and the potential impact of such vesicles on prospective recipient cells (Coelho *et al.*, 2019). The protein content of MVs released by pathogenic mycobacteria under different growth conditions are well characterized as reviewed in Table 1. Some of biologic functions of these proteins include pathogen's adaptation and virulence in host tissues. The above vesicle-associated proteins of mycobacterial are not exhaustive. Prados-Rosales *et al.*; (2011) utilized LC-MS/MS to identify 48 unique proteins within MVs found in *in vitro* culture of *M. tuberculosis*. Some of these proteins included LpqH, MPB83, LpqL, LppX, LppZ, LpqN, LprA, LprF, LprG, PBP-1, PSTS3, and phoS1 with immune modulatory and adaptive functions that are very critical to intracellular survival and tissue dissemination by the pathogen. In addition to this, 66 different proteins were found in membrane vesicles released by *M. bovis* (BCG) under the similar growth many of which demonstrated features of virulence and adaptations (Prados-Rosales *et al.*, 2011). Moreover, extracellular vesicles released by *M. avium*-infected macrophage (THP-1) were found to contain immunodominant proteins like MPT51, MPT63, ESAT-6, Ag85-C and SodA with both virulent and anti-inflammatory consequences (Wang *et al.*, 2015).

Table 1: *Mycobacterial proteins in pathogen-associated membrane vesicles (Wang et al., 2019).*



Bacterium type	Host cells or body fluids	Proteins	References
<i>M. tuberculosis</i>	Macrophages (<i>in vitro</i>)	Lipoprotein, GPL phosphatidylmyoinositol mannosides (PIM)	(17)
BCG	BCG (<i>in vitro</i>)	LpqH, MPB83, FEIII, LpqL, LppX, LppZ, LpqN, LprA, LprF, LprG, PBP-1, PSTS3, phoS1	(27)
<i>M. tuberculosis</i>	<i>M. tuberculosis</i> (<i>in vitro</i>)	LpqH, FEIII, LprA, LprG	(27)
<i>M. tuberculosis</i>	Macrophages (<i>in vitro</i>)	Antigen 85C, PckA, GabD1, GabD1, DnaK, LpdC, TB27.3, Cfp29, GltA2, PstS1, TB8.4, LprA, Tal, Cfp17, GlcB, Mpt32, Apa, BfrA, Antigen85B, Rv1906c, KatG, Mpt63, Cfp20/Tpx, Mpt64, HspX, PrcB, GlnA1, AcpM, Cfp2, PepN, Ald, Mpt53, TB22.2, SahH, SapM, GroES, Rv3587c, Mpt51, Antigen 85A, BfrB, ESAT-6	(59)
<i>M. tuberculosis</i>	Human serum (<i>in vivo</i>)	Antigen 85B, Antigen 85C, Apa, BfrB, GlcB, HspX, KatG, Mpt64	(60)
<i>M. tuberculosis</i>	Human serum (<i>in vivo</i>)	Cfp2, Mpt32, Mpt64, MrsA, BfrB, Esat-6, GroES, Ag85c, SahH, Ag85a, DnaK, GlnA1, GlcB, AcpM, PpiA, Ag85b, Cfp10, GarA, HspX, MrsA	(61)
<i>M. tuberculosis</i>	RAW264.7, HEK293 (<i>in vitro</i>)	KatG, HspX, and GroES	(62)
<i>M. tuberculosis</i>	<i>M. tuberculosis</i> (<i>in vitro</i>)	LppX, LpqH (19 kDa lipoprotein), LpqN, LprA, LprF, LprG, PstS1, PstS2, PstS3, HbhA, TatA, Hup, Acn, FbpA (Ag85A), FbpB (Ag85B), FbpC (Ag85C), SodB	(63)
<i>M. tuberculosis</i>	Macrophages (<i>in vitro</i>)	LpqH (19-kDa lipoprotein)	(64)
<i>M. tuberculosis</i>	<i>M. tuberculosis</i> (<i>in vitro</i>)	LppX, PstS 1, LpqH, Apa, Esx, Rv1435c, LprA, LprG, MPT63, LprF, EsxA, PstS 2, CysA2, Rv0954, MPT53, Rv1987, Rv0309, MPT64, LpqN, EspC, Cut2, Rv1269c, EspA, TB22.2, Rv1984c, HbhA, TatA, PstS 3, Rv2668, Rv043, Rv1488, Rv2091c, Rv1410c, Hup, MlhF, Can, Enolase, MmsA, Rv0315, Gnd2, IlvX, AtpD, Rv0296c, gmdA, Rv0248c, Rv2251, Rv3671c, aldC, icd2, Rv0063, Rv0148, AtsA, MycP3, AtpA, Gor, Ag85A, FadA3, Ag85C, AccA3, Ag85B, MPT51, FadA2, EchA21, FadD13, Rv1544, FadE4, maA4, FadB, SodB, HspX, EphG, Rv3722c, Rv0831c, Rv2159c, ppe41, Rv3099c, Rv3717, Rv3169	(65)
<i>M. bovis</i> BCG	Bronchoalveolar lavage fluid of mice (<i>in vivo</i>)	19-kDa lipoprotein	(66)
<i>M. bovis</i> BCG	Macrophages, mice (<i>in vitro</i> and <i>in vivo</i>)	Ag85A, HspX	(67)
<i>M. tuberculosis</i>	B16 melanoma cells (<i>in vitro</i>)	ESAT-6	(68)

Furthermore 287 proteins, some with virulent impact, are reported in MVs released by *M. tuberculosis* (Lee *et al.*, 2015). Considering all these data, it can be suggested that antigenic proteins can confer specific biologic functions on MVs and that these proteins may further provide unique Pathogen Associated Molecular Pattern (PAMPs) in host's tissues that could be harnessed for potential clinical applications.

2.7.5. Applications of Membrane Vesicles from Mycobacterial Pathogens

Pathogenic vesicles of mycobacteria are usually stable in biological systems and often exist with unique antigenic repertoire in plasma, broncho-alveolar lavages and urine under different disease conditions (Mehaffy *et al.*, 2017; Wang *et al.*, 2019). This was first demonstrated when

an immunoblot was used to demonstrate immunological recognition to 23 kDa, 25 kDa and 36 kDa proteins found in membrane vesicles of *M. bovis* and *M. tuberculosis*. These proteins were recognized by the sera of 28 patients with active tuberculosis. Healthy control sera failed to demonstrate this immunological response. This suggested the differentiating power of such mycobacteria antigens as potential disease biomarkers (Ziegenbalg *et al.*, 2013). Such an interesting concept led to the subsequent discovery of 19 unique proteins of *M. tuberculosis* (Table 2) in sera of patients with active pulmonary tuberculosis. These peptides were associated with MVs confirming the vesicles as genuine source of knowledge on disease pathogenesis and potential diagnostics target (Mehaffy *et al.*, 2017).

Furthermore, additional insight was provided when Dahiya *et al.* (2019) found the exclusive presence of mycobacterial protein, 10 kDa Culture Filtrate Protein (CFP), in membrane vesicles that were separated from urine of patients with active tuberculous infections (Dahiya *et al.*, 2019). More recently, unique of mycobacterial glutamine synthase (GlnA1, highlighted in Table 2) in serum vesicles of individuals with latent tuberculous infections was established (Mehaffy *et al.*, 2020). This suggests GlnA1 as a unique biomarker that can help to differentiate cases of active pulmonary tuberculosis from latent cases and further help to identify people at risk of the disease for timely intervention (Mehaffy *et al.*, 2020). In brief, proteins in vesicles of mycobacteria present unique PAMPs to serve useful clinical applications in the management of such infectious diseases (Mehaffy *et al.*, 2020; Wang *et al.*, 2019).

Table 2: Unique mycobacterial peptides in extracellular vesicles separated from sera of patients with active pulmonary tuberculosis (Mehaffy *et al.*, 2020)

Protein	Peptide	Protein	Peptide
Ag85c (Rv0129c)	VQFQGGGPHAVYLLDGLR NDPMVQIPR ITQDLLDR FLEGLTLR	DnaK (Rv0350)	TTPSIVAFAR
		Ag85B (Rv1886c)	PGLPVEYLQVPSPSMGR
GclB (Rv1837c)	VVADLTPQNQALLNARD FALNAANAR NYTAPGGGQFTLPGR	Cfp10 (Rv3874)	AADMWGPSSDPAWER QELDEISTNIR

Mpt32 (Rv1860)	TTGDPPFPQGPPPVANDT LYASAEATDSK	EsxA (Rv3875)	LAAAWGGSGSEAYQGVQK
Mpt64 (Rv1980c)	SLENYIAQTR FLSAATSSTPR	GlnA1 (Rv2220)	SVFDDGLAFDGSIR GGYFPVAPNDQYVDLR
HspX (Rv2031c)	AELPGVDPDK TVSLPVGADEDDIK	MrsA (Rv3441c)	YVLEELR TAVEQAAAELGDTGR
Cfp2 (Rv2376c)	GSLVEGGIGGTEAR SLADPNVSFANK	PpiA (Rv0009)	IALFGNHAPK VIQGFMIQGGDPTGTGR
SahH (Rv3248c)	GVTEETTTGVLR IHVEALGGHLTK	AcpM (Rv2244)	HTIFGEVIDAESQR IPDEDLAAGR
GroES (Rv3418c)	DVLAVVSK RIPLDVAEGDTVIYSK		TVGDVVAYIQK LEENPEAAQALR
BfrB (Rv3841)	EALALALDQER AGANLFELENFVAR	Ag85A (Rv3804c)	NDPLLNVGK FLEGFVR
		GarA (Rv1827)	FLLDQAITSAGR LVFLTGP

2.7.6 Effects of Mycobacterial Membrane Vesicles on Immune Cell Functions

Functional effects of extracellular vesicles from mycobacterial pathogens are mostly immunomodulation. Vesicle-associated PAMPs are often recognized by Pattern Recognition Receptors (PRRs) on immune cells leading to either pro- or anti-inflammatory responses that play to positive selection of the pathogens (Layre, 2020; Singh *et al.*, 2012; Smith *et al.*, 2017).

2.7.6.1. Pro-inflammatory Impact of Membrane Vesicles from Mycobacterial Pathogens

This stimulatory impact was first described when Prados-Rosales *et al.*; (2011) observed that membrane vesicles of *M. bovis* and *M. tuberculosis* could engage Toll-Like Receptor 2 (TLR-2) on murine Bone Marrow-Derived Macrophage (BMDM) to induce persistent secretion of IL-12, IL-1 β , IL-6, IL-12, TNF- α , CXCL1, and MIP-1 α into culture supernatant together with increase intracellular activity of COX-2 enzymes. Such a stimulatory impact, which persisted on exposed cells even after 48 hours of incubation were absent in controlled untreated cells (Prados-Rosales *et al.*, 2011). Consistent with this evidence was subsequent report that HEK293 monolayer could respond to 17.5 microliters (μ L) vesicles from *M. tuberculosis* by secreting high levels of IL-8 chemokine in growth medium (Athman *et al.*, 2015).

The same vesicle load increased the expression of TNF- α and IL 12 p40 in cultured BMDM (Athman *et al.*, 2015). A similar stimulatory impact was observed when cultured human macrophage cells (THP-1) was exposed to 50 μ g of MVs found in growth medium of *M. avium*-infected macrophage cells (Wang *et al.*, 2015). The vesicles could induce macrophage cells to differentiate with enhanced phagocytic function and increased secretions of IL-6, IL-8, TNF- α and INF- γ into culture supernatants (Wang *et al.*, 2015). Moreover, MVs from *M. tuberculosis*-infected RAW 264.7 cells were found to elicit high IL-8 secretion from stimulated murine endothelial cells (SVEC4-10). This chemokine promoted migration of activated macrophage cells through the vesicle-treated endothelial monolayer (Li *et al.*, 2018). The isolated vesicles also enhanced the expressions of VCAM 1 and TLR-2 in exposed macrophage cells (Li *et al.*, 2018). More recently, MVs from wild *M. ulcerans*, was found to contain 2 μ g mycolactone for high secretion of pro-inflammatory IL-1 β from human macrophage cells injurious to mouse footpath (Foulon *et al.*, 2020).

Many of these reports ascribed the stimulatory impact to lipoproteins and other TLR-2 agonists found in exogenic vesicles from pathogenic mycobacteria (Athman *et al.*, 2015). Such a claim was supported by report that LprG and LpqH, which are membrane-associated lipoproteins can engage TLR-2 on CD4⁺ T-cells and act in synergy with anti-CD3 or anti-CD28 to stimulate T-cells secretion of IL-2 and INF- γ cytokines required for effective immune response (Lancioni *et al.*, 2011). Among others, LprG, LprA, LprF, LpqH are enriched in MVs from pathogenic mycobacteria. These lipoproteins are immune-reactive and could induce florid pro-inflammatory response in immune cells leading to host's cell death and tissue injury (Athman *et al.*, 2015;Palacios *et al.*, 2019;Prados-Rosales *et al.*, 2011;Wang *et al.*, 2015). Interestingly, mycolactone requires an unknown intermediary TLR-2 ligands (PAMPs) in isolated *M.*

ulcerans MVs to provide first signal before it can induce strong, secondary pro-inflammatory response in host macrophage cells via IL-1 secretion. (Foulon *et al.*, 2020). This suggests that *M. ulcerans* MVs, without mycolactone, can impact on host's macrophage cell functions.

Tissue homeostasis requires tight regulation of pro-inflammatory cells like macrophages to minimize oxidative stress and enhance efficient tissue recovery, especially in skin or muscle injuries. Oxidative imbalance can create chronic skin conditions like Buruli ulcer (Van den Bossche *et al.*, 2017). The high content of TLR-2 agonists in mycobacterial MVs (Prados-Rosales *et al.*, 2011) may potentially induce oxidative stress in host's cells. Therefore, it may be worthwhile to evaluate how *M. ulcerans* MVs influence internal generation of ROS in host's macrophage cells.

2.7.6.2. Anti-inflammatory Impact of Membrane Vesicles from Mycobacterial

Pathogens

Conversely, MVs from pathogenic mycobacteria also possess inhibitory impact on host's immune cells. Complexes of lipoproteins, lipoglycans and glycolipids often found in these membrane vesicles can interact with specific PPRs to limit immune potency against invading microbes (Layre, 2020). Evidence to this concept is found in previous report which indicated that extracellular vesicles from *M. tuberculosis* stifled activation and proliferation of cultured CD4⁺ T-cells and prevented secretion of IL-2; a cytokine which enhances host's immune response to antigens (Athman *et al.*, 2017). This vesicle-induced inhibition on adaptive immune function was strong enough to defy repeated lymphocytes' stimulations with anti-CD28 and anti-CD $\square$ meant to relieve the inhibition on them (Athman *et al.*, 2017). Also, MVs from the same pathogen de-sensitized macrophage cells and made them insensitive to INF- $\square$ activation required for pro-inflammatory functions against mycobacterium pathogens in host's tissues (Wang *et al.*, 2019).

The anti-inflammatory impact of MVs was further consolidated in a recent study which indicated that MVs from *M. tuberculosis*-infected J774A.1 cell line contained phosphatidylserine and four (4) unique proteins (22 kDa, 40 kDa, 50 kDa and 100 kDa) which limited *in vitro* macrophage activation and blocked secretion of MCP-1 and TNF- α from the phagocytes (García-Martínez *et al.*, 2019). Moreover, physiologically active Lipoarabinomannan (LAM) enriched in MVs from mycobacteria pathogens can limit signaling cascades downstream of T-cell receptor (TCR) to downregulate phosphorylation of critical molecules like proximal lymphocyte specific tyrosine kinase (Lck), zeta-chain-associated protein kinase 70 (ZAP-70) and linker of activated T-cell (LAT) necessary for T-cell proliferation and IL-2 secretion (Mahon *et al.*, 2012). In summary, the above reports indicate strong immune-regulatory functions of extracellular vesicles released from mycobacterial pathogens either to selective advantage of the pathogens (immune evasion) or against their successful tissue colonization (immune protection) (Wang *et al.*, 2019).

Study's Rationale

Therefore, it may be imperative to inquire more about how such vesicles can assist *M. ulcerans* to adapt to host's tissues and induce disease. Almost all the existing data in this regard originates from two clinically significant tuberculous strains; *M. tuberculosis* and *M. bovis*. Data on virulent functions of *M. ulcerans* and *M. marinum* is very limited. Also, there is limited knowledge on how such *M. ulcerans* vesicles influence innate immune cell function. *M. ulcerans* survives and multiplies within host's phagocytes, even at low infectious doses (Torrado *et al.*, 2007). Whether this transient intracellular lifestyle goes with the release of unique pathogen-specific antigens via membrane vesicles is unknown.

A study reported of some vesicular bodies within extracellular matrix (ECM) of 6 weeks cultured *M. ulcerans* (Marsollier *et al.*, 2007). The vesicles contained 57 proteins none of which

had a virulent function. Also, the vesicles were cytotoxic and could induce necrosis in more than 80% of cultured macrophage, HeLa and Cos cells. The cytotoxicity was explained to have been caused by mycolactone believed to be contained in the vesicles just because the vesicles originated from mycolactone-producing *M. ulcerans* and that vesicles from mycolactone-deficient *M. ulcerans* did not show cytotoxic effect. Therefore, the authors suggested, and recently confirmed that membrane vesicles from growing *M. ulcerans* mediate transport of mycolactone for Buruli ulcer pathogenesis (Foulon *et al.*, 2020; Marsollier *et al.*, 2007).

A careful look at the study's outcome reveals some gaps in the study's experimental design. First, technical details on how such vesicles were separated from the matrix and the growth medium of the pathogens were not reported. Only the final ultra-centrifugation force of 40,000 x g for 3 hours was reported (Marsollier *et al.*, 2007). Centrifuge type, specified rotor and adjusted K-factor (ie; relative pelleting efficiency) were all not mentioned in the study's methods. Also, whether the final centrifugal force was sufficient to pellet such nanostructures remains a question. There is a standardized guideline on Minimal Information for Studies of Extracellular Vesicles, 2018 (MISEV2018) issued by the International Society of Extracellular Vesicles (ISEV). The document admits that there is currently no single optimal separation technique for extracellular vesicles. Therefore, it enjoins authors to report details of separation techniques so as to allow for reproducibility (Théry *et al.*, 2018). Secondly, as was graphically shown, the reported responses of macrophage, HeLa and Cos cells lines (different cells) exposed to same concentrations of vesicles was the same (Marsollier *et al.*, 2007). This is quite surprising because the calorimetric MTT redox assay the authors used is limited by variance in physiologic state of different cells due to varied activities of targeted mitochondrial dehydrogenases (Jaszczyszyn *et al.*, 2008; Wang *et al.*, 2011). Therefore, it may be worthwhile to re-evaluate macrophage response to MVs of *M. ulcerans* with a more sensitive fluorometric

assay like Microplate AlamarBlue Assay (MABA). This may provide additional insight into how such vesicles influence immune cell functions in Buruli ulcer pathogenesis. Thirdly, the authors reported 80% cell death by necrosis (lysis) due to the vesicles (Marsollier *et al.*, 2007). However, the MTT assay has loss or gain in cell's metabolic function as its endpoint and not direct cell lysis. Finally, none of the proteins found in the isolated vesicles had its biological activities tested to confirm virulence function (Marsollier *et al.*, 2007). This is in spite of previous shreds of evidence that virulent proteins existed in MVs from *M. tb*, *M. bovis* and *M. avium* (Prados-Rosales *et al.*, 2011) and also, such proteins found in growth medium of growing *M. ulcerans* (Dobos *et al.*, 2001; Dobos *et al.*, 2000; Franco-Paredes *et al.*, 2019; Gomez *et al.*, 2001). In view of all these gaps in previous studies, the current study aimed to confirm the *in vitro* release of EMVs by viable cells of *M. ulcerans* and assess how the pathogen may utilize these exogenic vesicles to mediate virulence on innate macrophage cells through their protein content.



CHAPTER THREE (3)

MATERIALS AND METHODS

3.0.0 Source of Mycobacterial Strains and Growth Conditions

Three (3) strains of mycobacteria were used for the current study; *M. ulcerans* strain (Nm 209), *M. marinum* (Sa200695) and *M. smegmatis*. *M. ulcerans* (Nm 209) is a clinical strain originally isolated from wound swab from a patient in Ghana with PCR confirmed Buruli ulcer case. The isolate was initially cultured on Middlebrook 7H11 agar with 10% OADC (0.6 mls Oleic acid, 50 g bovine albumin, 20 g dextrose, 0.03 g Catalase), passed through Middlebrook 7H9 base broth (BD Difco™, Fischer Scientific) with 10% OADC and 0.2% glycerol, before frozen stocks were prepared and kept at the microbiology laboratory of Animal Experimental Unit, Noguchi Memorial Institute for Medical Research (NMIMR), Ghana. Provision for the study was a kind gesture from Dr. Phyllis Addo of the Unit. Strain of *M. marinum* (Sa 200695), which also conducts virulence in host's tissues with mycolactone congener F, is a fish pathogen isolated from Sea bream, *Sparus aurata* (Red Sea, Israel). This pathogen and the control avirulent, *M. smegmatis* were provided for the current study by Dr. Lydia Mosi, Department of Biochemistry, Cell and Molecular Biology (BCMB), University of Ghana.

Initial stock cultures were prepared from frozen stocks of each strain in M 7H9 base broth with 10% OADC and 0.2% glycerol. Cultures were made in vented T-25 cm² culture flasks, Nunc™ None-treated (Thermo Scientific™) and kept in standing position unagitated at 32°C in a humidified 5% carbon dioxide (CO₂) incubator (Eppendorf, New Brunswick S41i, version 1.98). Incubation continued till cultures were needed for subsequent *in vitro* experiments. All the biological samples were processed in two separate laboratories under supervision and strict adherence to safety protocols. They included Biosafety level 3 (BSL 3) laboratory, Virology department, NMIMR- Legon, Ghana and Microbiology laboratory, BCMB, Legon.

