University of Liège
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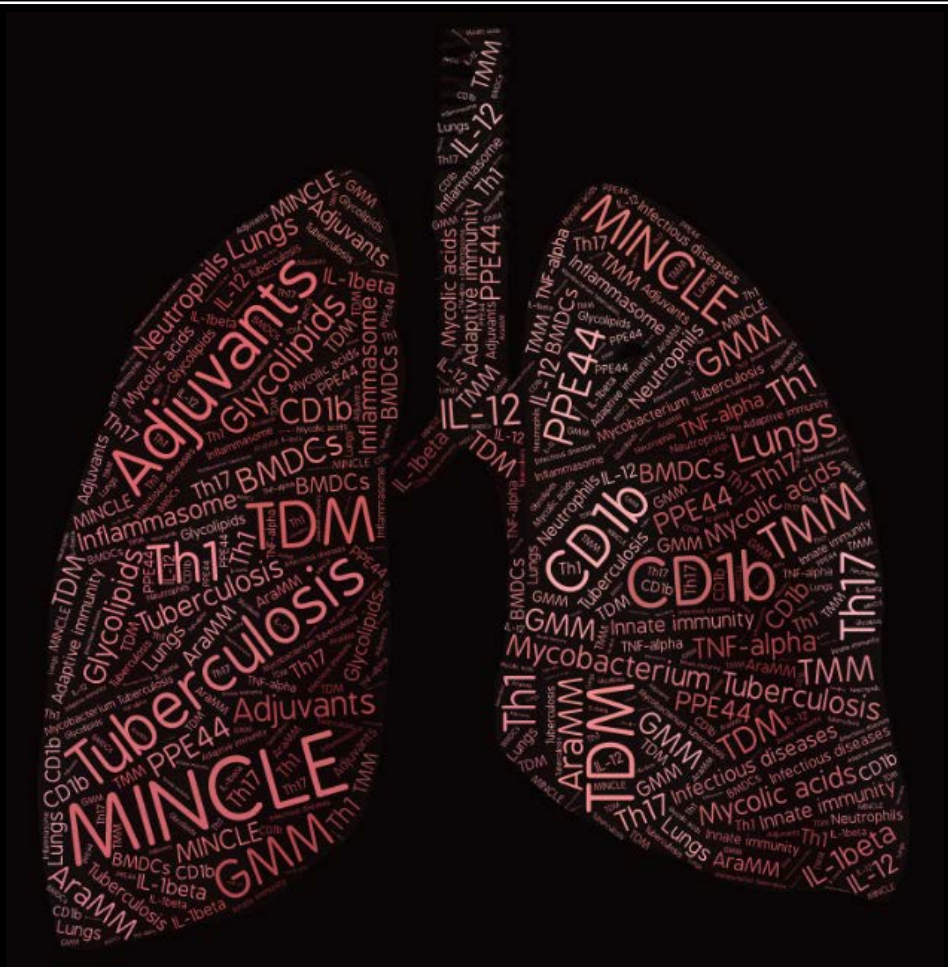
Scientific service Immunology

Faculty of Sciences
GIGA-Research

Innate signaling by mycolate esters of the cell wall of *Mycobacterium tuberculosis* and relevance for development of adjuvants for subunit vaccines



Giresse TIMA

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Sciences
Academic year 2016-2017

Promotor : Jacques PIETTE

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Kris HUYGEN

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“What cannot be eschew’d must be embraced “

“Ce qui ne peut pas être évité il faut l’embrasser”

William Shakespeare

REMERCIEMENTS

Il m'est agréable d'adresser mes sincères remerciements à tous ceux qui m'ont apporté de près ou de loin, aide et conseils lors de ces travaux de thèse de doctorat.

Je remercie le Docteur Kris Huygen pour avoir cru en moi en m'accueillant au sein du laboratoire d'Immunologie de l'Institut Scientifique de la Santé Publique. Merci pour votre soutien, votre disponibilité et vos précieux conseils.

Mes plus vifs remerciements au Docteur Marta Romano pour m'avoir encadré tout au long de ces années, pour sa patience et sa disponibilité (même pendant son congés de maternité), pour tous ses précieux conseils aussi bien sur le plan scientifique que personnel. Sa compétence et sa rigueur scientifique m'ont beaucoup appris. Merci du fond du cœur pour ton soutien au cours de ces années, je n'aurais pas rêvé mieux comme co-promoteur.

J'adresse mes remerciements au Professeur Jacques Piette pour avoir accepté d'être mon promoteur de thèse, pour son aide, ses conseils et sa disponibilité.

J'exprime mes remerciements à l'ensemble des membres de mon comité d'accompagnement, le Docteur Sylvie Legrand et le Professeur Bernard Joris pour leurs remarques et conseils avisés.

Je remercie l'ensemble des membres de mon jury de thèse, le Professeur Johan Grooten de l'Université de Gent, le Docteur Jérôme Nigou de l'Institut de Pharmacologie et de Biologie Structurale de Toulouse et le Docteur Christophe Desmet de l'Université de Liège pour avoir accepté d'évaluer mon travail de thèse.

Je remercie le Docteur Roland Lang et le Docteur Rudy Beyaert pour nous avoir respectivement fournis les souris Mincle et MALT-1 knock out. Le Docteur Laurent L'homme pour tous ses conseils et son aide technique pour les expériences sur l'inflammasome. Le Professeur Mark Baird et toute son équipe pour la synthèse de glycolipides.

Je tiens particulièrement à remercier le Docteur Olivier Denis pour qui notre formation scientifique est une priorité. Merci pour ta rigueur scientifique, tes précieux conseils et pour ton soutien qui sont aussi au-delà de l'aspect scientifique.

J'adresse toute ma gratitude à tous les membres du laboratoire qui ont contribué à ce travail, Christophe Van den Poel, Fabienne Jurion, Sofie De Prins et plus particulièrement Pauline Lehebel. Merci pour votre excellente aide technique et pour votre bonne humeur qui ont contribué à ces années agréables. Merci Pauline pour ces longues pauses café et ces discussions constructives. Merci également aux étudiants qui ont contribué à ce travail, Antoine Francotte, Natacha Motteu, Georges Potemberg, Lorien Bogaert et Jeremy Franche.

Je remercie également tous les autres membres du service d'Immunologie qui ont partagé mes moments au cours de ces années, Martine Clinet, Muriel Vincent, Caroline Rodeghiero, Greta Treutens, et Nadège Caboré.

Je remercie mes parents et ma famille sans qui je ne serais probablement pas là aujourd'hui. Merci pour votre tendresse, votre amour, votre soutien et votre présence. Merci également à Zoe, Winter, Jacques et Thi Hue et à mes amis Kevin, Elisa, Pauline, Jean-Michel, Antoine, Nicolas… pour leur soutien.

Je remercie également le FRIA, l'Institut de la Santé Publique, le projet DISCOBEL (BELSPO) et le projet européen TBVAC2020 qui ont contribué partiellement financièrement à ce projet.

Mention spéciale à mes Amours Virginie et Gabriel. Vi pour m'avoir supporté et motivé pendant toutes ces années de thèse. Merci pour le temps que tu as pris pour m'écouter ou pour me relire. Merci également pour ton soutien et pour tous tes encouragements. Merci Gabriel pour ton sourire qui a suffi à me booster. Merci parce que tu m'as ramené à l'essentiel.

1. INTRODUCTION

1.1 Introduction

1.1.1 The history of tuberculosis

Tuberculosis (TB) is an infectious disease that has plagued humankind throughout the known human history [1]. This disease, caused by infection with bacteria from the *Mycobacterium tuberculosis (M. tuberculosis*) complex, is indeed an ancient scourge. By using techniques of molecular genetics and the sequencing of the genome of several strains of *M. tuberculosis*, Gutierrez *et al* have demonstrated that human tubercle bacilli from East Africa represented the progenitor species. It is estimated that this species originated 3 million years ago and may have infected hominids at that time [2]. Subsequently, throughout all history of humankind traces of *M. tuberculosis* infections can be found. Some evidences document the presence of tuberculosis more than 5000 years ago. Indeed, some abnormalities characteristic to tuberculosis, such as Pott's deformities, have been discovered in Egyptians. These observations were confirmed later when Nerlich and co-workers amplified *M. tuberculosis* DNA from tissues of Egyptian mummies [3]. DNA of *M. tuberculosis* has also been found in Peruvian mummies [4]. In Europe, there is archaeological evidence of tuberculosis on widespread locations since the 5th century, during the millennium that followed the fall of Rome [1].

In Europe and America, the peak of mortality due to TB was observed during the 18th century [5]. Annual death rates in London, Stockholm, Hamburg and American cities approached 800 to 1000/100 000 inhabitants at that time [1]. The 19th century is characterized by improvements in our knowledge of this disease. The first important advance in the understanding of this disease was made in 1819 by Laënnec, best remembered today for the invention of the stethoscope. This doctor had enormous experience in autopsies of patients who died of tuberculosis. Laënnec undoubtedly elucidated the pathogenesis of tuberculosis [1]. Later in 1865, Villemin demonstrated the infectious nature of tuberculosis by inoculating rabbits and guinea pigs with purulent liquid from a tuberculous cavity from a human corpse [6]. The 24th March 1882 is a date of a dramatic change in the history of tuberculosis. Indeed, Hermann Heinrich Robert Koch demonstrated that he had discovered and cultured the infectious agent of the disease. He also showed that the inoculation of the bacillus into healthy laboratory animals could reproduce the disease. Koch clearly established the principle according to which a specific

microorganism can cause a particular disease (Koch's postulate). In 1905, Koch was awarded with the Noble prize in Medicine or Physiology for his elucidation of the aetiology of tuberculosis [6].

1.1.2 Diagnosis, vaccination, treatment strategies

During the early 19th century, tuberculosis continued to kill a lot of persons and accounted for up to 25% of deaths in Europe [5]. In 1890, Koch isolated a substance from tubercle bacilli and the use of this substance, known now as tuberculin was initially tested as a treatment for tuberculosis [7]. However, this treatment was rapidly discredited by its ineffectiveness to cure TB patients and actually in contrast exacerbated the disease. Indeed, in 1891, treatment with tuberculin failed to threat more than 1700 ill individuals [8]. It was discovered later that tuberculin induces tissue destruction and sometimes leads to systemic dissemination of *M. tuberculosis* infection, which sometimes resulted in the death of the patient [9]. Later, Charles Mantoux demonstrated that tuberculin could be used to diagnose latent TB, which is asymptomatic infection with *M. tuberculosis* [10]. This test consists in monitoring development of a delayed-type hypersensitivity (DTH) response at the site of intradermal administration of tuberculin. It is a test that is still used to identify *M. tuberculosis* infected individuals and that can be replaced by more specific IFN-gamma release assay (IGRA) tests recently developed (QuantiFERON-TB, T-SPOT-TB) [11]. These tests are *in vitro* assays based on measurement of specific responses of PBMCs stimulated with *M. tuberculosis* specific antigens (such as ESAT-6, CFP10 or TB7.7) [11]. Microscopic examination, culture of sputum and chest radiography remain nowadays the major tools used to diagnose active pulmonary TB. In more recent years, these tools have been improved by the development for example of molecular tests to determine antibiotic resistance of the mycobacteria causing disease [12]. Nevertheless, application of these new detection and diagnostic tools needs laboratories with dedicated infrastructures and experienced staff. This is not always the case in most of the laboratories in high incidence TB countries.

In the mid-19th century, already before the advent of a vaccine and anti-tuberculosis drugs the death rates from tuberculosis began to fall. A clear explanation for this decline is not available, but some hypotheses have been formulated. These include:

- the discovery that TB is an infectious disease and the related diagnostic tools that allowed for the isolation of patients in hospitals or sanatoria,
- improved social and living conditions,
- improved nutrition,
- natural selection of a genetically more resistant population [1,5].

Nevertheless, none of these hypotheses can fully explain the decline of tuberculosis death rates observed at that time.

In the 1880s, Louis Pasteur developed the concept of deliberately attenuating the virulence of a pathogen to produce a vaccine [13]. Based on this approach, Albert Calmette and Camille Guérin of the Pasteur Institute in Lille (France) began their research on an anti-tuberculosis vaccine. They cultured the tubercle bacilli on a glycerine and potato medium which resulted in a suspension of non-homogeneous bacilli with clumps. They added ox bile to the medium to counter the formation of clumps and they found that subculturing lowered the virulence of the bacteria. Starting with a virulent bovine strain of the tubercle bacilli and after 13 years and 230 subcultures, they produced a strain that failed to induce progressive tuberculosis in guinea pigs, rabbits, cattle and horses. They named it Bacille Bilié Calmette-Guérin and later Bacille Calmette-Guérin (BCG) [14]. In 1921, BCG was first tested in humans in a healthy infant born of a mother who died of pulmonary tuberculosis and there were no undesirable sequelae [15]. In 1948, UNICEF and the Danish Red Cross sponsored the first campaign to control tuberculosis. During 3 years, approximately 14 million of people were vaccinated with BCG [16]. In 1974, the WHO expert committee on tuberculosis provided policy guidelines for the control of tuberculosis. They strongly recommended BCG vaccination for all persons under 20 years of age [17]. Today, BCG vaccination is no longer recommended in the industrialized world but it is still part of the Expanded Program on Immunization of WHO and administered to new-borns in endemic countries. So far, BCG is the only vaccine available for TB and constitutes the most widely used vaccine worldwide with more than 4 billion doses administered.

Regarding the treatment, the history of the disease changed dramatically with the development of antibiotics. Indeed, in January 1944 Schatz, Bugie and Waksman discovered streptomycin and demonstrated its potency against 22 different species of bacteria, including *M. tuberculosis* [18]. Streptomycin was rapidly used to treat TB patients. The results were good

but far from perfect. Streptomycin had significant nerve toxicity, provoked deafness and strains resistant to streptomycin appeared rapidly [5]. Few years later, Jörgen Lehman developed para-aminosalicylic acid (PAS) which like streptomycin produced only transient clinical benefit before the apparition of resistant bacteria [19]. Afterwards, a clinical trial in the United States demonstrated the effectiveness of combining several antibiotics to avoid the apparition of resistance [20]. Several years later, the antibiotic panel against TB was expanded with the discovery of isoniazid in 1951 and rifampicin in the mid-1960s [21]. Nowadays, the recommended treatment against active TB is two months of four first-line generic drugs, namely isoniazid, rifampicin, ethambutol and pyrazinamide, followed by a four month regimen with isoniazid and rifampicin. WHO reported that the treatment success rate of active TB is 83% [22].

1.1.3 Tuberculosis epidemiology

The progress realized over the centuries in terms of diagnosis, vaccination and treatment of TB improved the control of this disease in many parts of the world. Nevertheless, TB remains a major global health problem and the leading cause of morbidity and mortality due to a single bacterial pathogen in the world. In 2015, 1.8 million people have died of TB (among HIV-negative and HIV-positive individuals) and 10.4 million estimated new TB cases occurred worldwide [22]. Most of the cases occur in Asia and Africa (Figure 1). Indeed, 60% of the global total estimated new cases are located in India, Indonesia, China, Nigeria, Pakistan and South Africa. The incidence of TB cases in high-income countries including Western Europe, Canada, United States of America, Australia and New Zealand is relatively low [22]. In these areas, the incidence of TB is less than 10/100 000 inhabitants per year. In Belgium, the incidence in 2015 was of 9.4/100 000 with a total of 988 reported cases [23]. Nevertheless, even in Belgium a higher incidence of approximately 20/100 000 inhabitants is reported in large cities such as Brussels, Antwerp and Liège (Table 1) [24]. TB remains a disease related to poverty, as 22 low-income and middle-income countries account for about 80% of the global number of TB cases. Several factors can explain this unacceptable high number of morbidity and mortality, including (besides the socio-economic factors) vaccine limitations, the HIV epidemic and the growing challenge of drug resistance.

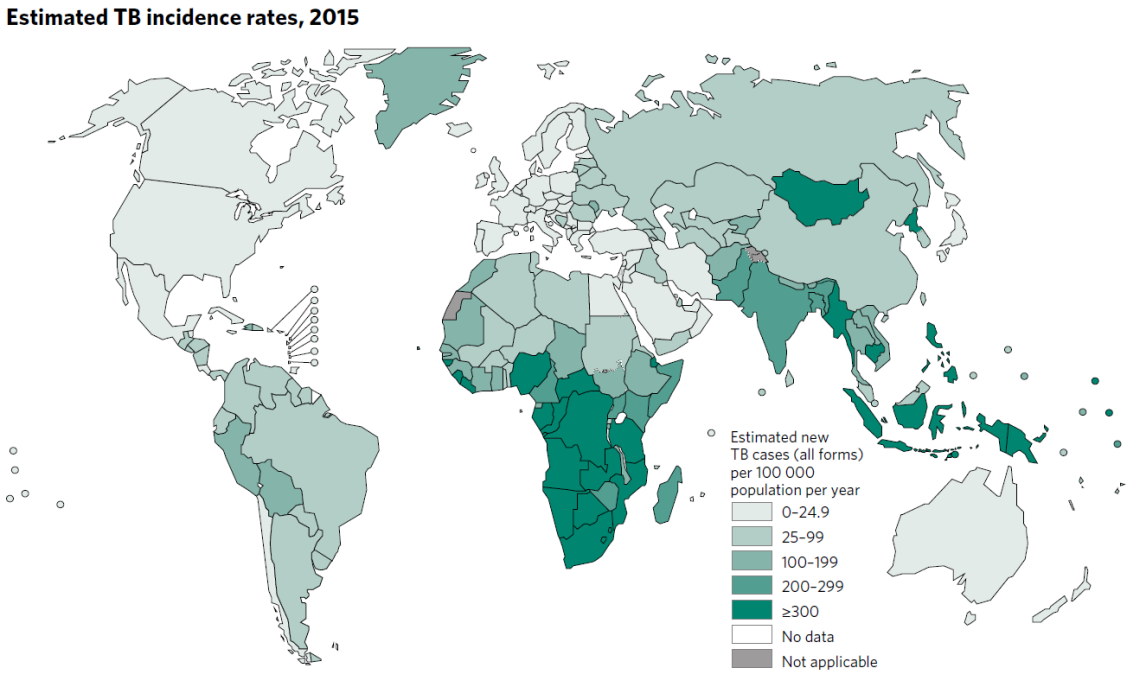


Figure 1: Estimated tuberculosis incidence rates, 2015.
The number of incident TB cases varies widely among countries. The lowest rates are predominantly in high-income countries including most countries in Western Europe, Canada, the United States of America, Australia and New Zealand (with incidence rates of less than 10 cases per 100 000 population in 2015). In most of the 30 high TB burden countries, incidence is of 150-300/100 000 and reaches levels of 500 / 100 000 in some countries such as Lesotho, Mozambique and South Africa [22]

Estimates of TB burden*, 2015	Number (thousands)	Rate (per 100 000 population)
Mortality (excludes HIV+TB)	0.054 (0.052–0.055)	0.47 (0.46–0.49)
Mortality (HIV+TB only)	0.011 (<0.01–0.055)	0.1 (0–0.49)
Incidence (includes HIV+TB)	1.1 (0.92–1.2)	9.4 (8.1–11)
Incidence (HIV+TB only)	0.084 (0.069–0.099)	0.74 (0.61–0.88)
Incidence (MDR/RR-TB)**	0.023 (<0.01–0.038)	0.2 (0.08–0.34)

Estimated TB incidence by age and sex (thousands)*, 2015			
	0-14 years	> 14 years	Total
Females	0.028 (0.013–0.044)	0.35 (0.23–0.46)	0.38 (0.25–0.51)
Males	0.041 (0.028–0.055)	0.65 (0.51–0.79)	0.69 (0.54–0.84)
Total	0.07 (0.052–0.088)	1 (0.91–1.1)	1.1 (0.92–1.2)

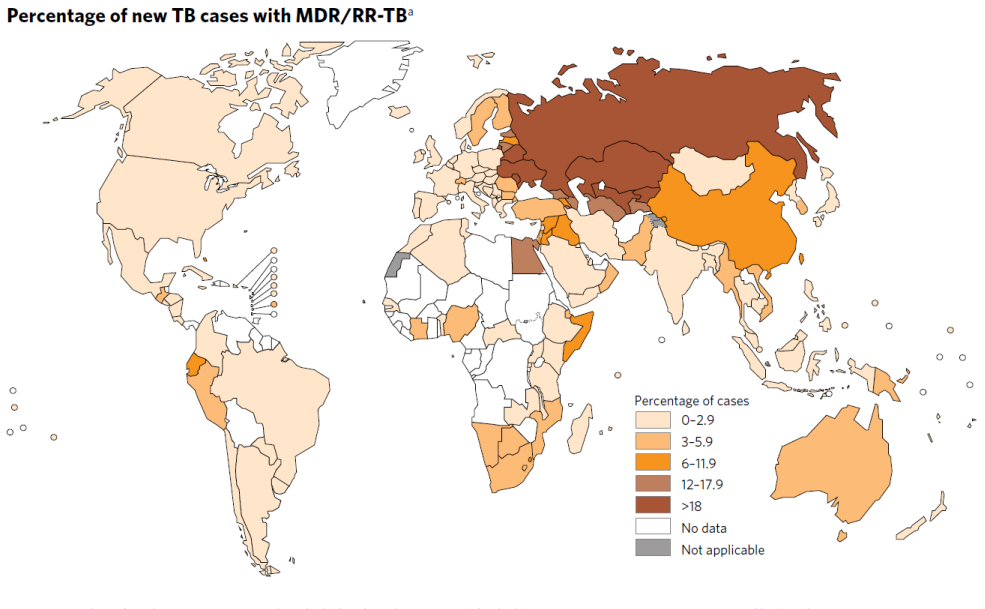
Table 1: TUBERCULOSIS IN BELGIUM: estimates of disease burden and incidence for 2015 (11 million inhabitants)[23]

a. Drug resistance and HIV epidemic

The past two decades have faced the worldwide appearance of multidrug-resistant (MDR) isolates, defined as *M. tuberculosis* strains resistant to at least isoniazid and rifampicin, extensively drug-resistant (XDR) isolates, known as *M. tuberculosis* strains resistant to isoniazid, rifampicin, any fluoroquinolone and one of the three injectable drugs (capreomycin, kanamycin and amikacin), and most recently, strains which are resistant to all available anti-tuberculosis drugs. Drug resistant tuberculosis represents a very significant public health problem. WHO estimated in 2015 that 3.9% of new TB cases were MDR-TB and that only 21% of these MDR-TB cases occurred in individuals previously treated for the disease (Figure 2) [22,25]. Duration of treatment of MDR-TB is at least three times as long as for drug-susceptible TB and can cost up to 25 times more. Indeed, the recommended regimens to treat MDR-TB and XDR-TB are very complex, take at least 18 months and require second line drugs that are generally toxic, difficult and painful to administer [25].

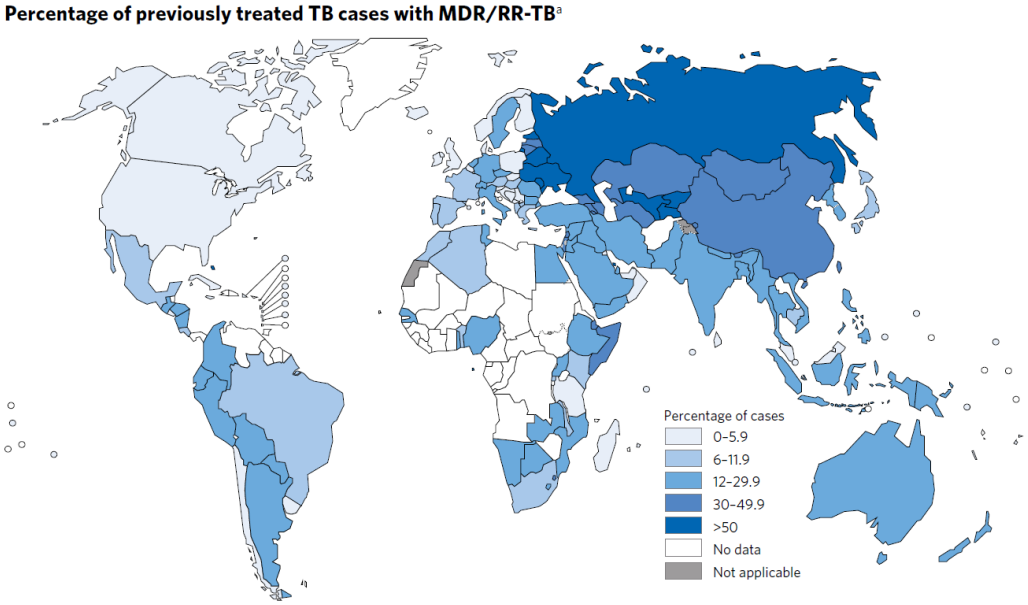
The recommended treatment for MDR-TB is a treatment with at least five effective TB drugs during the intensive phase of the disease. These five medicines comprise pyrazinamide and four second line drugs. This treatment has to be followed by a high dose of rifampicin and/or ethambutol. Beside this problem of drug-resistant TB, the HIV epidemic contributes to the TB epidemic. Co-infection with HIV increases the susceptibility to TB. Indeed, HIV positive individuals are estimated to be 26 times more susceptible to develop active disease in comparison to HIV negative people [22], with an annual risk for latently *M. tuberculosis* infected individuals to develop active TB of 10% if HIV co-infected as compared to 0.1%-1.5% in HIV negative individuals. An estimated 11% of the new cases in 2015 were in HIV positive persons and about 390 000 died of TB (Figure 3) [22]. HIV co-infection accounts for the elevated TB incidence in many countries with high HIV prevalence, with most of the cases of TB in HIV positive individuals occurring in countries located in sub-Saharian Africa and Southeast Asia [22]. In addition, HIV positive individuals treated for active TB are also predisposed to having sub-therapeutical concentrations of anti-tuberculosis drugs in the circulation because of deficient drug absorption or drug-drug interactions and this can favor the development of drug-resistant strains [26,27].

A



[a] Figures are based on the most recent year for which data have been reported, which varies among countries. Data reported before the year 2001 are not shown.

B



[a] Figures are based on the most recent year for which data have been reported, which varies among countries. Data reported before the year 2001 are not shown. The high percentages of previously treated TB cases with MDR-TB in Bahamas, Bahrain, Belize, Bonaire – Saint Eustatius and Saba, French Polynesia and Sao Tomé and Principe refer to only a small number of notified cases (range: 1-8 notified previously treated TB cases).

Figure 2: Percentage of new TB cases (A) or previously treated TB cases (B) with Multidrug resistant tuberculosis.

Eastern European and central Asian countries have the highest levels of MDR-TB. Among new cases, the proportions with MDR-TB were highest in Belarus, Estonia, Kazakhstan, Kyrgyzstan and Uzbekistan. Among previously treated TB cases, the proportions with MDR-TB were highest in Belarus , Estonia, Kazakhstan, Kyrgyzstan, the Republic of Moldova, Tajikistan, Ukraine and Uzbekistan [22].

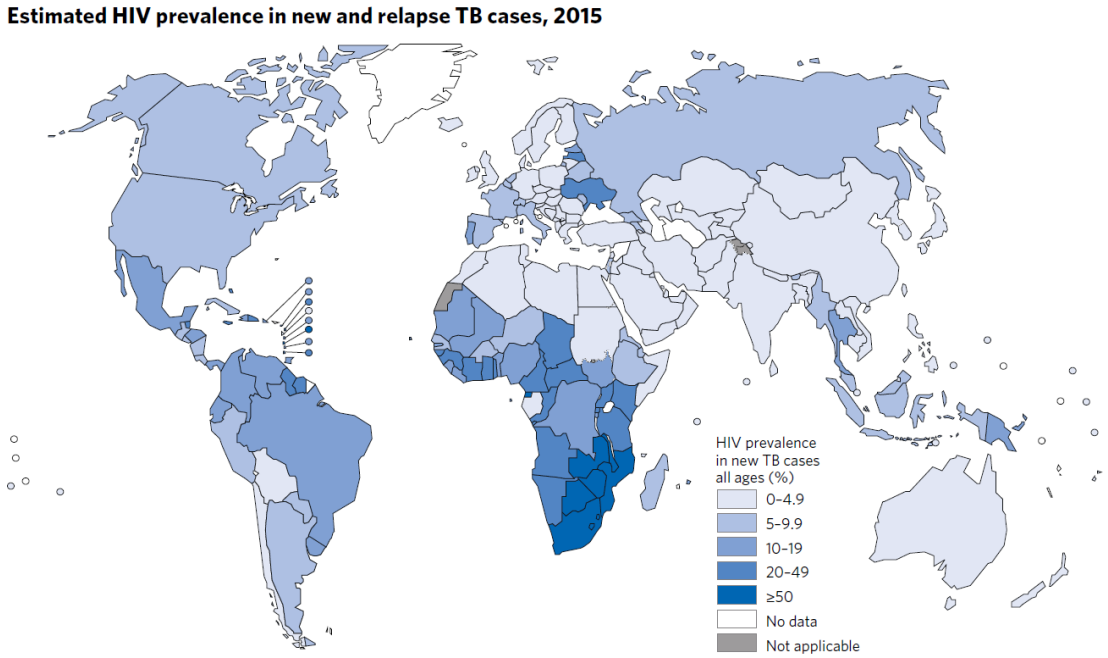


Figure 3: Estimated HIV prevalence in new and relapse TB cases, 2015.
11% of the incident TB cases in 2015 were among people infected with HIV. In some countries with high prevalence of TB in African Region, this proportion exceeded 50% [22].

b. Vaccine limitations

As previously mentioned, BCG is the only vaccine against TB available so far. This vaccine provides protection against severe forms of TB, particularly miliary and meningitis, in childhood [28]. In contrast, the reported efficacy of the vaccine against the pulmonary contagious form of TB in adults varies enormously, from 0 to 80% [29,30]. A meta-analysis calculated an overall efficacy of the vaccine of 50% against pulmonary TB in adults [29]. Numerous hypothesis have been formulated to explain this poor efficacy of BCG

- Presence of environmental mycobacteria, which can provide some protection against TB and mask the protection conferred by BCG [30]
- Population genetics [31]
- Variations in virulence of the *M. tuberculosis* strains [31,32]
- Difference between *M. bovis* (parental strain of BCG) and *M. tuberculosis*
- Exposure to chronic helminth infections that can reduce the host immune responses [31,33]
- Nutritional status [31]

- Variations in BCG strains that differ in terms of immune responses induced [31]
- Waning of BCG-induced immune responses over time
- Low $CD8^+$ responses

All these factors have shown that TB remains a global health problem that continues to kill and cause disease in millions of people each year. This mortality and morbidity are unacceptably high and in order to control TB, development of better tools to diagnose individuals with active TB on time is needed. This should lead to limit the transmission of the infection. Better and shorter treatments as well as a better vaccine able to confer protective immunity against pulmonary TB are also needed. In order to reach these objectives, an improvement of our knowledge of the bacterial physiology and of our understanding of all the mechanisms related to the pathogenicity of the bacteria and its interaction with the immune host are instrumental. This PhD thesis project fits into these objectives in two aspects. Indeed, on the one hand we have contributed to a further analysis of the host-pathogen interactions during *M. tuberculosis* infection and on the other hand we have explored the design of novel adjuvants, which can be used in subunit vaccines against diseases such as TB. In this regard, in the introduction we will discuss some selected pathogenesis determinants secreted by or found in the cell wall of *M. tuberculosis* and how these factors influence the host immune responses. We will next introduce current knowledge on protective immunity against *M. tuberculosis* infection. Finally, we will touch on development of subunit vaccines in general and TB subunit vaccines currently evaluated in clinical trials.

1.2 Host-pathogen interaction: the *Mycobacterium tuberculosis* perspective

1.2.1 Microbiology of *Mycobacterium tuberculosis*

M. tuberculosis is a facultative intracellular pathogen, aerobic, non-motile, not encapsulated and non-spore forming bacillus [34]. *M. tuberculosis* belongs to the *Mycobacterium* genus, from the family of *Mycobacteriacea,* in the *Corynebacterinae* sub-group of the Actinomycete line [35]. The members of the genus *Mycobacterium* are generally separated in two major groups based on their growth rate on solid medium. Among the slow-growing mycobacteria (generation times 24h or more) which are pathogenic in immunocompetent hosts, *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium ulcerans* are

respectively responsible for TB, leprosy and Buruli Ulcer [36]. In contrast, with the exception of *Mycobacterium abscessus* that is known now as an emerging human pathogen in cystic fibrosis patients, the fast-growing mycobacteria such as *Mycobacterium smegmatis* are generally non-pathogenic [36,37]. According to their phylogenetic position, Mycobacteria belong to the Gram-positive bacteria regrouped within the phylum of *Firmicutes.* Nevertheless, Mycobacteria are characterised by a very complex cell wall envelope that has been shown to form a sort of inner and outer membrane (a so-called mycomembrane) [38–40]. Therefore, the mycobacterial envelope resembles more closely the one of Gram-negative than the one of Gram-positive bacteria (Figure 4) [37]. This complex and unique cell wall envelope, particularly rich in lipids (30 to 60% of dry weight) [41], is responsible for the characteristic differential staining procedure known as Ziehl-Neelsen acid-fast stain [36]. Indeed, the cell wall of Mycobacteria resists to the decolourisation of pink fuchsine by acidic alcohol. In addition, a polysaccharide-based capsule is part of the cell wall envelope [42]. While the great majority of species in the genus *Mycobacterium* are environmental saprophytes abundant in soil and water, some mycobacteria such as members of *Mycobacterium tuberculosis* complex (MTBC) have evolved and acquired numerous molecular mechanisms to circumvent host defence systems and are able to establish long-term infections.

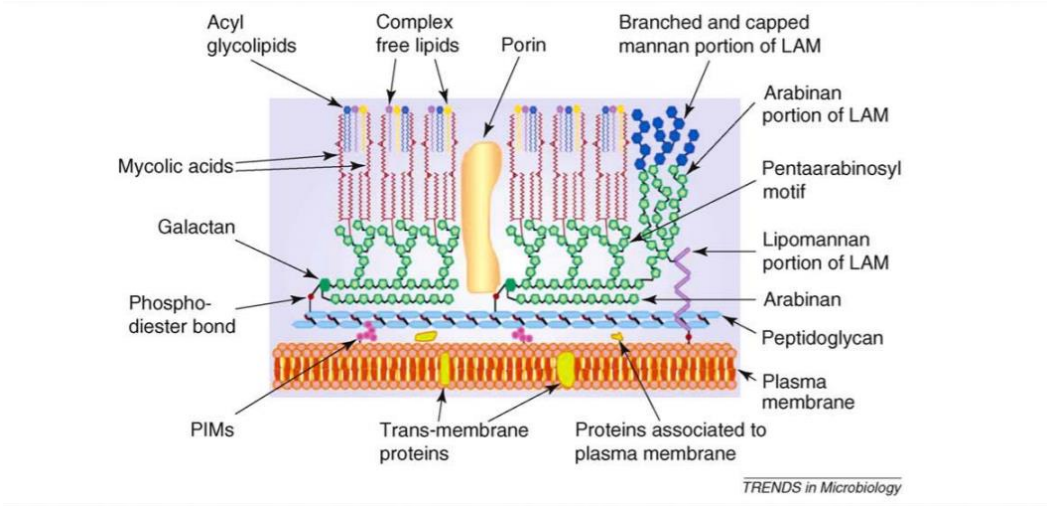


Figure 4: Schematic representation of the mycobacterial cell wall.
The cell wall of *M. tuberculosis* is characterized by the presence of a thick mycolate-rich outer layer constituting an extraordinary efficient permeability barrier. The cell wall of *M. tuberculosis* is rich in complex lipids such as lipoarabinomannam (LAM), phosphatidylinositol mannosides (PIM), mycolic acids and acyl glycolipids (TDM, TMM, GMM) [40].

1.2.2 *Mycobacterium tuberculosis* complex

M. tuberculosis belongs to the group of species know as *Mycobacterium tuberculosis* complex (MTBC) that comprises mycobacterial pathogens causing tuberculosis in humans and animals. MTBC consists of mycobacteria that are genetically very similar, with 99.9% similarity at the nucleotide level and with practically identical 16S rRNA sequences [43]. MTBC comprises *Mycobacterium tuberculosis, Mycobacterium canetti, Mycobacterium africanum, Mycobacterium bovis, Mycobacterium caprae, Mycobacterium pinnepedii, Mycobacterium orygis* and *Mycobacterium mungi* [37]. It has been proposed that members of MTBC evolved from a common ancestor via successive DNA deletions/insertions and acquired by this way the present speciation and their differences in pathogenicity.

1.2.3 TB pathogenesis

TB is an airborne disease transmitted by inhalation of aerosol droplets spread when patients with active disease cough, speak, spit or sneeze [44]. *M. tuberculosis* aerosol inhaled by a human subject can reach the alveolar spaces in the lung resulting in one of three possible outcomes: elimination of *M. tuberculosis*, establishment of a latent infection or development of active disease (Figure 5). It is estimated that in 70-90% of exposed subjects the bacteria are eliminated via the innate anti-microbial defences (detailed in chapter 1.3.1) while 10-30% of the exposed subjects will become effectively infected. Indeed, in the lung *M. tuberculosis* is phagocytosed by alveolar macrophages, in which the mycobacteria are contained in the endocytic compartment. Normally, phagosomes are fused with lysosomes to destroy the phagosomal content with lysosomal hydrolases, reactive oxygen and nitrogen species but *M. tuberculosis* possesses several mechanisms to counteract this fusion and is able to establish an infection inside the hostile macrophage environment [45]. Due to an unique ability of *M. tuberculosis* to delay the initiation of an adaptive immune responses during the early stages of the infection, the replication is very active [46,47]. After a few weeks, *M. tuberculosis* can reach high numbers in the lung (pulmonary TB) and can disseminate through the lymph and blood circulation to other parts of the body (extra-pulmonary TB). After the establishment of the adaptive immune response (detailed in chapter 3.2), in 90-95% of cases in immuno-competent people the infection is contained by the formation of a structure called a granuloma, characterized by a dynamic equilibrium between the bacilli and the host. In this granuloma,

there are dormant and replicating bacilli that continuously stimulate T-cell responses and on the other hand, T-cell responses control bacterial replication and prevent the emergence of active disease: this phenomenon is known as latent tuberculosis infection (LTBI) (Figure 6) [48]. WHO estimates that one third of the world population (2-3 billion people) is latently infected by *M. tuberculosis*. Latently infected individuals can potentially develop the disease especially if they become immuno-deficient [22]. An estimated 5-15% of people with LTBI will develop TB disease during their lifetime and this percentage significantly increases in HIV positive patients [22].

M. tuberculosis has numerous strategies (arrest of phagosome maturation, delay of the adaptive immune response, establishment of dormancy…) to avoid its complete elimination by the immune responses of the host. A great majority of components of *M. tuberculosis* that allow this persistence in the host are located in the cell wall envelope or are secreted proteins which are in close contact with its external environment. The characterization of these components associated with the cell wall is critical for a better understanding of bacterial survival and immune modulation in the host.

