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PHENOTYPIC CHARACTERIZATION OF HOST-PATHOGEN INTERACTION IN Mycobacterium africanum

By

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF PHD MICROBIOLOGY DEGREE

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DECLARATION

This thesis was presented by me to the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences, College of Health Sciences, under the supervision of Professor Mercy Jemima Newman, Professor Dorothy Yeboah-Manu and Professor Ben Gyan. This work has never in part or in whole been submitted to any University or by any other person for the purpose of the award of the degree.

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DEDICATION

This study is dedicated to God for His unmeasuring love, grace, wisdom and guidance that empowered me to go through this study to completion.

Secondly, I dedicate it to my beloved wife, Mrs. Janet Ama Amissah Tetteh for her love and support including my delightful and wonderful children, Julia Afua Ofeibea Tetteh, John Kuuku Amissah Tetteh and Jude Papa Ekow Elinam Tetteh.

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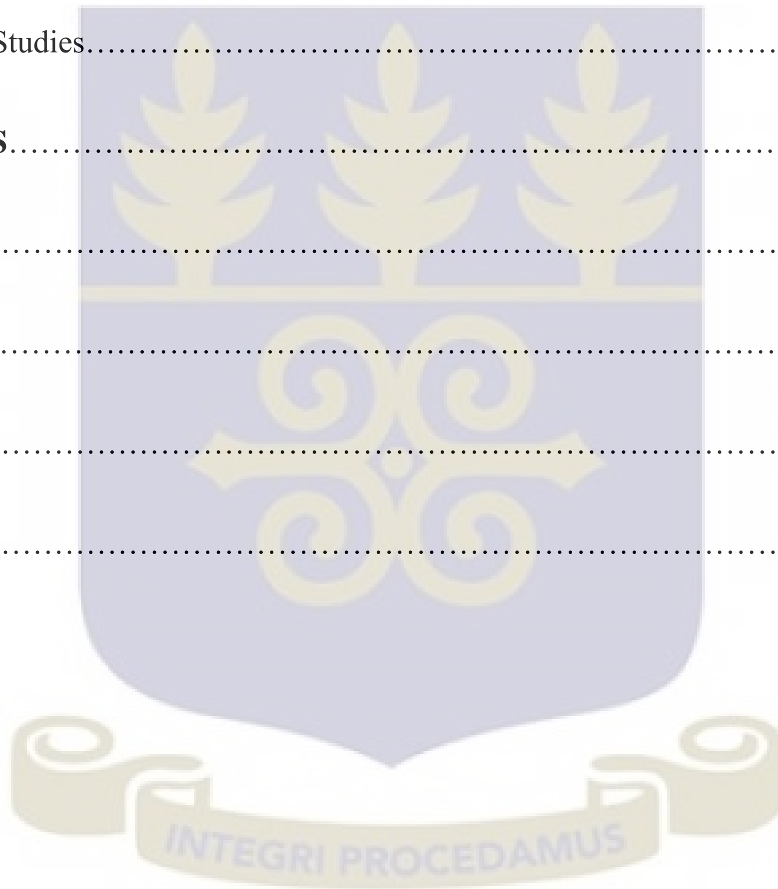

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LIST OF ABBREVIATIONS

1. ADC Albumin – Dextrose - Catalase
2. AIDS Acquired Immunodeficiency Syndrome
3. BCG Bacillus Calmette-Guérin
4. BD: Becton Dickinson
5. CD Cluster of Differentiation
6. CDC Centers for Disease Control and Prevention
7. CFP Culture Filtrate Protein
8. CFUs Colony Forming Units
9. CLRs C-type Lectin Receptors
10. CRs Complement Receptors
11. DCs Dendritic Cells
12. DNA Deoxyribonucleotide Acid
13. DOTS Directly Observed Therapy, Short-Course
14. EBV Epstein–Barr Virus
15. EDTA Ethylenediamine Tetra-Acetic Acid
16. ELISA Enzyme-Linked Immunosorbent Assay
17. ELISPOT Enzyme-Linked Immunospot
18. ESAT Early Secreted Antigenic Target
19. ETH Ethambutol
20. FCS Foetal Calf Serum
21. FITC Fluorescein Isothiocyanate
22. FSC Forward Scatter
23. GM-CSF Granulocyte Macrophage Colony Stimulating Factor
24. HIV Human Immunodeficiency Virus

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25. IFN-γ Interferon Gamma
26. Ig Immunoglobulin
27. IGRA Interferon Gamma Releasing Assay
28. INH Isoniazid
29. iNOS Inducible Nitric Oxide Synthase
30. IS Insertion Sequence
31. IL- Interleukin
32. kDa Kilo Dalton
33. LAM Liparabinomannan
34. LSP Large Sequence Polymorphism
35. LTBI Latent Tuberculosis Infection
36. mAbs Monoclonal Antibodies
37. MACS Magnetic Cell Separation System
38. MAF *Mycobacterium africanum*
39. MBLs Mannose-Binding Lectins
40. MDM Monocyte-Derived Macrophage
41. MDR-TB Multi-Drug Resistant Tuberculosis
42. MHC Major Histocompatibility
43. MIRU-VNTR Multiple Interspersed Repetitive Unit-Variable Number Tandem Repeat
44. MOI Multiplicity of Infection
45. MOTT *Mycobacterium* Other Than Tuberculosis
46. MRs Macrophage Mannose Receptors
47. MTB *Mycobacterium tuberculosis*
48. MTBC *Mycobacterium tuberculosis* Complex

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49. MTBss *Mycobacterium tuberculosis* Sensu Stricto
50. NAAT Nucleic Acid Amplification Tests
51. NLRs Nucleotide-Binding Like Receptors
52. NMIMR Noguchi Memorial Institute for Medical Research
53. NOD Nucleotide-Binding Oligomerization Domain
54. NTM Non-Tuberculous Mycobacteria
55. NTP National Tuberculosis Program
56. OADC Oleic Acid-Albumin–Dextrose-Catalase
57. PAS P-Aminosalicylic Acid
58. PBS Phosphate Saline Buffer
59. PBMC Peripheral Blood Mononuclear Cells
60. PCR Polymerase Chain Reaction
61. PE Phycoerthyrin
62. PerCP Peridinin Chlorophyll Protein
63. PGL Phenolic Glycolipid
64. PPD Purified Protein Derivative
65. PZA Pyrazinamide
66. QFT-IT QuantiFERON-TB Gold In-Tube
67. Q-RT RT-PCR Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction
68. RD Regions of Difference
69. rESAT-6/CFP-10 recombinant early secreted antigenic Target–6 and culture filtrate protein–10
70. RIF Rifampin
71. RPMI Rose Pillar Medical Institute

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72. rRNA Ribosomal Ribonucleic Acid

73. SEB *Staphylococcal* enterotoxin B

74. SEM Standard Error of Mean

75. SC Side Scatter

76. SM Streptomycin

77. Sp-A Surfactant Protein A

78. SRs Scavenger Receptors

79. TAT Twin Arginine Translocation

80. TB: Tuberculosis

81. Th T Helper

82. TbD1 *Mycobacterium tuberculosis* Specific Deletion 1 Gene

83. TLRs Toll-Like Receptors

84. TNF-α Tumour Necrosis Factor Alpha

85. TST Tuberculin Skin Test

86. USAID United States Agency for International Development

87. WGS Whole Genome Sequence

88. WHO World Health Organization

89. XDR-TB Extensively Drug-Resistant Tuberculosis

90. ZN Ziehl-Neelsen

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Abstract

Background: *Mycobacterium africanum* (MAF) and *Mycobacterium tuberculosis* sensu strico (MTBss) are two members of a closely related bacterial species of *Mycobacterium tuberculosis* complex (MTBC) that causes tuberculosis (TB) in humans. However MAF is known to cause up to 50% of human pulmonary TB in West Africa only. MAF has been sub-divided into MAF West African 1 (MAF1) (Lineage 5) and MAF West African 2 (MAF2) (Lineage 6), as two distinct phylogenetical lineages within MTBC. Subsequently the absence of *Mycobacterium tuberculosis* deletion gene (TbD1) strains in MTBss has been referred to as modern lineage whilst ancient lineage (MAF1 and MAF2) have the presence of TbD1. Ghana represents one of the few countries within Central West Africa known to have this unique genetic diversity of MAF1, MAF2 and MTBss that causes TB cases in significant proportions. While previously it was thought MAF is genetically very closely related to MTBss such that there are no important phenotypic differences between the two species, current advance in molecular biology indicate that substantial genetic difference exit between the two that can translate into significant phenotypic differences including immunogenicity and virulence.

Aim: The aim of the study was to analyze the phenotypic features of host-pathogen interaction in *Mycobacterium africanum* and compared to *Mycobacterium tuberculosis* sensu strico strains.

Methodology: The study was embedded in 2 different projects. Retrospective archived cryopreserved peripheral blood mononuclear cells (PBMCs) of MAF-infected and MTBss-infected patients were stimulated with growth medium (negative control), *Staphylococcus enterotoxin B* (SEB, positive control) and recombinant early secreted antigenic protein 6 kiloDalton/culture filtrate protein 10 kiloDalton fusion protein (rESAT-6/CFP-10), surface

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stained for T-subsets (CD4 and CD8) and intracellular cytokine, interferon gamma (IFN-γ), and acquired with FACS Calibur flow cytometer. The second study used characterized large sequence polymorphism (LSP) clinical isolates identified as MAF1, MAF2 and MTBss to determine intracellular growth assay in human monocyte–derived macrophages (MDM), mean doubling time and pro-inflammatory tumour necrosis factor–alpha (TNF-α), interleukin 6 (IL-6) and 12p70 cytokines by enzyme-linked immunosorbent assay (ELISA).

Results: The percentage frequencies of CD4+IFN-γ+ and CD8+ IFN-γ+ T cells of MAF-infected patients did not differ from the percentage frequencies CD4+ IFN-γ+ and CD8+ IFN-γ+ T cells of MTB-infected patients in response to rESAT-6/CFP-10 fusion protein ($p>0.05$). Uptake of MAF1, MAF2 and MTBss representing modern and ancient strains respectively at 4hours was not significant ($p>0.05$). Mean intracellular growth index from 24hours to 72hours was significantly rapid for MTBss (modern) lineage compared to MAF1 and MAF2 (ancient) lineages ($p<0.05$). In contrast the mean doubling time of MTBss (modern) lineage was significantly lower compared to MAF1 and MAF2 (ancient) lineages ($p<0.05$). Levels of pro-inflammatory cytokines released into the supernatants by MTBss, MAF1 and MAF2 at 4hours was not statistically significant ($p>0.05$). However at 24hours to 72hours levels released by MAF1 and MAF2 (ancient) lineages was significantly higher than MTBss (modern) lineage ($p<0.05$).

Conclusion: The study has shown that MAF-infected patients had similar T subset response to rESAT-6/CFP-10 fusion protein relative to MTBss-infected patients. Furthermore MAF had reduced uptake, low intracellular growth rate and a higher doubling time in MDM. Likewise MAF (ancient) lineages have hyper-inflammatory response thereby inducing a 'slow growth' phenotype highlighting the point that MAF indeed has lower virulence and longer latency leading to slower progression to active disease in the host.

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CHAPTER ONE

1. INTRODUCTION

1.1 Background

Tuberculosis (TB) is a primeval airborne contagious disease, which still remains a major public health emergency (WHO, 2014), accounting for an estimated 9.0 million incident cases and 1.5 million deaths annually; with 30% of the global burden of TB occurring in Africa (WHO, 2014). At the same time approximately 2 billion individuals (one third of the total world population) are latently (asymptomatically) infected, of whom 5-10% individuals progresses to TB disease in their lifetime, with higher rates of progression in immune compromised people (WHO, 2014). The global TB epidemic is further exacerbated by a strong synergy with HIV/AIDS, which is a particular problem in sub - Saharan Africa (Corbett *et al.*, 2003), as well as diabetes whose impact is increasing in many rapidly growing world economies such as India (Stevenson *et al.*, 2007) and Mexico (Ponce-De-Leon *et al.*, 2004). Furthermore, the long - term neglect of basic TB research and product development have relied on the 100 – year - old diagnostic method for active TB infection (i.e. sputum smear microscopy) of poor sensitivity. The QuantiFERON-TB Gold In-Tube [an alternative to tuberculin skin test (TST)] for diagnosis of latent and active TB is not *Mycobacterium tuberculosis* sub-specie specific, an 80-year old and largely ineffective vaccine (*Mycobacterium bovis* Bacillus Calmette-Guérin [BCG]) and few decades old drugs (streptomycin, rifampicin, isoniazid, ethambutol, pyrazinamide) have remained unchanged (Young *et al.*, 2008). Consequently the worldwide emergence of multidrug - resistant (MDR) and extensively drug-resistant (XDR) forms of TB is threatening to make this human infectious disease incurable (Raviglione & Smith, 2007). Thus, in the face of modern epidemics of HIV/AIDS, diabetes, XDR and MDR and tools still in use today, all of which

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contribute to susceptibility to TB, the global TB control by Stop TB Partnership 2006, have outlined plans to halve TB prevalence and mortality by 2015 and eliminate the disease as a public health problem by 2050 (WHO, 2006). This vision hopes to depend on the development of improved diagnostics, simpler treatment, and more effective vaccination, which will in turn require enhancement in the knowledge of the disease and the biology of the pathogen (Young *et al.,* 2008). Although a lot of resources are being invested into the development of new TB diagnostics, drugs and vaccines, it is not clear how strain diversity of the pathogen will affect the effectiveness of these new control tools.

TB in humans is caused mainly by two members of *Mycobacterium tuberculosis* complex (MTBC) known as *Mycobacterium tuberculosis* sensu stricto (MTBss) and *Mycobacterium africanum* (MAF). MAF since its first description in Senegal (Castets *et al.*, 1968) has traditionally been identified by phenotypic criteria, occupying an intermediate position between MTBss and *M. bovis* based on biochemical characteristics (David *et al.*, 1978). Typically, MAF was subdivided by geographic origin and biochemical properties into two major subgroups: MAF subtype I originating from West Africa and MAF subtype II from East Africa (David *et al.*, 1978; Niemann *et al.,* 2002). Recent advances in molecular strain-typing techniques and comparative genomics have shown that MAF subtype I (West African clade) comprises of 2 separate lineages which are genetically diverse; i.e. MAF I, West African type 1 (MAF1), prevalent around the Gulf of Guinea and MAF I, West African type 2 (MAF2), prevalent in western part of West Africa (Gagneux *et al.*, 2006). The MAF type II (East African clade) has been reclassified into MTBss as indicated as ‘‘Uganda’’ genotype (Mostowy *et al.*, 2004) (Figure 1.1).

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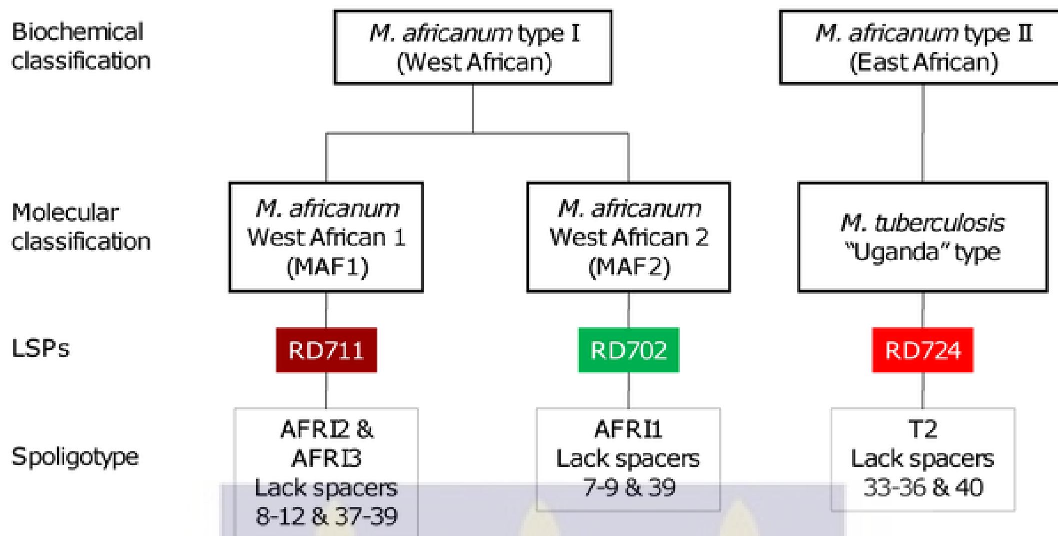

Figure 1.1 Nomenclature of MAF as related to its biochemical and molecular classification. Source: (de Jong *et al.,* 2010)

Though, MAF has been sporadically identified in other regions of the world such as England (Grange & Yates 1989), California (Desmond *et al.*, 2004), France (Frottier *et al.*, 1990), Spain (Perez-de Pedro *et al.*, 2008) and Germany (Jungbluth *et al.*, 1978; Schroder, 1982) both from pulmonary and extra-pulmonary sources, the main burden occur in West-Africa, where it causes up to 50% of TB cases in some of the countries (Källenius *et al.*, 1999; de Jong *et al.*, 2009)., The first evolutionary reconstructions of the genetic population structure of the human-adapted members of MTBC highlighted a group of strains harbouring a deletion in the genomic region known as *M. tuberculosis* specific deletion 1 gene (TbD1) (Brosch *et al.,* 2002). TbD1-deleted strains have been referred to as evolutionarily "modern" (MTBss) compared to the strains without this deletion as evolutionarily "ancestral" or "ancient" (MAF) strains. Using large sequence deletion analysis, Gagneux *et al.,* 2006 grouped the human adapted strains into 6 main phylogenetic lineages (L1 to L6), which showed that each lineage was associated with distinct human populations and geographical regions. MTBss was subdivided into L1 to L4 while MAF was divided into L5 and L6 or

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MAF1 and MAF2. However, Africa is the only continent where all six lineages of human-adapted MTBC lineages occur, which also carries a disproportionally large share of the global TB burden and harbours the largest genetic diversity of the human-adapted MTBC lineages (Figure 1.2) (Gagneux *et al.*, 2006).

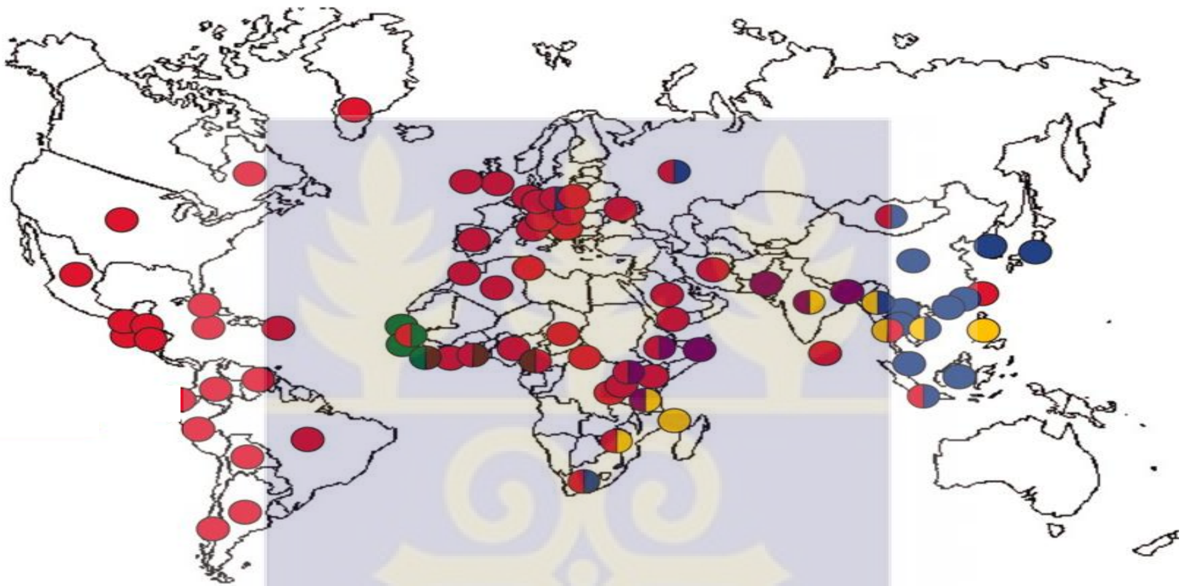

Figure 1.2 The global population structure and geographical distribution of human adapted MTBC. Source: (Gagneux *et al.*, 2006)

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MTBC an obligate, aerobic, Gram-positive acid-fast intracellular human bacteria has a preference for the lung tissue rich in oxygen (Galagan, 2014). TB is transmitted when an individual with active pulmonary TB expels droplet nuclei containing infectious pathogens through actions such as coughing, spitting, singing and talking. Inhaled droplets are deposited in the alveolar spaces of the lungs, where the bacteria are phagocytosed by phagocytic cells, mainly alveolar macrophages (Mehta *et al.,* 1996; Andersen, 2002). In humans, large numbers of alveolar macrophages are not readily available for *in vitro* study, therefore matured human monocyte derived macrophages (MDM) derived from peripheral blood

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mononuclear cells (PBMCs) are used as an *in vitro* model to analyze the virulence of *M. tuberculosis* infection in human cells (Gordon & Taylor, 2005). The bacteria are phagocytosed in a process that is initiated by bacterial contact with macrophage mannose and/or complement receptors (Schlesinger, 1993). Surfactant protein A, a glycoprotein found on alveolar surfaces enhances the binding and uptake of *M. tuberculosis* by up-regulating mannose receptor activity (Gaynor *et al.,* 1995). Intracellular mycobacterial growth (Silver *et al.,* 1998), cytokine expression (Falcone *et al.,* 1994), or combinations of the two parameters (Manca *et al.,* 1999) have been used as a measure of virulence in isolated macrophages. Infected alveolar macrophages release a panel of antimicrobial effector molecules, cytokines and chemokines, which govern innate immune responses and initiate specific immunity. The cytokine network plays a central role in the inflammatory response and the outcome of mycobacterial infections (van Crevel *et al.,* 2002). Early pro-inflammatory cytokine secretion is a hallmark of *M. tuberculosis* infection (van Crevel *et al.,* 2002). A crucial role in protective immunity and pathophysiology against tuberculosis is performed by tumour necrosis factor alpha (TNF-α) (Jo *et al.,* 2003). It synergizes with interferon gamma (IFN-γ) to increase the production of nitric oxide metabolites and facilitate mycobacterial killing and is essential for granuloma formation for the containment of mycobacterial infection (Jo *et al.,* 2003). Interleukin (IL)-12 productions is essential to induce a protective Th1 response (Cooper *et al.,* 1997), while IL-6 has also been suggested to be a pivotal pro-inflammatory cytokine during acute infection (Ladel *et al.,* 1997).

The outcomes of TB infections in humans are extremely variable, ranging from lifelong latent infection to active disease with variable degrees of extra-pulmonary involvement (Gagneux & Small, 2007; Malik & Godfrey-Faussett, 2005). This variation has primarily been attributed to host and environmental factors and the general belief that genetic strain diversity within MTBC was too limited to account for the differences in virulence (Sreevatsan *et al.,*

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1997). However, with the advancement in genomic analysis, more data are mounting to support the fact that substantial genetic polymorphism (driven by large deletions and single nucleotide polymorphisms) exist within the MTBC and some of the phenotypic variations in outcome of infections may be due to strain genetic variability (Gagneux & Small, 2007; Nicol & Wilkinson, 2008; Kato-Maeda *et al.*, 2001). For example, experimental studies using macrophage and animal models have shown that strains of human MTBC differ in virulence and immunogenicity. Previous studies demonstrated that virulent *M. tuberculosis* strains grow more rapidly than avirulent or attenuated strains within human phagocytes (Silver *et al.,* 1998; Zhang *et al.,* 1998). Individual strains from L2 (Beijing) have been shown to grow more rapidly than comparator strains in *in vitro* human cell culture models using MDM or monocyte or human macrophage cell lines (Li *et al.,* 2002; Zhang *et al.,* 1999; Theus *et al.,* 2005). Similarly, animal studies comparing *M. africanum* (L6) from Senegal to *M. tuberculosis* showed that *M. africanum* was less virulent (Castets & Sarrat, 1969), however *M. africanum* (L5) have not been well studied. The lack of virulence in attenuated strains such as the BCG vaccine reflects the loss of genetic material encoding cytotoxic materials including culture filtrate protein 10 (CFP-10) and ESAT-6 proteins (Guinn *et al.*, 2004; Lewis *et al.*, 2003). In contrast, a clinical strain HN878 (East Asia/Beijing strain) that caused disease outbreaks in Los Angeles and Houston, has been shown to exhibit a consistent hyper-virulent phenotype in various experimental models, including human monocyte-derived macrophages (Zhang *et al.*, 1999), mice (Reed *et al.*, 2004; Manca *et al.*, 1999; Manca *et al.*, 2001; Manca *et al.*, 2005), and rabbits (Tsenova *et al.*, 2005).

Limited evidence suggests that TB caused by MAF is clinically indistinguishable from disease caused by MTBss, and the same treatment regimens are used to treat both conditions (de Jong *et al.*, 2007; Meyer *et al.*, 2008). However, a study from the Gambia reported an association between MAF and HIV and that TB patients and their household contacts

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infected with MAF exhibited an attenuated T-cell response to the major MTBC antigen, early secreted antigenic target 6 (ESAT-6) protein (de Jong *et al.,* 2006), whereas another study in Ghana was unable to replicate these findings (Meyer *et al.*, 2008). Furthermore, another study in the Gambia, (de Jong *et al.*, 2008) showed that the primary disease progression of HIV-negative household contacts of active TB patients exposed to MAF were significantly lower than contacts exposed to other lineages. These results indicated that the strain diversity in MTBC may account for differences in how the human host perceive MAF and hence the observed phenotypic variability.

MTBC has evolved multiple mechanisms to interfere with the host immune system (Doherty & Andersen, 2005; Flynn & Chan, 2005). A number of studies suggest that particular strains of MTBC differ qualitatively or quantitatively in some of these features of immune-modulation. A rapid growth phenotype is associated with reduced TNF-α secretion, whereas robust TNF-α secretion inhibits mycobacterial replication (Theus *et al.,* 2005). For example, the hyper-virulence of strain HN878 mentioned earlier has been linked to the production of phenolic glycolipid (PGL), which has immune-modulatory effects (Reed *et al.*, 2004; Manca *et al.*, 2005). Studies in human monocytes and mice have shown that while other strains induce a strong expression of pro-inflammatory cytokines, including TNF-α, IL-1 beta (β), IL-12, and IFN-γ, strain HN878 was associated with a reduced expression of pro-inflammatory cytokines and an elevated production of macrophage deactivating cytokines such as IL-11 and IL-13 (Manca *et al.*, 2005).

The Euro-American lineage (L4), one of the 4 lineages of MTBss, and the East-Asian lineage (Beijing) are both considered as ''modern'' *M. tuberculosis* lineages, lacking in particular the TbD1 genomic region (Gagneux *et al.,* 2006; Brosch *et al.,* 2002) while the "ancient" *M. tuberculosis* lineages to which *M. africanum* lineages belongs have the TbD1 genomic region (Brosch *et al.,* 2002). Modern lineages were shown to induce lower levels of pro-

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inflammatory cytokines when compared with ancient lineages (Portevin *et al.,* 2011). The low inflammatory response induced by evolutionary modern strains has been associated with an enhanced ability to cause early progressive disease (Manca *et al*., 2004; Newton *et al*., 2006).

Although Ghana is not among the World Health Organization (WHO) twenty-two high-burden countries for TB, the disease is a major public health problem in the country. In 2013, WHO then estimated a drop in the cases of TB in Ghana at 72 per every 100,000 persons, but a prevalence survey conducted in 2014 has revealed that TB burden in Ghana is three times higher than the WHO estimate (WHO, 2014). Prior to the survey, WHO estimates showed that TB cases in Ghana were below 92 per every 100,000 people but the survey across the country showed that there were 286 cases per every 100,000 people in Ghana (WHO, 2014). Previous study published by Yeboah-Manu *et al.* (2011), showed that human TB is caused by six out of the seven MTBC lineages, with 20% of all cases attributed to MAF1/L5 and MAF2/L6 while 65% was by the Cameroon sub-lineage of MTBss/L4, the dominant cause of human TB in Ghana. MAF is an important TB causing pathogen in Ghana and therefore makes it imperative to explore whether strain-specific diversity of MAF1/L5 and MAF2/L6 may translate into phenotypic differences. Hence this study proposed to take advantage of the unique TB epidemiology in Ghana to characterize MAF in detail, using a variety of phenotypic assays to compare host-pathogen interactions of MAF and MTBss strains.

1.2 Problem Statement and Justification

MTBss and MAF are the two main causative species of human TB; while MTBss is globally distributed, for yet unknown reason (s) MAF is restricted to West Africa. MAF1/L5 is prevalent around the Gulf of Guinea (Nigeria and Cameroon) while MAF2/L6 is prevalent in western West Africa (Senegal, The Gambia, Guinea Bissau and Mali) (Gagneux *et al,* 2006).

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However within central West Africa, Ghana represent one of the few countries (Sierra Leone, Ivory Coast and Benin) that are known to have both distinct phylogenetic lineages, i.e. MAF1/L5 and MAF2/L6 that cause TB in significant proportions (Figure 1.3). Previous study has shown in Ghana that 20% of human TB is caused to MAF1/L5 and MAF2/L6 (Yeboah-Manu *et al,* 2011).

For a long time the general dogma was that these species together with five others that make-up the *Mycobacterium tuberculosis* complex (MTBC) are genetically similar (Brosch *et al*. 2002). The assumption therefore was that genetic diversity in the MTBCs has no significant consequence on the outcome of host-pathogen interaction. However, this notion relied on the findings of early DNA sequencing studies that were conducted using limited and often biased strain collections (Brosch *et al*. 2002).. Conversely, with the advancement in molecular biology, more data is accumulating indicating that at the genomic level, substantial strain diversity exists among the different members of MTBC and this could have functional implications (Gagnuex *et al*. 2006). However, this strain diversity has not been taken into consideration in all the numerous investment in works going on in vaccine, diagnostic and drug development, all studies are centered on MTBss and MAF is largely neglected due to the unique epidemiology of MAF. In Ghana, the genetic diversity of MAF have been studied, but the effect of MAF diversity on host-pathogen interactions and the specific host immune response they induce have not been well studied. Therefore understanding the host-pathogen interactions may help to gain insight into the intracellular growth dynamics and virulence of the 2 MAF lineages in the host. This will enable us better assess the response of MAF-infected individuals to recombinant ESAT-6/CFP-10 fusion protein in infected Ghanaians. This will finally aid in better understanding and management of MAF diversity on host-pathogen interactions.

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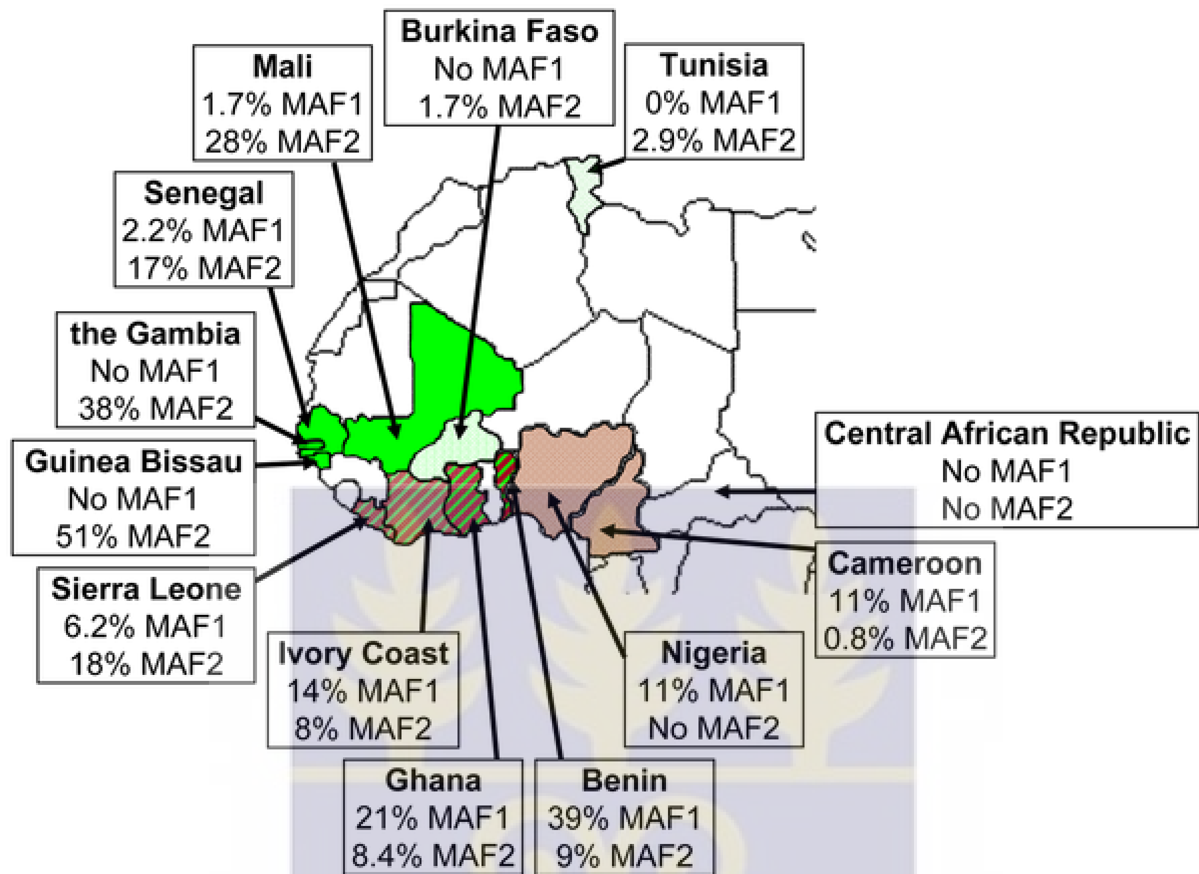

Figure 1.3 *M. africanum* prevalence in Western African countries. Source: (de Jong *et al.*, 2010)

The study hypothesis is *M. africanum* lineages will have reduced ESAT6/CFP10-specific interferon gamma expression, a slower growth rate and an increase early inflammatory cytokines compared to MTBss

1.3 Aim

The aim of the study was to analyze the phenotypic features of host-pathogen interaction of MAF compared to MTBss strains.