3.1.0 Culture of Test Mycobacterial Strains for Membrane Vesicle Exploration

This was done based on a combined and slightly modified protocol developed by Dobos *et al.*, (2000) and Marsollier *et al.*, (2007). Aliquots from each stock culture were inoculated in freshly prepared M7H9 broth, 10% OADC with or without 0.05% Tween-80 at initial inoculation load of 1 mg/ml. Cultures in standing position were kept at 32°C, 5% CO₂ for 8 to 12 weeks with intermittent agitation at 40 rpm. At week 4 or 5 of incubation, growing cells of *M. ulcerans* (Nm 209) in clumps were de-clumped by passaging (15 times) through a sterile 1 ml tuberculin syringe fitted with 25 guage (25G) needle (BIOLINE, China) (Nazarova *et al.*, 2017). At this point, the growth medium was refreshed with additional freshly prepared 7H9 broth. This led to a total culture volume of around 45 milliliters (mls) in standing vented T-25 cm² culture flasks (Dobos *et al.*, 2000). Since more individual bacilli were observed with cultures of *M. marinum* (Sa 200695) under the same growth conditions, the growing cells were not de-clumped as was done with *M. ulcerans* cells. Further incubation ensued until the 8th to 12th week of incubation. Fast-growing *M. smegmatis* cells were rather incubated over a short period of 2-3 days under the same growth condition as *M. ulcerans* and *M. marinum* until culture Optical Density (D₆₀₀) of 0.6 was obtained. Finally, the growth medium of each strain was harvested and explored for extracellular membrane vesicles.

The release of extracellular membrane vesicles is a conserved phenomenon among prokaryotes (Coelho *et al.*, 2019). To avoid or minimize possible confounding outcomes due to other contaminated bacteria, the cultured strains were monitored by physical inspections on two alternate's days for evidence of mixed growth. This was complemented with culture purity check by Ziehl Nelsen staining technique (concentrated method) and spread culture on LB agar at 37°C. All contaminated cultures of the test strains were excluded from the study. At the

end of incubation mycobacterial cell viability was assessed prior to membrane vesicle exploration.

3.2.0 Assessment of Mycobacterial Cell Viability at End of Incubation

The viability of cultured mycobacteria was assessed by a two-dye fluorescent assay, LIVE/DEAD® BacLight™ Viability kit (Molecular Probes, Invitrogen, USA). Manufacturer's instructions were strictly followed. Briefly, 1 ml aliquot of a well-mixed culture suspension was washed in sterile 0.85% sodium chloride (NaCl) solution and recovered at 3900 x g, 4°C for 10 minutes. Washed cells were then stained with 3 µl of working fluorescent mixture (1:1 v/v), incubated in the dark at room temperature for 15 minutes. Stained cells were trapped between a sterile glass slide and 18 mm square coverslip in mounting medium.

After brief incubation in the dark at room temperature, the slides were finally mounted on stage and examined with Fluorescent Microscope (Axiol Vert A1, Zeiss, Germany). Viable *M. ulcerans* cells stained with green SYTO9 and were observed and captured under excitation/emission wavelengths of 480 nm/500 nm. Dead mycobacterial cells stained with red propidium iodide (PI) and appeared under 490 nm/635 nm excitation/emission. Captured images were documented and deconvoluted with Image J software (version 1.52a, National Institute of Health, USA). Final assessment of overall mycobacterial cell viability following 8-12 weeks of incubation was made under a merged channel output. Growth medium of *M. ulcerans* culture with >90% cell viability was harvested and processed by differential centrifugations with final ultra-centrifugation stage.

3.3.0 Harvest of Culture Medium for Membrane Vesicles Isolation

Growth media of selected cultures of *M. ulcerans*, *M. marinum* and *M. smegmatis* were harvested by gentle pipetting into sterile centrifuge tube and processed using an experimental

“cut-off-size” model of sedimentation. This model was proposed for efficient separation of extracellular vesicles from biological fluids (Livshits *et al.*, 2015) based on selected parameters from a web-based centrifugation calculator deposited at <http://vesicles.niifhm.ru/> by Research Institute of Physical-Chemical Medicine, Extracellular Vesicle Research Group, Moscow, Russia. The model is also recommended by the International Society of Extracellular Vesicles (Théry *et al.*, 2018).

Briefly, after initial filtration, the culture suspension in a 50 ml centrifuge tube was clarified at 3,000 x g, 4°C for 5 minutes. The ensuing supernatant was gently pipetted into another sterile tube and further spun at an increased speed of 6500 x g, 4°C for 10 minutes to remove residual cells and debris. These two (2) steps of centrifugations were provided by Sorvall Legend X1/X1R centrifuge fitted with FiberLite™ F15-8x50c fixed-angled rotor (Thermo Scientific, UK). Where required, swinging bucket rotor, Tx-200 SW was used with estimated parameters from <http://vesicles.niifhm.ru/>. The final high-speed supernatant was carefully transferred into a 13.7 ml Poly-Allomer Re-seal™ tubes (Seton Scientific, USA) and spun at an ultra-speed of 120,000 x g, 4°C for 70 minutes using Sorvall MTX150 micro-ultracentrifuge equipped with S58-A fixed-angled rotor (Thermo Fisher Scientific, UK). This force of centrifugation provided a medium pellet expected to contain extracellular nano-membrane vesicles with a “cut-off-size” range of 30 nm to 765 nm. The separated pellets were then washed in 1X PBS (pH 7.4, no magnesium, no calcium, Gibco, UK) and recovered at 120,000 x g, 4°C for 70 minutes. The final pellet of vesicles was then resuspended in 100µl PBS and stored at -80°C for downstream biophysical and proteomic analysis. Total protein concentration was used to quantify isolated vesicles from growth medium of *M. ulcerans* (Théry *et al.*, 2018). This was initially assessed by NanoDrop ONE (Thermo Fisher Scientific, Wilmington, USA) at 280 nm wavelength and confirmed with Pierce™ BCA protein assay kit at 562 nm (Thermo Scientific,

Illinois, USA). Also, the ensued supernatant was concentrated by Amicon® Ultra-15 10K cut-off filter (M, Carrigtwohill Ireland) and stored at -80°C for proteomic analysis.

3.4.0 Extraction of Extracellular Matrix (ECM) For Membrane Vesicles Exploration

Extracellular matrix (ECM) of cultured *M. ulcerans* is reported to contain extracellular vesicles (Marsollier *et al.*, 2007). Therefore, such matrix was explored in the current study. Harvested cells of *M. ulcerans* were resuspended in 1X PBS and briefly treated with 2 mm glass beads for 15 seconds vortex. This was to mechanically disrupt and extract ECM from the cultured cells (Marsollier *et al.*, 2007). Matrix suspension was gently pipetted into sterile centrifuge tubes and further processed by differential ultra-centrifugation as described above. The final pellets and supernatant concentrates were stored at -80°C for downstream analysis.

3.5.0 Biophysical Analysis by Transmission Electron Microscopy (TEM)

Biophysical analysis on the separated medium pellet from cultures of *M. ulcerans*, *M. marinum* and *M. smegmatis* was done by TEM using negative staining technique. First, a 400 mesh grid with collodium-carbon coat was mounted onto tweezer dumount No. 5. Then 15 μ L sample from a well-mixed EMVs suspension was aliquoted unto the coated surface and kept at room temperature for 5 minutes. Excess samples on the grids were blotted out with wet filter paper. The grids were then stained with 15 μ L Phosphotungstic acid (PTA) and kept at room temperature for 30 seconds. Excess stain was also blotted out. The stained grids were then allowed to air-dry for 45 minutes before image analysis. Grids were imaged using JEOL-JEM1400 TEM working at an accelerated Voltage of 120 kV on 4x4k CCD camera. Nominal magnification was 8000X. This corresponded to pixel size of 1.36 nm. Electron micrographs growing bacterial cells and medium pellets were observed, captured, processed, and analyzed

with Image J software (version 1.52a, National Institute of Health, USA). Structure and morphology of observed features membrane vesicles were documented.

3.6.0 Detection of Mycolactone in Extracellular Vesicles by *M. ulcerans* (Nm 209) and *M. marinum* (Sa 200695).

The potential presence of mycolactone in discharged membrane vesicles from *M. ulcerans* and *M. marinum* was evaluated by the slightly modified protocol of Folch *et al.*, (1956) for acetone soluble lipids (ASL) as reported by (George *et al.*, 1999). After 10 weeks of incubation at 32°C, the obtained cell pellet of *M. ulcerans* and *M. marinum* were homogenized in chloroform/methanol (2/1) mixture at a ratio of 1-part bacterial cells to 20-part mixture. Similar preparation was made for isolated membrane vesicles from the pathogens' growth media. The homogenates were continuously mixed at room temperature for 12 hours and then span to remove bacterial debris.

The ensuing supernatant was thoroughly mixed with 0.6% sodium chloride (NaCl) solution. This provided a final ration of 8:4:3, chloroform: methanol: NaCl respectively. A biphasic separation of lower immiscible chloroform (organic phase) and upper aqueous phase was obtained. The organic phase was then transferred into glass vials, dried with rotary evaporator (EYELA-CCA1112A) and then dissolved in ice-cold acetone to precipitate phospholipids. The resulting acetone-soluble supernatant (15 μ L) was ran on a thin layer silica chromatographic (TLC) plate, alongside synthetic mycolactone A/B (5 μ L of 50 ng) as control. A mixture of chloroform: hexane: methanol (5:4:1) was used as the mobile phase. Finally, the plate was quickly immersed in 0.1M 2-naphthalene boronic acid, heated at 100°C for 60 seconds and irradiated with 365 nm UV lamp for image documentation. Detection limit of 2 ng mycolactone A/B was assumed (Spangenberg *et al.*, 2010).

3.7.0 A Dose-Sensitivity Analysis: Macrophage Cells Verses Extracellular Vesicles from *M. ulcerans*

The impact of *M. ulcerans* vesicles on viability of RAW 264.7 cells was assessed by non-toxic, fluorescent-based Microplate AlamarBlue Assay (MABA). This technique quantified overall metabolic activities as index of cell viability. The selected protocol was based on recommended reports from (Ayupova *et al.*, 2019) and (Niepel *et al.*, 2019).

3.7.1 Depletion of Potential Mycolactone in *M. ulcerans* Vesicles by Photo-degradation

First, the presence of cytotoxic mycolactone in extracellular vesicles from *M. ulcerans* could confound actual macrophage response to the vesicles (Marsollier *et al.*, 2007). Hence, harvested vesicles were divided into two portions. One portion was kept in 5 mm, 1.75 ml volume semi-micro white wall quartz cuvette (Jay-Hely, Ningbo, China) and exposed to long Ultra-Violet A (UV-A) wavelength (365 nm) using SpectroLine lamp EA-160/FE (Spectronics, Westbury, New York, USA). The essence was to degrade and reduce levels of all native mycolactones possibly associated with the vesicles (Marion *et al.*, 2012). This sample became a UV-A treated sample. However, UV-A can induce structural change to tertiary structure of proteins and cause significant loss in antigenicity and protein's function (Feys *et al.*, 2014; Kristo *et al.*, 2012). Thus, the photo-degradation process was carried out over a maximum exposure period of one (1) hour (Marion *et al.*, 2012). Eventually, native and UV-A irradiated membrane vesicles of *M. ulcerans* were obtained for subsequent dose-sensitivity assessment on RAW264.7 cells.

3.7.2 Optimization Experiment for Dose Sensitivity Period

Secondly, the exact period for efficient response of the macrophage cells to vesicles was optimized. Briefly, frozen stock of RAW 264.7 cells with passage number 8 was sub-cultured 3 times in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine serum at 37°C, 5% CO₂. Afterwards 100 μ L suspension from 2 x 10⁴ cells/ml suspension at 98.9% cell viability was made in flat-bottom 96-well plates (Greiner-bio one, USA) and incubated at 37°C, 5% CO₂ overnight for adherence. Vesicles' concentration was determined by total protein quantification. Subsequently, 20 μ L of either native or UV-A irradiated vesicles at serial protein concentrations of 0.01 μ g/ml, 0.1 μ g/ml, 1.0 μ g/ml and 10 μ g/ml were added to their respective macrophage monolayers in triplicates wells. This gave a total test volume of 120 μ L per test well. Untreated adherent cells and positive control cells containing only 100% reduced resazurin dye were also set up in each experimental plate.

Four (4) different experimental plates for either vesicle type were set up and incubated at 37°C in a humidified 5% CO₂ incubator (HERACELL, 24i, Thermo Scientific). These plates were kept at four (4) different time-points: 6 hours, 12 hours, 18 hours and 24 hours. Four (4) hours prior to end of each incubation period, 12 μ L alamarBlue™ dye (Thermo scientific, USA) representing 10% of total culture volume was then added to each well and incubated further. At end of incubation at each time-point, Relative Fluorescent Unit (RFU) representing change in metabolic activities in vesicle-exposed RAW 264.7 cells were quantified at excitation/emission wavelengths of 560 nm_{EX}/ 590 nm_{EM} using Varioskan LUX, Multimode Microplate Reader (Thermo Scientific). The optimized data was finally analyzed for the most efficient time required to assess the response of RAW 264.7 cells to membrane vesicles from *M. ulcerans*.

3.7.3 Exposure-Response Analysis by Double Dilution

Following the optimization outcome, exposure periods of 24 hours and 48 hours were selected for subsequent dose sensitivity assay using double dilution of test samples. As described above, a “healthy” RAW 264.7 monolayer with 99.2% cell viability in 96-well plate was exposed to double-diluted concentrations of either native or UV-A irradiated vesicles from *M. ulcerans*. The twelve (12) test concentrations ranged from 6 mg/ml through to 0.0029 mg/ml. Technical triplicate wells for each test in different replicate plates were made. Some of the untreated cells were treated with 5 mg/ml of Amphotericin B (potent cytolytic agent) to serve as positive control for acute cell lysis. Experimental plates were incubated at 37°C, 5% CO₂ for 24 hours and 48 hours. Four (4) hours prior to end of incubation, alamarBlue dye (10% total culture volume) was added to each experimental well. At the end of incubation, RFU was quantified as described above and analyzed. An increase in activity was expected. Hence, average RFU of untreated cells was deducted from RFU of each test well (A_0). Also, the average untreated RFU was deducted from average RFU of 100% reduced resazurin dye (A_1). The percentage (%) cell viability of treated RAW 264.7 cells was then calculated using the formula $(A_0/A_1) \times 100$. Additionally, the morphology and number of vesicle-treated macrophage cells were respectively captured by phase contrast microscopy and counted using the multi-point built-in function of Image J software. The outcome was compared with amphotericin B treated and untreated RAW 264.7 cells.

3.7.4 Total Intracellular Reactive Oxygen Species in Vesicle-Treated RAW 264.7 Cells

The impact of *M. ulcerans* vesicles on redox environment of exposed RAW 264.7 cells was evaluated by a cell permeate dye, 2',7'-dichlorofluorescein diacetate, DCFDA cellular ROS detection kit (Abcam, New Zealand). The manufacturer's instructions were strictly followed. Briefly, 100 µL macrophage cells at 3×10^4 cells/ml was seeded in a flat-bottom 96-well plate

and were allowed 12 hours to adhere at 37°C, 5% CO₂. The monolayer was then exposed to either untreated or UV-A irradiated membrane vesicles from *M. ulcerans* at concentrations of 3.0 mg/ml, 1.5 mg/ml, 0.75 mg/ml, 0.375 mg/ml, 0.188 mg/ml and 0.094 mg/ml and incubated at 37°C, 5% CO₂ for 24 hours or 48 hours. Untreated cells serving as negative control and a positive control cell meant for treatment with 500 μ M Tert-butyl Hydrogen Peroxide (TBHP) were also set up. Duplicate wells were prepared in experimental replicates. Four (4) hours prior to end of incubation, the positive control cells were treated with 11 μ L of TBHP prepared in 1X supplemented buffer. In some circumstance, 50 μ M TBHP (10X dilution of initial 500 μ M) was used. Forty-five (45) minutes prior to end of incubation, each of the vesicle-treated and control wells were treated with 100 μ L of 40 μ M DCFDA dye prepared in 1X buffer followed by incubation in the dark. At end of incubation, fluorescent intensity of oxidized dichlorofluorescein products in the treated RAW 264.7 cells as well as control cells were quantified with Varioskan LUX, Multimode Microplate Reader (Thermo Scientific) at an excitation/emission wavelength of 495 nm/529 nm, analyzed and compared.

3.8.0 Proteomic Analysis

3.8.1 Preparation of *M. ulcerans* Lysates

Recovered cells of *M. ulcerans* were resuspended in 200 μ L of RIPA lysis buffer (50 mM Tris pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS). The mixture was then sonicated on ice for 45 minutes at 35% amplitude (5 seconds ON cycle, 10 seconds OFF cycle) using Q500 Sonicator with micro-tip probe 1/16 (1.6 mm) (Qsonica, USA). Lysates were clarified at 13,000 rpm for 10 minutes, 4°C. Supernatants were quickly aliquoted, mixed with 1X protease inhibitor cocktail (Sigma-Aldrich) and stored at -20°C.

3.8.2 One (1)-Dimensional Gel Electrophoresis for Mass Spectrometry

Different protein concentrations of *M. ulcerans*' lysates, isolated membrane vesicles and concentrated supernatants were normalized to 2.0 mg protein load and each sample reduced using 1X SDS-sample loading buffer (1.67% SDS, 1.67% β -mercaptoethanol, 5% glycerol, 50 mM Tris pH 6.8 and 0.0062% bromophenol blue) at 95°C for 8 minutes. The mixture was then clarified at 13,000 x g for 10 minutes. Twenty microliters (20 μ L) of each reduced sample was then loaded onto a 15-well, 4 -12% Bis-Tris Plus precast gel (Invitrogen, USA) for electrophoresis at 90 volts. Each electrophoretic run was marked with standard broad ranged molecular weight marker, P7712 (Biolabs, New England). Resolved protein bands were then stained with SimplyBlue™ Safestain (Invitrogen, USA) for 1 hour following the manufacturer's instruction and imaged using Amersham Imager 600 (Buckinghamshire, UK) for documentation.

3.8.3 In-Gel Digestion for Mass Spectrometry

Tryptic digestion of separated proteins was based on recommended protocol for rapid protein identification (Shevchenko *et al.*, 2006). Initially, brief electrophoresis for stacked protein bands was done. Stacked bands were then excised from the gel slab against white background, further sliced into smaller gel pieces (1 x 1 mm) into 1.5 ml deplasticized tube and de-stained with 100 mM ammonium bicarbonate with neat acetonitrile (1:1, vol/vol) for 30 minutes coupled with occasional vortex. Destained solution was pipetted out and the process repeated until the gel pieces shrink and got adequately destained. The gel pieces were then dried in Savant Speed-Vac and covered with 13 ng/ μ L sequencing grade trypsin (Roche Molecular Biochemicals), in 1:20 trypsin : protein ratio at room temperature until the trypsin solution was adsorbed into the gels to swell. Total digestion volume was topped-up with additional 0.2M ammonium bicarbonate to cover gels and further incubated at 37°C for 16 hours.

Afterwards, 100 μ L 0.1% trifluoroacetic acid in 60% acetonitrile (v/v) was added to the gels, briefly vortexed and further incubated at 37°C for 40 minutes, with gentle agitation. After a brief centrifugation period to collect condensations on the tube's lid, peptide-containing supernatant was transferred into sterile deplasticized tubes using pipette with fine gel loader tips. The extraction process was repeated three times. All the ensued supernatants were then pooled and dried in Savant speed-vac. Digested peptides were then mixed with MS buffer A (0.1% formic acid, 3% acetonitrile and 96.9% water) at 1 μ L/ μ g. Following 5 minutes of centrifugation, the extract was finally pipetted into autosampler vial, labelled and sent for analysis by linear ion trap LC/MS-MS. Residual extracts and gel pieces were stored at -20°C as contingency.

3.8.4 LC-MS/MS

Proteins were analyzed on Orbitrap Velos MS platform coupled with nano-HPLC instrument (Thermo Scientific) as recently reported (Ramirez *et al.*, 2019). Briefly, digested peptides (0.5 μ g) were randomly injected, in duplicate, into an EASY nanoLC-II system (Thermo Scientific, San Jose, CA), purified and concentrated on an on-line enrichment column (EASY-Column, 100 μ m ID x 2 cm ReproSil-Pur C18). Chromatographic separation was then carried out on a reverse phase nanospray column (EASY-Column, 3 μ m, 75 μ m ID X 100 mm ReproSil-Pur C18) using a linear gradient of 5% to 45% solvent B (100% Acetonitrile, 0.1% formic acid) at a flow rate of 400 nL/min for 90 minutes. The peptides were then directly eluted into the Orbitrap Velos MS (Thermo Scientific) and analyzed in an Orbitrap-LTQ mode. Precursor ions were acquired in Orbitrap with 60,000 resolution. Top 20 tandem MS/MS spectra were also acquired in the LTQ ion trap using a normalized collision energy of 35%.

3.8.5 Database Searching

ProteoWizard (MSConvert version 3.0) was used to convert the tandem mass spectra data into mzXML files and analyzed on an integrated platform consisting of Sorcerer2™ (version 5.0.1, Sage-N Research, Milpitas, CA, USA) and SEQUEST (version 3.5, Thermo Fisher Scientific, San Jose, CA, USA). SEQUEST was primed to search UniProt, Agy99 database. This included all reverse entries as decoys, trypsin as digestion enzyme and a maximum of two missed cleavage sites. A fragment ion with mass tolerance of 1.00 Da and parent ion tolerance of 20 ppm were searched. Specified variable modifications in SEQUEST included methionine oxidation (15.99 amu) and carbamidomethylation of cysteine (57.02 amu) (Ramirez *et al.*, 2019).

3.8.6 Protein Identification

Peptides and proteins identified were validated by Scaffold (version Scaffold_4.11.1, Proteome Software Inc. Portland). Peptide identity was accepted at more than 95% probability using Scaffold Local FDR algorithm at 1%. Protein probability was assigned by Protein Prophet algorithm against Agy99 database. Protein identity was accepted if it could be established at greater than 99% probability with at least two (2) unique peptides. Proteins which could not be differentiated by MS/MS analysis alone due to similar peptide profile were grouped to satisfy the principle of parsimony. To compare protein compositions in the analyzed samples, protein quantities were expressed as normalized spectra abundance factor (NSAF). Fold difference of individual protein (vesicle count/lysate count) between samples was then computed. Absence of a protein in either compartment was represented by factor of 1×10^{-8} spectra count. This was to avoid the issue of dividing by zero. Protein abundance, in terms of fold difference, was aligned to assess the most abundant vesicular proteins.