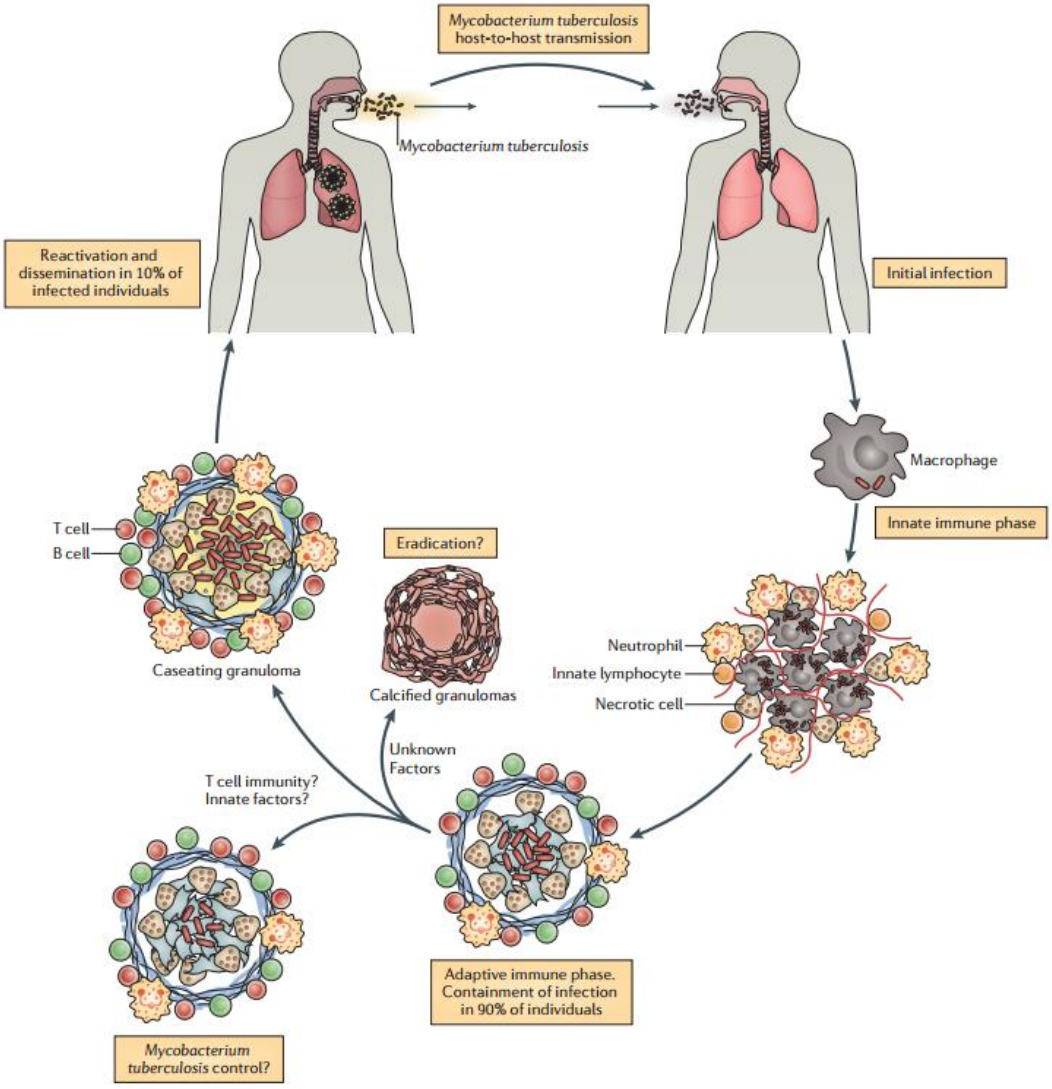


Figure 5: Tuberculosis pathogenesis.

Individuals with active pulmonary TB can generate by coughing infectious droplets that can transmit the infection to others by the inhalation of aerosol droplets that contain mycobacteria. The initial stages of infection are characterized by an innate immune response that involves the recruitment of inflammatory cells to the lung. Following migration of dendritic cells to the draining lymph node, dendritic cell presentation of bacterial antigens leads to T cell priming and triggers an expansion of antigen-specific T cells, which are recruited to the lung. The recruitment of T cells, B cells, activated macrophages and other leukocytes leads to the establishment of granulomas, which can contain *Mycobacterium tuberculosis*. Most infected individuals will remain in a 'latent' state of infection, in which no clinical symptoms are present. A small percentage of these people will eventually progress and develop active disease, which can lead to the release of *M. tuberculosis* from granulomas that have eroded into the airways [44].

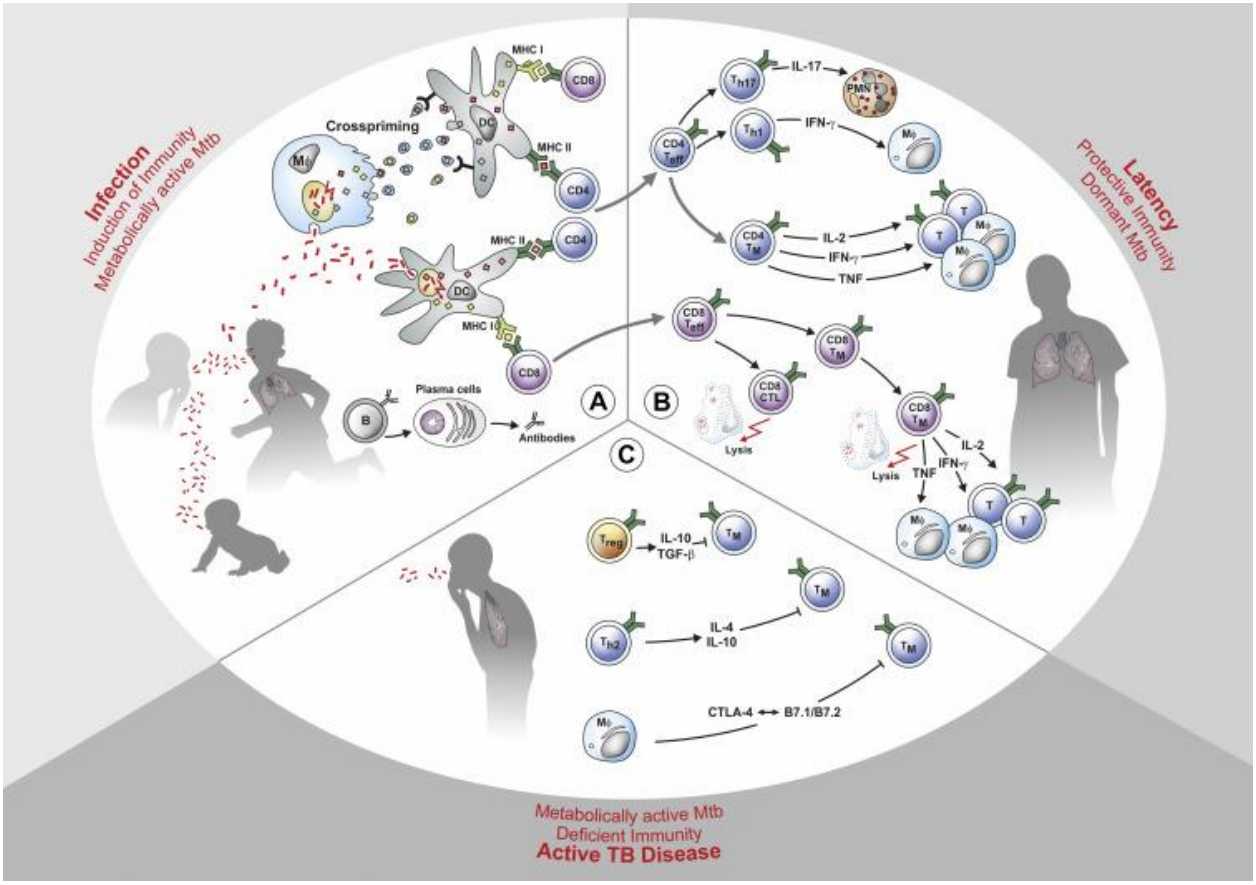


Figure 6: Immunity to TB.
(A) Antigen presentation by dendritic cells (DCs) results in stimulation of CD4 and CD8 T-cells. These T cells develop into memory T cells performing multiple functions. During the infection state, Mtb is metabolically active. (B) As long as memory T cells can sustain the solid granuloma, Mtb is controlled and individuals remain healthy but latently infected. During latency, Mtb enters into, and remains in, a dormant stage. (C) Immuno-regulatory mechanisms impair T cell responses, thereby allowing caseation of granulomas and progression to active disease. During disease, Mtb regains metabolic and replicative activity.
Abbreviations: B, B cell; CD4, CD4 T cell; CD8, CD8 T cell; CTL, cytotoxic T lymphocyte; CTLA-4, CTL-associated protein 4; IFN-γ, interferon gamma; IL, interleukin; MΦ, macrophage; MHC, major histocompatibility complex; Mtb, Mycobacterium tuberculosis; PMN, polymorphonuclear neutrophil; T, T cell; TB, tuberculosis; Teff, effector T cell; TGF-β, transforming growth factor beta; Th, T helper cell; TM, memory T cell; TNF, tumor necrosis factor; Treg, regulatory T cell [48].

1.2.4 *Mycobacterium* cell wall

In this chapter, we will introduce some selected cell wall components of *M. tuberculosis,* which have been associated directly or indirectly with *M. tuberculosis* pathogenesis (defined as PAC for pathogenesis associated components) as well as enzymes involved in their synthesis. The PACs introduced here are involved in the activation or in the modulation of immune responses (introduced in section 1.3). Deletion of some of these PACs results in mutants with attenuated replication potential *in vitro* and *in vivo.* The complete sequencing by S. Cole *et al* in 1998 of the genome of the laboratory reference strain *M. tuberculosis* H37Rv has greatly contributed to a better understanding of the pathogenicity of *M. tuberculosis* [49]. The genome of *M. tuberculosis* H37Rv has a size of 4.4 megabase (Mb) and it encodes approximately 4000 proteins and 50 RNA molecules [49] (Figure 7). Comparative genomic and functional analysis has allowed for example to identify 14 regions known as regions of difference (RD-1 to RD-14) which are involved in the pathogenesis of *MTBC* [50]. The RD-1 locus of *M. tuberculosis* H37Rv is absent in all BCG vaccine strains [51].

The selected PACs present in the cell wall envelope introduced hereafter in more detail are divided in proteins, lipids and carbohydrates.

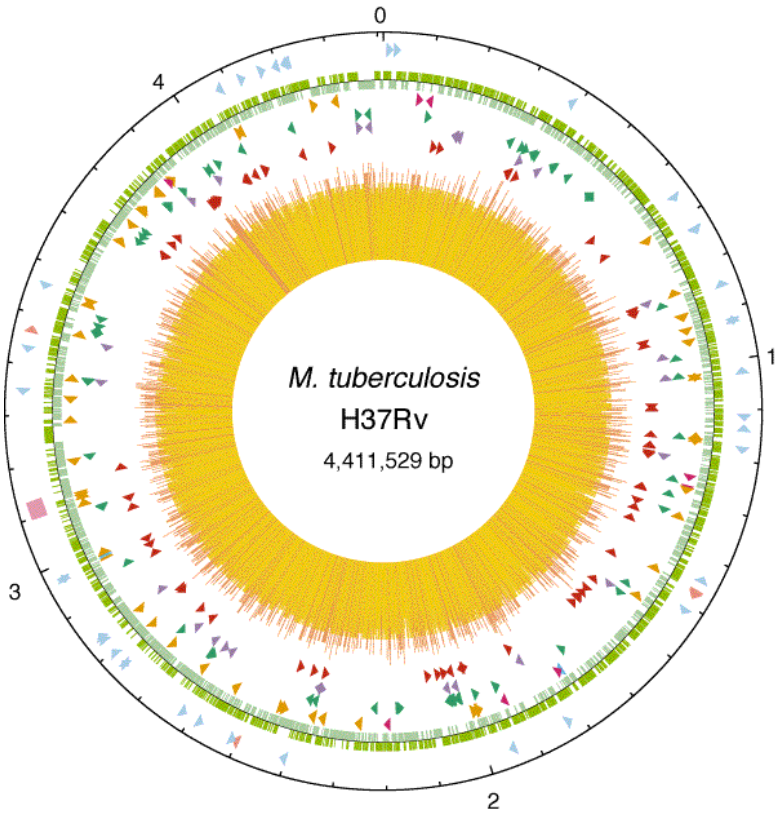


Figure 7: The complete genome of *M.tuberculosis* H37Rv.
0 represent the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, others are pink) and the direct repeat region (pink cube); the second ring inwards shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (centre) represents G + C content, with <65% G + C in yellow, and >65% G + C in red [48].

a. Cell wall and secreted proteins

As we previously mentioned, the cell wall envelope of *M. tuberculosis* is highly complex and is composed in majority of lipids, but also of proteins. By using proteomic approaches, Wolfe and co-workers identified 528 proteins in the cell wall of *M. tuberculosis* [52]. Most of these proteins are involved in metabolism and cell wall processes. About 15% of these proteins are involved in the lipid metabolism [53]. 87 proteins are predicted to have secretion signals. Hereafter, cell wall and secreted proteins involved in the virulence of *M. tuberculosis* or in the synthesis of PACs are briefly introduced.

❖ PE/PPE family

PE/PPE proteins belong to a family of 169 proteins, which are found only in mycobacteria. These proteins represent 7.1% of the genome of *M. tuberculosis* H37Rv strain. The name PE and PPE derives respectively from the Pro-Glu (PE) and Pro-Pro-Glu (PPE) sequences found in their conserved N-terminal regions. Most of these proteins are secreted by a dedicated type VII secretion system [54,55] (encoded by ESX-5) and induce strong immune responses [56,57]. Therefore, they are included as antigens in different subunit vaccines in clinical and preclinical development.

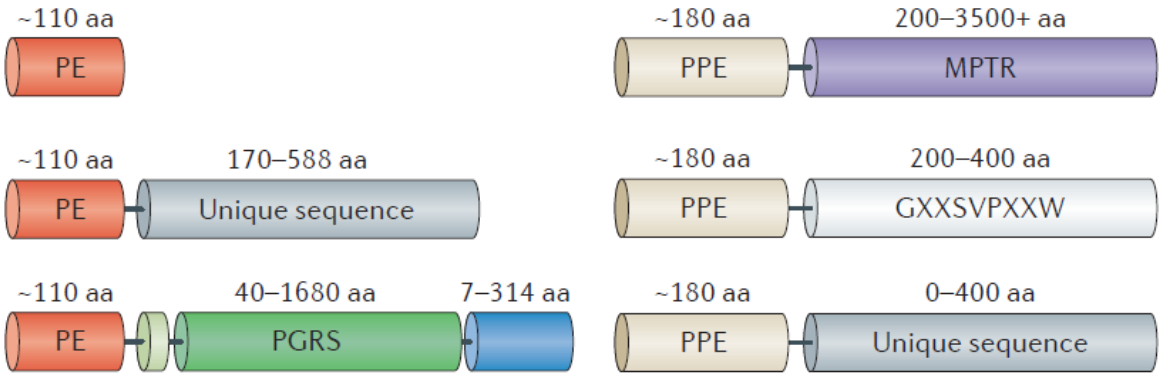


Figure 8: Structures of PE/PPE-secreted proteins.
PE/PPE proteins are characterized by a conserved amino-terminal region that comprises the Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs. These motifs are respectively approximately 110 and 180 amino acids long. The PE and PPE domains are fused to diverse central and carboxy-terminal sequences that may contain repeated motifs (such as polymorphic GC-rich-repetitive sequences (PGRS) or major polymorphic tandem repeats (MPTR) [273].

The functions of these PE/PPE proteins are not yet elucidated but they appear somehow to be related to pathogenicity. Indeed, slow-growing mycobacteria possess larger and more complex PE/PPE proteins compared to fast-growing mycobacteria. Slow-growing

mycobacteria contain at the C-terminus of some of their PE and PPE proteins a PGRS and MPTR domain respectively (Figure 8). PGRS is a polymorphic G+C rich sequence and MPTR is a major polymorphic tandem repeat sequence [58]. The direct role of PE/PPE families in the pathogenicity was demonstrated by Dheenadhayalan and co-workers who showed that PE_PGRS 33 (Rv1818c) exerts a cytotoxic effect on host cells. In fact, they have expressed the *PE_PGRS 33* gene in the non-pathogenic fast-growing *Mycobacterium smegmatis* [59]. Their results showed that the recombinant strain induced more TNF-α and cell necrosis *in vitro* in macrophages compared to the parental *Mycobacterium smegmatis* strain transformed with non-coding control vector or a strain expressing only the PE domain of PE_PGRS 33. Further, the mutant strain survives better in macrophage cultures as well as in mice after intraperitoneal injection compared to the parental *M. smegmatis* strain. Later, it was demonstrated that the induction of cell death by PE_PGRS 33 was related to its co-localization to the mitochondria of transfected cells [60]. As necrosis of macrophages is important for the dissemination of *M. tuberculosis* during natural infection, PE_PGRS 33 might be involved in the pathogenesis of TB. Two other members of the PE/PPE families PE_PGRS51 (Rv3367) and PPE46 (Rv3018) have been shown to be essential for the full replication of *M. tuberculosis* in the lungs of mice [61,62]. Finally, deletion of the five adjacent genes encoding PPE25 to PE19 (Rv1787 to Rv1791) in the ESX-5 type VII secretion operon results in a *M. tuberculosis* mutant strain of considerable decreased virulence in bone marrow derived macrophages and SCID mice [63]and with an interesting vaccine potential [64]. In our laboratory, we investigated the vaccine potential of a member of the PE/PPE proteins, namely PPE44 (Rv2770c) [65]. We found that mice vaccinated with a plasmid DNA vaccine coding for PPE44 or with recombinant PPE44 formulated in cationic liposomes induced strong cellular and humoral immune responses. Further, we demonstrated that these vaccinations are protective in a mouse model of *M. tuberculosis* challenge. This result suggests that PPE44 of *M. tuberculosis* can be used as a protective antigen that can be incorporated in tuberculosis subunit vaccine. Thus, in this PhD thesis, we used PPE44 as a model antigen for protection experiments.

❖ Fibronectin binding proteins

Fibronectin binding proteins (Fbp) are three proteins known as FbpA (Rv3804), FbpB (Rv1886) and FbpC2 (Rv0129c), encoded by genes on distinct locations in the genome of *M.*

tuberculosis. The name comes from the ability of these proteins to bind to fibronectin. Although not physically organized in a complex, the three proteins are commonly also referred to as the antigen 85 complex (Ag85), composed of Ag85A (FbpA), Ag85B (FbpB) and Ag85C (FbpC2). Ag85A, Ag85B and Ag85C are major secreted proteins of *M. tuberculosis* [66] and are also present in the cell wall envelope [67]. The secretion of these proteins is dependent on a Twin-Arginine translocation (TAT) secretion system [68]. Just like members of the PE/PPE family, members of the Ag85 family are highly immunogenic. Ag85A and Ag85B are used in preclinical and clinical studies for the development of new TB subunit vaccines. The Ag85 proteins play an essential function in the pathogenesis of the bacilli. Indeed, they can alter the host immune response by binding to fibronectin or immunoglobulin G in the blood [69]. Armitige and co-workers have reported that the deletion of *fbpA* and *fbpB* results in strongly reduced growth of *M. tuberculosis* in macrophage cell lines J774 and THP-1 [70,71]. Ag85 proteins play an essential enzymatic function and are shared among all mycobacteria. These enzymes are mycoloyltransferases involved in the synthesis of trehalose dimycolate (cord factor TDM) by coupling a second mycolic acid to trehalose monomycolate and in the coupling of mycolic acids to the arabino-galactan of the cell wall [72]. These mycolate esters play an important role in the pathogenesis of *M. tuberculosis* and will be discussed hereafter. Antigen 85C inhibition restricts *M. tuberculosis* growth by inducing the disruption of trehalose dimycolate biosynthesis [73,74].

❖ ESAT6 family

The ESAT-6 (Early secreted antigenic target 6 kDa) family represents a group of small proteins composed of about 100 aa [75]. These proteins lack the classical signal sequence necessary for secretion via the Sec2 pathway or the TAT system and similarly to PE/PPE proteins, these proteins are secreted via type VII secretion systems [76]. ESAT-6 family proteins are characterized by an amino acid (aa) motif Trp-Xaa-Gly (WXG) located in the central part of the protein [68]. In *M. tuberculosis*, the ESAT-6 family is constituted by 23 members. The genes of this family are located in tandem pairs at 11 loci in the *M. tuberculosis* H37Rv genome and are often preceded by *pe/ppe* gene pairs [75]. ESAT-6 (Rv3875 or *esxA*) and CFP-10 (Rv3874 or *esxB*) are the most studied proteins of this family. *esxA* and *esxB* are both encoded by the region of difference 1 (RD1) [36,75,77]. This region is absent in the vaccine strain *M. bovis* BCG and

also in another *MTB*C member of attenuated virulence, namely *Mycobacterium microti* [78] (Figure 9). *M. microti* has been used as a live attenuated vaccine in the 1960s [79]. These observations clearly suggested the importance of RD1 in the virulence of *M. tuberculosis*. The relevance of this region in the virulence of *M. tuberculosis* was demonstrated by functional genomics. Indeed, Pym and colleagues have demonstrated that re-introduction of the RD-1 locus in *M. bovis* BCG and *M. microti* resulted in a strain with increased virulence, characterized by more vigorous growth of the BCG::RD1 and *M. microti*::RD1 knock-ins than the parental strains in immunodeficient SCID mice [78]. In C57BL/6 and BALB/c mice BCG::RD1 persists while the parental BCG strain is cleared. In addition, the importance of RD1 in the virulence was also demonstrated using transposon mutants and/or genetic deletion of RD1 in *M. tuberculosis* [80].

The RD1 locus is a region of 9.5 kilobases of *M. tuberculosis* composed of 9 genes from *Rv3871* to *Rv3879c* (Figure 9) [81]. Several studies using genetic approaches and biochemical analysis demonstrated that the genes within the RD1 region are important for the secretion of ESAT-6 and CFP-10. This secretion system is known as ESX-1. Brodin and co-workers have investigated the contribution of each member of the ESX-1 secretion system in the secretion of ESAT-6 and CFP-10 [82]. They have demonstrated that with the exception of *Rv3873* and *Rv3876*, the inactivation of all the other genes of the RD1 locus results in an impairment of the secretion of ESAT-6. Further, they have clearly demonstrated the correlation between the secretion of these proteins and the virulence in SCID mice [82].

In addition, ESAT-6 plays an important role in cytosolic egress of *M. tuberculosis* and in inflammasome activation (discussed in detail in section 1.3.1.b).

Beside this role of ESAT-6 and CFP-10 as PACs, these proteins have also been reported to be strongly immunogenic in several animal species including mice [83,84], cattle [85], guinea pigs [86] and humans [87]. Karlsson et *al.* have shown that up to 35% of splenic T cells respond to ESAT-6 in *M. tuberculosis* infected C57BL/6 mice [88]. Given that ESAT-6 and CFP-10 are immunogenic proteins specific for *M. tuberculosis* and are not expressed by the vaccine strain *M. bovis* BCG, they can be used in *in vitro* blood test to specifically detect *M. tuberculosis* infection without cross-reaction in BCG vaccinated individuals. In 2001 indeed, a study of Lalvani and co-workers analyzed peripheral blood T cell responses to ESAT-6 using IFN-γ ELISPOT in different groups

of individuals [89]. They observed a sensitivity of 96% for detecting *M. tuberculosis* in patients with bacteriologically confirmed tuberculosis as compared to patients suffering from other illnesses. In addition, they could detect ESAT-6 specific responses in skin test positive exposed household contacts, while no ESAT-6 specific responses could be detected in BCG vaccinated individuals [89]. Nowadays, interferon release assays (IGRAs) to measure the amount of IFN-γ released in response to ESAT-6 and CFP-10 in peripheral blood derived cells are used to detect LTBI (confer section 1.2) [90].

Several other members of this family are known to be secreted and to be immunogenic, including TB10.4 (Rv0288 or *esxH*), Rv0287 (*esxG*) and Rv3019c (TB10.3). ESAT-6, TB10.4 and TB10.3 are components of several subunit vaccines against TB in preclinical studies and clinical trials [47,91].

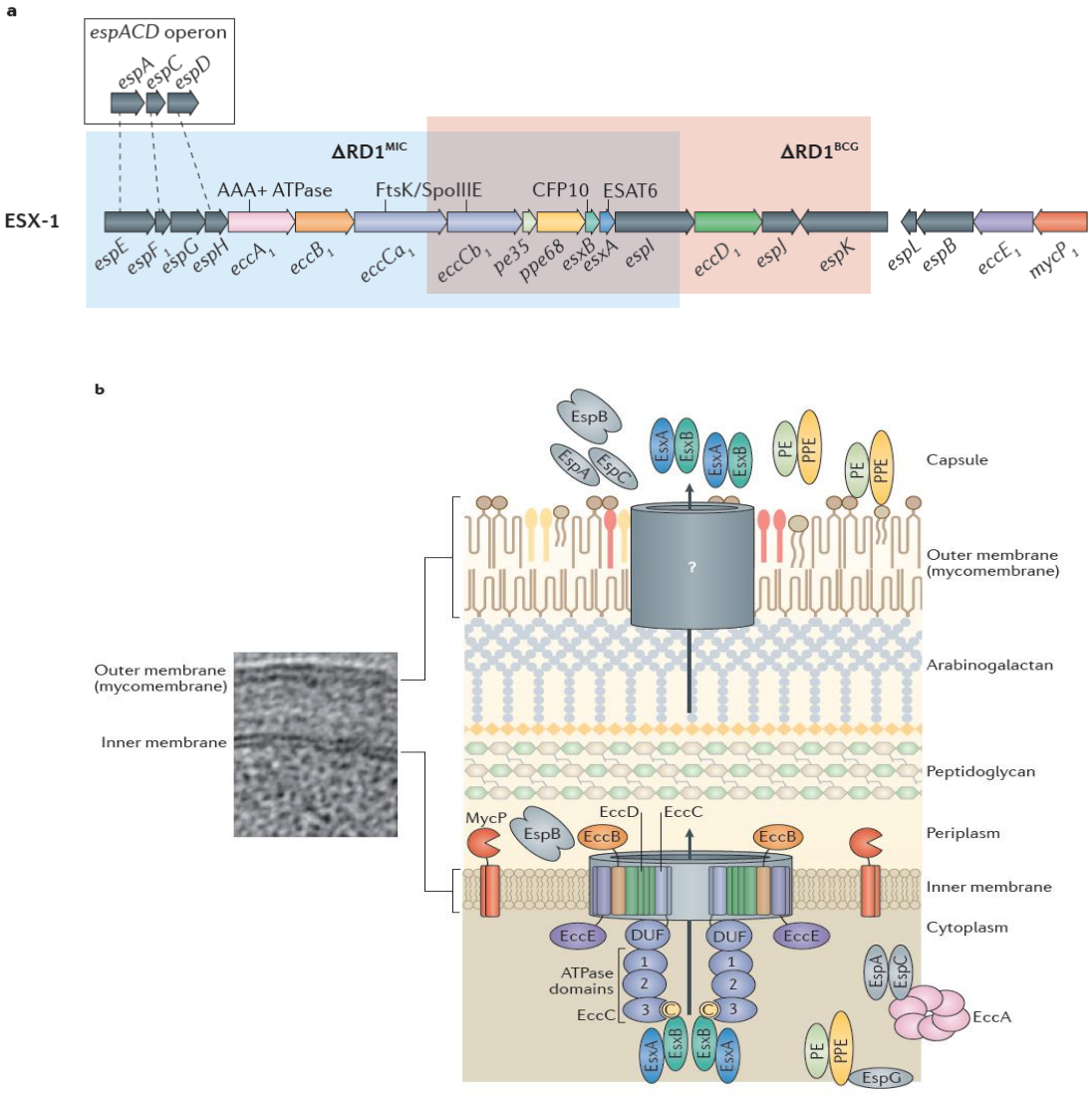


Figure 9: Genetic and structural architectures of ESX systems.
a) Genetic organization of the *esx*-1 locus. This locus comprised three group of genes: *esx* genes*, ecc* genes and *esp* genes. The *esx* genes encode for the two effector proteins EsxA and EsxB. ΔRD1^{MIC} represents the region absent in *M. microti* and ΔRD1^{BCG} the region absent in BCG strain. These regions are thought to be associated with the attenuation of the strains. espACD operon shares sequence homology with *espE, espF* and *espH* located in the *esx-1.* **b)** Electron microscopy image of the inner and outer membranes of mycobacteria cell envelope and model of the secretion apparatus of secreted substrates of ESX systems. EccB, EccC, EccD and EccE form the core structure of the ESX systems. EccC is thought to be a translocase that provides the energy for the secretion of EsxB. EsxB is cosecreted with EsxA. Heterodimers that are formed from PE and PPE family proteins are also secreted by ESX secretion system [273].

b. Lipids, sulfolipids and glycolipids

As previously mentioned, the cell wall of *M. tuberculosis* is complex and extremely rich in lipids and glycolipids (30 to 60% of its dry weight). Three different compartments can be distinguished: the plasma membrane, the cell wall skeleton and the capsule (Figure 4) [35]. Several lipids found in these 3 compartments are characteristic of mycobacterial species [92]. These include:

- Phosphatidylinositol mannosides (PIMs), lipoarabinomannan (LAM) and lipomannan (LM) anchored in the plasma membrane (Figure 10).
- Phthiocerol containing lipids, trehalose containing lipids and mycolic acids (MAs) found in the cell wall skeleton (Figure 10).

Several of these components are associated with the immune response to *M. tuberculosis* and this aspect is discussed in chapter 1.3. In this chapter we introduce their fine structure and differences between pathogenic and non-pathogenic mycobacteria.

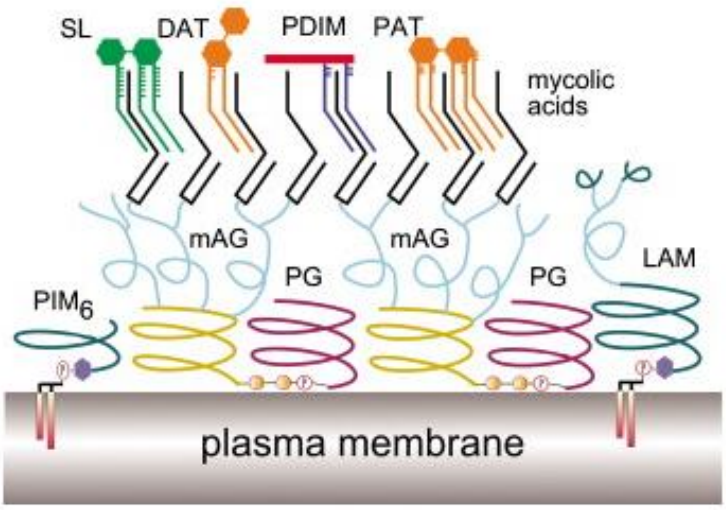


Figure 10: Structural components in the cell envelope of *M. tuberculosis.*
Mycolyl arabinogalactan (mAG) is connected by a phosphoryl linker unit to peptidoglycan (PG). Complex free lipids (PDIM, DAT, PAT and SL) interact with mAG. LAM and PIM_6 are shown anchored in the plasma membrane [518].

❖ PIMs, LM and LAM

Phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) are glycolipids found in the cell wall and are involved in the pathogenicity of mycobacteria. The phosphatidyl-*myo*-inositol mannosides (PIMs) are composed of a phosphatidyl-*myo*-innositol (PI) anchor that can carry one to six mannose groups and up to four acyl chains [93]. Thus, the family of PIMs is composed of PI mono-, di-, tri-, tetra-, penta- and hexamannosides with different degrees of acylation. In mycobacteria, the acyl groups are predominantly composed of palmitic, tuberculostearic (10-methyl-octadecanoic), myristic and octadecenoic acids [94]. Some traces of stearic, hexadecenoic and heptadecanoic acids are also present [94]. PIM_2 and PIM_6 are the predominant classes found in *M. tuberculosis*, *M.bovis* BCG and *M. smegmatis* [95]. PIMs are the structural basis of the lipoglycans (LM and LAM) [94]. By their PI moiety, these compounds are non-covalently attached to the inner and outer membranes of the mycobacterial envelope [96,97]. LM is a polymannosylated form of PIM [98], while LAM is known as LM with an arabinan domain. LAM is formed by three domains, a glycosylphosphatidyl anchor, a D-mannan core and a large terminal D-arabinan (Figure 11). The D-arabinan can be capped by mannosyl (ManLAM) or phosphoinositol (PILAM) or uncapped (AraLAM) [99]. Mannosyl caps of ManLAM seem to be related to the virulence. Indeed, ManLAM has been found in pathogenic mycobacteria, such as *M. tuberculosis* and *M. kansasii* [100], whereas PILAM has been found in non-pathogenic species, such as *M. smegmatis* [101,102]. AraLAM has been isolated from *M. chelonae* [99].

In addition to their physiological function, namely maintaining cellular integrity and acting as a permeability barrier of the cell envelope, lipoglycans have been implicated in the immune-modulation of the immune responses. Indeed, Man-LAM and PIMs have been shown to be involved in several mechanisms used by *M. tuberculosis* to avoid its elimination and favor spreading. These mechanisms include: arrest of phagosome-lysosome fusion, escape from phagosome to the cytosol and inhibition of the production of pro-inflammatory cytokines (discussed in chapter 1.3.1)[94,103–105].

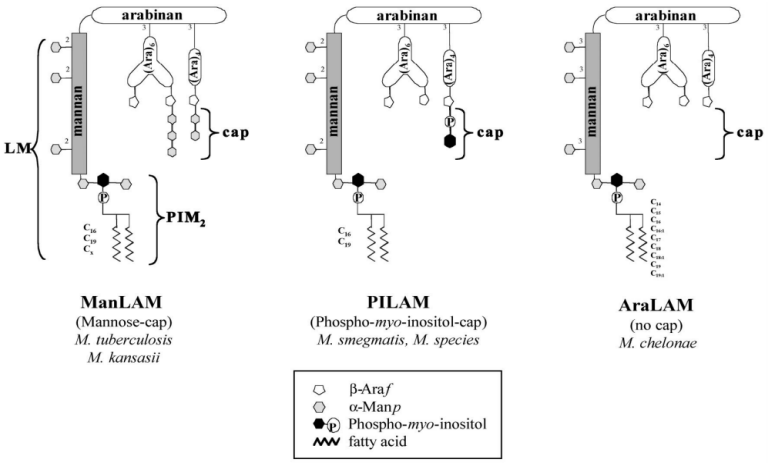


Figure 11: Structures of the three representative families of LAM molecules isolated from different mycobacterial species [105].

❖ Phthiocerol containing lipids (PDIM and phenolic glycolipids)

Phthiocerol containing lipids comprise phthiocerol dimycocerosates (PDIM) and phenolic glycolipids (PGL) [35]. PDIMs are composed of long chain β-diol (C33-C41)–phthiocerol-esterified by long chain, multiple methyl branched fatty acids known as mycocerosic acids (C27-C31) [36]. PGL have a similar core as PDIM, with the exception that the phthiocerol chain is ended by a glycosylated phenolic part [35]. This glycosylated moiety varies between different mycobacterial species [106]. PDIMs have been isolated from pathogenic mycobacteria, such as *M. africanum, M. bovis, M. leprae, M. marinum, M. ulcerans, M. kansasii, M. haemophilum, M. microti, M. gastri* and *M. tuberculosis*. PGL are produced by the same pathogenic species except the fact that only certain *M. tuberculosis* strains isolated from infected patients produce PGL. Several studies have demonstrated the importance of phthiocerol lipids in the virulence of *M. tuberculosis*. Using transposon mutagenesis, Cox and co-workers have identified three mutants of *M. tuberculosis* that are not able to generate or transport PDIM in the cell wall. They have found that these three mutants are attenuated compared to WT strain [107]. Later, it was reported that Δ*drrc* MT103, a mutant strain of *M. tuberculosis* MT103 incapable to translocate PDIM to the mycobacterial cell wall, is also attenuated and more protective than *M. bovis* BCG in a mouse model [108]. Recently, Day et *al.* have demonstrated that PDIMs are important for the protection of *M. tuberculosis* against early innate immune responses [109]. Interestingly, the deletion of *phop* and *fadD26* genes results in complete abrogation of the synthesis of PDIM. This mutant, namely MTBVAC, is highly attenuated compared to WT strain and a promising new TB vaccine candidate [110]. The role of PGL in virulence of *M. tuberculosis* is not so clear. Indeed, several clinical isolates, such as CDC1551

or MT103, are naturally deficient in PGL production [111,112]. The deletion of PGL in *M. tuberculosis* HN878, an isolate known to produce both PDIM and PGL and exhibiting a hyper-virulent phenotype in infected animals, decreases the virulence of the mutant to the same level as strains that do not produce PGL (CDC1551, H37Rv) [111,113]. From these observations, it was suggested that PGL is associated to the hyper-virulence of some strains of *M. tuberculosis* such as the Beijing family. However, H37Rv strain that is genetically modified to produce PGL does not seem to be more virulent than the parental strain in mice and rabbit [114]. On the other hand, it was reported that *M. marinum* mutants deficient in the production of PDIM and/or PGL are attenuated in zebrafish [115]. In addition, Tran and colleagues have reported recently that *M. bovis* BCG deficient in the production of PDIM and PGL was more attenuated in SCID mice [116].

❖ Trehalose containing lipids (Sulfolipid and poly-acyl trehalose)

Trehalose containing lipids comprise sulfolipid 1 (SL-1), diacyltrehaloses (DAT), triacyltrehaloses (TAT) and penta-acyltrehaloses (PAT) (Figure 12). SL-1 is composed of sulphated trehalose which is esterified at position 2 by four fatty acids [117]. DAT are constituted of stearic acid at position 2 and a methyl acid at position 3 of trehalose. Finally, PAT are formed by trehalose esterified by five fatty acids [41]. The distribution of these lipids in different mycobacterial species suggests a potential contribution of these compounds to the pathogenesis of mycobacteria. DAT and PAT have been isolated only in members of *Mycobacterium tuberculosis* complex [118]. Sulfolipids (SL) have only been isolated from *M. tuberculosis* and *M. canetti.* Purified SL but also DAT and PAT have been shown to regulate host immune responses *in vitro* [119–123]. Nevertheless, the contribution of these molecules in the pathogenesis of *M. tuberculosis* is not clear *in vivo.* Indeed, it was described that deletion of *pks2-3/4* gene (involved in the synthesis of trehalose containing lipids) in *M. tuberculosis* H37Rv does not result in the attenuation of the strain [124,125]. It cannot be excluded that this lack of attenuation is due to compensatory PACs that complemented the absence of trehalose derived lipids. Recently, Passemar and colleagues analyzed the individual and combined contributions of SL, DAT/PAT, and PDIM to the pathogenesis of *M. tuberculosis* [118]. They have found that the single deficiency of PDIM affects the replication of *M. tuberculosis* in lung and spleen of mice in comparison to WT strains. Further, they have reported that an additional

deficiency of DAT/PAT and SL further increased the attenuated phenotype of the PDIM mutant. Interestingly, they have observed this increased attenuation of DAT/PAT and SL mutant on the PDIM deficiency background also in human monocyte-derived macrophages [118]. Taken together, these results demonstrated that PDIM is a dominant PAC that can mask the function of trehalose containing lipids.

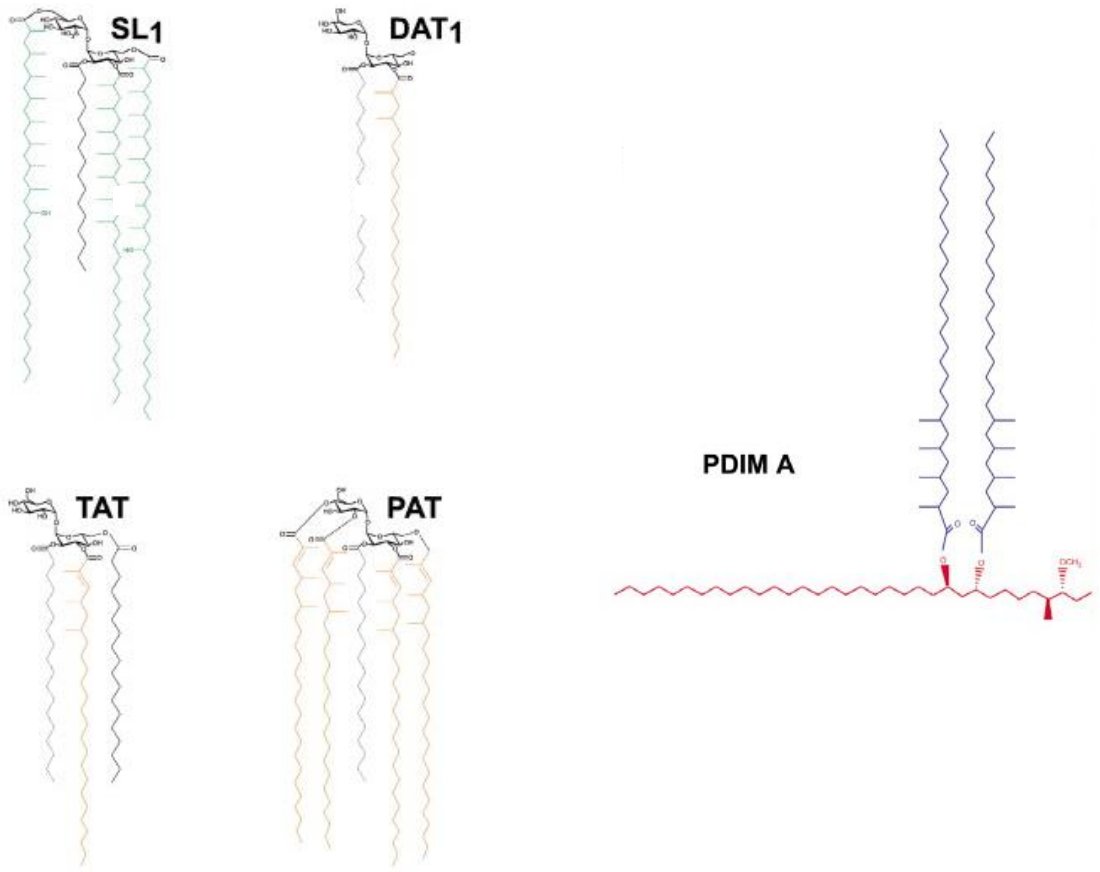


Figure 12: Structures of complex lipids of the cell envelope of *M. tuberculosis.*
SL1 (sulfated tetra-acyl trehalose based on phthioceranic and hydroxypthioceranic acids, DAT (diacyl trehalose based on mycosanoic acids, TAT (triacyl trehalose) and PAT (pentaacyl trehalose) based on mycolipenic acids [518].