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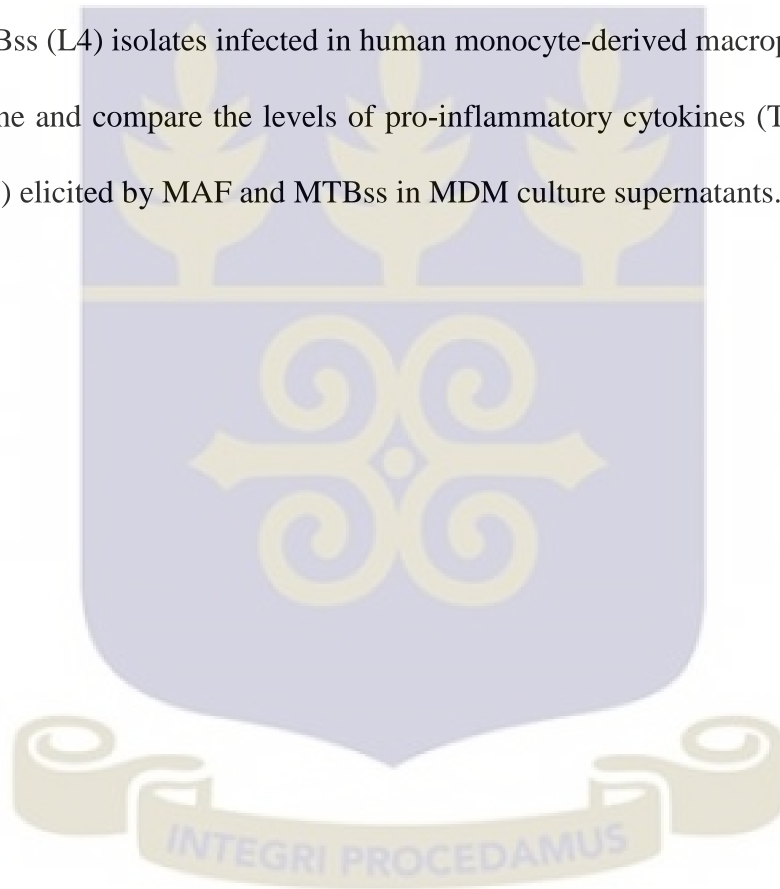
1.4 Specific Objectives

The specific objectives of the study were to:

1. Determine and compare the frequencies of rESAT-6/CFP-10 fusion protein-specific interferon gamma (IFN-γ) expressing T cell subsets (CD4+ and CD8+) from MAF-infected compared to MTB-infected patients.
2. Analyze and compare the mycobacterial intracellular growth rate of MAF (L5 and L6) and MTBss (L4) isolates infected in human monocyte-derived macrophages (MDM).
3. Determine and compare the levels of pro-inflammatory cytokines (TNF-α, IL-12p70 and IL-6) elicited by MAF and MTBss in MDM culture supernatants.



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CHAPTER TWO

2 Literature Review

2.1 Historical Facts and Public Health Importance of Tuberculosis

Tuberculosis or TB (short for *tubercle bacillus*) is one of the primeval and deadliest diseases of mankind (WHO, 2012). Bone TB was identified in old skeletons from Europe and Middle East 4000 years ago as the cause of death, showing that this disease was already a widespread health problem. In ancient times, Ibn Sina (Avicenna adopted from the Greeks), was of the view that TB is caused by pollution in the air (*miasma*, a noxious form of "bad air") (Byrne, 2008). It was later described by Hippocrates as *phthisis* (Greek term for consumption) as bloody cough, fever, pallor, and long relentless wasting or white plague because afflicters appear markedly pale (Haas & Haas, 1996). It was also known as “The King’s Evil” because of the myth that it could be cured by the touch of reigning monarch on scrofula (lymphatic system resulting in swollen neck glands in adults) (Talavera *et al.,* 2001). Other various forms of extra pulmonary TB included Potts’ disease (spinal tuberculosis), meningitis (tuberculosis of the central nervous system), miliary tuberculosis (now commonly known as disseminated TB of the circulatory system), cutaneously in the form of *lupus vulgaris,* TB of the skin and TB in the urogenital tract (Talavera *et al.,* 2001). TB is also called Koch’s disease, after the scientist Robert Koch, who identified the *tubercle bacillus* as the etiologic agent (Koch, 1882).

Treatment options were limited. Albert Calmette and Camille Guerin provided bacillus calmette guerin protection (BCG) against *Mycobacterium tuberculosis* (MTB) infection when animals were injected with MTB; after refinement and clinical trials, it became widely utilized as a human vaccine bearing their initials, BCG with its greatest efficacy in preventing children under 5 years of age from extra-pulmonary TB (Bloom and Murray, 1992). Earlier

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on ineffective treatments were rapidly abandoned once effective chemotherapy was introduced while sanatorium became obsolete. In 1946, an aminoglycoside antibiotic, streptomycin (SM), which inhibits protein synthesis, was shown effective in reducing early mortality in clinical trials (Musser, 1995). Combination of SM with p-aminosalicylic acid (PAS), a folic acid inhibitor, reduced the development of resistance (Mitchison and Davies, 2012). Isoniazid (INH), a bactericidal agent against replicating MTB and bacteriostatic against latent MTB, was introduced in 1952 allowing for effective, 3 drug combination regimens (SM, PAS, INH) which was widely adopted in Europe. In 1968, Rifampin (RIF), known as Rifampicin in Europe, was introduced clinically. It was shown that pyrazinamide plus rifampicin added to a six month regimen of INH was effective and became the standard in the 1970s (Saltini, 2006).

TB was on the decline until the late 1980s and 1990s due to lack of political commitment, neglect by control programs and when the human immunodeficiency virus (HIV) epidemic hit, causing a resurgence in TB due to the synergy between HIV and TB infection (CDC, 2012). In 1993, World Health Organization (WHO) declared TB epidemic a global emergency. Furthermore, the long - term neglect of basic TB research and product development has led to reliance on the 100 – year - old diagnostic tool for active TB infection (i.e. sputum smear microscopy), which is of poor sensitivity; an 80-year old and largely ineffective vaccine, BCG and few decades old drugs [SM, RIF, INH, ethambutol (ETH), pyrazinamide (PZA)] that have remained unchanged (Young *et al.*, 2008). To control the upsurge of TB and drug resistance TB, the WHO introduced the directly observed therapy, short-course (DOTS) strategy which allows patient to take his daily drug under supervision of a health worker or community treatment support. Underlying this strategy is renewed political commitment, effective case detection supported by the laboratory, standardized treatment, effective drug supply, and standard monitoring and evaluation system. Multidrug

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resistant TB (MDR-TB), defined as MTB resistant to INH/RIF, became a challenge in the early 1990s, especially in the HIV positive population (Pozniak, 2001; CDC, 1993; CDC, 1992). The emergence of extremely drug resistant TB (XDR-TB) (defined as MTB resistant to INH and RIF, the fluoroquinolones, and at least one of the injectables: amikacin, capreomycin, or kanamycin) in March 2005, pose greater treatment challenges (Jassal and Bishai, 2009) and threaten to make TB a curable disease incurable (Raviglione & Smith, 2007). Moreover anti-TB drug pipeline has been thin since the elucidation of RIF. It is only recent that two new anti-TB drugs which have been shown to be effective against MDR-TB is the newly approved antituberculous medication bedaquiline, a diarylquinoline (Voelker, 2013).

Thus, in the face of modern epidemics of HIV/AIDS, diabetes, XDR, MDR, and tools which are still in use today contributes to susceptibility to TB. The global TB control by Stop TB Partnership 2006, have outlined plans to halve TB prevalence and mortality by 2015 and eliminate the disease as a public health problem by 2050 (WHO, 2006). This vision hopes to depend on the development of improved diagnostics, simpler treatment, and more effective vaccination, which will in turn require enhancement in the knowledge of the disease and the biology of the pathogen (Young *et al.*, 2008).

2.2 Global Burden of TB

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TB remains the leading cause of adult death by a single infectious disease world-wide (WHO, 2014). One-third of the world's human population is latently infected with MTB with only 5% to 10% of infected immunocompetent individuals' progress from initial infection to active disease (WHO, 2014). In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease; 1.1 million cases and 360 000 of the deaths were HIV-positive respectively (WHO, 2014). Close to two-thirds of the cases and deaths occurred among men and an estimated 510 000 women died as a result of TB, more than one third of whom were

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HIV-positive. There were 80 000 deaths from TB among HIV-negative children in the same year. The number of people dying from HIV-associated TB has been falling for almost a decade. Of the estimated 9 million people who developed TB in 2013, more than half (56%) were in the South-East Asia and Western Pacific Regions. Further close to 30% was in the African Region, which also had the highest rates of cases and deaths relative to population. India and China alone accounted for 24% and 11% of total cases, respectively. The African region accounts for about four out of every five HIV-positive TB cases and TB deaths among people who were HIV-positive (WHO, 2014) as shown in Figure 2.1.

Globally, the TB mortality rate fell by an estimated 45% between 1990 and 2013 and the TB prevalence rate fell by 41% during the same period. Worldwide, TB incidence fell at an average rate of about 1.5% per year between 2000 and 2013. The 2015 Millennium Development Goal (MDG) of halting and reversing TB incidence has been achieved globally, in all six WHO regions and in most of the 22 high TB burden countries. Progress needs to accelerate to reach the Stop TB Partnership targets of a 50% reduction by 2015 (WHO, 2014).

2.3 TB in Ghana

Although Ghana is not among the WHO twenty-two high-burden countries for TB, the disease is a major health problem. A 1997 estimated TB burden by the WHO showed a TB prevalence of 260 per every 100,000 persons and a TB incidence of 165 per 100,000 persons. According to WHO in 2006, the TB incidence rate in Ghana was 90 cases per 100,000 populations. In 2008, 14,022 new cases of all forms of TB were officially recorded in Ghana (USAID Ghana, 2008). Approximately 12 percent of adult TB cases were HIV positive. Impact modeling showed that HIV infection among TB patients increased to 59 percent by 2009, and by 2015 an additional 30,000 new TB cases could be attributable to HIV/AIDS

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annually (WHO, 2010). According to the WHO in 2011 there were approximately 20,000 new TB cases diagnosed in the country but only 78 per cent were treated. In 2013, WHO then estimated a drop in the cases of TB in Ghana at 72 per every 100,000 persons, but a prevalence survey conducted in 2014 has revealed that TB burden in Ghana is three times higher than the WHO estimate (WHO, 2014). Prior to the survey, WHO estimates showed that TB cases in Ghana were below 92 per every 100,000 people but the survey across the country showed that there were 286 cases per every 100,000 people in Ghana. The TB mortality rate in Ghana is considered high at 7.5 per every 1,000 infected and the prevalence is 264 per 100,000 in general population (WHO, 2014).

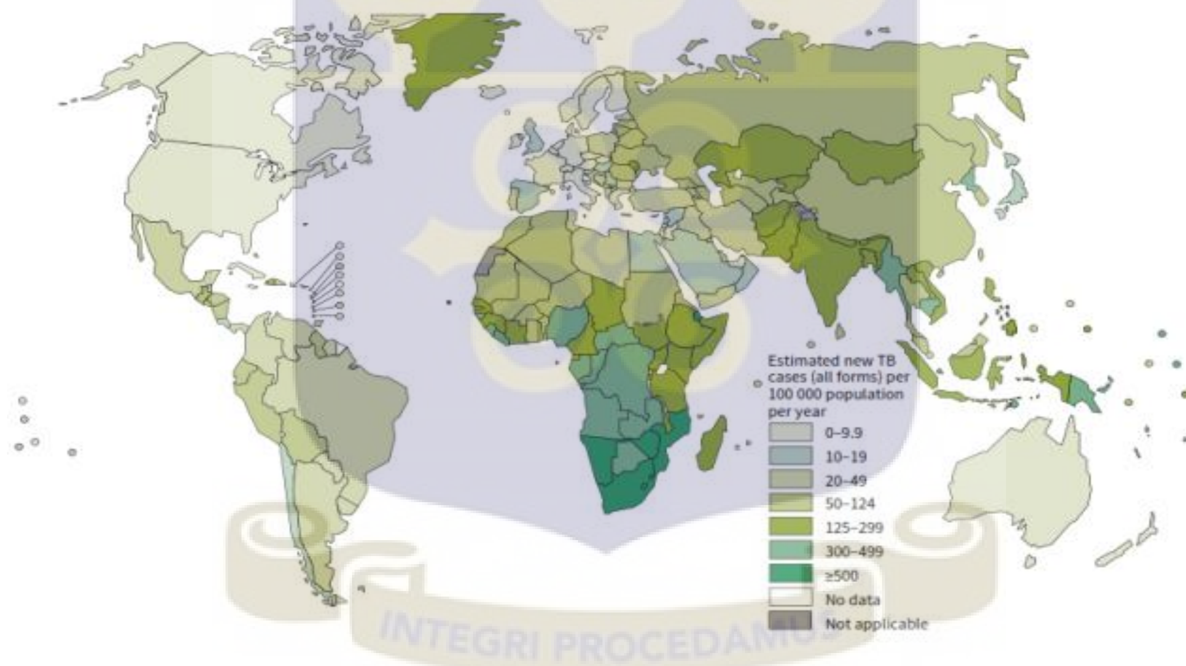

Figure 2.1 Global trend in estimated rates of TB incidences, 2013. Source: (WHO, 2014)

2.4 The Causative Agent of TB

The bacillus causing TB in humans belongs to the genus *Mycobacterium* that comprises mostly soil dwelling saprophytes and cause diseases of diverse nature and varying severity (Cosma *et al.,* 2003). The species that causes TB belongs to the *Mycobacterium tuberculosis* complex (MTBC). The MTBCs have a high GC content genome and are phylogenetically closely related (Comas *et al.,* 2013). They share about 99% similarity at the nucleotide level

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and identical in 16S ribosomal ribonucleic acid (rRNA) sequences (Böddinghaus *et al.*, 1990; Sreevatsan *et al.*, 1997).However they differ widely in terms of their host tropisms, phenotypes and pathogenicity (Wayne & Kubica, 1986). The classical species of MTBC include, *M. tuberculosis* sensu stricto (MTBss) (Brosch *et al.,* 2002), *M. africanum* (MAF) (Castets *et al.*, 1968) (causative agents of TB in humans), *M. bovis* (pathogen of cattle along with the use of vaccine strain *M. bovis* BCG) (Garnier *et al.,* 2003; Bloom and Murray, 1992), *M. microti* (pathogen of voles) (Frota *et al.*, 2004). There were newly recognized additions which include *M. caprae* (pathogen of goats) (Aranaz *et al.*, 2003) and *M. pinnipedii* (pathogen of seals and sea lions) (Cousins *et al.*, 2003). *M. bovis* was once a common cause of tuberculosis, but the introduction of pasteurized milk has largely eliminated this as a public health problem in developed countries (Garnier *et al.,* 2003; Bloom and Murray, 1992). *M. canetti* an outlier of the complex is rare and causes a limited number of cases in the horn of Africa, although a few cases have been seen in African emigrants (van Soolingen *et al.*, 1997). *M. microti* is mostly seen in immunodeficient people, although it is possible that the prevalence of this pathogen has been underestimated (Frota *et al.*, 2004).

These species are unique in that they possess a high thick fatty cell wall layer rich in mycolic acids outside the normal peptidoglycan layer and accounts for most of their unique clinical characteristics (Southwick, 2007). They are referred as slow growers with a generation time of 18–24hrs under optimal availability of oxygen and nutrients at 37 °C. They form a white to light-yellow colony on agar within 3–4 weeks (Cox, 2004). Unlike many other bacteria, the MTBCs do not form spores but has the capacity to become dormant–a non-replicating state characterized by low metabolic activity and phenotypic drug resistance. Due to their high impermeable cell wall the MTBC are relatively resistant to acids and bases (acid-fast bacilli), which forms the basis of the staining protocol. They are typically visualized by Ziehl–

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Neelsen staining and appear as a rod-shaped red bacillus (Madison, 2001; Kumar *et al.,* 2007).

The main virulence factors of the pathogenic bacteria, including the MTBCs are protein secretion systems of five extended ESX systems (ESX1-5) of which ESX1 consists of early secreted antigenic target-6 kiloDalton (kDa) (ESAT-6) and culture filtrate protein-10 kDa (CFP-10) secretion systems (Abdallah *et al.,* 2007). The Rv3874 and Rv3785 genes which encode ESAT-6 and CFP-10, two small highly immunogenic proteins, are noted to be co-transcribed in *M. tuberculosis* (Berthet *et al*., 1998); when co-expressed in *E. coli*, they form a tight 1:1 complex (Renshaw *et al*., 2002) (Figure 2.2).

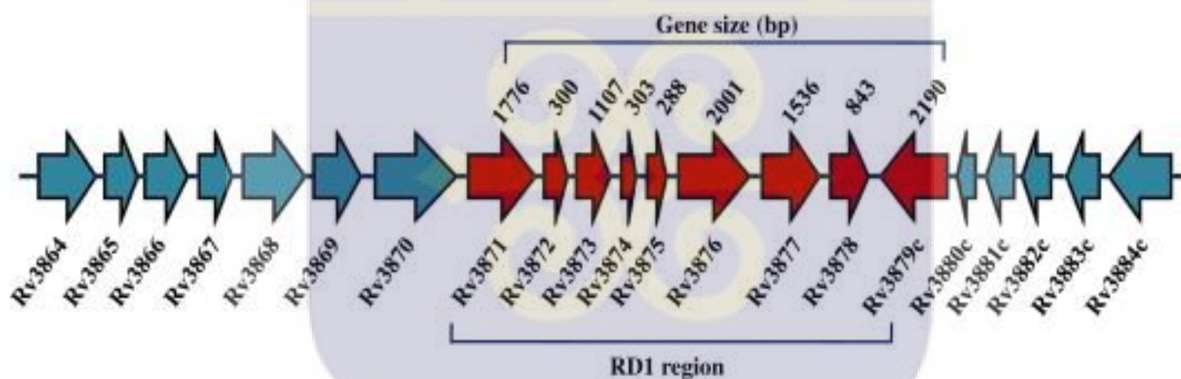

Figure 2.2 Diagram of the region of difference 1 (RD1) region of *Mycobacterium tuberculosis* along with flanking genes. Arrows show the transcriptional direction of genes. Rv3874 and Rv3875 gene loci encode CFP-10 and ESAT-6, respectively. Source: (Rai *et al.,* 2012)

These antigens are extracellular secretory proteins with low molecular weight, encoded by the region of difference 1 (RD1) genomic region of virulent strains of MTB, MAF and *M. bovis* (Mahairas *et al.,* 1996; Berthet *et al.,* 1998; Behr *et al.,* 1999; Brosch *et al.,* 2002). They are however absent from *M. bovis* BCG sub-strains and most environmental mycobacteria. RD1 secretion systems are associated with virulence of the MTBC (Brodin *et al.,* 2006; Guinn *et al.,* 2004) and are effective stimulators of T cells (Horwitz *et al.,* 1995; Kamath *et al.,* 1999; Brandt *et al.,* 2000). The secretion systems play diverse roles in the pathogenesis of

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TB, from virulence to immune-modulation (Ganguly *et al.,* 2008) and include the formation of granulomas (Volkman *et al.,* 2010).Others are inhibition of phagosomal maturation (Clemens & Horwitz, 1995) and trans-migration of bacteria from the phagosome to the cytosol (van derWel *et al.,* 2007). It also down regulates the functions of dendritic cells, macrophages and T cells by inhibiting secretion of cytokines that are important for immune cell activation. They are used in interferon gamma releasing assays (IGRAs) (Pai *et al.,* 2004) which have been widely utilized for the diagnosis of TB because they are more specific than conventional tuberculin skin test (TST) (Pai *et al.,* 2004). IGRAs have advantages over the TST, in terms of higher specificity, better correlation with exposure to MTB, and less cross-reactivity due to BCG vaccination and non-tuberculosis mycobacterial infection. However, IGRAs use RD1 antigens in isolation and may maximize specificity at the cost of sensitivity (Pai *et al.,* 2004). The rESAT-6/CFP-10 fusion protein, a dominant interferon gamma (IFN-γ) inducing antigen, of live and actively metabolizing MTB.

2.5 Phylogenetic Lineages of the Human Adapted *Mycobacterium tuberculosis* complex

Based on comparative whole genome sequence (WGS) analyses, the human adapted MTBC have been divided into 7 phylogenetic lineages (5 from MTBss and 2 from MAF) (Comas *et al.,* 2013). Many studies have shown that the human-adapted MTBC lineages show a strong phylogeographical population structure; meaning distinct lineages are associated with specific geographical regions (Hirsh *et al.,* 2004; Gagneux *et al.,* 2006; Filliol *et al.,* 2006; Baker *et al.,* 2004; Hershberg *et al.,* 2008; Wirth *et al.,* 2008; Reed *et al.,* 2009) (Figure 2.3A). Specifically, the most widely distributed groups are L2 (also known as East-Asian lineage, includes the Beijing family of strains) predominates in East Asia, but is also present in Central Asia, Russia and South-Africa) and L4 (also known as the Euro-American lineage which occurs frequently in populations from Asia, Europe, Africa and America) (Figure

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2.3B). Lineages 1 and 3 show a more restricted geographical distribution limited to East Africa, Central-, South- and South-East Asia (Figure 2.3C). The most geographically restricted lineages are L5–7, which are all associated with specific regions of Africa. L5 and L6 also known as *M. africanum* West Africa 1 and West Africa 2, respectively, and almost exclusively occur in West Africa (de Jong *et al.,* 2010), while L7 is confined to Ethiopia (Firdessa *et al.,* 2013) (Figure 2.3D). Thus, Lineages 1 - 4 together with Lineage 7 are collectively known as MTBss.

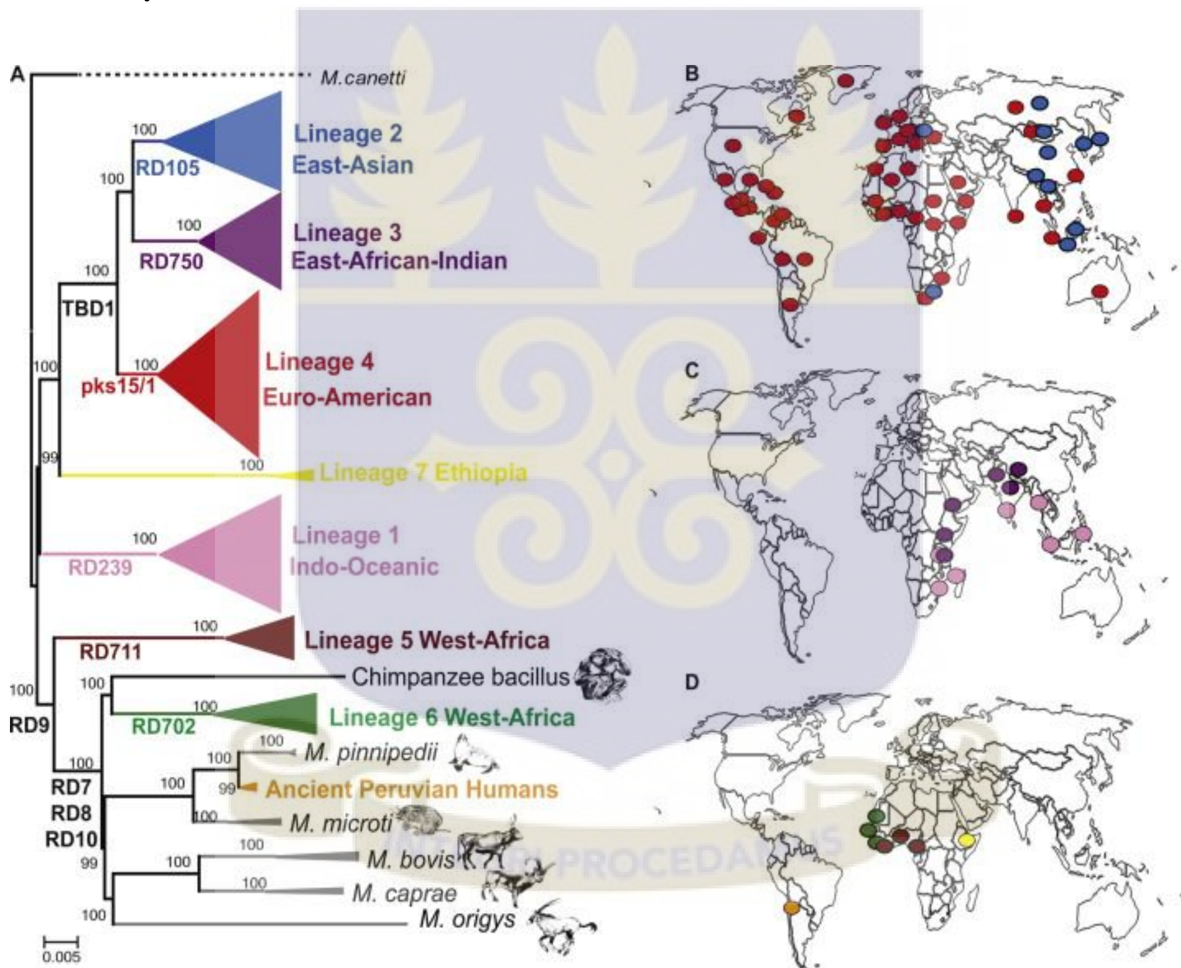

Figure 2.3 Human-adapted MTBC lineages showing phylogeographical population structure, with the different lineages associated with distinct geographical regions. Source: (Coscolla & Gagneux, 2014)

One of the first evolutionary reconstructions of the genetic population structure of the whole MTBC highlighted a group of strains harbouring a deletion in the genomic region known as *M. tuberculosis* specific deletion 1 gene (TbD1) (Brosch *et al.,* 2002). TbD1-deleted strains

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have been referred to as evolutionarily "modern" compared to the strains without this deletion, which collectively have been referred to as evolutionarily "ancestral" or "ancient". Consequently the evolutionarily "modern" strains consist of L1 – L4 and L7 collectively known as MTBss while the evolutionarily "ancestral" or "ancient" strains exist as *M. africanum* West Africa 1 and West Africa 2, respectively.

2.5.1 *Mycobacterium africanum*

M. africanum (MAF) was first described as a distinct sub-species within the MTBC by Castets and colleagues in 1968 in Senegal (Castets *et al.*, 1968). It has traditionally been identified by phenotypic criteria, occupying an intermediate position between MTBss and *M. bovis* based on biochemical characteristics (David *et al.*, 1978). Typically, MAF was subdivided by geographic origin and biochemical properties into two major subgroups: MAF subtype 1 originating from West Africa, exhibiting *M. bovis*-like properties, while MAF subtype 2, from East Africa, exhibits MTBss-like properties (David *et al.*, 1978; Niemann *et al.,* 2002). Recent advances in molecular strain-typing techniques and comparative WGS have reclassified MAF type II (East African clade) as MTBss (Sola *et al.,* 2003) genotype ''Uganda'', a sub-lineage of MTBss (L4) due to the presence of TB specific deletion and other single nucleotide polymorphisms. Thus the true MAF is MAF subtype 1 (West African clade) which occurs exclusively in West-Africa, has been sub-divided into 2 separate lineages which are genetically diverse. These are, MAF subtype 1, West African type 1 (MAF1) or L5, characterized by RD711, prevalent around the Eastern part of West Africa (Gulf of Guinea) (Gagneux *et al.*, 2006) and MAF subtype 1, West African type 2 (MAF2) or L6 characterized by RD702, (Gagneux *et al.*, 2006; Mostowy *et al.,* 2004) prevalent in Western part of West Africa.

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2.5.2 Prevalence and Distribution of *Mycobacterium africanum*

MAF is the cause of up to 40% of all TB cases in West Africa (de Jong *et al.*, 2010) though actual prevalence varies from country to country. The distributions of MAF1 and MAF2 overlap in Central West Africa, particularly in Ghana (Goyal *et al.*, 1999), Benin (Affolabi *et al.*, 2009), Sierra Leone and Ivory Coast (de Jong *et al.*, 2010). Based on the genetic variability of MAF in Ghana, the strain-specific diversity of MAF1 and MAF2 may translate into phenotypic differences. Likewise this unique TB epidemiology in Ghana may have phenotypic variations in the host-pathogen relationship in *M. africanum.* MAF1 prevalence (based on phenotypic identification) in Cameroon was estimated to be 56% in 1971 (Huet *et al.*, 1971) but a decrease to 9% was observed in 1997–1998 (Niobe-Eyangoh *et al.*, 2003). A current study from 2013 suggested that MAF1 has almost disappeared from the Cameroon (Koro Koro *et al.*, 2013). In contrast, two recent studies covering several regions of neighboring Nigeria identified a high MAF1 prevalence, ranging from 14% to 33% (Lawson *et al.*, 2012; Thumamo *et al.*, 2012), and detected active foci of recent MAF1 transmission in 2009–2010 (Lawson *et al.*, 2012). In like manner a prevalence of MAF1 recorded in Benin is around 28% (Affolabi *et al.*, 2009) and in Ghana a recent report showed that human TB is caused by six out of the seven MTBC lineages, with 20% of all cases attributed to MAF1 and MAF2 (Yeboah-Manu *et al.*, 2011). However, relatively little is known about MAF in Ghana in terms of virulence and immunogenicity.

MAF has also been sporadically identified in other regions of the world such as England (Grange & Yates 1989), California (Desmond *et al.*, 2004), France (Frottier *et al.*, 1990), Spain (Perez-de Pedro *et al.*, 2008) and Germany (Jungbluth *et al.*, 1978; Schroder, 1982). These were from pulmonary and extra-pulmonary sources. However, in most cases, TB patients carrying MAF were immigrants from West Africa.

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Most of the studies on MAF have been conducted in the Gambia, where MAF2 is prevalent. Findings from these studies suggest that MAF is less virulent compared to MTBss. MAF2 infected patients were more likely to be older and HIV co-infected (de Jong *et al.*, 2005); less immunogenic phenotype (de Jong *et al.*, 2006); severely malnourished (de Jong *et al.*, 2010); and associated with slower progression to active disease (de Jong *et al.*, 2008).

2.6 Pathogenesis of *M. tuberculosis* Infections

2.6.1 Transmission of *M. tuberculosis*

TB is transmitted when an individual with active pulmonary TB expel infectious aerosol droplets 0.5 to 5 μm in diameter through coughing, singing, talking and other forced respiratory maneuvers and inhaled by an individual. The risk of infection is dependent on several factors such as the infectiousness of the source case, the closeness of contact, the bacillary load inhaled, and the immune status of the potential host (Frieden *et al.,* 2003; Mathema *et al.,* 2008). Inhaled droplet are deposited in the alveolar spaces of the lungs, where the bacteria are engulfed by phagocytic immune cells, mainly alveolar macrophages and dendritic cells (Teitelbaum *et al.,* 1999), an event which induces a rapid inflammatory response and accumulation of cells.

2.6.2 Disease Progression of *M. tuberculosis*

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Contact with the TB bacilli may lead to several outcomes. In some individuals, the host innate immune system eliminates the bacilli with no memory of exposure. However in most individuals infection is established needing the host adaptive mechanism, championed by the Th1 arm for host defense. In about 90% of infected individuals between 3 to 8 weeks after MTB contained in inhaled aerosols becomes implanted in alveoli the host immune system comprising both the innate and adaptive arm will wall off the site if infection in a granuloma (ghon complex), such individuals are asymptomatic and have latent TB infection (LTBI).

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LTBI is tested by delayed hypersensitive reaction through assays such as tuberculin skin test. Among the remaining 10% individuals, 5% will develop active TB disease within 2-3 years while the remaining 5% in their lifetime. However these proportions can be different in individuals with immune suppression conditions/disease as occur in HIV positive individuals. Disease can be confined to the lungs, which is called pulmonary TB, while there can be spread to other body parts outside the pulmonary to form extra-pulmonary or disseminated disease (Smith, 2003). Symptoms of TB include fatigue, wasting, night sweats, chronic cough with blood and chest pains.

The extra pulmonary forms are:

The first lasting about 3 months, is marked by hematogenous circulation of bacteria to many organs including other parts of the lung; at this time in some individuals, acute and sometimes fatal disease can occur in the form of tuberculosis meningitis or miliary (disseminated) tuberculosis.