3.9.0 Statistical Analysis

All statistical analysis was made with GraphPad Prism version 8.1.1 (224). Differences in size distribution of isolated vesicles from *M. ulcerans*, *M. marinum* and *M. smegmatis* were analyzed and compared by one-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison tests. Mean (SD or SEM) and median (interquartile range) at 95% confidence interval were computed where appropriate. Also, pairwise analysis on grouped dataset on optimized cell viability was done for both native and UV-A irradiated vesicles. Subsequent dose-stimulation curves, based on % cell viability were generated with XY datasets. The best fit model of [Agonist] vs. Response---Variable slope (four parameters) was generated for both native and UV-A irradiated vesicles. EC₅₀ was extrapolated from the appropriate sigmoid response curve where necessary. Then a two-way ANOVA followed by Tukey's multiple post-hoc tests on grouped datasets on macrophage's cell viability after exposure to membrane vesicles was done. Moreover, heatmap analysis on column datasets of relative fluorescent unit (RFU) following treatment with membrane vesicles was done. Finally, the number of vesicle-treated cells were counted, analyzed and compared by one-way ANOVA. A p-value < 0.05 was considered statistically significant. This was grouped as moderately significant, $p = 0.0332$ to 0.05 (*), highly significant, $p = 0.002$ to 0.029 (***) and extremely significant, $p < 0.0001$ (****)



CHAPTER FOUR (4)

4.0 RESULTS

4.1.0 *M. ulcerans* Growth And Viability at EMVs harvest

Two (2) pre-requisites for release of extracellular vesicles from bacteria cells include cell viability and growth (Athman *et al.*, 2015;Palacios *et al.*, 2019;Prados-Rosales *et al.*, 2011). First, we sought to establish these cellular processes in the test strains. Despite its slow growth rate, growth of *M. ulcerans* (Nm 209) occurred with steady increase in the cells' biomass from the day of inoculation through to the 8th -12th weeks of incubation at 32°C (**Figure 4.1.0, a-d**).

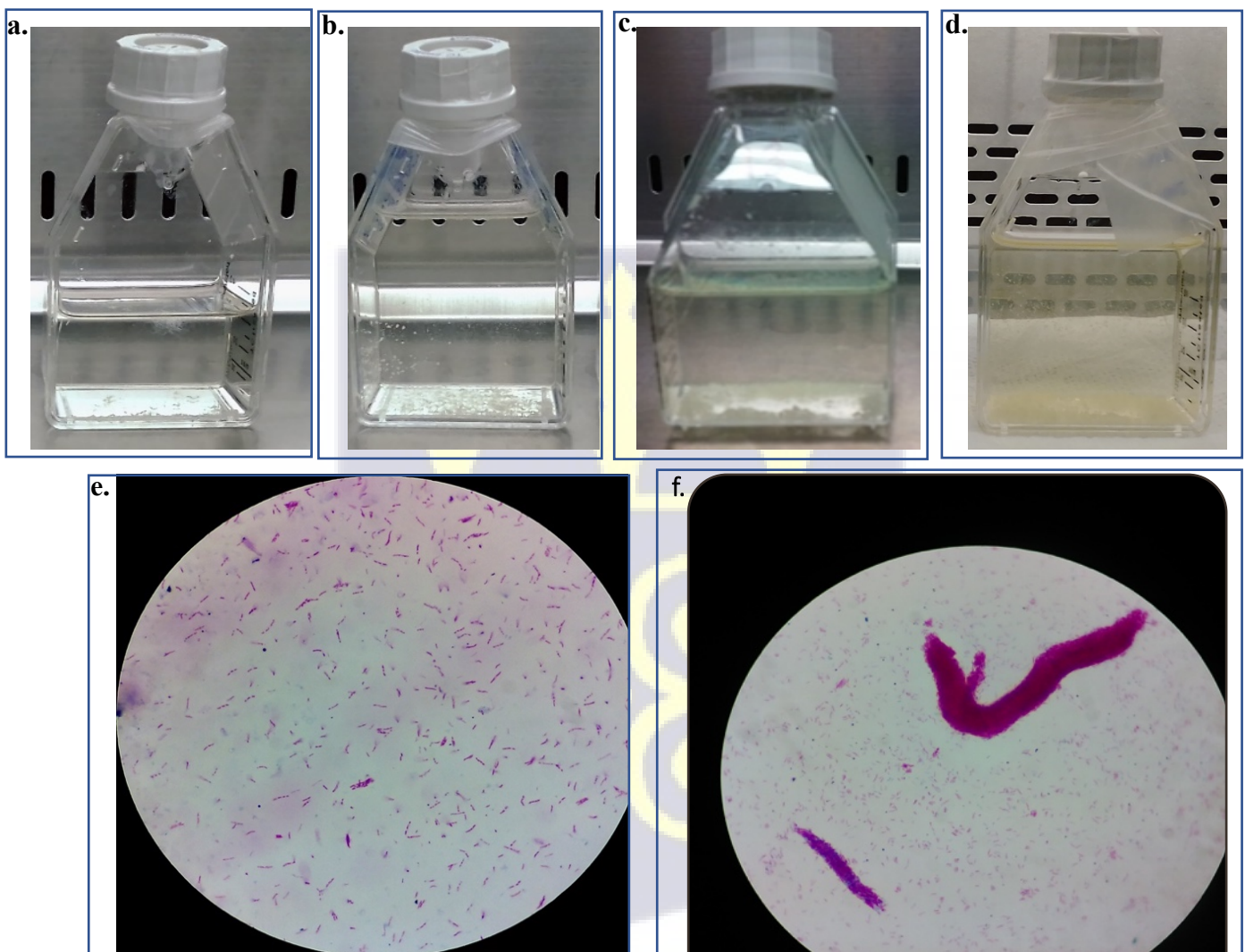


Figure 4.1.0: Dynamic growth of *M. ulcerans* in M7H9 broth supplemented with/without Tween-80

(a to c) A consistent increase in bacterial cell biomass; (a) initial inoculum (b) culture at week 3 (c) culture in Tween-80 supplemented medium at week 9 (d) culture in medium without Tween-80 at week 9. (e) Cultures in Tween-80 supplemented medium showed a more dispersed Acid-Fast Bacilli (AFBs) compared to (f) “clumpy”, rod-like, serpentine characteristics in medium without Tween-80. Magnification X1000

Also, *M. ulcerans* growth occurred in response to medium constituents. Although growth characteristics was similar between Tween-80 supplemented cultures (**Figure 4.1.0, c**) and cultures without the detergent (**Figure 4.1.0, d**), cultures with Tween-80 produced a more dispersed individual Acid-Fast Bacilli (AFBs) (**Figure 4.1.0, e**). The dispersive force of Tween-80 was able to minimize “clumpy” aggregation of mycobacterial cells in culture. In contrast, cultures in medium without Tween-80 existed with clumpy, rod-like, serpentine cords during the 8-12 weeks of incubation (**Figure 4.1.0, f**). Absence of Tween-80 allowed enhanced expression of cohesive trehalose-6’6’-dimycolate (a membrane glycolipid also called cord factor) which promotes cording of pathogenic mycobacterial cells in cultures (Caceres *et al.*, 2013). Similar growth kinetics was observed with cultures of *M. marinum* (Sa 200695) but the level of bacterial cell clumping was minimized and almost non-existent in cultures of the controlled avirulent *M. smegmatis*.

Invariably, *in vitro* growth of *M. ulcerans* is associated with the pathogen’s cell viability, most especially during the first 8 weeks of incubation. Beyond this point, an inverse relation was observed between cell viability and age of culture (**Figure 4.1.1**). Compared to control heat-killed cultures under excitation/emission wavelengths of 490/635 nm (**Figure 4.1.1a**), cultures at week 8 showed many viable mycobacterial cells with intact cell envelop. This allowed the cells to exclude red DNA-binding Propidium Iodide (PI) and stained with green SYTO9 under 480/500 nm excitation/emission wavelengths (**Figure 4.1.1b**). Similar enhanced cell viability was observed at week 9 of culture (**Figure 4.1.1c**). Many live cells occurring in satellites clumps existed. However, when the period of incubation at 32°C, 5% carbon dioxide was extended to week 12, the number of dead *M. ulcerans* cells began to accumulate showing many red-stained mycobacteria and a reduced number of live, green-fluorescent cells (**Figure 4.1.1d**).

This growth dynamics provided more than 95% cell viability at week 8 and 9 of culture suggesting potential exponential growth phase of *M. ulcerans* at weeks during these periods while week 12 marked possible onset of stationary phase as nutrients in the growth medium depleted and toxic wastes accumulated to initiate bacterial cell death.

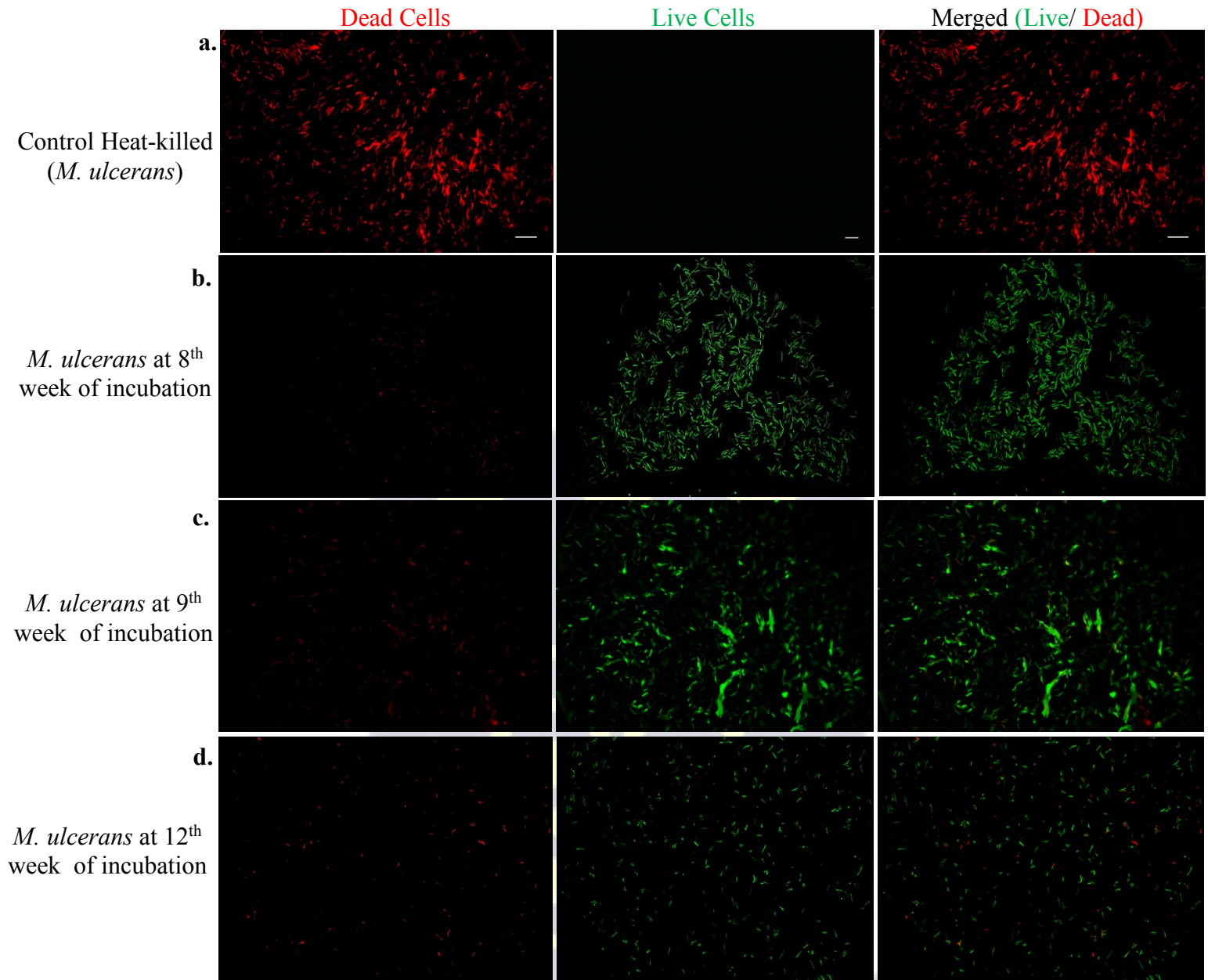


Figure 4.1.1: An inverse relation between the number of LIVE *M. ulcerans* cells under 480_{EX}/500_{EM} nm and age of culture. (a) DEAD control cells under 490_{EX}/635_{EM}, (b) more viable, less dead bacterial cells (>95% cell viability) at culture week 8, (c) dead cells begins to accumulate at week 9, (d) Many dead cells at week 12 as number of viable cells dwindles (~ 75% cell viability). Scale bar: 10 μ m.

4.2 Differential Centrifugations of Growth Medium Yields a Unique Pellet

Growth media of cultures at weeks 8 and 9 were then harvested and processed to explore traces of growing *M. ulcerans* (Nm 209). Following series of selective separations under increasing centrifugal forces (Livshits *et al.*, 2015;Théry *et al.*, 2018), no pellet was found in the controlled un-inoculated medium that was incubated under the same experimental conditions as cultures of the pathogen (**Figure 4.2, a**). But after the final force at 120,000 x g for 70 minutes, growth media from *M. ulcerans* cultures yielded a unique, white pellet with yellowish taint at the bottom-up of each centrifuge tube (**Figure 4.2, b**). Similarly, medium pellets were separated from growth media of *M. marinum* (Sa 200695) and *M. smegmatis*. This suggested that such medium pellets, as in suspension (**Figure 4.2, c**), originated from the growing mycobacterial cells.

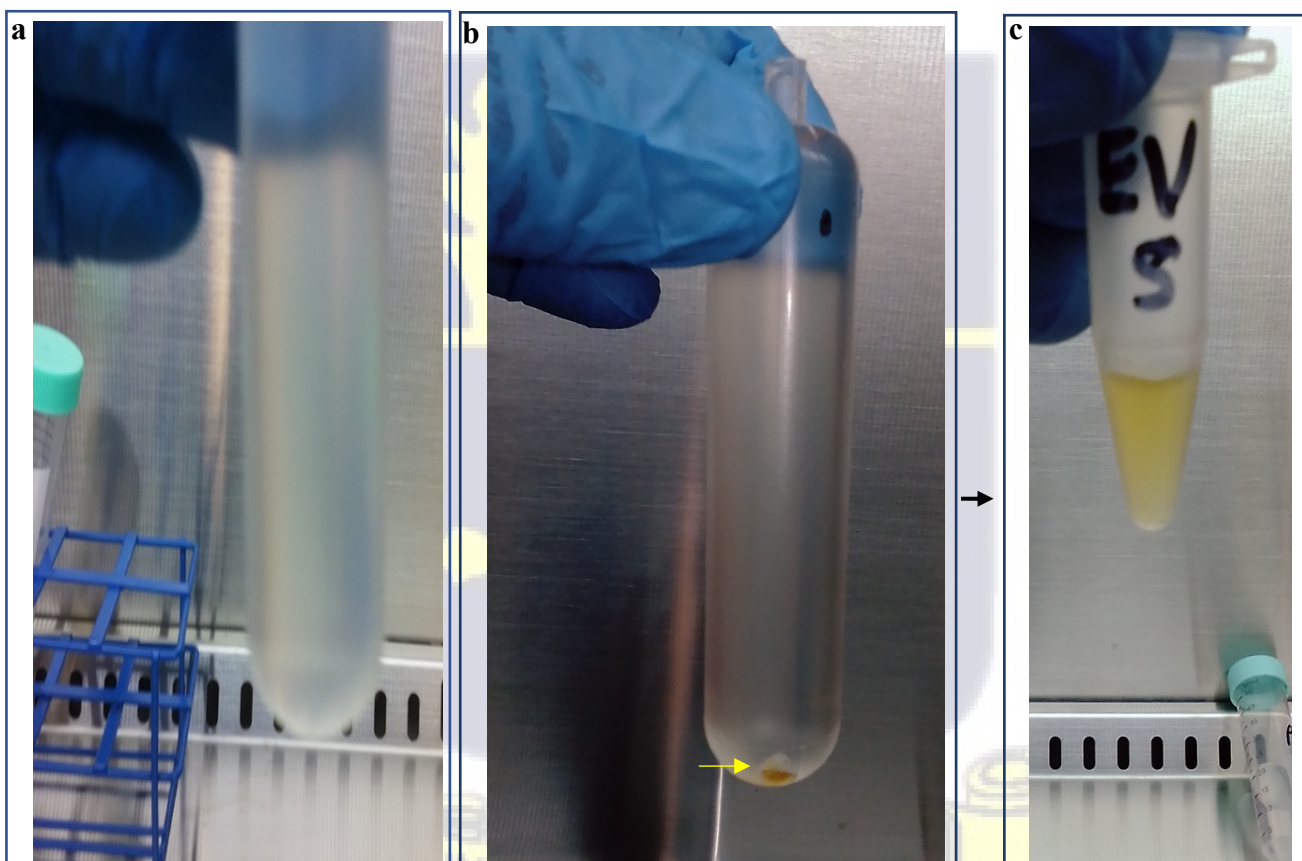


Figure 4.2.0: Outcome of differential centrifugations with final ultra-centrifugation by model of sedimentation “cut-off-size” (Livshits *et al.*, 2015). (**a**) No pellet was obtained from the controlled, uninoculated medium, (**b**) recovered pellet (arrowed) from growth medium of *M. ulcerans* after final ultra-centrifugation, (**c**) pellet suspension for downstream analysis.

4.3 Pellets from Growth Media of *M. smegmatis*, *M. marinum* (Sa 200695) and *M. ulcerans* (Nm 209) Contained Intact Extracellular Membrane Vesicles.

First, we sought to confirm the release of extracellular membrane vesicles by *M. smegmatis* as reported in literature (Prados-Rosales *et al.*, 2011). Transmission Electron Microscopy (TEM) on the medium pellet revealed many intact, membrane-bound, nano-vesicles scattered within the field of view of the pellet (**Figure 4.3.1**). These membrane vesicles appeared similar in structure and morphology to the previously reported EMVs in literature and confirmed *in vitro* release of these vesicles by the control strain. More importantly, the evidence demonstrated how effective our vesicles-separation technique was, especially for separating mycobacterial EMVs, although the model was initially developed for isolation of mammalian EVs (Livshits *et al.*, 2015) as recommended by the International Society of Extracellular Vesicles (Théry *et al.*, 2018).

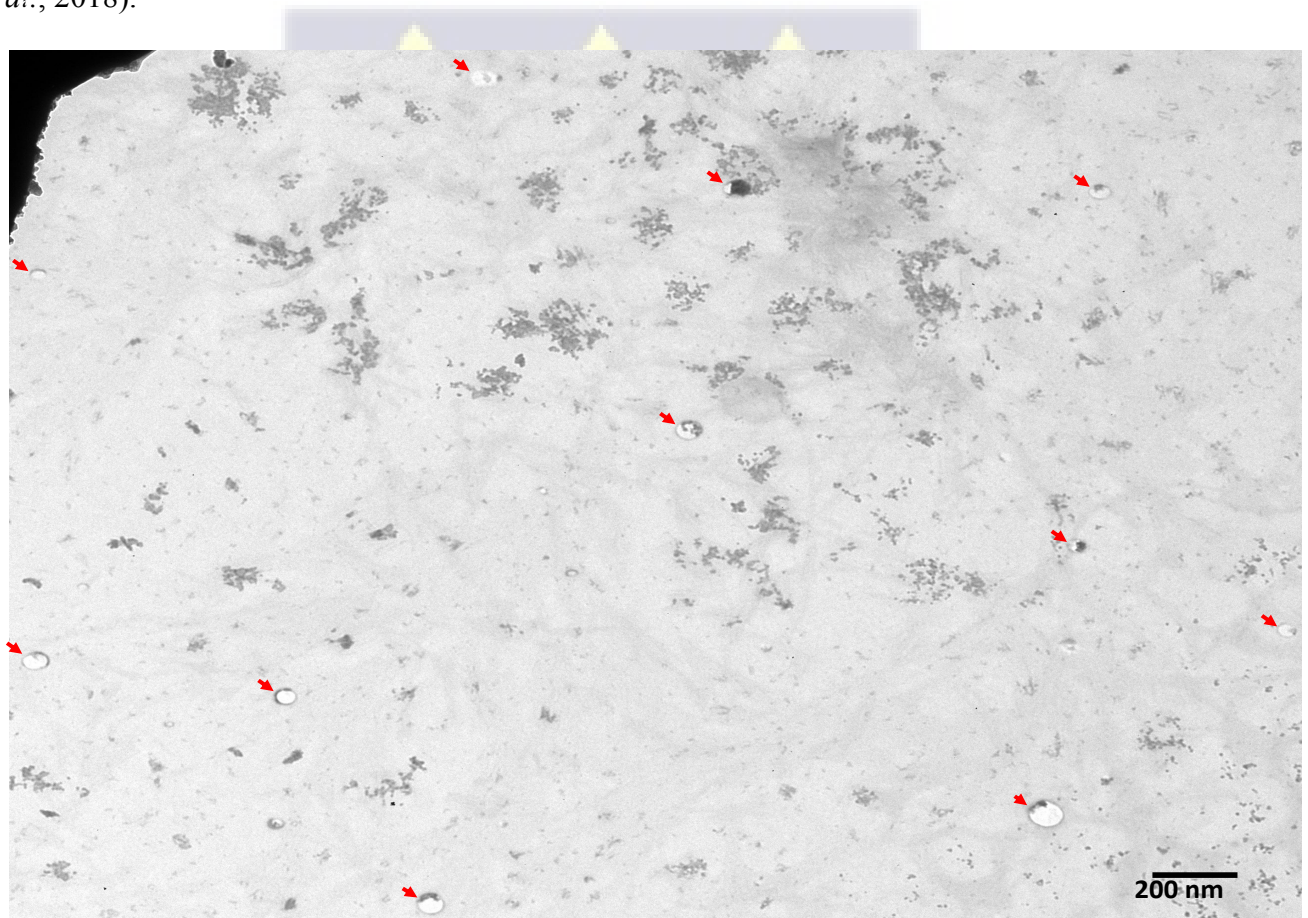


Figure 4.3.1a: Transmission electron micrograph showing scattered membrane vesicles (arrowed) of *M. smegmatis* used to model the current study.