❖ Mycolic acids and esters.

- Structural features

In 1950, Asselineau and Lederer described the first structure of MAs (Figure 13) [126]. MAs are long chain α-alkyl β-hydroxy fatty acids that constitute up to 60% of the dry weight of the cell wall [127]. They are found mostly esterified with the terminal penta-arabinofuranosyl units of arabinogalactan (AG), present as free mycolic acids or as non-bound extractable lipids esterified with different sugars (trehalose, glucose) and glycerol [128,129]. MAs represent the hallmark of the cell wall of mycobacterial species but are also present in some other bacteria of the order of *Corynebacteriales*. The chain lengths of MAs and their complexity vary depending on the species and are largely used as taxonomic markers. In fact, in *Corynebacteria,* they consist of 22 to 38 carbon atoms, 30 to 36 in *Amycolicicoccus* and *Hoyosella,* 34 to 36 in *Dietzia,* 34 to 52 in *Rhodococcus,* 46 to 60 in *Nocardia*, 46 to 66 in *Gordonia*, 64 to 78 in *Tsukamurella,* 60 to 90 in *Mycobacterium* and finally up to 100 in *Segniliparus* [127,128]. In addition to the chain length, MAs of mycobacteria vary in terms of chemical groups present in the long alkyl chain, known as the meromycolate chain, which define the different classes of MAs. α-MAs are the most apolar MAs with generally two *cis*-cyclopropyl groups or two double bonds in *cis* or *trans* configuration. α-MAs with three double bonds and longer chains [130] or with double bond and shorter chain (known as α'-MA) have also been reported [128]. In addition, several classes of MAs with oxygen groups located in the distal part of the meromycolic chain are also present in the cell wall of some mycobacteria species. These MAs are methoxy-, keto-, wax-ester-, epoxy- and hydroxyl- (Figure 14) [127–129]. Further, cyclopropane rings present in the proximal position of the meromycolate chain can have both *cis*- or *trans*- stereochemistry [131]. The composition of MAs in mycobacteria consists of a very complex mixture and can be used as a fingerprint [128]. A study that demonstrated clearly this complexity of the composition of MAs is the one of Watanabe *et al.* published in 2001 [132]. By using chromatography, mass spectrometry and ^{1}H-NMR techniques, they analyzed the composition in MAs of 24 representative samples of *M. tuberculosis* mycobacterial strains including *M. bovis* BCG, *M. microti* and *M. avium*. Later, Laval et *al.* have shown that a relationship between the growth rate of mycobacteria and the composition of MAs [133]. Indeed, slow-growing mycobacteria like *M. ulcerans* or *M. tuberculosis* produce a series of odd carbon numbers of α-MAs while fast-growing mycobacteria synthesize both even

and odd carbon numbers. Further, they have reported that the meromycolate chain of oxygenated MAs of slow-growing mycobacteria are 6-8 carbon atoms longer than the corresponding α-MAs, while in fast-growing mycobacteria oxygenated and α-MAs possess the same chain lengths [133]. Others reported that the fast-growing and saprophytic strain *M. smegmatis* possesses a majority of α-alkene MAs [134].

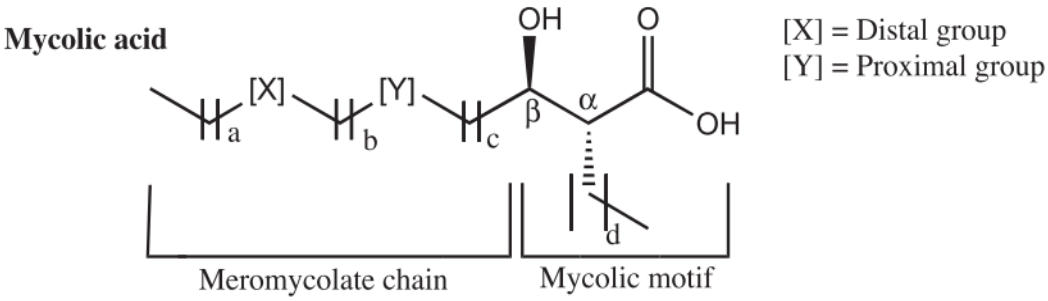


Figure 13: General structure of mycolic acids [129].

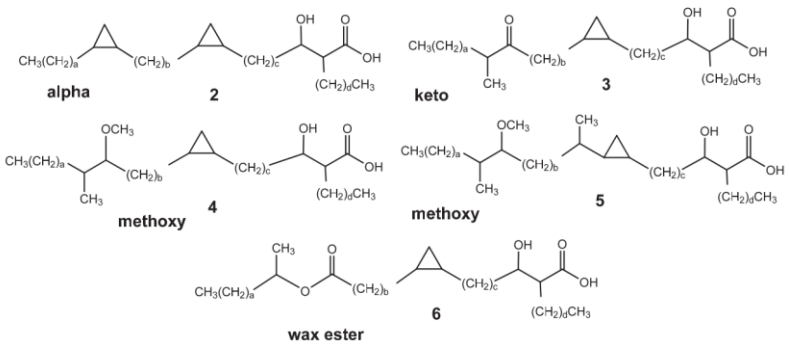


Figure 14: Major mycolic acids of *Mycobacterium tuberculosis complex* and wax ester from *Mycobacterium paratuberculosis* [129].

- Mycolic acids

The most important aspect regarding the contribution of MAs in the virulence of *M. tuberculosis* is related to their physiological function in maintaining the structure of the cell wall. Indeed, MAs are responsible for the extremely low permeability of the mycomembrane to hydrophilic molecules, such as antibiotics. In addition to this structural role, MAs are also involved in the virulence of *M. tuberculosis* by inducing foamy macrophages (FM) and by participating in the formation of biofilm.

- Foamy macrophages (FM) are a characteristic of *M. tuberculosis* infection and are formed by an accumulation of lipid droplets in the macrophages. Korf and colleagues have reported that MAs formulated in phosphatidylcholine liposomes induced the formation of FM when administered in C57BL/6 mice [135]. The induction of this specific cell population has been shown to be related to the class of MAs. Indeed, by using *M. smegmatis* (that does not produce oxygenated MAs), *M. smegmatis* that overexpressed *hma gene* (responsible for the introduction of keto-MAs in the strain) and *M. tuberculosis,* Peyron *et al* have shown that only strains with oxygenated MAs induced FM [136].These results suggested that oxygenated MAs, especially keto-MAs, were responsible for the induction of FM. This observation was later confirmed by Dhkar and co-workers who demonstrated that keto-MAs induce the formation of foamy macrophages while alpha- and methoxy MAs do not [137]. Recently, Vermeulen and colleagues showed that keto-MA induced accumulation of cholesterol esters and lipid droplets that facilitates BCG proliferation in macrophages. In addition, they found that methoxy-MA induces the formation of vacuoles without changing the cholesterol ester levels and does not facilitate mycobacterial growth [138].
- The formation of biofilms is known to be implicated in the chronicity and transmission of several infections. The formation of biofilms *in vitro* has been reported for several mycobacteria [139–141]. The ease to culture mycobacteria as a surface pellicle on synthetic Sauton medium (eg. the classical production method for BCG vaccine) is a reflection of this property. These biofilms are rich in free MAs released after bacterial lysis due to long culture time or by enzymatic hydrolysis of TDM [141]. A correlation between MA biosynthesis and the formation of biofilms has been reported [142,143]. In this context, the classes of MAs also appear to be important. Indeed, Sambandan and co-workers have shown that the

deletion of *hma* gene results in a mutant strain defective in the formation of biofilm and highly sensitive to antibiotics [144].

MAs also mimick certain immunological aspects of *M. tuberculosis* infection including the production of pro-inflammatory chemokines and cytokines and a transient neutrophil influx. This pro-inflammatory property of MAs has also been reported to be dependent on the class of MAs [135,145]. In fact, Vander Beken *et al* have reported that intra-tracheal instillation of liposomes containing single isomers of MAs induces lung inflammation that depends on the class of MAs. Alpha-MA appears to be inert while *cis*-keto and *cis*-methoxy induce respectively mild and solid inflammation. Interestingly, they have reported that the stereochemistry of MAs also influences the ability to induce inflammation. Indeed, they have shown that *trans*-methoxy MA partially loses its inflammatory potential while *trans*-keto MA appears to be anti-inflammatory by inhibiting inflammation induced by *cis*-methoxy MAs [145].

- Mycolate esters

As previously mentioned, MAs are also found in the cell wall esterified to several sugars or to glycerol (Figure 15) [128,129]. These mycolate esters are located in the outer membrane of the mycobacterial cell wall [128]. Trehalose dimycolate (TDM) or ''cord factor'' is the best studied glycolipid present in the cell wall of *M. tuberculosis*. The name ''cord factor'' comes from the ability of *M. tuberculosis* to form ''cords'' *in vitro* under proper conditions [126]. Trehalose dimycolate is constituted by two mycolic acid chains that form 6,6'-diesters bonds with a α-D-trehalose [146]. Trehalose with one chain of mycolic acid, known as trehalose monomycolate (TMM), has also been found in the cell wall. Bloch was the first to describe TDM as toxic lipid that characterized virulent mycobacteria. Nowadays, TDM and TMM have been reported in the cell wall of pathogenic as well as non-pathogenic mycobacteria [129]. Glucose monomycolate (GMM) that consists of one mycolic acid molecule bound to glucose, is a glycolipid characteristic of pathogenic mycobacteria. Indeed, Moody and colleagues have reported that the source of glucose necessary for the production of GMM is non-mycobacterial [147]. They have demonstrated that *M. tuberculosis* can produce GMM only if glucose is added to culture medium at physiological concentrations found in mammalian tissues or after *in vivo* infection [147]. Glycerol monomycolate (GroMM) is a mycolate ester that has been reported in the cell

wall of most mycobacterial species. GroMM has been shown to be present in the form of two stereoisomers, (S) - and (R)-1-O-mycoloyl-glycerol, present in equal amounts in bacilli [148].

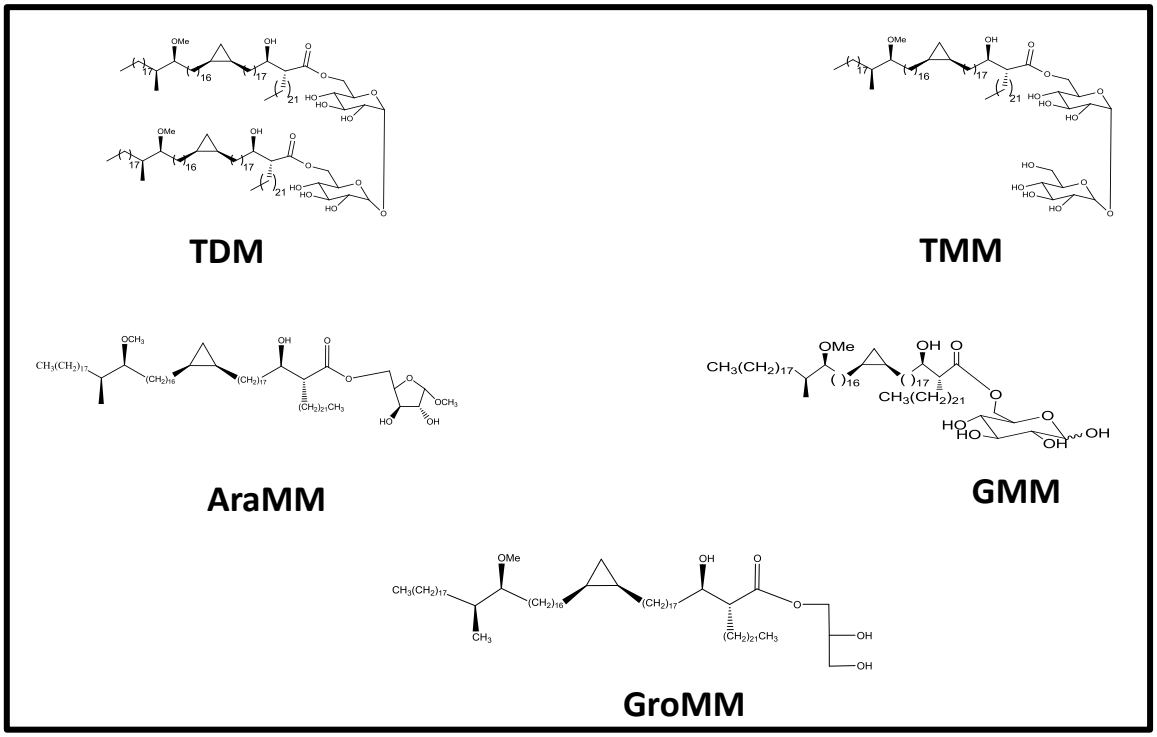


Figure 15: Structure of mycolate esters of *M. tuberculosis*.
TDM: Trehalose dimycolate, TMM: Trehalose monomycolate, GMM: Glucose monomycolate, AraMM: Arabinose monomycolate, GroMM: Glycerol monomycolate [519].

The esterification of MAs induces compounds with biological properties completely different from free MAs. For example, TDM is unable to induce the formation of foamy macrophages [135]. The identification of TDM as a PAC has been known since the 1950's when Bloch *et al.* showed that the extraction of TDM from the cell wall of *M. tuberculosis* diminishes the virulence of the strain in mice [149]. Later, Indrigo and co-workers have reported that removal of lipids including TDM with petroleum ether from the surface of *M. tuberculosis* induced cell death of the bacteria by over 99% in macrophages within three days [150,151]. The ability of *M. tuberculosis* to resist this killing by macrophages was restored by the reconstitution of the bacilli with purified TDM. These results were confirmed using genetic deletion of *fpbA,* a gene that codes for Ag85A responsible for the final esterification step in the synthesis of TDM. The ΔfbpA strain was attenuated compared to the WT strain [71]. Later on, several virulence mechanisms related to TDM were described. These mechanisms include the arrest of

phagosome-lysosome fusion, the toxicity in macrophage and the induction of granuloma [152,153].

As previously mentioned, a granuloma is a structure that allows the containment of mycobacteria and prevents the spread of the infection [152]. Two different types of granuloma have been described: one caused by a T-cell dependent hypersensitivity reaction and one characterized as a T-cell independent foreign body type [154]. These two types of granuloma contribute to the tuberculous granuloma [152]. Interestingly, it has been demonstrated that intravenous injection of TDM in oil emulsion induces transient granulomas resembling those seen after *M. tuberculosis* infection within a week in lungs, spleen and liver of mice [152,155–159]. The formation of these granulomas appears to involve the pro-inflammatory cytokines (TNF-α, IL-6) and the activation of the complement system (C5) [157]. Regarding other mycolic acid containing glycolipids, trehalose monomycolate and glucose monomycolate have also been reported to induce granulomas as TDM [160–163].

In addition to these biological properties of mycolic acids and mycolate esters in the virulence of *M. tuberculosis*, these compounds possess also the capacity to induce the activation of both innate and adaptive immune responses. Indeed, it has been shown that MAs, TDM, TMM, GMM and GroMM have adjuvant properties and can be used as adjuvant to induce antibody and cell-mediated immune responses (discussed in chapter 4.4). Furthermore, MAs, GMM and GroMM are also known to be lipid *antigens* [148,164–167].

- Does structure matter?

The nature of the mycolate moiety of a given mycolate ester seems to modulate the induced inflammatory response. In 2001, Ueda and colleagues have compared the granulomatogenic property of TDM, TMM, GMM, mannose monomycolate and fructose monomycolate isolated from *Rhodococcus* sp. 4306 (short chain of MAs C34-C52) and TDM of *M. tuberculosis* [168]. They found that only TDM isolated from *Rhodococcus* sp.4306 and TDM from *M. tuberculosis* induce granulomas. However, the granulomatogenic activity of TDM from *Rhodococcus* sp.4306 was significantly lower than that of *M. tuberculosis* suggesting that the carbon chain length and/or the type of sugar influences the inflammatory potential of mycolate esters. Later, Fujita and co-workers reported that the classes of MAs bound to trehalose can influence the toxicity and

granulomatogenic property of TDM [169]. In fact, they have divided TDM isolated from different species in two groups: group A with TDM composed of alpha-, keto- and methoxy- MAs isolated from *M. tuberculosis*, *M. bovis* and *M. kansasii* and group B with TDM composed of alpha-, keto- and wax-ester MAs isolated from *M. phlei, M. flavescens* and *M. avium.* Group B TDM showed a higher granulomatogenic activity but low toxicity while group A TDM showed moderate granulomatogenic property and low to high toxicity. This team has also suggested that the abundance of alpha-MAs affects the toxicity of TDM in BALB/c mice. Indeed, TDM from *M. tuberculosis* with a higher amount of alpha-MAs (47.3%) showed higher toxicity while TDM from *M. phlei* with a low amount of alpha-MAs (11.6%) showed almost no toxicity [169]. Nevertheless, it is important to note that *M. tuberculosis* has also a higher amount of methoxy-MAs that have an impact on toxicity. Using Δ*mmaA4* mutant strain obtained by transposon insertion mutation, Dao et *al.* have reported that TDM with oxygenated MAs are implicated in the virulence of *M. tuberculosis* by inhibiting IL-12p40 (involved in the polarization of an appropriate Th1 immune response) [170]. Indeed, bone marrow derived macrophages stimulated with TDM from Δ*mmaA4* mutant strain produce more IL-12p40 and TNF-α compared to TDM from WT or complemented strain. Further by using the same strategy, it was reported that macrophages stimulated with TDM from *ΔcmaA2* mutant (involved in *trans*-cyclopropanation of MAs) secrete more TNF-α compared to TDM from the WT or complemented strain [171]. In contrast, TDM devoid of both *cis*- and *trans*-cyclopropanated MAs induces a delayed production of TNF-α by macrophages [172]. Altogether, these studies indicated that the class of MAs seems to influence the biological properties of TDM. Nevertheless, despite the fact that these studies with mutant *M. tuberculosis* strains have given some indications on the structure-activity relationship of TDM, it is important to note that these studies were performed using complex mixtures of TDM isolated from different mycobacterial strains. **As the synthesis of pure TDM molecules has become feasible [173], the use of synthetic TDMs now makes it possible to analyze in more detail and with more precision this structure-activity relationship and this was one of the aims of this PhD thesis.**

1.3 Host-pathogen interactions: The host perspective

As introduced in the previous chapter, *M. tuberculosis* possesses many components involved in its pathogenicity. Nevertheless, the host immune system has several mechanisms which

allow the detection, the control and in some cases the elimination of the bacillus or its containment to a non-pathogenic level (latency). These mechanisms can be roughly divided into two categories: mechanisms of innate and mechanisms of adaptive immunity.

1.3.1 Innate immune responses to mycobacteria

Innate immune responses are the first line of defense against microbial infections. The initial step of these responses is characterized by the recognition and phagocytosis of the mycobacteria by macrophages and dendritic cells (DCs). This recognition is mediated by several receptors, known as pattern recognition receptors (PRRs) that decorate innate immune cells. These cells possess also numerous mechanisms that allow the elimination of the mycobacteria. Moreover, the innate immune response contributes to the generation of the appropriate adaptive immune responses. However, it is important to keep in mind that the involved innate immune cells are also potential niches for *M. tuberculosis* replication and are readily manipulated by the mycobacteria (such as macrophages as discussed later in chapter 1.3.1). In this chapter, we will focus on the specific host receptors that mediate the detection of mycobacterial pathogen associated molecular patterns (PAMPs), most of which have been described in section 1.2.4. In addition, we discuss the role of some innate immune cells in the clearance of *M. tuberculosis* and the mechanisms involved.

a. Pattern recognition receptors

Pattern recognition receptors (PRRs) implicated in the recognition of PAMPs of *M. tuberculosis* comprise cell surface receptors (Toll like receptors (TLR2, 4), C-type lectin receptors (CLRs) and scavenger receptors) and intracellular receptors (TLR9, Nod like receptors (NLRs), Retinoid acid-inducible gene I like receptors (RLRs)).

❖ Toll like receptors

TLRs are characterized by an ectodomain composed of leucine rich repeats (LRR) that are responsible for the recognition of PAMPs [174] and a cytoplasmic domain Toll/Interleukin-1 receptor (TIR) domain [174]. The recognition of PAMPs by TLRs induces an intracellular cascade that results in the activation of the transcription factor NF-κB and interferon regulatory factors (IRFs) (Figure 16) [175]. These transcription factors will trigger the expression of several pro-

inflammatory cytokines and chemokines as well as type I interferons (IFNs) that are involved in the innate immune response as well as in adaptive immunity [176]. TLR2, TLR4 and TLR9 have been reported to be involved in the recognition of PAMPs of *M. tuberculosis* [177]. The signaling pathways of all these TLRs are dependent on the adaptor protein Myeloid Differentiation primary response gene 88 (MyD88). In addition, TLR4 activates the pathway of the TIR domain containing adapter-inducing interferon-β (TRIF) pathway [177], a MyD88 independent signaling pathway.

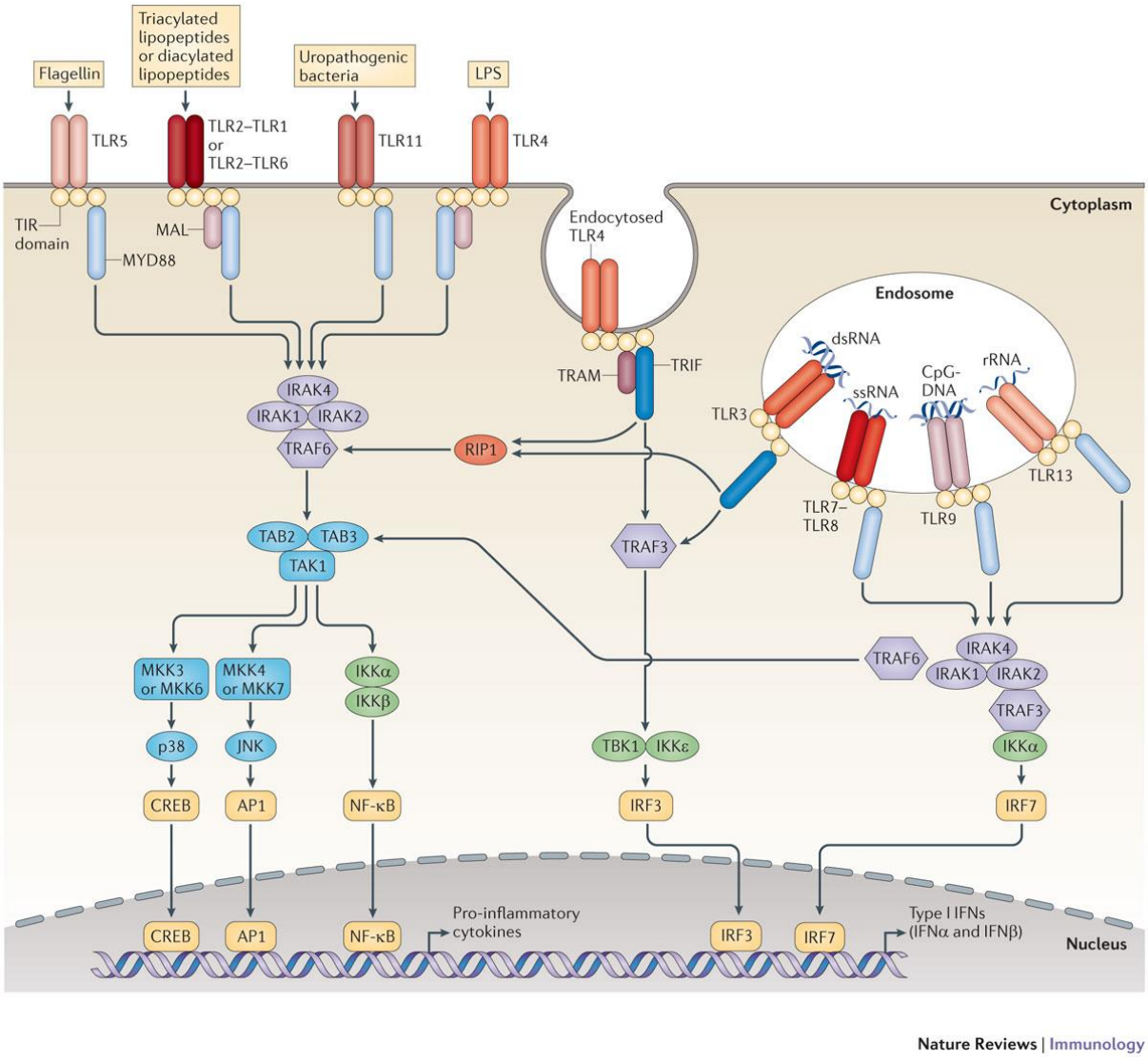


Figure 16: Mammalian Toll-like receptor (TLR) signaling pathways.
TLR1, TLR2, TLR4, TLR5 and TLR11 are located on the cell surface while TLR3, TLR7, TLR8, TLR9 and TLR13 are present in the endosomes. With the exception of TLR3, all TLRs signal via the intracellular adaptor protein MyD88 to activate the transcription factor NF-κB. Other transcription factors, such as activator protein 1 (AP1) and cyclic AMP-responsive element-binding protein (CREB) are also activated. TLR3 activates a TRIF dependent pathway that mediates the activation of the transcription factor IRF3. The activation of TLR pathways leads to the production of pro-inflammatory cytokines and in the case of the endosomal TLRs, also the induction of type 1 Interferons [177].

TLR2 recognizes a wide range of PAMPs of mycobacteria. These PAMPs include lipids such as LM, uncapped LAM and PIMs. Brightbill et *al.* reported that 19kD lipoprotein induces the production of IL-12p40 by mechanisms dependent on TLR-2 [178]. They observed that RAW 264.7 macrophages transfected with a TLR-2 dominant negative mutant showed an inhibition in the production of IL-12p40 upon stimulation with 19kD lipoprotein. Quesniaux et *al.* have reported that LM induces the upregulation of cell surface expression of CD40/CD86 and the production of TNF-α and NO in bone marrow derived macrophages (BMDMs). This activation of macrophages by LM is completely lost in TLR2$^{-/-}$BMDMs [179]. Similarly, uncapped LAM as well as PIM have been demonstrated to activate macrophages by mechanisms dependent on TLR2 [180]. In addition, it was reported that LM is a stronger activator of TLR2 than LAM [181]. *M. tuberculosis* and *M. bovis* BCG have been shown to release vesicles, known as membrane vesicles (MVs) *in vitro* and *in vivo* in murine phagocytic cells. These MVs are enriched in lipoproteins (LpqH and LprG) and induce the secretion of pro-inflammatory cytokines and chemokines by BMDMs [182]. This production is abrogated in BMDMs deficient for TLR2 [182]. In addition to lipids, lipoproteins and non-acylated proteins have been suggested to interact also with TLR2. Indeed, Chatterjee and co-workers have reported that BMDCs pulsed with ovalbumin and treated with ESAT-6 induce the production of TGF-β and IL-6 [183]. Co-culture of these BMDCs with naïve OT-II TCR Tg CD4^{+} T cells induces the production of IL-17 by mechanisms dependent on TLR2 and MyD88 [183]. Another member of the ESAT-6 family, TB10.4, has been demonstrated to activate RAW264.7 macrophages and induce the production of TNF-α, IL-6 and IL-12p40. Using TLR2 neutralizing antibody, it was shown that this activation of RAW264.7 macrophages was dependent on TLR2 [184].

Using BMDMs derived from WT and TLR4 KO mice, Bulut and co-workers demonstrated that heat shock proteins (hsp65 and hsp70) induce the production of TNF-α and IL-6 by mechanisms dependent on TLR4 [185]. In addition, human monocytes stimulated with 38-kDA glycoprotein PstS1 (Rv0934) produce IL-6 and TNF-α that can be significantly reduced with TLR2- as well as TLR4-blocking antibodies [186].

TLR9 is known to recognize unmethylated CpG motifs (CpG) in bacterial DNA. In this regard, *M. tuberculosis* DNA has been shown to activate human monocyte-derived macrophages as well as human and mouse alveolar macrophages [187].

Studies in animal models have yielded conflicting results on the importance of these TLRs in the control of *M. tuberculosis* infection. Indeed, two different studies have demonstrated that mice deficient for TLR4 are not more susceptible than WT mice [188,189] while Abel and co-workers have observed that TLR4 mutant C3H/HeJ mice are more susceptible to *M. tuberculosis* than control C3H/HeN mice [190]. Similarly, some studies have reported that TLR2 or TLR9 KO mice are more susceptible to *M. tuberculosis* infection [191,192] and others have observed that the deficiency of these receptors does not increase the susceptibility to *M. tuberculosis* infection [193,194]. Reiling *et al*. observed that the susceptibility in mice depends on the dose of infection. Indeed, at a low dose of 100 CFU, TLR2 deficient mice are not more susceptible than WT mice to *M. tuberculosis* infection, while at a higher dose of 2000 CFU, TLR2 deficient mice succumb more rapidly than WT mice [194]. Interestingly, in humans, TLR2, TLR4 and TLR9 polymorphisms have been found to be associated with differences in susceptibility to *M. tuberculosis* [195–200].

❖ Scavenger receptors

Scavenger receptors (SR) are a class of phagocytic receptors expressed on the surface of cells and secreted by monocytes and macrophages [177]. SR have been reported to be involved in the protective immunity against mycobacterial infection. These receptors include scavenger receptor A (SRA), CD36, MARCO and the secreted SR AIM (apoptosis inhibitor of macrophages). SRA has been shown to be upregulated after exposure to *M. tuberculosis* and is highly expressed on macrophages within *M. bovis* BCG-induced granulomas [201]. Bowdish and colleagues have reported that Chinese hamster ovary cells expressing SRA bind to TDM coated beads [202]. They have also found that HEK293 cells expressing MARCO, TLR2 and CD14 can recognize TDM-coated beads and induce the activation of NF-κB [202]. Peritoneal macrophages from $MARCO^{-/-}$ and $SRA^{-/-}$ mice are impaired in the secretion of pro-inflammatory cytokines in comparison to WT peritoneal macrophages upon TDM stimulation [202]. CD36 is a class B scavenger receptor reported to recognize mycobacterial lipoglycans such as ManLAM and LM. Indeed, Jozefowski et *al*. have reported that J774 cells and peritoneal murine macrophages stimulated with ManLAM and LM produceTNF-α, which can be blocked by neutralizing anti-CD36 antibody [203]. Regarding the secreted scavenger protein AIM, increased serum levels of AIM were found after infection with *M. tuberculosis*. Furthermore,

THP-1 macrophages stably transfected with AIM, control better the infection with *M. tuberculosis* [204]. The deletion of neither of these receptors increased the susceptibility to *M. tuberculosis* infection *in vivo*, on the contrary. SRA and CD36 deficiencies confer increased *in vivo* resistance to mycobacterial infection. Indeed, $SRA^{-/-}$ mice showed a prolonged survival (430 days) compared to WT mice (230 days) after aerosol infection with *M. tuberculosis* [205]. $CD36^{-/-}$ mice peritoneally infected with *M. bovis* BCG showed a reduced bacterial burden in liver and spleen (83%) in comparison to WT mice [206]. In humans, a single nucleotide polymorphism (rs17009726) in MARCO has been associated with susceptibility to *M. tuberculosis* infection [207].

❖ C-type lectin receptors

C-type lectin receptors (CLRs) are receptors characterized by the presence of one or several carbohydrate recognition domains (CRD) that can bind conserved carbohydrate moieties on the surface of pathogens by mechanisms dependent on Ca^{2+} [208]. CLRs are known to be involved in maintenance of homeostasis as well as in the anti-microbial defense [208]. Additionally, these receptors can bind several compounds, such as proteins, lipids and inorganic compounds. CLRs are a large family of receptors composed of soluble receptors (also known as collectins) and transmembrane receptors. This last group is divided according to their downstream signaling pathways into:

- CLRs that signal via immunoreceptor tyrosine-based activation motif (ITAM) domain such as Dectin-1. Binding to CLRs that signal via ITAM motifs leads to the phosphorylation of ITAM by a kinase of the Src family and to the recruitment of the protein spleen tyrosine kinase (Syk) and the activation of NF-κB via the complex formed by caspase recruitment domain-containing protein 9 (CARD9), B cell lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT-1).
- CLRs signaling via an immunoreceptor tyrosine-based inhibitory motif (ITIM) domain such as Siglec-G. Ligation of receptor with ITIM motifs leads to its phosphorylation and the recruitment of the phosphatases Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1), 2 (SHP-2), and/or SH2 domain-containing inositol phosphatase (SHIP-1).

- CLRs that do not possess any ITAM or ITIM motifs such as Mincle. Interestingly, these receptors have been demonstrated to associate with ITAM-bearing adaptors such as FcRγ, DAP10, or DAP12 and mediate signaling through Syk-CARD9-Bcl10-MALT-1 complex.

Soluble CLRs or collectins have been known to enhance the aggregation, uptake and neutralization of pathogens through the attraction of phagocytes and the activation of complement.

Mannose receptor (MR), DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), collectins, Dectin-1, Dectin-2, MCL and Mincle are CLRs known to be involved in the recognition of *M. tuberculosis* PAMPs (Figure 17).

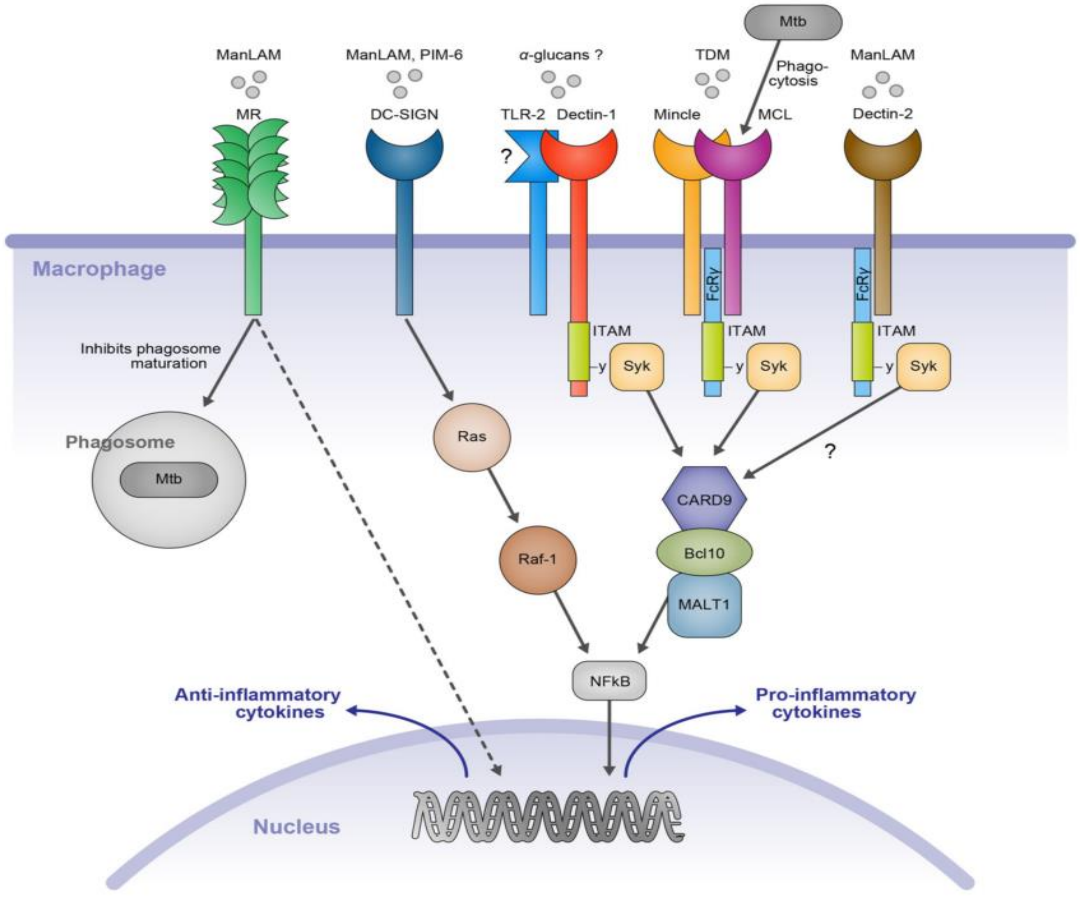


Figure 17: Recognition of *M. tuberculosis* by membrane-bound CLRs.
Dectin-2 recognizes ManLAM and activates Syk-CARD9-Bcl10-MALT1 pathway that leads to the production of pro- and anti-inflammatory cytokines. Mincle and MCL form heterodimers and lead to the production of pro-inflammatory cytokines via Syk-CARD9-Bcl10-MALT1. MCL has been reported to play a role in bacterial phagocytosis. Dectin-1 forms a heterodimer with TLR2 and recognizes an unknown component of *M. tuberculosis.* DC-SIGN recognizes ManLAM and PIM_6 and activates Raf-1 pathway. Mannose receptor has been shown to recognize mannose containing molecules, such as ManLAM. This recognition induces bacterial internalization and may also contribute to the secretion of anti-inflammatory cytokines [208].

- MR and DC-SIGN

Mannose receptor, also known as CD206, is a type I transmembrane and highly effective endocytic receptor expressed on alveolar macrophages as well as on monocyte-derived DCs and non-vascular endothelium [209,210]. The signaling pathway downstream of MR is not well known. MR is an endocytic receptor involved in the internalization of ligand and antigen presentation via MHC class II and CD1b [211]. MR binds several PAMPs of *M. tuberculosis* including ManLAM, LM and PIM. Chieppa and colleagues showed that binding to MR induces the production of anti-inflammatory cytokines IL-10, IL-1R antagonist and of the non-signaling IL-1R type II [212]. ManLAM from *M. tuberculosis* and *M. bovis* BCG were reported to inhibit LPS-induced production of IL-12 by a mechanism dependent on MR in human DCs [213]. These observations suggest that binding to MR promotes *M. tuberculosis* infection by inhibiting inflammatory responses. However, Court et *al.* have reported that MR-deficient mice are not more resistant to *M. tuberculosis* infection [214]. Nevertheless, some human polymorphisms in MR are associated to susceptibility to *M. tuberculosis* infection in Chinese populations [215,216].

DC-SIGN or CD209 is a type II transmembrane receptor expressed mainly on myeloid cells, some macrophage populations in humans and B cells [217,218]. DC-SIGN recognizes ManLAM, LM, PIM, α-glycans as well as LpqH and 45-kDa glycoprotein [219–222]. Geijtenbeek et *al.* have reported that DC-SIGN is an important receptor involved in the internalization of *M. bovis* BCG through its binding to ManLAM. Further, they have observed that ManLAM suppressed DC maturation induced by *M. bovis* BCG or LPS in terms of upregulation of CD80, CD83, CD86 and HLA-DR. This maturation of DC is restored after treatment with antibodies blocking DC-SIGN [221]. MR together with DC-SIGN interfere with Dectin-1 induced Th17 responses, which are responses involved in *M. tuberculosis* control. These results suggest that similarly to MR, DC-SIGN promotes the survival of mycobacteria by modulating the host immune responses. To address the relevance of DC-SIGN in *M. tuberculosis* infection *in vivo,* Schaefer and co-workers generated transgenic mice that expressed human DC-SIGN (hSIGN) under the control of the CD11c promoter. They observed that DCs from transgenic mice upon *M. tuberculosis* infection produced less IL-12p40 in comparison to WT DCs [223]. No difference in terms of IL-10 production was observed. Further, they found that after high dose aerosol infection, transgenic mice showed a reduction in tissue damage and prolonged survival in comparison to control mice. They proposed that hSIGN may be involved in the limitation of the pathology

induced by *M. tuberculosis* rather than be involved in promoting *M. tuberculosis* infection [223]. There are 8 genetic homologs of hSIGN in mice (SIGNR1 to SIGNR8) and which one is the direct ortholog is not clear. However, SIGNR3 has been reported to bind to LM, LAM and LpqH [177]. SIGNR3$^{-/-}$ mice showed increased bacterial growth early upon *M. tuberculosis* infection but no difference in terms of survival [224].