The second form, pleurisy or inflammation of the pleural surfaces occur lasting 3 to 7 months and causing severe chest pain, but this stage can be delayed for up to 2 years. It is thought that this condition is caused by either hematogenous dissemination or the release of bacteria into the pleural space from sub-pleural concentrations of bacteria in the lung. The free bacteria or their components are thought to interact with sensitized CD4 T lymphocytes that are attracted and then proliferate and release inflammatory cytokines (Kamholz, 1996).

The last stage or resolution of the primary complex, may take up to 3 years, where the disease does not progress. In this stage, more slowly developing extra-pulmonary lesions, e.g. those in bones and joints, frequently presenting as chronic back pain, can appear in some individuals.

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2.7 Host Immune Response

2.7.1 Phagocytosis

Alveolar resident macrophages are the primary cell of the host innate defense system involved in the initial uptake of *M tuberculosis*, an intracellular pathogen (Houben *et al.,* 2006). Infection with *M. tuberculosis* starts with phagocytosis of the bacilli by phagocytic antigen-presenting cells in the lung including alveolar macrophages and dendritic cells (Henderson *et al.,* 1997). The mycobacteria are phagocytosed in a process that is initiated by bacterial contact with macrophage mannose receptors (MRs) and/or complement receptors (CRs) (Schlesinger, 1993). Mycobacteria invade the host macrophages after opsonization with complement factor C3, which is followed by binding and uptake through CR1, CR3, and CR4 (Aderem and Underhill, 1999; Hirsch *et al.,* 1994; Schlesinger, 1993). The relative importance of the various receptors for complement factor C3 is apparent from experiments in vitro, in which in the absence of CR3, phagocytosis of MTB by human macrophages and monocytes is reduced by approximately 70 to 80% (Schlesinger, 1993; Schlesinger *et al.,* 1990). For opsonization with C3, the split product C3b should first be generated by activation of the complement system. M. tuberculosis also utilizes part of the classical pathway of complement activation by direct binding to C2a, even in the absence of C4b; in this way the C3b necessary for binding to CR1 is formed (Schorey *et al.,* 1997). This mechanism facilitates mycobacterial uptake in environments low in opsonins, such as the lung. Nevertheless, nonopsonized M. tuberculosis can bind directly to CR3 (Cywes *et al.,* 1997) and CR4 (Zaffran & Ellner, 1997).

However, the best-characterized receptor for non-opsonin-mediated phagocytosis of *M. tuberculosis* is the MR, which recognizes terminal mannose residues on mycobacterium (Schlesinger, 1993; Schlesinger *et al.,* 1996). When uptake by CRs and MR is blocked,

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macrophages may also internalize MTB through the type A scavenger receptor (Zimmerli *et al.,* 1996). Fcγ receptors, which facilitate phagocytosis of particles coated with antibodies of the immunoglobulin G class, seem to play little role in tuberculosis (Armstrong & Hart, 1975).

Enhanced binding of MTB to alveolar macrophages may represent a risk factor for developing clinical tuberculosis. Collectins, a structurally related group of proteins that includes surfactant proteins, mannose-binding lectins (MBLs), and C1q, seem to be important in this respect. Surfactant protein A (Sp-A) facilitates the uptake of MTB (Pasula *et al.,* 1999), through binding to either the macrophages (Gaynor *et al.,* 1995) or neutrophils (Ernst, 1998). Interestingly, it has been reported that human immunodeficiency virus-infected individuals have increased levels of Sp-A in the lungs, and this results in a threefold-greater attachment of *M. tuberculosis* to alveolar macrophages (Downing *et al.,* 1995). In contrast, another surfactant protein, Sp-D, has been found to block the uptake of pathogenic strains of *M. tuberculosis* in macrophages (Ferguson *et al.,* 1999).

Thus, there are multiple mechanisms for the uptake of MTB, involving a number of different host cell receptors. Most of these interactions have been demonstrated *in vitro*, and their relative importance in vivo remains to be shown. Distinct routes of entry of MTB may lead to differences in signal transduction, immune activation, and intracellular survival of M. tuberculosis. For example, Fcγ-receptor-mediated phagocytosis is directly linked to an inflammatory response, and binding to CR is not (Aderem and Underhill, 1999). Survival of MTB after binding to CR1 is better than that after binding to CR3 or CR4 (da Silva *et al.,* 1998). Likewise, virulent strains of *M. tuberculosis* H37Rv are phagocytosed through MR, while attenuated strains H37Ra are not (Schlesinger, 1993), suggesting that this route of entrance is advantageous to the mycobacterium.

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The recognition of pathogen-associated molecular patterns (PAMP) by specific pathogen recognition receptors (PRRs) is central to the initiation and coordination of the host innate immune response (Akira *et al.,* 2006). The MTB or MTB components are recognized by host receptors that include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-(NOD-) like receptors (NLRs), and C-type lectins (Jo, 2008; Harding & Boom, 2010). Some of these receptors (such as TLRs) are expressed on both, immune cells (such as macrophages, dendritic cells, B cells, and specific types of T cells) and nonimmune cells (like fibroblasts and epithelial cells). TLRs are phylogenetically conserved mediators of innate immunity which are essential for microbial recognition on macrophages and dendritic cells (Belvin & Anderson, 1996; Medzhitov *et al.,* 1997; Visintin *et al.,* 2001). Members of the TLR family are transmembrane proteins containing repeated leucine-rich motifs in their extracellular domains, similar to other pattern-recognizing proteins of the innate immune system. To date, at least 10 TLRs have been identified; of those TLR2, TLR4, and TLR9 seem responsible for the cellular responses to peptidoglycan and bacterial lipopeptides (Yoshimura *et al.,* 1999), endotoxin of gram-negative bacteria (Schlesinger *et al.,* 1990), and bacterial DNA (Hemmi *et al.,* 2000), respectively TLRs are also involved in cellular recognition of mycobacteria. Apparently, TLRs play an important role in innate recognition of mycobacteria, and this also holds for humans (Figure 2.4).

As this pathogen has co-evolved with humans, it has attained several immune-evasion tactics to subvert the host immune response that targets its destruction (Brites & Gagneux, 2012). The pathogen protects itself from destruction by macrophages through various mechanisms such as prevention of phagosome maturation, inhibition of fusion of the phagosome with lysosomes, and interference with the antigen processing and presentation machinery (Schluger & Rom, 1998). The cell wall of MTB, which is abundant in mycolic acids, assists the organism in remaining dormant for many years (Peyron *et al.,* 2008). MTB also secretes

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many proteins that play critical roles in modulating host immune responses that favour its survival inside host macrophages (Chatterjee *et al.,* 2011; Wang *et al.,* 2009). Thus, MTB has evolved multiple mechanisms to promote its survival inside the host. During the early stages of infection, the surface-exposed and secretory proteins of MTB are the primary targets of the host immune response. Studies on ESAT-6, a secretory mycobacterial protein, have suggested that its incorporation in the vaccine strain BCG or as part of a subunit vaccine can improve vaccination strategies against TB. The immunological role of ESAT-6 was demonstrated by its capacity to induce IFN-γ production in T cells isolated from immune mice challenged with MTB (Sorensen *et al.,* 1995).

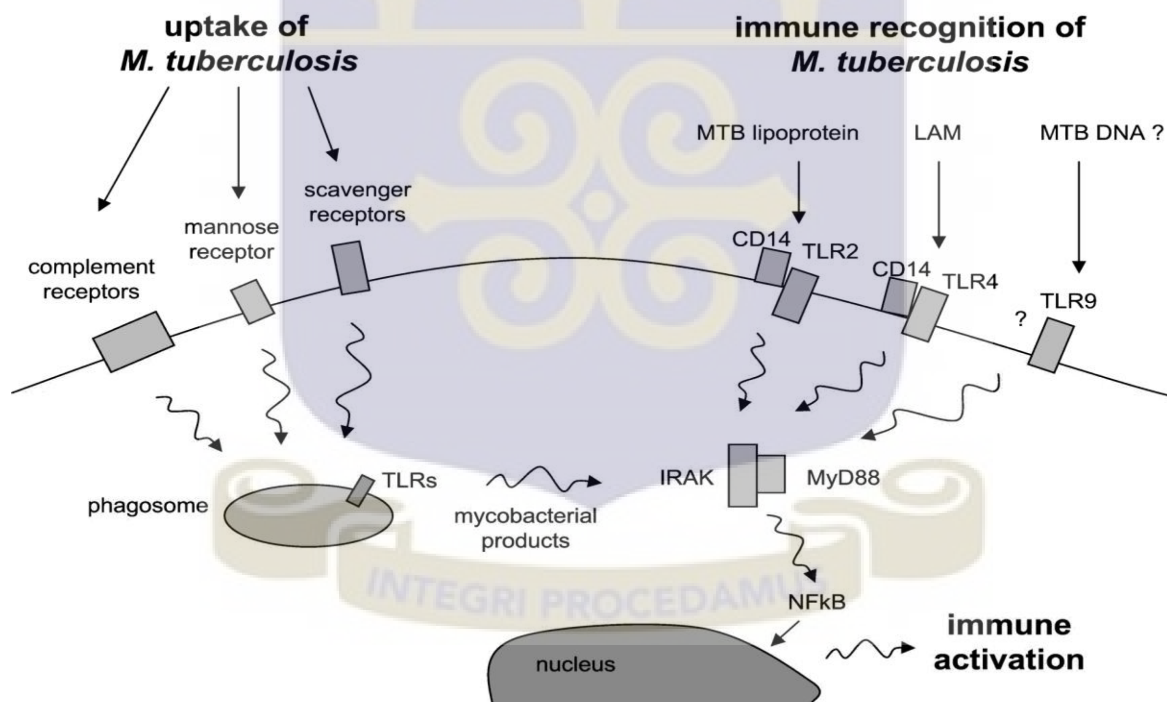

Figure 2.4 Phagocytosis/uptake and immune recognition of *M. tuberculosis*. Source: (van Crevel *et al.,* 2002)

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2.8 Innate Immune Response of the Host to *M. tuberculosis*

Innate immune mechanisms are essential to limit growth of mycobacteria in the initial phase of the infection. The interplay between the host innate immune response and the bacterial mechanisms in play to offset this response is of considerable importance indicating the course of the disease. In order to gain an understanding of this interplay it is of importance to analyze how MTB interacts with innate immune receptors and makes its entry into macrophages, how it subverts the bactericidal effects of macrophages, and dampens processes required for protective immunity, including cytokine and chemokine induction (Figure 2.5).

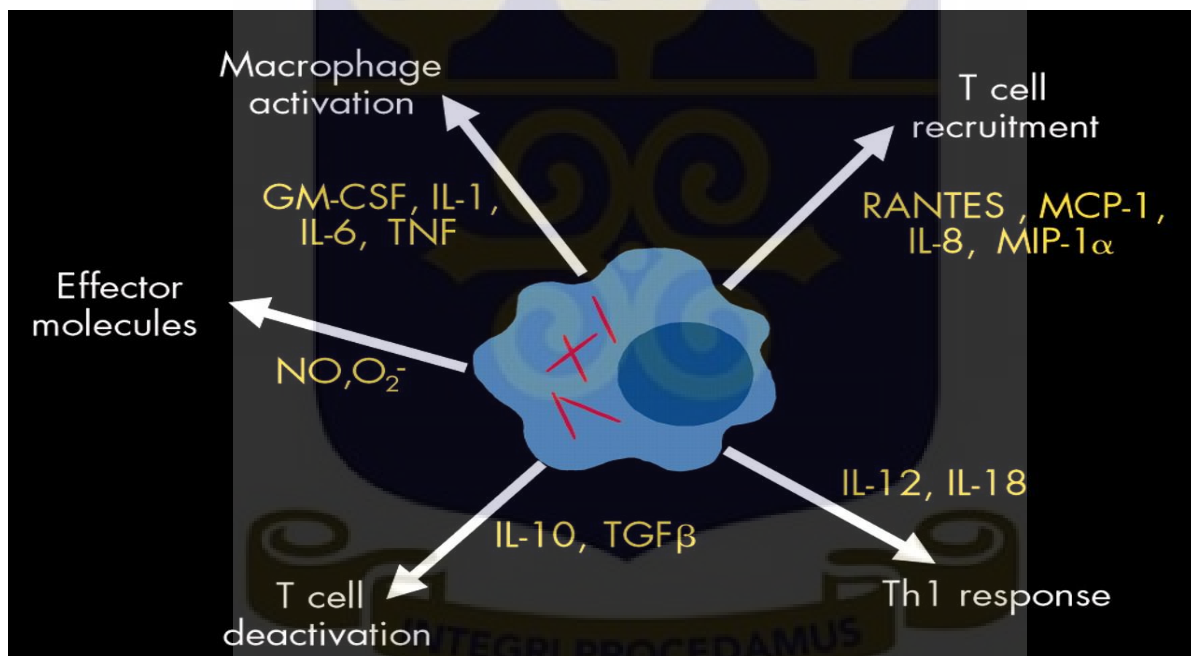

Figure 2.5 Innate immunity in Tuberculosis. Source: (Stenger, 2005) [GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RANTES, Regulated on Activation, Normal T Expressed and Secreted; TGF, transforming growth factor; Th, T helper; TNF, tumour necrosis factor]

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2.8.1 Macrophages

Macrophages are an important component in the first line of defense of the innate immune system and play a unique role in host response to mycobacterial infections. These cells represent both the primary effector cell for killing and the primary site of intracellular replication of MTB (van Crevel *et al.,* 2002).

In humans, large numbers of alveolar macrophages are not readily available for *in vitro* study, therefore matured human monocyte derived macrophage (MDM) derived from peripheral blood mononuclear cells (PBMCs) are used as an *in vitro* model to analyze the virulence of MTB infection in human cells (Gordon & Taylor, 2005). The macrophage model has the advantage of allowing us to study the interaction of MTB within the host in the early phase of human infection (van Crevel *et al.,* 2002). *In vitro* assays of virulence that reflect the pathogenesis of tuberculosis in humans are required to understand disease dynamics. Intracellular mycobacterial growth (Silver *et al.,* 1998), cytokine expression (Falcone *et al.,* 1994), or combinations of the two parameters (Manca *et al.,* 1999) have been used as a measure of virulence in isolated macrophages. However, there is evidence of variation in phagocytosis by different strains (Torrelles *et al.,* 2008). Numerous receptors expressed by phagocytic cells, namely the C-type lectin receptors (CLRs), scavenger receptors (SRs), and CRs, bind mycobacteria (Philips & Ernst, 2012). Many ligands present within the mycobacterial outer surface tend to engage host receptors and activate multiple pathways of recognition and signaling leading to the induction of innate immune response (Mourao-Sa *et al.,* 2013). Schlesinger L (1993) found that phagocytosis of two virulent strains (H37Rv and Erdman) by human MDM is mediated by the mannose receptor in addition to receptor (CR1, CR3 and CR4) whereas the avirulent strain H37Ra uses only complement receptor for phagocytosis.

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Assessments of intracellular growth of MTB in human macrophages as a marker of virulence have been reported in previous studies (Li *et al.,* 2002; Zhang *et al.,* 1999; Hoal-van Helden *et al.,* 2001; Wong *et al.,* 2007; Sarkar *et al.,* 2012). Zhang *et al.,* 1999 utilized the monocyte derived macrophage model to study the correlation between the extent of the spread of MTB strains in a Los Angeles community setting and the ability of the strains to grow in human macrophages. It has also been suggested that the intracellular growth of clinical isolates of *M. tuberculosis* in host macrophages is associated with their virulence, which is defined as the capacity for causing tuberculosis (Theus *et al.,* 2005; Zhang *et al.,* 1999). Previous studies demonstrated that virulent MTB strains grow more rapidly than avirulent or attenuated strains within human phagocytes (Silver *et al.,* 1998; Zhang *et al.,* 1998). Individual strains from lineage 2 (Beijing) have been shown to grow more rapidly than comparator strains in *in vitro* human cell culture models using MDM or monocyte or human macrophage cell lines (Li *et al.,* 2002; Zhang *et al.,* 1999; Theus *et al.,* 2005). Multiple isolates of *M. tuberculosis* strain 210 (a Beijing-family strain [lineage 2] responsible for an outbreak in Los Angeles), grew more rapidly than small cluster or unique cluster strains in human MDM (Theus *et al.,* 2005).

2.8.2 Pro-inflammatory Mediators of the Host due to *M. tuberculosis* Infection

The central role in the inflammatory response and the outcome of mycobacterial infections involves the cytokine network. The cytokine network plays a central role in the inflammatory response and the outcome of mycobacterial infections (van Crevel *et al.,* 2002). Early pro-inflammatory cytokine secretion is a hallmark of MTB infection (van Crevel *et al.,* 2002).

2.8.2.1 Tumour necrosis factor alpha (TNF-α)

Tumour necrosis factor alpha (TNF-α) is critical for sustained protective immunity against *Mycobacterium tuberculosis* infection. Stimulation of monocytes, macrophages (Valone *et al.,* 1988), and dendritic cells (Henderson *et al.,* 1997) with mycobacteria or mycobacterial

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products induces the production of TNF-α, a prototype pro-inflammatory cytokine. It plays a crucial role in protective immunity and pathophysiology against tuberculosis (Jo *et al.,* 2003); synergizes with IFN-γ to increase the production of nitric oxide metabolites which facilitate mycobacterial killing. TNF-α, plays a key role in granuloma formation for the containment of mycobacterial infection (Kindler *et al.,* 1989; Senaldi *et al.,* 1996), induces macrophage activation, and has immune-regulatory properties (Orme & Cooper, 1999; Tsenova *et al.,* 1999). In mice, TNF-α, is also important for containment of latent infection in granuloma (Mohan *et al.,* 2001). In tuberculosis patients, TNF-α, production is present at the site of disease (Barnes *et al.,* 1993; Casarini *et al.,* 1999, Law *et al.,* 1996).

The expression level of TNF-α induced by MTB correlated with the level of bacterial growth (Theus *et al.,* 2005). Previous work has shown that low pro-inflammatory innate immune responses to MTBC infection were associated with a higher virulence in animal models (Reed *et al.,* 2004; Tsenova *et al.,* 2005). A rapid growth phenotype is associated with reduced TNF-α secretion, whereas robust TNF-α secretion inhibits mycobacterial replication (Theus *et al.,* 2005).

2.8.2.2 Interleukin 6 (IL-6)

IL-6 has both pro- and anti-inflammatory properties (VanHeyningen *et al.,* 1997). During mycobacterial infection, IL-6 is produced early at the site of infection (Law *et al.,* 1996). IL-6 may be harmful in mycobacterial infections, as it inhibits the production of TNF-α and IL-1β (Schindler *et al.,* 1990) and promotes *in vitro* growth of *M. avium* (Shiratsuchi *et al.,* 1991). Other studies support a protective role for IL-6: IL-6-deficient mice display increased susceptibility to infection with MTB (Ladel *et al.,* 1997), which seems related to a deficient production of IFN-γ early in the infection, before adaptive T-cell immunity has fully developed (Ladel *et al.,* 1997).

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2.8.2.3 Interleukin 12 (termed IL-12p70 and commonly designated IL-12)

Interleukin 12 (termed IL-12p70 and commonly designated IL-12) is an important immune-regulatory cytokine a product of phagocytic antigen-presenting cells and acts as a pro-inflammatory cytokine (Chehimi & Trinchieri, 1994; Beadling & Slifka, 2006). IL-12 is part of a family of heterodimeric cytokines that comprises: IL-12p70, which is a heterodimeric cytokine composed of the p35 and p40 subunits (p35/p40); IL-23, which shares the p40 chain with IL-12, but this subunit is associated with a new protein, termed p19 chain (p19/p40); IL-27, which is a heterodimeric cytokine composed of the Epstein–Barr virus (EBV)-induced molecule 3 that associates with the IL-27 p28 chain (EBI-3/p28) (Trinchieri, 2003; Kang & Kim, 2006) and IL-35, which is composed of p35 and EBI-3 (Collison *et al.,* 2007). Similar to IL-12, IL-23 and IL-27 are produced predominantly by macrophages and dendritic cells (Hunter, 2005). IL-12 cytokine family, which is evolutionarily linked to the IL-6 cytokine superfamily, is composed of IL-12, IL-23, IL-27, and the newly identified IL-35 (Hunter, 2005; Beadling & Slifka, 2006; Collison *et al.,* 2007) are new family members that play distinct cellular and functional roles in Th1 cell development (Beadling & Slifka, 2006; Brombacher *et al.,* 2003)

IL-12 bridges the early nonspecific innate response and the subsequent antigen-specific adaptive immunity (Chehimi and Trinchieri, 1994). The expression of IL-12 during infection regulates innate responses and determines the type of adaptive immune responses. IL-12 induces IFN-γ production and triggers CD4+ T cells to differentiate into type 1 T helper (Th1) cells (Trinchieri, 1995). Previous reports involving TB infection models showed that the development of Th1 cells in response to IL-12 production and subsequent induction of IFN-γ are key players in immunity to TB (Orme *et al.,* 1992). Exogenous IL-12 application was found to result in lower bacterial load and increased incidence of survival (Flynn *et al.,* 1995). Dendritic cells (DCs) and Th1 activation and migration to the infected lungs were

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shown to control bacterial growth via IFN-γ–induced activation of phagocytes (Cooper & Khader, 2008). More recently, data from Khader *et al.,* 2005 have indicated that IL-23 compensates for the absence of IL-12p70 and that this IL-12 cytokine family member is essential for the IL-17 response during tuberculosis (Khader *et al.,* 2005). This observation was supported by the fact that the continuous presence of IL-12 is needed for maintenance of pulmonary Th1 effector function in chronic tuberculosis (Feng *et al.,* 2005).

Modern lineages were shown to induce lower levels of pro-inflammatory cytokines when compared with ancient lineages (Portevin *et al.,* 2011). Another study has found that Beijing strains (L2), irrespective of subfamily, showed an immune phenotype of low levels of TNF-α, IL-6, IL-10 and GRO-α production (Wang *et al.,* 2010) as compared to H37Rv and other genotypes of MTB in human macrophages. The low inflammatory phenotype of modern strains is in agreement with previous studies of individual Beijing strains (Manca *et al*., 2004; Tanveer *et al*., 2009) and other strains (Newton *et al*., 2006) belonging to the modern lineages. Previous report suggests that a low inflammatory response may lead to a reduction in the adaptive response (Rakotosamimanana *et al*., 2010). By contrast, the low inflammatory response induced by evolutionary modern strains has been associated with an enhanced ability to cause early progressive disease (Manca *et al*., 2004; Newton *et al*., 2006).

2.9 Adaptive Immunity by Host to *M. tuberculosis* Infection

Reports in animal models and in humans have shown that a wide range of immune components are involved in an effective immune response against MTB. These include, macrophages and DCs, αβ-T cells (both CD4+ and CD8+), CD1 restricted T cells, γδ-T cells, and cytotoxic T cells, as well as the cytokines produced by these immune cells (Tufariello *et al*., 2003; Chan & Flynn, 2004; Beetz *et al*., 2008). The most important among these are CD4+ T cells and the cytokine IFN-γ. Although CD4+ T cells along withCD8+ T cells and the natural killer (NK) cells are the major producers of IFN-γ, studies carried out in CD4+

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deficient mice have shown that it is the early production of IFN-γ by CD4+ T cells and subsequent activation of macrophages that determine the outcome of infection (Caruso *et al*., 1999; Cooper, 2009). The CD4+ T cells also play other roles in the defense against infection that is independent of IFN-γ production. Depletion of CD4+ T cells was associated with the reactivation of infection in a chronically infected mouse and resulted in increasing pathological features and death. However IFN-γ levels would still be high due to a strong response from CD8+ T cells and normal levels of inducible nitric oxide synthase (iNOS) (Scanga *et al.,* 2000). Thus, IFN-γ from CD8+ T cells may not be as effective as that from CD4+ T cells.

2.9.1 CD4 T cells

The CD4+ T cells carry out several functions that are important to control infection in the granuloma. These include apoptosis of infected macrophages through Fas/Fas ligand interaction, production of other cytokines (such as IL-2 and TNF-α), induction of other immune cells (macrophages or dendritic cells) to produce other immune-regulatory cytokines such as IL-10, IL-12, and IL-15, and activation of macrophages through direct contact via CD40 ligand (Chan & Flynn, 2004; Cooper, 2009; Cella *et al.,* 1996; Oddo *et al.,* 1998). The CD4+ T cells also appear to be critical for the cytotoxic function of CD8+ T cells that is mediated by IL-15 (Cooper, 2009; Serbina *et al.,* 2001). It has also been shown thatCD4+ T cells can control the intracellular growth of MTB by a nitric oxide-dependent mechanism that is independent of IFN-γ production (Cooper, 2009; Cowley & Elkins, 2003). Thus, CD4+ T cells, in addition to early production of IFN-γ appear to have several other secondary functions that are critical in the control of *M. tuberculosis* infection

2.9.2 CD8 T cells

The CD8+ T-cells, in addition to producing IFN-γ and other cytokines, may also be cytotoxic for *M. tuberculosis*-infected macrophages, and thus play an important role in providing

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immunity to TB. The CD8+ T-cells can directly kill *M. tuberculosis* via granulysin, and facilitate the control of both the acute as well as chronic infection (Cooper, 2009; Grotzke & Lewinsohn, 2005). The abundant presence of *M. tuberculosis*-specific CD8+ T cells in latently infected individuals shows that the CD8+ T cells also have a role in the control of latent infection. This is also supported by reactivation of latent infection following depletion of CD8+ T cells in the Cornell model of latent TB (Van Pinxteren *et al.,* 2000).

2.10 Biomarkers of *M. tuberculosis*

A biomarker (biological marker) is defined as a characteristic that is objectively measured and evaluated as an indicator of a biological or pathological process or pharmacological response(s) to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). A profile of combined biomarkers is called a biosignature. A clinically useful biomarker needs to fulfill three important criteria: (i) provide accurate, repeated measurements at reasonable cost and with a short turnaround time; (ii) provide information not available from clinical assessment; (iii) assist in medical decision making (Biomarkers Definitions Working Group, 2001). Although biomarkers can be studied in any tissue or body fluid (including urine, saliva, sputum and breath), peripheral blood is the most widely used source in clinical practice. TB diagnostic tests that rely on detection of host immunological markers currently in use include the tuberculin skin test (TST) (Huebner *et al.,* 1993; Lee & Holzman, 2002) and interferon gamma release assays (IGRAs) (Andersen *et al.,* 2000; Pai *et al.,* 2008).

2.10.1 Diagnosis of *M. tuberculosis*

Diagnosis is the crucial first step to effectively reduce TB cases. Hence, rapid and sensitive TB diagnostics play an important role in detecting and preventing the disease. In this regard, existing tests for diagnosis of TB vary in sensitivity, specificity, speed and cost. TB control still relies on tests such as smear microscopy, microbiological culture and chest radiographs, despite their known limitations.

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2.10.1.1 Sputum Smear Microscopy

Sputum smear microscopy still remains the most available diagnostic tool, because it is requires inexpensive infrastructure, easy to perform, inexpensive, and rapid choice for diagnosis of TB (Foulds & O'Brien, 1998). This is especially true for laboratories in developing countries, where there are limited resources (Steingart *et al.,* 2006). The greatest drawback is that it lacks sensitivity and specificity (Perkins and Cunningham, 2007). It therefore provides false-negative results in 30 to 50% of individuals partially due to the need for at least 5000 bacilli/mL of sputum (Steingart *et al.,* 2006). However it is good and can the most infectious cases. The principal method for testing acid fast bacilli in sputum is the Ziehl-Neelsen (ZN) staining technique, which is an affordable method involving hot carbol fuchsin staining, followed by decolorization with acid-alcohol with the acid fast bacilli retaining the red color (Foulds and O'Brien, 1998). Fluorescence microscopy using auramine-stained requires less time to read. Now it is being used, since it requires trained personnel and is quite costly. In addition, slides testing positive in the fluorescent smear microscopy need to be confirmed through ZN staining (Foulds and O'Brien, 1998).

2.10.1.2 Microbiological Culture

Microbiological culture is the gold standard diagnostic tool as it detects viable organism and hence provide the final proof of infection. It also has the added advantage of allowing the identification of the species and/or of the isolated complex, and the determination of the sensitivity of the microorganism to chemotherapeutic agents for TB. The main drawback is due to the slow growth nature of the MTBCs; it cannot be used as the principal diagnostic tool for case detection but is used mainly to support case management especially in individuals with treatment failures/ drug resistant TB. Furthermore due to the infectiousness of the bacilli, it requires high level laboratory facility and well-trained personnel and the test do not always present 100% positivity (Frieden *et al.,* 2003). The principal solid culture

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media used are Löwenstein-Jensen (egg-based solid medium) and Middlebrook (7H10 or 7H11 in agar medium) takes about 4 – 6 weeks and liquid culture medium, Middlebrook 7H9 which takes 3 weeks to grow (Morgan *et al.,* 1983). Automated systems for the detection of mycobacteria, such as the BACTEC 460 TB®, BACTEC 9000® and the MGIT®, which use enriched media that promote the acceleration of bacterial growth, are promising, although they can also produce false-positive results due to contamination by other bacteria (Sociedade Brasileira de Pneumologia e Tisiologia, 2004). Nevertheless the long culture period is still a requirement for definitive diagnosis of TB and in drug-susceptibility testing (Fadda & Sanguinetti, 1998).

2.10.1.3 Chest Radiographs

Chest X-rays are indicated as an auxiliary method in the diagnosis of TB in symptomatic and smear-negative patients, contacts of individuals with active tuberculosis, and even in those suspected of having extra-pulmonary TB. The method is based on the presence of characteristic radiographic opacities and is useful in the diagnosis of primary pulmonary TB (more homogeneous opacity and increase in the volume of regional lymph nodes) and secondary pulmonary TB (heterogeneous opacity, cavities, and nodules) (Teixeira *et al.,* 2007). The radiologic analysis, however, is not a specific test to detect patients with TB, since pulmonary lesions similar to those caused by MTB can occur in other diseases. In practice chest X-rays and sputum tests are applicable in patients suspected of having pulmonary TB (Teixeira *et al.,* 2007). Although they are quite costly and are only available at referral centers, computed tomography scans of the chest are high-resolution radiologic tools, more sensitive than chest X-rays (Teixeira *et al.,* 2007)

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2.10.1.4 Tuberculin Skin Test (TST)

For centuries, the TST or Mantoux test has been the oldest diagnostic test used for the diagnosis TB infection and disease (Huebner *et al.,* 1993; Lee & Holzman, 2002). The TST attempts to measure cell-mediated response in the form of a delayed-type hypersensitivity reaction to the purified protein derivative (PPD). The PPD is a crude mixture of antigens, many of which are shared among MTB, *M. bovis* BCG, and several non-tuberculous mycobacteria (NTM) (Huebner *et al.,* 1993; Lee & Holzman, 2002; Andersen *et al.,* 2000).

In the TST, a very small amount of PPD, is injected intradermally into the forearm and read 48 to 72 hours later to see whether a hypersensitivity reaction occurs (Farhat *et al.,* 2006). The TST is based on the principle that T cells of individuals sensitized with mycobacterial antigens produce IFN-γ when they re-encounter these antigens (Figure 2.6) (Andersen *et al.,* 2000). The reaction is read by measuring the diameter of induration across the forearm, perpendicular to the long axis in millimeters. A positive reaction usually appears 3-4 days after PPD is administered. A positive result (in the form of erythema and/or vesiculation) results when an individual has active TB, was recently vaccinated with BCG, or has been cured of TB. The TST cannot distinguish latent from active TB, and a positive result can occur even if an individual is infected with another Mycobacteria species. The main drawback with the clinical use of the TST is the lack of specificity due to cross-reactivity with proteins present in other mycobacteria, such as BCG or *Mycobacterium* other than tuberculosis (MOTT) (American Thoracic Society, 2000; Farhat *et al.,* 2006).

Regardless of these limitations, the TST is still widely used because of its ability to predict active infection in latently infected individuals, and the fact that trials have shown that management of latent tuberculosis, diagnosed on the basis of TST results, reduces the risk of active disease by about 60% (American Thoracic Society, 2000; Jasmer *et al.,* 2002).