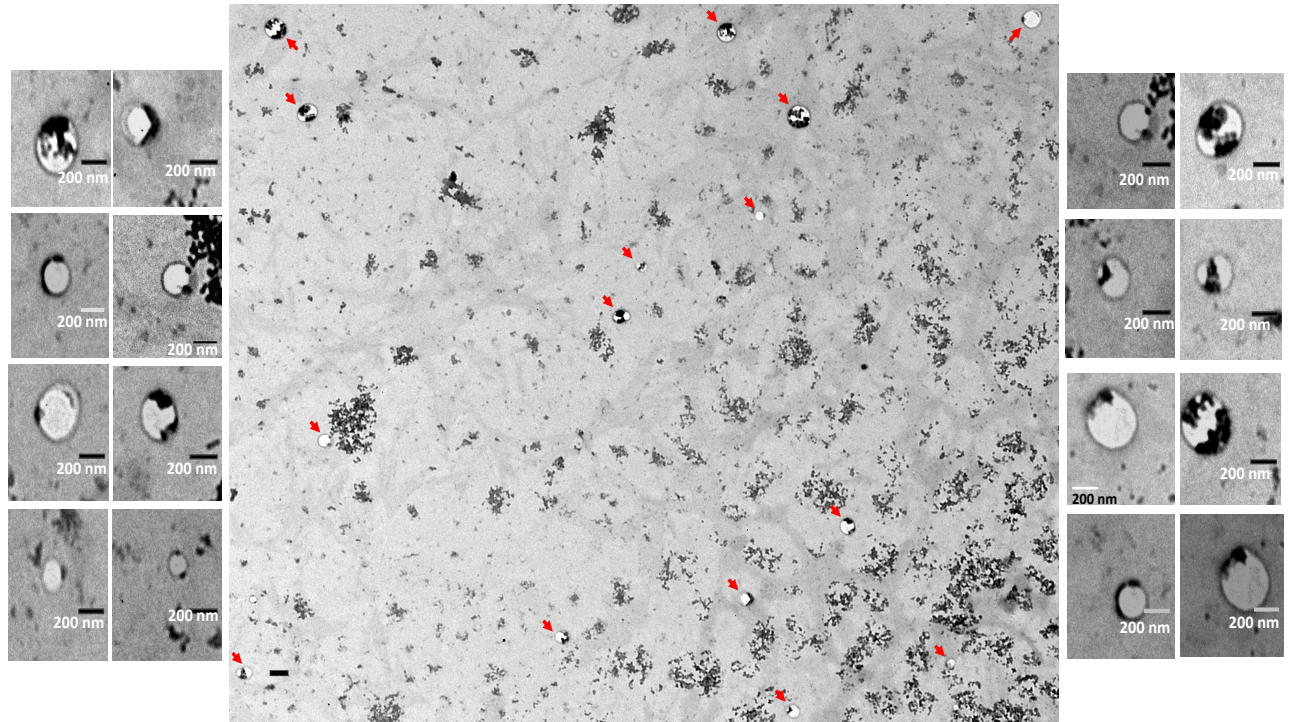


Figure 4.3.1b: Transmission electron micrograph showing scattered membrane vesicles (arrowed) of *M. smegmatis* used as a model to the current study. The micrograph represents two independent experiments. JEOL JEM-1400, 120kV Nominal Magnification 8000x, scale bar 200 nm.

Next, we sought to establish whether non-tuberculous *M. marinum* (Sa 200695) releases extracellular membrane *in vitro*. Interestingly, observation of separate medium pellet by TEM revealed scattered, intact, membrane-bound vesicles from the pathogen (Figure 4.3.2). These extracellular vesicles appeared similar in structure and morphology to the vesicles found in the growth medium of *M. smegmatis*. However, slight differences were observed between the vesicle cohorts. Vesicles from *M. marinum* appeared larger in dimensions with some irregular outlines and areas of flattened surfaces. Also, the relative number of vesicles released by *M. smegmatis* appeared more than vesicles found in growth medium of *M. marinum*. Notwithstanding, this piece of evidence strongly indicates that pathogenic *M. marinum* (Sa 200695) which also secretes mycolatone F releases extracellular vesicles just like *M. avium*, *M. bovis*, *M. kansasii* and *M. tuberculosis* (Palacios *et al.*, 2019; Prados-Rosales *et al.*, 2011).

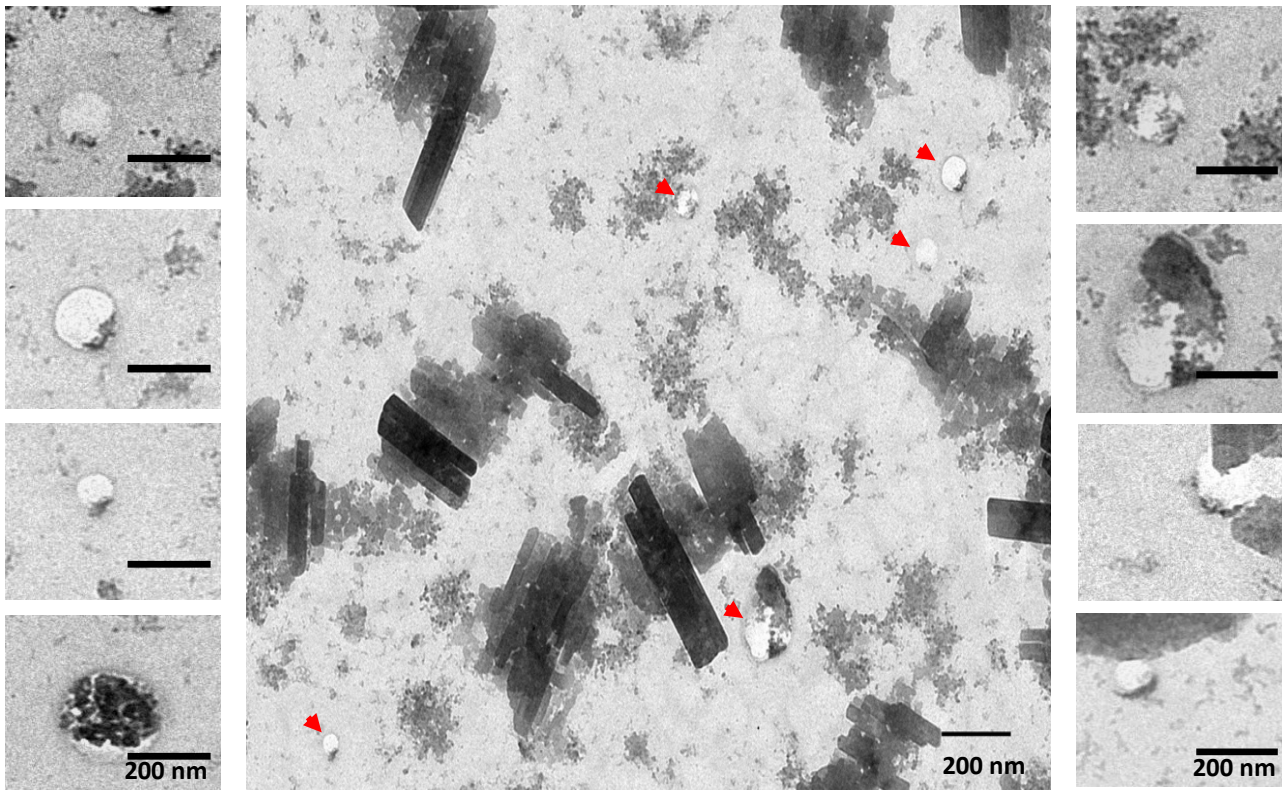


Figure 4.3.2: Transmission electron micrograph showing scattered membrane vesicles (arrowed) released from *M. marinum* (Sa 200695) into the growth medium. Black bars are potential crystals deposits from phosphotungstic acid.

M. marinum remains the ancestral lineage of *M. ulcerans* and shares more than 97% nucleotide identity. The level of similarity was consolidated by the fact that *M. marinum* (Sa 200695) utilized a similar plasmid-mediated mechanism of virulence just like *M. ulcerans*. Therefore, we sought to confirm whether this genetic linkage could support the hypothesis of active membrane vesicle release by clinical strain of *M. ulcerans* (Nm 209).

To this end, growing viable cells of *M. ulcerans* (Nm209) were first subjected to morphological examination by TEM. Stepwise scrutiny along the periphery of a bacillus revealed two (2) remarkable membrane protrusions at the lower epical end of the pathogen (**Figure 4.3.3a**). A blow-out on this part of the mycobacterium revealed three (3) impressive membrane vesicles emerging from the cell envelop of the bacillus (**Figure 4.3.3b**).

Two of such vesicles were found to be released from the laterals as the third vesicles originated from the lower epical end. This biophysical evidence strongly suggested that the growing *M. ulcerans* cells were in active process of releasing extracellular vesicles into the growth medium.

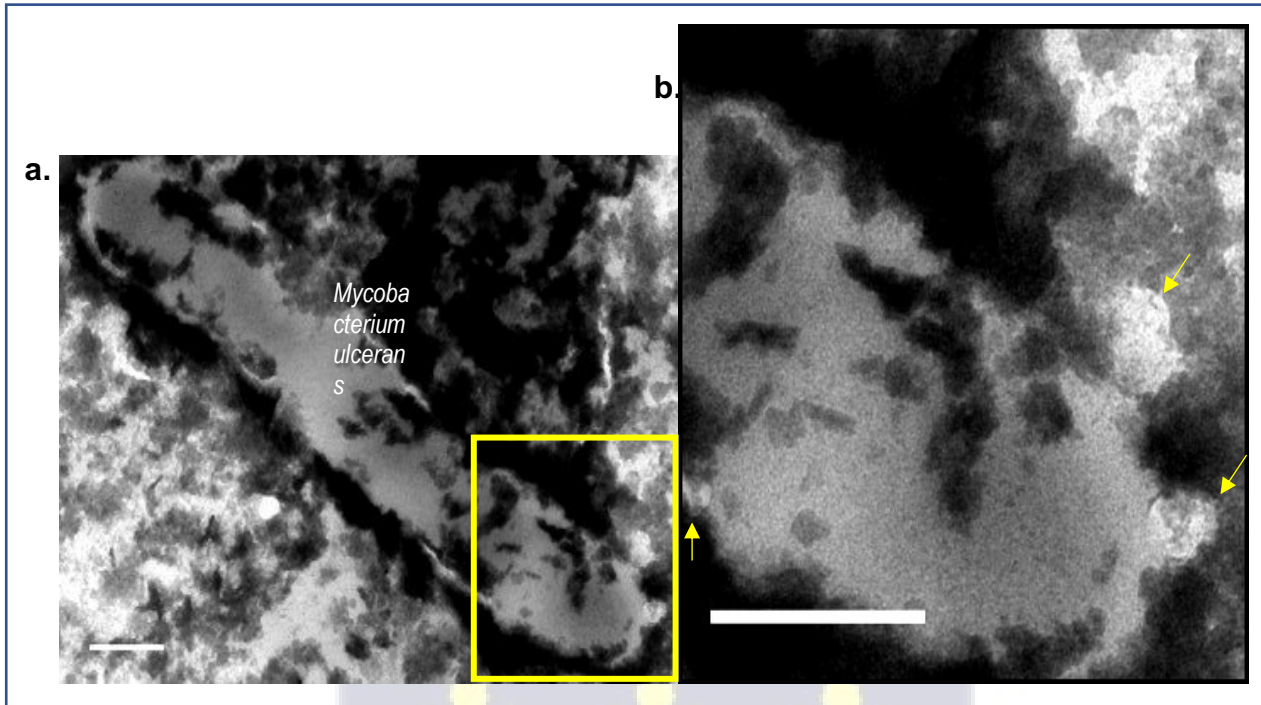


Figure 4.3.3a: Transmission electron micrograph showing membrane vesicles being released from *Mycobacterium ulcerans* (Nm 209). (a) Initial micrograph revealed two (2) membrane bulge-outs at lower epical end of *M. ulcerans*, (b) a blow-out image (insert) confirmed three (3) unique membrane vesicles (red arrowed) emerging from the lower end of the bacilli. JEOL JEM-1400, 120kV, Nominal magnification 8000x. Scale bar: 200 nm

Although Hayman *et al*; (1985) made no indication of this cellular process in their previous report, the current evidence is similar to captured micrograph of the pathogen in Buruli ulcer lesion (Figure 4.3.3b).



Figure 4.3.3b: Inset: Electron micrograph of *M. ulcerans* in smear obtained from Case 9 (EM x45,000) (Hayman and McQueen., 1985)

Following this evidence, we sought to establish whether the emerging vesicles from the bacillus could eventually get released into the growth medium. Advanced microscopy on separated medium pellets from growing *M. ulcerans* cells revealed many liberated, intact, lipid-bilayer, membrane-enclosed nano-vesicles (**Figure 4.3.4**). These vesicles varied in size from 16 nm to 87 nm, as was measured with the Line plug-in function of image J software. Also, the vesicles appeared similar in structure and morphology to vesicles that were found to be released from the bacillus (**Figure 4.3.3a**) suggesting that such liberated membrane vesicles originated from the growing cells of *M. ulcerans*. Moreover, the structure and morphology of such vesicles appeared similar to membrane vesicles observed in growth media of *M. marinum* (Sa 200695) (**Figure 4.3.2**) and *M. smegmatis* (**Figure 4.3.1**).

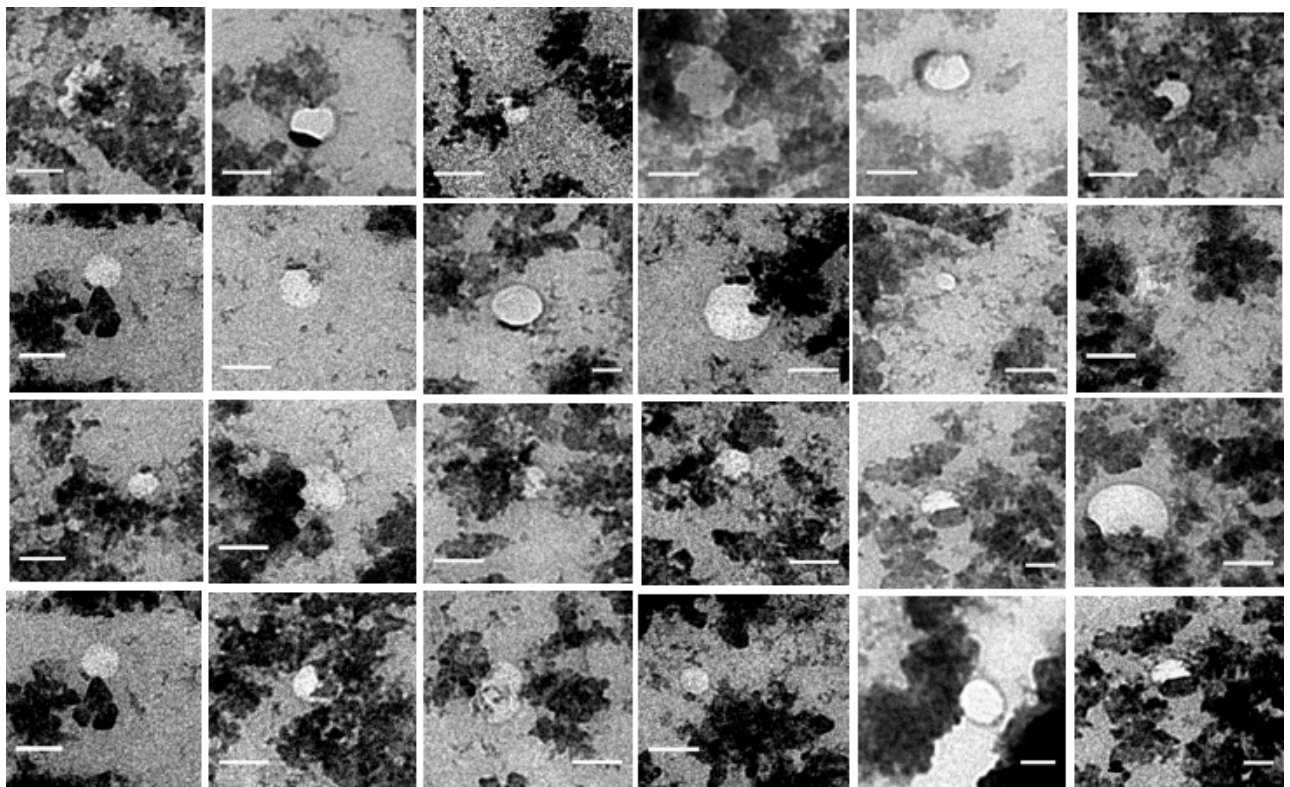


Figure 4.3.4: Transmission electron micrograph showing scattered intact membrane vesicles released from growing *M. ulcerans* (Nm 209) into growth medium. Scale bar:200 nm

Similar vesicles were also observed in pellets that were processed from the extracellular matrix samples from cultured *M. ulcerans* as previously reported (Marsollier *et al.*, 2007). However, the number of such membrane vesicles were very few compared to vesicles found in the growth medium of the pathogen. This may suggest that cultured *M. ulcerans* can release extracellular vesicles to transit the protective matrix and eventually get discharged into the environment.

Finally, we sought to determine and compare size distribution of vesicles released by *M. marinum* and *M. ulcerans* using one-way ANOVA followed by Tukey's multiple post-hoc test. The overall size distribution of vesicles released by *M. ulcerans* (Nm 209) ranged from 16 nm to 87 nm. This provided a median diameter of 50 nm (interquartile range, 24.3 nm – 66 nm). (Figure 4.3.5). Vesicles from *M. marinum* (Sa 200695) appeared larger in dimension with an overall size range of 31 nm to 117 nm.

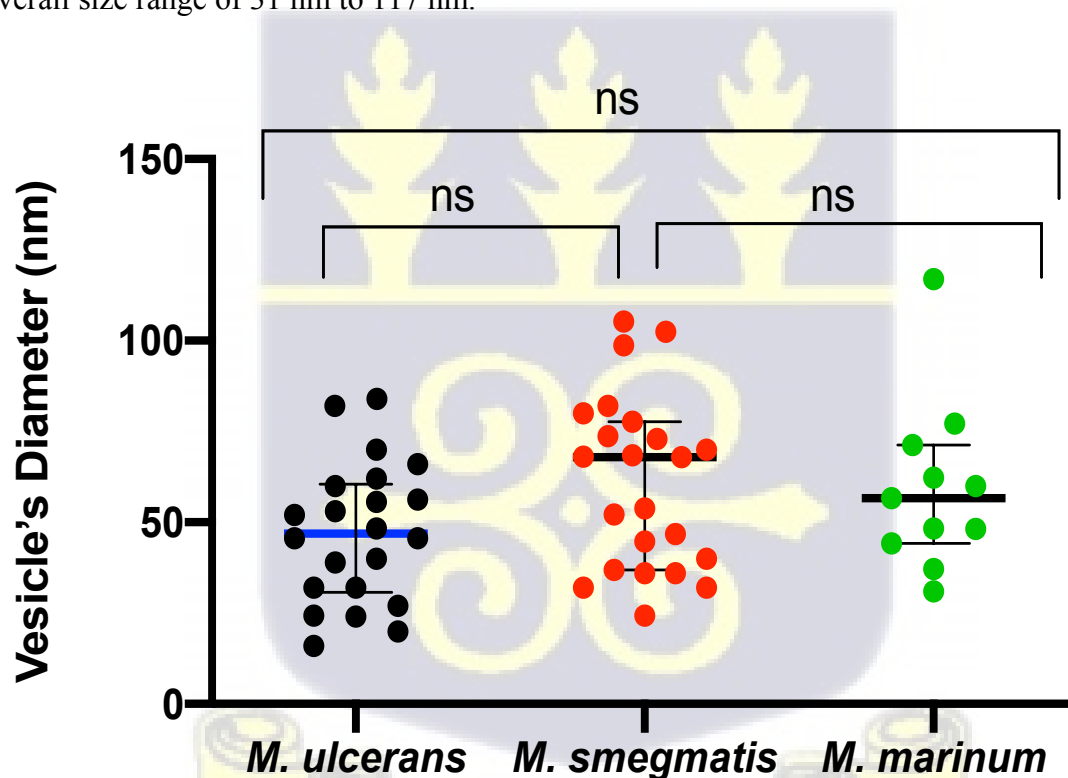
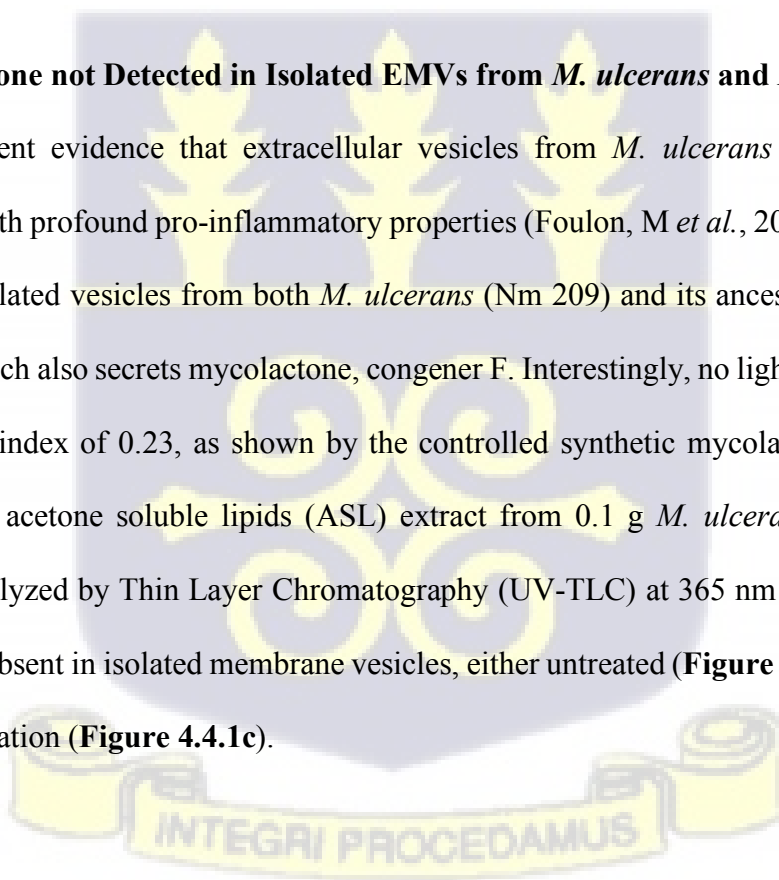


Figure 4.3.5: Scatter plots showing size distributions of membrane vesicles released by *M. ulcerans* (Nm 209), *M. marinum* (Sa200695) and *M. smegmatis*. Size distribution of vesicles released by *M. ulcerans* and *M. marinum* were similar. No statistically significant difference (ns) between median size values of the two strains ($^{ns}P > 0.05$).