- Dectin-1 and Dectin-2

Dendritic cell-associated C-type lectin 1 (Dectin-1) is a type II transmembrane receptor that belongs to the Dectin-1 cluster in the natural killer complex (NKC) on chromosome 12 in humans. Dectin-1 is a non-classical CLRs that bears a hemITAM motif (Figure 17). The signaling downstream of Dectin-1 is very complex and involves several transcription factors such as NF-κB, MAPKs, NFAT, IRF1 and IRF5. The components of *M. tuberculosis* that ligate Dectin-1 are still undefined. Nevertheless, Yadav and colleagues demonstrated that the production of TNF-α by BMDMs stimulated with non-pathogenic mycobacteria such as *M. smegmatis, M. phlei* or *M. bovis* BCG or attenuated mycobacteria like *M. tuberculosis* H37Ra can be significantly reduced by anti-Dectin-1 antibody [225]. They observed that this production of TNF-α is also dependent on TLR2. Indeed, TLR2$^{-/-}$BMDMs had a reduced production of TNF-α after stimulation with the same mycobacteria and pretreatment with anti-Dectin-1 did not increase this reduction. These data indicate that Dectin-1 mediated production of TNF-α is dependent on TLR2, suggesting that Dectin-1 collaborates with TLR2 to induce the production of TNF-α after stimulation with mycobacteria [225]. This observation was confirmed later by Lee *et al.* who showed that Dectin-1 is an inducible receptor in human alveolar epithelial cell line (A549) that collaborates with TLR2 to induce the generation of reactive oxygen species and the production of pro-inflammatory cytokines upon infection with *M. tuberculosis* [226]. In contrast to these studies, it was reported that production of IL-12p40 by mouse splenic DCs stimulated with *M. tuberculosis* can be reduced with laminarin or glucan phosphate (2 molecules known to block the activity of Dectin-1). This reduction appears to be independent of TLR2 receptor [227]. Further studies demonstrated that Dectin-1 engagement in human monocyte-derived DCs promotes Th1 and Th17 polarization [219]. Dectin-1 deficient mice infected with *M. tuberculosis* by the aerosol route showed a reduction in pulmonary bacterial burden, but their survival time was not different from WT mice[228].

Dectin-2 is a type II transmembrane receptor that belongs to the Dectin-2 cluster in the Natural Killer Complex region on chromosome 12 in humans. The transmembrane domain of Dectin-2 possesses an ITAM motif involved in its signaling pathways. Recently, Yonekawa *et al.* have identified the PAMP recognized by Dectin-2. Indeed, they reported that WT BMDCs stimulated with ManLAM produce pro- as well as anti-inflammatory cytokines and that this production is abrogated in Dectin-2$^{-/-}$BMDCs [104]. Further, they showed that in a model in which OVA-pulsed BMDCs stimulated with ManLAM are co-cultured with OT-II TcR Tg naive CD4^{+} T cells, Th17 responses are induced. If Dectin2$^{-/-}$ BMDCs are used in this model, induced Th17 responses are significantly reduced [104]. Finally, Dectin-2$^{-/-}$ mice infected with *M. avium* complex (MAC) showed increased lung pathology, higher chemokine concentrations and antigen-specific T cell responses in comparison to WT mice. Altogether these results suggested that Dectin-2 could be involved in host defense to mycobacteria [229].

- Mincle and MCL

Macrophage inducible C-type lectin (Mincle) and macrophage inducible receptor (MCL) belong also to the Dectin-2 cluster. Mincle is expressed on monocytes, macrophages, neutrophils, myeloid DCs and some subsets of B cells while MCL is expressed by neutrophils, monocytes and some DC subsets [208]. Mincle and MCL do not have the ITAM domain in their transmembrane region and recruit the adaptor FcRγ to activate NF-κB [230,231]. These two receptors have been reported to recognize TDM. Mincle recognizes in addition other mycolate esters of *M. tuberculosis* cell wall such as TMM and GroMM [232–235]. In 2009, Ishikawa *et al.* as well as Schoenen and co-workers demonstrated that TDM is recognized by Mincle [232,233]. Ishikawa and colleagues reported that the granulomatogenic and pro-inflammatory properties of TDM are totally abrogated in Mincle deficient mice. By using reporter cell systems, they have shown that TMM can also bind to Mincle but with lower binding than TDM [232]. Schoenen described that the adjuvant potential of trehalose dibehenate (TDB), a structural analogue of TDM with shorter acyl (C22) chain, is completely lost in Mincle deficient mice [233]. Later, Lee and co-workers showed that neutrophils are involved in the generation of TDM-induced granulomas [156]. TDM-Mincle signaling on neutrophils increases the expression of CR3, ROS and TNF-α in collaboration with TLR2 [156]. It was also reported that TDM and TDB induce the activation of the NLRP3 inflammasome [236] which is completely abrogated in Mincle$^{-/-}$

BMDCs [237]. However, Lee and colleagues reported that TDM induces the production of nitric oxide that inhibits the activation of NLRP3 inflammasome induced by ATP or Monosodium urate [238]. Further, iNOS inhibitors (1400W and l-NMMA) inhibit Mincle-mediated downregulation of IL-1β secretion, suggesting that NO is responsible for the inhibition of NLRP3 inflammasome mediated by TDM and dependent on Mincle pathway.

Until recently, it was not known whether human antigen presenting cells (APC) use the same mechanisms to respond to TDM/TDB as murine APCs. The group of Roland Lang investigated whether primary human monocytes, macrophages and dendritic cells respond to TDM/TDB stimulation similarly to mice. Using small interfering RNA, they have found that TDB or TDM induce cytokines and chemokines by mechanisms dependent on SYK, Mincle and MCL [239]. However, the magnitude of the response to TDM and TDB differs in primary human macrophages compared to DCs. Indeed, macrophages stimulated with cord factor induce more IL-8, IL-6, CCL3, CCL4 and CCL2 while DC respond more strongly to TDB [239]. **This observation suggests that the chain length of the acyl group bound to trehalose can influence the inflammatory responses of human APCs but it is not known if the classes of MAs bound to trehalose can influence the binding to Mincle and the inflammatory and adjuvant potential of TDM in humans as well as in mice**. Nevertheless, the type of compound that forms the mycolate ester (sugar or glycerol) seems to influence its recognition by Mincle. As mentioned previously, TMM has also been shown to be recognized by Mincle [232]. Further studies demonstrated that TDM with shorter acyl chain length can also bind to Mincle [240,241]. Regarding Gro-MM, it was reported that this compound can bind to human Mincle but not to mouse Mincle [234]. However, Gro-MM as well as glycerol-esters with shorter acyl chain length can be used as adjuvants to trigger Th1 and Th17 responses in mice, suggesting that these compounds are recognized by a different receptor [242,243]. Results concerning interaction of GMM with Mincle are not clear. Indeed, Ishikawa and colleagues have shown that GMM obtained by trehalase digestion of TDM is not able to bind to Mincle [232], while Van der Peet *et al*. recently showed that synthetic GMM with shorter chain length (C32) can bind to Mincle [241]. This discrepancy of the results may be explained by the fact that the longer acyl chain of GMM influences the binding to Mincle or by the fact that trehalase may have unspecific activity (separation of MAs from trehalose). **To investigate this difference, in this PhD thesis we used synthetic GMMs from mycobacteria and analyzed their binding to Mincle**.

TDM has been reported to increase the expression of Mincle [156,233] whereas Mincle itself is barely detectable on resting myeloid cells. These observations suggested that another receptor upstream of Mincle recognizes TDM and induces the expression of Mincle. Miyake *et al*. addressed this issue and found that MCL, which is constitutively expressed in myeloid cells, can recognize TDM and induce the expression of Mincle [244]. This team showed that the granulomatogenic and adjuvant potential of TDM are severely impaired in MCL knock-out mice. Later, Lobato-Pascual et *al.* demonstrated that Mincle and MCL form a heteromer on the cell surface and this complex is optimal for their function [245]. Recently, it was reported that even if MCL is constitutively expressed in myeloid cells, it is retained in the intracellular compartment. Miyake *et al.* demonstrated that Mincle and MCL co-regulated their surface expression through the stalk region of MCL [246,247]. This co-regulation of surface expression depends on MyD88 pathway [248].

The studies which investigated the relevance of Mincle in the control of *M. tuberculosis* infection in mice are contradictory. Heitmann and co-workers have shown that Mincle deficient mice infected by low or high dose aerosol route showed no difference in terms of bacterial burden in lung and spleen, inflammatory responses, T cell responses in lungs and draining lymph nodes or formation of granulomas in comparison to control mice. Lee and colleagues however reported that low dose aerosol infected Mincle$^{-/-}$ mice have an increased bacterial burden and a stronger expression of inflammatory cytokine genes in lungs. Finally, Behler et *al.* infected Mincle$^{-/-}$ or WT mice with *M. bovis* BCG by the intratracheal or intravenous route. They found that Mincle$^{-/-}$ mice had a higher bacterial burden in lung and draining lymph nodes than control mice upon intravenous infection [249]. More recently, the same team reported that Mincle$^{-/-}$ mice intravenously infected with *M. bovis* BCG showed a reduction of Th1 cytokine production as well as a reduction in IFN-γ positive T cells. Further, using a series of adoptive transfers, they have demonstrated that the expression of Mincle in splenic classical DC but not macrophages is important for the control of infection [250]. Regarding MCL, Wilson *et al.* clearly demonstrated the non-redundancy of the role of MCL in the control of *M. tuberculosis* infection [251]. Indeed, compared to WT, MCL$^{-/-}$showed an enhanced neutrophil recruitment, increased pro-inflammatory cytokine production, higher bacterial burden and increased mortality upon low dose aerosol infection with *M. tuberculosis*. Further, they found that MCL is strongly upregulated in whole blood patients

with pulmonary TB compared to healthy control subjects. Finally, they reported a polymorphism in MCL that is associated with susceptibility to *M. tuberculosis* [251].

❖ NOD like receptors and inflammasome

Nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) are intracellular sensors characterized by the presence of a central nucleotide-binding and oligomerization (NACHT) domain, a common C-terminal leucine-rich repeats (LRRs) and a N-terminal caspase recruitment (CARD) domain or pyrin domain (PYD) [252]. NLRs are divided in three families : NODs, NLRPs (NACHT, LRR and PYD domains-containing proteins) and IPAFs (ICE-protease activating factors) [252]. NOD2 and NLRP3 are known to be involved in the recognition of mycobacterial PAMPs [253].

NOD2 is a NLR expressed in epithelial cells and APCs such as macrophages. NOD2 (CARD15) has been reported to recognize muramyl dipeptide (MDP), a component of peptidoglycan [254]. NOD2 senses MDP through its LRRs domain. This recognition leads to a signaling cascade which results in the activation of NF-κB and the synthesis of pro-inflammatory cytokines. *In vitro*, NOD2 deficient peritoneal macrophages infected with *M. tuberculosis* showed a significant decrease in the production of TNF-α compared to WT infected macrophages [255]. Synergistic effects on the cytokine production between NOD2 and TLR2 have been reported [255]. Gandotra and colleagues also reported an impairment in the production of TNF-α, IL-12p40, RANTES and NO in NOD2 KO mice following *M. tuberculosis* infection [254]. Knock-down of NOD2 expression by using small interfering RNA (siRNA) in human monocyte-derived macrophages leads to a reduction of the secretion of pro-inflammatory cytokines [256]. Similarly, PBMC isolated from patients with Crohn's disease who have a NOD2 mutation (NOD2fs) showed a significant decrease in the production of TNF-α following *M. tuberculosis* infection in comparison to PBMC from healthy volunteers [255]. $NOD2^{-/-}$ mice are not affected in their susceptibility during the first two months of the mycobacterial infection (4 to 8 weeks) [254,257]. However, in this early phase, $NOD2^{-/-}$mice showed a decrease in the production of IL-12p40 as well as a reduced recruitment of $CD8^+$ and $CD4^+$ T cells in the lungs, suggesting impaired innate and adaptive immune responses. To investigate whether this diminution of the immune response can have an impact on the control of the infection later, they analyzed the susceptibility of $NOD2^{-/-}$mice after 6 months of infection with *M. tuberculosis*. They observed

a higher bacterial burden in the lungs and a reduced survival time, indicating that NOD2 is important and non-redundant in the later stages of *M. tuberculosis* infection [257].

NLRP3 is an important protein involved in the formation of a multiprotein complex known as the inflammasome (Figure 18). Inflammasomes are high molecular weight platforms activated upon microbial infection such as *M. tuberculosis* infection or cellular stress that trigger the activation of caspase-1 and the maturation and secretion of IL-1β and IL-18 [252]. Indeed, the production of an active IL-1β molecule is tightly regulated at the expression level as well as at the maturation and secretion levels. The pro-forms of these cytokines (pro-IL-1β and pro-IL-18) are synthesized in the cytosol upon the activation of PRRs such as TLRs and these pro-forms are not secreted via the classical Golgi pathways. The maturation and secretion of IL-1β/IL-18 requires the activation of caspase-1 which depends on the inflammasome activation [252]. IL-1 cytokine family has been reported to be indispensable for the control of TB. In fact, IL-1β, IL-1α, IL-1R and MyD88 (involved in the signaling of IL-1R) KO mice succumb rapidly after low-dose aerosol infection with *M. tuberculosis* [258,259] suggesting an important role of the inflammasome activation in the control of *M. tuberculosis*. However, it is important to note that inflammasome-independent production of IL-1β by neutrophils has also been reported [260,261]. The general structure of the canonical inflammasome complex is composed of a sensor protein such as NLRP3, the adaptor-associated speck-like protein containing a caspase-activation recruitment domain (ASC) and caspase-1. Three different sensor proteins are involved in *M. tuberculosis* infection: NLRP3, NLRP7 and AIM2 [262,263].

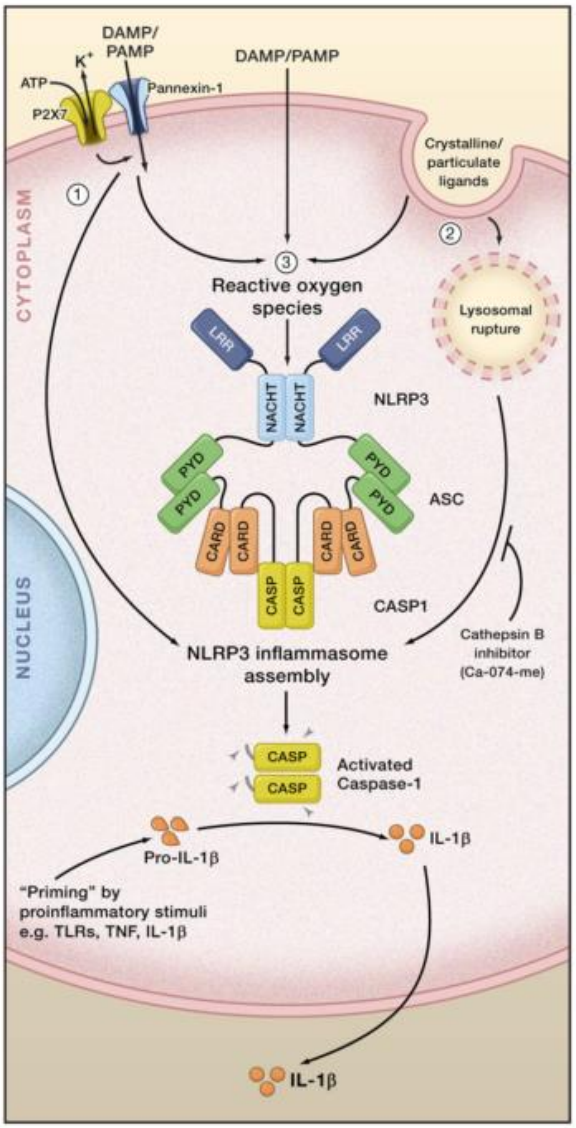


Figure 18: NLRP3 inflammasome. Three major models for NLRP3 inflammasome activation are favored in the field, which may not be exclusive: (1) The NLRP3 agonist, ATP, triggers P2X7-dependent pore formation by the pannexin-1 hemichannel, allowing extracellular NLRP3 agonists to enter the cytosol and directly engage NLRP3. (2) Crystalline or particulate NLRP3 agonists are engulfed, and their physical characteristics lead to lysosomal rupture. The NLRP3 inflammasome senses lysosomal content in the cytoplasm, for example, via cathepsin- B-dependent processing of a direct NLRP3 ligand. (3) All danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), including ATP and particulate/crystalline activators, trigger the generation of reactive oxygen species (ROS). A ROS-dependent pathway triggers NLRP3 inflammasome complex formation. Caspase-1 clustering induces auto-activation and caspase-1-dependent maturation and secretion of pro-inflammatory cytokines, such as interleukin-1b (IL-1β) and IL-18 [252].

NLRP3

Several studies have shown an activation of NLRP3 inflammasome in macrophages following infection with *M. tuberculosis*. The precise ligand that can directly bind NLRP3 is not yet

identified, and might not exist. Nevertheless, it was reported that THP-1 macrophages activate NLRP3 inflammasome following infection with WT *M. tuberculosis*. ΔRD1 *M. tuberculosis* fails to induce the secretion of IL-1β and the activation of caspase-1. Further, it was shown that recombinant ESAT-6 is sufficient to activate NLRP3 inflammasome, suggesting that a functional ESX-1 system is required for the activation of NLRP3 inflammasome [264]. TDM has also been reported to activate NLRP3 inflammasome in BMDCs [236]. Nevertheless, NLRP3$^{-/-}$ mice do not show an increased susceptibility in terms of survival upon *M. tuberculosis* infection [265].

AIM2

Also absent in melanoma 2 (AIM2) inflammasome activation has been associated with mycobacterial infection. AIM2 recognizes double stranded DNA and leads to the activation of caspase-1 and finally the maturation and secretion of IL-1β. ESX-1 has been shown to mediate rupture of the phagosomal membrane inside host phagocytes, resulting in the release of mycobacterial compounds such as mycobacterial DNA into the host cell cytosol [266]. It was described that pathogenic mycobacteria inhibit the activation of AIM2 inflammasome and that this inhibition was dependent on ESX-1 secretion system [267]. In contrast to this, Saiga *et al.* have reported the critical role of AIM2 in the control of *M. tuberculosis* infection. Indeed, they have demonstrated that AIM2 deficient macrophages infected with *M. tuberculosis* showed a significant decrease in the secretion of both IL-1β and IL-18 [268]. Further, they have shown that DNA extracted from *M. tuberculosis* activates AIM2 inflammasome leading to the secretion of IL-1β and IL-18. Finally, AIM2 KO mice showed an impairment in the production of IL-1β and IL-18 as well as Th1 immune response. These mice are more susceptible to *M. tuberculosis* infection than control mice [268]. These data clearly reveal the critical role of AIM2 inflammasome in the control of *M. tuberculosis*.

NRLP7

Zhou and colleagues recently reported that *M. bovis* Beijing strain activates the NLRP7 inflammasome [262]. Indeed, they showed that THP-1 macrophages infected with virulent *M. bovis* induce the activation of caspase-1 and the secretion of IL-1β. Knock down of NLRP7 with siNLRP7 results in a significant reduction of the secretion of IL-1β and the activation of caspase-1. The PAMPs of *M. tuberculosis* involved in the activation of NLRP7 inflammasome are not yet elucidated. However, NLRP7 is known to sense lipopeptides [269].

❖ Cyclic guanosine mono-phosphate-AMP (cGAMP) synthase (cGAS)

cGAS is a member of the nucleotidyltransferase family located in the cytosol that recognizes cytosolic dsDNA [270]. Binding of dsDNA to cGAS leads to the production of the second messenger cGAMP from adenosine triphosphate (ATP) and guanosine triphosphate (GTP) [271]. cGAMP in turn binds and activates the ER STING pathway, leading to the production of type I interferons through IRF3 (Figure 19) [272,273]. This receptor has been known to be absolutely required for the type I IFN induced protection in viral infection [274]. Recently, in addition to sensing of *M. tuberculosis* DNA by AIM2, it has been shown by three independent studies that cGAS is also involved in the recognition of the mycobacterial DNA [275–277]. These three studies have shown that the cGAS is essential in type I IFNs induced by mycobacterial DNA. This process appears to be dependent on ESX-1 secretion system given the fact that *M. tuberculosis* mutants deficient for ESX-1 cannot induce type I IFNs. Further, Collins and colleagues reported that mice deficient for cGAS are more susceptible upon *M. tuberculosis* infection in comparison to WT mice. In addition, Watson and co-workers showed that cGAS activation leads to the antibacterial selective autophagy pathway. Given the fact that the production of type I IFNs in *M. tuberculosis* infection has been shown to be detrimental for the host via the secretion of anti-inflammatory cytokine IL-10 and the fact that autophagy plays a role in anti-mycobacterial protective immunity, these data suggest that cGAS activation can be important to determine the balance between pathogenesis and protection [278].

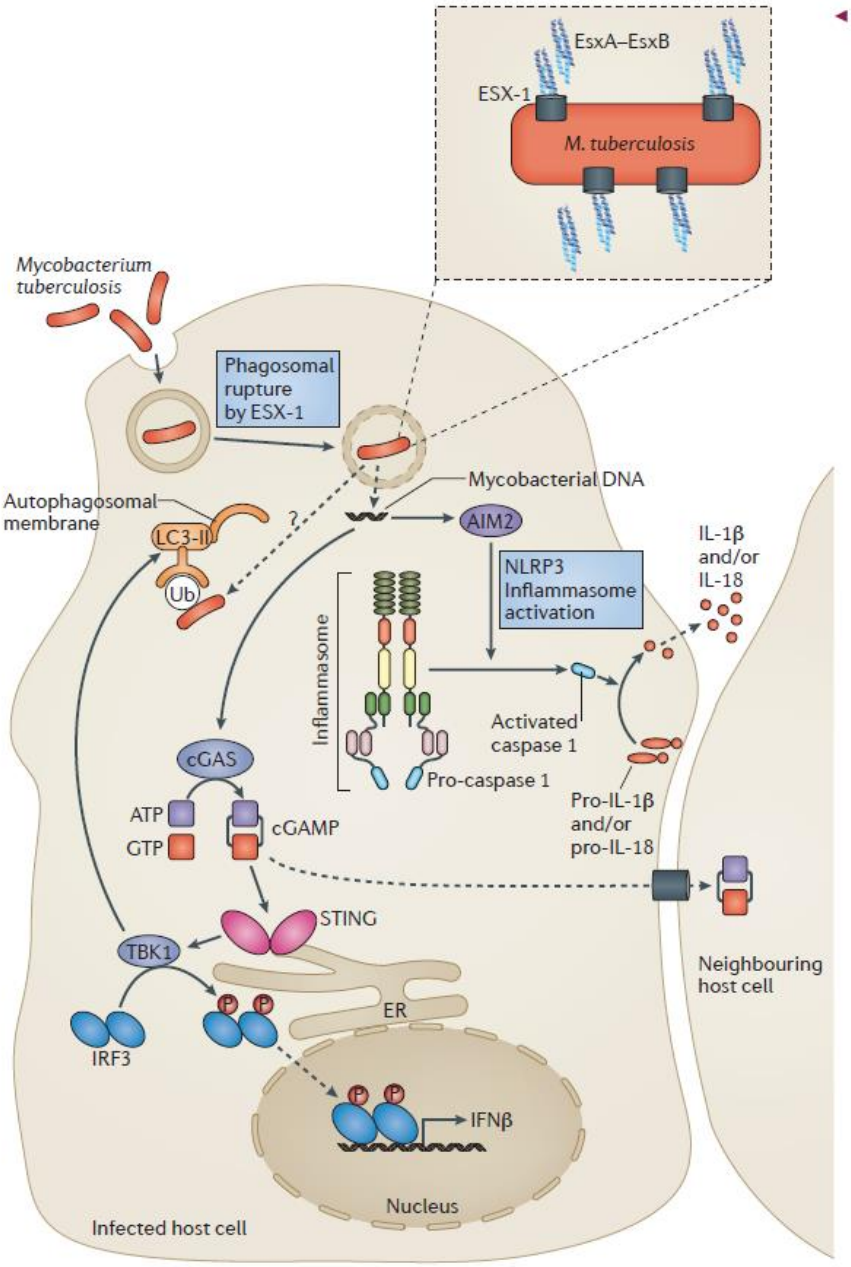


Figure 19: Downstream effects of phagosomal membrane rupture by ESX-1.
Esx-A mediates the rupture of the phagosomal membrane that releases mycobacterial components such as DNA or perhaps intact mycobacteria in the cytosol. In the cytosol, DNA is recognized by cyclic GMP-AMP synthase (cGAS) and AIM2. The recognition of DNA by cGAS leads to the production of the second messenger cGAMP that activates STING pathway and the production of type I interferons. The recognition of mycobacterial DNA by AIM2 induces the activation of the inflammasome that triggers that maturation of the caspase-1. Caspase-1 will induce the maturation of pro-IL-18 and pro-IL-1β. Serine/threonine-protein kinase (TBK1) downstream STING may activate autophagy [273].

❖ Liver X receptors

Liver X receptors (LXRs) are important nuclear receptor transcription factors that have been shown to regulate macrophage function such as lipid homeostasis, inflammatory responses and glucose metabolism. We distinguish two isoforms of LXRs: LXRα and LXRβ. One hallmark of *M. tuberculosis* infection is the induction of foamy macrophages characterized by the accumulation of lipid droplets. These lipid droplets contain neutral lipids such as triglycerides and cholesteryl esters [279]. In macrophages, the influx and efflux of cholesterol is regulated by the nuclear receptors LXRs. Indeed, several genes involved in cholesterol trafficking and efflux such as the genes encoding for lipid transporters such as ATP-binding cassette transporter A1, G1, G4 (ABCA1, ABCG1, ABCG4) and lipid carrier proteins like apolipoprotein E (apoE) are induced by LXRs activation [280,281]. Cholesterol constitutes the major component of lipid droplet and has been reported to be essential for the persistence of the bacteria. Indeed, *M. tuberculosis* can use cholesterol as main carbon source in nutrient-deficient macrophages [282]. In addition, cholesterol plays a role in the phagocytosis of the bacteria and the inhibition of the fusion of phago-lysosome [283–285]. Our laboratory has shown that mice deficient for both isoforms of LXR are more susceptible to *M. tuberculosis* infection. These KO mice are characterized by a decreased clearance of bacterial load in the lung and showed bigger and more granulomatous lesions in comparison to WT mice [286]. It was recently suggested that LXR can negatively regulate the formation of foamy macrophages and mycobacterial growth [138]. Indeed, it was found that macrophages from LXR KO mice showed an increase in cholesterol ester accumulation and BCG growth. Altogether, these data demonstrated the importance of LXRs in the control of TB.

b. Manipulation of host's innate immune responses

We have seen in the previous chapter that host innate immune cells possess several receptors involved in the recognition of *M. tuberculosis.* This recognition will induce several mechanisms that aim to eliminate the pathogen. These mechanisms include: phagocytosis and phagosome maturation, production of pro-inflammatory cytokines, the induction of toxic compounds such as reactive nitrogen species and anti-microbial peptides, autophagy and efferocytosis. However, *M. tuberculosis* has acquired during its evolution mechanisms to counter-balance these host's immune defense strategies in order to survive and establish an infection. The

host's innate immune defenses involve several innate immune cells, including macrophages, dendritic cells, neutrophils and natural killer cells. In this chapter, we will discuss the different mechanisms acquired by *M. tuberculosis* to counter-balance host's innate immune responses with a focus on macrophages.

❖ Phagocytosis and phagosome maturation

Phagocytosis is a hallmark of anti-bacterial host defense. Binding of PAMPs to certain PRRs leads to internalization of the pathogen. After its phagocytosis, in normal circumstances, the engulfed pathogen will be destroyed following the fusion of the phagosome with lysosomal contents. However, *M. tuberculosis* possesses several mechanisms to avoid the recognition by PRRs and to inhibit the maturation of the phagosome and its fusion with lysosome. For example, receptors involved in the phagocytosis of *M. tuberculosis* seem to be important for the fate of the infection. Thus, *M. tuberculosis* has been reported to manipulate mechanisms involved in the recognition of PAMPs by PRRs. Indeed, it was shown that PDIM can mask the recognition of mycobacteria by TLRs [287]. This recognition by TLRs is known to trigger production of toxic reactive nitrogen and oxygen species [288]. In addition, the binding of ManLAM (present in virulent mycobacteria) to MR contributes to arrest the maturation of the phagosome [289], whereas, uptake via DC-SIGN or Fc receptors is known to induce lysosomal delivery of the mycobacteria [290]. Several molecules of *M. tuberculosis* inhibit the maturation of the phagosome and the fusion with lysosome. Indeed, it was reported that approximately 70% of phagosomes containing mycobacteria do not fuse with lysosomes [290]. These molecules included TDM, ManLAM, PknG, LpdC, Zmp1, PtpA, NdkA and SapM. The molecular mechanisms involved in the arrest of phagosomal maturation are not clear for all these molecules. The maturation of the phagosome involves the conversion of Rab5 to active GTP-bound Rab7 and the production of phosphatidyl-inositol-3-phosphate (PI3P). Two proteins of *M. tuberculosis* are involved in the inhibition of the production of Rab7: PtpA, a tyrosine phosphatase and NdkA a guanosine triphosphatase [77]. SapM, a PI3P phosphatase of *M. tuberculosis,* has been reported to block *in vitro* the maturation of the phagosome [77]. ManLAM was shown to disrupt a calcium-dependent calmodulin signaling pathway involved upstream of the PI3P production [77].

If the blocking of the maturation of the phagosome fails, the mycobacteria possess additional mechanisms to resist destruction by lysosomal hydrolases, reactive nitrogen and oxygen species. AhpC, SodC, KatG and TpX proteins have been reported to actively participate in the detoxification of reactive oxygen and nitrogen intermediates (ROI and RNI) [36].

M. tuberculosis can also escape from phagosome-lysosome fusion by damaging the phagosomal membrane. Even if this egress of *M. tuberculosis* from phagosome has been proposed by several studies in the 1980's and 1990's, this concept remained a matter of debate until recently. Indeed, by using human monocyte-derived dendritic cells, macrophages and a cryo-electron microscopy, Van der Wel and colleagues demonstrated that *M. tuberculosis* translocates from phagolysosome to cytosol [291]. Further, they demonstrated that this mechanism is dependent on the secretion of CFP-10 and ESAT-6 [291]. This observation was later confirmed by Simeone et *al* [292]. In addition, this team showed that the escape from phagosome to cytosol is mediated only by ESAT-6. Indeed, an *M. marinum* mutant defective in the ESX-1 secretion system is impaired in phagosome escape. Further, purified ESAT-6, but not other ESX-1 secreted proteins, is able to trigger dose-dependent pore formation in host cell membranes [293]. ESX-1 secretion system also plays an unknown role in the arrest of phagosome maturation [294]. This escape of *M. tuberculosis* to cytosol has been shown to favor cell to cell spreading through cytolysis of infected cells. In fact, mutants of *M. tuberculosis* defective in ESX-1 are impaired in cell to cell spreading and macrophage cytolysis [293,295]. Nevertheless, the escape of *M. tuberculosis* to the cytosol is not necessarily beneficial for the mycobacteria. Indeed, in the cytosol *M. tuberculosis* is recognized by intracellular receptors such as NOD-2, cGAS, AIM2 and NLRP3 which leads to the production of type I IFN cytokines, inflammasome activation and autophagy (Figure 19). Autophagy is a process whereby cytoplasmic contents such as damaged organelles, protein aggregates and intracellular invading pathogens are engulfed in a double membrane structure, known as the autophagosome, and directed to the lysosome for degradation [177]. Yoshinori Ohsumi won the Nobel Prize in Physiology or medicine 2016 for his discovery of the autophagy mechanisms. The formation of autophagosome depends on protein-protein (Atg5/atg12) and protein-lipid (Atg8/LC3) conjugation systems [77]. Autophagy is used by the host as a defense mechanism against *M. tuberculosis* by sequestering mycobacteria that access to the host cell cytosol. Indeed, LpqH isolated from *M. tuberculosis* H37Ra has been reported to activate the

autophagy by mechanisms dependent on TLR2. Binding of MDP to NOD2 improves the capacity of *M. tuberculosis* infected human alveolar macrophages to control infection by increasing TNF-α and IL-6 production as well as by increasing autophagocytosis of *M. tuberculosis* [296]. By inhibiting the fusion of phagosomes with lysosomes, *M. tuberculosis* also inhibits MHC class II antigen presentation. However, autophagy contributes to T cell activation by enhancing antigen presentation [297].

❖ Pro-inflammatory cytokine production and DC maturation

As previously introduced, binding of *M. tuberculosis* PAMPs to several PRRs leads to the production of pro-inflammatory cytokines that contribute to the clearance of the bacteria. *M. tuberculosis* counteracts this production of pro-inflammatory cytokines by inducing anti-inflammatory cytokines as well. Indeed, hsp60 and PPE18 are two mycobacterial secreted proteins which trigger the production of the anti-inflammatory cytokine IL-10 in THP-1 cells. Blocking TLR2 signaling with anti-TLR2 blocking antibody or siRNA against TLR2 inhibit the production of IL-10, suggesting that IL-10 production induced by hsp60 and PPE18 is dependent on TLR2 [177]. In addition, ESAT-6 and TDM composed of oxygenated mycolates have been shown to inhibit the production of IL-12 by mouse macrophages [170,298]. Doz and colleagues showed that PIMs regulate negatively the activation of NF-κB and the surface expression of co-stimulatory molecules (CD40 and CD86) induced by LPS in BMDMs. Similarly, human DCs stimulated with ManLAM increase the expression of IL-10 and decreases the expression of IL-12 by mechanisms dependent on DC-SIGN [299]. ManLAM has also been reported to inhibit DC maturation following infection with *M. tuberculosis* or treatment with LPS [299].

❖ Apoptosis

Apoptosis is a programmed cell death process by which cytoplasmic content of the dying cells is confined within membrane-bound structures, known as apoptotic bodies. In contrast, necrosis is a form of cell death by autolysis that results in the release of the cytoplasmic contents in the extracellular medium. Increasing evidences indicate that apoptotic cell death is more favorable for the host than necrosis. Indeed, it was reported that cells dying by apoptosis release less viable mycobacteria than if dying by necrosis [77]. Keane and colleagues

have compared the apoptotic activity of attenuated mycobacteria compared to virulent mycobacteria in human alveolar macrophages. They showed that attenuated mycobacteria (*M. tuberculosis* H37Ra, *M. bovis* BCG and *M. kansaii)* induce significantly more apoptosis than virulent strains (*M. tuberculosis* H37Rv, Erdmann, *M. bovis),* suggesting that virulent mycobacteria possess mechanisms to modulate apoptotic response of alveolar macrophages [300]. Further, several proteins (SecA2, nuoG, Ndk and PtpA) of *M. tuberculosis* have been shown to inhibit apoptosis [301]. Indeed, the inactivation of the genes coding for these proteins results in a pro-apoptotic phenotype. These mutants show also an attenuated phenotype compared to wild type [301]. Interestingly, several studies demonstrated that virulent mycobacteria manipulate host cell death by using arachidonate products. Indeed, comparison between *M. tuberculosis* H37Ra and H37Rv showed that the attenuated strain H37Ra induced the synthesis of proapoptotic prostaglandin E2 (PGE2) while H37Rv induced production of lipoxin A4 (LXA4) [302]. In addition, mice deficient for 5-lipoxygenase, involved in the production of LXA4, are more resistant to infection with H37Rv, indicating that LXA4 production is detrimental for the host. Similarly, zebrafish carrying a mutation in the leukotriene A4 hydrolase (the phenotype being overproduction of LXA4) are more susceptible to *M. marinum* infection and show an accelerated necrosis of infected macrophages and a release of a large numbers of viable mycobacteria [303]. In addition to the control of mycobacterial multiplication, apoptosis is widely accepted to also facilitate mycobacterial antigen cross presentation through delivery of apoptotic bodies to non-infected DCs [304,305].

In summary, *M. tuberculosis* possesses multiple overlapping strategies to manipulate innate immune responses in order to establish an infection. In addition, this mycobacterium also subverts adaptive immune response. Indeed, in *M. tuberculosis* infection in humans and mice, a delay in the induction of specific adaptive immune response compared with the time required to develop adaptive immune response to other pathogens is observed. This is probably related to the manipulation by *M. tuberculosis* of the innate immune response (antigen presentation, maturation of DCs…) or others unknown factors. However, as previously mentioned, 90 to 95 % of *M. tuberculosis* infected individuals control the infection in the granuloma after the establishment of specific adaptive immune responses. In the following chapter, we introduce what is known about the adaptive immune response involved in the control of *M. tuberculosis.*

1.3.2 Adaptive immune response

Several actors are involved in the adaptive immune response against *M. tuberculosis.* These actors include $CD4^+$, $CD8^+$ T cells and B cells.

a. $CD4^+$ T cells

The critical role of $CD4^+$ T lymphocytes in the protective immunity to *M. tuberculosis* infection in humans and in animal models is clearly demonstrated. First, the studies in HIV and TB coinfected individuals showed that the rate of progression from latent to active TB increases by 5 to 10 fold compared to non HIVinfected individuals. Moreover, antiretroviral therapy, that reconstitutes $CD4^+$ T cells, decreases the incidence of active TB to the same level as in HIV negative individuals [306]. Second, the depletion of $CD4^+$ T cells or knock out of MHC class II results in the impairment of the control of bacterial growth and accelerated mortality of infected mice [307,308]. Finally, several studies indicate that *M. tuberculosis* possesses mechanisms to inhibit stimulation of $CD4^+$ T cells as well as MHC class II antigen presentation [77].

Specific $CD4^+$ T cell mediated immune responses are generated when activated *M. tuberculosis*-infected or cross-primed DCs migrate from lungs to the draining lymph nodes to activate naïve T cells. The activation of naïve T cells depends on 3 signals. The first signal is given by peptide-MHC II complex to T cell receptor (TCR). The second signal depends on the binding of B7 molecules to CD28 on T cells and the last signal that triggers the polarization of T cells depends on the cytokine environment. Several types of T helper cells have been associated with the immune protection against *M. tuberculosis.*

❖ T helper 1 T cells

T helper 1 (Th1) T cells are characterized by the expression of the transcription factor T-bet and the secretion of IFN-γ. These cells are induced by the cytokine IL-12. In addition, these cells also secrete TNF-α, IL-2, lymphotoxin and some CCL chemokines. The critical role of this subset of T cells in the control of *M. tuberculosis* infection is related to increased bactericidal effector mechanisms of infected macrophages by IFN-γ. Indeed, IFN-γ activates several mechanisms involved in the elimination of *M. tuberculosis*, including iNOS pathway, acidification and maturation of the phagosome and autophagy [309]. It is important to keep in mind that other immune cells such as $CD8^+$ T cells, NK cells, γδ T cells and CD-1 restricted T

cells also produce IFN-γ [309]. However, $CD4^+$ T cells seem to be the main producers of this cytokine [310]. The importance of Th1 T cells in the protective immunity against TB has been widely investigated in experimental models and in humans. Mice deficient for T-bet, IL-12 and IFN-γ are highly susceptible to *M. tuberculosis* infection [311–314]. Similarly, humans with mutations in the IL-12/IFN-γ axis develop disseminated mycobacterial infections caused by non-pathogenic mycobacteria such as BCG as well as non-tuberculous mycobacteria species [315]. Nevertheless, IFN-γ alone is clearly not sufficient to prevent active disease. For example, it has been reported that most of the individuals who develop active TB have specific mycobacterial IFN-producing T cells. In addition, Dieli and co-workers showed that patients whose T cells produce higher amount of IFN-γ are more likely to progress to active disease than patients with weaker responses [309]. Thus, the production of IFN-γ is indispensable but not sufficient alone to prevent active disease. In addition to IFN-γ, studies using other genetically deleted mice demonstrated that TNF-α, GM-CSF, IL-1 as well as IL-6 are also essential for the protective immunity against TB [309]. TNF-α for example has been shown to act in synergy with IFN-γ to stimulate the production of NO by macrophages. This cytokine is also involved in the formation and maintenance of granuloma. Indeed, treatment of chronic auto-immune diseases with anti-TNF-α therapy considerably increased the risk of reactivation of latent *M. tuberculosis* [316].