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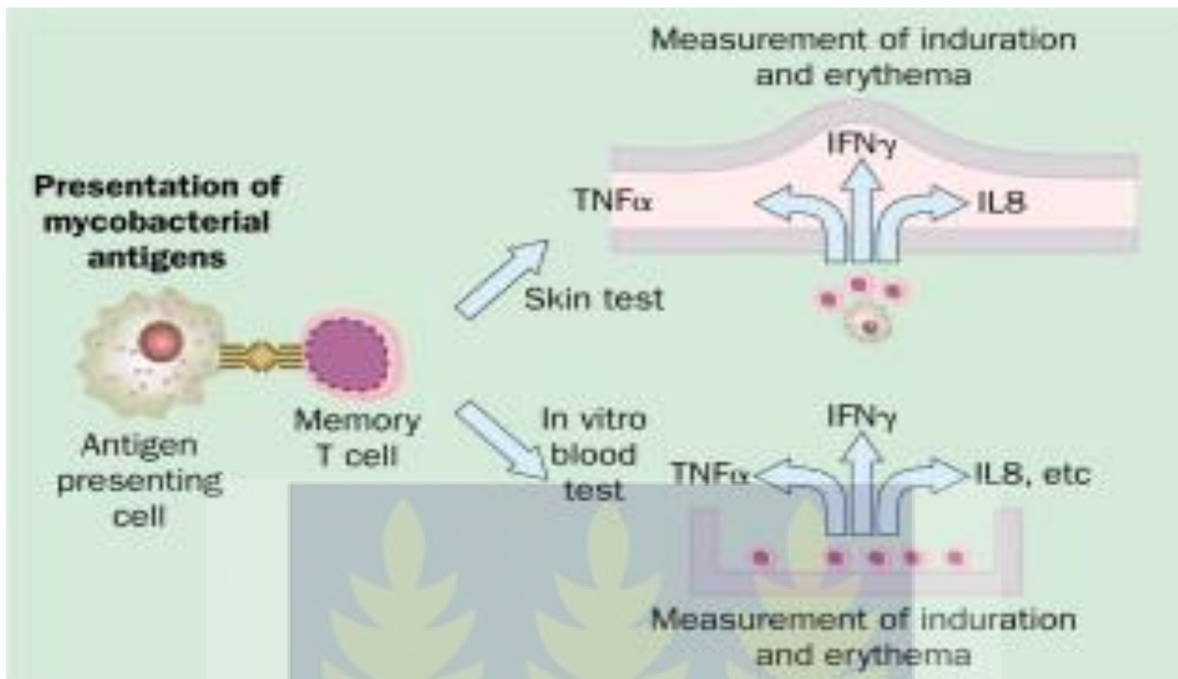

Figure 2.6 Biological basis of the tuberculin skin test and interferon-γ assay. Source: (Pai *et al.*, 2004)

2.10.1.5 Interferon Gamma Releasing Assay (IGRA)

Interferon gamma releasing assay or IGRA became the first assay as an alternative for TST in detection of latent TB. This assay is a relatively new type of *in-vitro* analysis based on cell mediated immune reaction. The assay is based on the same principle as the TST, in that T cells of individuals sensitized with MTB produce IFN-γ when they re-encounter mycobacterial antigens (Figure 2.6). However, it cannot discriminate LTBI from active disease.

There are currently two commercially available assays: QuantiFERON-TB Gold In-Tube (QFT-IT) assay (Cellestis Ltd, Carnegie, Australia) and the T-SPOT.TB assay (Oxford Immunotec, Oxford, UK). The first assay quantifies IFN-γ released when whole blood is incubated with peptides of CFP-10, ESAT-6 and TB7.7 antigens of TB in an enzyme linked immunosorbent assay (ELISA) format. T-SPOT.TB is enzyme-linked immunospot (ELISPOT) assay that measures cells themselves releasing IFN-γ when treated with ESAT-6 and CFP-10 (Pai *et al.,* 2004). These proteins (ESAT-6 and CFP-10) are encoded by genes

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located within the RD1 of the MTB genome, but absent from *M. bovis* BCG sub-strains and most environmental mycobacteria but present in *M. kansasii, M. szulgai, M. marinum,* and *M. riyadhense* (Mahairas *et al.,* 1996; Berthet *et al.,* 1998; Behr *et al.,* 1999; Brosch *et al.,* 2002).

There have been concerns about lack of sensitivity of IGRAs in detection of LTBI; these assays have shown to be unable to distinguish active TB from LTBI (Ngeow *et al.,* 2011; Nguyen *et al.,* 2012). In order to overcome disadvantages of CFP-10/ESAT-based ELISPOT, other proteins (Rv1978, NrdF1, Mpt64, CFP-21, Ppe57 and Ppe59) were expressed and used in ELISPOT. This report showed that they are specifically recognized by human PBMC in both active and latent TB patients and thus ELISPOT and probably other IGRAs can be further improved (Chen *et al.,* 2009)

2.10.1.6 Biosensors

A biosensor is an analytical device that incorporates a biological sensing element and an electrical transducer to measure a biological result using electrical output (Cooper & Hall, 1988). The detection of disease-related proteins requires sensitive, specific, and low cost assays. Numerous biosensors have been developed for the detection of such molecules, and each differs in terms of specificity, composition, and price.

Lately, a waveguide-based optical biosensor platform has been designed for the direct detection of three TB-specific bio-markers, namely liparabinomannan (LAM), ESAT-6 and Ag85 (Mukundan *et al.,* 2012). The whole system is based on a sandwich immunoassay consisting of a biotinylated capture antibody, the TB antigen, and a fluorophore-labeled reporter antibody located on the sensor's surface. The generated evanescent field and a spectrometer are used to detect fluorescence in the proximity of the sensor surface. The proposed sensor has a detection limit of 0.5 pM for ESAT-6, 100 pM for Ag85, and 1 pM for

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LAM. However, poor antibody stability precludes the use of this assay on clinical samples at present.

2.10.1.7 Molecular Methods

PCR method is more sensitive than culture, and the results are available more quickly. However, during analysis some false positive results might occur that can lower specificity (FIND, 2006) and in this case, culturing remains to be the standard to establish definitive diagnosis of primary tuberculosis infection. PCR remains to be a gold standard method; however reagents and equipment used are costly.

The Xpert MTB/RIF detects DNA sequences specific for *Mycobacterium tuberculosis* and rifampicin resistance by polymerase chain reaction (Van Rie *et al.,* 2010; Helb *et al.,* 2010). It is based on the Cepheid GeneXpert system, a platform for rapid and simple-to-use nucleic acid amplification tests (NAAT). The Xpert® MTB/RIF purifies and concentrates *Mycobacterium tuberculosis bacilli* from sputum samples, isolates genomic material from the captured bacteria by sonication and subsequently amplifies the genomic DNA by PCR. The process identifies all the clinically relevant rifampicin resistance inducing mutations in the RNA polymerase beta (rpoB) gene in the *Mycobacterium tuberculosis* genome in a real time format using fluorescent probes called molecular beacons. Results are obtained from unprocessed sputum samples in 90 minutes, with minimal biohazard and very little technical training required to operate (Boehme, *et al.,* 2010). This test was developed as an on-demand near patient technology which could be performed even in a doctor's office if necessary.

2.11 Treatment of *M. tuberculosis*

Treatment for TB uses antibiotics to kill the bacteria. Effective TB treatment is difficult, due to the unusual structure and chemical composition of the mycobacterial cell wall, which makes many antibiotics ineffective and hinders the entry of drugs (Brennan & Nikaido,

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1995). Currently, more than 20 drugs are available for the treatment of TB. Many of them, however, have considerable side effects. The current first line drug treatment regime comprises an initial intensive phase with a cocktail of four drugs (INH, RIF, ETH and PZA) for two months, followed by a continuation phase with two drugs (RIF and INH) for four months. However, in some individuals depending on response to therapy as assessed by bacteriological test, the treatment can be extended. Such a demanding treatment is poised for poor patient compliance (Parida & Kaufmann, 2010).

Individuals with fully susceptible TB may develop secondary resistance (acquired resistance) during TB therapy because of inadequate treatment, not taking the prescribed regimen appropriately, or using low-quality medication. Drug-resistant TB is a public health issue in many developing countries as treatment is longer and requires more expensive drugs with more toxic side effect (O'Brien, 1994). Multi-drug resistant TB (MDR-TB) is defined as resistance to the two most effective first-line TB drugs (INH and RIF). Furthermore essentially untreatable outbreaks of extensively drug-resistant XDR-TB, is defined as MDR-TB plus resistance to a quinolone and one of the second-line anti-TB injectable drugs (Amikacin, Kanamycin, and Capreomycin) (CDC, 2006).

The WHO introduced the directly observed therapy, short-course DOTS program as the internationally recommended strategy for TB control (Raviglione & Pio, 2002), which demands a patient to take the anti-TB drugs under the supervision of a health worker and or family supported. The DOTS program is based on sustained political and financial commitment; diagnosis by quality-ensured sputum-smear microscopy; standardized short-course anti-TB treatment given under direct and supportive observation; a regular, uninterrupted supply of high-quality anti-TB drugs; and standardized recording and reporting (Frieden & Munsiff, 2005). However, the well-designed DOTS program faces higher failure rates, due in part to increasing incidences of multidrug-resistant-TB (Caminero, 2008).

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2.12 Vaccines against TB

2.12.1 The *M. bovis* Bacillus Calmette-Guerin (BCG) Vaccine

The current vaccine BCG is the most widely used vaccine in the world. Despite a difficult start, it remains the only licensed TB vaccine, and has been given to 4 billion individuals. It is administered soon after birth and can prevent severe forms of childhood TB (Kaufmann *et al.,* 2010). Robert Koch (1843-1910) elucidated the etiology of TB (Koch, 1882) and Albert Calmette (1863-1933) together with Camille Guerin (1872-1961), by attenuation of *M. bovis* between 1906 and 1919, developed the BCG vaccine and first tested it in humans in 1921 (Calmette *et al.,* 1927). The first clinical studies took place in the 20th century in France and Belgium, and showed that BCG was highly efficient in protecting against TB in children (Calmette & Plotz, 1929).

The BCG vaccine, since 1974, has been administered to infants in high-risk TB populations as part of the WHO Expanded Program on Immunization (Brenzel & Claquin, 1994). However, a number of epidemiological studies and controlled clinical trials have shown that the BCG vaccine has numerous limitations (Mangtani *et al.,* 2014). The BCG vaccine induces an immune response that can prevent miliary and meningeal TB but cannot prevent or eliminate TB infection. The vaccine's effectiveness depends on several factors such as: patient age, TB localization, the geographic area in which the vaccine is administered, previous sensitization to mycobacteria, and the patient's immune status. The protection conferred by the BCG vaccine is significantly greater when the vaccine is administered to infants (Colditz *et al.,* 1995; Colditz *et al.,* 1994). Protection against pulmonary TB, which accounts for the majority of TB mortality and morbidity globally, is significantly lower and clearly age dependent (Colditz *et al.,* 1995; Colditz *et al.,* 1994). In children, protection against pulmonary TB can reach up to 80% (Mangtani *et al.,* 2014); however, only 50% of adults are protected, and some studies have reported no real preventive effects (Trial of BCG

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vaccines in south India for tuberculosis prevention, 1979; Fine, 1988). Finally, an important limit of the BCG vaccine is its inability to induce long-term protection, even when booster doses are administered. Subjects who receive the vaccine soon after birth are not protected during adolescence and adulthood (Rodrigues *et al.,* 2005). Thus, the BCG vaccine can protect against TB in infants but not adults, the major source of TB transmission. Consequently, the performance of the BCG vaccine is far from satisfactory, and this explains why BCG vaccination has been discontinued or has been limited to children at risk in a growing number of countries with low to intermediate TB prevalence. Consequently, the development of new vaccines more effective and safer than the BCG vaccine is considered a priority (Manissero *et al.,* 2008).

2.13. Development of New *M. tuberculosis* Vaccines

2.13.1 BCG Replacements

2.13.1.1 Recombinant BCG Strains

The first recombinant BCG vaccine is rBCG30; rBCG30 was made by inserting the plasmid pMBT30 into BCG, resulting in the overexpression of the 30-kDA protein α antigen or antigen 85b (Ag85b) (Horwitz *et al.,* 2000). This protein (mycolyl transferase) is the most abundant protein secreted by Mtb into broth culture and released into the MTB phagosome in infected human macrophages (Horwitz *et al.,* 1995; Lee & Horwitz, 1995; Harth *et al.,* 1996). The immunogenicity and efficacy of rBCG30 have been assessed in experimental studies. The immune response and protection induced by rBCG30 has been shown to be significantly greater in animals that have received this vaccine compared to those that received the BCG vaccine (Harth *et al.,* 1996). Moreover, rBCG30 has been found to be equally safe and well tolerated as the BCG vaccine and also has similar antibiotic susceptibility (Horwitz & Harth, 2003). In a phase 1 clinical trial of tuberculin-negative adults, rBCG30 was shown to be safe

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and immunogenic (Hoft *et al.,* 2008). The vaccine was able to induce increased Ag85b-specific T cell lymphoproliferation, IFN-γ secretion IFN-γ enzyme-linked immunospot responses, and direct ex vivo intracellular IFN-γ responses (Hoft *et al.,* 2008). Moreover, it significantly enhanced the population of Ag85b-specific CD4+ and CD8+ T cells that were capable of concurrent expansion and effector function. Importantly, rBCG30 significantly increased the number of Ag85b-specific T cells that were capable of inhibiting intracellular mycobacteria. However, despite these promising results, rBCG30was not pursued further (Hoft *et al.,* 2008)..

The second recombinant BCG vaccine, VPM1002 (Montagnani *et al.,* 2014), is based on live Mtb with two modifications. The immunogenicity, protection and tolerability of VPM1002 have primarily been tested in animals, with very encouraging results (Montagnani *et al.,* 2014). Compared to the BCG vaccine, VPM1002 evoked superior protection due to its abilities of stimulating both type 1 and type 17 cytokine responses (Desel *et al.,* 2011) and of generating apoptotic vesicles in infected macrophages, which induce more profound CD4 and CD8 T cell responses (Farinacci *et al.,* 2012). In human trials, VPM1002 has been found to be immunogenic (as measured by IFN-γ production), safe, and well tolerated in adults (Farinacci *et al.,* 2012). The vaccine was able to induce multifunctional CD4+ and CD8+ T cell subsets and exhibited a trend of being better than a comparable dosage of BCG (Grode *et al.,* 2013). Despite favorable results in animals the administration of this vaccine to humans has recently been halted due to safety concerns in a phase I clinical trial in the US (Kaufmann, 2012).

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2.13.1.2 Attenuated *M. tuberculosis* Strains

MTBVAC01 is the only vaccine based on an attenuated MTB strain of human origin in phase 1 clinical trials (Kupferschmidt, 2011; Kaufmann & Gengenbacher, 2012). This vaccine should be both safer and more effective than the BCG vaccine. The *PhoP* and *fadD26* genes of the MTB strain were inactivated to increase safety. This prevents the secretion of ESAT-6, the most important MTB virulence factor (Kaufmann & Gengenbacher, 2012), and reduces the synthesis of phtiocerol dimycocerosates, a component of cell envelope that protects MTB from host defenses (Perez *et al.,* 2011). MTBVAC01 does not produce complex lipids regulated by *PhoP*, which interfere with the host immune response (Perez *et al.,* 2011). Furthermore, the antigenic properties of MTBVAC01 are enhanced by silencing Mcr7, an antisense RNA that regulates the secretion of the twin arginine translocation (TAT) protein of Mtb (Perez *et al.,* 2011). MTBVAC01 can be considered the next iteration of the live attenuated MTB vaccine SO_2, which inserted a kanamycin-resistance cassette into *PhoP* (Arbues *et al.,* 2013).

2.13.2. Viral vectored *M. tuberculosis* Vaccines

MVA85A is a recombinant strain of the modified Vaccinia Ankara virus that expresses the immunodominant Mtb protein antigen 85A. MVA85A was developed as a heterologous boost for the BCG vaccine in 2002 (McShane *et al.,* 2004). Boosting the BCG vaccine with MVA85A improved BCG-induced protection against mycobacterial challenge in several animal studies (Verreck *et al.,* 2009; Vordermeier *et al.,* 2009; Williams *et al.,* 2005). Currently, MVA85A is the MTB vaccine with the most studies in humans. Several clinical trials have shown that MVA85A is safe and well tolerated. However, data on MVA85A immunogenicity and efficacy are conflicting. The first study of MVA85A in humans found that MVA85A was highly immunogenic in all vaccinated subjects; however, the CD4+ T cell response was significantly higher in BCG-vaccinated subjects than in BCG-naive subjects

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(McShane *et al.,* 2004). This difference was not observed in further studies in Africa, which suggests that the cumulative mycobacterial immunity in adults living in tropical climates was significantly lower than that in adults living in more temperate climates; this may be due to higher exposure to mycobacteria antigens in these tropical climates (Hawkridge *et al.,* 2008). Nonetheless, the vaccine-induced responses were higher than baseline in adults, adolescents, and children, although the vaccine's efficacy in children was disappointing.

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CHAPTER THREE

3.0 Materials and Methods

3.1 Ethical Statement and Subject Recruitment

This study was embedded in 2 different projects. Each project was approved by Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, Accra.

The first study used retrospective archived cryopreserved PBMCs of nine *Mycobacterium africanum* (MAF) and ten *Mycobacterium tuberculosis* sensu stricto (MTBss) individuals in liquid nitrogen. These were made up of 19 newly diagnosed sputum smear-positive TB patients who were adults of mean age (±standard deviation [SD]) was 34.05 (±9.25) years yet to begin therapy, and were recruited consecutively from three health facilities in Accra, Ghana during May to June 2011. Each participant donated heparinized venous blood from which peripheral blood mononuclear cells were isolated and cryopreserved in liquid nitrogen. Their sputum samples were characterized by HAIN Test into MAF and MTB lineages. The original study entitled "Host immunological profiling from exposure to MTB to active disease, a tuberculosis case contacts study in Ghana" had approval from the Ministry of Health and ethical clearance by the IRB of NMIMR, University of Ghana, Legon, Accra (Federal wide Assurance number FWA00001276) and Certified Product Number (CPN) 030/10–11 and funded by the Wellcome Trust (WT087535MA).

The second study which had to do with intracellular growth assay in human monocyte – derived macrophages had IRB approval. The standard procedure for sampling as outlined by the National Tuberculosis Program (NTP) for the routine management of TB in Ghana was used in the study. Written (in the case of literate participants) or oral (for illiterates) informed

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consent was sought from all participants before inclusion in the study. For minors (below sixteen years of age) consent was sought from their parents/guardians before enrolment into the study. In the case of minors between sixteen and eighteen years, in addition to parental consent, assent was sought from them before enrolment into the study. As per the guidelines of the IRB of NMIMR, information confidentiality was strictly adhered to. In addition, objectives and benefits of the study were explained to all participants. For the human monocytes derived macrophages component, all donors who enrolled were examined and found not to be on any immunosuppressive drugs by a physician. Blood was obtained from all donors after ethical approval and informed consent had been granted. A total of six adult males of ages between 38 years and 42 years old from the Institute were recruited for the study.

3.2 Analysis for Differences in Host Response to Distinct Mycobacterial Lineages

3.2.1 Antigens for Stimulation

The test antigens evaluated in this study were MTB-specific recombinant early secreted antigenic Target–6 and culture filtrate protein–10 (rESAT-6/CFP-10) fusion protein [a combination of the two most immunodominant proteins of the RD1] were provided by the Department of Infectious Diseases, Leiden University Medical Centre, Leiden, The Netherlands (courtesy K.L.M.C Franken) (Franken *et al.*, 2000). The positive control antigen, the super antigen, was *Staphylococcal* enterotoxin B (SEB) (Sigma-Aldrich, St. Louis, USA) while the negative control was complete medium [RPMI-1640 supplemented with 1% L-glutamine, 10% foetal calf serum (FCS) and 1% penicillin/streptomycin all from Sigma-Aldrich, St. Louis, USA]. The MTB antigen and SEB were received in a dehydrated form and reconstituted with sterile complete medium and used at a final concentration of 5µg/ml

3.2.2 Thawing and Counting of Retrospective Archived Cryopreserved PBMCs

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Each selected single vial of cryopreserved PBMCs from MAF- and MTBss-infected individuals was rapidly thawed by immersion in a water bath at 37°C with gentle agitation until a small amount of ice crystals remained. The content of each vial were transferred into a 15ml centrifuge tube containing complete medium [RPMI-1640 supplemented with 10% FCS and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, USA)] and centrifuged at 300 x g for 10 minutes. After the supernatant was carefully removed, the cells were washed again and finally resuspended in complete medium for cell counting. Cell count and recovery was done using Neubauer improved haemocytometer while viability was determined using the 0.4% trypan blue dye exclusion technique (Weinberg *et al.*, 2000). All thawed PBMCs evaluated for viability prior to flow cytometric assay were ≥ 90% viable.

3.2.3 *In vitro* Antigen Stimulation

Each assay contained 5 x 10^5 cells per FACS tube (BD Pharmingen, New Jersey, USA) and suspended in 500μl warm complete medium (RPMI-1640 supplemented with 10% heat inactivated FCS, 1mM glutamine, 1% penicillin/streptomycin) and stimulated with SEB at a final concentration of 5μg/ml, (positive control), rESAT-6/CFP-10 fusion protein at 5μg/ml and complete medium which served as unstimulated negative control. After 2 hours of antigen stimulation, 10μg/ml of Brefeldin A (Sigma-Aldrich, St Louis, USA) [final concentration] was added to each culture i.e. negative control, SEB and rESAT-6/CFP-10 fusion protein to inhibit the secretion of IFN-γ into the medium. The tubes were incubated overnight for 16 hours at 37°C in 5% CO_2 humidified incubator.

3.2.4 Monoclonal Antibodies for Surface Phenotypic and Intracellular Staining

Monoclonal antibodies (mAbs) used for surface staining were mouse anti–human cluster of differentiation (CD)4 peridinin chlorophyll protein (PerCP) (Becton Dickinson (BD)/Pharmingen, clone RPT4; Ref: 345570, New Jersey, USA), mouse anti–human CD8

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allophycocyanin (APC) (BD/Pharmingen, clone RPA-T8; Ref: 561421, New Jersey, USA) and intracellular cytokine, fluorescein isothiocyanate (FITC) anti–human interferon gamma (IFN-γ) (BD/Pharmingen, clone 4S.B3; Ref: 554551, New Jersey, USA) and their isotype controls respectively; mouse immunoglobulin (Ig) G2a PerCP, mouse IgG1 kappa APC (Ref: 554681) and mouse IgG1 kappa FITC (Ref: 554679) were all obtained from BD/Pharmingen, New Jersey, USA.

3.2.5 Flow Cytometric Staining for Cell Surface Markers and Intracellular Cytokine.

After overnight antigen stimulation, each set of stimulated PBMCs was washed with 2ml cold FACS buffer (filtered phosphate buffer saline (PBS), supplemented with 1% heat inactivated FCS and 0.1% sodium azide), supernatant discarded and resuspended in 100µl FACS buffer. Cells were then stained with surface antibodies as follows; 3µl anti-human CD4 APC, 2µl anti–human CD8 PerCP and incubated for 30 minutes at 4°C. After incubation, 2ml FACS buffer was added and washed and supernatant decanted as before. The cell pellet was then re-suspended in 250µl of Cytofix/Cytoperm (BD/Pharmingen, Ref: 554714, New Jersey, USA) and kept at room temperature in the dark for 15 minutes to fix the cells for intracellular staining. After fixing, cells were permeabilized by washing 2 times with 1ml of 1X BD Perm/Wash buffer (BD/Pharmingen, Ref: 554723, New Jersey, USA). For the second wash, the cells were incubated with the perm wash for 25 minutes prior to centrifugation at 300 x g. The supernatant was decanted, the pellet re-suspended in the residual perm wash (approx. 50µl) and 2µl of anti-human IFN-γ FITC or its isotype control was added and incubated for 30 minutes in the dark. After incubation, 2ml perm wash was added and centrifuged as before and the supernatant decanted. The pellet was then resuspended in 0.3ml of FACS flow solution and immediately analyzed on a FACS Calibur (BD, San Jose, USA) using CELLQuest software version 3.3.

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3.2.6 Flow cytometric analysis

FACS data was reanalyzed using the FlowJo software version 7.6.2 (TreeStar Inc. Calif., USA). The lymphocytes gate (R1) was defined in a dot plot of forward scatter channel (FSC) versus side scatter channel (SSC) by the light scattering properties. The fluorescence of CD4+ or CD8+ T cells was defined in a plot of CD4-PerCP or CD-8 APC vs. SSC gated on R1 to be R2. Finally, the percentage frequencies of CD4+IFN-γ+ or CD8+IFN-γ+ in response to GM, SEB and rESAT-6/CFP-10 fusion protein was determined on R2. Background fluorescence was determined using isotype controls. Compensation settings were defined using FACS Comp Beads (BD Biosciences) stained with each fluorochrome–conjugated antibody. The frequencies or proportions of IFN-γ+ producing antigen specific CD4+ or CD8+ T cells was defined as percentage CD4+IFN-γ+ or CD8+ IFN-γ+ in response to SEB and rESAT-6/CFP-10 fusion protein subtracted from percentage CD4+IFN-γ+ or CD8+ IFN-γ+ in response growth medium. A threshold of >0.44% of CD4+IFN-γ+ or CD8+ IFN-γ+ T cells defined positive T cell responses against antigens.

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Representative flow cytometric analyses of frozen PBMCs from either MAF- or MTBss-infected TB patients after overnight stimulation with growth medium (GM), SEB and rESAT-6/CFP-10 fusion protein.

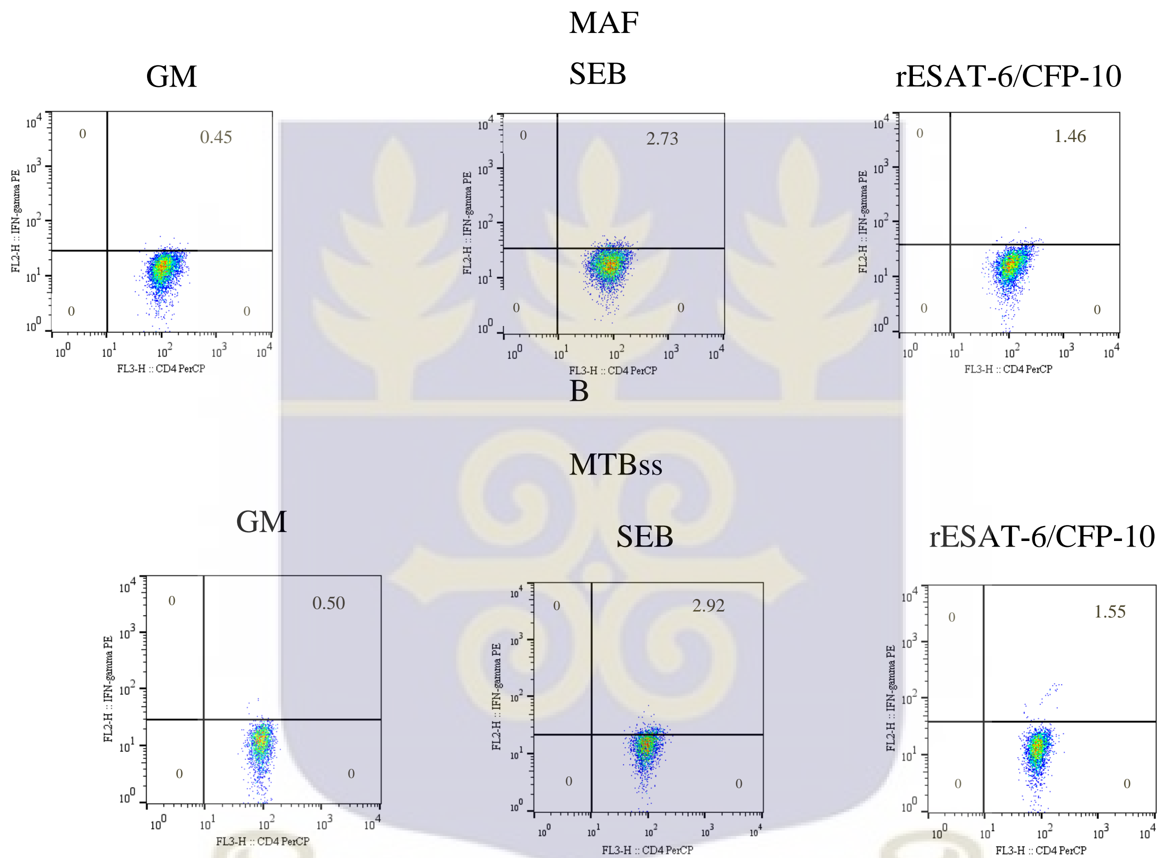

Figure 3.1 Representative Dot Plots showing percentage frequencies of CD4+IFN-γ+ T cells from PBMCs of MAF- and MTBss-infected patients after stimulation with GM, SEB and rESAT-6/CFP-10 fusion protein.

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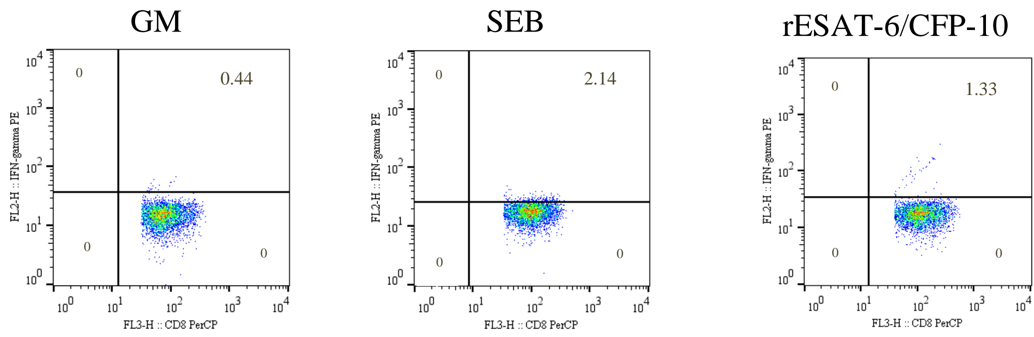
MAF



MTBss

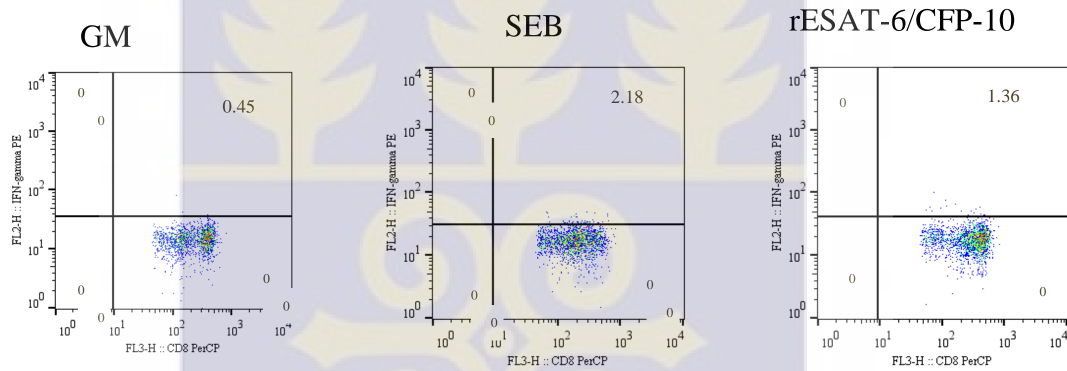


Figure 3.2 Representative Dot plots showing percentage frequencies of CD8+IFN-γ+ T cells from PBMCs of MAF- and MTBss-infected patients after stimulation with GM, SEB and rESAT-6/CFP-10 fusion protein.

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3.3 Analysis of Mycobacterial Growth in Macrophages

3.3.1 *Mycobacterium tuberculosis* complex isolates

This study used a total of 18 archived MTBC clinical isolates comprising of 6 *Mycobacterium tuberculosis* sensu stricto (MTBss) (Lineage 4), 6 *Mycobacterium africanum* West African 1 (MAF1) (Lineage 5) and 6 *Mycobacterium africanum* West African 2 (MAF2) (Lineage 6) for the intracellular growth assay.

Briefly, these isolates were selected from the large collection of tuberculosis (TB) strains generated from sputum of acid fast TB patients who attended four main health centres namely, Agona Swedru Government Hospital, Winneba Government Hospital and Saint Gregory Catholic Clinic at Budumbura refugee camp covering three different districts in the Central Region; Effia-Nkwanta Regional Hospital in Western Region over a period of 17 months from October 2007 to February 2009 as published by Yeboah-Manu *et al*., 2011. These isolates had been analyzed by large sequence polymorphism (LSP) analysis at various regions of difference (RD) for the presence of main lineages within MTBC as previously described by Yeboah-Manu *et al*., 2011 using RD9, RD702, RD711 and RD726 PCR (de Jong *et al.,* 2009; Brosch *et al.,* 2002).

3.3.2 *Mycobacterium tuberculosis* sensu stricto (MTBss) (L4)

Six of the selected clinical isolates analyzed by LSP showed the presence of RD9 and was identified by deletion of RD726 PCR as belonging to MTBss/L4 (Hershberg *et al.,* 2008; Gagneux *et al.,* 2006). The absence of *M. tuberculosis* specific deletion-1 (TbD1) gene defined MTBss/L4 as a 'modern' lineage (Brosch *et al.,* 2002).

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3.3.3 *Mycobacterium africanum* West African 1 (MAF1) (L5)

Six of the isolates were classified as MAF1/L5 by deletions at RD9 and identified by RD711 using PCR analysis (Hershberg *et al.,* 2008; Gagneux *et al.,* 2006). However, the presence of TbD1 gene characterized these clinical isolates as 'ancient' lineages (Brosch *et al.,* 2002).

3.3.4 *Mycobacterium africanum* West African 2 (MAF2) (L6)

Furthermore, six clinical isolates were classified as MAF2/L6 by the absence of RD9 and identified by RD702 by PCR analysis (Hershberg *et al.,* 2008; Gagneux *et al.,* 2006), characterized by the presence of TbD1 gene as 'ancient' lineages (Brosch *et al.,* 2002).

3.4 Preparation of Mycobacterial Cells for Internalization/Intracellular Growth Assay

3.4.1 Single Colony Mycobacterial Cultures

Mycobacterial cultures of each of the selected characterized clinical isolates were obtained from single colony units. The cultures were performed in a restricted access, category 3 biosafety facility at NMIMR.