However, such vesicles were not statistically different from vesicles released by *M. ulcerans* ($P = 0.2941$) as they provided a median size of 56.6 nm (interquartile range, 36 nm to 62.3 nm). Also, vesicle size distribution of *M. marinum* was not different from that of *M. smegmatis* ($P = 0.0975$) which in turn provided a median vesicle size of 68 nm (interquartile range, 48.2 nm to 82 nm) and an overall size range of 24 nm to 110 nm. All these shreds of evidence harmonize to suggest that pathogenic *M. ulcerans* (Nm 209) and *M. marinum* (Sa 200965), in addition to the fact that they are genetically linked, may utilize similar process to generate and release intact membrane vesicles from their surfaces into their respective environment and that the discharged vesicles may serve as transport medium of specific virulence factors as recently reported for *M. ulcerans* by Foulon *et al.*, (2020) and also confirmed by extracellular vesicles from *M. tuberculosis*, *M. avium* and *M. bovis* (Athman *et al.*, 2017; Prados-Rosales *et al.*, 2011).

4.4.1 Mycolactone not Detected in Isolated EMVs from *M. ulcerans* and *M. marinum*

In view of recent evidence that extracellular vesicles from *M. ulcerans* contained 2 μ g mycolactone with profound pro-inflammatory properties (Foulon, M *et al.*, 2020), we explored this toxin in isolated vesicles from both *M. ulcerans* (Nm 209) and its ancestral *M. marinum* (Sa200695) which also secretes mycolactone, congener F. Interestingly, no light-yellowish band with refractive index of 0.23, as shown by the controlled synthetic mycolactone A-B, was observed when acetone soluble lipids (ASL) extract from 0.1 g *M. ulcerans* cells (**Figure 4.4.1a**) was analyzed by Thin Layer Chromatography (UV-TLC) at 365 nm wavelength. The toxin was also absent in isolated membrane vesicles, either untreated (**Figure 4.4.1b**) or treated by photodegradation (**Figure 4.4.1c**).



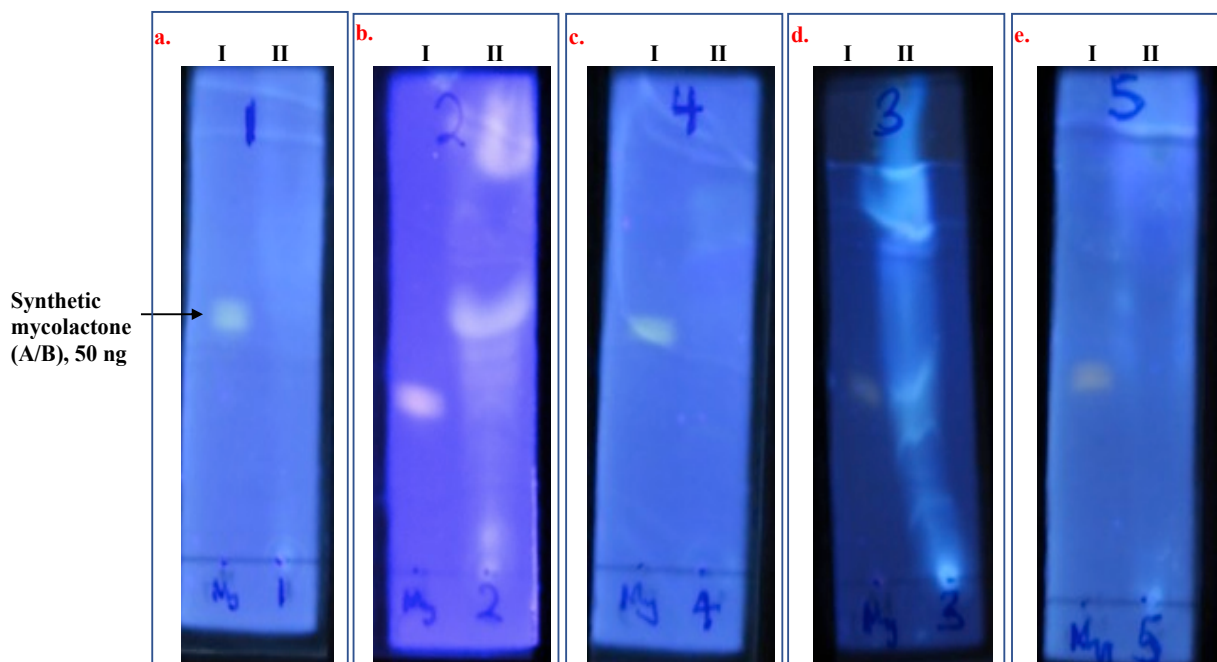


Figure 4.4.1: TLC plates on acetone soluble lipids (ASL) from *M. ulcerans* (Nm 209) and *M. marinum* (Sa200695) and their respective extracellular vesicles. (a) homogenate of 0.1 g of cultured *M. ulcerans* cells, (b) pooled native vesicles from *M. ulcerans*, (c) UV-A irradiated membrane vesicles from *M. ulcerans*. The macrolide was also absent in (d) homogenate of 0.1g *M. marinum* (Sa200695) (e) native membrane vesicles from *M. marinum* (Sa200695). Lane I - 5 μ L synthetic mycolactone A/B (50 ng) positive control; lane II - samples (cell homogenate or isolated membrane vesicles respectively).

Similarly, ASL profile of *M. marinum* (Sa200695) and its isolated native vesicles did not show mycolactone. The test strain, *M. marinum* (Sa200695) secretes mycolactone, congener F to conducts virulence in host's tissues. But we did not detect the toxin in 0.1 g bacterial cells (**Figure 4.4.1d**). Neither was it found in its isolated membrane vesicles (**Figure 4.4.1e**). Low sensitivity of TLC used for the study or low bacterial cells mass may have contributed to our inability to detect mycolactone in the EMVs. Notwithstanding, the current evidence potentially support that extracellular membrane vesicles from *M. ulcerans* (Nm 209) and *M. marinum* (Sa200695) may be released by a mechanism independent of mycolactone secretion and that EMV from the pathogen may exist without mycolactone.

4.4.2 *M. ulcerans* EMVs Reduced Viability of Interacting RAW 264.7 Cells

Our inability to detect mycolactone in the isolated vesicles led us to further explore the potential impact of untreated and UV-A irradiated membrane vesicles on viability of RAW 264.7 cells. This was based on reports that membrane vesicles from *Mycobacterium tuberculosis* can elicit pro-inflammatory response from innate macrophage cells (Athman *et al.*, 2015; Prados-Rosales *et al.*, 2011) and also, anti-inflammatory reaction from adaptive T-lymphocytes (Athman *et al.*, 2017).

Initial optimization data from fluorescent, non-cytotoxic resazurin assay revealed a dose-dependent stimulation of the macrophage metabolic activities by membrane vesicles from *M. ulcerans* (**Figure 4.4.2**). This stimulatory impact was similar between native and mycolactone-depleted vesicles. The reactions were also prominent at 18 hours and 24 hours of co-incubation suggesting that extended period of interactions between macrophage cells and pathogenic vesicles has initial stimulatory impact on baseline metabolic activities of macrophage cells. Based on this unique observation, an extended period of 24 hours and 48 hours were chosen for subsequent dose-stimulation analysis based on double dilution of test samples.

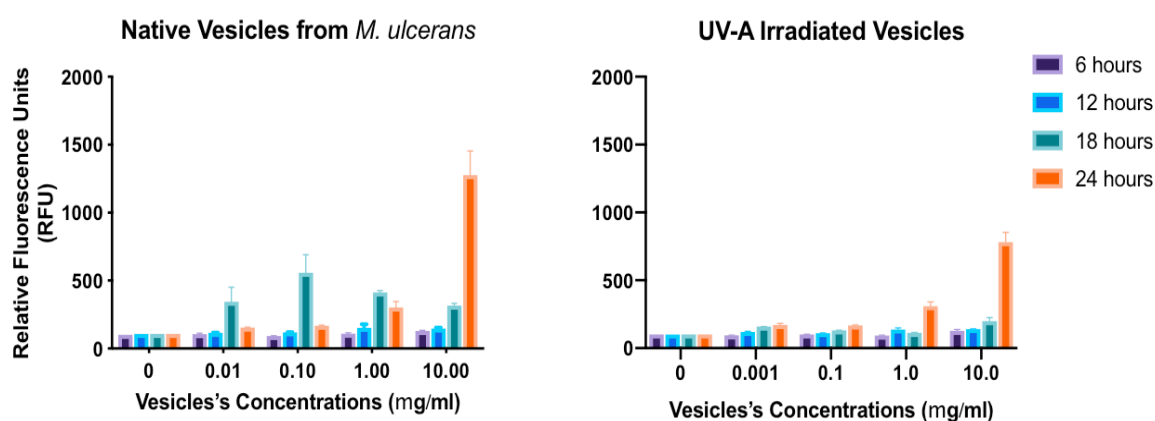


Figure 4.4.2: Optimization experiment for dose-sensitivity assay: monolayers of RAW 264.7 cells versus native and mycolactone-depleted membrane vesicles from *Mycobacterium ulcerans*. Relative Fluorescence Unit (RFU) by resazurin assay under 560_{EX} – 590_{EM} . Bars represent mean (SD) of three data points from triplicates wells

Interestingly, a 24-hour interaction between monolayers of RAW 264.7 cells and double-diluted samples of *M. ulcerans*' vesicles confirmed the initial observation with concentration-dependent stimulation of the macrophage's metabolism (**Figure 4.4.3**). When compared to baseline metabolism, membrane vesicle load ranging from 3 $\mu\text{g/ml}$ to 47 $\mu\text{g/ml}$ had minimal stimulatory impact on metabolic activities of the macrophage cells. However, increased vesicles' load of 94 $\mu\text{g/ml}$ ignited the phagocyte's baseline metabolism in a dose-dependent manner giving a steady increase in Relative Fluorescence Unit (RFU) from around 500 to above 1,500 at vesicle load of 6 mg/ml (**Figure 4.4.3**). This stimulatory pattern was similar between UV-A irradiated and untreated vesicles from *M. ulcerans* (**Figure 4.4.3**).

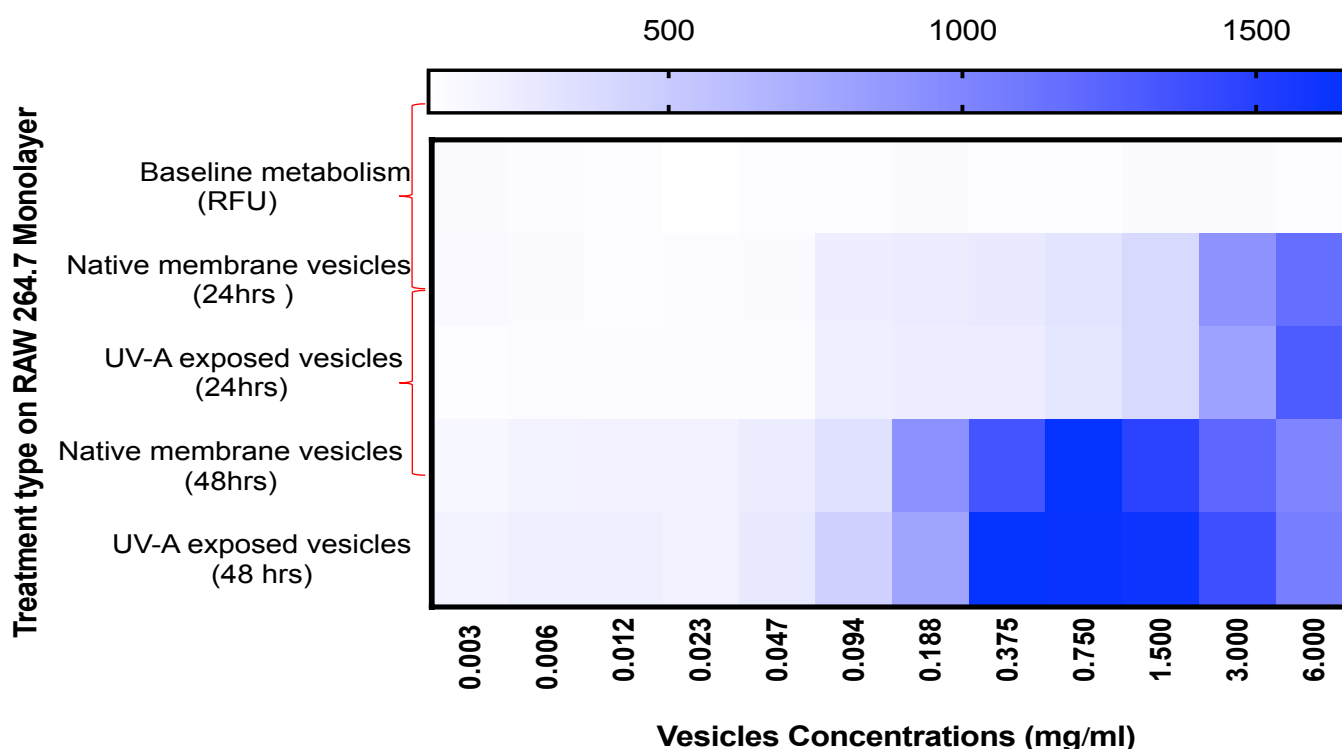
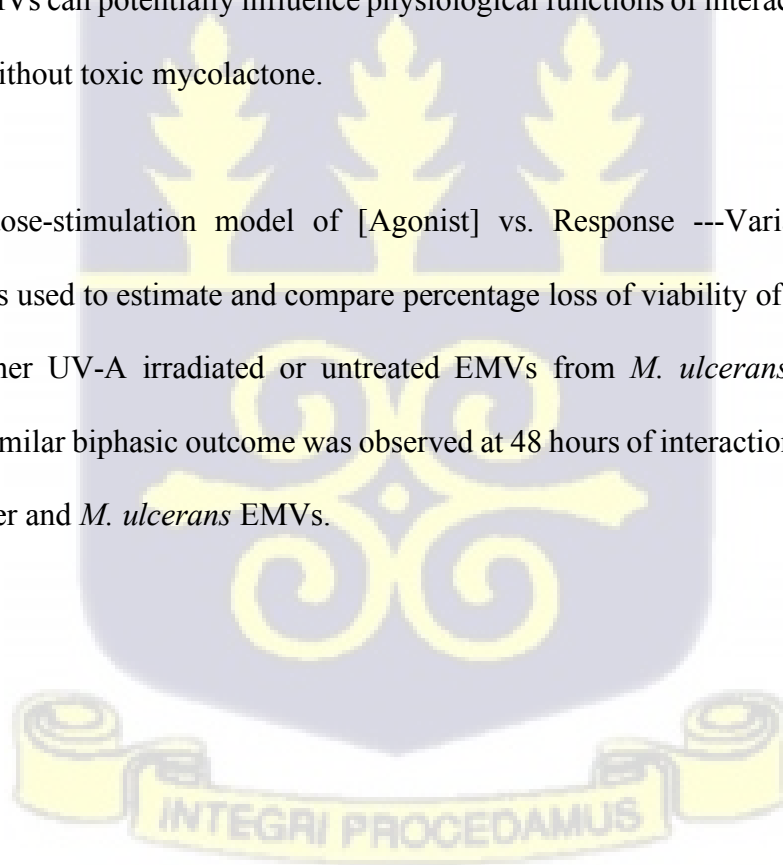


Figure 1.4.3: Extracellular vesicles from *M. ulcerans* modulated metabolic activities of RAW 264.7 cells by resazurin assay. Concentration-dependent stimulation of the macrophage metabolism occurred at 24 hours and 48 hours, respectively. At 48 hours, a dose-dependent inhibition of metabolism activities ensued beyond 750 $\mu\text{g/ml}$ vesicle load. This modulatory impact was similar between untreated and UV-A irradiated vesicles from the pathogen. Each grid represents the mean ($\pm$ SD) of triplicate wells from two independent experiments.

An extended 48-hour period of interaction between RAW 264.7 cells and membrane vesicles demonstrated potential influence of vesicles. As low as 6 µg/ml EMVs altered baseline metabolism of the phagocytes. However, concentration-dependent stimulation of RAW264.7 metabolism was prominently initiated at EMVs load of 47 µg/ml. Once again, the pattern of elicited metabolic response either by UV-A irradiated or untreated *M. ulcerans* EMVs was similar. RAW 264.7 cells metabolism was consistently increased to its peak at vesicle's load of 750 µg/ml. Beyond this load, series of addition EMVs led to potential toxicity; a dose-dependent inhibition RAW264.7 cells metabolism was observed (**Figure 4.4.2**). Baseline metabolism of the controlled RAW 264.7 cells without exposure to the pathogen's EMVs remained unaltered under the same experimental conditions (**Figure 4.4.2**). This suggested that *M. ulcerans* EMVs can potentially influence physiological functions of interacting macrophage cells, with or without toxic mycolactone.

Therefore, a dose-stimulation model of [Agonist] vs. Response ---Variable slope (four parameters) was used to estimate and compare percentage loss of viability of RAW264.7 cells exposed to either UV-A irradiated or untreated EMVs from *M. ulcerans* (**Figure 4.4.4**). Interestingly, similar biphasic outcome was observed at 48 hours of interactions between RAW 264.7 monolayer and *M. ulcerans* EMVs.



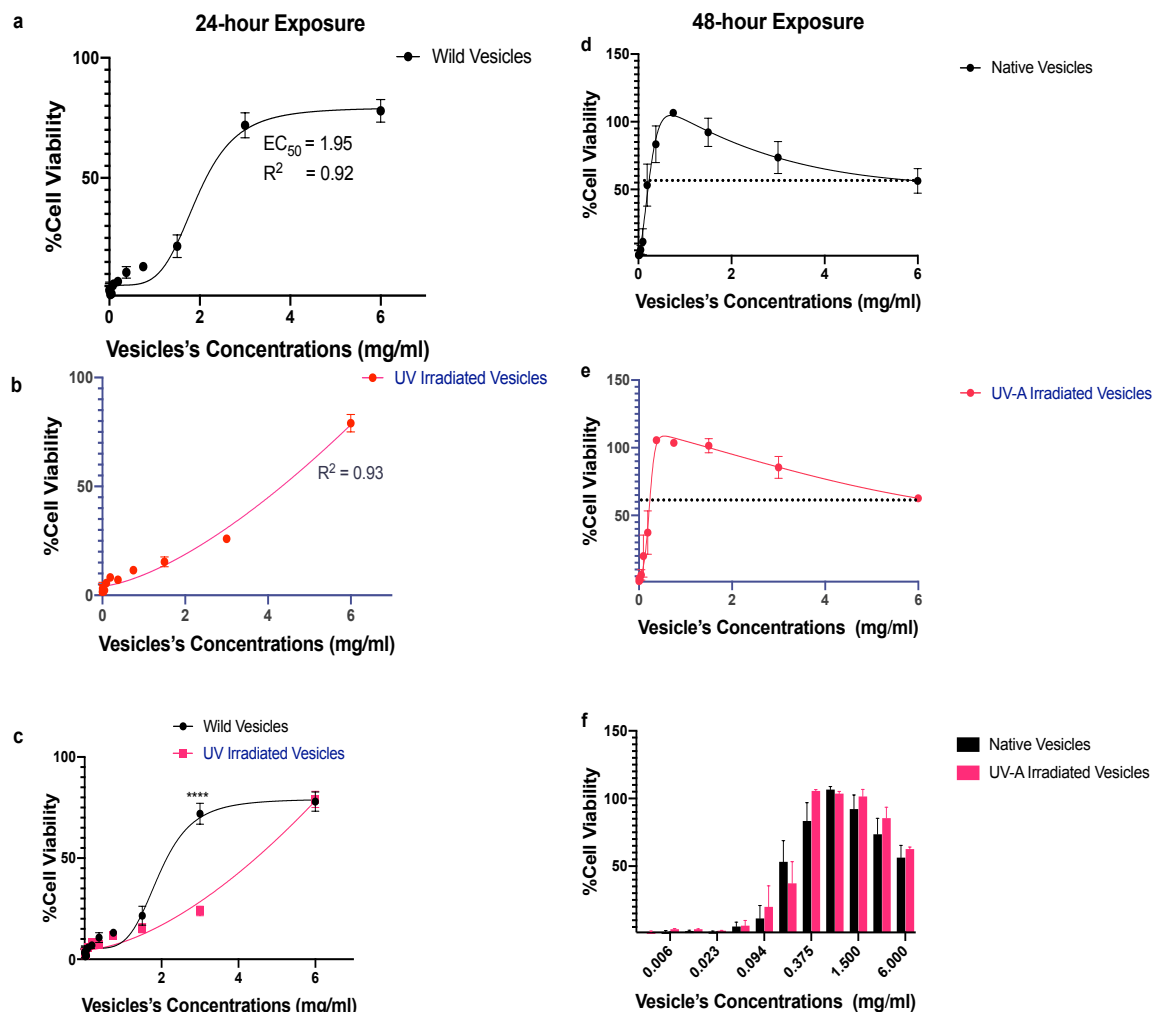


Figure 4.4.4: Percentage loss RAW264.7 cells induced by *M. ulcerans* (Nm 209) vesicles as assessed by [Agonist] vs. Response—variable slope (four parameters). **(a)** Increasing vesicle concentrations proportionally increased % viability of the phagocytes at 24 hours of interactions. **(b)**, UV-A irradiation on the vesicles significantly altered the threshold of cell survival at vesicle load of 3 mg/ml ($p < 0.0001$). **(c)** UV-A irradiation on vesicles did not alter the overall stimulatory impact. **(d-f)** Similar dose-dependent response with a biphasic output was observed at 48 hours; a steady viability enhancement peaked around 0.75 mg/ml vesicles. Macrophage viability then dropped to about 60% at vesicles load of 6 mg/ml. Cell viability was significantly influenced by vesicles concentrations at both 24 hours and 48 hours (**** $p < 0.0001$). Each point represents the mean (SD) of two (2) biological replicates, each with triplicate data.

The initially enhanced level of RAW264.7 cells viability was followed with a dose-dependent decline in the phagocytes viability. Untreated *M. ulcerans* EMVs at concentrations of 1.5 mg/ml, 3 mg/ml and 6 mg/ml associated with induced 7%, 25% and 45% loss in RAW 264.7 cells viability, respectively (**Figure 4.4.3d**).