❖ T helper 17 T cells

The differentiation of Th17 cells is mediated by TGF-β and IL-6 cytokines through STAT-3. These T cells are characterized by the expression of the transcription factor RORγt and the secretion of pro-inflammatory cytokines IL-17 and IL-22. IL-21 and IL-23 also produced by Th17 are involved in the differentiation as well as the maintenance of Th17 T cells. The role of Th17 cells in the immune protection against natural infection of *M. tuberculosis* is debated. It has been reported that mice deficient for IL-23 are unable to maintain Th17 cells but are not more susceptible following infection with *M. tuberculosis* H37Rv, suggesting that Th17 cells are dispensable for the control of *M. tuberculosis* infection. In the same line, Cruz et *al.* described that IFN-γ deficient mice showed an increased number of Th17 T cells upon infection with *M. bovis* BCG. In addition, they have demonstrated that IFN-γ modulates the frequency of Th17 cells. Further, they have shown that Th1 cells impair the survival of neutrophils (recruited by

IL-17 produced by Th17 cells) and the accumulation of these cells in the lungs. In this way, Th1 cells control the IL-17 mediated immunopathology of TB [317]. In contrast, others have demonstrated a protective role of Th17 cells in TB. Wozniak and colleagues showed that transfer of Th17 cells, isolated from BCG immunized mice, in IFN-γ$^{-/-}$ recipient results in a significant prolongation of the survival of mice in comparison to control IFN-γ$^{-/-}$ mice following infection with *M. tuberculosis* [318]. Khader et *al.* reported that IL-17-producing CD4^{+} T cells are required for the recruitment of Th1 cells in the lung. Indeed, depletion of Th17 cells reduces the recruitment of IFN-γ-producing CD4^{+} T cells in the lung of vaccinated mice following *M. tuberculosis* infection [319]. Similarly, Gopal and co-workers demonstrated that Th17 cells promote Th1 cell responses by counteracting the inhibitory effect of IL-10 following *M. bovis* BCG vaccination [320]. More recently, Gopal and colleagues have shown that in contrast to the laboratory strain H37Rv, clinically isolated hypervirulent *M. tuberculosis* W-Beijing strain HN878 requires IL-17 responses for the early protective immune response. In our laboratory, we investigated the role of IL-17 receptor A in the control of *M. tuberculosis* using C57BL/6 IL-17RA KO mice [321]. We observed that IL-17RA$^{-/-}$ mice were more susceptible than WT mice following *M. tuberculosis* infection. In addition, these IL-17RA$^{-/-}$ mice showed a decreased in the control of *M. tuberculosis* infection at the later stages. Indeed, bacterial burden increased by about 10 fold at 12 weeks and 70-fold at 20 weeks post infection.

Even if the role of Th17 cells in the natural control of *M. tuberculosis* is debated, it is accepted that Th17 cells play an important role in vaccine induced protection [322–324].

❖ Regulatory T cells

Regulatory T lymphocyte (Treg) CD4^{+}CD25^{+} cells are characterized by the expression of the transcription factor FoxP3. In contrast to effector T cells, this population recognizes self-peptide antigens on APC. The role of this population of T cells is to counter-regulate pro-inflammatory immune responses by producing anti-inflammatory cytokines (IL-10, TGF-β), in order to prevent excessive inflammation and tissue damage. This inhibition of the pro-inflammatory immune response could also be a mechanism to dampen excessive pathogen induced inflammation. In *M. tuberculosis* infection, increasing evidence supports the important contribution of this population in TB immunity. Some studies suggest a detrimental role of Treg cells in TB. Indeed, Treg cells are more frequently observed in patients with active

TB and the specific depletion of this cell population results in higher levels of IFN-γ production [302,325]. The contribution of Treg cells in the reactivation of latent TB has also been reported [309]. Shafiani and colleagues showed that early after infection with *M. tuberculosis*, *M. tuberculosis*-specific Treg cells can delay the priming of effector $CD4^+$ T cells in pulmonary lymph nodes [326]. The same team demonstrated later that this population of *M. tuberculosis*-specific Treg cells is eliminated by Th1 cells [327]. This elimination probably prevents the suppression of immune responses in the later stages of the infection. Using adoptive transfer of $CD4^+$ effector T cells and Treg cells in $Rag^{-/-}$ mice, Kursar and colleagues demonstrated that Treg cells prevent the eradication of mycobacteria by suppressing the response of effector T cells following *M. tuberculosis* infection [328]. In conclusion, Treg cells can promote *M. tuberculosis* infection but this population is also clearly involved in the prevention of excessive inflammation. More studies need to be done in order to understand the contribution of Treg cells in the balance between protection and pathology in TB.

b. $CD8^+$ T cells

Conventional $CD8^+$ T lymphocytes recognize peptide epitopes bound to classical MHC class I molecules. This subset of T cells contributes to the protective immunity against TB by producing IFN-γ, IL-2, IL-17 and TNF that are known to be critical for the control of *M. tuberculosis.* $CD8^+$ T cells are also able to kill *M. tuberculosis*-infected cells via granule-mediated function (via perforin, granzymes and granulysin) or via Fas-Fas ligand interaction to induce apoptosis [329].

It was thought for a long time that $CD8^+$ T cells did not play a major role in the control of TB. Nevertheless, several studies using antibody-mediated depletion, adoptive transfer of purified T cells or the use of knock-out mice have demonstrated convincingly that $CD8^+$ T cells are necessary for optimal control of *M. tuberculosis* infection. Thus, Müller and co-workers have demonstrated that depletion of $CD8^+$ T cells in thymectomized mice infected with *M. tuberculosis* H37Rv and BCG results in an impaired control of infection. Further, Feng et *al.* reported that the adoptive transfer of $CD8^+$ T cells from *M. bovis* BCG-infected mice in $RAG^{-/-}$ deficient mice, exposed to aerosol infection with *M. bovis* BCG, results in the reduction of splenic CFU [330]. Regarding KO mice, in 1992 Flynn and co-workers showed that β2-microglobulin (β2m) deficient mice die rapidly following *M. tuberculosis* Erdman infection.

β2m is a protein involved in the assembly and trafficking of the class I heavy chain, so no class I MHC molecule is expressed on the surface of cells in the absence of β2m [331]. Subsequently, studies using TAP-1$^{-/-}$, CD8$^{-/-}$ and MHC heavy chain KO ($K^bD^{b-/-}$) mice confirmed that CD8$^+$ T cells play a crucial role in the immunity to *M. tuberculosis* in mice [329]. In humans, it is much more complicated to determine the role of CD8$^+$ T cells in the protection against TB. Nevertheless, it is known that *M. tuberculosis* infection induces specific CD8$^+$ T cells and these cells produce cytokines and are cytotoxic [329]. Studies in rhesus macaques, a model that recapitulates almost all aspects of human TB, showed that depletion of CD8$^+$ T cells results in a significant decrease in the BCG vaccine induced protection [332]. The team of Stenger clearly reported the role of CD8$^+$ T cells in the control of TB in humans. It is known that anti-TNF therapy for autoimmune diseases increases the reactivation of latent TB infection, and they demonstrated that this therapy reduced the expression of perforin and granulysin in CD8$^+$ lymphocytes. Further, they showed that anti-TNF-α treatment specifically depleted CD8$^+$ effector memory T cells that expressed cell surface TNF-α. Precisely these CD8$^+$ effector memory T cells express the highest levels of granulysin, lysed *M. tuberculosis* and infected macrophages [333].

In addition to conventional MHC class I restricted CD8$^+$ T cells, non-classical CD8$^+$ T cells can also recognize peptide or lipid antigens presented by non-polymorphic class Ib molecules such as HLA-E molecules, group 1 CD1 molecules, and MHC I-related molecules (MR1) such as mucosal associated invariant T cells (MAIT) [329]. The contribution of these populations will be described in the next chapter.

c. Non-classical T cells

MHC class Ib molecules are structurally similar to MHC class Ia molecules but are less polymorphic[334]. These MHC class Ib molecules possess a range of functions and are considered as part of the innate and adaptive immune responses [335]. MHC class Ib molecules comprise H2-M3, Qa-1/HLA-E, Qa-2/HLA-G, CD1 and MR1 molecules in mice and/or humans [335]. Most of these molecules are involved in the host defense against *M. tuberculosis* [335]. Indeed, H2-M3 restricted CD8$^+$ T cells have been shown to recognize *N*-formylated peptides derived from *M. tuberculosis.* MR1-restricted mucosal-associated invariant T cells (MAIT) recognize vitamin B metabolites produced by a broad range of bacteria including *M. tuberculosis* [336]. Gold and

co-workers reported that *M. tuberculosis* reactive MR1 restricted T cells are found in healthy individuals. Further, they showed that these *M. tuberculosis*-reactive MAIT cells are decreased in PBMC of patients with active TB but accumulate in the lungs of TB patients [337]. Later, it was shown that in mice MAIT cells can mediate protection against infection with *Mycobacterium abscessus.* Further, $MR1^{-/-}$ mice infected with BCG showed higher bacterial burdens in the lung in comparison to WT mice [336]. HLA-E restricted $CD8^+$ T cell responses to *M. tuberculosis* have been reported in humans [338].

Until recently, the relative contribution of various MHC class Ib restricted T cells to anti- *M. tuberculosis* immunity was not clear. Shang and colleagues interestingly addressed this issue by using a series of knock-out mice [339]. They used mice lacking MHC class Ia ($K^{b-/-}D^{b-/-}$), MHC class Ia/H2M3 ($K^{b-/-}D^{b-/-}M3^{-/-}$) or β_2M ($\beta_2M^{-/-}$). By comparing the proportion of $CD8^+$ T cells and analyzing the bacterial burden in the lungs and spleens of these 3 KO mice following *M. tuberculosis* infection, they observed that H2M3-restricted $CD8^+$ T cells represent only a small proportion of the total $CD8^+$ T cells and play a minor role in the protection against *M. tuberculosis*. However, they showed that MHC class Ib restricted $CD8^+$T cells (non H2M3-restricted T cells) recognize several *M. tuberculosis*-derived antigens at a higher frequency that MHC class Ia-restricted $CD8^+$ T cells. Those MHC class Ib restricted $CD8^+$ T cells have polyfunctional capacities (produce TNF-α, IL-2 and IFN-γ) and confer protection against *M. tuberculosis* [339]*.* Finally, they demonstrated that MR1 T cells represent only a small proportion of these MHC class Ib restricted $CD8^+$ T cells that recognize protein antigens from *M. tuberculosis.* Collectively, these data indicate that other MHC class Ib restricted $CD8^+$ T cells than the known H2M3, CD1 or MR1 T cells also contribute to the host defense against *M. tuberculosis* [339]*.*

In addition to protein antigens, CD1 molecules also recognize a wide variety of lipids found in *M. tuberculosis.* In humans two major groups of CD1 isoforms have been identified: group 1 CD1 (CD1a, CD1b and CD1c) and group 2 (CD1d) [340]. Mice express only CD1d molecules which complicates the use of the mouse model to study the role of group 1 CD1 in the control of *M. tuberculosis.* However, humanized mice that express group 1 CD1 have been created and used to address the contribution of group 1 CD1 molecules in immunity against mycobacteria. In

addition, guinea pigs, rabbits, non-human primates and cows that express some of group 1 CD1 molecules are also used for this purpose.

Regarding group 2 CD1 molecules, it was reported that CD1d-restricted invariant natural killer cells (iNKT cells) recognize *in vitro M. tuberculosis*-infected macrophages and this leads to the control of the infection by producing GM-CSF [341,342]. In addition, adoptive transfer of CD1d-restricted iNKT cells obtained from uninfected mice significantly diminishes the bacterial burden in the lungs of mice infected by aerosol with *M. tuberculosis* [341].

Regarding group 1 CD1 molecules, individuals exposed to *M. tuberculosis* showed higher frequencies of group-1 CD1-restricted T cells compared to control population, suggesting that these cells are activated following *M. tuberculosis* infection [148,165,343]. In addition, Felio and co-workers have shown that in human group 1 transgenic mice infected with *M. tuberculosis* or vaccinated with lipid antigens robust group 1 CD1-restricted *M. tuberculosis* lipid-specific T cell responses are induced [344].

CD1b molecules present a large variety of *M. tuberculosis*-derived lipids such as MAs, GMM, GroMM, diacylated sulfoglycolipids, LAM and PDIM [345]. CD1a molecules have been shown to present lipopeptide [345]. Until recently, it was not known whether T cell populations that recognize lipid antigens presented by group 1 CD1 molecules play a protective role during *M. tuberculosis* infection. To address this question, Zhao et *al.* made transgenic mice that expressed mycolic acids-specific CD1b-restricted TCR (DN1tg) and human group 1 CD1 molecules (hCD1Tg) [346]. They have found that DN1 T cells were more rapidly activated in mediastinal lymph nodes compared to Ag85B-specific $CD4^+$ T cells following *M. tuberculosis* aerosol infection. These DN1 T cells are cytotoxic, polyfunctional and contribute to the control of the infection, as shown by the reduction of the bacterial load in the lung, spleen and liver of infected mice following transfer of DN1 T cells in infected hCD1Tg/$Rag^{-/-}$ mice [346].

d. B cells and antibodies

M. tuberculosis is an intracellular facultative organism and as previously introduced, cell-mediated immune responses are necessary for the control of the infection. B cells and antibodies have long been thought to play no role in immunity to tuberculosis. This is partly due to the studies in animals and humans in the late 19th and early 20th century in which the

use of sera of infected animals in the therapy of TB did not lead to conclusive results [347]. It has been also reported that patients with active TB showed higher antibody titers against mycobacterial antigens compare to individuals with LTBI [348–350], supporting the concept that antibodies must be non-protective. Furthermore, the notion that *M. tuberculosis* is an intracellular pathogen and that antibodies are extracellular molecules also contribute to the fact that studying the role of B cells and antibodies in the control of TB has been neglected.

However, increased evidence demonstrated a role for B cells and antibody in the control of intracellular pathogens. These mechanisms vary from the classical opsonization/activation of FcR to interaction between B cells and other immune cells. For example, regarding TB, several authors have reported that intradermal BCG vaccination induces IgG and IgM antibodies to several mycobacterial antigens such as LAM [351]. Some of these antibodies were shown to enhance both innate and cell-mediated immune responses to *M. tuberculosis* [352]. Several laboratories showed that transfer of monoclonal antibodies to mycobacterial antigens, ranging from surface proteins to polysaccharides, can ameliorate the course of *M. tuberculosis* infection in mice. In addition, Balu et *al.* evaluated the combined role of administration of IFN-γ alone and/or monoclonal antibody 2E9IgA1 (that recognizes mycobacterial α-crystallin) in the outcome of *M. tuberculosis* infection [353]. They observed a reduction in lung CFUs 4 weeks following *M. tuberculosis* infection in the combined group compared to IFN-γ or monoclonal antibody alone [353], suggesting a potential synergistic effect between cytokine responses and antibodies in the protection against *M. tuberculosis* infection. Furthermore, in humans, serological studies have shown that adults and children with miliary, meningeal and pleural TB have lower antibody levels against mycobacterial antigens compared to latently infected individuals[349]. B cell as well as IgA deficient mice show an increased susceptibility to *M. tuberculosis* infection [354,355].

B cells have also been reported to contribute to the host defense against *M. tuberculosis* through interaction with other immune cells. For example, B cells can modulate the activation of T cells through antigen presentation as well as cytokine production [356]. Studies using B cells deficient mice (the μMT strain) point to the importance of B cells for the development of an optimal immune response to *M. tuberculosis.* For example, by modulating neutrophilic response. Indeed, these μMT mice have enhanced lung inflammation, neutrophil recruitment

and local increases in IL-10 production upon *M. tuberculosis* infection [357]. This increase of neutrophil infiltration is associated with an excessive lung pathology and poor control of *M. tuberculosis* infection in susceptible mice [358]. Adoptive transfer of B cells in µMT mice reverses this phenotype [357]. Kozakiewicz and co-workers analyzed mechanisms by which B cells modulate neutrophilic responses using µMT mice and the depletion of B-cell in C57BL/6 [359]. They found that B cells regulate neutrophil recruitment by modulating IL-17 responses following *M. tuberculosis* infection and BCG immunization. Further, they observed that the increased neutrophil infiltration observed in µMT mice negatively affects the migration of DCs to lymph nodes, and by this way impairs the development of vaccine-induced Th1 response. In addition, they observed that the administration of sera obtained from *M. tuberculosis*-infected C57BL/6 mice reverses the recruitment of neutrophils in the lungs of infected µMT mice [359].

In conclusion, we have seen in this chapter that immunity to *M. tuberculosis* is highly complex and involves numerous cell subsets, cytokines and effector mechanisms. However, while our understanding of the mechanisms of protection and pathogenesis has been improved, a lot needs to be done in order to understand all details. For example, it is still not clear why some people, particularly children, immediately develop active TB upon infection and others not or why despite all these mechanisms the immune system contains but cannot clear *M. tuberculosis.* In addition, as we have previously mentioned in the chapter 1.1.3 (vaccine limitation), we need a new effective vaccine against this disease but the major problem is the absence of good correlates of protection. Nevertheless, in the last two decades, substantial progress have been made in the TB vaccine field with 14 candidate vaccines, which have reached the level of clinical trial analysis (Figure 20) [360].

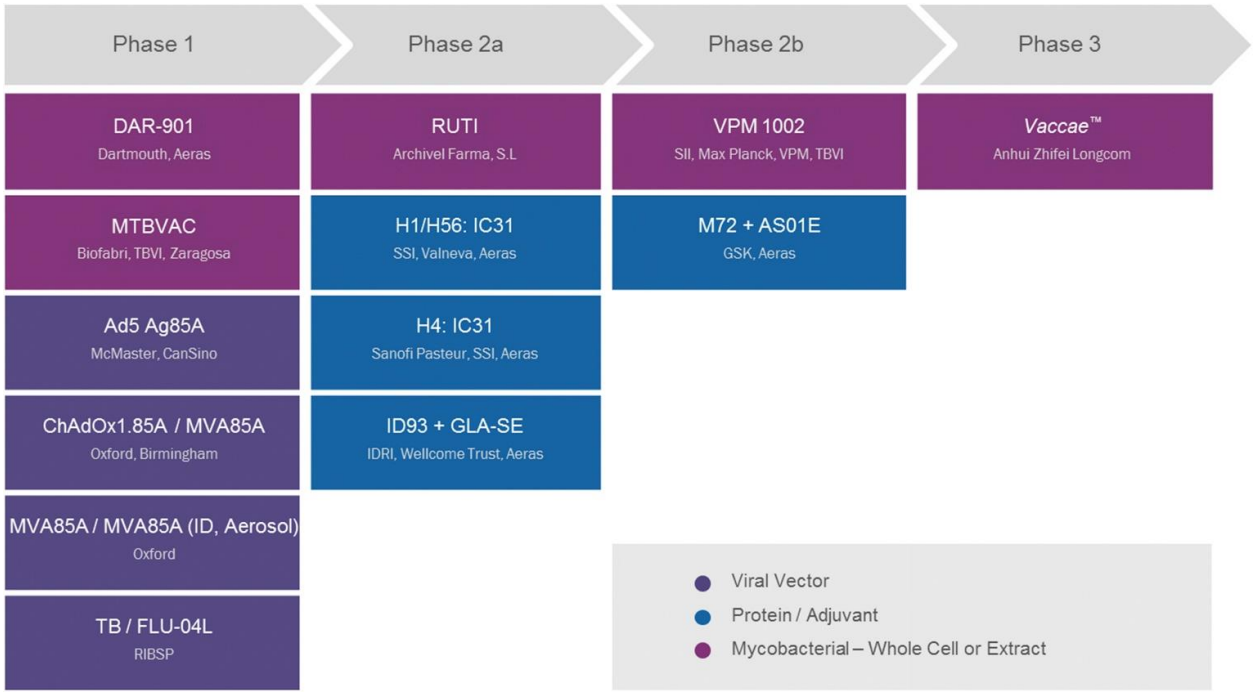


Figure 20: Pipeline of tuberculosis vaccine candidates in ongoing clinical trials [360]

Two types of preventive vaccines are actually in the pipeline:

- Subunit vaccines which are generally designed to be used as vaccines for boosting BCG-primed individuals. These subunit vaccines can be used as pre-exposure vaccination in new-borns but also for post-exposure vaccination in adults.
- Whole cell vaccines that comprise either *M. tuberculosis* attenuated by genetic manipulation or recombinant BCG with improved efficiency and/or safety to replace the BCG vaccine currently used.

Several approaches are used to ameliorate or develop vaccines against TB and this is also true for several other infectious diseases and cancer. As said, one of these approaches is the development of subunit vaccines. These vaccines are generally considered to be safer than live attenuated vaccines, especially for immuno-deficient individuals. Given the fact that the second aim of this project was the design of adjuvants that can induce cell-mediated immune responses for subunit vaccine against diseases such as TB, in the next chapter, we will discuss in more detail subunit vaccine formulation and subunit vaccine candidates which are currently investigated in clinical trials for TB.

1.4 Subunit vaccines

Subunit vaccines contain generally only a portion of the microbe that can be presented as antigens to the immune system in order to induce specific immune responses against the pathogens. For this purpose, antigens known as immuno-dominant i.e. that best activate the immune response in a large portion of subjects, are usually selected. However, in contrast to whole organism vaccines (inactivated or attenuated) that possess several immuno-stimulatory PAMPs and antigens, subunit vaccines are generally poorly immunogenic and need additional constituents to help to stimulate protective immunity [361]. These additional components are called adjuvants. The term adjuvant comes from the Latin word ''*adjuvare*'' that means to help/aid. These compounds are defined as molecules, compounds or macromolecular complexes added in vaccine formulation to stimulate, reinforce or modulate the immune response to a co-administrated antigen. Adjuvants are generally used in subunit vaccine for different purposes:

- Improve the efficacy of the vaccine in individuals with a weakened immune system such as elderly, new-borns or immuno-compromised individuals [362].
- Increase the breadth, magnitude and the durability of the immune responses by inducing long term memory responses [363].
- Reduce the costs by inducing equivalent responses with lower amount of antigen [364–367].
- Reduce the number of doses required to induce protection [365,368].
- Modulate the quality of the immune responses induced (e.g. by inducing the appropriate type of immune responses [363]: Th1 or Th2 immune response, cytotoxic immune response, specific antibody isotype…). This process is dependent on the type of innate immune responses induced (discussed below).

Mechanisms of action of several empirical adjuvants, such as alum, are complex and remain poorly understood. However, given the critical function of antigen presenting cells (APC) in the induction of specific immune response in the lymph node, it becomes easier to elucidate some mechanisms by which adjuvants work. These mechanisms comprise:

- Attraction of APC to the site of vaccine administration through inflammatory responses induced by the adjuvants.

- Formation of a depot at the site of the injection. This process will favour a longer exposure of the antigen to the immune system, therefore increasing the likelihood of APC-antigen interaction

- The induction of an appropriate immune response that is dependent on the activation profile of APC and co-stimulatory signals given by APC to naïve B and T cells.

Adjuvants can be classified based on their mechanisms of action. Thus, we distinguish immuno-stimulatory compounds or delivery systems. Some adjuvants such as ISCOMATRIX can be considered both as immuno-stimulatory compounds and as delivery systems.

1.4.1 Immuno-stimulatory compounds

Immuno-stimulatory compounds are compounds that can activate innate immune responses through binding to PRRs or directly (e.g. cytokines). As previously described, there are several families of PRRs that differ depending on the downstream signalling pathway that they activate. During the last decades, several studies have improved our understanding of the mechanisms of activation and regulation of these PRRs. As a consequence, this allows a rational design of new immuno-stimulatory adjuvants that are safer and quantitatively and qualitatively more immuno-potentiating. One interesting example is the development of monophosphoryl lipid A (MPLA), a structural analogue of LPS. LPS is a TLR4 agonist present on the outer membrane of Gram negative bacteria. This molecule comprises 3 different domains: the O-specific chain, the core domain and the lipid A domain [369]. Binding of LPS to TLR4 leads to the activation of MyD88 dependent and TRIF/TRAP dependent signalling pathways [370,371]. Unfortunately, LPS cannot be used as adjuvant because it is highly reactogenic and can induce septic shock [372]. Thus, MPLA has been developed by chemically removing a phosphate group on the Lipid A from *Salmonella Minnesota* 595 [369]. It has been reported that as an adjuvant MPLA increases serum antibody titers and induces strong cellular immune responses [373,374]. In addition, MPLA is substantially better tolerated in humans in comparison to wild-type lipid A [375]. This can be explained by the fact that MPLA predominantly activates the TRIF/TRAP pathway [376]. MPLA has been approved for use in the United States in the vaccine Cervarix.

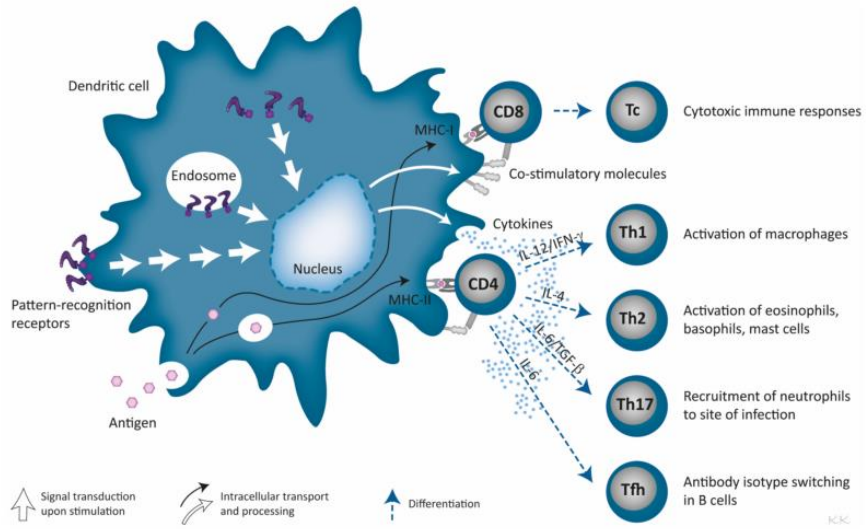


Figure 21: Major immunological responses derived by activation of DCs through cell surface, cytosol or endosome PRRs.
The antigen is phagocytosed, processed and presented on MHC-I molecules via cross-presentation or on MHC-II molecules to antigen-specific $CD8^+$ and $CD4^+$ T cells respectively. Activation of the DC upregulates co-stimulatory molecules and secretion of cytokines that provide activation signals to the antigen-specific T cells. Activation of $CD8^+$ T cells may require additional IL-2 from $CD4^+$ T cells (not shown) to become cytotoxic T cells. Activated $CD4^+$ T cells differentiate to distinct effector populations depending on the received cytokine signals; Th1 requires secretion of IL-12/IFN-γ; Th2 requires IL-4; Th17 requires IL6/TGF-β; and follicular T-helper cells secretion (Tfh) requires IL-6 [406].

In general, activation of PRRs by PAMPs on APCs, leads to the maturation of the APC, its migration to lymph nodes, cytokine/chemokine production, ultimately resulting in induction of appropriate adaptive immune responses (Figure 21). Thus, depending on the correlates of protection against the disease, the choice of immuno-stimulatory compounds is critical for the orientation of the vaccine-induced immune responses. For example, it is well known that $CD4^+$ T cells producing IFN-γ and $CD8^+$ T cells are essential for the protective immunity against intracellular pathogens and cancer. Thus, adjuvants that trigger Th1-biased immune responses such as TLR agonists are suitable for vaccines against these types of disease. Indeed, several clinical and preclinical studies have demonstrated the protective role of TLR agonists against cancer and intracellular pathogens [377]. In addition, Th17 immune responses have been shown to contribute to vaccine induced protection in *M. tuberculosis* infection [378]. Thus, an adjuvant such as CAF01 (cationic liposome DDA formulated with TDB) that triggers this type of immune response can be useful against TB.

MPLA is so far the only immune-stimulatory compound that has been licenced for human use. However, several PRR agonists are tested in clinical trials. Selected studies are summarized in Table 2.

Adjuvants	Innate immune receptors	Type of responses	References
Poly(I:C)	TLR3	Antibody, Th1 and CD8+ T cell immune responses. NK cell activation	[366]
MPLA	TLR4	Antibody, Th1 T cell immune responses	[367]
Flagellin	TLR5	Antibody, Th1 and Th2 T cell immune responses	[368]
Imiquimod	TLR7/8	Antibody, Th1 T cell immune responses	[369]
Unmethylated CpG oligodeoxynucleotides	TLR9	Antibody, Th1 and CD8+ T cell immune responses	[370]
TDB	Mincle	Antibody Th1 and Th17 immune responses	[89]

Table 2: Adjuvants currently tested in clinical trials. TLR: Toll-like receptor, NK: Natural Killer cell, TDB: Trehalose DiBehenate, Mincle: Macrophage Inducible C-type Lectin, Th: T-helper lymphocyte [519].

1.4.2 Delivery system

Delivery systems are also important in the development of subunit vaccines because they can increase antigen presentation and consequently enhance induced immune responses. In addition, they can reduce the toxicity of immuno-stimulatory compounds. There is a range of different delivery systems including aluminum salts, emulsions, liposomes, microparticles, and saponin. Most of these delivery systems have also immune-stimulatory properties [111].

a. Aluminum salts

Aluminum salts (Alum) were the first adjuvant/delivery system discovered in the 1920s. Since this period some billions of Alum adjuvanted vaccine doses have been administrated to humans. Alum is a particulate adjuvant composed of crystalline nanoparticles (hydrated potassium aluminum sulfate) that aggregate to form a heterogeneous dispersion of several microns. Alum is known to contribute to induction of higher antibody responses but not of cell mediated immune responses. The mechanisms of action of Alum are not fully understood. It was first proposed that Alum induces a depot at the injection site that releases gradually the adsorbed antigen. A study showed that Alum is detected in the muscles of immunized Cynomolgus monkey up to 6 months after intramuscular injection [379]. Nevertheless, this

hypothesis has been challenged by several studies. For example, Holt et *al.* have reported that excision of the injection site and removal of Alum does not alter the immune response to the co-administrated antigen [380]. A second proposed mechanism of action of Alum is that it can facilitate the uptake of antigen by APCs by presenting the antigen in a multivalent form. Furthermore, Alum was shown to induce inflammation at the injection site and to induce the maturation of APC (upregulation of co-stimulatory molecules) [111]. Regarding the receptor involved in the activation of APCs by Alum, it was first shown using different KO mice that the immunogenicity of Alum is independent of TLR-pathways [381]. In 2007, Li et *al.* reported that Alum induces the secretion of IL-1β by mechanism dependent on caspase-1 activation [382]. Later, several *in vitro* studies demonstrated that Alum activates NLRP3 inflammasome [383–386]. However, the role of NLRP3 activation in the adjuvant properties of Alum *in vivo* is a matter of debate in the field. Indeed, by using mice deficient for NLRP3, ASC or caspase-1, Einsenbarth and co-workers showed that the activation of NLRP3 inflammasome is necessary for the adjuvant potential of Alum [384]. This result was confirmed later on by two others studies [385,387]. In contrast, several others studies demonstrated an NLRP3 inflammasome independent adjuvant activity of Alum. In fact, Franchi and co-workers reported that NLRP3$^{-/-}$ mice produce similar level of antigen-specific IgG response following vaccination with human serum albumin and Alum [383]. Marichal et *al*. showed that Alum induces cell death that triggers the release of DAMPs such as DNA. Then, by mechanisms dependent on IRF3 pathway, DNA induces the activation of iDC and induction of Th2 responses. They also reported that Alum induces specific IgG1 responses by mechanism independent of IRF3 [386]. Recently, others confirmed that the adjuvant properties of Alum are independent of NRLP3 inflammasome activation [388]. Nevertheless, even if the adjuvancity is not clearly understood, most of these studies agree on the fact that Alum induces the recruitment of inflammatory cells at the site of injection or cell death and the liberation of DAMPs [383,385,386,389,390]. DAMPs will further induce the activation of APCs.

b. Emulsions

There are two different types of emulsion: oil in water emulsions (o/w) and water in oil emulsions (w/o). These two emulsions have been shown to induce strong antibody responses.

However, w/o emulsion is much more reactogenic in comparison to o/w [391]. In 1997, in Europe, one o/w emulsion has been approved in vaccines for human use: MF59. This adjuvant contains small (250nm), uniform and stable droplets and is composed of a squalene, polyoxyethylene sorbitan monooleate (Tween™ 80) and sorbitan triolate (Span85) [362]. Squalene is a fully metabolized oil obtained from shark liver. This compound is also found in humans as a natural metabolite of cholesterol. MF59 has been shown to enhance immunogenicity of influenza vaccine in small animal models, baboons and humans [392–394]. Several clinical trials demonstrated the safety and immunogenicity of MF59 in elderly persons and newborns [395,396]. In addition to induction of strong Th2 responses, MF59 formulated with oligomeric V2 loop-deleted gp140Env protein has been reported to enhance lymphoproliferative responses and frequency of T cells secreting IFN-γ in plasmid DNA primed rhesus macaques [397]

Another emulsion that has been developed is the adjuvant system 03 (AS03). This is a w/o emulsion composed of α-tocopherol, squalene and polysorbate 80. The diameter of the droplets in AS03 is smaller than in MF59 (150-155 nm as compared to 250nm). The addition of α-tocopherol in this o/w emulsion increases antigen uptake and presentation in draining lymph nodes [398]. α-tocopherol is one of the 8 isoforms of vitamin E and the most widely distributed in the nature. AS03 has an acceptable safety profile and was used in the H1N1 vaccine produced by GSK during the 2009-2010 influenza pandemic [399].

c. Liposomes

Liposomes are double- or multi-layered, biodegradable carrier vesicles that mimic the structure of natural bilayer membranes. These vehicles have the advantage of being able to carry insoluble antigens or immuno-stimulatory compounds. In addition, a same liposome formulation can carry different types of molecules (lipid immuno-stimulatory compound and protein antigen). Physico-chemical properties of liposomes, which depend on the lipid composition and the method of preparation, have a significant impact on the induced immune responses. Indeed, it has been reported that the size of the particle, the surface charge and surface modifications of the liposomes can have an impact on the immune response induced.

- The particle size has been shown to influence the draining kinetics of liposomes [400,401]. For example, Oussoren and co-workers showed that larger particles are retained longer in the site of injection following subcutaneous injection compared to smaller particles [401]. Brewer et *al.* reported that particle size of liposome determines the type of immune response induced. Larger liposomes (>250 nm) induce Th1 immune response in mice while smaller liposomes (<150 nm) induce Th2 response [402].
- Surface charge of liposomes depends on the composition of the lipids in the liposome bilayers. Liposomes based on dimethyldiacylammonium (DDA), a cationic lipid, are charged positively while liposomes based on phosphatidylserine are charged negatively. Positively-charged liposomes have been demonstrated *in vitro* to be taken up to a much higher degree than neutral or negatively charged liposomes by APCs [403–405]. This higher uptake by APCs was correlated *in vivo* with a higher induction of antibody responses in mice immunized subcutaneously. Furthermore, surface charge was also shown to influence the retention of liposomes at the site of injection. Indeed, neutral liposomes distearoyl phosphatidylcholine-TDB drained from the site of injection faster than cationic CAF01 liposomes (DDA-TDB) and only CAF01 induced cell-mediated immune responses [406].
- One strategy used to improve the adjuvant capacity of liposomes is to modify the surface of the liposome. For example, addition of polyethylene glycol (PEG) can affect its biodistribution. PEG-CAF01 liposomes administered intramuscularly increase the draining from the site of injection in comparison to non-PEGylated CAF01 [407].

Virosomes are liposomal formulations that contain viral envelope proteins anchored in their lipid membrane. This formulation boosts the immune system by delivering material that mimics certain virus-like particles. Indeed, virosomes maintain a morphology and cell-penetrating ability similar to infectious viral particles [408] and strongly induce both cellular and humoral immune responses [409–411]. Approved vaccines Epaxal and Inflexal V for hepatitis A are virosome-based vaccines.

d. Saponins

Saponins are triterpenoid glycosides extracted from *Quillaja saponaria* bark. These compounds have been shown to intercalate into cell membranes and to form pores [412]. It is not clear if the pore forming effect of saponins is related to its adjuvant potential. However,

one proposed mechanism is the fact that saponins allow antigen to have access to endogenous pathway of antigen presentation and by this way promote $CD8^+$ T cell responses. QS-21 is a fraction isolated from Quil A (partially purified saponin). QS-21 has been reported to stimulate both humoral and cell-mediated immune responses to subunit antigen in several animal models such as mice, dogs and primates [413,414]. This compound has been used in a number of clinical trials for cancer vaccines as well as infectious diseases (HIV, HSV, malaria and hepatitis B) [415]. However, the doses of 200 µg or higher appear to induce necrotic areas with inflammatory reactions in muscles at the injection site [416]. However, QS-21 formulated in liposome with MPL-A showed acceptable lack of toxicity for human use [417].

ISCOMATRIX is an immune-stimulating complex composed of saponin (QuilA), cholesterol and phospholipid [418]. This compound promotes antigen delivery as well as immune-stimulation [418]. ISCOMATRIX induces high titers of long-lasting functional antibodies and potent $CD8^+$ T cell responses specific to the viral antigen [419,420,421]. One interesting capacity of ISCOMATRIX is the induction of mucosal immunity in mice after intranasal [422] or intravaginal immunizations [421].

e. Micro-particles

Micro-particles like Alum are particulate delivery system. Poly-lactide-co-glycolides (PGLs) are the first class of micro-particles that have been developed as delivery system for vaccines. These micro-particles have been used in humans for decades as biodegradable suture material and as controlled-release drug delivery systems [423,424]. PGLs in comparison to Alum particles have been shown to induce CTL responses in rodents [425,426] but not in non-human primates [416]. Like liposomes, the surface charge of micro-particles influences their adjuvant property. Indeed, coating PGLs with sodium dodecylsulfate provides overall negative charge to the particles and this increases the surface adsoption of the protein of interest. This approach has been used to adsorb tetanus toxoid in PGL for mucosal delivery [427]. In addition to antigen, micro-particles can be also used to entrap immune-stimulatory compounds in order to ensure long-term release of these agents [416].

To summarize, we have seen that a wide range of delivery system have been developed and that most of these delivery systems have also immuno-stimulatory properties. These different delivery systems also modulate the type of immune responses induced. So, the selection of

the type of delivery system to be used for a given subunit vaccine is very important. Some interesting studies that clearly demonstrate the important role of delivery systems in vaccine development, are those by GSK concerning development of malaria vaccines. Indeed, by comparing the formulation of RTS,S antigen in liposome formulation (AS01B) or in emulsion formulation (AS02A), they could demonstrate that RTS,S/AS01B induces higher cell-mediated immune responses and induces better clinical protection in human challenge compared to RTS,S/AS02A, indicating that for this vaccine a liposome formulation is better than an emulsion [428]. Similarly, the immuno-stimulatory effects of emulsion- as compared to liposome-based adjuvants on the tuberculosis polyprotein vaccine, Mtb72F has also been analyzed [429]. In this study, liposome delivery system appears again to be the best. This formulation induces higher T-cell responses as monitored by a stronger IFN-γ response and induction of CD8 T cell responses [429]. In contrast to these studies, Fox and co-workers showed in the mouse model that a malaria antigen containing vaccine formulated in oil-in water emulsion is better in terms of induced cell-mediated responses as compared to a liposome formulation or an aqueous suspension [430]. In a direct comparison, Alum combined with glucopyranosyl lipid adjuvant (GLA is a TLR4 agonist) and ID93 (a TB vaccine antigen) appears to be less protective compared to GLA formulated in an oil in water emulsion [431]. Further, they showed that the oil component used in oil in water emulsion is critical for the adjuvant activity [431]. In addition, it has been reported that delivery systems can reduce the toxicity of PAMPs by for example limiting the systemic exposure [378,417].

To conclude, these studies demonstrate that the choice of adjuvant formulation is critical for the development of potent vaccines, as they can be used to enhance the level of response, reduce the toxicity of PAMPs and skew the induced response through a desired pathway.

1.4.3 Next generation of adjuvants – Tuberculosis

In contrast to first licensed adjuvants (Alum, virosome…), the current strategy in the development of adjuvants consists in a combination of one or several immune-stimulatory molecules with one or several delivery systems. These combinations are developed because they can act synergistically to enhance the antigen-specific immune responses or to stimulate several types of immune responses. For example, the Cervarix vaccine, used for the vaccination against human papillomavirus, contains AS04 adjuvant. AS04 is composed of a

TLR4 agonist MPLA and aluminum hydroxide salt. Formulation in AS04 induces higher antibody titers and Th1 immune responses than Alum alone [432,433]. It has also been reported that addition of CpG ODN (TLR9 agonist) to MF59 in an influenza vaccine study in mice increases antibody titers and shifts the immune response to a Th1-type as compared to both adjuvant and delivery system alone [434].