Briefly, the archived clinical isolates were thawed and sub-cultured for single colonies by streaking on Difco™ Middlebrook Mycobacteria 7H11 agar (BD, Maryland, USA) containing either 0.5% glycerol (Fisher Scientific, Loughborough, UK) for (MTBss/L4) or 40mM sodium pyruvate (Sigma-Aldrich, St. Louis, USA) (final concentration) for (MAF1/L5 and MAF2/L6), supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) growth enrichment media (BD, Maryland, USA) in 90 mm three compartment petri dish (Greiner Bio-One, Vilvoode, Belgium). Plates were incubated for 4–6 weeks at 37°C for microscopic growth.

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3.4.2 Amplification of Viable Mycobacterial Cells

A single colony obtained for each clinical isolate was inoculated in 10ml Difco™ Middlebrook Mycobacteria 7H9 broth (BD, Maryland, USA) containing either 0.2% glycerol or sodium pyruvate 40mM (final concentration) supplemented with 0.05% Tween 80 (Fisher Scientific, USA) and 5% albumin-dextrose-catalase (ADC) growth enrichment media (BD, Maryland, USA) in 30ml sterile graduated square plastic translucent bottles-PETG (Fisher Scientific, USA) at 37°C for 14 days for mid-log phase cultures. Mycobacterial cell density was measured at 600 nm using 8000 Biowave Cell Density Meter (Biochrom Ltd, England, United Kingdom). One millitre of the mid log phase culture was then inoculated into 100ml of the same medium in the absence of detergent (0.05% Tween 80) and incubated for 10 days at 37°C. Gentle culture dispersion was performed manually every 48 hours. The cultured suspension for each mycobacterium was pelleted at 4500 x g for 30 minutes, supernatants discarded and pellets mechanically dispersed by manual shaking for 1 minute with equal volumes of 3mm glass beads (MERCK, Damstadt, Germany). Each dispersed mycobacterium was resuspended in PBS and centrifuged at 260 x g for 10 minutes to sediment cell clumps. The supernatant which contains single cells were enumerated by adding volume/volume ratio 50% glycerol and PBS, serial diluted and plated on Difco™ Middlebrook Mycobacteria 7H11 agar containing either 0.5% glycerol (L4) or sodium pyruvate 40mM (final concentration) (MAF1/L5 and MAF2/L6), supplemented with 10% OADC growth enrichment media before and after freezing at -80°C. Multiple vials were stored at -80°C until further use.

3.4.3 Determination of Colony-Forming Unit (CFU) Concentration of Stock Vials

Following incubation of serially diluted mycobacterial cells in triplicates in petri dishes for 4-6 weeks, the number of cells per milliliter of mycobacterial suspension was computed as

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follows: average number of CFU counted from the 3 plates multiplied by the dilution factor and a constant (20 units) as the volume plated per well was 50µL [Average CFU counted (3 plates) X dilution factor X 20 units per 1mL (volume per well = 50µL X 20 units)]. The suspension then was diluted to get a mycobacterial cell concentration of cells per milliliter for the intracellular growth assay.

3.5 Human Blood Processing, Monocyte Isolation, Culture and Maturation to Human Monocyte-Derived Macrophages

This process used Ficoll density gradient centrifugation to isolate PBMCs from venous whole blood. The Miltenyi Biotec magnetic cell separation system (MACS) was then used to isolate the monocyte fraction (CD14+) from the PBMCs. Subsequent culture with human granulocyte macrophage colony stimulating factor-2 (GM-CSF-2) differentiated the CD14+ cells into macrophages.

3.5.1 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood was obtained by venipuncture using vacutainer blood collection set (BD, New Jersey, USA) into sodium heparinized vacutainer blood collection tubes (BD, New Jersey, USA). PBMCs were isolated from heparinized venous blood over Ficoll Paque™ Plus density gradient (GE HealthCare, Uppsala, Sweden) by centrifugation (800 X g for 30 minutes at room temperature). Recovered PBMCs were washed twice in RPMI, pooled and counted. Surface staining of T-cells and monocytes by BD FACS Calibur flow cytometry on PBMCs isolated using FITC–labeled anti-human CD3 (Beckman Coulter, ref; A07746, Marseille, France) and phycoerthyrin (PE) labeled mouse anti-human CD14 (BD/Pharmingen, Ref: 555398, San Jose, USA) monoclonal antibodies.

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3.5.2 Monocyte Isolation, Culture and Maturation to Monocyte-Derived Macrophages

Monocytes were purified from the counted PBMCs resuspended in cold monocyte buffer (PBS [pH7.0] supplemented with FCS and ethylenediamine tetra-acetic acid [EDTA]) by positive sorting using anti-CD14-conjugated magnetic microbeads (Milteny Biotec, Auburn, USA) and MACS LS separation columns (Miltenyi Biotec, Auburn, USA) according to manufacturers' recommendations. The recovered monocytes was >90% CD14^{+} as determined by BD FACS Calibur flow cytometry using FITC labeled anti-human CD3 and PE-Cy-7 labeled mouse anti-human CD14 (M5E2) monoclonal antibodies as shown in Figure 3.3.

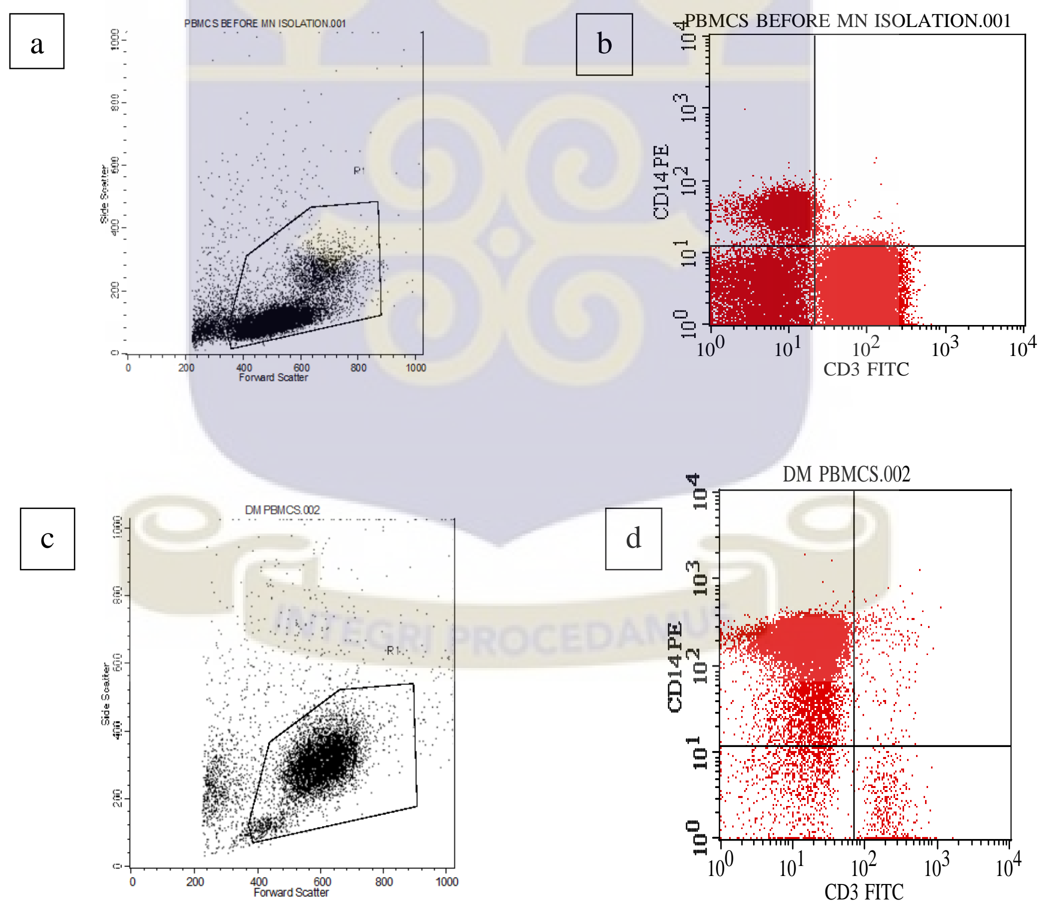

Figure 3 3 Flow cytometric analysis of whole blood from healthy individual stained with CD3 FITC and CD14 PE showing Dot Plots of light scattering of lymphocytes and monocytes gated R1 in a forward vs. side scatter before depletion (a) and after depletion of monocytes (c) and its fluorescence (b) and (d).

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Cells were resuspended in RPMI-1640 supplemented with 10% FBS and 5mM L-glutamine at 2 x 10^6 per 3 ml per well supplemented with GM-CSF-2 (50U/mL) (Peprotech, New Jersey, USA) in Falcon® Multiwell™ Primaria™ 6 well tissue culture plate flat bottom with low evaporation lid (treated) (BD, New Jersey, USA) and incubated for 7 days at 37°C in 5% CO_2 humidified incubator (Ikemoto Rika Kogyo, Tokyo, Japan) to mature to macrophages. The MDM were recovered after 20 minutes treatment with Trypsin/EDTA (2mM) (Sigma-Aldrich, St. Louis, USA) , harvested with undiluted FCS, pooled and then resuspended in RPMI-1640 supplemented with 10% FCS and 5mM L-glutamine to be counted. The counted MDM were evenly distributed at 1 X 10^5 MDM per 0.3 ml culture medium per well in Costar® 96 well sterile cell culture cluster round bottom plate with lid (Fisher Scientific, Loughborough, UK) before mycobacterial infection at a multiplicity of infection (MOI) of 1:1 unless specified otherwise

3.5.3 Infection of Human Monocyte-Derived Macrophage with Mycobacterial Cells.

Frozen aliquots of the representative lineage that is MTBss/L4, MAF1/L5 and MAF2/L6 of known concentration were freshly thawed at room temperature at the time of infection. The hourly rested MDMs were co-cultured in triplicate with each bacillus (1 X 10^5 bacilli) at MOI 1:1 ratio in RPMI-1640 and 10% FCS in a 96 well tissue culture plate with lid. Control set-up included MDM without bacilli for each strain. After 4 hours incubation, cultured supernatants of each strain were harvested gently, filtered individually using 0.20µm millipore (Sartorius stedim, Goettingen, Germany) and stored frozen at -30°C. The infected MDM were subjected to complete lysis by mixing with sterile distilled water supplemented with 0.01% Tween 80 and incubated for 20 minutes at 37 °C in 5% CO_2 humidified incubator. Likewise after 24, 48 and 72 hours, cultured supernatant for each strain infected MDM of each time point were harvested and filtered; their lysates mixed thoroughly, serially diluted, and plated in triplicate

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on 7H11 agar dishes. Plates were incubated for 4-6 weeks at 37°C and CFUs formed enumerated in triplicate plated in petri dishes. All cultures were conducted in a category 3 biosafety facility.

3.5.4 Determination of Colony Forming Units

One part lysate and its unfiltered cultured supernatant for each strain infected MDM were mixed thoroughly ten times to 10 parts of PBS supplemented with 0.05% Tween 80 separately. Each was plated with sterile disposable L-shaped microbiological spreader (Greiner Bio-One, Vilvoode, Belgium) in triplicate on Difco™ Middlebrook Mycobacteria 7H11 agar containing either 0.5% glycerol (MTBss/L4) or sodium pyruvate 40mM (final concentration) (MAF1/L5 and MAF2/L6), supplemented with 10% OADC growth enrichment media. The 90mm three compartment petri dish (94 x 15) with vents were incubated for 4–6 weeks at 37°C and CFUs enumerate by counting the triplicate petri dishes.

3.6 Enzyme-Linked Immunosorbent Assay (ELISA) for Determination of Pro-inflammatory Cytokines in Culture Supernatants of Strain–Infected MDM Cells.

Culture supernatants from the control and infected MDM cells harvested at time points 4hrs, 24hrs, 48hrs, and 72hrs, sterile-filtered were assayed with commercial sandwich ELISA kit (Peprotech, New Jersey, USA) according to manufacturer' instructions to measure levels of tumor necrosis factor alpha (TNF-α), interleukin 12p70 (IL-12p70) and IL-6 cytokines. The assay sensitivities were 16 ρg/ml for TNF-α and 32 ρg/ml for IL-6 and IL-12p70 respectively.

Briefly, Nunc MaxiSorp 96 well plates (Fisher Scientific, Loughborough, UK) were coated separately with 1μg/ml in PBS coating buffer of capture antibody for TNF-α, IL-12p70 and IL-6 in duplicates at a volume of 50μl/well and sealed with a plate sealer. Overnight

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incubation was done at 4^oC. Plates for each cytokine were washed to remove unbound capture antigen and blocked with 100μl/well of 1% bovine serum album (BSA) (Sigma-Aldrich, St. Louis, USA) in PBS for 1 hour. Then 50μl/well of cultured supernatants, diluted 1:3 in complete medium [RPMI-1640 with L-glutamine, 1mM sodium pyruvate and 10% FCS]) and cytokine standard for each, TNF-α, IL-12p70 and IL-6 (diluted 2 fold from 2000 ρg/ml to 5.859 ρg/ml) were added in duplicates and incubated at room temperature for 2 hours. Bound antibodies for each cytokine were detected by incubation at room temperature for another 2 hours with TNF-α detection antibody (diluted 1:200 in diluent buffer [Tween 20 (0.05%) and BSA (0.1% in PBS]); IL-12p70 and IL-6 (diluted 1:400 each respectively in diluent buffer). Affinity for the antibodies for each cytokine were detected by incubating at room temperature for 30 minutes with avidin horse radish peroxidase (HRP) conjugate (diluted 1:2000 in diluent buffer). Colour development was at room temperature by adding 2, 2'–azino–bis (3–ethylbenzothiazoline–6–sulphonic acid) (ABTS) liquid substrate (Sigma-Aldrich, St. Louis, USA), incubated in the dark for 40 minutes and the reaction was stopped by adding 50μl/well of 2N sulphuric acid. The OD was measured with an Absorbance Microplate Reader ELx 808 (BioTek Instruments, Vermont, USA) at 405nm, with wavelength correction set at 650nm. After each incubation step, contents within the microtitre plate wells were decanted and washing of the microtitre plate wells was done with 250μl/well of 0.05%Tween–20 in PBS 4 times. The sandwich ELISA for measuring the levels of TNF-α, IL-12p70 and IL-6 cytokines in culture supernatants of the different strain infected MDM was repeat for the other time points i.e. 24, 48, and 72 hours.

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3.7 Data Analysis

Data was entered in Microsoft Excel 2007 (Microsoft Corp., USA) and imported into GraphPad Prism 5.03 (GraphPad Software Inc., California, USA) for statistical analysis while graphs were plotted with Microsoft Excel 2007 (Microsoft Corp., USA). All results were represented as means and standard error of means (means ± SEM).

Flow cytometric data was analyzed using FlowJo software Version 7.6.2 (Tree Star Inc., USA). Results were expressed as percentage IFN-γ+CD4+/ IFN-γ+CD8+ T cells in response to GM, SEB and rESAT-6/CFP-10 fusion protein. Background values obtained for the unstimulated conditions were subtracted from the antigen-stimulated responses to yield antigen-specific cytokine production. The flow data were then represented by mean column bar while the error bars represented the standard error of the mean (SEM). Descriptive analysis and differences in the IFN-γ+CD4+/ IFN-γ+CD8+ T-cells to antigenic stimulation in MAF and MTBss were analyzed using unpaired t-test, where $p<0.05$ was considered as significant.

Initial strain uptake by MDM after 4hrs indicated the number of CFU at the 4 hour time point from MDM lysates as a percentage of the initial inoculum. The summarized intracellular growth data at 4hrs for MDM infected with MTBss/L4, MAF1/L5 and MAF2/L6 were represented by mean bar graph while the error bars represented by SEM. Growth index was determined by calculating the Log10 [number of CFU at each time point (i.e. 24hrs, 48hrs or 72hrs) divided by number of CFU at its 4hrs time point]. Line graph and error bars as SEM were plotted for at 24hrs, 48hrs and 72hrs for MDM infected with MTBss/L4, MAF1/L5 and MAF2/L6. Line graph and its error bars as SEM were plotted for at 24hrs, 48hrs and 72hrs for MAF (ancient) and MTBss (modern) lineages. The mean doubling time was calculated based on the CFU at 4hrs divided by log 10 (CFU at its 72hrs) for each individual clinical

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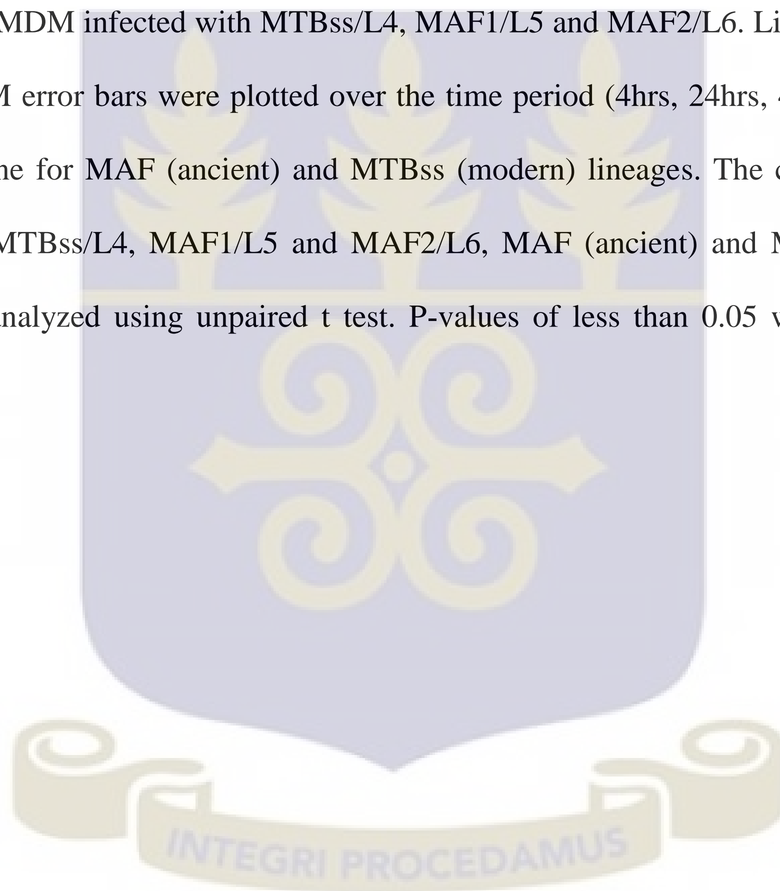
isolates MTBss/L4, MAF1/L5 and MAF2/L6 and for MAF (ancient) and MTBss (modern) lineages. The intracellular growth index was compared amongst different groups using unpaired t test at each time point, where $p<0.05$ was considered as significant.

The minimum detectable concentrations of the cytokines in this assay were 16ρg/ml, 32ρg/ml and 32ρg/ml for TNF-α, IL-6 and IL-12p70 respectively. Mean bar graphs and its error bars as SEM were plotted for at 4hrs, 24hrs, 48hrs and 72hrs for each cytokine released in the supernatant for MDM infected with MTBss/L4, MAF1/L5 and MAF2/L6. Likewise mean bar graphs and SEM error bars were plotted over the time period (4hrs, 24hrs, 48hrs and 72hrs) for each cytokine for MAF (ancient) and MTBss (modern) lineages. The cytokines for the various group MTBss/L4, MAF1/L5 and MAF2/L6, MAF (ancient) and MTBss (modern) lineages were analyzed using unpaired t test. P-values of less than 0.05 were regarded as significant.



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CHAPTER FOUR

4.0 Results

4.1 Characteristics of Study Participants

In all a total of nineteen (19) sputum smear-positive pulmonary TB patients were investigated. Nine (9) individuals infected with *Mycobacterium africanum* (MAF) strains, were made up of six (66.7%) males and three (33.3%) females. Likewise individuals infected with *M. tuberculosis* sensu stricto (MTBss) were made up of ten patients with the male being six (60%) and the female four (40%). Mean age (± standard error of mean [SEM]) of MAF-infected patients was 34.6 (± 2.4) while that of MTBss-infected patients was 31.2 (± 2.9). The ages of cases infected with MAF did not differ from that of MTBss (p=0.3833) as shown in Table 4.1. However, an HIV sputum smear-positive pulmonary TB patient was excluded from the study.

Table 4.1 Characteristics of study participants

Characteristics	MAF-infected patients	MTBss-infected patients
Sample size (n)	9	10
Mean age years (SEM)	34.6 (2.4)	31.2 (2.9)
Male (%)	6(66.7)	6(60)
Female (%)	3(33.3)	4(40)

n: number; SEM: standard error of mean; %: percentage

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4.2 Interferon Gamma (IFN-γ) Production by T Cells in Response to Mycobacterial Antigens in TB Patients.

Peripheral blood mononuclear cells (PBMCs) from participants infected with the 2 different species were stimulated *in vitro* with a mitogen, *Staphylococcal* enterotoxin B (SEB) and Mtb-specific recombinant early secreted antigenic target – 6 and culture filtrate protein – 10 (rESAT-6/CFP-10) fusion proteins and assessed through flow cytometry for the production of IFN-γ. The ability of the participants infected with the 2 different species response to replicating associated antigens was analyzed.

4.2.1 Percentage Frequency of CD4+IFN-γ+ T Cells Production in Response to Mycobacterial Antigens in TB Patients

The percentage frequency CD4+IFN-γ+ T cells in MAF-infected patients in response to the growth medium (negative control) were 0.4544 ± 0.03845. Likewise the percentage frequency CD4+IFN-γ+ T cells in MTBss-infected patients were 0.4970 ± 0.01325. This background of unstimulated condition was subtracted from the antigen-stimulated responses to yield antigen-specific IFN-γ production.

However in response to SEB, the percentage frequency CD4+IFN-γ+ T cells in MAF-infected patients (2.278 ± 0.1142) was lower than the percentage frequency CD4+IFN-γ+ T cells in MTBss-infected patients (2.415 ± 0.1320) and was not statistically significant ($p=0.45$).

Likewise the percentage frequency CD4+IFN-γ+ T cells in response to rESAT-6/CFP-10 fusion protein in MAF-infected patients (1.009 ± 0.004547) though lower was statistically not significant from the percentage frequency CD4+IFN-γ+ T cells in MTBss-infected patients (1.049 ± 0.02019) ($p=0.08$) as shown in Figure 4.1.

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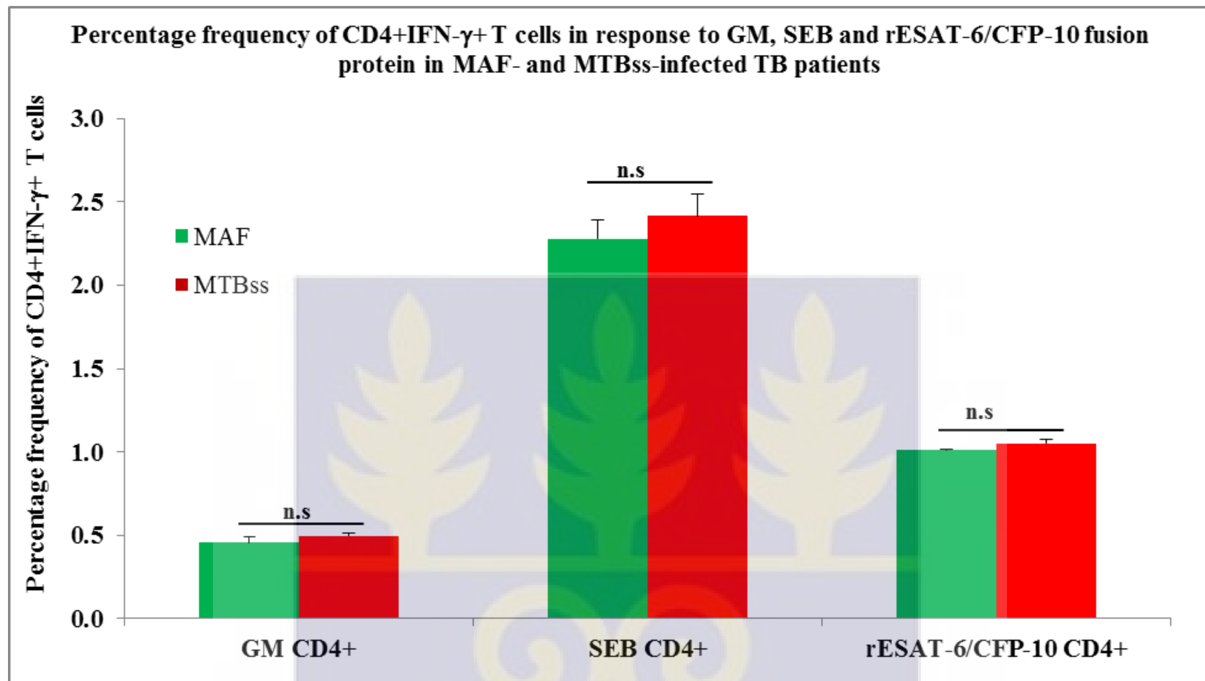
Figure 4.1 Bar graph of percentage frequency CD4+IFN-γ+ T cells in TB patients infected with 2 different strains after *in vitro* stimulation with mycobacterial antigens. Each error bar represents standard error of mean. (n.s, not significant)



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4.2.2 Percentage Frequency of CD8+IFN-γ+ T Cells Production in Response to Mycobacterial Antigens in TB Patients.

Likewise CD8+ T cell activation of the different hosts in response to testing antigens was analyzed. The percentage frequency of CD8+IFN-γ+ T cells in response to GM (negative control) in MAF-infected patients was 0.4400 ± 0.03678 while that of MTBss-infected individuals was 0.4530 ± 0.01713. This background was subtracted from the antigen-stimulated responses to yield antigen-specific IFN-γ production.

The percentage frequency of CD8+IFN-γ+ T cells in response to SEB in MAF-infected patients (1.696 ± 0.1472) was lower when compared to the percentage frequency of CD8+IFN-γ+ T cells in response in MTBss-infected patients (1.732 ± 0.1413) but the difference was not statistically significant (p=0.86).

Likewise the percentage frequency of CD8+IFN-γ+ T cells in response to rESAT-6/CFP-10 fusion protein in MAF-infected patients (0.8900 ± 0.02461) was lower than the percentage frequency of CD8+IFN-γ+ T cells in response in MTBss-infected patients 0.9080 ± 0.02843 was not statistically significant (p=0.64) as shown in Figure 4.2

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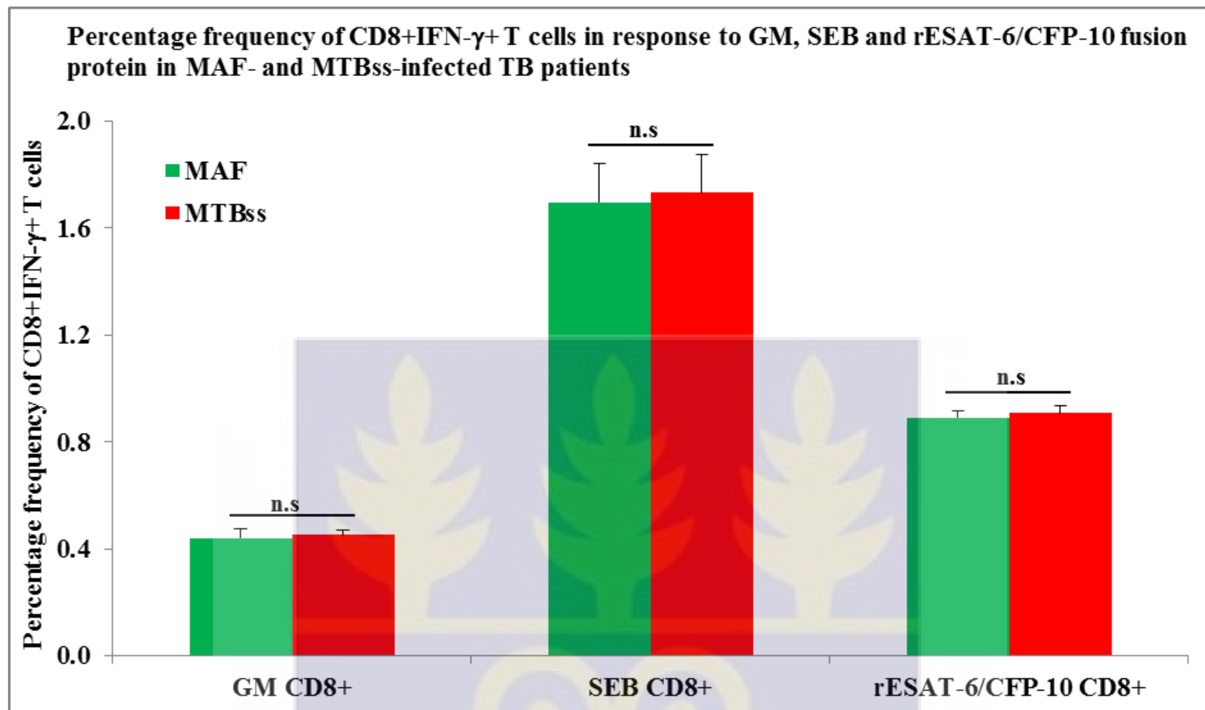
Figure 4.2 Bar graph of percentage frequency of CD8+IFN-γ+ T cells in response to 2 different hosts after *in vitro* stimulation with mycobacterial antigens. Each error bar represents SEM. (n.s, not significant).



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4.3 Mycobacterial Isolates

Mycobacterial isolates used were from a molecular epidemiological study that was sampled from the Greater-Accra and the Central Regions. The isolates were all cultured from sputum samples and have been well characterized to the three distinct lineages by IS*6110*, spoligotyping and single nucleotide polymorphism. These 3 distinct lineages of *Mycobacterium tuberculosis* complex were made up of *M. africanum* West African 1 (MAF1) (Lineage 5), *M. africanum* West African 2 (MAF2) (Lineage 6) and Lineage 4 of *M. tuberculosis* sensu stricto (MTBss). They were assayed for intracellular growth index in MDM infection model.

4.3.1 Uptake of Different Lineages by Monocyte-Derived Macrophages (MDM) at 4hrs

The MDM infection model was obtained from different adult human donors (n=6) whose PBMCs was converted to MDM by recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (Peprotech, USA) for 7 days. The initial strain uptake (after 4hrs) was expressed by the number of colony forming units (CFUs) at the 4 hour time point as a percentage of the initial bacteria inoculum i.e. 1×10^5 bacteria per milliliter.

The mean uptake of MTBss/L4 by MDM was 6.333 ± 0.2108 after 4hrs. That of MAF1/L5 was 6.167 ± 0.3073 while MAF2/L6 was 6.167 ± 0.3073. Though MTBss/L4 mean uptake was higher than MAF1/L5 (p=0.66) and MAF2/L6 (p=0.66) respectively, it was not statistically significant. Likewise MAF1/L5 and MAF2/L6 were also not statistically significant (p=1.00) as shown in Figure 4.3

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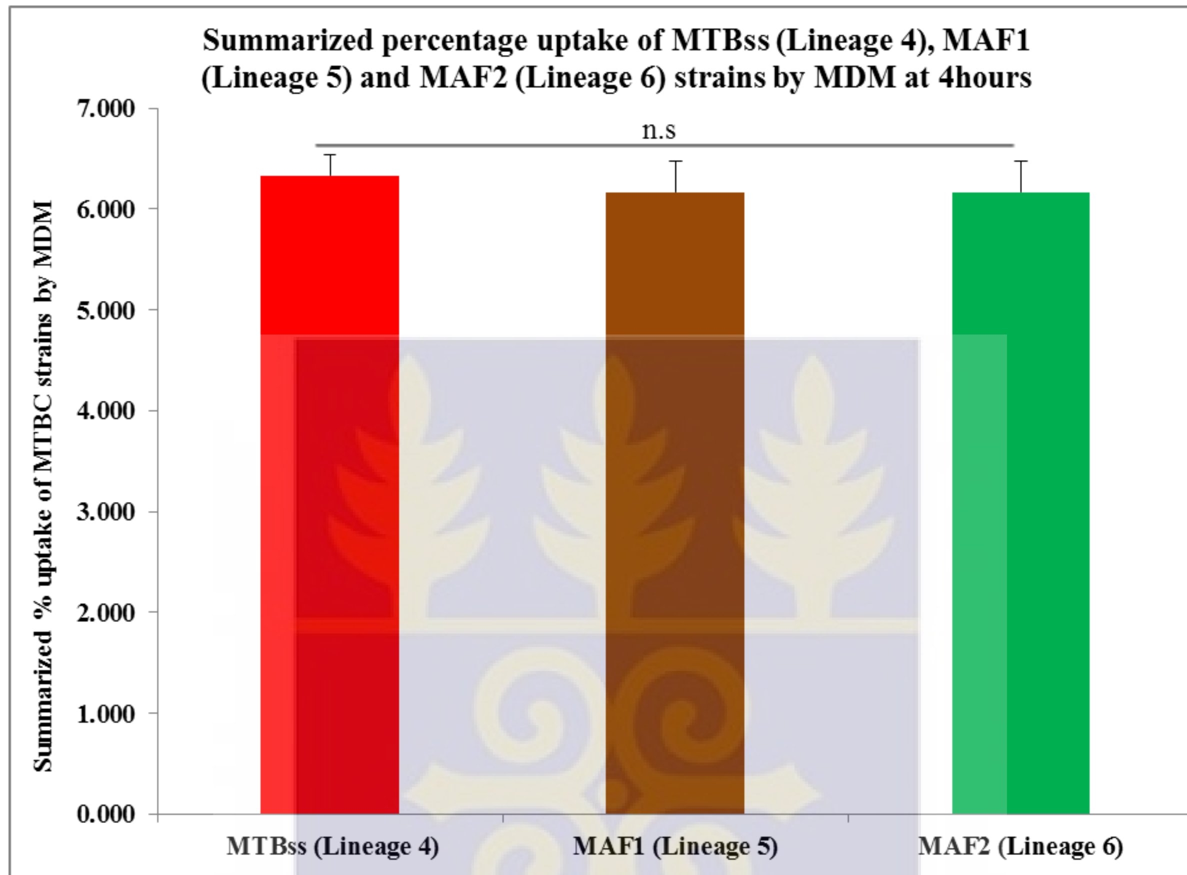
Figure 4.3 Summarize percentage uptake of the different mycobacterial isolates by MDM at 4hrs. Data represents the mean and error bars as SEM. (n.s, not significant)



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4.3.2 Intracellular Growth Index of the Three Distinct Lineages from 24hrs to 72hrs

The set of three distinct lineages made up of MTBss/L4, MAF1/L5 and MAF2/L6 were determined by their growth index in MDM. The growth index was based on calculating the Log10 [number of colony forming units (CFUs) of each strain at each time (24hrs, 48hrs, or 72hrs) point divided by the number of CFUs of that strain at its 4hrs time point].