Similarly, UV-A irradiated vesicles from the pathogen, at concentrations of 3 mg/ml and 6 mg/ml elicited around 15% and 40% loss in RAW 264.7 cells viability, respectively (**Figure 4.4.3e**). Comparatively, we did not observe significant difference in pattern of percentage viability loss when RAW264.7 cells were exposed to either UV-A irradiated or untreated *M. ulcerans* EMVs ($P = 0.7493$) (**Figure 4.4.3f**). However, a significant difference in percentage viability loss ($P < 0.001$) was observed when the mammalian monolayers were exposed to different concentrations of the two vesicle types.

Interestingly, viability of unexposed RAW264.7 cells, used as negative control, remained unaltered under same experimental conditions. This suggests strong association between presence of *M. ulcerans* EMVs (either native or UV-A irradiated) and macrophage cell death at 48 hours of interaction indicating that the pathogen's EMVs, with or without mycolactone, can potentially engage pernicious interaction with host's macrophage cells.

4.5 Loss in RAW 264.7 Cell Viability is Not Associated with Direct Cell Lysis

Next, we investigated the kind of cytotoxicity induced by extracellular vesicles from *M. ulcerans* by phase contrast microscopy and direct cell count. After 48 hours of incubation at 37°C, 5% CO₂, the “healthy” untreated RAW 264.7 cells showed normal morphology with increased cell density and evidence of macrophage activation (**Figure 4.5a**). These cells showed complete lysis, within the same time point, when they were treated with membrane-active amphotericin B as positive control for cell lysis. No intact macrophage cell was observed (**Figure 4.5 b**). When phagocytes were then exposed to 0.0234 mg/ml of *M. ulcerans* vesicles, they appeared activated, some existing in clump, suggesting their possible exposure to PAMPs in the interacting vesicles. However, no significant differences in cell count and cell morphology were observed when compared to untreated cells (**Figure 4.5c**).

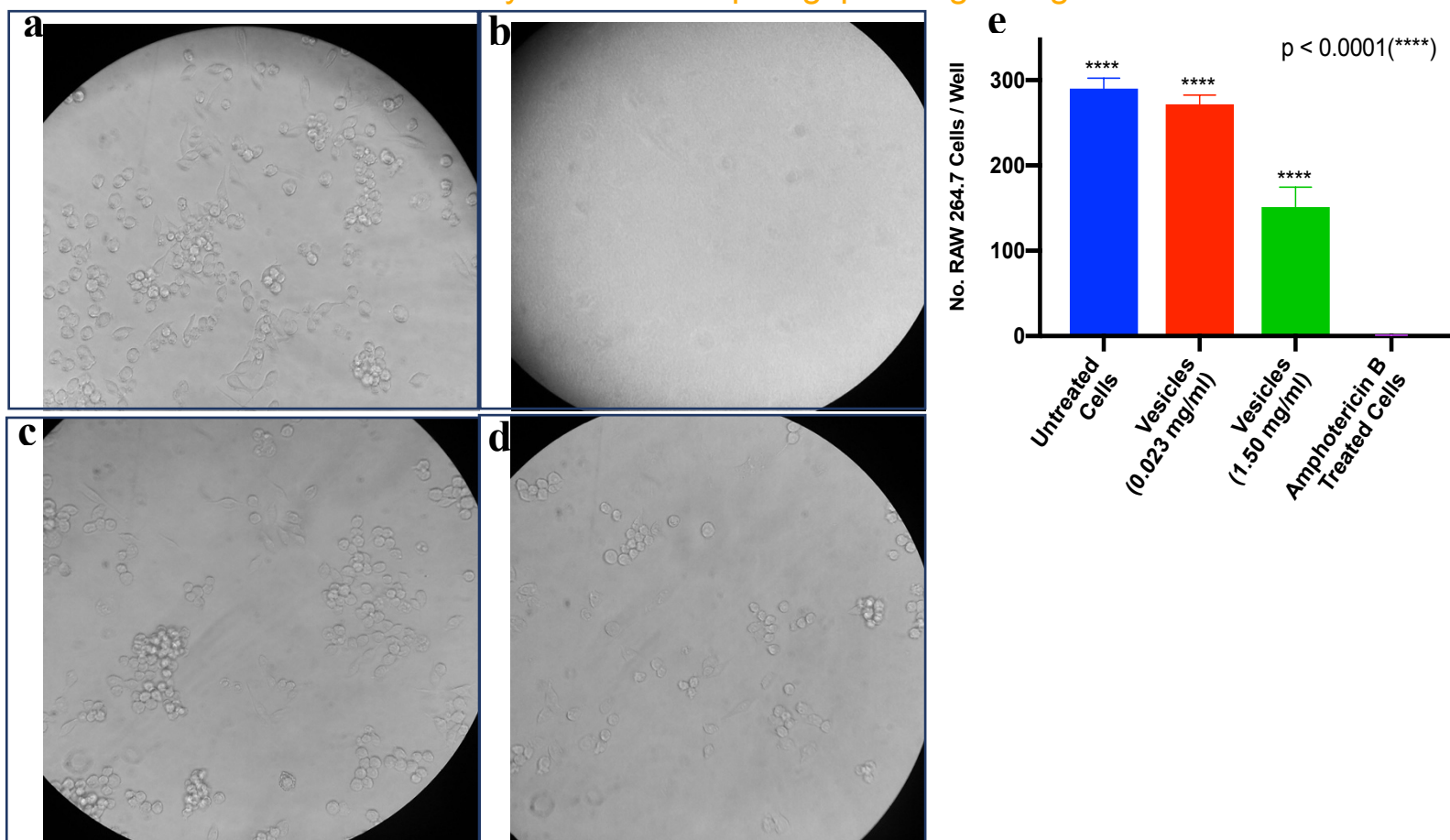


Figure 4.5.0: Apoptotic features in RAW264.7 monolayer ($100\mu\text{l}$ of 2×10^4 cells/ml) when it was exposed to *M. ulcerans* vesicles after 48 hours of co-incubation. **(a)** Healthy untreated cells. **(b)** Absolute lysis of the macrophage cells after treatment with 5 mg/ml amphotericin B. **(c)** Macrophage cells after treatment with 0.0234 mg/ml vesicles appeared activated compared to untreated cells. **(d)** RAW 264.7 cells treatment with 1.50 mg/ml vesicles triggered apoptotic changes in the cells. **(e)** Significant reduction in macrophage cell count after treatment with 1.50 mg/ml vesicles.

macrophage monolayer was exposed to an increased membrane vesicle load of 1.50 mg/ml for 48-hour interactions (**Figure 4.5d**). Residual phagocytes rounded up and exhibited wrinkled cell membrane under phase contrast microscopy suggestive of apoptosis. Also, decline in the macrophage cell count was significantly pronounced, when compared to 0.0234 mg/ml vesicle load ($p < 0.0001$), after 48 hours of co-incubation with 1.50 mg/ml vesicles (**Figure 4.5e**).

This suggests that extracellular vesicles from *M. ulcerans* (Nm 209), without cytotoxic mycolactone, can interact and trigger apoptotic mechanism in host's macrophage cells as opposed to the recently published evidence that vesicles from the pathogen require mycolactone for cytotoxicity (Foulon, M. *et al.*, 2020).

4.6.0 RAW 264.7 Cell Stimulation Associated with Increased Intracellular Level of Reactive Oxygen Species (ROS).

Following this apoptotic impact, we sought to elucidate which cellular process triggered such a gradual death in interacting RAW 264.7 cells. Unrestrained macrophage activation can lead to oxidative stress in macrophage cells as metabolic activities get reprogrammed and mitochondrial functions repurposed (Van den Bossche *et al.*, 2017). Therefore, we quantified total intracellular ROS in RAW264.7 cells after 24 hours (**Figure 4.6 a**) and 48 hours (**Figure 4.6b**) of interactions with either native or UV-A irradiated EMVs from *M. ulcerans*. At 24 hours of interactions, UV-A irradiated vesicles induced a higher total intracellular ROS in RAW264.7 cells above the average untreated level. On the contrary, native vesicles from the pathogen appeared to suppress internal ROS generation during the same period (**Figure 4.6a**). A significant difference in total intracellular ROS was observed between RAW 264.7 cells exposed to UV-A irradiated vesicles and cells exposed to untreated vesicles ($p < 0.03$).



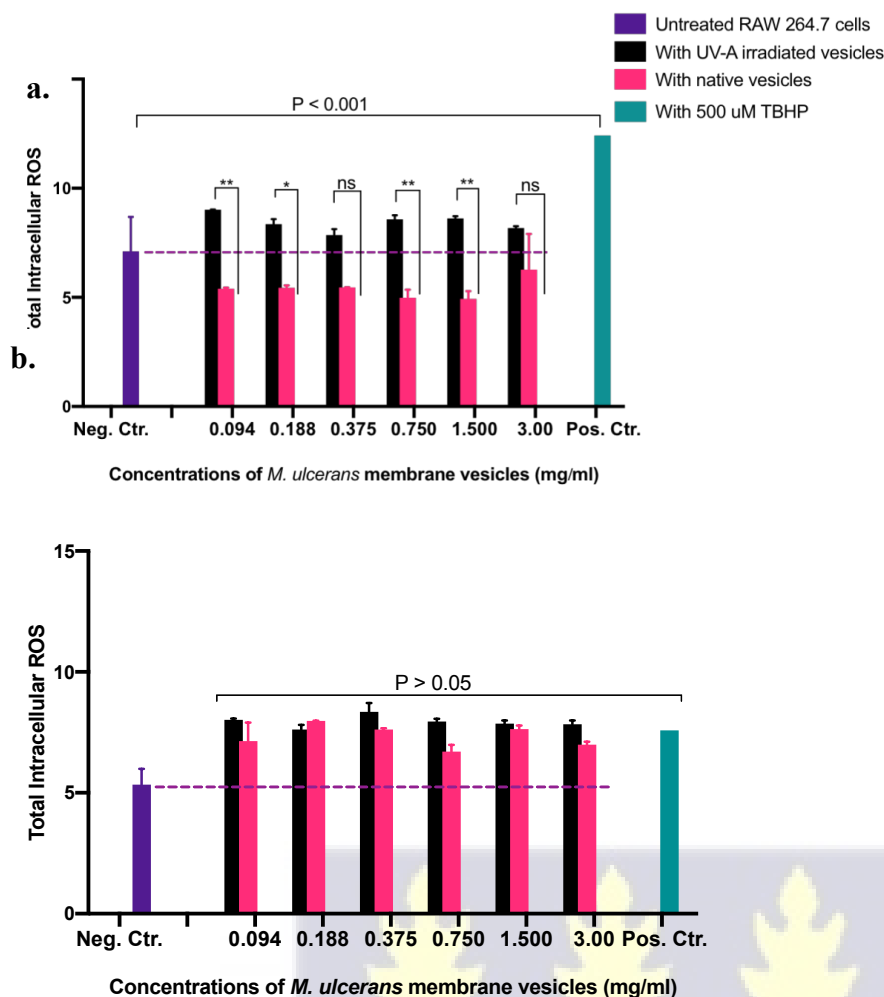


Figure 4.6: Influence of *M. ulcerans* membrane vesicles on intracellular generation of Reactive Oxygen Species (ROS) in RAW 264.7 cells after 24 hours (a) and 48 hours (b) of interactions. (a) Total intracellular ROS in macrophage cells by varied concentrations of *M. ulcerans* vesicles after 24 hours controlled by average untreated cells (violet dotted lines) and 500 μ M Tert-butyl Hydrogen Peroxide-TBHP. (b) Total intracellular ROS after 48-hour interactions with varied membrane vesicle concentrations. Fluorescence intensity quantified by multimode VARIOSKAN LUX plate reader at excitation/emission wavelength of 495 nm/529 nm. Each bar represents the mean (SD) of duplicate wells. **P = (0.001 to 0.002), *P = (0.01 to 0.03), ns P > 0.05

This created an initial suggestion that extracellular vesicles from *M. ulcerans*, without mycolactone, can possibly induce oxidative stress in interacting host cells. Therefore, we repeated the experiment at an extended period of 48 hours to assess the impact of *M. ulcerans* vesicles on the redox environment of host cells. Remarkably, the effect of native vesicles was comparable to that of UV-A irradiated vesicles in terms of redox imbalance. At 48 hours of interactions, the two vesicle types demonstrated equal potential to trigger higher internal generation of ROS in interacting RAW 264.7 cells (**Figure 4.6b**) consistent with positive control cells treated with an oxidizing agent, tert-butyl hydrogen peroxide.

More interestingly, this redox imbalance was not influenced by the vesicles' load but rather period of exposure to cells. Vesicle load of 0.094 mg/ml induced similar oxidative stress just as load of 1.5 mg/ml (**Figure 4.6, b**). Therefore, it is possible that oxidative stress was constitutively induced in the macrophage cells by interacting *M. ulcerans* EMVs. Not in a dose-dependent fashion.

In retrospect, the observed 40% and 35% loss in RAW264.7 cells' viability after 48 hours of interactions with native and UV-A irradiated vesicles (**Figure 4.4.4**) respectively, coincided well with the apoptotic changes induced in the mammalian cells (**Figure 4.5d**) and also the high total intracellular ROS generated in the interacting macrophage cells (**Figure 4.6b**). This demonstrated that extracellular vesicles from *M. ulcerans*, with or without mycolactone, can interact to induce oxidative stress, with evidence of apoptosis, in the host's macrophage cells.

4.7.0 Proteomic Analysis on Membrane Vesicles from *M. ulcerans*

Proteomic analysis on native vesicles released by growing *M. ulcerans* cells revealed an array of prominent proteins which migrated within a range of six (6) prominent spots on 1 D SDS-gel electrophoresis (A-E, **Figure 4.7.0**). By label-free quantitative proteomics (LC-MS/MS), the two prominent proteins at spot **A** (between 100 kDa and 135 kDa) were identified as a conserved protein (MUL_2313) and Integral membrane indolylacetyltransferase, EmbA/B (**Table 3**). The other complex bands between spots **B** and **C** (46 kDa to 58 kDa) were identified as mixtures of amidase amiC, aldehyde dehydrogenase, maltokinase and a conserved protein (MUL_2373). Protein bands at spot **D** (around 27 kDa) were identified as indole-3-glycerol phosphate synthase and two component system response phosphate regulon transcriptional regulator, PhoP.

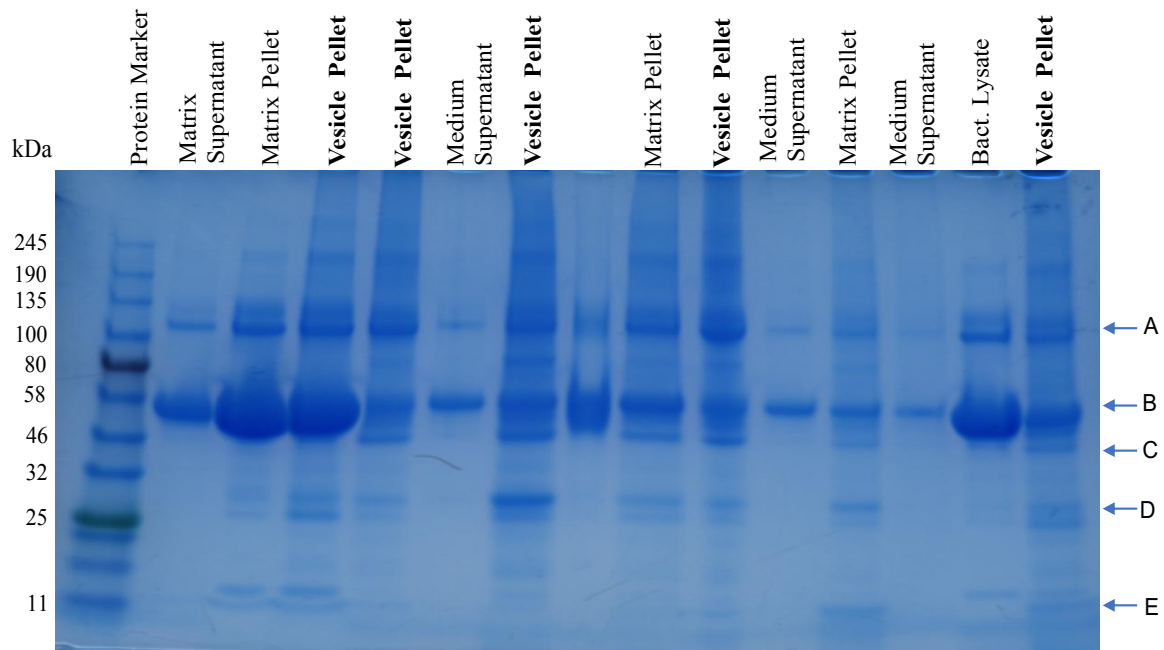


Figure 4.7.0: Proteomic profile of cultured *M. ulcerans* (Nm 209) lysate, extracted extracellular matrix from culture and isolated membrane vesicles from growth medium of the pathogen. Besides the five prominent bands (arrowed A – E), less prominent and many faint background bands (range within 11 kDa to 245 kDa) were observed on the gel. The gel is a representative of five (5) independent experiments.

Finally, less prominent migrations between spots **D** and **E** were found as iron-regulated conserved protein (MUL_1619), conserved proteins (MUL_0465, MUL_1619), transcriptional repressor, NrdR and phosphoriboisomerase B. In addition, many faint background of proteins within migrating within a broad range, from 11 kDa to 245 kDa were also observed (**Figure 4.7.0**). Moreover, some of the proteins found in the separated vesicles were also found in samples prepared from extracellular matrix (ECM) of cultured *M. ulcerans* as well as cultured supernatants after the final ultra-centrifugation (**Figure 4.7.0**). This confirmed the potential trajectory of such proteins as they got packaged into extracellular vesicles to transit the protective matrix into the surround medium (Marsollier *et al.*, 2007).

4.7.1 Quantitative Analysis on Proteins found in Membrane Vesicles from *M. ulcerans*

In all, we detected 695 total proteins in mid-log liquid cultures of *M. ulcerans*. About 99.6% (692/695) of these proteins were present in bacterial cell lysate, 60% (417/695) present in extracted extracellular matrix from *M. ulcerans* and 45.6% (317/695) in isolated membrane vesicles from the bacilli (**Figure 4.7.1**). Thirty six (36) proteins detected in bacterial cell lysate were also detected in the isolated EMVs. Only one, identified as a conserved hypothetical protein (MUL_2313), existed in the vesicles but could not be traced to the bacterial cell lysate. Possibly, this suggest its unique mechanism of package into the secreted membrane vesicles. The other 36 proteins were traced to *M. ulcerans* lysate and not in the surrounding matrix (**Figure 4.7.1**) suggesting the possibility of direct protein package into isolated EMVs as was confirmed by higher concentrations of 57% (21/37) proteins in the discharged EMVs compared to the bacterial cell lysate showing a positive log 2-fold change in their respective Normalized Spectra Abundance Factor (NSAF) (**Table 3**). A conserved protein (MUL_2313), amidase *amiC*, integral membrane indolylacetylinoitol arabinosyltransferases (*EmbA* and *EmbB*), MCE-family lipoprotein, *LprK*, cell division protein *FtsZ*, maltokinase and S-adenosylmethionine synthase. Each of these vesicle-associated proteins had more than 0.50-fold increase in NSAF compared to their respective quantities in *M. ulcerans* lysate (**Table 3**). A conserved protein (MUL_2313), found only in the vesicles, had the highest log₂ fold change of 11.92. Amidase *amiC*, a cell wall remodeling hydrolase, was the second most enriched protein in *M. ulcerans* EMVs with log₂ fold change of 4.19.

The remaining 43% (16/37) showed decreased quantities in isolated EMVs but increase quantities in *M. ulcerans* lysate; a negative log 2-fold change in NSAF values (**Table 3**). These proteins are mostly cytosolic and included a conserved protein (MUL_3046), an uncharacterized protein (MUL_2484), indole-3-glycerol phosphate synthase, Adenylate

kinase, Lipase/esterase LipN, Transcription elongation factor GreA, Glycine--tRNA ligase, NADH dehydrogenase Ndh, Proteasome-associated ATPase (**Appendix D**, Table 4). All these shreds of evidence suggest *M. ulcerans* EMVs to be more enriched with membrane proteins than cytosolic proteins. They suggest active release of the vesicles and that EMVs may not have resulted as mere cell fragments or self-aggregating lipid bodies from dying *M. uclerans* cells in culture.