Hereafter, given the scope of this PhD project, we will elaborate on four TB protein-based subunit vaccines currently in clinical trials and more in particular on the four adjuvants included in these subunit vaccines (Figure 20).

a. M72 + AS01E

AS01 is a liposome developed by GSK that contains MPL and QS21. AS01E is the same adjuvant but with a lower dose of adjuvant for pediatric use. M72 is a fusion protein composed of a putative serine protease Mtb32A (Rv0125 encoding PepA) and Mtb39A (Rv1196 encoding PPE18). This formulation has been shown to be protective in several animal models including mice, guinea pigs and rabbits [429,435,436]. Several clinical trials have demonstrated the safety of this vaccine [437–439] even in HIV positive individuals [440]. Regarding the type of immune response induced, AS01 induces a strong Th1 immune response with polyfunctional $CD4^+$ T cells, $CD8^+$ T cells and the activation of NK cells.

b. ID93 + GLA-SE

Glucopyranosyl lipid adjuvant (GLA) is a hexa-acylated lipid A recognized by TLR4 and signals via MyD88 and TRIF pathways. This compound is a synthetic analog of MPL that possesses even more immuno-stimulatory properties in human DCs in comparison to natural MPL [441]. ID93 is a fusion protein composed of antigens involved in the virulence of *M. tuberculosis* (Rv2608, Rv3619 and Rv3620) and a latency antigen (Rv1813). ID93 formulated in a stable emulsion with GLA (GLA-SE) has been shown to be highly protective in several animal models (mice, guinea pigs and cynomolgus macaques) [442]. This vaccine has recently been reported to be also protective against hypervirulent Beijing *Mtb* strains in mice [443]. ID39/GLA-SE induces a potent Th1 responses with polyfunctional $CD4^+$ T cells with a predominant contribution of IFN-

γ/IL-2/TNF-α triple positive cells and IFN-γ/TNF-α double positive cells [444]. This vaccine is currently in phase IIa clinical testing (NCT02465216).

c. H1/H56, H4 + IC31

IC31 is an immuno-stimulatory compound composed of a single-stranded oligodeoxynucleotide containing deoxy-Inosine/deoxy-Cytosine (ODN1a) and the antimicrobial cationic peptide $KLKL_5KLK$ [445]. This compound is recognized by the innate immune receptor TLR9 and has been shown to induce long-term cell-mediated immune responses that are highly protective in mice and non-human primates [446,447]. This adjuvant has been tested in several clinical trials in association with three fusion proteins H1 (Ag85B and ESAT-6), H4 (Ag85B and TB10.4) and H56 (Ag85B, ESAT-6 and a latency antigen Rv2660). These candidates are in phase IIa clinical trial [378]. Regarding safety, vaccination with IC31 caused minimal adverse effect even in HIV-positive individuals. It has also been reported that vaccination with this adjuvant in combination with fusion proteins induces a Th1 response that persists throughout 2.5 year and consists of polyfunctional T cells triple positive for IFN-γ/IL-2/TNF-α or IL-2/TNF-α double positive cells [448–451]. In addition, the multistage vaccine H56+IC31 has been reported to prevent reactivation in cynomolgus macaque [447].

d. CAF01-H1

Cationic adjuvant formulation 01 (CAF01) is an adjuvant composed of cationic liposome DDA combined with the immuno-stimulatory compound trehalose dibehenate (TDB). As discussed in section 1.3.1.1, TDB is a structural analogue of the cord factor of *M. tuberculosis* with short acyl chain (C22) and with a more acceptable toxicity [452]. TDB like cord factor is recognized by the C-type lectin receptor Mincle [233]. TDB incorporated in DDA liposomes is highly stable with a shelf life of more than 2 years at 4°C. This adjuvant is the only adjuvant in clinical trials which can induce not only long-lived Th1 but also Th17 immune responses [233,453]. This response is also characterized by the induction of central memory T cells that are double positive for TNF-α/ IL-2 [454,455]. CAF01/H1 protect against *M. tuberculosis* infection in several animal models [378]. Several phase I trials have reported that CAF01/H1 has an excellent safety profile in humans [91].

1.4.4 *M. tuberculosis* and adjuvants

Since the use of Alum as adjuvant more than 80 years ago [380], there have been few adjuvants licensed for human use that can promote cell mediated immune responses. However, great progress has been made in the field of adjuvant research and as mentioned there are many candidates in clinical trials. Nevertheless, few of these new candidates can induce Th17 responses, known to be important in the protective immunity against some infection diseases such as tuberculosis. In addition, most of these candidates target the induction of classical T cell responses. Non-classical T cells such as MAITs, CD1 or γ/δ T cells are still relatively unexplored. So, new candidates that can induce these types of responses are needed.

The cell wall of *M. tuberculosis* has been known for decades to have many biologically active compounds with an adjuvant potential. To this introduction chapter a published review concerning the adjuvant potential of cell wall components of *M. tuberculosis* is attached. In this review, we discussed of recent understanding of the interactions between mycobacterial cell wall components and PRRs, with a particular emphasis on CLRs. We also discuss the relevance of these interactions for the development of novel adjuvants for subunit vaccines and novel TB vaccines based on lipid antigens.

a. Review

Expert Review of Vaccines

ISSN: 1476-0584 (Print) 1744-8395 (Online) Journal homepage: http://www.tandfonline.com/loi/ierv20

Innate signaling by mycobacterial cell wall components and relevance for development of adjuvants for subunit vaccines

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To cite this article: Hermann Giresse Tima, Kris Huygen & Marta Romano (2016): Innate signaling by mycobacterial cell wall components and relevance for development of adjuvants for subunit vaccines, Expert Review of Vaccines, DOI: 10.1080/14760584.2016.1187067

To link to this article: http://dx.doi.org/10.1080/14760584.2016.1187067

Accepted author version posted online: 20 May 2016.
Published online: 30 May 2016.

EXPERT REVIEW OF VACCINES, 2016
http://dx.doi.org/10.1080/14760584.2016.1187067

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REVIEW

Innate signaling by mycobacterial cell wall components and relevance for development of adjuvants for subunit vaccines

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ABSTRACT

Introduction: Pathogen recognition receptors (PRRs) recognize pathogen-associated molecular patterns, triggering the induction of inflammatory innate responses and contributing to the development of specific adaptive immune responses. Novel adjuvants have been developed based on agonists of PRRs.

Areas covered: Lipid pathogen-associated molecular patterns (PAMPs) present in the cell wall of mycobacteria are revised, with emphasis on agonists of C-type lectin receptors, signaling pathways, and preclinical data supporting their use as novel adjuvants inducing cell-mediated immune responses. Their potential use as lipid antigens in novel tuberculosis subunit vaccines is also discussed.

Expert commentary: Few adjuvants are licensed for human use and mainly favour antibody-mediated protective immunity. Use of lipid PAMPs that trigger cell-mediated immune responses could lead to the development of adjuvants for vaccines against intracellular pathogens and cancer.

ARTICLE HISTORY
Received 6 January 2016
Accepted 3 May 2016
Published online 30 May 2016

KEYWORDS
Mycobacteria; mycolic acids; mycolate esters; lipoarabinomannan; C-type lectin receptors; inflammasome; cell-mediated immunity; adjuvants; sub-unit vaccines

1. Introduction

Inducing protective immunity using vaccines is one of the major medical achievements ever developed, which has resulted in considerable reduction of morbidity and mortality caused by infection. The term *vaccine* derives from the Latin word *vacca* for cow and was coined after Edward Jenner's research, a medical doctor who at the end of the eighteenth century inoculated humans with cowpox to protect them against smallpox [1]. Jenner's research led two centuries later to the worldwide eradication of smallpox and paved the way to the development of vaccines against other infections. Vaccines are a part of multiple national health programs and are the most effective and cost-efficient method for preventing infectious diseases and their complications. However, new or better vaccines for some infectious diseases such as HIV, tuberculosis, and malaria, that continue to kill millions of people each year, are still needed.

Three major types of vaccines have been developed:

(1) Live attenuated vaccines, based on pathogens in which virulence has been reduced or removed, such as *the Mycobacterium bovis* BCG (Bacille Calmette-Guérin) vaccine against tuberculosis.
(2) Inactivated/killed whole pathogen vaccines, such as inactivated poliovirus vaccine.
(3) Subunit vaccines, which are based on components of a pathogen (such as protein antigens, polysaccharide antigens, or inactivated pathogen toxins). Notable example is the DTaP (Diphteria, Tetanus, acellular Pertussis) vaccine, based on diphtheria and tetanus toxoids and three to five protein antigens of *Bordetella pertussis*.

Live attenuated vaccines have the advantage of inducing strong humoral and cell-mediated immune (CMI) responses but they are not safe for administration to immunodeficient persons. Inactivated whole pathogen vaccines or subunit vaccines are noninfectious and therefore safer, but also induce weaker and mainly humoral immune responses. Vaccination with these latter types of vaccines hence involves administration of several doses to achieve protective immunity against the targeted pathogen [2].

This review focuses on the development of subunit vaccines based on protein antigens formulated with adjuvants derived from the cell wall of mycobacteria. These compounds have the potential to contribute to the induction of humoral and, more importantly, cellular protective immune responses. The word 'adjuvant' derives from the Latin word *adjuvare*, which means 'to help/to favor'. Adjuvants are defined as molecules, compounds, or macromolecular complexes used to improve the immune response to coadministered antigens. The choice of an adjuvant is very important in the development of subunit vaccines. Indeed, it is well known that adjuvants can act at the level of the innate immune response induced and can activate the appropriate class of immune effector responses.

There are many mechanisms involved in the ability of adjuvants to promote immune responses [3]:

- The formation of a depot is a common mechanism to many adjuvants. It will lead to the gradual release of the adsorbed antigen at the injection site. This will promote a longer interaction between the immune cells and the antigen.

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Table 1. Clinically approved adjuvants.

Adjuvants	Composition	Formulation	Mechanisms or receptor	Type of induced immune responses	Target infection/disease (example)	References
Aluminum salts	Aluminum hydroxide or oxide or phosphate or sulfate of hydroxyphosphate	Suspension	NLRP3, antigen delivery	Antibody, Th2	Diphtheria, tetanus, and whooping cough (Boostrix)	[4,5]
MF59	Squalene, sorbitan trioleate, polysorbate 80	Oil in water emulsion	Antigen uptake, ASC, recruitment of monocytes and neutrophils	Antibody, Th1, Th2	Influenza (Fluad)	[6,7]
AS04	MPL + aluminum hydroxide	Liposome	TLR4	Antibody, Th1	Human Papilloma Virus (Cervarix)	[8]
AS03	Squalene, α-tocopherol, polysorbate 80	Oil in water emulsion	Antigen uptake	Antibody, Th1, Th2	Influenza (Pandemrix)	[9]
Virosome	Lipid vesicle with influenza membrane protein	Liposome	Antigen delivery	Antibody, Th1, Th2	Hepatitis A virus (Epaxal)	[10]

NLRP3: Nucleotide-binding oligomerization domain, Leucine rich repeat and Pyrin domain containing 3; ASC: apoptosis-associated speck-like protein containing a CARD; TLR4: Toll-like receptor 4; AS: adjuvant system; Th: T-helper lymphocyte.

Table 2. Adjuvant currently tested in clinical trials.

Adjuvants	Innate immune receptors	Type of responses	Reference
Poly(I:C)	TLR3	Antibody, Th1 and $CD8^+$ T cells immune responses. NK-cell activation	[13]
MPLA	TLR4	Antibody and Th1 T cells immune responses	[14]
Flagellin	TLR5	Antibody, Th1 and Th2 immune responses	[15]
Imiquimod	TLR7/TLR8	Antibody and Th1 T cells immune responses	[16]
Unmethylated CpG oligodeoxynucleotides (ODN)	TLR9	Antibody, Th1 and $CD8^+$ T cells immune responses	[17]
TDB	Mincle	Antibody, Th1 and Th17 immune responses	[18]

TLR: Toll-like receptor; NK: natural killer cell; TDB: trehalose dibehenate; Mincle: macrophage inducible c-type lectin; Th: T-helper lymphocyte.

- The induction of inflammatory responses at the site of vaccine administration attracts antigen presenting cells (APCs) leading to improved antigen uptake/presentation.
- The induction of specific immune responses dependent on the type of co-stimulatory signals given by APCs to T and B lymphocytes.

There are currently only a few adjuvants licensed for human use (summarized in Table 1), such as aluminum salts, AS04 (Alum + monophosphoryl lipid A [MPLA]), squalene-based oil in water emulsion (MF59 and AS03), and virosomes (liposomes with fusogenic viral proteins). In addition, these adjuvants mainly contribute to the induction of antibody-mediated protective responses in vaccines for infections such as pertussis and hepatitis A and B. However, they only marginally contribute to the induction of CMI responses required for protection against intracellular pathogens and cancers (such as type 1 T helper (Th1), type 17 T helper (Th17), and cytotoxic T lymphocyte (CTL) responses).

Recent advances in our understanding of the role of innate receptors – the so-called Pathogen Recognition Receptors (PRRs) – in the development of adaptive immune responses have contributed to the rational development of novel adjuvants. These adjuvants are based on compounds similar to the so-called pathogen-associated molecular patterns (PAMPs) able to interact with PRRs. There are several families of PRRs: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and retinoic acid inducible gene I-like receptors (RLRs) [11,12]. All these receptors recognize PAMPs, which are highly conserved molecular structures found on pathogens. As said, many adjuvants in clinical development are PAMPs or their synthetic analogs and a non-exhaustive list of examples is provided in Table 2.

Mycobacterium tuberculosis (*Mtb*), the causal agent of tuberculosis, is a major human pathogen that in 2014 caused disease in about 10 million people and killed 1.5 million individuals [19]. Infection by *Mtb* induces humoral and cellular immune responses (recently revised by Mascart and Locht [20]). Induction of these cellular immune responses can be in part attributed to the abundance in the cell wall of *Mtb* of specific PAMPs, which are characteristic of bacteria of the *Mycobacteriaceae* family. Notable examples include lipomannans, lipoarabinomannans (LAMs), and their mannosylated forms (ManLAM), lipoproteins, phtiocerol dimycocerosates (PDIM), mycolic acids (MAs), and mycolate esters (Figure 1). The interactions of these compounds with PRRs have been recently reviewed [21]. For many decades, mycobacteria and mycobacterial components have been used for their ability to induce innate immune responses. For instance, *M. bovis* BCG has been used for nearly 40 years as an immunotherapy to treat superficial bladder cancer [22]. Complete Freund's Adjuvant (CFA) contains killed mycobacteria and stimulates strong antibody as well as Th1/Th17 immune responses [23,24]. The RIBI adjuvant system (RAS) is a water in oil emulsion containing MPLA, a nontoxic homolog of bacterial lipopolysaccharide (LPS) combined with TDcM (trehalose dimycolate of Corynebacterium) and RAS has been shown to induce strong Th1 responses at levels similar to CFA [25].

In this review, we provide an update on our recent understanding of the interactions between mycobacterial cell wall components and PRRs, with a particular emphasis on CLRs. We

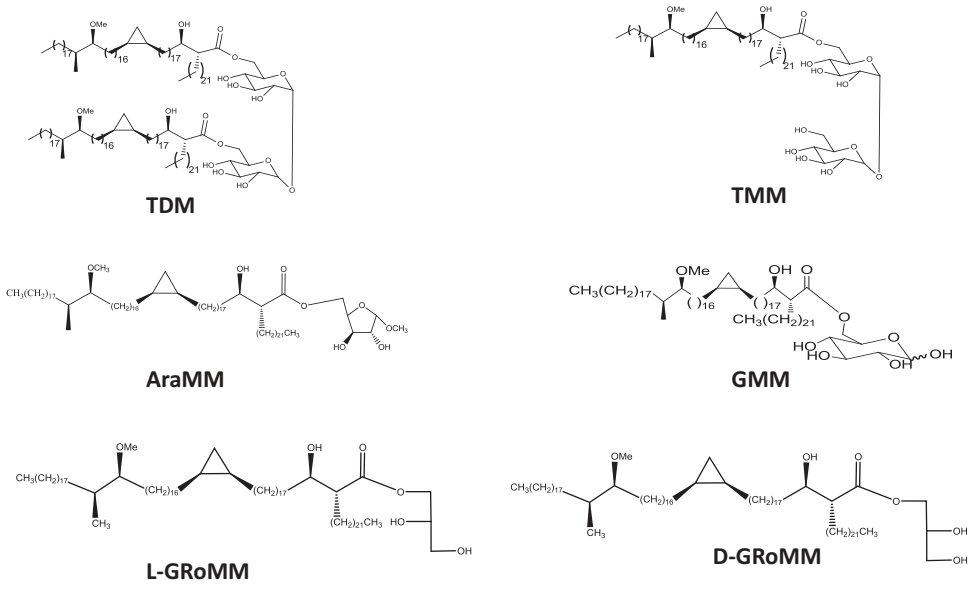

Figure 1. Representation of the structure of mycolate esters. TDM: Trehalose dimycolate, TMM: Trehalose monomycolate, GMM: Glucose monomycolate, AraMM: Arabinose monomycolate, GroMM: Glycerol monomycolate.

also discuss the relevance of these interactions for the development of novel adjuvants for subunit vaccines and novel TB vaccines based on lipid antigens.

2. Mycolic acids

MAs are high molecular weight C-60 to C-90 α-alkyl-β-hydroxy fatty acids characteristic of mycobacteria. They are the major lipids of the mycobacterial cell wall [26] and are predominantly bound to the cell wall with arabinogalactan as penta-arabinose tetramycolates. In addition, MAs are also present as free (non-glycosylated) MAs [27] or as non-bound solvent extractable mycolate esters, such as trehalose mono- and dimycolates (TMMs and TDMs), glucose monomycolates (GMMs), and glycerol monomycolates (Gro-MMs) [26]. It has been reported that purified MAs formulated in liposomes have an inflammatory potential when instilled in the airways and that they can mimic certain aspects of *Mtb* infection including recruitment of neutrophils, monocytes, lymphocytes and production of the inflammatory cytokines IL-12 and IL-6 [28]. MAs also have the potential to induce foamy macrophages. Foamy macrophages are characterized by the intracellular accumulation of lipid droplets. These macrophages have been described as favoring the persistence of the bacilli by providing lipid nutrients and by conferring protection against antibiotic treatments [26,28–31]. Pending the functional group(s) present on their meromycolate chain, MAs are subdivided in different classes. The three principal classes are the alpha-, keto-, and methoxy-MAs [26]. Current evidence strongly suggests that the classes of MAs can influence their biological activities [26,32–34]. Indeed, Vander Beken *et al*. have shown that alpha-MA was inert while methoxy-MA-induced solid inflammation and keto-MA with cis-cyclopropane stereochemistry-induced mild inflammation in the lungs of mice after intratracheal instillation. In addition, trans-keto-MA was reported to be anti-inflammatory and to inhibit the inflammation induced by cis-methoxy-MA [34]. Using an *in vitro* model of human granuloma, it was also shown that oxygenated (keto- and methoxy-) MAs play a crucial role in the induction of foamy macrophages [30]. Regarding the receptor involved in the recognition of MAs, it was recently reported that the host lipid-sensing nuclear receptor TR4 (Testicular receptor 4) specifically recognizes keto-MA and is involved in the induction of foamy macrophages by oxygenated MAs [29].

Besides their pro-inflammatory capacity and their ability to induce foamy macrophages, MAs are also known for their antigenic potential. Indeed, they are lipid antigens that can be presented by human dendritic cells (DCs) in the context of CD1b molecules [35]. CD1-restricted T cells have been reported to be involved in the protective immunity to tuberculosis [35]. The antigenic potential of MAs could be used in the development of subunit vaccines against tuberculosis especially because in contrast to classical MHC molecules, CD1 proteins show very limited polymorphism and may induce immunity in genetically diverse human populations. A study has shown that immunization of guinea pigs with total lipid of *Mtb* formulated in liposomes with adjuvant (DDA and squalene) reduced bacterial burdens in the lung and spleen and also reduced the lung pathology after *Mtb* challenge [36].

3. Trehalose mono- and dimycolates

TDM, also known as cord factor, is the best studied mycolate ester found in several species including Mycobacteria, Nocardiae, Corynebacteria, *Arthrobacter*, and *Brevibacterium* [37]. TDM is an α-α-trehalose which forms 6,6′-diester bonds with two MA molecules [37]. TDM is the most abundant glycolipid released by virulent mycobacteria and has several biological activities. Indeed, TDM has been reported in mouse models to mimic certain aspects related to *Mtb* infection including the production of pro-inflammatory cytokines as

well as the induction of granuloma and procoagulant activity [38,39]. Additionally, several pieces of evidence suggest that TDM may also inhibit phagosome-lysosome fusion [40,41]. Moreover, TDM has a well-known adjuvant potential. CFA, a water in oil emulsion containing heat-killed *Mtb* strain H37Ra or *M. butyricum* in mineral oil, efficiently induces humoral and CMI responses [42]. Shenderov *et al*. have recently reported that the ability of CFA to stimulate Th17 responses is linked to the presence of TDM in CFA. Indeed, two components of CFA (TDM and peptidoglycan [PGN]) formulated in Incomplete Freund's Adjuvant (IFA)-induced Th17 polarization at levels comparable to CFA in an ovalbumin model. Interestingly, in this study, these components formulated separately in IFA were ineffective in inducing substantial Th17 responses [43]. Nevertheless, TDM is considered as toxic and this has obvious implications for TDM to be used as an adjuvant for vaccines. However, this toxicity was reported in the mouse model when TDM was administered intravenously and at high doses (300 µg) [44,45] but was not observed in several models including rats and guinea pigs [37]. Of note, it was reported that this toxicity of TDM is dependent on its conformation. Indeed, in aqueous solution, TDM forms cylindrical micelles that are nontoxic, but in oil-water, plastic-water, or air-water interfaces, TDM forms monolayers that are toxic [46]. Hunter *et al*. have reported that TDM toxicity can be attributed to its interaction with lipids of the host [47]; hence, in the development of novel adjuvants based on TDM, possible toxicity of the formulation should be carefully analyzed. Purified TDM used in these different studies was generally a mixture of alpha-, keto-, and methoxy-MAs in different proportions and with varying carbon chain lengths. Interestingly, the classes of mycolate bound to the trehalose seem to influence the inflammatory potential of this glycolipid as reported in different studies using different mycobacterial strains or *Mtb* mutants [45,48–50]. Likewise, when *Mtb* mutants or different mycobacterial strains were used to study the link between classes of mycolates present in TDM and inflammatory potential of TDM, the TDM preparations tested actually consisted of a complex mixture of molecules. Therefore, synthetic analogs can be very useful to analyze the inflammatory potential and the toxicity of different classes of TDM. This could be an approach for the development of new adjuvants for human vaccines.

Another approach already used is the development of less toxic structural analogs. Trehalose dibehenate (TDB) is a structural analog of TDM with acceptable toxicity. The adjuvant property of this compound has been extensively reported [51–53]. TDB formulated in cationic liposomes with dimethyl dioctadecyl ammonium bromide (DDA) and with H1 antigen (a fusion of two mycobacterial antigens, namely ESAT-6 and Ag85B)-induced strong Th1 and Th17 responses protecting against experimental *Mtb* infection in a mouse model at levels comparable to the protective immunity induced by BCG vaccination. This so-called CAF01 adjuvant is currently evaluated in phase I clinical trials for the development of subunit vaccines against HIV and tuberculosis [18,54].

Recently, the innate immune receptor responsible for the recognition of TDB and TDM was identified, namely the macrophage inducible CLR Mincle or Clec4e [55,56]. This CLR can recognize damage-associated molecular patterns (DAMPs) and PAMPs from bacteria and fungi [57]. Binding of a ligand to Mincle leads to the activation of the adapter protein FcRγ which is essential for the recruitment of spleen tyrosine kinase (SYK). Activation of SYK leads to the recruitment of a complex formed by the caspase recruitment domain-containing protein 9 (CARD9), B-cell lymphoma, and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1). Recruitment of this complex will then lead to the production of pro-inflammatory cytokines and chemokines via the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [57]. However, Mincle is barely expressed on resting macrophages. This suggested the existence of another TDM receptor upstream of Mincle. Indeed, Miyake *et al*. have shown that *in vivo* injection of TDM strongly induced the expression of Mincle, barely detectable in unstimulated conditions. They identified the macrophage CLR macrophage C-type lectin (MCL) or Clec4d as the primary receptor for TDM and demonstrated that MCL is required for the induction of Mincle in the mouse model [58]. MCL is a transmembrane protein belonging to the CLRs family. This receptor is lacking an arginine residue in its transmembrane domain and arginine is known to be required for the association with the adapter protein FcRγ. However, MCL can activate SYK [59]. The mechanism by which mouse MCL (mMCL) induces the expression of mouse Mincle (mMincle) was recently elucidated, again by Miyake *et al*. [60]. This team has shown that mMCL regulates mMincle at the protein level. Further, they have shown that mMincle and mMCL co-regulate their expression on the cell surface (Figure 2). mMCL is composed of four domains: a cytoplasmic, a transmembrane, a stalk, and a carbohydrate recognition domain. Finally, they observed that mMCL but not human MCL (hMCL) interact with mMincle. So, they generated a series of chimeric proteins by replacing different mMCL domains by human MCL domains. Using this strategy, they identified the stalk domain of MCL to be necessary for the association with Mincle. It was recently reported that the expression of MCL on leukocytes in the lung is very low in naïve mice but significantly upregulated during inflammation and infection. These data show that MCL is also an inducible receptor like Mincle on myeloid cells [61].

While the role of Mincle during *Mtb* infection is not clearly demonstrated, it has recently been shown by Wilson *et al*.that MCL plays an important and nonredundant role in the control of *Mtb* infection [62]. Indeed, MCL deficient mice have higher bacterial burdens, exacerbated pulmonary inflammation, and increased mortality after *Mtb* infection compared to control mice. Finally, these authors identified an MCL polymorphism in humans associated with an increased susceptibility to pulmonary tuberculosis [62]. However, the regulation of Mincle expression by MCL was not confirmed in the guinea pigs. Indeed, Toyonaga and coworkers [63] have shown that gpMincle is detected in resting condition in the spleen, lymph nodes, and peritoneal macrophages and that the expression of gpMincle is upregulated after stimulation by TDM. Using two 2B4-NFAT-GFP reporter cells expressing Mincle or MCL together with FcRγ, they showed that TDM can bind to gpMincle and activate the transduction of the signal while TDM is unable to bind to gpMCL.

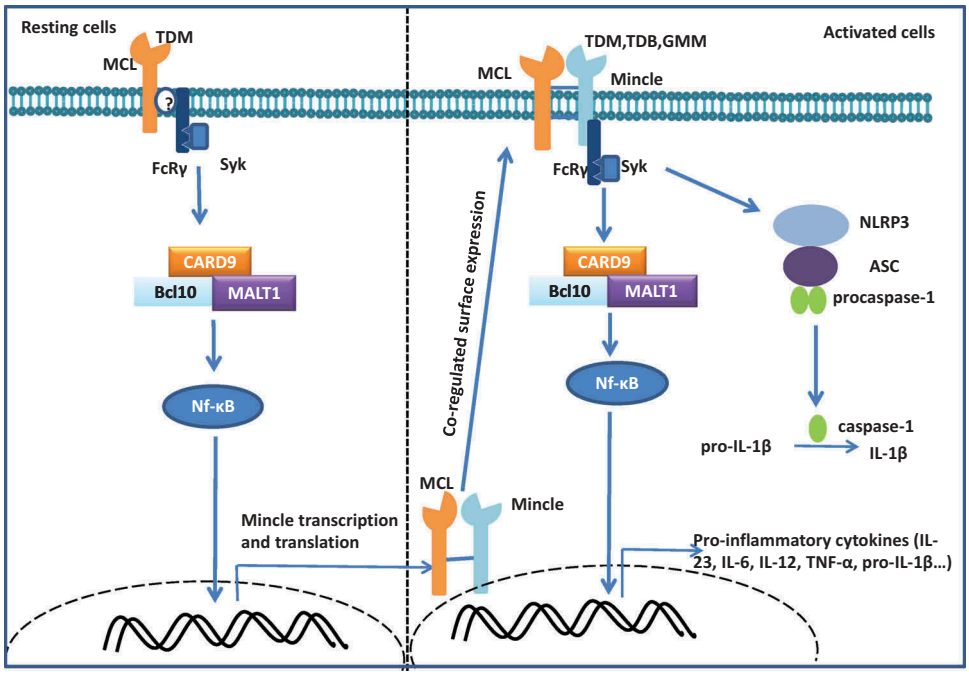

Figure 2. Regulation of Mincle and MCL expression in mouse. MCL is constitutively expressed on myeloid cells. Binding of cord factor (TDM) leads to Mincle expression through the CARD9-Bcl10-MALT1 signalosome. Mincle and MCL form a heterodimeric complex and mutually co-regulate their surface expression. TDM, trehalose dibehenate (TDB) and glucose monomycolate (GMM) bind to Mincle and induce the production of pro-inflammatory cytokines and the activation of NLRP3 inflammasome. This activation triggers the maturation of pro-IL-1beta.

Until recently, it was not known whether TDB and TDM activated human immune cells by the same mechanisms as in rodents. This issue was investigated by Ostrop *et al*. [64] who analyzed *in vitro* the activation of primary human monocytes, macrophages, and DCs by TDB and TDM. In this study, different cell populations were generated from $CD14^+$ monocytes: GM macrophages (differentiated with granulocyte-macrophage colony-stimulating factor [GM-CSF]), M macrophages (differentiated with M-CSF), and GM + 4 DCs (differentiated with GM-CSF and IL-4). Upon stimulation with TDB and TDM, all three cell types secreted pro-inflammatory cytokines and chemokines but the level induced depended on the cell type. Indeed, M-CSF macrophages produce significantly more IL-8, IL-6, CCL3, CCL4, and CCL2 upon stimulation with TDM compared to TDB while GM + 4 DCs produce more of these cytokines and chemokines upon stimulation with TDB compared to TDM. No differences in terms of cytokine and chemokine productions were observed in GM macrophages stimulated with TDM or TDB. Inhibition of SYK with a pharmacological inhibitor R406 significantly reduced the secretion of IL-8 induced by TDB and TDM. These results suggest a potential role of CLRs in the activation of primary human cells by TDB and TDM. In resting cells, hMincle and hMCL are highly expressed at the mRNA level and this expression is only marginally induced upon stimulation with TDB/TDM. However, only a low level of hMCL is detected on the cell surface. Further, siRNA knockdown in GM-CSF macrophages of SYK and CARD9 significantly reduced the release of IL-8. Finally, by using retroviral transduction of DCs from $mMincle^{-/-}$ with cDNA encoding hMincle or hMCL, Ostrop *et al*. have shown that hMincle on its own is sufficient to trigger a response to TDB/TDM in contrast to hMCL. Nevertheless, the response is higher when the cells co-expressed hMincle and hMCL suggesting a possible interaction of these receptors.

In addition to TDM, monoesters of trehalose (TMM) are found in the cell wall of mycobacteria. This compound is produced from trehalose-6-phosphate. TMM is the precursor of TDM and the mycolyl transferases of the Ag85 complex that catalyzes the synthesis of TDM from TMM [65]. TMM has been shown to have similar biological activity as TDM, including the induction of pro-inflammatory cytokines [50,66], tumor regression [67], and granulomatogenesis [68]. TMM has been reported to be less toxic than TDM [68,69]. Ishikawa *et al*. have demonstrated that TMM from *Mtb*, obtained by partial alkaline de-acylation, can bind to Mincle but with less affinity than TDM [55]. Later, it was reported that synthetic analogs of TMM with shorter chain length can also bind to Mincle [70,71]. We have analyzed the adaptive immune responses induced by synthetic TMM and TDM formulated in water in oil emulsion with ovalbumin. We have seen that the level of Th1 and Th17 responses induced with TMM was comparable to that induced by the corresponding TDM (manuscript in preparation). These data suggest that TMM may also induce Th1 and Th17 responses through Mincle binding and could be used for the development of new adjuvants.

4. Glucose monomycolate

GMM is another glycolipid naturally found *in vivo* after infection with *Mtb* [72]. Together with TDM and TMM, GMM contributes to the hydrophobic barrier, which protects mycobacteria from chemical attack by the host [72]. GMM is considered a hallmark for pathogenic mycobacteria, as only

intracellular mycobacteria are thought to have access to host glucose to synthetize GMM by a competitive substrate selection by antigen 85A [72]. Matsunaga *et al*.observed that macrophages stimulated with GMM produced lower levels of nitric oxide than macrophages stimulated with TDM. They deduced from these observations that the preferential production of GMM *in vivo* may be a mechanism developed by mycobacteria to subvert the defense mechanisms of the host. On the other hand, like MAs, GMM is recognized by the acquired immune system in the context of CD1-restricted responses [73]. GMM-specific T-cell responses have been reported in several species including human, rhesus macaque, cattle, and guinea pigs [74–77].

Given this capacity to induce T-cell responses, GMM has been investigated as a lipid antigen for novel subunit vaccines against mycobacterial infections. In cattle immunized with GMM or keyhole limpet hemocyanin (KLH) formulated in DDA, comparable levels of GMM and KLH-specific T-cell proliferative responses were observed after antigen stimulation of freshly isolated peripheral blood mononuclear cell (PBMC). However, only weak antibody responses against GMM were induced while a strong antibody response to KLH was detected [75]. Kawasaki *et al*. recently developed liposome nanoparticles decorated with high affinity glycan ligand of sialic acid-binding immunoglobulin-like lectins (Siglecs) to target human $CD1b^+$ DCs. They have found that monocyte-derived DCs pulsed with liposome nanoparticles composed of Siglecs- and GMM-activated CD1b-restricted cell lines more efficiently than DCs pulsed with GMM alone [78].

Besides this antigenic role of GMM, an inflammatory potential has been reported. Indeed, GMM from *Nocardia rubra* is able to activate macrophages and induce granulomas in a mouse model as efficiently as TDM [44]. Regarding the innate immune receptor involved in the recognition of GMM, Ishikawa *et al*. have shown that GMM, obtained by hydrolysis of TDM from *Mtb* using trehalase, cannot bind to the Mincle CLR [55]. This observation was contradicted by a recent study, in which van der Peet *et al*. have shown using a cell reporter system that GMM from Corynebacteria is able to bind Mincle and activate cells [71]. Our team analyzed the inflammatory potential of synthetic GMMs homologous to those found in *Mtb in vitro* and *in vivo*. We observed that bone marrow-derived dendritic cells (BMDCs) stimulated with GMM strongly induced the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6). This response was totally abrogated in Mincle, FcRγ, or MALT-1 deficient BMDCs. Our data confirm that GMM induces inflammatory responses by a mechanism dependent on the Mincle pathway. Further, we observed that GMM can induce Th1 and Th17 responses in mice (manuscript in preparation). These results indicate the feasibility of developing novel subunit vaccines against mycobacterial infections, in which mycobacterial lipids would be both pro-inflammatory and antigenic at the same time.

5. Glycerol monomycolate

GroMM, composed of a mycolate covalently bound to a glycerol head group, is another mycolate ester found in the cell wall of *Mtb* [79]. This mycolate ester is present in two stereoisomers s(R) and s(S) in equal proportions. It has been reported that DCs infected with *Mtb* can present GroMM in a CD1b dependent manner and activate CD1b restricted T cells [79]. The ability to activate CD1b-restricted T cells has been shown to be dependent on the stereochemistry of the hydrophilic head and the mycolate chain length. Indeed, the R stereoisomer and C80 GroMM have been shown to be more active than the S stereoisomer and C32 GroMM. Several studies have reported that GroMM can activate innate immune cells [71,80–82]. Andersen and coworkers reported in 2009 that GroMM and its C32 synthetic analog can induce the production of IL-6 and TNF-α as well as the upregulation of co-stimulatory molecules on human PBMC-derived immature DCs [81]. Further, immunization of mice with H1 fusion protein in GroMM-DDA liposomes induced the production of Th1/Th17 responses to levels comparable to those induced by TDB-DDA [80,81]. These responses could confer significant protection against *Mtb* infection. Later, it was reported that a shorter GroMM, based on octadecanoic acid (C18), could also stimulate strong Th1 responses. A formulation of peptide antigen TB10.3-P1 ($Rv3019c_{1\text{-}20}$) in this shorter GroMM combined with DDA and Poly(I:C) (CAF09) induced significant $CD8^+$ T responses after intraperitoneal but not subcutaneous immunization in mice [83].

Regarding the innate immune receptor involved in the recognition of GroMM, it was shown that GroMM can bind to hMincle but not mMincle [82]. In fact, reporter cells which expressed hMincle are activated by TDM and GroMM while cells expressing mMincle are only activated by TDM. Moreover, bone marrow-derived macrophages (BMDMs) obtained from human Mincle transgenic mice produce TNF-α after stimulation with TDM and GroMM whereas BMDMs from wild-type mice only respond to TDM. Finally, when liposomes containing GroMM are injected in the skin of mice, a local inflammation characterized by an infiltration of macrophages and eosinophils is observed in human Mincle transgenic mice but not in wild-type mice. These results have been confirmed by van der Peet *et al*. [71], who observed that S and R GroMCM (GroMM from *Corynebacterium*) can activate a reporter cell line expressing hMincle but not mMincle. In contrast to their ability to activate CD1-restricted T cells, the S stereoisomer is comparatively more active than the R stereoisomer in terms of interaction with Mincle [71]. Taken together, these findings indicate that the adjuvant potential of GroMM could be dependent on Mincle in humans, but the innate receptor involved in its adjuvant properties observed in mice still remains unidentified. Noteworthy, the contribution of MCL in this context has not yet been investigated; it is possible that MCL is involved in the recognition of GroMM *in vivo*.

6. Arabinose mycolate

Cell-wall skeleton (CWS) of BCG has been known for decades to have adjuvant potential [84–87]. It has been shown that CWS-BCG induces the production of pro-inflammatory cytokines by mechanisms dependent on TLR2 receptor [88]. CWS-BCG is composed of PGN, arabinogalactan, and MAs. Miyauchi *et al*. have investigated the contribution of arabinose mycolate in the inflammatory potential of CWS-BCG [88]. By acid

hydrolysis, they have isolated a fraction consisting of mono-arabinose monomycolate, tetra-arabinose tetra-mycolate, penta-arabinose tetra-mycolate, and hexa-arabinose tetra-mycolate. These arabinose mycolates stimulate the production of TNF-α in RAW264.7 cells and peritoneal macrophages. However, they are less inflammatory than TDM in terms of TNF-α production and this production is totally abrogated in TLR2 and MyD88 deficient macrophages. Interestingly, it has been reported that the TLR2 activation of DC by CWS-BCG is dependent on phagocytosis [89]. The authors have shown that CWS-BCG after phagocytosis localizes in the lysosomes where a similar acid hydrolysis as in their experimental set-up can occur. Altogether, they suggest that after phagocytosis, CWS-BCG is degraded in lysosomes and that arabinose mycolates are generated. These arabinose mycolates will then activate cells through interactions with TLR2. Our team has recently analyzed the inflammatory potential of single synthetic arabinose monomycolates using Mincle$^{-/-}$ and MyD88$^{-/-}$ BMDCs. In contrast to the results of Miyauchi *et al*., we have found that arabinose monomycolates activate BMDCs by mechanisms dependent on Mincle and independent of MyD88 (manuscript in preparation). These differences may be explained by the fact that we have used pure synthetic arabinose monomycolates instead of a mix of natural arabinose mycolates with different structures.

7. Lipoarabinomannans

Other important immunomodulatory glycolipids found in the cell wall of mycobacteria are LAMs [90]. LAM is composed of three parts: an acylated glycosylphosphatidylinositol, a carbohydrate backbone, and a capping motif, the latter varying in the different mycobacterial species [91,92]. Indeed, mannose-capped LAM (ManLAM) is present only in slow-growing mycobacteria (such as *Mtb*), while phosphoinositol-capped (PILAMs) and LAM devoid of capping have been found in fast-growing mycobacteria (*Mycobacterium smegmatis, Mycobacterium chelonae*) [91]. The presence of mannose capping on LAM was reported to be associated with virulence [92]. Indeed, ManLAM has been shown to block phagosome–lysosome fusion, to inhibit the maturation of DCs, and to induce the secretion of anti-inflammatory and pro-inflammatory cytokines. However, it should be noted that most of these studies were *in vitro* studies, in which high concentrations of purified ManLAM were used. The role of the mannose capping of LAM was also investigated *in vivo* and *in vitro* using live bacteria [93,94]. It was shown that in fact mutant strains of BCG and *Mtb* that lack the mannose cap did not differ from wild-type strains in terms of survival and replication in macrophages, maturation of DC, bacterial loads, and immune responses induced in mice [93]. These data suggest that the mannose cap of LAM plays no major role in the interaction between mycobacteria and the host in mice.