At 24hrs, the mean growth index of MTBss/L4 was 0.5967 ± 0.02092 while MAF1/L5 was 0.3083 ± 0.04861 and MAF2/L6 0.2550 ± 0.02643. At this point the growth index of MTBss/L4 was statistically significant when compared to MAF1/L5 ($p=0.0030$) and MAF2/L6 ($p<0.0001$) respectively. In contrast the growth index of MAF1/L5 was statistically not significant when compared to MAF2/L6 ($p=0.36$).

Moreover, MTBss/L4 (0.9317 ± 0.02212) grew significantly faster over the next 48hrs when compared to MAF1/L5 (0.7033 ± 0.04544; $p=0.0011$) and more rapidly than MAF2/L6 (0.6483 ± 0.03842; $p<0.0001$). Though MAF1/L5 grew rapidly than MAF2/L6, mean growth index was not statistically significant ($p=0.3771$).

Furthermore, MTBss/L4 (1.102 ± 0.01046) strain grew significantly faster than MAF1/L5 (0.9967 ± 0.02186; $p=0.0015$) and MAF2/L6 (0.9350 ± 0.03149; $p=0.0005$) strains over the next 72hrs. Nonetheless the growth index of MAF1/L5 was higher when compared to MAF2/L6 strain at 72hrs but was not statistically significant ($p=0.1388$) as shown in Figure 4.4

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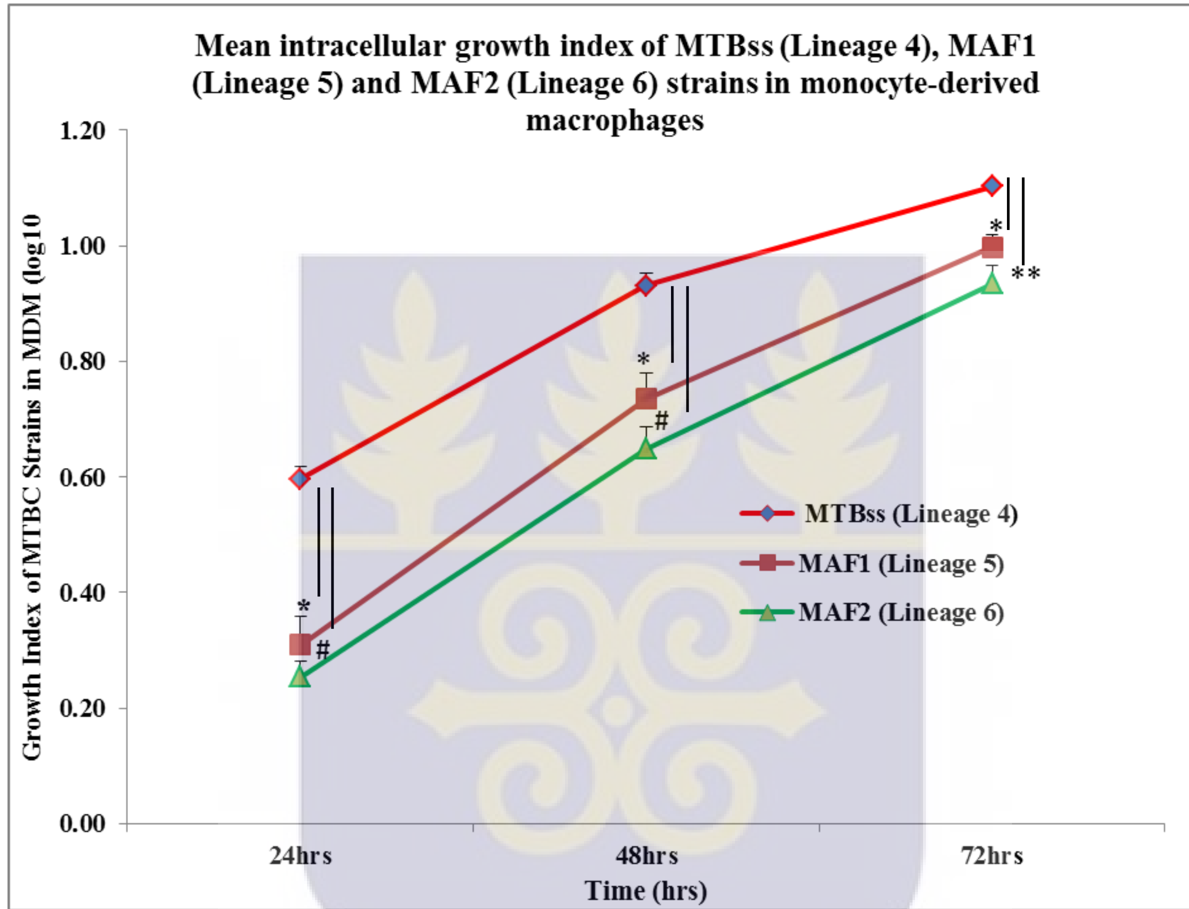
Figure 4.4 Summarized mean growth index of the distinct lineages in MDM from 24hrs to 72hrs. Data represent the mean and error bars as SEM. *p<0.01 compare to Lineage 4 at 24hrs, 48hrs and 72hrs; #p<0.0001 compared to MTBss/L4 at 24hrs, 48hrs and **p<0.01 compared to MTBss/L4 at 72hrs



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4.4 Mean Doubling Time for the Three Distinct Lineages in Monocyte-Derived Macrophages (MDM) at 4hrs and 72hrs

As an alternative approach to compare the growth rate among MTBss/L4, MAF1/L5 and MAF2/L6, the mean doubling time was determined. The mean doubling time was calculated based on the CFU at 4hrs divided by log 10 (CFU at its 72hrs) for each mycobacterial isolates.

As shown in Table 4.1, the mean doubling time of MTBss/L4 was 12.62 ± 0.065 while that of MAF1/L5 was 13.43 ± 0.115 and MAF2/L6, 13.88 ± 0.176 over the 72hrs period.

MTBss/L4 mean doubling time was statistically lower when compared to MAF1/L5 ($p=0.0001$) and MAF2/L6 ($p<0.0001$) respectively. On the contrary, though MAF1/L5 was lower when compared to MAF2/L6 the difference was not statistically significant ($p=0.0577$)

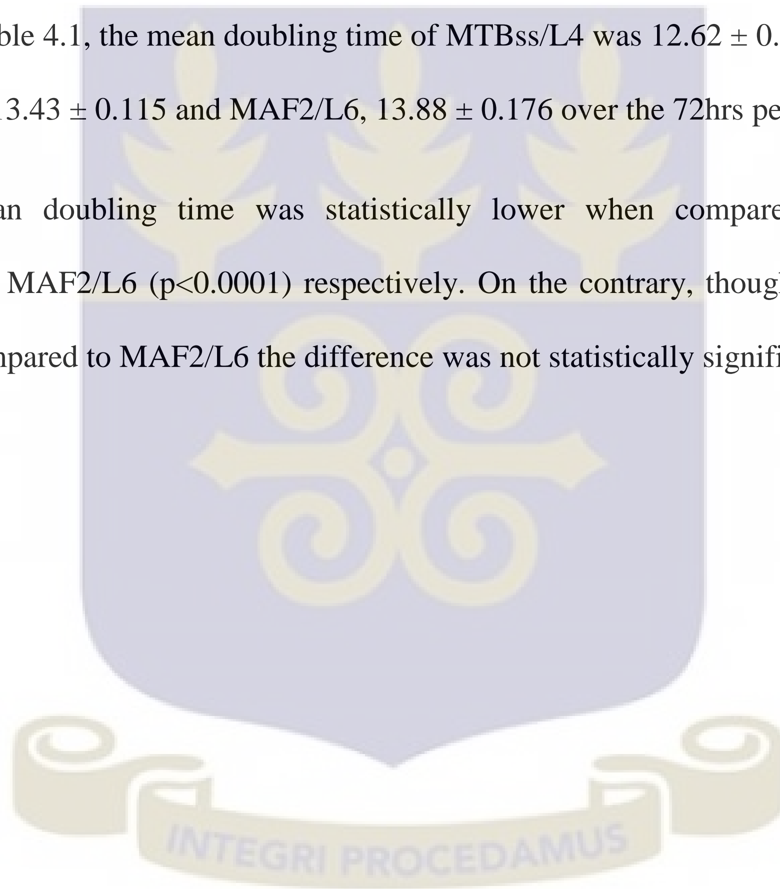

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Table 4.1 Mean doubling time for the three distinct lineages in MDM at 72hrs

			CFU X 10^3			
Nos	**Isolates**	**Lineage**	**4hrs**	**72hrs**	**DT**	**MDT±SEM**
1	TBNM094	MTBss/L4	6	72	12.9	12.62±0.065
2	TBNM103	MTBss/L4	6	78	12.7	
3	TBNM115	MTBss/L4	7	84	12.5	
4	TBNM143	MTBss/L4	7	84	12.5	
5	TBNM155	MTBss/L4	6	80	12.6	
6	TBNM167	MTBss/L4	6	82	12.5	
7	TBNM011	MAF1/L5	6	54	13.9	13.43±0.115
8	TBNM067	MAF1/L5	5	58	13.4	
9	TBNM070	MAF1/L5	6	64	13.3	
10	TBNM131	MAF1/L5	7	68	13.1	
11	TBNM135	MAF1/L5	7	58	13.6	
12	TBNM320	MAF1/L5	6	64	13.3	
13	TBNM009	MAF2/L6	6	52	14	13.88±0.176
14	TBNM176	MAF2/L6	6	60	13.5	
15	TBNM408	MAF2/L6	5	56	13.3	
16	TBNM451	MAF2/L6	6	45	14.5	
17	TBNM469	MAF2/L6	7	54	13.9	
18	TBNM802	MAF2/L6	7	50	14.1	

MTBss, *Mycobacterium tuberculosis* sensu stricto; MAF1, *Mycobacterium africanum* West African 1; MAF2, *Mycobacterium africanum* West African 2; L, Lineage; DT, Doubling time; MDT, Mean doubling time; data represented as means ± standard error of means (SEM)

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4.5 Inflammatory Cytokine Response during Mycobacterial Infection Assay

Innate immune response was measured by release of inflammatory cytokines [tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and IL-12p70] from monocyte-derived macrophages at various time points (4hrs, 24hrs, 48hrs and 72hrs) during the mycobacterial infection assay. Cytokines (TNF-α, IL-6 and IL12p70) released into harvested culture supernatants were measure by enzyme-linked immunosorbent assay (ELISA) using specific antibodies.

4.5.1 Pro-Inflammatory Cytokine (TNF-α) Released (at 4hrs, 24hrs, 48hrs, and 72hrs) in Harvested Culture Supernatants from MDM Infected Lineages

Mean levels of TNF-α released by MDM at 4hrs for MTBss/L4 was 225.5 ± 17.58 ρg/ml while that of MAF1/L5 was 242.3 ± 10.45 ρg/ml and MAF2/L6, 255.4 ± 19.59 ρg/ml respectively. However, when levels of TNF-α were compared between them, it was found not to be statistically significant; MTBss/L4 vs. MAF1/L5, ($p=0.4287$); MTBss/L4 vs. MAF2/L6, ($p=0.2817$) and MAF1/L5 vs. MAF2/L6, ($p=0.5688$).

At 24hrs mean levels of TNF-α induced by MAF1/L5 (677.4 ± 16.51 ρg/ml) increased and were significantly higher compared to MTBss/L4 (446.9 ± 18.76 ρg/ml, $p<0.0001$). Similarly levels of TNF-α elicited by MAF2/L6 (710.7 ± 23.35 ρg/ml) were also significantly higher when compared to MTBss/L4 ($p<0.001$). Although levels of TNF-α released by MAF2/L6 were higher when compared to MAF1/L5, it was not statistically significant ($p=0.2709$).

Likewise at 48hrs mean levels of TNF-α elicited by MAF1/L5 (1007 ± 29.65 ρg/ml) further increased and was significantly higher when compared to MTBss/L4 (650.2 ± 31.17 ρg/ml, $p<0.0001$). Similarly levels of TNF-α induced by MAF2/L6 (1073 ± 26.41 ρg/ml) was also significantly higher when compared to MTBss/L4 ($p<0.0001$), although levels of TNF-α

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induced by MAF2/L6 was higher when compared to MAF1/L5 it was not statistically significant (p=0.1282).

Similarly, at 72hrs the mean levels of TNF-α induced by MAF1/L5 (1335 ± 97.12 ρg/ml) was significantly higher compared to MTBss/L4 (926.3 ± 11.87 ρg/ml, p=0.0019). While levels of TNF-α released by MAF2/L6 (1495 ± 40.63 ρg/ml) was also significantly higher when compared to MTBss/L4 (p<0.0001), levels of TNF-α elicited by MAF2/L6 was higher when compared to MAF1/L5, which were not statistically significant (p=0.1606) as shown in Figure 4.5

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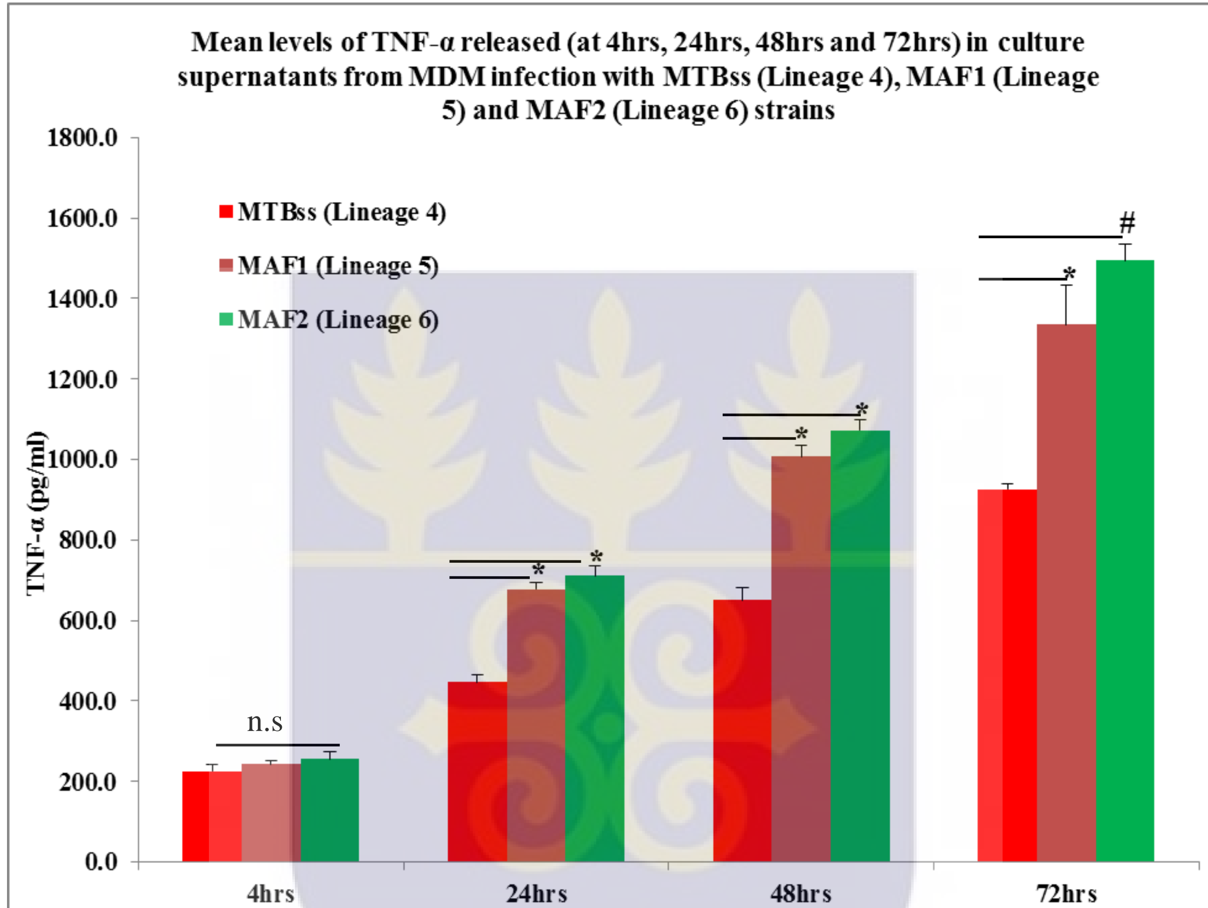
Figure 4.5 Mean levels of TNF-α released (at 4hrs, 24hrs, 48hrs and 72hrs) in culture supernatants from MDM infection with distinct lineages. Graph of data represents mean ± SEM. *p<0.0001 compared to Lineage 4 at 24hrs, 48hrs and 72hrs; #p<0.01 compared to Lineage 4 at 72hrs. (n.s, not significant)



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4.5.2 Pro-Inflammatory Cytokine (IL-6) Detected (at 4hrs, 24hrs, 48hrs, and 72hrs) in Harvested Culture Supernatants from MDM Infected Lineages

At 4hrs, the mean levels of IL-6 released by MTBss/L4 (143.0 ± 8.351 ρg/ml) was a bit lower than that from MAF1/L5 (160.3 ± 1.673 ρg/ml, p=0.0692) and MAF2/L6 (166.9 ± 21.26, p=0.3203) while levels of IL-6 released by MAF2/L6 was comparable to MAF1/L5 (p=0.7650).

However, at 24hrs the mean levels of IL-6 induced by MAF1/L5 (1687 ± 204.3 ρg/ml) was significantly higher compared to MTBss/L4 (1151 ± 33.61 ρg/ml, p=0.0271). Likewise levels of IL-6 released by MAF2/L6 (1727 ± 192.5 ρg/ml) was also significantly higher when compared to MTBss/L4 (p=0.0145). Although levels of IL-6 released by MAF2/L6 was higher when compared to MAF1/L5, it was not statistically significant (p=0.8871).

Likewise at 48hrs the mean levels of IL-6 induced by MAF1/L5 (2556 ± 206.7 ρg/ml) was significantly higher compared to MTBss/L4 (1924 ± 178.3 ρg/ml) (p=0.0434). Similarly levels of IL-6 elicited by MAF2/L6 (2568 ± 161.2 ρg/ml) was also significantly higher when compared to MTBss/L4 (p=0.0233). Mean levels of IL-6 of MAF2/L6 were comparable to MAF1/L5 (p=0.9643).

Also at 72hrs the mean levels of IL-6 induced by MAF1/L5 (3323 ± 136.0 ρg/ml) was significantly higher compared to MTBss/L4 (2517 ± 281.9 ρg/ml, p=0.0277). Likewise levels of IL-6 elicited by MAF2/L6 (3654 ± 166.6 ρg/ml) was also significantly higher when compared to MTBss/L4 (p=0.0060). Although levels of IL-6 released by MAF2/L6 was higher when compared to MAF1/L5, it was not statistically significant (p=0.1549) as shown in Figure 4.6.

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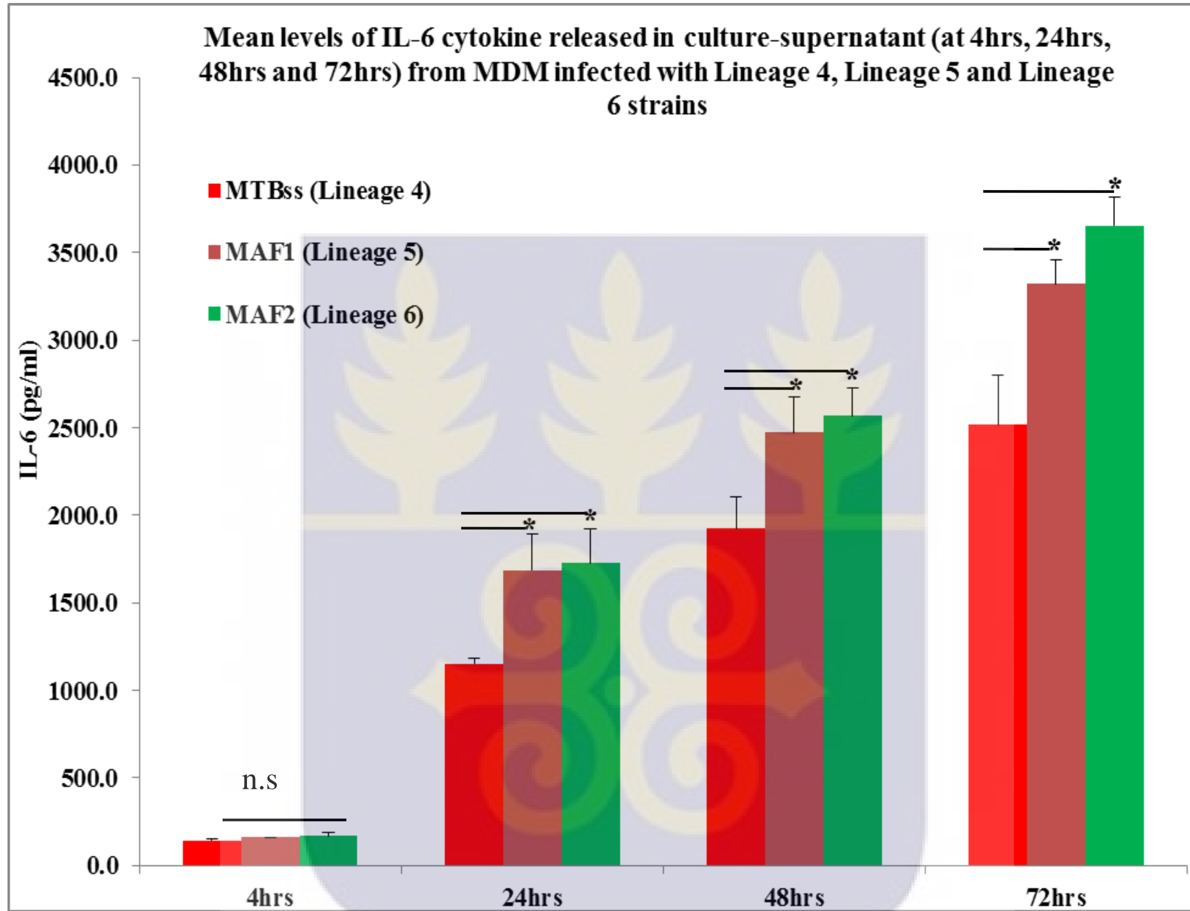
Figure 4.6 Mean levels of IL-6 detected in culture supernatants (at 4hrs, 24hrs, 48hrs and 72hrs) from MDM infection with distinct lineages. Graph of data represents mean ± SEM. *$p<0.05$ compared to Lineage 4 at 24hrs, 48hrs and 72hrs. (n.s, not significant).



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4.5.3 Pro-Inflammatory Cytokine (IL-12p70) Released (at 4hrs, 24hrs, 48hrs, and 72hrs) in Harvested Culture Supernatants from MDM Infected Lineages

At 4hrs mean levels of IL-12p70 released by MTBss/L4 (171.6 ± 11.74 ρg/ml) was not different from MAF1/L5 (186.3 ± 11.96 ρg/ml, p=0.4026) and MAF2/L6 (197.7 ± 12.98, p=0.1665). Though levels of IL-12p70 released by MAF2/L6 was higher compared to MAF1/L5, it was not statistically significant (p=0.0531).

Nevertheless at 24hrs mean levels of IL-12p70 induced by MAF1/L5 (1362 ± 42.27 ρg/ml) was significantly higher compared to MTBss/L4 (1044 ± 17.70 ρg/ml, p<0.0001). Likewise levels of IL-12p70 released by MAF2/L6 (1562 ± 79.67 ρg/ml) was also significantly higher when compared to MTBss/L4 (p<0.0001). In contrast, levels of IL-12p70 released by MAF2/L6 though higher when compared to MAF1/L5 it was not statistically significant (p=0.0506)

Likewise at 48hrs mean levels of IL-12p70 induced by MAF1/L5 (1964 ± 117.1 ρg/ml) was also significantly higher compared to MTBss/L4 (1237 ± 61.79 ρg/ml) (p=0.0003). Levels of IL-12p70 released by MAF2/L6 (2086 ± 175.3 ρg/ml) was also significantly higher when compared to MTBss/L4 (p=0.0010).

Similarly at 72hrs mean levels of IL-12p70 elicited by MAF1/L5 (2673 ± 70.43 ρg/ml) was significantly higher compared to MTBss/L4 (2211 ± 49.66 ρg/ml, p=0.0003). Likewise levels of IL-12p70 induced by MAF2/L6 (2754 ± 88.12 ρg/ml), was also significantly higher when compared to MTBss/L4 (p=0.0003). In contrast levels of IL-12p70 released by MAF2/L6 was statistically not significant from MAF1/L5 (p=0.4874) as shown in Figure 4.7.

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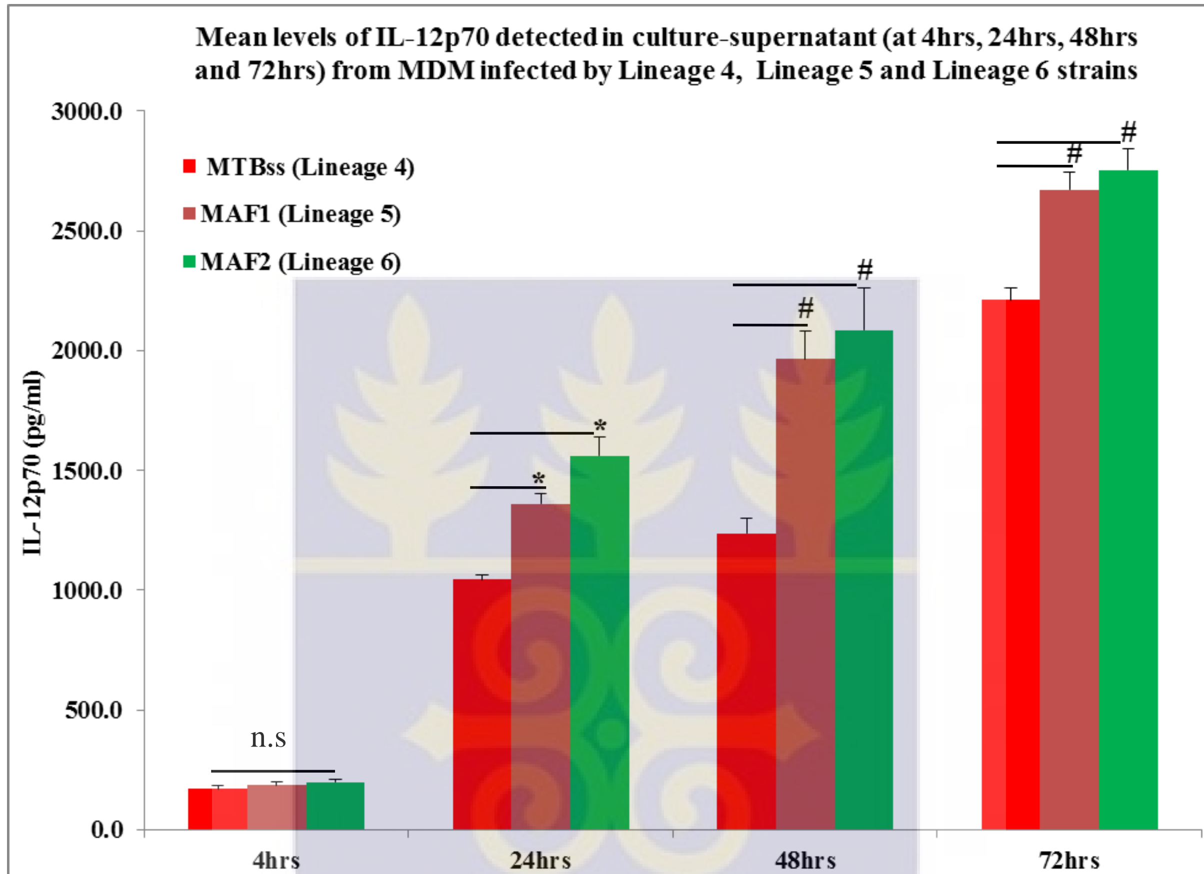
Figure 4.7 Mean levels of IL-12p70 detected in culture supernatants (at 4hrs, 24hrs, 48hrs, and 72hrs) from MDM infection with distinct lineages. Graph of data represents mean ± SEM. *p<0.0001 compared to Lineage 4 at 24hrs; #p<0.01 compared to Lineage 4 at 48hrs and 72hrs. (n.s, not significant)



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4.6 Uptake of MTBss (modern) and MAF (ancient) Strains by MDM at 4hrs

The mean uptake of MTBss (modern) isolates by MDM was 6.667 ± 0.2108 after 4hrs, while that of MAF (ancient) isolates was 6.083 ± 0.2876. Though MTBss (modern) strains mean uptake was higher than MAF (ancient) strains, it was not statistically significant (p=0.2011) as shown in Figure 4.8

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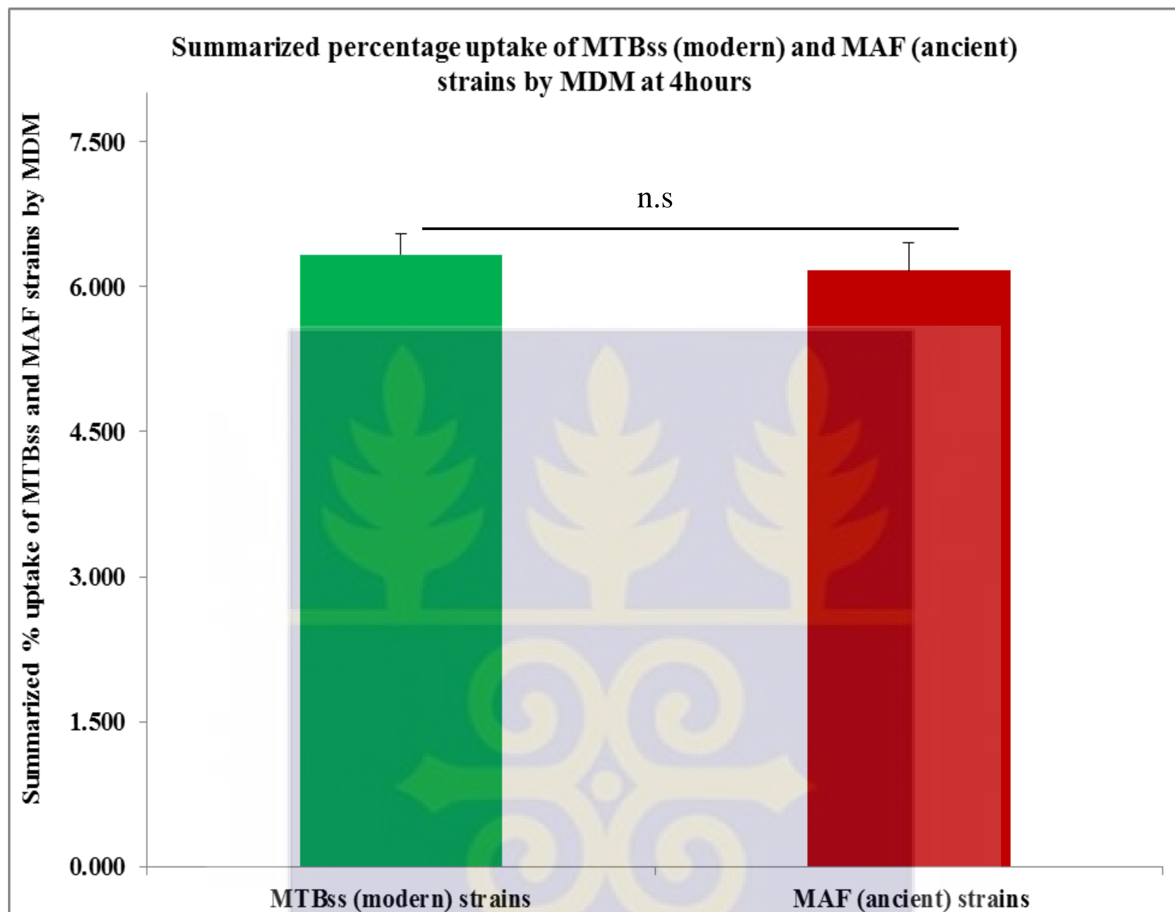
Figure 4 8 Summarize percentage uptake of MTBss (modern) and MAF (ancient) isolates by MDM at 4hrs. Data represents the mean and error bars as SEM. (n s, not significant).