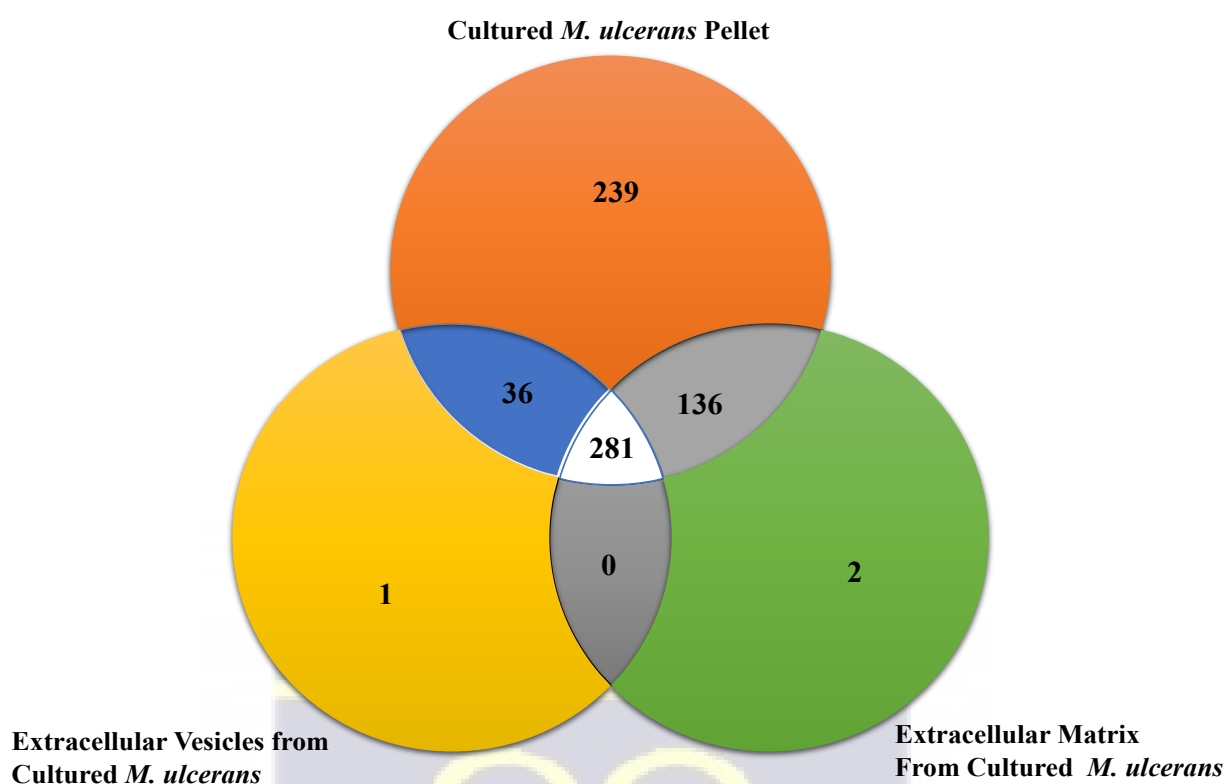


Figure 4.7.1: Quantitative distribution of proteins detected in *M. ulcerans* cell lysate (orange), extracted extracellular matrix from the mycobacterium (green), and isolated membrane vesicles from growth medium of the pathogen (yellow). A total of 281 proteins were common to the three cellular compartments of *M. ulcerans*. But one and two unique proteins were exclusively present in isolated vesicles and matrix from *M. ulcerans* respectively but not bacterial lysate. Proteins were identified by label-free quantitative proteomics (LC-MSMS)

Table 3: List of top 21 proteins from *M. ulcerans* enriched in extracellular vesicles, sorted by log₂ fold difference on normalized NSAF

Description				Quantitative Value (Normalized NSAF)		Fold Change	Log2 Fold Change
	UniProt ID	Gene	MW	Bact. Pellet	Vesicles		
<i>Mycobacterium ulcerans</i> (Nm 209)							
Conserved protein	A0PQR7_MYCUA	MUL_2313	124 kDa	3.8204E-08	0.00014794	3872.37	11.92
Amidase AmiC	A0PQ81_MYCUA	amiC	51 kDa	0.000038204	0.00069771	18.26	4.19
Aldehyde dehydrogenase	A0PLH7_MYCUA	MUL_0494	55 kDa	0.00024897	0.0012991	5.22	2.38
Integral membrane indolylacetyltransferase EmbA	A0PWY6_MYCUA	embA	118 kDa	0.00009721	0.00029588	3.04	1.61
MCE-family lipoprotein LprK (MCE-family lipoprotein Mce1E)	A0PMU1_MYCUA	lprK	43 kDa	0.00017988	0.00041062	2.28	1.19
S-adenosylmethionine synthase	METK_MYCUA	metK	43 kDa	0.00022373	0.00040858	1.83	0.87
Cell division protein FtsZ	A0PTI4_MYCUA	ftsZ	39 kDa	0.00023298	0.00042548	1.83	0.87
Maltokinase	MAK_MYCUA	mak	49 kDa	0.00079264	0.0010857	1.37	0.45
Integral membrane indolylacetyltransferase EmbB	A0PWY7_MYCUA	embB	116 kDa	0.00011742	0.00015317	1.30	0.38
Phosphoriboisomerase B	A0PU39_MYCUA	rpiB	17 kDa	0.0015878	0.0020712	1.30	0.38
Conserved protein	A0PLF0_MYCUA	MUL_0465	15 kDa	0.001884	0.0024576	1.30	0.38
UPF0336 protein MUL_0722	A0PM04_MYCUA	MUL_0722	19 kDa	0.00076041	0.00099192	1.30	0.38
Conserved protein	A0PWW1_MYCUA	MUL_4945	22 kDa	0.00067142	0.00087585	1.30	0.38
Iron-regulated conserved protein	A0PP51_MYCUA	MUL_1619	15 kDa	0.00098808	0.0011278	1.14	0.19
Epoxide hydrolase EphA	A0PV59_MYCUA	ephA	36 kDa	0.00044801	0.00051136	1.14	0.19
N5-carboxyaminoimidazole ribonucleotide mutase	A0PRI6_MYCUA	purE	18 kDa	0.00084362	0.00096292	1.14	0.19
Conserved protein	A0PQW8_MYCUA	MUL_2373	49 kDa	0.00063973	0.0007302	1.14	0.19
Conserved lipoprotein LppL	A0PQW3_MYCUA	lppL	37 kDa	0.00040522	0.00046253	1.14	0.19
DNA polymerase III subunit gamma/tau	A0PVG0_MYCUA	dnaZX	66 kDa	0.0002323	0.00026515	1.14	0.19
Transcriptional repressor NrdR	NRDR_MYCUA	nrdR	17 kDa	0.00093675	0.0010692	1.14	0.19
Sulfurtransferase	A0PLC3_MYCUA	cysA2	31 kDa	0.00057962	0.00058807	1.01	0.02

4.7.2. Functional Classification of Proteins Enriched in *M. ulcerans* Vesicles

Gene Ontology analysis revealed many of these vesicle-associated proteins from *M. ulcerans* (Nm 209) to be involved in “intermediary metabolism and respiration” (29.8%) and “cell wall and cell processes” (21.6%) (**Figure 4.7.2a**). Proteins in “information pathway” and “regulatory” arm comprised 16.2% and 5.4%, respectively. Interestingly, some proteins for virulence and adaptation (5.5%) including lipoprotein LprK and Maltokinase were identified in extracellular vesicles from *M. ulcerans*. The fact that 21.6% of these vesicle-associated proteins remain to be functionally categorized (**Figure 4.7.2a**) makes the current functional categorization inconclusive and that these proteins may add up to virulent composition of membrane vesicles *M. ulcerans*.

Undoubtedly, more than 50% of proteins found in *M. ulcerans* vesicles localized in the cell wall or cell membrane of the pathogen (**Figure 4.7.2b**). Only 21.6% of the proteins had cytosol as their cellular component. 21.6% remains uncharacterized with respect to their cellular component. These uncategorized proteins may have increased the overall membrane-localized proteome of the vesicles and contributed to the stimulatory effect on RAW264.7 cells. All these pieces of evidence establish that protein content of *M. ulcerans* vesicles varies, both in function and localizations and that EMVs are not mere cell fragments but intact vesicles which can convey functional virulent proteins to host's targets to play selective advantage to the pathogen. Possibly, the moonlighting function of these detected proteins or protein cross-linking may have mediated the observed vesicle-induced cytotoxicity on RAW264.7 cells.

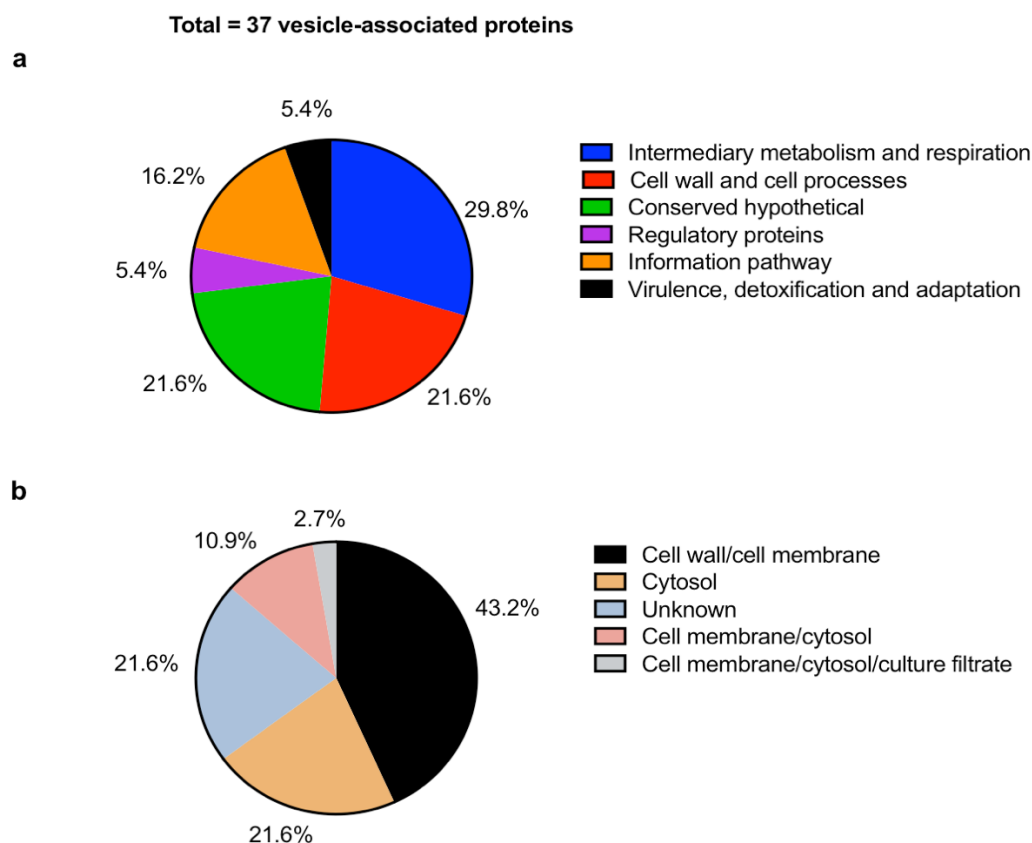


Figure 4.7.2: (a) Functional categories and (b) cellular components of membrane vesicle-associated proteins from cultured *M. ulcerans*.

CHAPTER FIVE (5)

5.0 DISCUSSION

To date, the pathophysiology of *Mycobacterium ulcerans* remains a grey area. Adopting the right experimental approaches to explore molecular traces of this “mysterious” pathogen may help to explain some of its virulent mechanism (s), particularly local immunosuppression. Also, this may create the platform to initiate search for potential fingerprint that may serve as molecular targets for early diagnosis of this debilitating skin disease.

To the best of our knowledge, the current study is the first to demonstrate that EMVs from viable *M. ulcerans* cells can decrease viability of host’s macrophage cells through oxidative stress. The evidence provides a an interesting new dimension on *M. ulcerans* pathophysiology. It details how this pathogen can potentially utilize its membrane components (EMVs), besides mycolactone, to circumvent proper functions of host’s macrophage cells and contribute to local immunosuppression in Buruli ulcer pathogenesis. It is relevant to global efforts to understand the biology of the pathogen for appropriate techniques to detect early Buruli ulcer stage for appropriate treatment.

The evidence of high *M. ulcerans* cells viability at log-phase of growth (**Figure 4.1.1, a – c**) and *de facto* mycobacterial EMVs as were captured being released from the bacillus (**Figure 4.3.3 a**) together with the vesicles’ subsequent release into growth medium of the pathogen (**Figure 4.3.4**) are consistent and comparable to previous reports including *in vivo* report by (Hayman *et al.*, 1985) (**Figure 4.3.4c**) and other studies suggesting that the pathogen can secrete EMVs *in vivo* or *in vitro* when most viable and actively growing (Marsollier *et al.*, 2007, Prados Rosales *et al.*, 2011, Palacios *et al.*, 2019 Palacios *et al.*, 2021) and that the discharged EMVs from *M. ucerans* may not have resulted and thus do not result as lipid bodies or mere cell

fragments from dying bacterial cells. Their release is evolutionary conserved and occurs in viable bacterial cells (Coelho *et al.*, 2019); the current study does not say otherwise

Also, the current evidence expands growing concept that pathogenic strains of mycobacterium may partly utilize the release of these EMVs to adapt to host's tissues and mediate virulence (Athman *et al.*, 2017; Brown, L. *et al.*, 2015; Gupta *et al.*, 2018; Prados-Rosales *et al.*, 2011). It further fits into the general thought that pathogens of bacterium discharge extracellular vesicles to colonize host's host tissues, induce virulence and cause various forms of infectious diseases (Briaud *et al.*, 2020). Exosomes from *M. avium*-infected macrophage cells, which contained specific vesicles from the pathogen (Athman *et al.*, 2015), stimulated metabolic functions of human macrophage cell lines (THP-1) within 24 hours of interactions but the authors could establish long-term consequences of these interactions (Wang *et al.*, 2015). We confirm this stimulatory impact with vesicles from *M. ulcerans* on RAW 264.7 cells within 24 hours (**Figure 4.4.4, c**) and further establish that such interactions can induce apoptosis via oxidative stress with more than 40% loss in the phagocyte's viability within 48 hours.

This may play out as an immune evasion process of *M. ulcerans*, particularly at the early phase of infections. Membrane vesicles from the pathogen can initially coy interacting phagocytes with stimulated metabolism (**Figure 4.3.3**) and gradually turn such an interaction into pernicious phase where they induce high intracellular reactive oxygen species to overwhelm the redox buffering system of the phagocytes and kill the mammalian cells by apoptosis (**Figure 4.5**). This proposed pathophysiology of *M. ulcerans* is consistent with several pathogens of bacterium. Outer membrane vesicles from *Neisseria meningitidis* initiated pro-inflammatory activities in human mononuclear cells and in the process induced higher intracellular reactive superoxide anion ($O_2^{\cdot-}$) to trigger cell death (Mirlashari *et al.*, 2002).

Also, 1 µg/ml of outer membrane vesicles from *Escherichia coli* Nissle 1917 initially activated metabolic activities and phagocytic functions of RAW 264.7 cells. This eventually led to the death of the phagocytes as was marked by increased levels of lactate dehydrogenase in culture supernatants of the mammalian cells (Hu, R. *et al.*, 2020). On the contrary, intracellular *Borrelia burgdorferi* utilized its discharged membrane vesicles to limit internal generation of ROS in host's macrophages cells so as to keep the phagocytes' viability and maintain their intracellular survival (Wawrzeniak *et al.*, 2020). The immunomodulatory impact of mycobacterial vesicles is well established and the current study does not show otherwise.

Another potential implication of stimulated metabolic activities of macrophage cells by membrane vesicles from *M. ulcerans* is the issue of chronic wound condition. An untamed macrophage activation can lead to oxidative stress at the site of infection via copious release of ROS. This prevents wound healing and delays tissue repair. Even in circumstances where healing occurs, massive scars ensue (Gensel *et al.*, 2015; Minutti *et al.*, 2017; Rendra *et al.*, 2019). These are hallmark features of Buruli ulcer. With the current evidence that exogenic vesicles from *M. ulcerans* induced oxidative stress in RAW 264.7 cells after 48 hours of interactions, we can conclude that such nano-vesicles create toxic redox imbalance at sites of infections where both interacting and neighboring host cells are possibly killed by post-apoptotic necrosis. This will release damage associated molecular pattern (DAMPs) which can in turn attract more immune cells to such a “slaughter site” to set up focal acellular necrotic areas marked by reduced inflammatory infiltrates as seen in histological lesions of Buruli ulcer.

The high level of intracellular hydroxyl radicals, singlet oxygen, superoxide anion, nitric oxide and hydrogen peroxide in RAW 264.7 cells induced by such noxious vesicles from *M. ulcerans* may have resulted from dysfunctions of mitochondrion, Transforming Growth Factor- α (TGF-

□)-Activated Kinase-1 (TAK-1), activated caspase functions, increased cytosolic activities of NADPH oxidases and Apoptosis Signal-regulating Kinase 1 (ASK-1). Some of these “suicidal” pathways often follow sustained activation of macrophage metabolism (Matsuzawa *et al.*, 2002; Rendra *et al.*, 2019) as was depicted in 48-hour interactions between RAW 264.7 monolayer and membrane vesicles from *M. ulcerans* (**Figure 4.6b**). Therefore, it was not much of a surprise that membrane vesicle load of 1.5 mg□ml, 3 mg□ml and 6 mg□ml, respectively induced 7%, 25% and 40% loss in the macrophage cells’ viability after 48 hours of interactions.

Moreover, the current findings fit the explanatory framework of paradoxical reactions (PR) described as pro-inflammatory rebound to either cellular fragments from dying *M. ulcerans* in host’s tissues or specific antigens from unrecognized foci of the pathogen which get released in response antimycobacterial agents (Frimpong *et al.*, 2019; Ruf *et al.*, 2011). The proposed antigens include lipoarabinomannan, (LAM), lipomannan (LM), phosphatidylinositol mannosides (PIMs) and other membrane-enriched glycolipids or lipoglycans (Ruf *et al.*, 2011). These molecules are enriched in membrane vesicles from *M. ulcerans* and considering the current evidence that such vesicles induced persistent macrophage activation, it is possible that tissue-resident *M. ulcerans* can release extracellular vesicles to counter antimicrobial agents as reported in entero-toxigenic *E. coli* and *Pseudomonas aeruginosa* (MacDonald *et al.*, 2013). In this phenomenon, the released vesicles may interact with invading immune cells, as the wound heals, to induce florid pro-inflammatory response which in turn trigger oxidative stress to compromise host’s cell viability. This process may underlie the issue of paradoxical reactions in some patients following initiation of antimicrobial treatment.

Certainly, some pathogen-specific molecules within the discharged vesicles from *M. ulcerans* cells may be responsible for the observed cytotoxicity on RAW 264.7 cells.

Interestingly, mycolactone may not have been the implicating agent. We probed the lipid content of separated membrane vesicles from *M. ulcerans* by UV-TLC and found no evidence of mycolactone in the vesicles (**Figure 4.4.1**). This is in sharp contrast to earlier suggestions recently buttressed in literature, that *M. ulcerans* discharges membrane vesicles to transport mycolactone and that such vesicles could contained as much as 2 μ g of the toxin (Foulon *et al.*, 2020). We may have missed mycolactone detection due to low sensitivity of the TLC assay. Cultured yields of both *M. ulcerans* (0.01 g) and *M. marinum* (0.002 g) may be too low to provide mycolactone's concentration above the detection limit of the assay. Probably, if we had eluted materials from the empty spot at 0.23 refractive index for a more sensitive mass spectrometry, we may have observed mycolactone in both mycobacterial cells and the discharge membrane vesicles (Kubicek-Sutherland *et al.*, 2019).

Strangely, the UV-A irradiated vesicles from *M. ulcerans* exhibited similar physiological impact on interacting macrophage cells just as native vesicles. Initially they stimulated metabolic activities of the phagocytes (**Figure 4.4.3**). This in turn compromised the cells' viability (**Figure 4.4.4**) via oxidative stress (**Figure 4.6**) and eventually induced apoptosis in the phagocytes (**Figure 4.5**). This potentially suggests that EMVs from *M. ulcerans* may act as independent virulent determinants on mammalian cells and that the vesicles may not necessarily require mycolactone to induce cytotoxicity during such interactions. This suggestion is supported by reports that EMVs from pathogenic strains which do not produce mycolactone, *Mycobacterium tuberculosis* and *Mycobacterium bovis*, can elicit florid inflammatory response from host's macrophage cells and that vesicle-associated lipoproteins were identified as implicating factors (Athman *et al.*, 2015; Prados-Rosales *et al.*, 2011). Foulon *et al.*, (2020) may have partly described this cytotoxic phenomenon with the suggestion that without mycolactone to supply the second signal for inflammation, other PAMPs in the isolated

EMVs cannot activate the inflammasome pathway (Foulon *et al.*, 2020). We think otherwise and suggest that other PAMS in the discharged EMVs from *M. ulcerans*, not necessarily mycolactone, have the potential to influence viability of interacting macrophage cells (**Figure 4.4.3**).

Potential mediators could have resulted from protein moon-lighting functions or cross-linking. Because more than 50% of the 37 proteins detected in the isolated EMVs were cell envelop localized and had activities related to either “cell wall and cell processes” or “intermediary metabolism and respiration”. Proteins with “virulence, detoxification and adaptation” constituted 5.4% (**Figure 4.7a**). This evidence agrees well with previous report that vesicles from virulent *M. tuberculosis* and *M. bovis* (BCG) were cytotoxic and contained 48 and 66 proteins, respectively, many of which were TLR-2 agonists involved in either “cell wall and cell processes” or “intermediary metabolism and respiration” (Prados-Rosales *et al.*, 2011). Proteins with virulence functions represented 9% and 13% for strains of *M. bovis* (BCG) and *M. tuberculosis*. Avirulent vesicles from *M. smegmatis* rather contained many proteins involved in “lipid metabolism” category.

Although few virulent protein cargoes in were found in the isolated *M. ulcerans* EMVs (Table 3), these proteins, together with amidase *amiC*, integral membrane indolylacetyltransferase *EmbA/B* and many of the conserved hypothetical proteins could have turned-out as effective TLR-2 agonists or shown moonlighting to induce the observed macrophage cytotoxicity (Jeffery, 2003).

Also, previous reports indicated that native vesicles from *M. ulcerans*, proposed to contain mycolactone, killed 80% of primary bone marrow derived macrophage (BMDM) cells within

24 hours and that cell death was by necrosis. Therefore, mycolactone-deficient vesicles were not cytotoxic (Marsollier *et al.*, 2007). In this study, we exposed RAW 264.7 cells to either native or UV-A irradiated vesicles from *M. ulcerans* for 24-hour interactions. Rather, an enhanced cellular metabolism was induced by the two vesicle types during the 24-hour interactions with RAW264.7 cells (**Figure 4.4.4, a to c**). Cytotoxicity was not observed, although it was by microscopy. However, extension of macrophage-vesicle interactions period to 48 hours demonstrated evidence of cytotoxicity on the mammalian cells, especially by UV-A irradiated vesicles to the extent that 6 mg/ml of such vesicles induced 35% loss in the phagocyte's viability. This was 5% less of 40% cell death induced by native vesicles from *M. ulcerans*. Interestingly, cell death was by apoptosis and not necrosis as previously reported by Marsollier *et al.*, 2007. Hence, extracellular vesicles from *M. ulcerans* may require an extended 48 hours of interaction with host macrophage cell to exhibit their cytotoxic impact.

One potential reason for the almost 40% difference in cell death between the current study and previous studies may have resulted from the different cell types used. The previous study used primary cells, BMDM (Marsollier *et al.*, 2007). Although useful in cytotoxicity studies, maintaining survival of these natural cells in the laboratory is quite difficult. Because, the cells have been separated from their natural micro-environment and deprived of critical nutrients and growth factors. Hence, they may become much more sensitive to noxious stimuli. Therefore, it could be reasoned that the previously reported 80% cell death by Marsollier *et al.*, may have resulted from traces of mycolactone discharged into the medium and possibly got contaminate in the isolated *M. ulcerans* EMVs. Poor centrifugation parameters in harvest of EMVs can contaminate the targeted vesicles with other unintended medium components like mycolactone (Arab *et al.*, 2019). Therefore, the reported 80% cell death by necrosis, by *M. ulcerans* EMVs (Marsollier *et al.*, 2007) might have been a confounding consequence of

contaminated mycolactone. Mycolactone is lipophilic and can inadvertently associate with structures like membrane vesicles (Kubicek-Sutherland *et al.*, 2019). We neither detected mycolactone in the isolated EMVs nor lysate of *M. ulcerans* cells which were the source of the vesicles. Yet, the isolated vesicle could induce macrophage cell death with evidence of oxidative stress.