Several innate receptors have been reported to interact with ManLAM including DC-SIGN, Macrophage Mannose receptor, surfactant protein (SP)-A, SP-D [92], and collectin 11 (CL-K1) [95]. None of these receptors could be linked to the ability of ManLAM to induce both anti-inflammatory (IL-10) and pro-inflammatory cytokines (TNF-α and IL-6). This has recently been elucidated by Yonekawa and coworkers [96], who reported that plate-coated ManLAM is able to activate BMDCs and induce a strong production of pro-inflammatory cytokines (MIF-2 and TNF) by a mechanism independent of Mincle, MCL, or MyD88 but dependent on FcRγ. Furthermore, using reporter cells, they have shown that ManLAM can bind directly to both murine Dectin-2 and human Dectin-2 and activate cells through this interaction. Stimulation of BMDCs by ManLAM triggered the production of pro- and anti-inflammatory cytokines (IL-10, IL-2, MIP-2, TNF-α, and IL-6) and the maturation of BMDCs (up-regulation of CD80 and CD40). These responses were abrogated in Dectin-2$^{-/-}$ mice. Finally, Yonekawa *et al*. reported that ManLAM is less inflammatory than LPS or TDM but is able to induce Th17 responses. Altogether, these data identified Dectin-2 as the receptor that binds directly ManLAM and triggers the secretion of pro- and anti-inflammatory cytokines. Interestingly, as compared to TDM, ManLAM does not induce skin inflammation in an experimental autoimmune encephalomyelitis (EAE) model. This might be due to its IL-10 stimulation. Taking in account this capacity to induce moderate inflammation, ManLAM could be used as a novel adjuvant for vaccines. Especially when considering this capacity to induce IL-2 in a Dectin-2 dependent manner, as it was reported that IL-2 produced by DCs plays a role in the induction of T-cell responses [97]. Finally, ManLAM in combination with IFA is able to induce antibody and CMI responses against ovalbumin protein in a cattle model [98].

8. Inflammasome and Th17 responses

In this review, we have listed and discussed several components of the mycobacterial cell wall that bind to CLRs and their potential adjuvant application. Almost all the compounds described have the ability to induce Th17 responses in common. Several studies have demonstrated the importance of Th17 response in the protection against bacterial and fungal infections. The fact that binding of agonists to CLRs induces Th17 responses, is tightly linked to inflammasome activation and IL-1β secretion. Indeed, IL-1β is an important cytokine in the induction of Th17 response [53]. Unlike other cytokines, IL-1β and IL-18 are not secreted by the classical Golgi pathway. These cytokines are produced in immature form (pro-IL-1β and pro-IL-18) and need processing by caspase-1, a process that is dependent on inflammasome activation. The inflammasome is a multi-protein platform, composed of a sensor protein (such as NLRP3 orNLRP1 or NLRC4 or AIM2), an adapter protein (ASC) and pro-caspase-1 [99]. Activation of the inflammasome leads to autoactivation and maturation of caspase-1, followed by caspase-1 cleavage of pro-IL-1β and pro-IL-18 and resulting in the secretion of the mature cytokines [99]. Activation of the NLRP3 inflammasome by particulate adjuvants such as Alum and ISCOMATRIX is a mechanism by which these adjuvants induce immune responses [100,101]. TDB as well as TDM have been reported also to activate NLRP3 inflammasome [102]. However, CARD9 appears only to induce the activation of NF-κB and the production of pro-IL-1β. It was recently shown that Mincle is indispensable for the activation of inflammasome by TDB [53]. Two parallel studies highlighted

Table 3. Adjuvants present in subunit TB vaccine currently under clinical investigation.

Adjuvants	Clinical phase	Types of adjuvant	Innate immune receptor	Type of immune response induced	Reference
IC31	Phase II a	CpG, cationic polypeptide	TLR9	Th1	[110]
AS01E	Phase II b	MPL, QS21, liposome	TLR4	Th1, CD8T cells, NK cells activation	[111]
GLA-SE	Phase II a	GLA, emulsion	TLR4	Th1	NCT02465216
CAF01	Phase I	TDB, cationic liposomes	Mincle	Th1, CD8T cells	[18]

AS: Adjuvant systems; TLR: Toll-like receptor; CAF: cationic adjuvant formulation; GLA: glucopyranosyl lipid; SE: stable emulsion; MPL: monophosphoryl lipid; NK: natural killer cells; Th: T-helper lymphocyte; Mincle: macrophage inducible c-type lectin.

the importance of inflammasome activation in the adjuvant potential of TDB and TDM [43,53]. Indeed, starting from the observation that the inflammatory response induced by TDB is independent of MyD88 *in vitro* but is reduced in MyD88 knockout mice, Desel and coworkers have investigated the mechanisms involved in this *in vivo* observation [53]. They have shown that Mincle$^{-/-}$ and MyD88$^{-/-}$ mice have an impairment of Th1 and Th17 responses. MyD88 is known to be involved in the signaling of IL-1 receptor. Using an inhibitor of IL-1-mediated signaling and different knockout mice (IL-1R, IL-18, IL-33, and ASC), they have demonstrated the importance of the inflammasome activation in Th17 response induced by TDB *in vivo*. Later, Shenderov *et al*. have shown that the Th17 response induced by CFA is dependent on inflammasome activation. They have identified the cord factor TDM and PGN as compounds responsible for the induction of Th17 response by CFA [43].

It remains to be demonstrated whether other compounds such as GMM and ManLAM that bind to CLR (Mincle and Dectin-2) can activate the inflammasome. However, the reports on TDB and fungal infections suggest that this may well be the case [103–105].

9. Expert commentary and 5-year view

Only few adjuvants are currently licensed for human use and many adjuvants are under experimental development (cfr. Tables 1 and 2). The licensed ones mainly favor induction of antibody-mediated protective immunity in subunit vaccines such as against pertussis or hepatitis A and B. Here, we have reviewed different compounds constituting the cell wall of mycobacteria that can favor induction of cell-mediated immunity, more in particular the induction of Th17 polarized cell-mediated responses through their binding to CLRs (Mincle and Dectin-2). Th1 and Th17 CD4$^+$ T-cell responses are involved in protection against infection with intracellular pathogens such as *Mtb*. The design of agonists of CLRs and their combination with TLR agonists is an interesting strategy in the development of new adjuvants. Indeed, the combination of several PAMPs that activate different signaling pathways could induce combinations of different types of adaptive immune responses, hence inducing enhanced efficacy of a given vaccine. Significant examples are CAF09 (combination of GroMM and Poly(I:C)) or CAF05 (combination of TDB and Poly(I:C)). These two adjuvants induce strong Th1 and Th17 as well as CD8$^+$ T-cell responses. However, it should be taken into account that combining several PAMPs could also lead to antagonistic effects. Indeed, negative cross regulation between PRRs has been described. For example, a TLR2 agonist has been shown to inhibit the induction of type I interferon and cross presentation induced by TLR9 agonist [106]. It has recently been reported that Mincle activation suppresses pro-inflammatory response mediated via TLR4 [107]. Therefore, it is very important to understand regulatory mechanisms when several PRR agonists are combined in an adjuvant formulation.

For the development of more efficient TB vaccines, induction of unconventional T-cell populations such as CD1 restricted T cells represent an interesting approach. Here, we have reviewed several compounds of the cell wall of *Mtb* that have an adjuvant potential and that are also nonprotein antigens presented in the context of CD1b restricted responses. It is tempting to speculate that in the context of the development of a TB subunit vaccine, the use of these compounds both as nonprotein antigens and as adjuvant could induce better protection against *Mtb*. In support of this, two recent studies have demonstrated the direct role of CD1b restricted T cells in the protection against TB [108,109]. Indeed, Busch *et al*. report of a correlation between the frequency of so-called polycytotoxic LAM-responsive T cells and the ability to control *Mtb* infection [108]. Moreover, Zhao *et al*. recently reported that MA-specific T cells protect against *Mtb* infection in a humanized transgenic mouse model. Subunit vaccines against TB currently under clinical evaluation are not expected to induce this type of unconventional T-cell responses (Table 3). Nevertheless, if mycobacterial cell wall components will be repeatedly administered in adjuvant + antigen formulations as vaccines against different infections, it is possible that interference to induction of antigen-specific responses will be observed and this should be carefully assessed. In the next 5 years, we expect that some of the adjuvants currently in clinical development will be licensed for human use. Based on the recent advances in our understanding of CLR pathways, we also expect that there will be more CLR agonists in clinical trials for the development of novel adjuvants for subunit vaccines. Finally, correlates of protection are not fully understood for many diseases, but further fundamental research and results of ongoing clinical trials will allow to progress in the field of adjuvant design.

Key issues

- The few adjuvants currently licensed for human use mainly favor induction of antibody-mediated immune responses.
- Recent advances in our understanding of the role of innate receptors – the so-called Pathogen Recognition Receptors (PRRs) – in the induction of adaptive immune responses are

contributing to the rational development of novel adjuvants.

- The cell wall of mycobacteria is rich in compounds that can bind PRRs.
- C-type lectin receptors Mincle and Dectin-2 bind mycolate esters and ManLAM.
- Binding to C-type lectin receptors drives induction of Th1 and Th17 responses through inflammasome activation.
- Glucose mono-mycolate is a non-protein lipid antigen recognized by CD1-restricted T cells generated during mycobacterial infection, which is also recognized by the innate receptor Mincle.
- Synthetic mycolate esters warrant further investigation as novel adjuvants for sub-unit vaccines inducing cell-mediated immune responses.

Declaration of interest

This work was partially funded by TBVAC2020 under grant agreement no. H2020-PHC-643381 and by the Interuniversity Attraction Pole Programme IAP 7/32. HG Tima holds a FRIA PhD fellowship. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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2. AIMS OF THE STUDY

AIMS OF THE STUDY:

Tuberculosis remains among the most deadly health threats to humankind. This disease, caused by bacteria of the *Mycobacterium tuberculosis* complex, kills each year more than 1.5 million people. It is estimated that a further 2 billion individuals are latently infected with *M. tuberculosis.* Most of them will never develop any clinical symptoms, although 5 to 10% of these latently infected individuals are at risk to develop TB in their lifetime. During the last decades the situation worsened because of deteriorating socio-economic conditions, the increased incidence of drug-resistant *M.tuberculosis* strains and the co-infection with HIV (a major risk factor for development of TB). Currently only one vaccine is available against TB, the Bacille of Calmette and Guérin (BCG). However, its efficacy is extremely variable against the contagious form of TB - pulmonary TB - in adults and adolescents (ranging from 0 to 80%). Therefore, to control and eliminate TB, a better vaccine, efficient drug treatments and more rapid and cheaper diagnostic techniques are needed. For that purpose, a better understanding of the mechanisms related to the pathogenicity of *M. tuberculosis* is needed. In this PhD thesis we have attempted to provide additional information to reach this goal.

The first part of this study was devoted to the analysis of the structure-activity relationship of mycolate esters, major components characteristic of the cell wall of mycobacteria. A great variety of mycolate esters are present in the cell wall of pathogenic and non-pathogenic mycobacteria. These compounds vary in terms of sugar moieties (trehalose, glucose or arabinose) and classes of mycolic acids (MA) bound to this sugar (alpha-, methoxy-, keto-, wax-ester-…). Several studies using mutated *M. tuberculosis* strains have shown that modification of the composition of the cell wall of *M. tuberculosis* in terms of classes of MA present can have an impact on virulence and inflammatory responses of the mutated strains. In addition, the modulation of the inflammatory responses by these mutated strains has been shown to depend on the classes of mycolate esters, more precisely on the classes of trehalose dimycolate present in the cell wall of these mutated strains. However, as these previous studies were performed using glycolipids purified from the cell wall of different strains and have mainly focused on contribution of trehalose dimycolates, they could not clearly elucidate the contribution of each specific class of glycolipids on the modulation of the immune systems.

In this study, we were interested in analysing more precisely the impact of the structure of mycolate esters on their inflammatory potential by using pure single synthetic compounds. We further analysed host pathways involved in the immune recognition of mycobacterial glucose and arabinose-based mycolates.

In the second part of this study, taking advantage of the inflammatory properties of synthetic mycolate esters, we have focused on the development of immuno-stimulatory adjuvants that can trigger cell-mediated immune responses. Indeed, the number of adjuvants licensed for human use that can induce cell-mediated immune responses is limited. But this type of immune responses is needed to achieve control of intracellular pathogens such as *M. tuberculosis* and for vaccines to treat cancer. Therefore, we analysed here the adjuvant potential of different classes of mycolate esters in two different formulations (emulsions and liposomes).

3. RESULTS

3.1 Synthesis of wax esters and related trehalose esters from *Mycobacterium avium* and other mycobacteria

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❖ Context

Mycolic acids (MAs) are long chain fatty acid α-alkyl and β-hydroxyl found in the cell wall of mycobacteria. It has been reported that the detailed composition of MAs can provide a fingerprint of the specific mycobacterium or even of different strains [129]. In this regard, the three majors mycolic acids found in the cell wall of *M. tuberculosis* are alpha-MA, methoxy-MA and keto-MA. In contrast, *Mycobacterium avium* lacks methoxy-MA but wax-esters-MA are abundantly present in its cell wall. Fujita and co-workers demonstrated that there are major differences in toxicity and granulomatogenic activity in mice of trehalose dimycolates (TDMs), dependent of the balance between classes of MA and wax-esters [169]. In this chapter, we aimed to analyze the inflammatory potential *in vitro* and *in vivo* of synthetic trehalose wax esters and compare this inflammation to those induced by synthetic TDMs with alpha-MA, methoxy-MA and keto-MA. This work was performed in collaboration with the group of Prof Mark Baird that synthetized wax-ester-MAs and related trehalose dimycolates (TDMs) and trehalose monomycolates (TMMs).

❖ Results

Our results showed that synthetic wax-ester-glycolipids from *M. avium* activate BMDCs by inducing the production of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in vitro. In addition, the level of pro-inflammatory cytokines triggered by wax-ester-TDM was higher to that of the corresponding wax-ester-TMM and TDB. These results indicate that the number of mycolate chains bound to trehalose influences the activation of BMDCs. Further, we compared the relationship between classes of MAs bound to trehalose and the activation of BMDCs. We observed that wax-ester-TDM induced a level of TNF-α production comparable to

the one achieved by methoxy-TDM and keto-TDM. These three compounds induced more TNF-α than alpha-TDM. In contrast to the *in vitro* results, when formulated in water in oil in water emulsion and injected in footpad of C57BL/6 mice, all the synthetic glycolipids induced a similar level of inflammation as determined by measuring footpad swelling.

❖ Conclusion

In this chapter, we demonstrated that pure synthetic glycolipids are immuno-stimulatory *in vitro* and *in vivo*. The data obtained here with pure synthetic glycolipids may allow a number of issues to be resolved. For example, analyze the toxicity or the granulomatogenic property of individual glycolipids or analyze the inflammatory and adjuvant potential of several classes of glycolipids that can vary in terms of the nature of the sugar but also the class of MAs bound to this sugar.

Tetrahedron 72 (2016) 3863–3876

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Synthesis of wax esters and related trehalose esters from *Mycobacterium avium* and other mycobacteria

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ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 17 April 2016
Accepted 3 May 2016
Available online 6 May 2016

Keywords:
Wax ester
Mycobacterium avium
TDM
TMM
Trehalose dimycolate
Trehalose monomycolate
Inflammation

ABSTRACT

The synthesis of mycobacterial mycolic acid related wax esters and of their trehalose di- and mono-esters is described. The trehalose dimycolates (TDMs) synthesised activated bone marrow derived dendritic cells (BMDCs) in vitro more strongly than trehalose dibehenate or the trehalose monomycolates (TMMs). The inflammatory effects were similar to those of TDM from either a synthetic keto- or methoxy-mycolic acid, but somewhat stronger than those of a TDM from an α-mycolic acid. In vivo, the effects of one wax ester TDM were similar to those of the methoxy-MA and α-mycolic acid TDMs and trehalose dibehenate.

1. Introduction

Mycobacterial cell walls show unusually low permeability, a factor which apparently contributes to their resistance to therapeutic agents. This is linked to an exceptionally thick monolayer formed by the packing of esters of C_{60}–C_{90} fatty acids.[1–7] These 'mycolic acids' (MA), exemplified by structures **1**–**3**, contain various structural features including *cis*-cyclopropanes, α-methyl-*trans*-cyclopropanes, α-methyl-β-methoxy and α-methyl-β-keto groups,[8–14] *cis*-alkene, α-methyl-*trans*-alkene and α-methyl-*trans*-epoxy fragments,[15,16] and they are generally present as mixtures of homologues differing by two methylene-units. Each contains a common *R*,*R*-β-hydroxy acid group,[14,17–20] though less is known about the absolute stereochemistries of the other groups. There is evidence that the 1-methyl-2-methoxy unit at the distal position from the hydroxy acid in mycolic acids **2** is *S*,*S*,[13,19–21] while other reports identify a *R*-stereochemistry for the three stereocentres of the α-methyl-*trans*-epoxy unit.[15,16] [but see also Al Kremawi et al.][22] In the case of *Mycobacterium tuberculosis*, the MA are of three main types, α-MA (**1**), methoxy-MA (**2**) and keto-MA (**3**) (Scheme 1), though other mycobacteria contain different functional groups in the mero-chain. The detailed composition of the mixture can provide a fingerprint of the specific mycobacterium, or even of different strains or stages of development of a single bacterium. The fingerprint can be used directly to demonstrate infection.[23]

In the case of *Mycobacterium avium* there are no methoxy-MA, but these are replaced by so called 'wax-esters'. Over 60 years ago, Anderson, in an epic series of papers, initiated studies on mycobacterial lipids and reported the isolation of two new optically active long-chain alcohols from the neutral fraction of the saponified waxes of the so called 'timothy bacillus',[2] later classified as *Mycobacterium phlei*.[1,2,4,6] These alcohols were identified as *d*-2-eicosanol ($[\alpha]_D$ +3.5) and *d*-2-octadecanol ($[\alpha]_D$ +5.7). It was noted that the acidic fraction from these saponified waxes contained a high molecular weight component, tentatively identified

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http://dx.doi.org/10.1016/j.tet.2016.05.004
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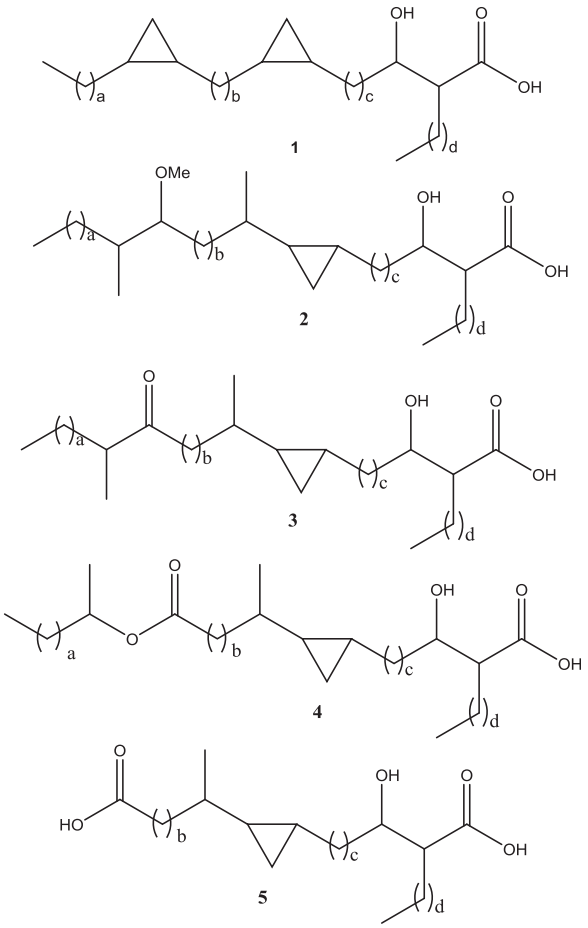


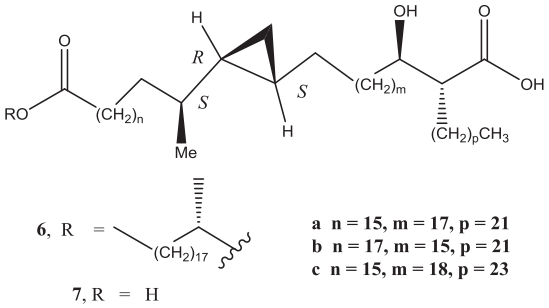
Scheme 1. Typical structures of mycolic acids **1**–**3**, and related wax esters **4** and wax dicarboxylic acid **5**.

as being dibasic.[24] A careful analysis of the firmly bound lipids from avian tubercle bacilli (*M. avium*) again yielded long-chain alcohols, with *d*-2-eicosanol as main component.[24] These lipid fractions also produced a long-chain di-acid, recognized for the first time as a mycolic acid derivative and given the title γ-mycolic acid ($[\alpha]_D$ +5.3).[24] The pioneering work of Etémadi et al.,[25–28] and of Minnikin and Polgar,[8,9] who isolated methyl avium mycolate ($[\alpha]_D$ +3.05) from *M. avium* strains Dn, 485 and 7169, and provided mass spectrometric evidence for structures and molecular weights, led to a clearer understanding of the presence of esters of these acids, apparently derived by a biological Baeyer-Villiger reaction on keto-MA.[4,13,29,31,32]

Wax esters from *Mycobacterium avium ssp. paratuberculosis* were partially characterised as **4** and the corresponding diacids **5**,[30] and found as a constituent of trehalose mycolates of *M. phlei*.[33,34] The relationship between the keto-MA and the wax ester MA in *M. phlei* and *Mycobacterium aurum* was examined by radiolabelling, which showed that the keto-MA appeared first in extractable lipids, and the wax ester MA first in wall-linked derivatives.[35] The mass relationship was probed directly by MALDI-MS.[36] In parallel studies, similar alcohols and acids were characterized from an organism claimed to be the causative agent of leprosy.[37] It is clear, however, that this bacterium was not the leprosy bacillus, as *Mycobacterium leprae* has not been cultivated to date and the mycolic acid composition of *M. leprae* is distinct.[38] The use of two dimensional TLC provided additional evidence of the presence of wax esters in other mycobacteria.[39] In this way, the distribution of various classes of MA could be characterised. Some mycobacteria, e.g., *Mycobacterium vaccae*, contain α, α′, keto and wax ester MA; others, such as *M. avium ssp. avium* and *M. avium ssp. paratuberculosis*, contain α, keto and wax ester MA. Others, such as *Mycobacterium komossense* and *Mycobacterium heckeshornense*, produce α, keto, methoxy and wax ester MA.[38,40] HPLC of MA from some strains of *Mycobacterium gordonae* showed double cluster patterns due to the presence of dicarboxy mycolates after saponification.[41] Wax esters MA are known to be a characteristic component in the *M. avium–Mycobacterium intracellulare* group and other rapidly growing bacteria.[42,43] Their presence in *Mycobacterium smegmatis* has been implied,[44] although not confirmed by a later study.[39] Usually they have been analysed as the corresponding α-methyl-alkanol and free acid after hydrolysis.[32,39,45] Some such wax esters, such as those from *M. aurum*, do not appear to contain cyclopropanes,[46] while in other cases the composition is not clear.[5,25–27,40,47–50]

The detection of intact trehalose dimycolate (TDM) containing a wax ester mycolate has been achieved by mass spectrometry. Three types of TDM were observed, one containing two α-mycolate residues, one two wax esters, and one having one residue of each type.[34] The analysis of the intact trehalose monomycolate (TMM) derivatives by MALDI-TOF mass spectrometry shows predominant ions due to C_{85} and C_{87} wax esters from *M. avium–M. intracellulare*, and C_{80}, C_{81}, C_{82} and C_{83} for *M. phlei* and *Mycobacterium flavescens*.[51] Direct observation of the corresponding wax ester trehalose dimycolate (TDM) species is also reported.[42]

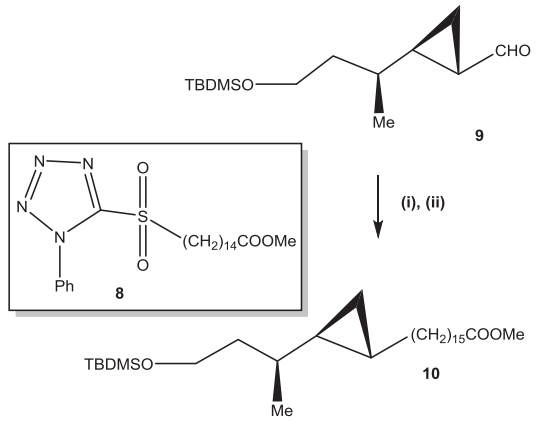
Yano et al. have reported that TDM isolated from *M. tuberculosis* is antigenic to antibodies present in the serum of patients infected with tuberculosis, and that the sub-class of TDM based on methoxy-MA is recognised more strongly than those based on α-MA or keto-MA.[52–55] Antibodies in the serum of patients infected with TB responded less strongly to TDM isolated from *M. avium* complex (MAC), while antibodies in serum of MAC infected patients showed the opposite response pattern. Disease caused by MAC is an important opportunistic pulmonary infection; the clinical symptoms of MAC pulmonary disease and TB resemble each other, but the treatment is different, so rapid distinction between the infections is desirable. The use of a serodiagnostic assay based on the selective antigenicity of TDM and TMM offers a clear opportunity to solve this problem, and considerable work has been carried out in this area, using extracts from different types of mycobacterial cell.[56–60] Although TDM from *M. tuberculosis* or *Mycobacterium bovis* induces significant resistance against influenza or *M. tuberculosis* infections when combined with muramyl dipeptide, TDM from *M. avium*, did not show the same effect. Moreover, while TDM from Mtb confers resistance to *Toxoplasma gondii* infections, that from *M. avium* does not.[61] There are major differences in toxicity and granulomatogenic activity in mice of TDMs from different mycobacteria, depending on the balance of different classes of MA and wax ester.[62] In addition to the problems in diagnosing MAC infection in humans, *M. avium ssp. paratuberculosis* is the etiologic agent of Johne's disease, one of the most widespread bacterial diseases of domestic animals.[63] Given that one major difference between the constituents of *M. tuberculosis* and *M. avium* is that the former contains methoxy-MA, but not normally wax ester MA, while the latter contains wax-ester MA but not methoxy-MA, the synthesis of both wax esters and of the corresponding sugar esters may allow a number of issues to be resolved. We have already reported the synthesis of the dicarboxylic acid fragment of a wax ester containing an α-methyl-*trans*-cyclopropane;[64] we now report the synthesis of three complete wax esters and of the derived trehalose esters. Compound **6a** (Scheme 2) is reported to be the major wax ester of *M. avium*;[43] compound **6b**, with reversed chain lengths is reported to be the major isomer in *M. gordonae*.[41] In addition, the longer chain wax ester **6c** was prepared in order to study the effects of chain length on bioactivity (Scheme 2).



Scheme 2. Typical chain lengths of some mycobacterial wax esters and di-acids.

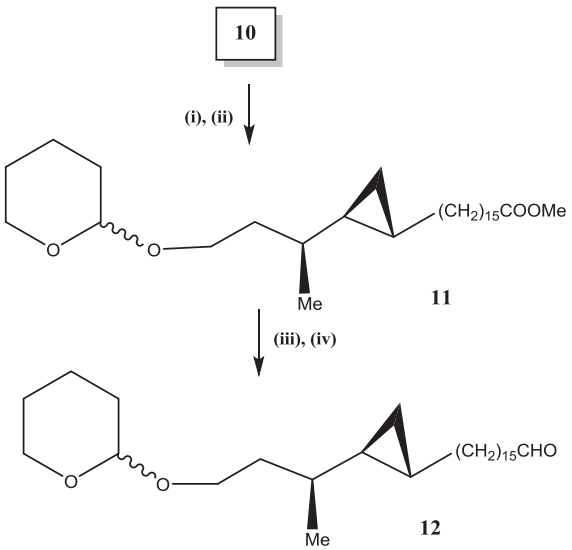
2. Results and discussion

The *trans*-cyclopropane fragment (**9**) (Scheme 3), prepared as described earlier was coupled to the sulfone (**8**) in a modified Julia-Kocienski reaction, followed by saturation of the derived mixture of *E*/*Z*-alkenes using di-imide. This led to the chain extended ester (**10**).



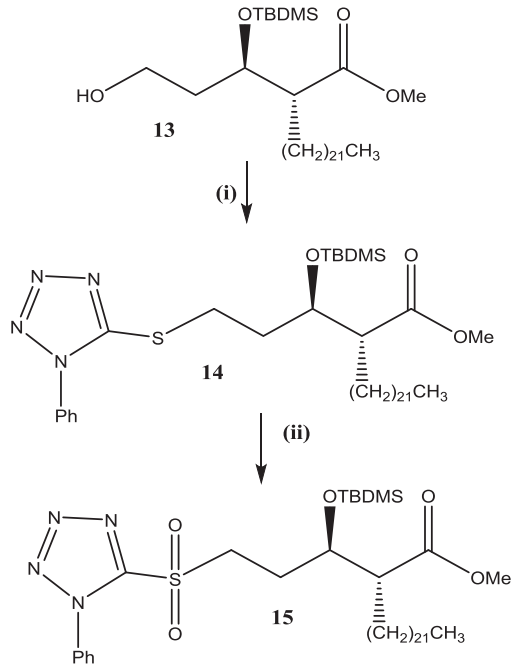
Scheme 3. (i) LHMDS, **8**, dry THF (85%); (ii) dipotassium azodicarboxylate, THF-methanol, CH_3COOH (96%).

Replacement of the silyl protecting group of (**10**) by a tetrahydropyranyl group, was followed by conversion of the ester into aldehyde (**12**) (Scheme 4).



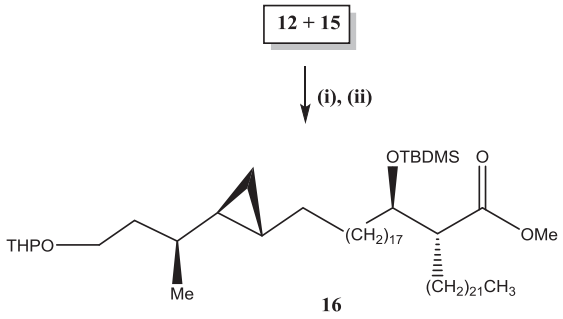
Scheme 4. (i) nBu_4NF, dry THF (96%); (ii) pyridinium-*p*-toluenesulfonate (PTSA), 3,4-dihydro-2*H*-pyran, dry CH_2Cl_2 (98%); (iii) $LiAlH_4$, dry THF (94%); (iv) PCC, CH_2Cl_2 (93%).

In order to generate the dicarboxylic acid part of the wax ester, the aldehyde (**12**) needed to be coupled to an *R*,*R*-β-hydroxyacid fragment (**15**), with a pendant C_{22} chain. This was prepared from **13**,[66] using the standard methods (Scheme 5).



Scheme 5. (i) 1-phenyl-1*H*-tetrazole-5-thiol, Ph_3P, DEAD, dry THF, (85%) (ii) MCPBA, CH_2Cl_2 (86%).

Reaction of the aldehyde (**12**) with sulfone (**15**) and base, followed by saturation of the derived alkenes provided the fragment (**16**) (Scheme 6).

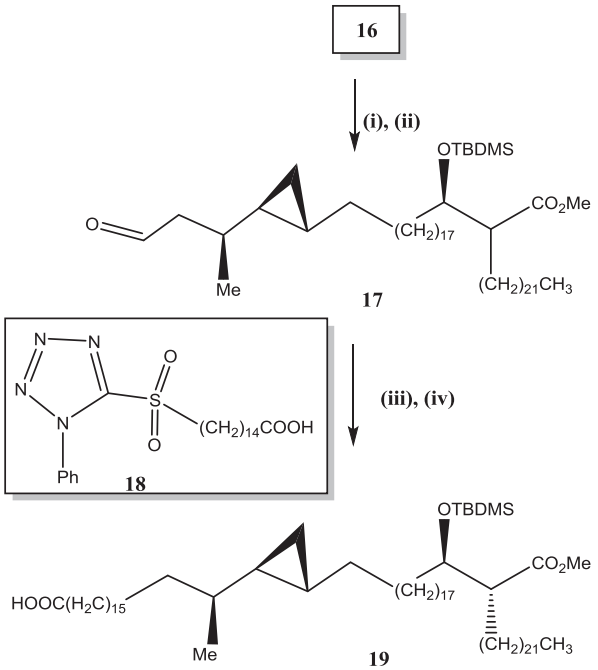


Scheme 6. (i) LHMDS, dry THF (78%); (ii) dipotassium azodicarboxylate, THF, methanol, CH_3COOH (91%).

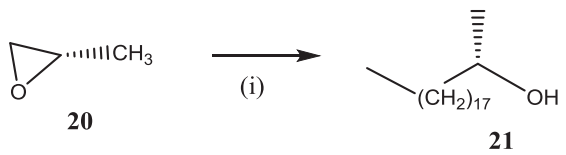
The tetrahydropyranyl protecting group was removed from ester (**16**) and the resulting alcohol was oxidised to produce aldehyde (**17**). Coupling of this with the sulfone (**18**) and base followed by saturation of the derived alkenes provided the ω-dicarboxylic acid mono-ester (**19**) (Scheme 7).

In order to generate the full wax ester, the ω-dicarboxylic acid mono-ester (**19**) needed to be coupled to (*S*)-2-eicosanol (**21**). This was synthesized by reacting the Grignard reagent from 1-bromoheptadecane with (*S*)-1-epoxypropane (**20**) to give the desired alcohol (**21**) (Scheme 8).

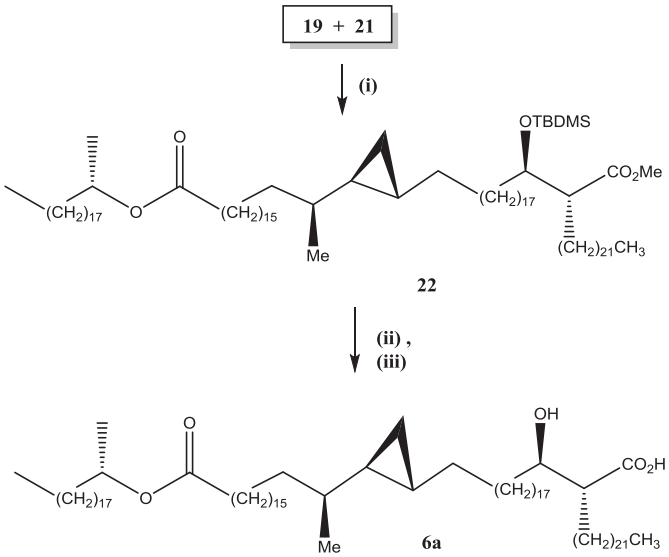
The ω-dicarboxylic acid mono-ester (**19**) was esterified with *S*-2-eicosanol (**21**) through a Steglisch reaction (Scheme 9). Removal of the silyl ether and methyl ester protection was achieved in two steps to produce the free wax ester mycolic acid (**6a**).



Scheme 7. (i) PTSA, MeOH (84%); (ii) PCC, dichloromethane (93%); (iii) LHMDS, **18**, dry THF (80%); (iv) dipotassium azodicarboxylate, THF, methanol, CH_3COOH (80%).



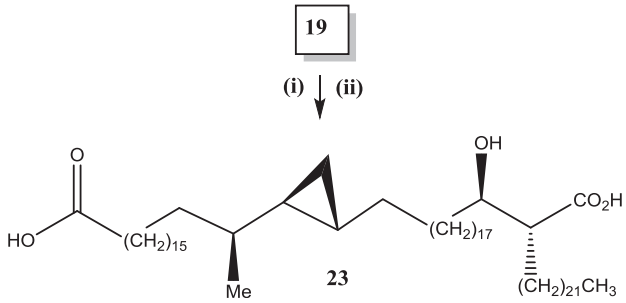
Scheme 8. (i) $CH_3(CH_2)_{16}MgBr$, CuI, dry THF (53%).



Scheme 9. (i) DCC, DMAP, dry CH_2Cl_2 (80%); (ii) HF-pyridine complex, dry THF (71%); (iii) 5% aq nBu_4NOH (60%).

The product **6a**, α_D^{21}+7.6 (*c* 0.40, $CHCl_3$), showed the characteristic apparent sextet for the hydrogen adjacent to the ester at δ 4.91 (1H, *J* 6.2 Hz), as well as the signals for the α- and β-hydrogens of the hydroxy acid at 3.72 (1H, dt, *J* 4.8, 9.2 Hz), 2.46 (1H, dt, *J* 5.3, 9.0 Hz), and a triplet for the methylene group adjacent to the ester carbonyl at 2.27 (2H, t, *J* 7.5 Hz).

The ω-dicarboxylic acid (**23**) has also been isolated from the cell wall as a component of a complex mixture.[43] Compound (**23**), α_D^{21}+5.8 (*c* 0.86, $CHCl_3$), was also synthesized starting from the ω-dicarboxylic acid mono-ester (**19**) after TBDMS deprotection and hydrolysis (Scheme 10). The acid showed the typical signals for the α-methyl-*trans*-cyclopropane in the ^{1}H NMR spectrum, together with the typical signals for the α- and β-hydrogens of the hydroxy acid.



Scheme 10. (i) HF-Pyridine, dry THF, 17 h (85%); (ii) LiOH, THF, water-MeOH, 17 h (85%).

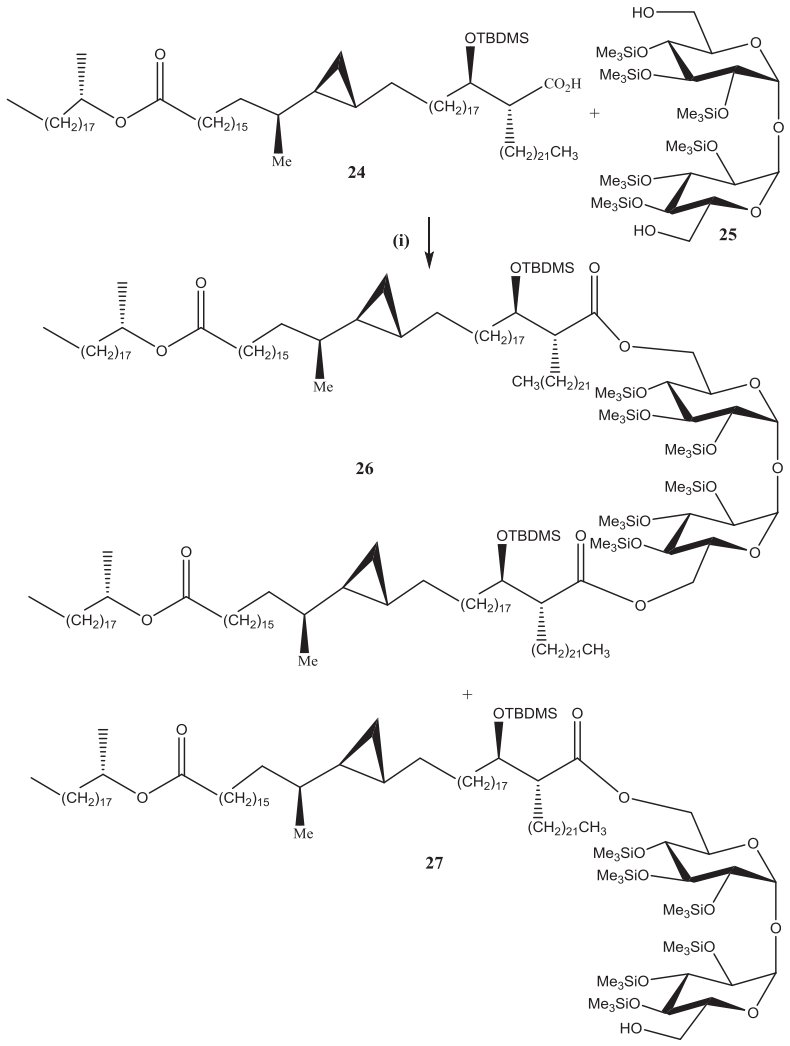
The acid **6a** was then converted into the corresponding TDM and TMM using methods described earlier.[67,68] The protected wax ester mycolate **24** was prepared from the corresponding free hydroxy wax ester **6a** by reaction with an excess of *tert*-butyldimethylsilylchloride and imidazole in the presence of 4-dimethylaminopyridine for 24 h at 70 °C, followed by hydrolysis of the TBDMS ester on the acid group by stirring in THF for 15 min with 4% aqueous tetrabutylammonium hydroxide. Compound **24** was coupled to protected trehalose (**25**) using 1-(3-dimethylaminopropyl-3-ethylcarbodiimide hydrochloride, DMAP and molecular sieves in CH_2Cl_2 (Scheme 11). This gave the protected TDM **26** (52%) and the protected TMM **27** (32%).