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4.6.1 Mean Intracellular Growth Index of MTBss (modern) and MAF (ancient) Strains from 24hrs to 72hrs

The intracellular growth index was compared between MAF (ancient) and MTBss (modern) strains. The intracellular growth index of MTBss (modern) strains at 24hrs were (0.5967 ± 0.02092) while that of MAF (ancient) strains was 0.3217 ± 0.05879. The growth index of MTBss (modern) strains was statistically faster when compared to MAF (ancient) strains (p=0.0013). Moreover, MTBss (modern) strains increased significantly by 0.9317 ± 0.02212 at 48hrs as compared with MAF (ancient) strains growth index of 0.7083 ± 0.04840 (p=0.0018) and at 72hrs MTBss (modern) strains further increased significantly by 1.102 ± 0.01046 as compared with MAF (ancient) strains growth index of 1.002 ± 0.02509 (p=0.0043) as shown in Figure 4.9

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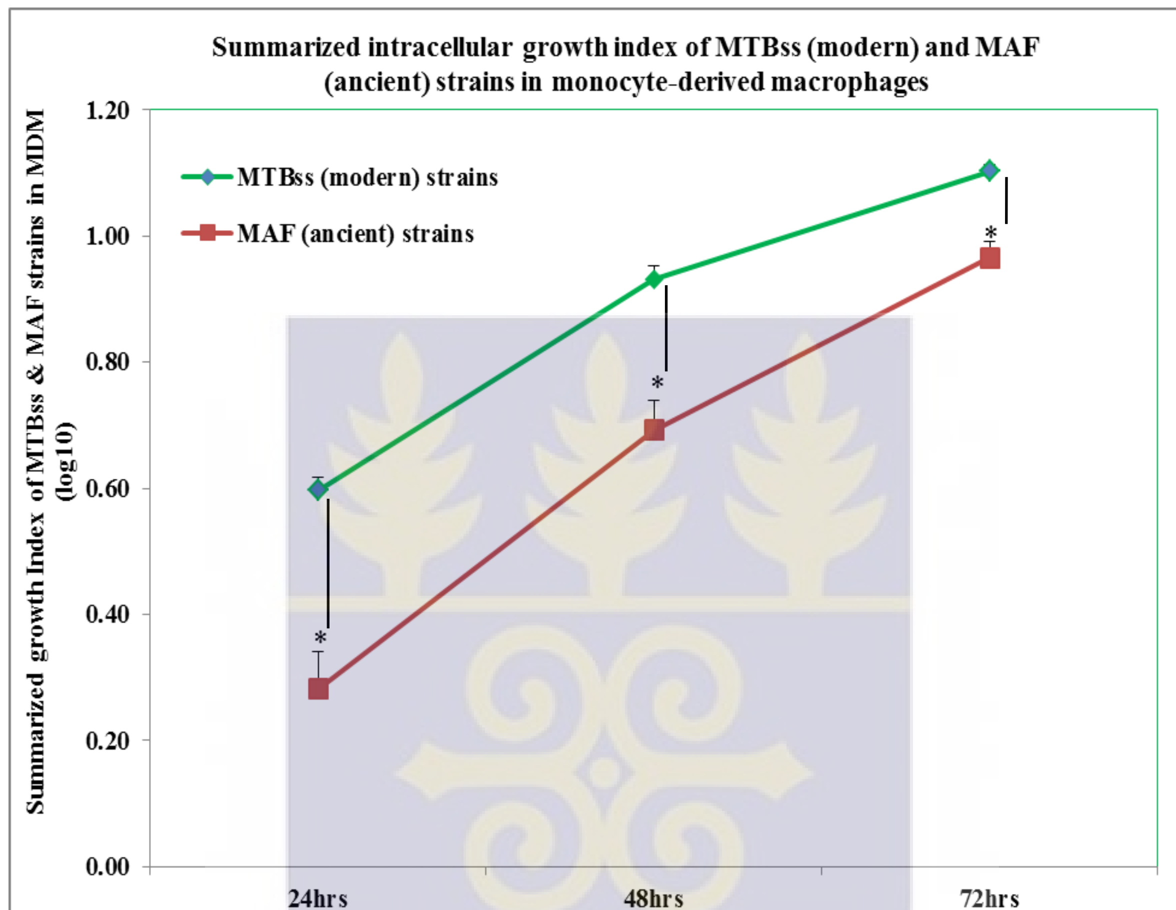
Figure 4.9 Summarized intracellular growth index of MTBss (modern) and MAF (ancient) strains from 24hrs to 72hrs. Graph of data represents mean ± SEM. *p<0.01 compared to modern strains at 24hrs, 48hrs and 72hrs.



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4.7 Mean Doubling Time for MTBss (modern) and MAF (ancient) Strains at 4hrs and 72hrs

Likewise the mean doubling time between MTBss (modern) and MAF (ancient) strains was also determined

As shown in Table 4.3, the mean doubling time of MAF (ancient) strains was 13.66 ± 0.121 while that of MTBss (modern) strains was 2.62 ± 0.065. Thus the doubling time of MTBss (modern) strains was significantly lower than MAF (ancient) strains over the 72hrs period ($p<0.0001$).

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Table 4.3 Mean doubling time for MTBss (modern) and MAF (ancient) strains in MDM at 72hrs

			CFU X 10^3			
Nos	**Isolates**	**Lineage**	**4hrs**	**72hrs**	**DT**	**MDT±SEM**
1	**TBNM094**	**MTBss (modern) strains**	**6**	**72**	**12.9**	**12.62±0.065**
2	**TBNM103**		**6**	**78**	**12.7**	
3	**TBNM115**		**7**	**84**	**12.5**	
4	**TBNM143**		**7**	**84**	**12.5**	
5	**TBNM155**		**6**	**80**	**12.6**	
6	**TBNM167**		**6**	**82**	**12.5**	
7	**TBNM011**	**MAF (ancient) strains**	**6**	**54**	**13.9**	**13.66±0.121**
8	**TBNM067**		**5**	**58**	**13.4**	
9	**TBNM070**		**6**	**64**	**13.3**	
10	**TBNM131**		**7**	**68**	**13.1**	
11	**TBNM135**		**7**	**58**	**13.6**	
12	**TBNM320**		**6**	**64**	**13.3**	
13	**TBNM009**		**6**	**52**	**14**	
14	**TBNM176**		**6**	**60**	**13.5**	
15	**TBNM408**		**5**	**56**	**13.3**	
16	**TBNM451**		**6**	**45**	**14.5**	
17	**TBNM469**		**7**	**54**	**13.9**	
18	**TBNM802**		**7**	**50**	**14.1**	

MTBss, *Mycobacterium tuberculosis* sensu stricto; MAF, *Mycobacterium africanum*; DT, Doubling time; MDT, Mean doubling time; data represented as means ± standard error of means (SEM)

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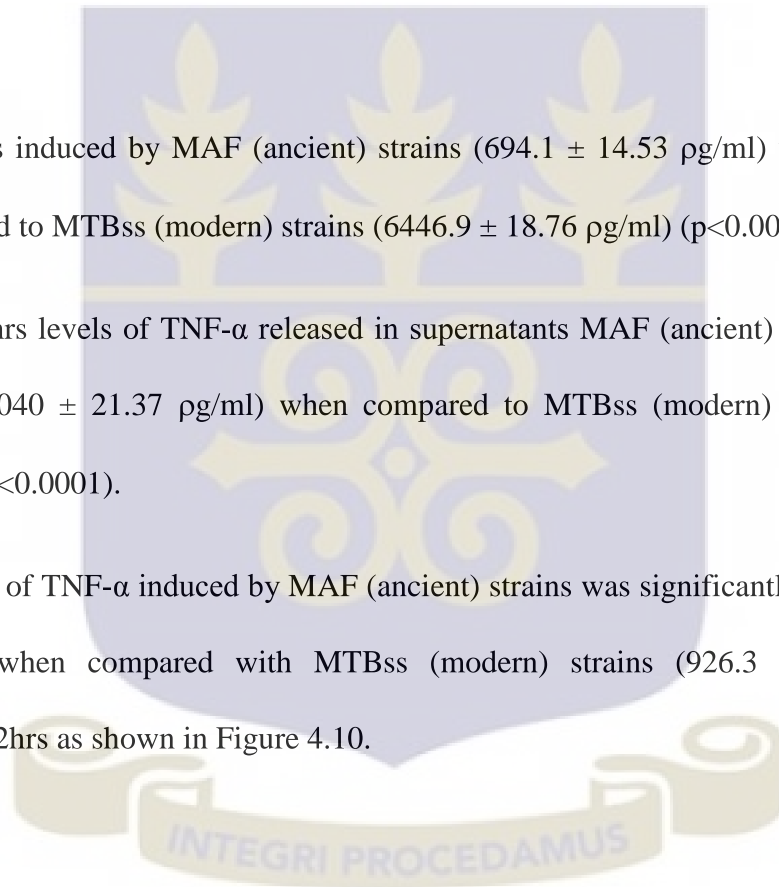
4.8 Inflammatory Cytokines (TNF-α, IL-6 and IL-12p70) Released at Various Time Points by MTBss (modern) and MAF (ancient) Strains.

4.8.1 Levels of TNF-α Induced at Various Time points (at 4hrs, 24hrs, 48hrs, and 72hrs) from MDM Infected with MTBss (modern) and MAF (ancient) Strains.

At 4hrs levels of TNF-α released by MAF (ancient) strains (248.9 ± 10.77 ρg/ml) was statistically not significant when compared to MTBss (modern) strains (225.5 ± 17.58 ρg/ml) ($p=0.2496$).



At 24hrs, levels induced by MAF (ancient) strains (694.1 ± 14.53 ρg/ml) was significantly higher compared to MTBss (modern) strains (6446.9 ± 18.76 ρg/ml) ($p<0.0001$).

Likewise at 48hrs levels of TNF-α released in supernatants MAF (ancient) strains increased significantly (1040 ± 21.37 ρg/ml) when compared to MTBss (modern) strains (650.2 ± 31.17 ρg/ml) ($p<0.0001$).

Similarly levels of TNF-α induced by MAF (ancient) strains was significantly higher (1415 ± 55.66 ρg/ml) when compared with MTBss (modern) strains (926.3 ± 11.87 ρg/ml) ($p<0.0001$) at 72hrs as shown in Figure 4.10.

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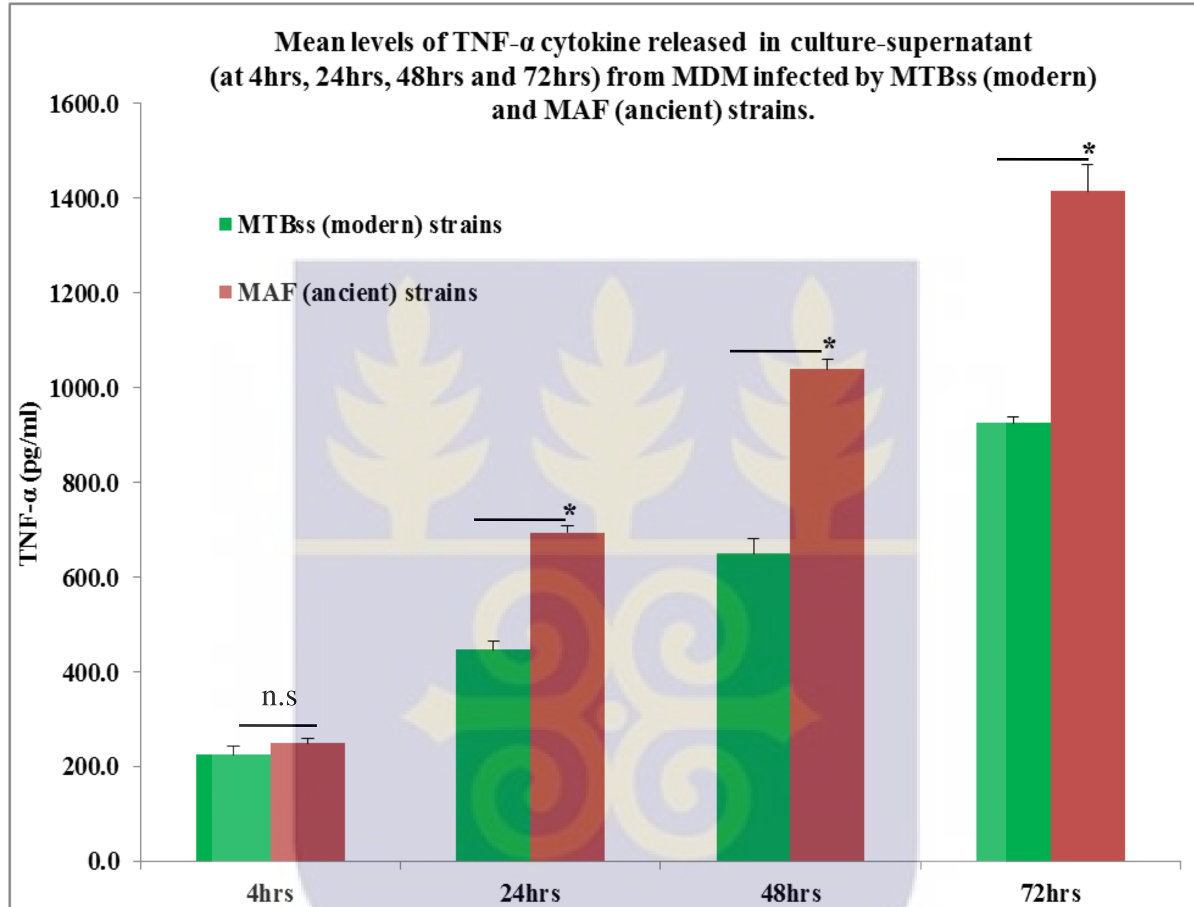
Figure 4.10 Mean levels of TNF-α released at various time points (4hrs, 24hrs, 48hrs and 72hrs) from MDM infected with MTBss (modern) and MAF (ancient) strains. Graph of data represents mean ± SEM. *$p<0.0001$ compared to MTBss (modern) strains at 24hrs, 48hrs and 72hrs. (n.s, not significant).



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4.8.2 Levels of IL-6 Elicited at Various Time Points (at 4hrs, 24hrs, 48hrs, and 72hrs) from MDM Infected with MTBss (modern) and* MAF (ancient)*) Strains.

At 4hrs levels of IL-6 released by MAF (ancient) strains (163.6 ± 10.21 ρg/ml) was statistically not significant when compared to MTBss (modern) strains (143.0 ± 8.351 ρg/ml; p=0.2090).

At 24hrs, levels of IL-6 induced by MAF (ancient) strains (1151 ± 33.61 ρg/ml) was significantly higher compared to MTBss (modern) strains (6446.9 ± 18.76 ρg/ml; p=0.0111).

Likewise at 48hrs levels of IL-6 detected in supernatants for MAF (ancient) strains increased significantly (2562 ± 125.0 ρg/ml) when compared to MTBss (modern) strains (1924 ± 178.3 ρg/ml; p=0.0097).

Similarly at 72hrs levels of IL-6 elicited by MAF (ancient) strains was significantly higher (3489 ± 114.0 ρg/ml) when compared with MTBss (modern) strains (2517 ± 281.9 ρg/ml; p=0.0015) as shown in Figure 4.11.

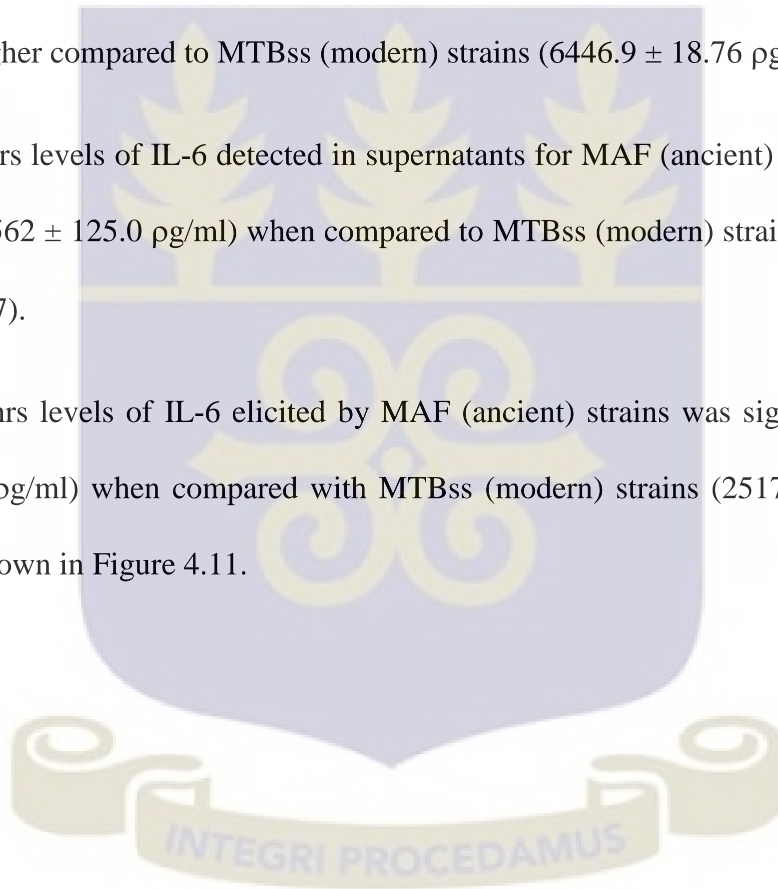

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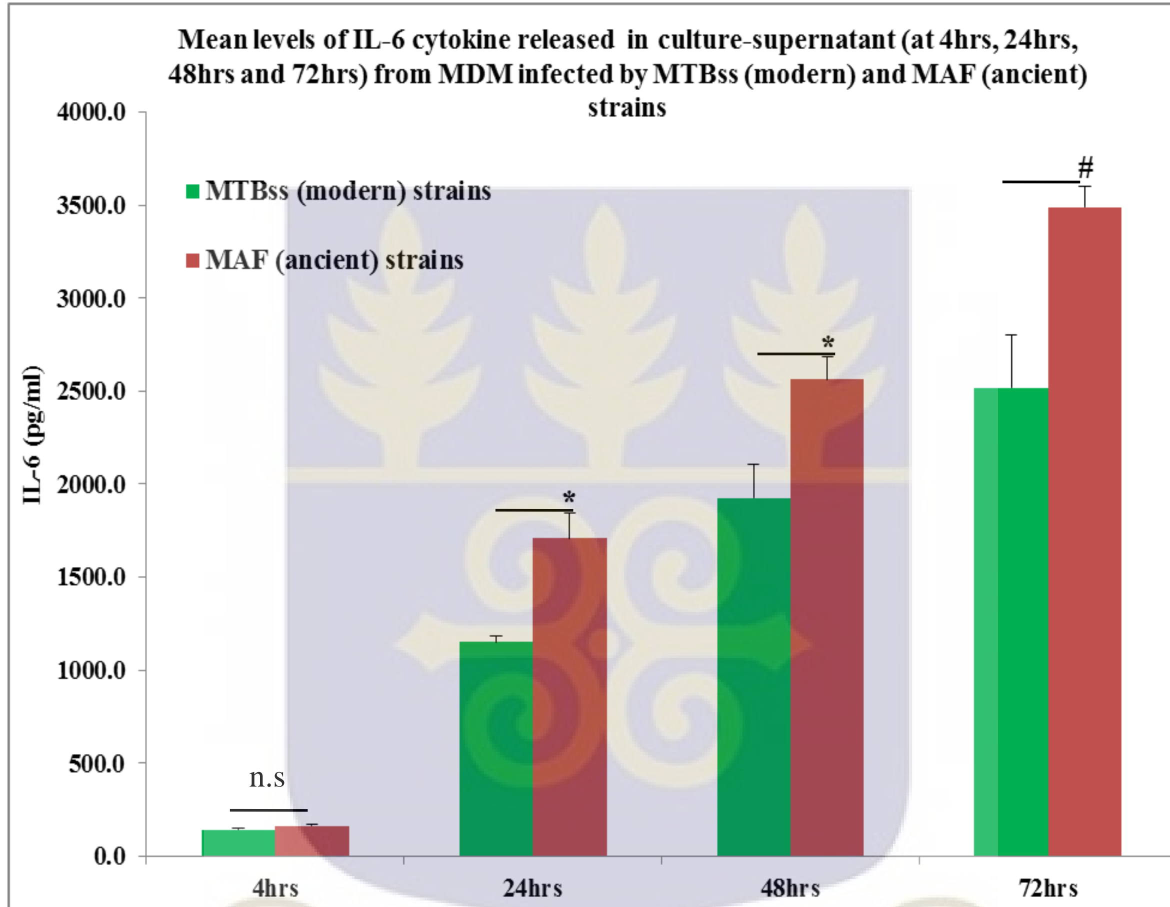
Figure 4.11 Mean levels of IL-6 released at various time points (4hrs, 24hrs, 48hrs and 72hrs) by MDM infected with MTBss (modern) and MAF (ancient) strains. Graph of data represents mean ± SEM. *p<0.05 compared to MTBss (modern) at 24hrs and 48hrs; #p<0.01 compared to MTBss (modern) at 72hrs. (n.s, not significant).



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4.8.3 Levels of IL-12p70 Released at Various Time Points (at 4hrs, 24hrs, 48hrs, and 72hrs) from MDM Infected with MTBss (modern) and MAF (ancient) Strains

At 4hrs levels of IL-12p70 released by MAF (ancient) strains (192.0 ± 8.591 ρg/ml) was not statistically significant when compared to MTBss (modern) strains (171.6 ± 11.74 ρg/ml; $p=0.1853$).

At 24hrs, levels of IL-2p70 released by MAF (ancient) strains (1462 ± 52.54 ρg/ml) was significantly higher compared to MTBss (modern) strains (1044 ± 17.70 ρg/ml; $p<0.0001$).

Likewise at 48hrs levels of IL-12p70 detected in supernatants for MAF (ancient) strains increased significantly (2025 ± 102.1 ρg/ml) when compared to MTBss (modern) strains (1237 ± 61.79 ρg/ml; $p<0.0001$).

Similarly at 72hrs levels of IL-12p70 induced by MAF (ancient) strains was significantly higher (2713 ± 55.16 ρg/ml) when compared with MTBss (modern) strains (2211 ± 49.66 ρg/ml; $p<0.0001$) as shown in Figure 4.12.

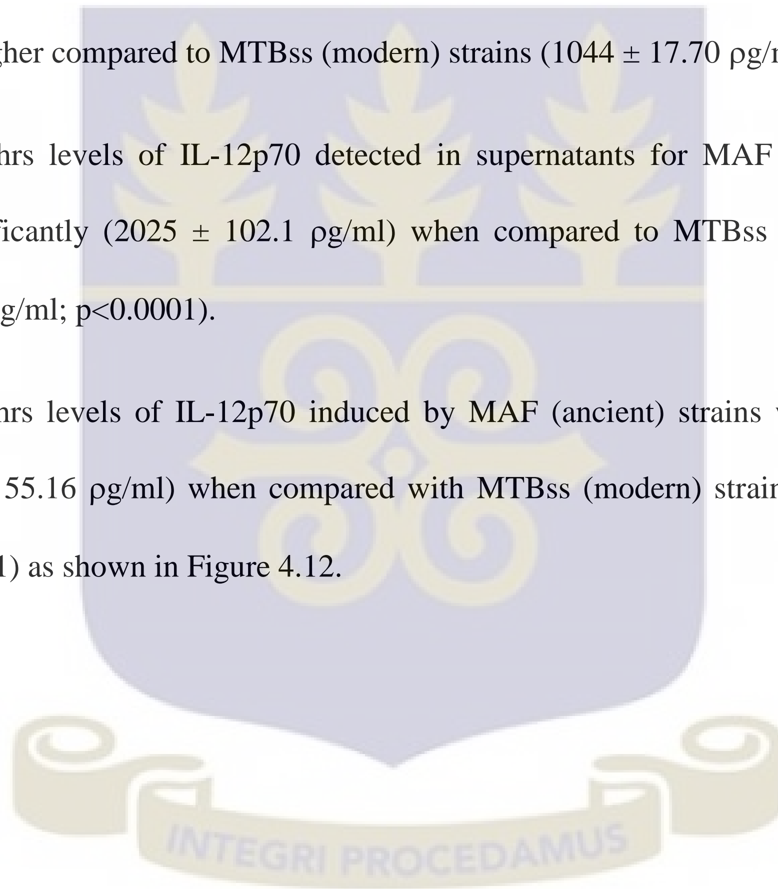

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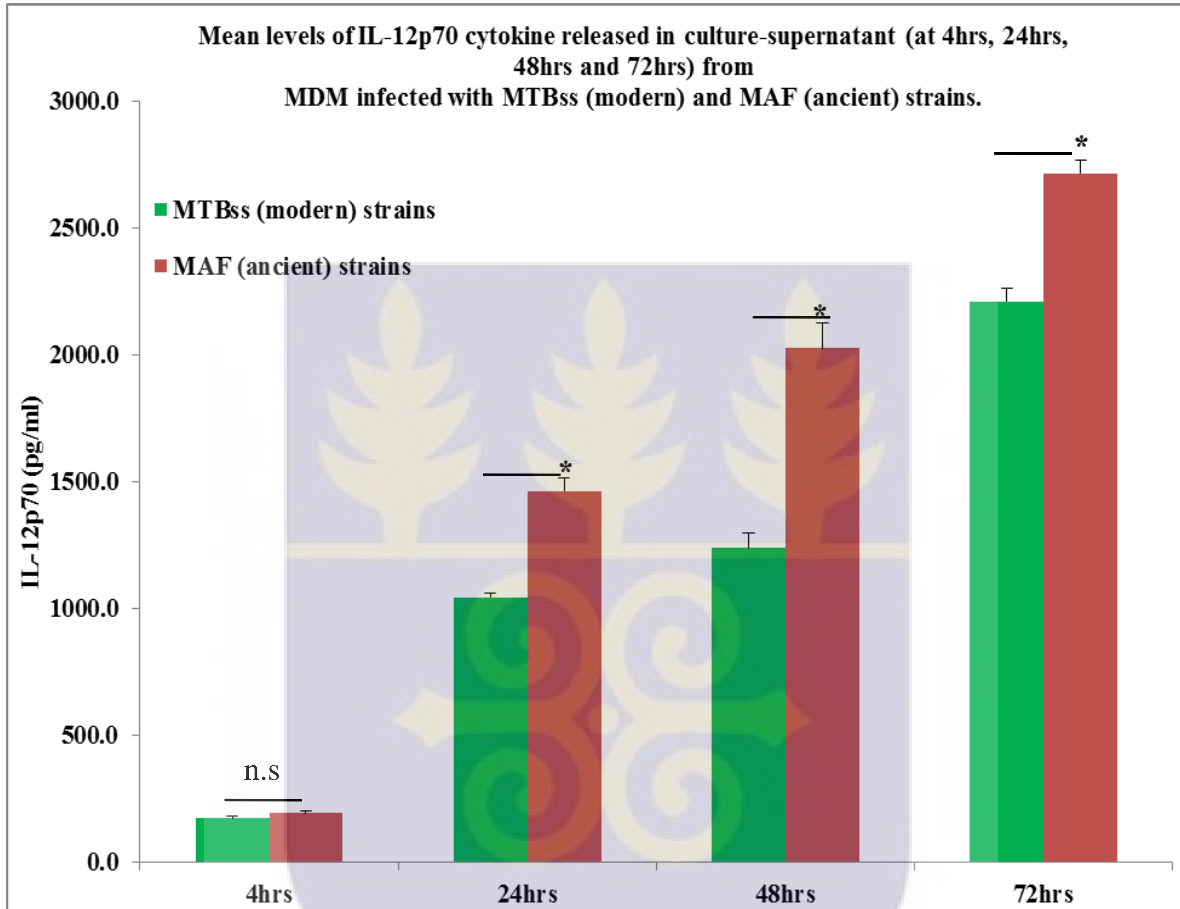
Figure 4.12 Mean levels of IL-12p70 released at various time points (4hrs, 24hrs, 48hrs and 72hrs) by MDM infected with MTBss (modern) and MAF (ancient) strains. Graph of data represents mean ± SEM *p<0.0001 compared to MTBss (modern) at 24hrs, 48hrs and 72hrs. (n.s, not significant).



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CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 Discussion

This study utilized immunological and bacteriological methods to analyze the phenotypic characteristics of host – pathogen interaction in *Mycobacterium africanum* (MAF). The phenotypic variation that includes immunogenicity, intracellular growth rate and innate immune phenotypes was assessed in the two lineages within MAF (MAF1/L5 and MAF2/L6) and compared to MTBss/L4.

The main findings for this study are; 1) MAF-infected patients had similar percentage frequencies of CD4+IFN-γ+ and CD8+IFN-γ+ T cell response to rESAT-6/CFP-10 fusion protein compared to MTB-infected individuals, 2) uptake of MAF strains by MDM was reduced and lower significantly as compared to MTBss, 3) virulence of MAF was slower significantly as compared to MTBss, 4) while the immune phenotype of MAF had significant hyper-inflammatory response with slow growth compared MTBss. Our findings show that MAF however have an attenuated response with rESAT-6/CFP-10 fusion protein; lower virulence and a slow growth phenotype were associated with MAF. Thus MAF may be considered to have a longer latency within the host furthermore leading to slower rate of progression to active disease.

The rESAT-6/CFP-10 fusion protein is a dominant IFN-γ inducing antigens of live and actively metabolizing MTB. The genes of ESAT-6 and CFP-10 are noted to be co-transcribed in *M. tuberculosis* (Berthet *et al*., 1998); when co-expressed in *E. coli*, they form a tight 1:1 complex (Renshaw *et al*., 2002). These antigens are extracellular secretory proteins with low molecular weight, encoded by the RD1 genomic region of virulent strains of MTB, *M. bovis*

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but absent from *M. bovis* Bacillus Calmette-Guerin (BCG) sub-strains and most environmental mycobacteria (Mahairas *et al.,* 1996; Berthet *et al.,* 1998; Behr *et al.,* 1999; Brosch *et al.,* 2002). The RD1 region is associated with virulence of MTB (Brodin *et al.,* 2006; Guinn *et al.,* 2004) and is effective stimulators of T cells (Horwitz *et al.,* 1995; Kamath *et al.,* 1999; Brandt *et al.,* 2000). They have been developed and are widely utilized for the diagnosis of TB because they are more specific than conventional tuberculin skin tests (Pai *et al.,* 2004). The T subset (CD4+ and CD8+) of MAF- and MTB-infected patient response to rESAT-6/CFP-10 fusion protein was analyzed to characterize its immunogenic difference. The finding from this study showed that the percentage frequency CD4+ IFN-γ+ T cells of MAF-infected patient were similar when compared to MTB-infected patients. Likewise a similar finding was observed for CD8+ T cells of MAF-infected patients when compared to MTB-infected patients. This is consistent with the studies by Bold *et al.,* 2012. However, de Jong *et al.,* 2006 showed that MAF-infected individuals had attenuated T cell response to the secreted and virulent factor ESAT-6. This attenuated T cell response of MAF-infected individuals was attributed to polymorphism in the ESAT-6 secretory proteins, lower amount of secretion by the secretory proteins, loss of RD8 gene and deletion of mammalian cell entry gene operon (Brodin *et al.,* 2006). However, the increase T cell responses of MTB-infected individuals may be due to genes of ESAT-6 and CFP-10 which are co-transcribed in MTB (Berthet *et al*., 1998). As the RD1 region is associated with virulence of MTB (Brodin *et al.,* 2006; Guinn *et al.,* 2004) and effective stimulators of T cells (Horwitz *et al.,* 1995; Kamath *et al.,* 1999; Brandt *et al.,* 2000), they have been developed and are widely utilized for the diagnosis of latent TB in interferon gamma releasing assays (IGRAs) as an alternative to TST. However, IGRAs cannot discriminate latent TB from active disease. Since IGRAs are unable to distinguish between LTBI and active TB, they will always have poor specificity in areas with high prevalence of LTBI. Consequently the attenuated rESAT-6/CFP-10 response

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induced by MAF against MTB antigens necessitates that a new generation of TB diagnostics and vaccines be developed from both MTB and MAF antigens to ensure efficacy in *M. africanum*–endemic areas. It should be noted that ESAT-6 being one of the key antigens in the development of new diagnostics and vaccines may have important implications in *M. africanum*–endemic areas (Doherty & Andersen, 2005; Pai *et al.,* 2004). Thus developing a universally effective vaccine which comprises of both MAF and MTB antigens might be the only way to eliminate TB in the future including MAF (Young *et al.,* 2008).