Also, the cytotoxic impact of UV-A irradiated EMVs on RAW264.7 cells was observed after 48 hours of incubation. Previous authors used EMVs from mycolactone-deficient *M. ulcerans* and found no such cytotoxicity after 24 hours of interactions (Marsollier *et al.*, 2007). Probably, if they had extended the interaction period to 48 hours, they could have observed some level of cytotoxicity from the perceived “avirulent” EMVs as the current study has clearly demonstrated. It is therefore possible that other PAMs in the isolated membrane vesicles from the mycobacterium, including proteins can confer cytotoxic impact on the EMVs.

5.1.0. CONCLUSION

Viable, actively growing cells of *M. ulcerans* (Nm 209) and its ancestral lineage *M. marinum* (Sa 200695) released extracellular membrane vesicles into their surroundings. Presence of *M. ulcerans* EMVs was associated with higher total intracellular reactive oxygen species which eventually compromised viability of 40% macrophage cell population within 48 hours as evidenced by apoptosis. The EMVs were cell wall/membrane localized enriched in proteins including virulent lipoprotein LprK, maltokinase, amidase amiC and other conserved hypothetical proteins. Though we could not establish direct contributions of these proteins to the vesicles' cytotoxicity, moon-lighting functions or possible protein cross-linking may have mediated the observed vesicle-mediated toxicity on host's RAW264.7 cells. Therefore, *M. ulcerans* can induce host's cells death through yet to be established mediators. This may partly

explain apoptosis, tissue necrosis, and local immune suppression observed in early stage Buruli ulcer and also provide additional insight on treatment-associated paradoxical reactions.

5.2.0. LIMITATIONS OF THE STUDY

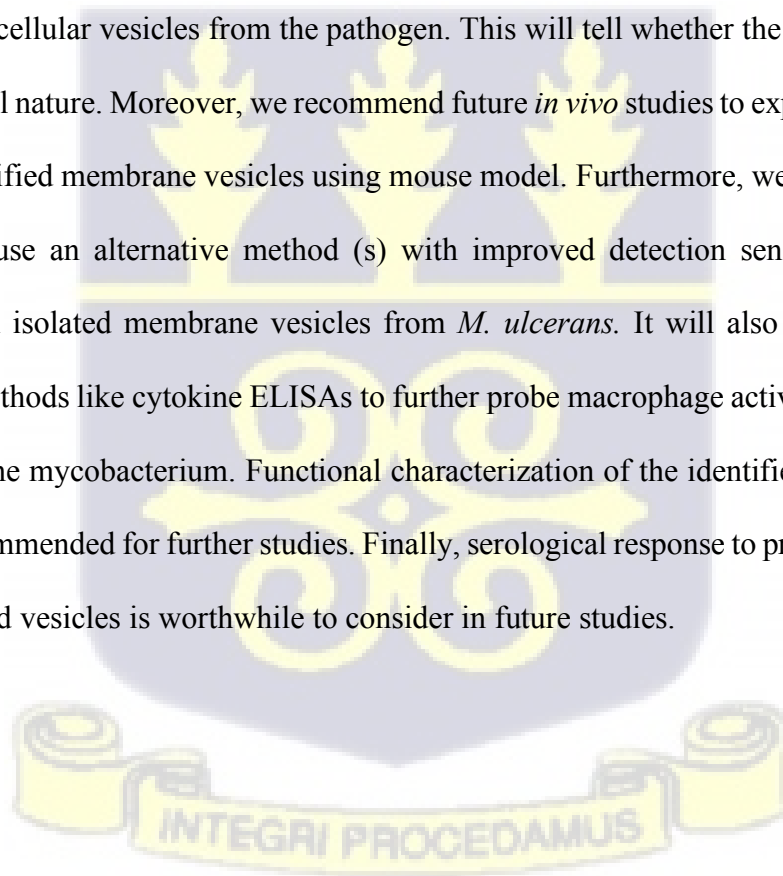
Since only macrophage cells were studied in relation to *M. ulcerans* EMVs, we cannot generalize the vesicle's cytotoxicity to include all host's cell types including keratinocytes, basal cells and other immune cells. We also take cognizance of the fact that the observed 40% cell death in RAW 264.7 cells, after 48 hours, may have resulted from medium nutrients depletion. However, this statement is likely to be a hyperbole since 100 μ L of 2×10^4 cells/ml cell suspension, in freshly prepared DMEM with 10% FBS was seeded. The cells' number in such a volume may be small to exhaust all the available nutrients in the medium within 48 hours. This statement is supported by evidence that 60% of the macrophage cells exposed to the isolated EMVs were still viable after 48 hours of interactions (Figure 4.4.4d).

Moreover, we cannot completely exclude the possibility that other molecules, related or unrelated to *M. ulcerans* cells, may have contributed to the observed cytotoxicity since isopycnic density gradient ultra-centrifugation was not employed in the current study (Arab *et al.*, 2019). Notwithstanding, *M. ulcerans* is non-flagellated, does not possess lipopolysaccharides -LPS (a common inducer of oxidative stress) and also lacks virulent ESX-1 secretory system (Huber *et al.*, 2008). More importantly, the separated vesicles were harvested from pure, growing cultures of *M. ulcerans* with high level of bacterial viability (**Figures 4.1 and 4.2**) using appropriate, recommended protocols for membrane vesicle separation from biological fluids (Livshits *et al.*, 2015; Théry *et al.*, 2018). Nonetheless, the observed cytotoxicity by the isolated vesicles from *M. ulcerans* was a reality. The possibility of moon-lighting effect from the identified proteins in the vesicles may exist but the current study did not consider that. Finally, the low sensitivity of UV-TLC may have led to our inability to prove the presence of

mycolactone in the separated vesicles from *M. ulcerans*. Also, we did not detect mycolactone in the vesicles possibly because the toxin was not possibly present in the mycobacterial cells. However, since 1-hour photo-gradation on UV-A irradiated vesicles did not demonstrate mycolactone presence but showed similar cytotoxicity on macrophage cells just as native vesicles, we can say that other PAMPs in the vesicles, not necessarily mycolactone, can play role (s) in Buruli ulcer pathogenesis and that mycolactone may be dispensable in vesicle-mediated cytotoxicity.

5.3.0. RECOMMENDATION

Further research on how other skin cells like basal cells, keratinocytes and primary human macrophage cells respond to purified vesicles from *M. ulcerans* is recommended. Also, we recommend an infection model to be included in future studies to explore events leading to release of extracellular vesicles from the pathogen. This will tell whether the released vesicles are of functional nature. Moreover, we recommend future *in vivo* studies to explore physiologic response to purified membrane vesicles using mouse model. Furthermore, we recommend that future studies use an alternative method (s) with improved detection sensitivity to assess mycolactone in isolated membrane vesicles from *M. ulcerans*. It will also be interesting to utilize other methods like cytokine ELISAs to further probe macrophage activation by isolated vesicles from the mycobacterium. Functional characterization of the identified proteins in the vesicles is recommended for further studies. Finally, serological response to proteins associated with the isolated vesicles is worthwhile to consider in future studies.



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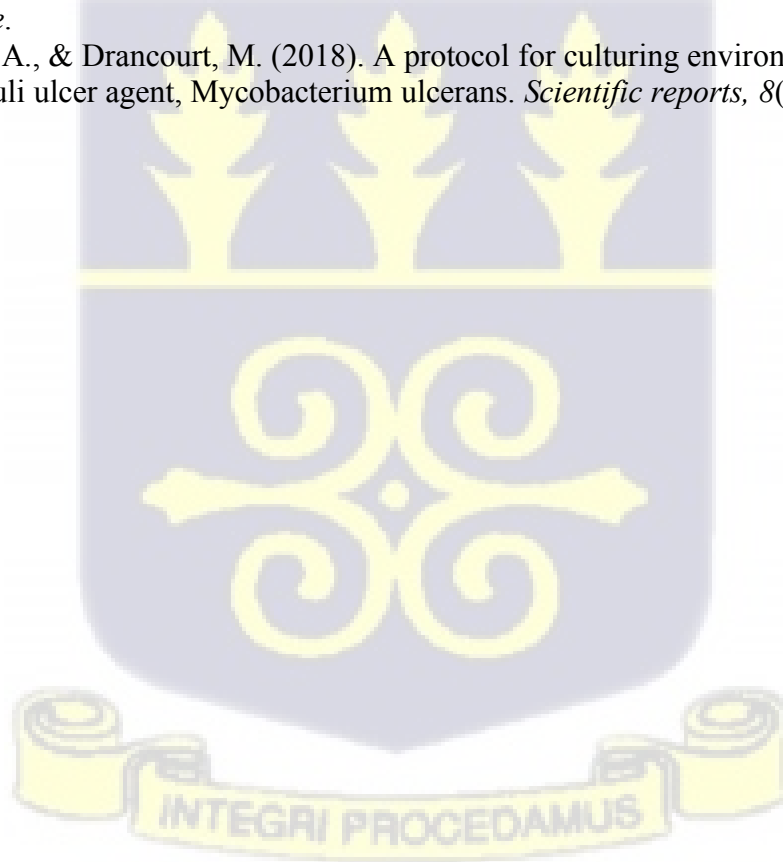
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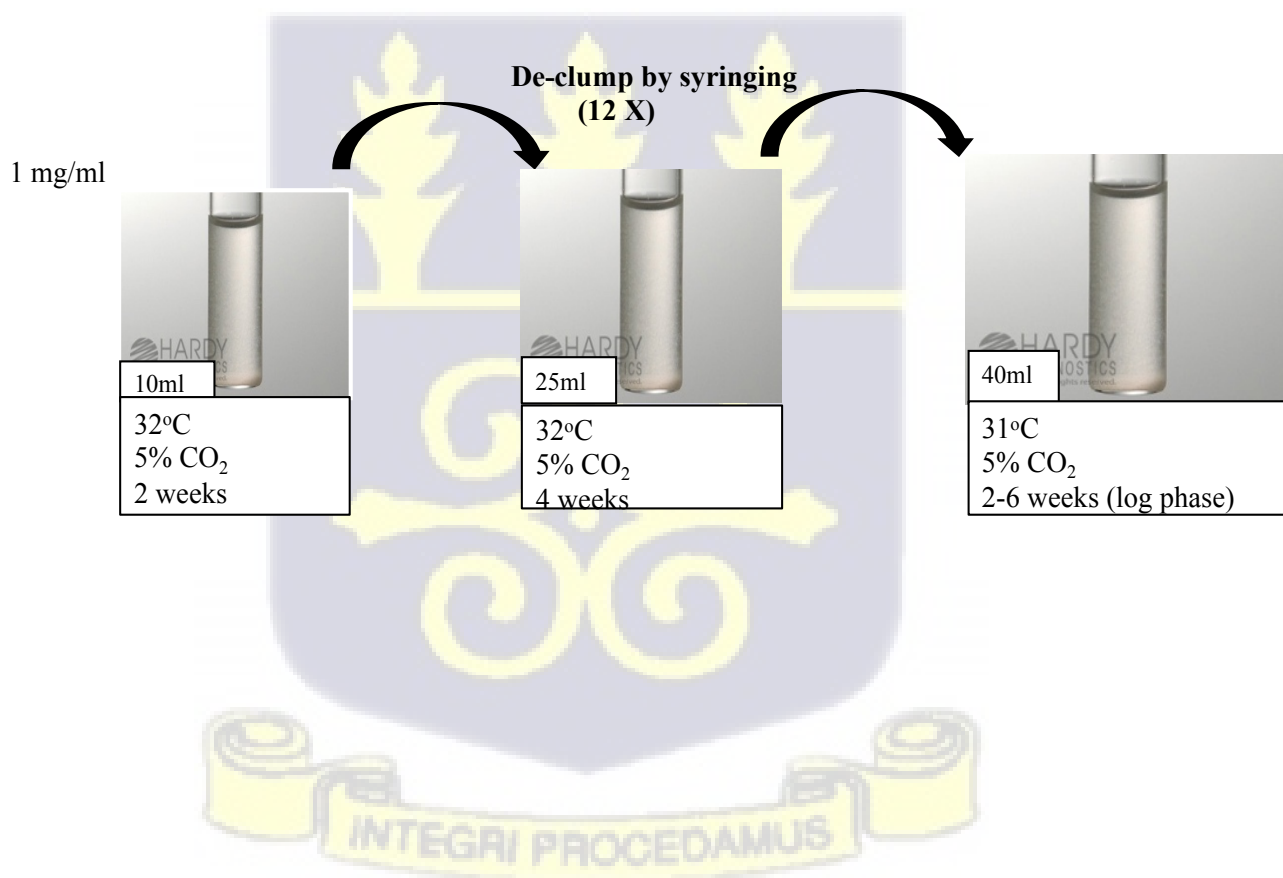
APPENDICES

Appendix A: *M. ulcerans* Culture for Membrane Vesicle Exploration

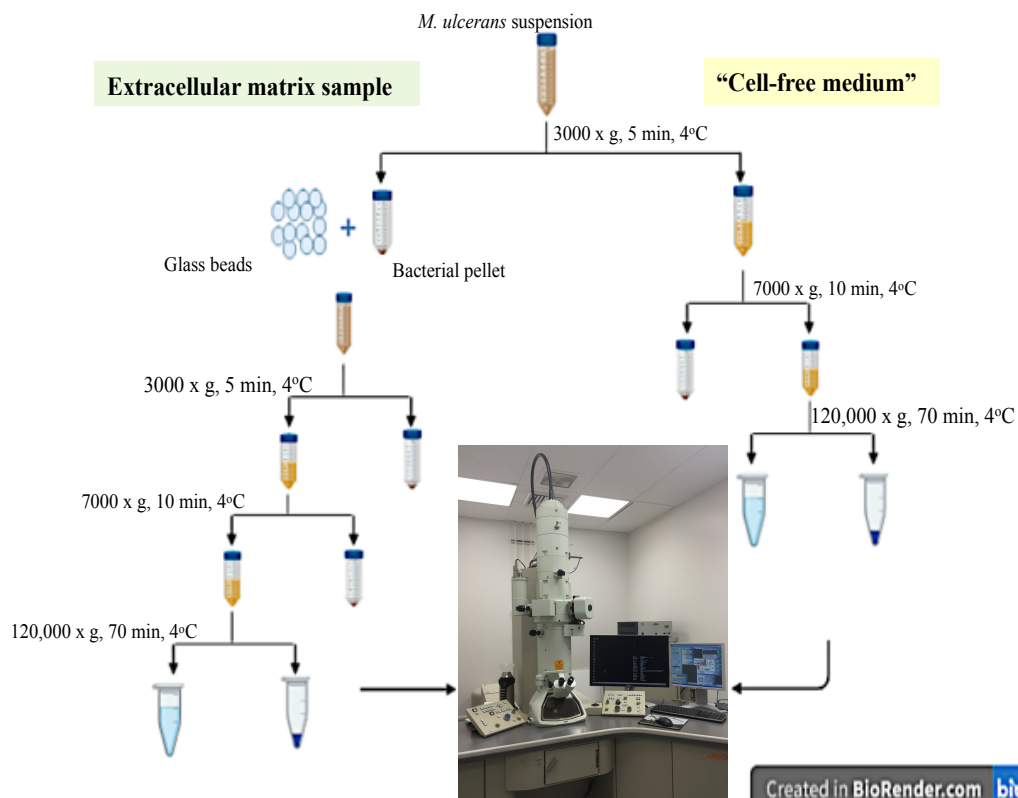
Strains: *M. ulcerans* (Nm 209) : Clinical isolate from Buruli ulcer lesion in Ghana
M. marinum (Sa 200695) : Fish pathogen from Sea bream *Sparus aurata* (Red Sea, Israel)
M. smegmatis : Positive control

Growth medium: Middlebrook 7H9 base broth + 10% OADC + 0.2% glycerol $\pm$ 0.05% Tween-80

Incubation Period: 8 – 12 weeks at 32°C



Appendix B: Harvest of Extracellular Vesicles in Growth Medium of *M. ulcerans*



✓ Research Institute of Physical-Chemical Medicine, Extracellular vesicles Research Group, Russia. <http://vesicles.niifhm.ru/>

ROTOR- S58A FIXED ANGLE

Parameters

R_{min}.....39.6 mm

R_{max}.....76.9 mm

Angle.....35°C

Tube diameter.....25 mm

Complete Sedimentation “cut-off” size.....34 nm

Diameter (nm)	50	70	100	120	150
Portion of pelleted vesicles	100%	100%	100%	100%	100%

Appendix C:

UV-A Irradiation (photo-degradation) on Harvested Vesicles form *M. ulcerans* And Dose-Sensitivity By Microplate AlarmaBlue Assay

1. Does mycolactone associates with vesicles?

2. How long to expose vesicles to RAW 264.7 monolayer?

Photo-degradation (Marion *et al.*, 2012)

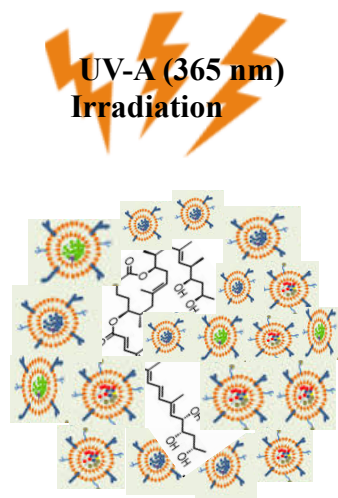
Optimization Experiments
(0.01 mg/ml, 0.1 mg/ml, 1.0 mg/ml and 10 mg/ml)

6 hours

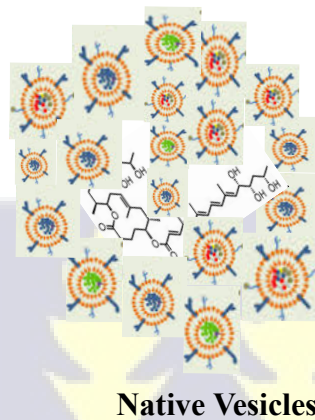
12 hours

18 hours

24 hours

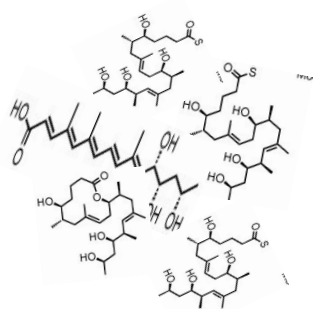


UV-A Irradiated vesicles

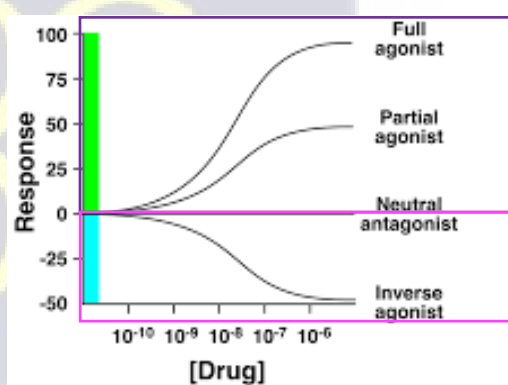


Native Vesicles

3. What will be macrophage response to these vesicles?



Degraded mycolactone



← Stimulatory ?

← Inhibitory ?

Appendix D: Down-regulated proteins in *M. ulcerans* membrane vesiclesTable 4: List of *M. ulcerans* proteins down-regulated in the pathogen's extracellular vesicles, sorted by log2 fold change.

<i>Mycobacterium ulcerans</i> (Nm 209)				Quantitative Value (Normalized NSAF)		Fold Change	Log2 Fold Change
	Description	UniProt ID	Gene	MW	Bact. Pellet	Vesicles	
	Inosine-5'-monophosphate (Imp) dehydrogenase, GuaB3	A0PMG1_MYCUA	guaB3	39 kDa	0.0010098	0.00087818	0.87
	Indole-3-glycerol phosphate synthase	TRPC_MYCUA	trpC	28 kDa	0.0013922	0.0012107	0.87
	NADH dehydrogenase Ndh	A0PSF8_MYCUA	ndh	50 kDa	0.00050851	0.00035718	0.70
	HpcH HpaI domain-containing protein	A0PRN5_MYCUA	MUL_2683	46 kDa	0.00054264	0.00038116	0.70
	Two component sensory transduction transcriptional regulatory protein MtrA	A0PRF1_MYCUA	mtrA	25 kDa	0.0011073	0.00072219	0.65
	Alpha-1,4-glucan:maltose-1-phosphate maltosyltransferase	A0PUI5_MYCUA	glgE	78 kDa	0.00036324	0.00023692	0.65
	Proteasome-associated ATPase	ARC_MYCUA	mpa	68 kDa	0.00079947	0.00054075	0.68
	Two component system response phosphate regulon transcriptional regulator, PhoP	A0PLE8_MYCUA	phoP	27 kDa	0.0012773	0.00068608	0.54
	50S ribosomal protein L7/L12	A0PM20_MYCUA	rplL	13 kDa	0.0047162	0.0025332	0.54
	Glycine--tRNA ligase	A0PTS7_MYCUA	glyS	52 kDa	0.00070716	0.00035874	0.51
	Glycerol kinase	A0PVD3_MYCUA	glpK	55 kDa	0.00063895	0.00032413	0.51
	Conserved lipoprotein LppZ	A0PPX7_MYCUA	lppZ	40 kDa	0.00083441	0.00042329	0.51
	Transcription elongation factor GreA	A0PKT2_MYCUA	greA	18 kDa	0.0021991	0.001004	0.46
	Lipase/esterase LipN	A0PQ13_MYCUA	lipN	39 kDa	0.001018	0.00044263	0.43
	Adenylate kinase	KAD_MYCUA	adk	20 kDa	0.0028892	0.00090972	0.31
	Uncharacterized protein	A0PR60_MYCUA	MUL_2484	42 kDa	0.0013982	0.00044027	0.31
	Conserved protein	A0PSH7_MYCUA	MUL_3046	17 kDa	0.0055656	0.0010164	0.18