Macrophages are the major population of tissue-resident mononuclear phagocytes and they are predominant targets for infection by intracellular pathogens including *M. tuberculosis*. They contribute to cell-mediated immunity and bacterial elimination but also provide an essential niche for intracellular bacterial survival and escape from host defense mechanisms (van Crevel *et al.,* 2002). In humans, large numbers of alveolar macrophages are not readily available for *in vitro* study, therefore matured human MDM derived from PBMCs are used as an *in vitro* model to analyze the virulence of *M. tuberculosis* infection in human cells (Gordon & Taylor, 2005). The macrophage model has the advantage of allowing us to study the interaction of *M. tuberculosis* within the host in the early phase of human infection (van Crevel *et al.,* 2002). *In vitro* assays of virulence that reflect the pathogenesis of tuberculosis in humans are required to understand disease dynamics. Intracellular mycobacterial growth (Silver *et al.,* 1998), cytokine expression (Falcone *et al.,* 1994), or combinations of the two parameters (Manca *et al.,* 1999) have been used as a measure of virulence in isolated macrophages. However, there is evidence of variation in phagocytosis by different strains (Torrelles *et al.,* 2008). Numerous receptors expressed by phagocytic cells, namely the C-type lectin receptors (CLRs), scavenger receptors (SRs), and complement receptors (CRs), bind mycobacteria (Philips & Ernst, 2012). Many ligands present within the mycobacterial outer surface tend to engage host receptors and activate multiple pathways of recognition and

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signaling leading to the induction of innate immune response (Mourao-Sa *et al.,* 2013). For example Schlesinger L (1993) found that phagocytosis of two virulent strains (H37Rv and Erdman) by human MDM is mediated by the mannose receptor in addition to receptor (CR1, CR3 and CR4) whereas the avirulent strain H37Ra uses only complement receptor for phagocytosis. This study shows that the initial uptake of MTBss/L4, MAF1/L5 and MAF2/L6 by human MDM at 4hrs was similar for all clinical isolates as well as MTBss (modern) and MAF (ancient) strains. This initial uptake of the clinical strains may have been due to the minimized infection rate of the strains by human MDM as a result of the short incubation period for the uptake.

Assessments of intracellular growth of MTB in human macrophages as a marker of virulence have been reported in previous studies (Li *et al.,* 2002; Zhang *et al.,* 1999; Hoal-van Helden *et al.,* 2001; Wong *et al.,* 2007; Sarkar *et al.,* 2012). Zhang *et al.,* 1999 utilized the monocyte derived macrophage model to study the correlation between the extent of the spread of *M. tuberculosis* strains in a Los Angeles community setting and the ability of the strains to grow in human macrophages. It has also been suggested that the intracellular growth of clinical isolates of *M. tuberculosis* in host macrophages is associated with their virulence, which is defined as the capacity for causing tuberculosis (Theus *et al.,* 2005; Zhang *et al.,* 1999). Previous studies demonstrated that virulent *M. tuberculosis* strains grow more rapidly than avirulent or attenuated strains within human phagocytes (Silver *et al.,* 1998; Zhang *et al.,* 1998). Individual strains from lineage 2 (Beijing) have been shown to grow more rapidly than comparator strains in *in vitro* human cell culture models using MDM or monocyte or human macrophage cell lines (Li *et al.,* 2002; Zhang *et al.,* 1999; Theus *et al.,* 2005). Multiple isolates of *M. tuberculosis* strain 210 (a Beijing-family strain [L2] responsible for an outbreak in Los Angeles), grew more rapidly than small cluster or unique cluster strains in human MDM (Theus *et al.,* 2005). The data from the study showed that the intracellular growth rate

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of MTBss (Lineage 4) grew significantly more rapidly from 24hrs to 72hrs than MAF (MAF1/L5 and MAF2/L6) strain. The finding from this study has been consistent with literature based on the virulent nature of MTBss. The possible mechanism has been the longer hours/days involved in the infection and the large numbers of mycobacterial strains that was recognized by the human MDM for uptake by the receptors. It seems to confirm that MTBss/L4 being a virulent strain was mediated by mannose receptor in addition to complement receptor which resulted in an increase in virulence as compared to MAF (MAF1/L5 and MAF2/L6) which used only complement receptor for phagocytosis. These findings indicate that MTBss/L4 high rate of virulence may contribute to more rapid disease progression as well as high rates of transmission, whereas the low virulence rate of MAF (MAF1/L5 and MAF2/L6) may be associated with longer latency and lower progression to disease. This is consistent with the study in The Gambia that confirmed that though *M. africanum* was less virulent than MTBss, both MTBss and *M. africanum* were equally able to infect household contacts but contacts exposed to MTBss progressed to active disease (de Jong *et al.,* 2008). The pattern of growth observed was MTBss/L4 grew faster than MAF1/L5 and MAF2/L6 but MAF2/L5 had intermediate growth phenotype between MTBss/L4 and MAF2/L6. The low growth pattern exhibited by MAF2/L6 may suggest it closer resembles to *M .bovis* than L5 (Gagneux & Small, 2007) Again this study may suggest that the MTBss/L4, MAF1/L5 and MAF2/L6 may possess distinct patterns of growth. To the best of my knowledge this is the first phenotypic analyses to be investigated in MAF1/L5 whereas more studies have been explored in MAF2/L6 (de Jong *et al.,* 2006; de Jong *et al.,* 2008; de Jong *et al.,* 2010; Bold *et al.,* 2012).

Alternatively, the mean doubling time was determined for the growth rate of MAF (MAF1/L5 and MAF2/L6) and MTBss/L4 and MTBss (modern) and MAF (ancient) lineages. MTBss (modern) lineage had a significantly shorter doubling time but grew faster compared

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to MAF (ancient) lineage respectively which had a longer doubling time and grew slower. The possible mechanism suggested by Gehre *et al.,* 2013 for the longer doubling time and slower growth for MAF (ancient) lineage has been due to the several mutations in genes that were previously associated with growth-attenuation and the higher mutation frequency which has been observed in functional groups of molecular membrane transport systems that translocate macromolecules and nutrients across the cell membrane into the bacterial cell.

The central role in the inflammatory response and the outcome of mycobacterial infections involves the cytokine network. The cytokine network plays a central role in the inflammatory response and the outcome of mycobacterial infections (van Crevel *et al.,* 2002). Early pro-inflammatory cytokine secretion is a hallmark of *M. tuberculosis* infection. The crucial role in protective immunity and pathophysiology against tuberculosis is performed by TNF-α (Jo *et al.,* 2003). It synergizes with IFN-γ to increase the production of nitric oxide metabolites and facilitate mycobacterial killing and is essential for granuloma formation for the containment of mycobacterial infection. IL-12 production is essential to induce a protective Th1 response (Cooper *et al.,* 1997). IL-6 has also been suggested to be a pivotal pro-inflammatory cytokine during acute infection (Ladel *et al.,* 1997). Studies have reported that heterogeneity exists in the cytokine response induced by various genotype of *M. tuberculosis* complex (Wang *et al.,* 2010; Newton *et al.,* 2006). Previous studies demonstrated that virulent *M. tuberculosis* strains grow more rapidly than avirulent strains within human phagocytes (Silver *et al.,* 1998; Zhang *et al.,* 1998). The expression level of TNF-α induced by MTB correlated with the level of bacterial growth (Theus *et al.,* 2005). Previous work has shown that low pro-inflammatory innate immune responses to MTBC infection were associated with a higher virulence in animal models (Reed *et al.,* 2004; Tsenova *et al.,* 2005). A rapid growth phenotype is associated with reduced TNF-α secretion, whereas robust TNF-α secretion inhibits mycobacterial replication (Theus *et al.,* 2005). The study finding

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involving the innate immune pro-inflammatory responses (TNF-α, IL-6 and IL-12p70) induced by MTBss (L4) in culture supernatant over time (4hrs, 24hrs, 48hrs and 72hrs) was lower compared with MAF (MAF1/L5 and MAF2/L6) . This also suggests that MTBss/L4, MAF1/L5 and MAF2/L6 possess distinct pattern of cytokine induction. The ability of MTBss/L4 to suppress the induction of TNF-α secretion becomes a property of the "rapid-growth" phenotype with an increase in mycobacterial replication. In as much as MAF induced a rapid and robust TNF-α secretion, the property of "slow-growth" phenotype was observed with restriction to mycobacterial growth. This immune subversion may be key characteristics to the success of these strains in different human populations.

The Euro-American lineage, to which MTBss belongs, and the East-Asian lineage (Beijing) are both considered as ''modern'' *M. tuberculosis* lineages, lacking in particular the TbD1 genomic region (Gagneux *et al.,* 2006; Brosch *et al.,* 2002) while the "ancient" *M. tuberculosis* lineages to which *M. africanum* lineages (MAF1/L5 and MAF2/L6) belongs have the TbD1 genomic region (Brosch *et al.,* 2002). Likewise a similar pattern of innate response was also observed between the MTBss (modern) lineages and MAF (ancient) lineages, whereas the immune response induced by MAF lineage were hyper-inflammatory . Modern lineages were shown to induce lower levels of pro-inflammatory cytokines when compared with ancient lineages (Portevin *et al.,* 2011). Another study has found that Beijing strains (L2), irrespective of subfamily, showed an immune phenotype of low levels of TNF-α, IL-6, IL-10 and GRO-α (Wang *et al.,* 2010) production as compared to H37Rv and other genotypes of *M. tuberculosis* in human macrophages. The study findings observed a statistically significant distribution towards more pro-inflammatory strains for the ancient lineages, and lower inflammatory responses to strains from the modern lineages. The low inflammatory phenotype of modern strains is in agreement with previous studies of individual Beijing strains (Manca *et al*., 2004; Tanveer *et al*., 2009) and other strains (Newton *et al*.,

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2006) belonging to the modern lineages. The study findings are also consistent with the previous suggestion that a low inflammatory response may lead to a reduction in the adaptive response (Rakotosamimanana *et al*., 2010). A possible model to rationalize this finding is that the respective high and low inflammatory responses could reflect different virulence strategies that emerged during the evolution of the ancient and modern lineages. The characteristic latency in TB has been suggested to represent an evolutionary adaptation to low host densities, with reactivation after several decades allowing the pathogen to access new susceptible birth cohorts (Hershberg *et al*., 2008; Blaser & Kirschner, 2007). By contrast, the low inflammatory response induced by evolutionary modern strains has been associated with an enhanced ability to cause early progressive disease (Manca *et al*., 2004; Newton *et al*., 2006). Such a strategy may be an advantage in the context of high human population densities, where the number of susceptible hosts is large, and rapid lethality does not threaten to exhaust the pool of new uninfected hosts. The overall lower inflammatory responses detected in the modern lineages of *M. tuberculosis* might be a result of their access to rapidly increasing numbers of susceptible hosts resulting in selection for faster progression to active disease. Taken together, these observations are consistent with the notion that 'modern' strains have evolved a strategy of higher virulence and shorter latency, perhaps to better adapt to the increasing availability of susceptible hosts brought by the large human population increases of the last few centuries. Answering them will help develop better tools and strategies to control one of the world's oldest and yet still most important human diseases.

Previous report by Yeboah-Manu *et al.,* 2011 showed that 17.1% MAF accounted for TB cases in Ghana while the Cameroon sub-lineage affected 65% cases. The dominance and success of the Cameroon sub-lineage in Ghana has been unclear but has been elucidated due to a founder effect and/or particular high fitness in the patient population (Asante-Poku *et al.,* 2015). The prevalence and possible stability of MAF in Ghana than in some other countries

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has been attributed to the bacterial lineage that might be particular well adapted to (some) human populations in Ghana (Asante-Poku *et al.,* 2015). Nonetheless its lower prevalence has been attributed to possible out-competition by MTBss (Bold *et al.,* 2012). Thus the phenotypic characteristic of host pathogen interaction of MAF compared to MTBss has been due to its dominance in Ghana as the leading sub-lineage to be spoligotype by Yeboah-Manu *et al.,* 2011.

Longer latency in *M. africanum* might be an adaptation to low host densities, whereas a reduced latency period (i.e., increased "virulence") in *M. tuberculosis* infections might be an adaptation reflecting the crowded conditions and high rates of tuberculosis. It seems to suggest that *Mycobacterium africanum* may be better controlled by innate immunity, leading to fewer bacteria and lower adaptive immune responses. The study highlights the advances toward a better understanding of the bacterial phenotypic virulence of MTBss, MAF and its interaction with a host's protective innate immune system in the fight against tuberculosis

The limitation to this study was the limited amount of cryopreserved PBMCs of sputum positive TB individuals (n=19) in Table 4.1. Moreover the commercial GenoType MTBC assay (Hain Lifescience, Nehern, Germany) was only used to differentiate the sputum samples of the sputum-positive TB individuals from MTB-infected individuals into MAF-infected individuals; whereas the sputum-positive samples of MAF-infected individuals did not undergo any spoligotype analysis and large sequence polymorphism to classify it into distinct lineages of MTBC but was later done whereas the limited amount of cryopreserved PBMCs had been depleted.

Secondly, the PBMC-derived human MDM from the whole blood differed phenotypically from the avelolar macrophages of the lungs (Gordon & Taylor, 2005). Though different donor PBMC-derived human MDM was utilized in the *in vitro* model whole blood from a

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single donor from the blood bank was unavailable and neither could the individual provide a lot of PBMC-derived human MDM for the same intracellular growth assay for the numerous lineages.

Thirdly, measurement of anti-inflammatory cytokines could have enriched our data interpretation. A lot more cytokines including anti-inflammatory could have been measured using Luminex bead assay.

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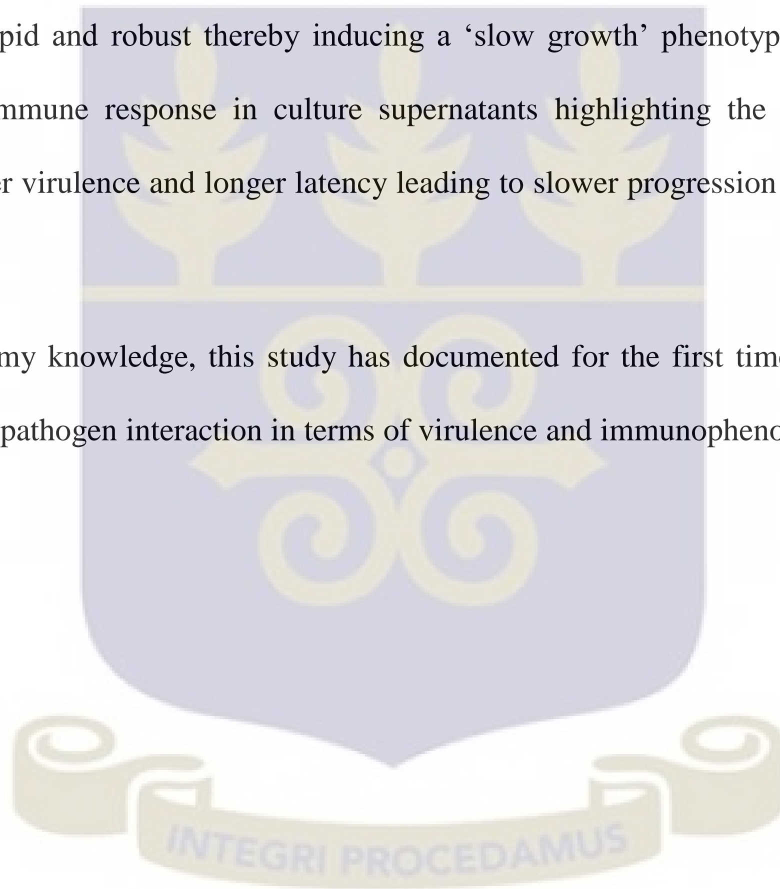
5.2 Conclusion(s)

In conclusion, consistent with previous studies, this study has shown that MAF-infected patients induced similar frequencies of T subset response to rESAT-6/CFP-10 fusion protein as MTB-infected patients.

Furthermore in this study, MAF had reduced uptake, low intracellular growth rate and a longer doubling time in MDM. Likewise the inflammatory response of MAF (ancient) lineages was rapid and robust thereby inducing a 'slow growth' phenotype with enhanced inflammatory immune response in culture supernatants highlighting the point that MAF indeed has lower virulence and longer latency leading to slower progression to active disease in the host.



To the best of my knowledge, this study has documented for the first time the phenotypic features of host pathogen interaction in terms of virulence and immunophenotypes in Lineage 5.

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5.3 Recommendation(s)

Isolates from mycobacteria infected individuals could further be typed into their various lineages instead of HAIN test

Cell lines (THP1-Human acute monocytic leukemia cell line) could be used as a macrophage model to study host pathogen interaction in clinical isolates of *Mycobacterium* in Ghana

Luminex assay may be used measure a lot of cytokines within the small volume cultured supernatants

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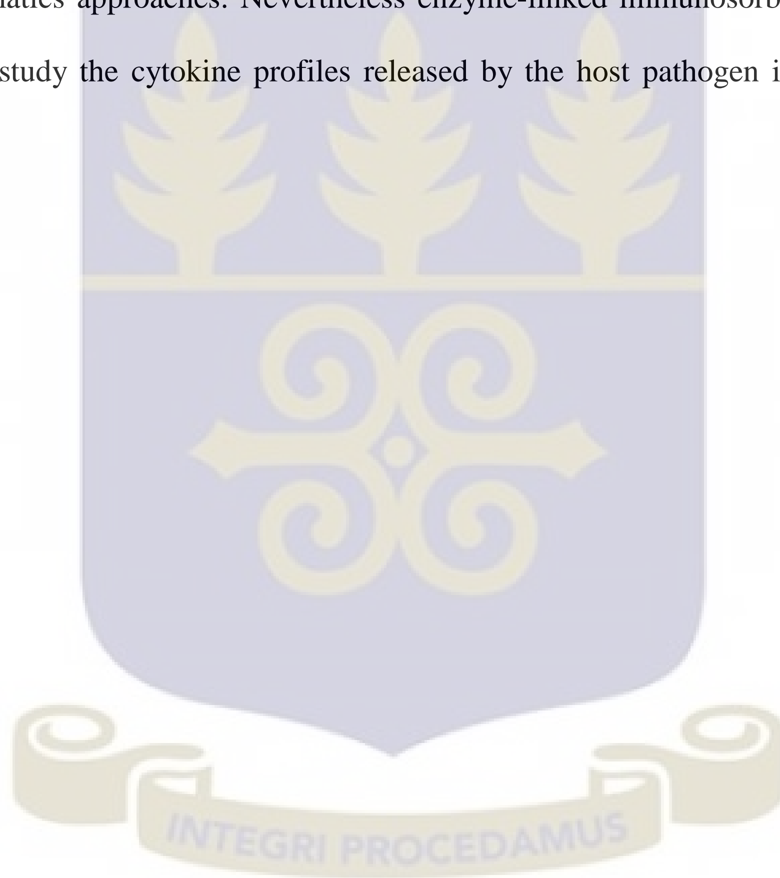
5.4 Further studies

The finding highlights the importance of investigating the cellular gene expression profile of host macrophages interaction with *M. africanum* by DNA array analyses over time; to study the transcription patterns of the host macrophages after infection with *M. africanum* and furthermore confirm signature genes by quantitative real-time reverse transcriptase polymerase chain reaction (q-rt RT-PCR) and proteomic techniques such as novel module-based bioinformatics approaches. Nevertheless enzyme-linked immunosorbent assay should be explored to study the cytokine profiles released by the host pathogen interaction in the supernatants.



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Appendix A

Reagents for flowcytometry

Complete culture medium

50mL of 10% foetal calf serum (FCS) together with 5mL 1mM glutamine (100X) and 5mL 1% penicillin/streptomycin (100X) was added to 440mL solution of RPMI to form a complete culture/growth medium. This was done aseptically in the biosafety hood.

Staphylococcus enterotoxin B (SEB)

The commercial product of SEB was received in a hydrated form and reconstituted with 1mL sterile culture medium and used at a final concentration of 5µg/mL.

Recombinant early secreted antigenic Target–6 and culture filtrate protein–10 (rESAT-6/CFP-10 fusion protein)

The commercial product of rESAT-6/CFP-10 fusion protein was received in a hydrated form and reconstituted with 1mL sterile culture medium to make a stock solution and used at a final concentration of 5µg/mL.

Brefeldin A (BFA)

The commercial product of BFA was reconstituted with 1mL dimethyl sulfoxide, aliquoted and frozen at 5mg/mL. The final concentration used was 5µg/mL.

Phosphate buffer saline (PBS)

One tablet of PBS was dissolved in 1000mL of distilled water and pH maintained at 7.2

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FACS buffer

5mL 1%FCS and 500µL 0.1% sodium azide was dissolved in 500mL PBS, filtered with a 0.20µm millipore filter and kept cold at $4^{O}C$.

BD Perm/Wash buffer (10X)

10mL of BD Perm/Wash buffer was dissolved in 90mL of distilled water to form 1X of BD Perm/Wash buffer

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Appendix B

Preparation of Difco™ Middlebrook Mycobacteria 7H11 agar for single colonies for MAF I and MTBss isolates in glycerol or sodium pyruvate

MTBss

2.1g 7H11 agar was dissolved in 90mL distil water containing 0.5mL 0.5% glycerol. The solution was sterilized at 121° C for 15minutes. 10ml BactoMiddlebrook OADC growth enrichment media was aseptically add to the media at 50° C–55° C. The solution was mixed well and aliquot into 90 mm three compartment petri dish

MAF I

2.1g 7H11 agar was dissolved in 90ml distilled water. The solution was sterilized at 121° C for 15minutes. 10ml BactoMiddlebrook OADC growth enrichment media and 4ml sterile 1M sodium pyruvate (final concentration of 40mM) was aseptically add to the media at 50° C–55° C. The solution was mixed well and aliquot into 90 mm three compartment petri dish

Culture of a single colony of MAF I and MTBss isolates in Difco™ Middlebrook Mycobacteria 7H9 broth/ADC/Tween80 in PBS in glycerol or sodium pyruvate

MTBss

0.47g of 7H9 broth was dissolved in 90ml distilled water containing 0.2ml of glycerol. The solution was sterilized at 121° C for 15minutes. 10ml BactoMiddlebrook ADC growth enrichment media and 10ml 0.05%Tween80 in PBS was added aseptically at 50° C–55° C. 10mL of the broth was aliquoted into 30ml sterile graduated square plastic translucent bottles-PETG.

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MAF I

0.47g of 7H9 was dissolved in 90ml distilled water containing 0.2ml of glycerol. The solution was sterilized at 121° C for 15minutes. 10ml BactoMiddlebrook ADC growth enrichment media, 10ml 0.05%Tween80 in PBS and 4ml sterile 1M sodium pyruvate (final concentration of 40mM) was added aseptically at 50° C–55° C. 10mL of the broth was aliquoted into 30ml sterile graduated square plastic translucent bottles-PETG.

Single mycobacteria suspension of MAF I and MTBss isolates in Difco™ Middlebrook Mycobacteria 7H9 broth/ADC in glycerol or sodium pyruvate

MTBss

0.94g of 7H9 broth was dissolved in 180ml distilled water containing 0.4ml of glycerol. The solution was sterilized at 121° C for 15minutes. 20ml BactoMiddlebrook ADC growth enrichment media was added aseptically at 50° C–55° C. 90mL of the broth was aliquoted into $75cm^3$ culture flasks.

MAF I

0.94g of 7H9 broth was dissolved in 180ml distilled water. The solution was sterilized at 121° C for 15minutes. 20ml BactoMiddlebrook ADC growth enrichment media and 8mL sterile 1M sodium pyruvate (final concentration of 40mM) was added aseptically at 50° C–55° C. 90mL of the broth was aliquoted into $75cm^3$ culture flask.

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Freezing media for MAF I and MTBss storage

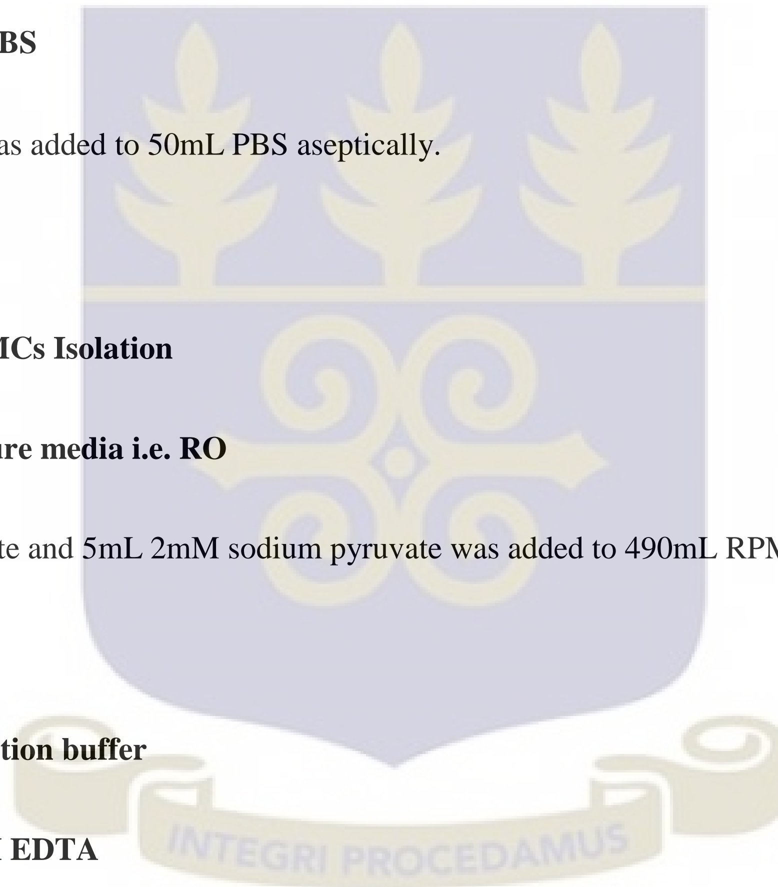
PBS in glycerol

5mL of PBS was added to 95mL of glycerol

Diluent buffer for MAF I and MTBss plating

0.5% FCS in PBS

25µL of FCS was added to 50mL PBS aseptically.



Media for PBMCs Isolation

Complete culture media i.e. RO

5mL L-glutamate and 5mL 2mM sodium pyruvate was added to 490mL RPMI aseptically.

Monocyte isolation buffer

PBS/FCS/0.5M EDTA

400µl of FCS and 500µl of 0.5M EDTA was added to 99.1mL of PBS aseptically and kept at 4° C.

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Culture media for macrophage growth

10% FCS in RO

10mL of FCS was added to 90mL of RO

Diluent buffer for infected macrophage lysing

0.01% Tween80 in distilled water

100µL of Tween80 was added to 100mL of distilled water aseptically.

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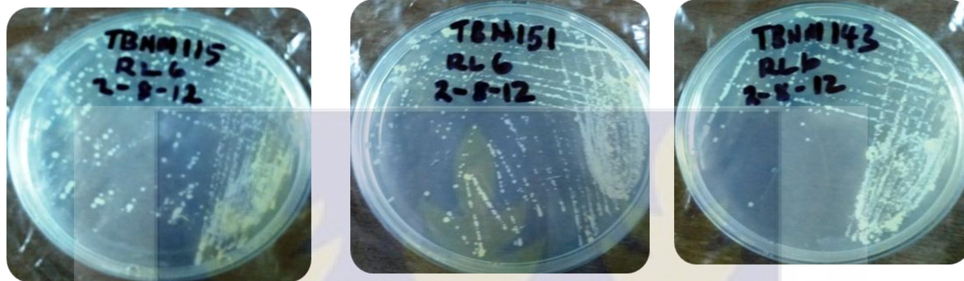
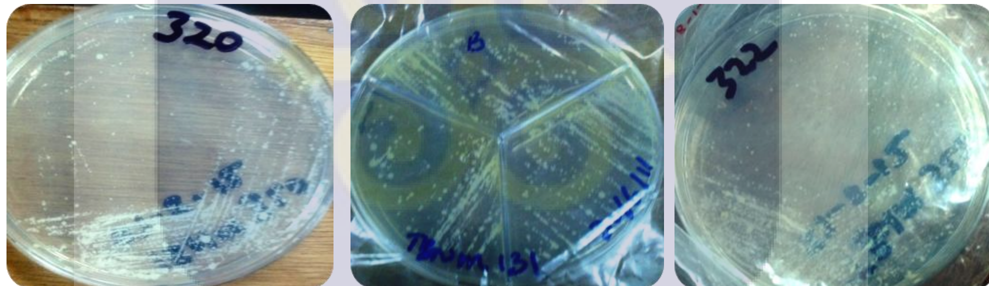
APPENDIX C

A. Single colonies of MTBss (Lineage 4), MAF1 (Lineage 5) and MAF2 (Lineage 6) on Difco™ Middlebrook 7H11 agar

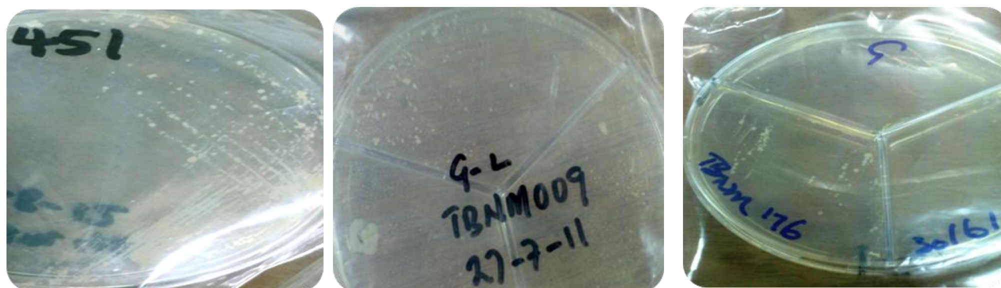
MTBss (Lineage 4)



MAF1 (Lineage 5)



MAF2 (Lineage 6)



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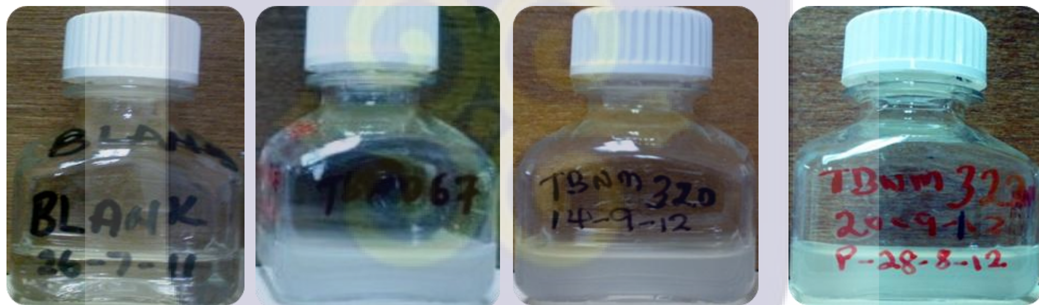
B. Cultures of MTBss, MAF1 and MAF2 in Difco™ Middlebrook 7H9 broth/ADC/Tween80 in PBS to a density corresponding to an absorbance of 0.7 – 0.8 against a blank at 600nm with a cell density meter

MTBss (Lineage 4)

Blank

MAF1 (Lineage 5)



MAF2 (Lineage 6)

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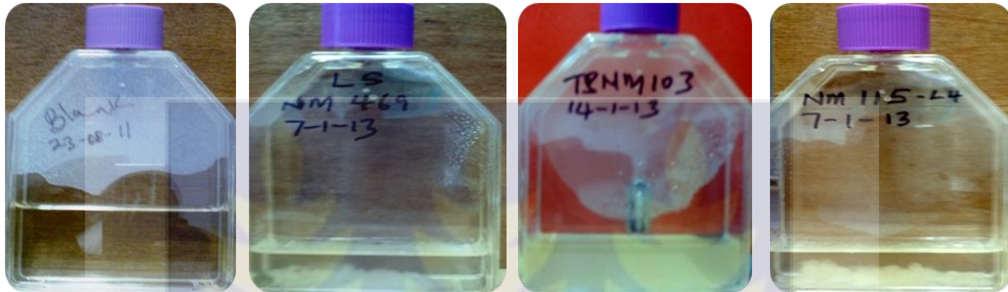
C. **Sub-culture of MTBss (Lineage 4), MAF1 (Lineage 5) and MAF2 (Lineage 6) suspension at a density corresponding to an absorbance at 0.7-0.8 in a freshly prepared 7H9/ADC and mechanically disrupted by 3mm beads for single bacteria suspension**

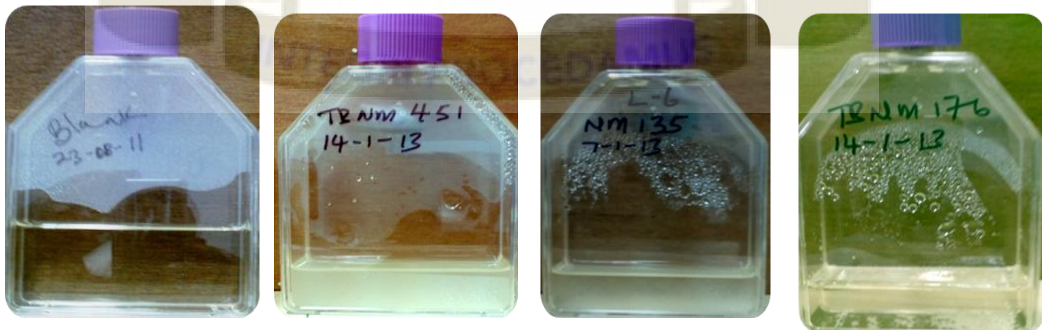
MTBss (Lineage 4)

Blank



MAF1 (Lineage 5)

MAF2 (Lineage 6)



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APPENDIX D

Detection of pro-inflammatory cytokines (TNF-α, IL-12p70 and IL-6) in harvested culture supernatant from infected MDM cells.

Commercial sandwich ELISA kit (Peprotech, New Jersey, USA) was used to measure levels of TNF-α, IL-12p70 and IL-6 cytokines in culture supernatants from the control and infected MDM cells harvested at time points 4hrs, 24hrs, 48hrs, and 72hrs. Manufacturer' instructions were followed accordingly. Capture antibodies, standards, detection antibodies and avidin horse radish peroxidase conjugate of TNF-α, IL-12p70 and IL-6 cytokines were supplied with the kit by the manufacturer.