The TDM was de-protected in two steps to give **28** and **29**, and the TMM was deprotected in the same way to give **30** and **31** (Schemes 12 and 13).

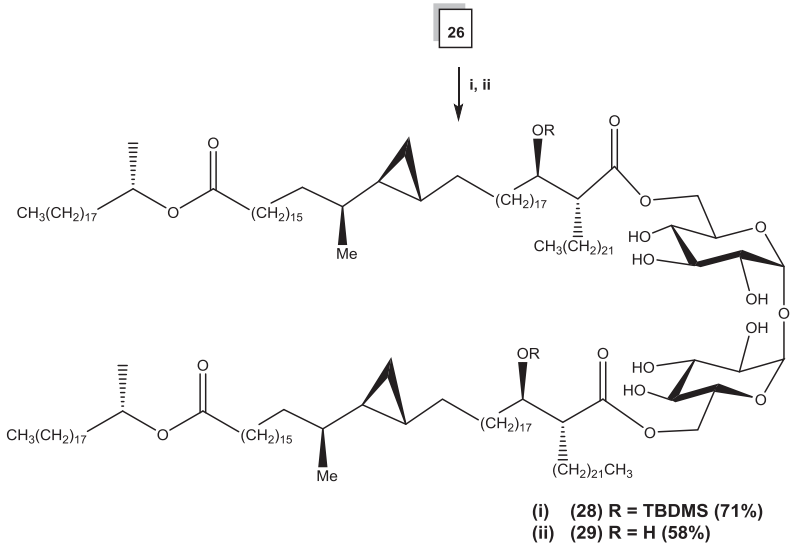
Using a similar method, the second wax ester, **6b** was prepared and converted into the corresponding TDM (**32**) and TMM (**33**) (Scheme 14), while wax ester **6c** was converted into the corresponding TDM (described in full in the Supplementary data).

3. Wax ester TDM and TMM are inflammatory in vitro and in vivo

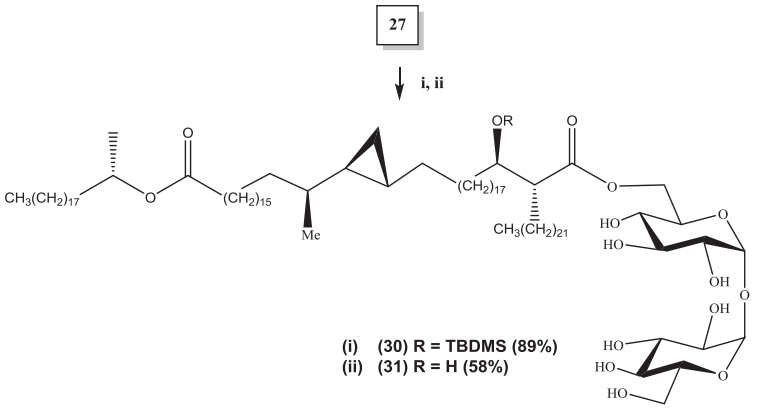
The ability of synthetic wax ester TDM and TMM to activate murine bone marrow derived dendritic cells (BMDCs) in vitro was examined. For that purpose, BMDCs were stimulated with 1 μM of the different wax esters and controls (evaporated isopropanol as a negative control and trehalose dibehenate as a positive control) for 24 h and the production of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) was analysed by ELISA in the supernatant. All the tested compounds induced the production of pro-inflammatory cytokines. The levels of TNF-α, IL-1β and IL-6 induced by wax ester TMMs **31** and **33** were significantly lower compared to the levels induced by the corresponding wax ester TDMs **29** and **32** and TDB (Fig. 1A), indicating that the number of mycolate chains bound to trehalose influences the activation of BMDCs in terms of pro-inflammatory cytokine secretion. When a dose-response analysis was performed using pairs of TMM and TDM composed of the same wax ester, we confirmed that at equimolar concentrations TDM is more inflammatory than TMM and TDB (Fig. 1B). These data are in



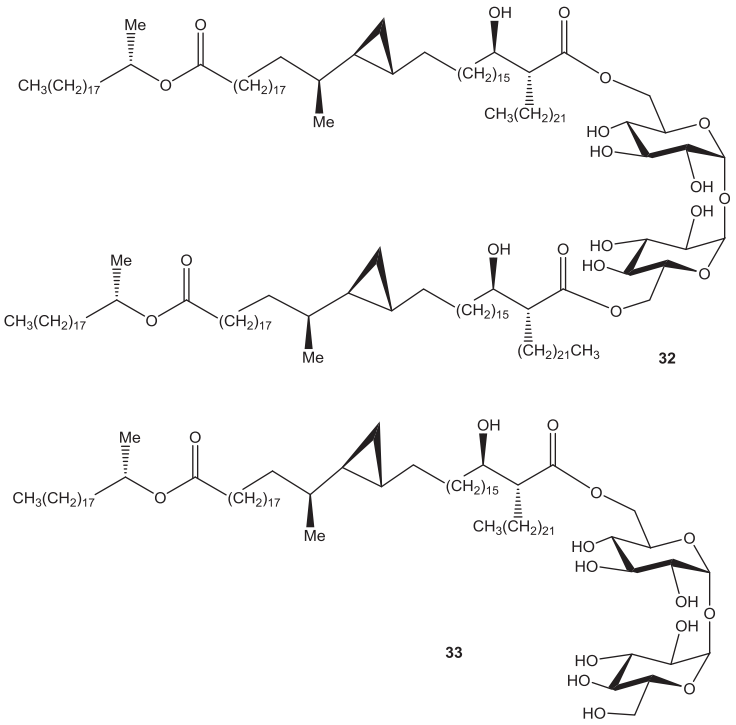
Scheme 11. EDCI, DMAP, 4 Å molecular sieves, CH_2Cl_2, 6 days at ambient temperature.



Scheme 12. (i) nBu_4NF, dry THF; (ii) HF-pyridine complex, dry THF.



Scheme 13. (i) nBu_4NF, dry THF; (ii) HF-pyridine complex, dry THF.



Scheme 14. TDM and TMM from **6b**.

accordance with our previous findings on trehalose and glucose mycolate esters.[71] Wax ester TDM **31** induced a comparable level of TNF-α to TDMs from a *cis*-cyclopropane containing methoxy-mycolic acid (Supplementary data, S1) or a *cis*-cyclopropane containing keto-mycolic acid (**S3**). These three compounds induced more TNF-α than a TDM from a di-*cis*-cyclopropane containing α-mycolic acid (**S2**) in vitro (Fig. 1C) (for structures see Supplementary data).

The in vivo inflammatory potential of synthetic wax ester TDM (**29**) was further compared to the synthetic *cis*-cyclopropane methoxy and α-TDMs. The compounds were formulated in a water in oil in water emulsion and the preparations were injected subcutaneously in the two hind footpads of C57BL/6 mice. The footpad swelling was measured with a caliper as read out for local inflammation. The data showed that all synthetic TDM compounds and TDB can induce a significant footpad swelling 3 days after injection as compared to vehicle control. The level of inflammation induced was comparable for the four compounds (Fig. 2).

4. Conclusion

We have described the first syntheses of complete mycobacterial wax esters in a process that can be adjusted to provide any required absolute stereochemistry or chain length. The wax esters are initially produced as methyl esters at the β-hydroxy-ester, but these may be selectively hydrolysed to the free β-hydroxy-acid without cleavage of the wax ester part, an eicosanol ester. Selective protection of the hydroxyl-group using trimethylsilyl allowed the coupling of the wax ester to a protected trehalose. Following

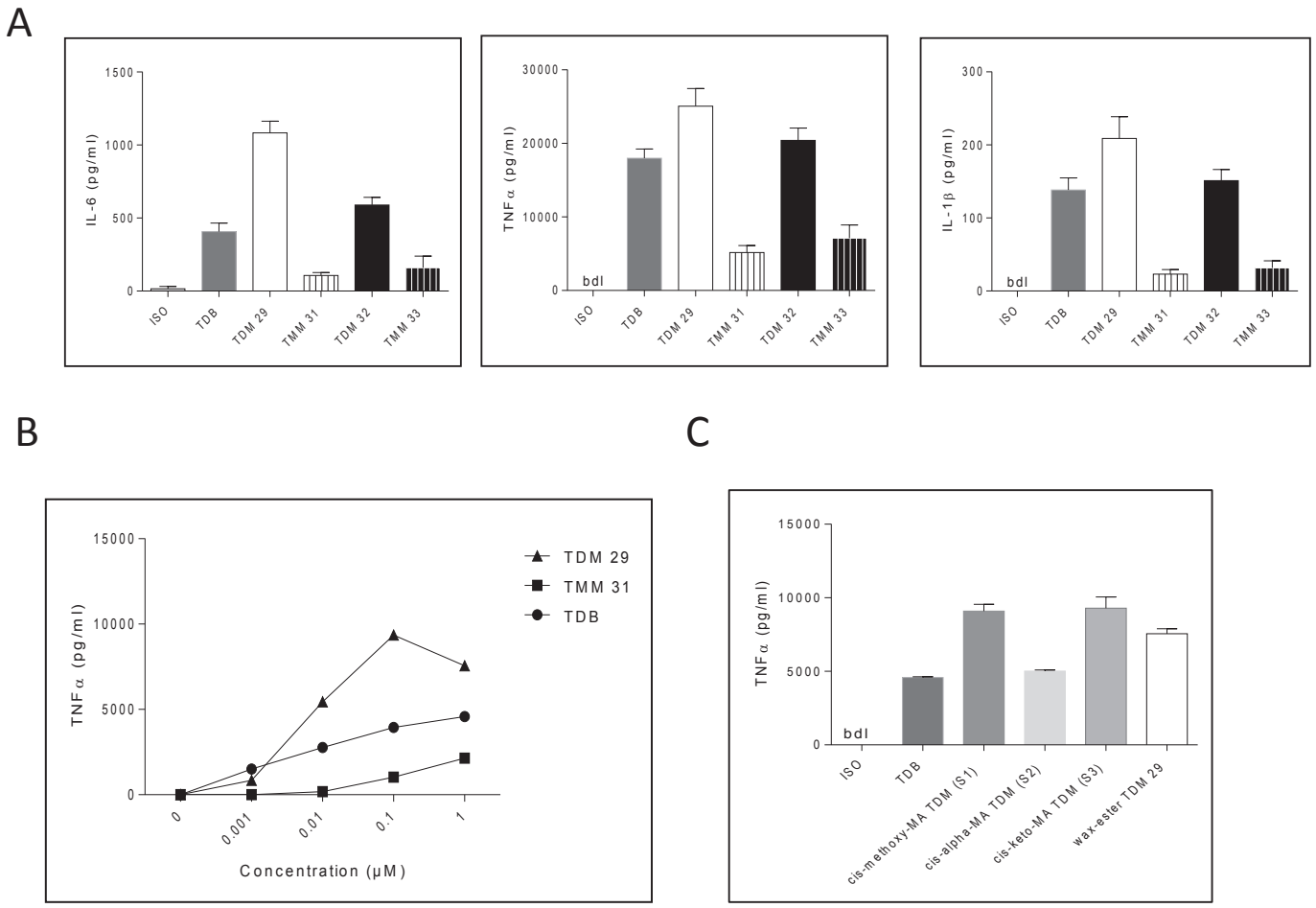


Fig. 1. Wax ester TDM and TMM activate BMDCs in vitro: BMDCs derived from C57BL/6 mice were stimulated for 24 h in triplicate with 1 μM or the indicated concentration of plate-coated synthetic wax ester TDMs **29** and **32** or TMMs **31** and **33**, synthetic TDMs containing *cis*-cyclopropane methoxy-MA (**S1**), keto-MA (**S3**) or α-mycolic acid (**S2**) (structures in Supplementary data), or controls (trehalose dibehenate (TDB) or evaporated isopropanol (ISO)). The supernatants were harvested separately and the amount of pro-inflammatory TNF-α, IL-1β and IL-6 was determined by sandwich ELISA. Results are expressed as mean pg/ml of cytokines ±SD and representative of at least three independent experiments (bdl: below detection limit).

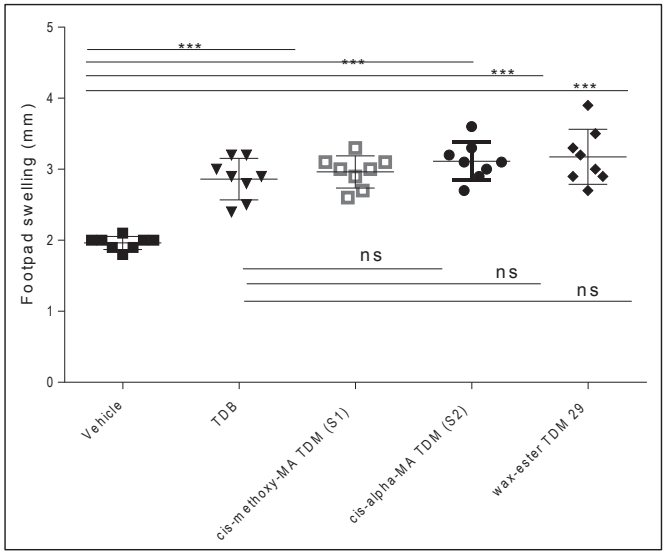


Fig. 2. Wax ester TDM induces inflammation in vivo: Groups of 4–5 C57BL/6 mice were injected s.c in the hind footpads with w/o/w emulsions composed of 3.2% Incomplete Freund's Adjuvant and 5 μg/footpad of synthetic mycolate esters **29**, **S1** and **S2**. In the emulsion of the vehicle control group no glycolipid was present, in the TDB control group 5 μg/footpad of TDB replaced synthetic glycolipid in the emulsion.

deprotection, this led to the free trehalose dimycolate and monomycolate of the wax ester.

The wax-esters are immuno-stimulatory in vitro and in vivo and the level of inflammation induced is at least comparable to that induced by TDB. The applications of the wax ester derivatives as antigens for the serodiagnosis of diseases caused by mycobacterial infections will be described elsewhere.

5. Experimental section

5.1. General

Chemicals used were obtained from commercial suppliers (Sigma, Aldrich, and Alfa Aeser) or prepared from them by the methods described. Solvents which were required to be dry, e.g., ether, THF were dried over sodium wire and benzophenone under nitrogen, while dichloromethane and HMPA were dried over calcium hydride. All reagents and solvents used were of reagent grade unless otherwise stated. Silica gel (Merck 7736) and silica gel plates used for column chromatography and thin layer chromatography were obtained from Aldrich; separated components were detected using variously UV light, I_2 or phosphomolybdic acid solution in IMS followed by charring. Anhydrous magnesium sulfate was used to dry organic solutions. Infra-red (IR) spectra were carried out on a Perkin–Elmer 1600 FTIR spectrometer as liquid films or KBr disc (solid). Melting points were measured using a Gallenkamp melting

point apparatus. NMR spectra were carried out on a Bruker Avance 400 or 500 spectrometer. Specific rotations were recorded in $CHCl_3$ on a POLAAR 2001 Optical Activity Polarimeter. Mass spectra were recorded on a Bruker matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) values are given plus sodium to an accuracy of 1 d.p.; accurate mass values obtained in Bangor were run on a Bruker LC-MS and those using MALDI-TOF MS were determined by Dr. Paul Gates in Bristol University.

5.2. Methyl 16-((1*S*,2*R*)-2-((*S*)-4-((*tert*-butyldiphenylsilyl)oxy) butan-2-yl)cyclopropyl)hexadecanoate (10)

LHMDS (17.8 mL, 18.9 mmol, 1.06 M) was added dropwise to a stirred solution of (1*S*,2*R*)-2-[(*S*)-3-(*tert*-butyldiphenylsilanyloxy)-1-methylpropyl]cyclopropanecarbaldehyde **9** (4.01 g, 10.5 mmol)[64] with 16-(1-phenyl-1*H*-tetrazole-5-sulfonyl)-pentadecanoic acid methyl ester **8** (5.86 g, 12.6 mmol)[65] in dry THF (75 mL) under nitrogen at −15 °C. The mixture was stirred for 1 h at room temperature, then cooled to 0 °C and quenched with satd aq ammonium chloride (30 mL). The organic layer was treated with petrol/ethyl acetate 10:1 (50 mL), and the aqueous layer was re-extracted with petrol/ethyl acetate 10:1 (2×25 mL). The combined organic layers were dried and evaporated; the product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a colourless oil, methyl (*E*/*Z*)-16-((1*R*,2*S*)-2-(4-((*tert*-butyldiphenylsilyl)oxy)-2-methylbutan-2-yl)cyclopropyl) hexadec-15-enoate as a 2:1 mixture (5.6 g, 85%). Dipotassium azodicarboxylate (5.03 g, 26.2 mmol) was added to a stirred solution of the above alkenes (5.55 g, 8.98 mmol) in THF (75 mL) and methanol (5 mL) at 5 °C. Glacial acetic acid (5 mL) in THF (5 mL) was added dropwise over 72 h. The mixture was poured into satd aq sodium bicarbonate, and then extracted with ethyl acetate (3×150 mL). The combined organic layers were dried and evaporated to give a thick oil; column chromatography eluting with petrol/ethyl acetate (20:1) gave compound **10** as a colourless oil (5.4 g, 96%), α_D^{21}+7.9 (*c* 0.91, $CHCl_3$) [Found $(M)^+$: 620.4645, $C_{40}H_{64}O_3Si$ requires: 620.4625]; δ_H (400 MHz, $CDCl_3$): 7.68–7.66 (4H, m), 7.42–7.36 (6H, m), 3.76–3.71 (2H, m), 3.67 (3H, s), 2.30 (2H, t, *J* 7.6 Hz), 1.7–1.48 (6H, m), 1.43–1.26 (26H, br m), 1.05 (9H, s), 0.89 (3H, s), 0.42 (1H, m), 0.13 (2H, m); δ_C (101 MHz, $CDCl_3$): 174.2, 135.9, 134.2, 129.57, 127.5, 62.4, 51.4, 40.2, 34.8, 34.4, 34.1, 29.7, 29.63, 29.6, 29.4, 29.3, 29.2, 26.9, 25.9, 25.0, 19.9, 18.6, 10.6; ν_{max}/cm^{-1}: 2925, 2854, 1742, 1428, 1111.

5.3. Methyl 16-((1*S*,2*R*)-2-((2*S*)-4-((tetrahydro-2*H*-pyran-2-yl) oxy)butan-2-yl)cyclopropyl)hexa-decanoate (11)

(i) Tetra-*n*-butylammonium fluoride (10.2 mL, 10.2 mmol) was added to a stirred solution of ester **10** (5.30 g, 8.54 mmol) in dry THF (70 mL) at 0 °C under nitrogen. The mixture was allowed to reach room temperature and stirred for 5 h, then cooled to 5 °C and quenched with satd aq ammonium chloride (30 mL). The product was extracted with ethyl acetate (3×150 mL), then the combined organic layers were washed with brine (100 mL), dried and evaporated to give a crude product, which was purified by column chromatography eluting with petrol: ethyl acetate (5:1) to give methyl 16-((1*S*,2*R*)-2-((*S*)-4-hydroxybutan-2-yl)cyclopropyl)hexadecanoate as a colourless oil (3.1 g, 96%), α_D^{21}+14 (*c* 0.93, $CHCl_3$) [Found $(M)^+$: 382.3467, $C_{24}H_{46}O_3$ requires: 382.3447]; δ_H (400 MHz, $CDCl_3$): 3.78–3.69 (2H, m), 3.67 (3H, s), 2.30 (2H, t, *J* 7.6 Hz), 1.76–1.67 (1H, m), 1.63–1.60 (2H, m), 1.59–1.52 (1H, m), 1.37–1.20 (26H, m), 1.18–1.11 (1H, m), 0.96 (3H, d, *J* 6.6 Hz), 0.90–0.81 (1H, m), 0.51–0.44 (1H, m), 0.25–0.13 (3H, m); δ_C (101 MHz, $CDCl_3$): 174.8, 61.4, 51.4, 40.4, 35.0, 34.4, 34.1, 29.7, 29.63, 29.6, 29.5, 29.3, 29.2, 26.6, 25.9, 25.0, 19.9, 18.8, 16.9, 10.6, 10.0; ν_{max}/cm^{-1}: 3369, 2923, 2853, 1742, 1459, 1170.

(ii) Pyridinium-*p*-toluenesulfonate (1.01 g, 4.01 mmol) was added to a stirred solution of the above alcohol (3.05 g, 7.98 mmol) and 3,4-dihydro-2*H*-pyran (1.67 g, 2.96 mmol) in dry CH_2Cl_2 (40 mL) under nitrogen at rt. The reaction was stirred for 30 min, then quenched with satd aq $NaHCO_3$ (20 mL). The product was extracted with CH_2Cl_2 (2×75 mL) and dried. The solvent was evaporated and the product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **11** as a colourless oil, a mixture of diastereoisomers (3.7 g, 98%), α_D^{21}+19 (*c* 0.75, $CHCl_3$) [Found $(M)^+$: 466.4011, $C_{29}H_{54}O_4$ requires: 466.4022]; δ_H (400 MHz, $CDCl_3$): 4.57 (1H, br m), 3.88–3.79 (2H, m), 3.68 (3H, s), 3.48 (2H, tdd, *J* 5.8, 10.2, 16.1 Hz), 2.31 (2H, t, *J* 7.6 Hz), 1.87–1.71 (1H, m), 1.69–1.64 (2H, m), 1.58–1.54 (7H, m), 1.37–1.20 (25H, m), 1.18–1.11 (1H, m), 0.95 (3H, d, *J* 6.8 Hz), 0.90–0.80 (1H, m), 0.51–0.43 (1H, m), 0.18–0.14 (3H, m); δ_C (101 MHz, $CDCl_3$): 174.4, 99.0, 98.9, 66.2, 65.9, 62.4, 51.4, 37.2, 37.1, 35.3, 35.2, 34.4, 34.1, 30.9, 29.74, 29.7, 29.62, 29.6, 29.5, 29.3, 29.2, 26.0, 25.9, 25.5, 25.0, 19.83, 19.8, 18.62, 18.6, 10.6; ν_{max}/cm^{-1}: 2924, 2853, 1743, 1454.

5.4. 16-((1*S*,2*R*)-2-((2*S*)-4-((Tetrahydro-2*H*-pyran-2-yl)oxy)butan-2-yl)cyclopropyl)-hexadecanal (12)

(i) The ester **11** (3.63 g, 7.78 mmol) in THF (10 mL) was added to a stirred suspension of $LiAlH_4$ (0.440 g, 11.7 mmol) in THF (60 mL) at −10 °C under nitrogen and then refluxed for 1 h, then quenched with satd aq sodium sulfate decahydrate at −10 °C until a white precipitate had formed. The mixture was stirred at rt for 30 min, filtered through a pad of Celite and the solvent was evaporated to give the product which was purified by column chromatography eluting with petrol/ethyl acetate (3:1) to give 16-((1*S*,2*R*)-2-((2*S*)-4-((tetrahydro-2*H*-pyran-2-yl)oxy)butan-2-yl)cyclopropyl)hexadecan-1-ol as a colourless oil, a mixture of diastereoisomers (3.4 g, 94%), α_D^{21}+19 (*c* 0.94, $CHCl_3$) [Found $(M)^+$: 438.4084, $C_{28}H_{54}O_3$ requires: 438.4073]; δ_H (400 MHz, $CDCl_3$): 4.57 (1H, br m), 3.94–3.77 (2H, m), 3.64 (2H, t, *J* 6.6 Hz), 3.56–3.39 (2H, m), 1.92–1.64 (3H, m), 1.59–1.54 (7H, br m), 1.34–1.26 (28H, br m), 1.17 (1H, m), 0.96 (3H, d, *J* 6.7 Hz), 0.90–0.77 (1H, m), 0.58–0.40 (1H, m), 0.28–0.07 (3H, m); δ_C (101 MHz, $CDCl_3$): 99.0, 98.9, 67.7, 66.2, 65.9, 63.0, 62.4, 37.2, 37.1, 35.3, 35.2, 34.4, 32.8, 30.9, 30.8, 29.7, 29.65, 29.62, 29.6, 29.4, 26.0, 25.9, 25.8, 25.5, 19.82, 19.81, 19.8, 18.64, 18.6, 10.6; ν_{max}/cm^{-1}: 3392, 2923, 2852, 1468.

(ii) The above alcohol (3.25 g, 7.41 mmol) in CH_2Cl_2 (10 mL) was added to stirred suspension of PCC (3.99 g, 18.6 mmol) in CH_2Cl_2 (80 mL) and stirred for 2 h, then diluted with petrol/ethyl acetate 10:1 (50 mL), and filtered through a pad of silica gel and Celite. The solvent was evaporated and the product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **12** as a colourless oil, a mixture of diastereoisomers (3.0 g, 93%); α_D^{21}+5.23 (*c* 1.01, $CHCl_3$) [Found $(M)^+$: 436.3911, $C_{28}H_{52}O_3$ requires: 436.3916]; δ_H (400 MHz, $CDCl_3$): 9.77 (1H, t, *J* 1.9 Hz), 4.61–4.53 (1H, br m), 3.95–3.77 (2H, m), 3.56–3.40 (2H, m), 2.42 (2H, td, *J* 7.4, 11 Hz), 1.80–1.75 (3H, m), 1.59–1.53 (7H, br m), 1.34–1.26 (26H, br m), 0.96 (3H, d, *J* 6.9 Hz), 0.91–0.78 (1H, m), 0.59–0.41 (1H, m), 0.29–0.10 (3H, m); δ_C (101 MHz, $CDCl_3$): 203.0, 99.0, 98.9, 66.2, 65.9, 62.4, 37.2, 37.0, 35.3, 35.2, 34.4, 30.9, 30.8, 29.73, 29.7, 29.65, 29.62, 29.6, 29.4, 25.9, 25.5, 19.82, 19.8, 18.61, 18.6, 10.6; ν_{max}/cm^{-1}: 2923, 2852, 1728, 1455.

5.5. Methyl (*R*)-2-((*R*)-1-((*tert*-butyldimethylsilyl)oxy)-3-((1-phenyl-1*H*-tetrazol-5-yl)-thio)propyl)tetra-cosanoate (14)

Diethyl azodicarboxylate (DEAD) (2.38 g, 13.7 mmol) in dry THF (8 mL) was added to a stirred solution of methyl (*R*)-2-((*R*)-1-((*tert*-

butyldimethylsilyl)oxy)-3-hydroxypropyl)tetracosanoate **13** [66] (6.0 g, 10.5 mmol), triphenylphosphine (3.58 g, 13.7 mmol) and 1-phenyl-1*H*-tetrazole-5-thiol (2.43 g, 13.9 mmol) in dry THF (70 mL) at 0 °C under nitrogen atmosphere. The mixture was allowed to reach rt and then stirred for 3 h. The solvent was evaporated and the residue was stirred with petrol/ethyl acetate (10:1, 150 mL) for 30 min and then filtered through a pad of Celite. The filtrate was evaporated and the crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **14** as a thick pale yellow oil (6.5 g, 85%), α_D^{21}−13.2 (*c* 1.06, $CHCl_3$) [Found $(M-Bu^t)^+$: 673.4535 calculated for $C_{37}H_{65}N_4O_3SiS$: 673.4547]; δ_H (400 MHz, $CDCl_3$): 7.59–7.52 (5H, m), 4.07 (1H, m), 3.67 (3H, s), 3.51–3.44 (1H, m), 3.40–3.34 (1H, m), 2.59 (1H, ddd, *J* 4.8, 6.9, 11.4 Hz), 2.16–2.09 (1H, m), 2.01–1.93 (1H, m), 1.59–1.21 (42H, br m), 0.90–0.87 (12H, m, including t at 0.87, *J* 6.9 Hz), 0.08 (3H, s), 0.06 (3H, s); δ_C (101 MHz, $CDCl_3$): 174.4, 154.1, 130.1, 129.8, 123.8, 72.0, 51.5, 51.48, 33.1, 31.9, 29.7, 29.66, 29.6, 29.5, 29.4, 28.6, 27.9, 27.1, 25.7, 22.7, 14.1, −4.4, −4.9; ν_{max}/cm^{-1}: 2918, 2852, 1741, 1498, 1459, 1161.

5.6. Methyl (*R*)-2-((*R*)-1-((*tert*-butyldimethylsilyl)oxy)-3-((1-phenyl-1*H*-tetrazol-5-yl)-sulfonyl)propyl)-tetracosanoate (15)

m-Chloroperoxybenzoic acid (7.87 g, 32.1 mmol) was added to a stirred solution of ester **14** (7.80 g, 10.7 mmol) in CH_2Cl_2 (50 mL), followed by addition of $NaHCO_3$ (3.59 g, 42.7 mmol). The mixture was stirred for 24 h at rt, then poured into sat.aq $NaHCO_3$ (150 mL), and stirred for 2 h. The product was extracted with CH_2Cl_2 (3×100 mL), and the combined organic layers were dried and evaporated to give a crude product which was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **15** as a white solid (5.0 g, 86%), α_D^{21}−14.4 (*c* 1.12, $CHCl_3$) [Found $(M-Bu^t)^+$: 705.4431; calculated for $C_{37}H_{65}N_4O_5SiS$: 705.4445]; δ_H (400 MHz, $CDCl_3$): 7.72–7.7 (2H, m), 7.65–7.61 (3H, m), 4.15 (1H, m), 3.81 (2H, m), 3.69 (3H, s), 2.53 (1H, ddd, *J* 4.0, 7.4, 11.0 Hz), 2.16–2.09 (2H, m), 1.59–1.21 (42H, br m), 0.90–0.87 (12H, m, including t at 0.87, *J* 6.9 Hz), 0.08 (3H, s), 0.06 (3H, s); δ_C (101 MHz, $CDCl_3$): 174.1, 153.2, 133, 131.5, 129.7, 125.0, 70.9, 51.8, 51.6, 51.4, 31.9, 29.7, 29.66, 29.62, 29.6, 29.5, 29.4, 29.3, 27.7, 27.4, 26.2, 25.7, 22.70, 14.1, −4.5, −5.1; ν_{max}/cm^{-1}: 2920, 2852, 1729, 1497, 1467, 1155.

5.7. Methyl (2*R*)-2-((1*R*)-1-((*tert*-butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((2*S*)-4-((tetra-hydro-2*H*-pyran-2-yl)oxy)butan-2-yl)cyclopropyl)nonadecyl)tetracosanoate (16)

LHMDS (11.1 mL, 11.7 mmol, 1.06 M) was added dropwise to a stirred solution of sulfone **15** (5.97 g, 7.84 mmol) and aldehyde **12** (2.85 g, 6.53 mmol) in dry THF (50 mL) under nitrogen at −15 °C. The mixture was stirred for 1 h at room temperature, then cooled to 0 °C and quenched with satd aq ammonium chloride (30 mL). The organic layer was extracted with petrol/ethyl acetate 10:1 (50 mL), and the aqueous layer was re-extracted with petrol/ethyl acetate (10:1, 2×25 mL). The combined organic layers were dried and evaporated to give a crude product, which was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give methyl (*E*/*Z*)-(2*R*)-2-((1*R*,*E*)-1-((*tert*-butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((2*S*)-4-((tetrahydro-2*H*-pyran-2-yl)oxy)butan-2-yl)cyclopropyl)-nonadec-3-en-1-yl)tetracosanoate as a diastereomeric mixture in ratio 2:1 (5.0 g, 78%). Dipotassium azodicarboxylate (2.52 g, 13.2 mmol) was added to a stirred solution of the above alkene mixture (4.95 g, 5.09 mmol) in THF (75 mL) and methanol (5 mL) at 5 °C. Glacial acetic acid (5 mL) in THF (5 mL) was added dropwise over 72 h. The mixture was poured into satd aq sodium bicarbonate solution, and extracted with ethyl acetate (3×100 mL). The combined organic layers were dried and evaporated to give a thick oil residue, which was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give the *title* compound **16** as a mixture of diastereoisomers (4.6 g, 91%), α_D^{21}−8.6 (*c* 0.91, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 997.8949, $C_{62}H_{122}NaO_5Si$ requires: 997.8954], δ_H (400 MHz, $CDCl_3$): 4.58 (1H, br m), 3.97–3.76 (3H, m), 3.67 (3H, s), 3.56–3.40 (2H, m), 2.53 (1H, ddd, *J* 3.7, 7.1, 10.8 Hz), 1.75–1.64 (3H, m), 1.61–1.06 (84H, br m), 0.96 (3H, d, *J* 6.6 Hz), 0.90–0.87 (12H, m, including t at 0.87 with *J* 6.9 Hz), 0.57–0.40 (1H, m), 0.27–0.11 (3H, m), 0.05 (3H, s), 0.02 (3H, s); δ_C (101 MHz, $CDCl_3$): 175.2, 99.0, 98.9, 73.2, 66.2, 65.9, 62.4, 51.5, 51.4, 37.4, 37.2, 37.1, 35.3, 35.2, 34.4, 32.8, 31.9, 30.8, 29.8, 29.7, 29.63, 29.60, 29.5, 29.4, 29.3, 27.9, 27.7, 27.6, 25.9, 25.7, 25.5, 23.7, 22.7, 22.6, 20.4, 19.8, 11.2, 10.0, −4.4, −4.9; ν_{max}/cm^{-1}:, 2924, 2853, 1741, 1464.

5.8. Methyl (*R*)-2-((*R*)-1-((*tert*-butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((*S*)-4-oxobutan-2-yl)cyclo-propyl)nonadecyl)tetracosanoate (17)

(a) Pyridinium-*p*-toluenesulfonate (0.58 g, 2.3 mmol) was added to a stirred solution of methyl tetrahydropyranyl acetal **16** (4.51 g, 4.62 mmol) in THF (50 mL), methanol (10 mL) and stirred at 45 °C for 6 h. Satd aq $NaHCO_3$ (20 mL) and water (20 mL) were added and extracted with ethyl acetate (3×50 mL). The combined organic layers were dried and the solvent was evaporated. Column chromatography eluting with petrol/ethyl acetate (12:1) gave *methyl* (*R*)-2-((*R*)-1-((*tert-butyldimethylsilyl*)*oxy*)-19-((1*S*,2*R*)-2-((*S*)-4-*hy-droxybutan*-2-*yl*)*cyclopropyl*)*nonadecyl*)*tetracosanoate* (3.5 g, 84%), α_D^{21}+7.9 (*c* 1.1, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 913.8369; $C_{57}H_{114}NaO_4Si$ requires 913.8384]; δ_H (400 MHz, $CDCl_3$); 3.92 (1H, ddd, *J* 4.9, 7.6, 11.8 Hz), 3.80–3.68 (2H, m), 3.66 (3H, s), 2.53 (1H, ddd, *J* 3.8, 7.1, 10.9 Hz), 1.72 (1H, td, *J* 6.8, 13.5 Hz), 1.54–1.26 (81H, br m), 0.96 (3H, d, *J* 6.5 Hz), 0.91–0.84 (12H, m, including t at 0.87 with *J* 6.9 Hz), 0.55–0.44 (1H, m), 0.29–0.13 (3H, m), 0.05 (3H, s), 0.03 (3H, s); δ_C (10 MHz, $CDCl_3$): 175.2, 73.2, 61.4, 51.6, 51.2, 40.4, 35.0, 34.4, 33.7, 31.9, 31.6, 29.8, 29.72, 29.7, 29.63, 29.6, 29.59, 29.5, 29.4, 29.1, 27.8, 27.7, 27.65, 27.5, 25.9, 25.8, 25.3, 23.7, 22.7, 22.66, 21.0, 20.5, 19.8, 18.8, 17.9, −4.4, −4.9; ν_{max}/cm^{-1}: 3584, 2924, 2853, 1740, 1463, 1066.

(b) The above alcohol (3.25 g, 3.65 mmol) in CH_2Cl_2 (20 mL) was added to a stirred suspension of PCC (2.36 g, 10.9 mmol) in CH_2Cl_2 (120 mL), stirred for 2 h, then diluted with petrol/ethyl acetate (10:1, 100 mL), and filtered through a pad of silica gel and Celite. The solvent was evaporated and the product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **17** as a colourless oil (3.0 g, 93%), α_D^{21}+1.6 (*c* 1.06, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 912.2; $C_{57}H_{112}NaO_4Si$ requires 911.8]; δ_H (400 MHz, $CDCl_3$): 9.79 (1H, t, *J* 2.5 Hz), 3.92 (1H, dt, *J* 5.9, 7.9 Hz), 3.66 (3H, s), 2.50–2.48 (2H, m), 2.38 (1H, ddd, *J* 2.5, 7.7, 15.7 Hz), 1.54–1.26 (79H, br m), 1.03 (3H, d, *J* 6.8 Hz), 0.92–0.80 (12H, m, including t at 0.87 with *J* 6.9 Hz), 0.50–0.48 (1H, m), 0.38–0.19 (3H, m), 0.05 (3H, s), 0.02 (3H, s); δ_C (101 MHz, $CDCl_3$): 202.9, 73.2, 51.6, 51.5, 34.1, 33.9, 33.7, 31.9, 29.8, 29.73, 29.7, 29.6, 29.5, 29.4, 27.8, 27.72, 27.7, 27.5, 25.9, 25.8, 25.3, 23.7, 22.7, 22.66, 21.0, 20.5, 19.8, 18.82, 14.1, 11.4, −4.4, −4.9; ν_{max}/cm^{-1}: 2924, 2853, 1738, 1464.

5.9. 15-((1-Phenyl-1*H*-tetrazol-5-yl)sulfonyl)pentadecanoic acid (18)

(a) Aq sodium hydroxide (8M) 100 mL was added with stirring to methyl 15-((1-phenyl-1*H*-tetrazol-5-yl)thio)pentadecanoate (10.5 g, 93.8 mmol)[69] in THF (100 mL), followed by addition of MeOH (15 mL). The mixture was refluxed for 30 min, then the solvent was evaporated, and the residue was dissolved in water (50 mL), and then acidified with aq HCl (2M) to pH 2. The aqueous layer was extracted with ethyl acetate (3×100 mL), and the combined organic layers were dried and evaporated to give crude product. Re-crystalization from ethyl acetate gave 15-((1-

phenyl-1*H*-*tetrazol*-5-*yl*)*thio*)*pentadecanoic acid* as a white solid (9.0 g, 89%), mp 78–80 °C {[MALDI-Found] $(M+Na)^+$: 441.4; $C_{22}H_{34}N_4O_2S$ requires 441.2}; δ_H (400 MHz, $CDCl_3$): 7.74–7.59 (5H, m), 3.40 (2H, t, *J* 7.4 Hz), 2.36 (2H, t, *J* 7.5 Hz), 1.82 (2H, quintet, *J* 7.4 Hz), 1.66–1.64 (2H, quintet, *J* 7.4 Hz), 1.48–1.41 (2H, quintet, *J* 7.4 Hz), 1.30–1.26 (19H, m); δ_C (101 Hz, $CDCl_3$): 130.1, 129.8, 123.9, 33.7, 33.4, 29.6, 29.5, 29.49, 29.4, 29.3, 29.28, 29.1, 29.04, 28.6, 24.7; ν_{max}/cm^{-1}: 3436, 2918, 2849, 1725, 1594, 1496, 1463, 1413, 1381, 1172.

(b) A solution of ammonium molybdate (VI) tetrahydrate (7.60 g, 6.14 mmol) in 35% H_2O_2 (15 mL) was prepared and cooled in an ice bath, it was added to a stirred solution of the above acid (5.01 g, 12.3 mmol) in THF (30 mL) and IMS (30 mL) at 10 °C, and stirred at rt for 2 h. A further solution of ammonium molybdate (VI) tetrahydrate (3.80 g, 3.07 mmol) in 35% H_2O_2 (8 mL) was added and the mixture was stirred at rt for 18 h. The mixture was poured into water (250 mL) and extracted with ethyl acetate (3×75 mL). The combined organic layers were dried and the solvent was evaporated. The product was purified by recrystallisation from MeOH/acetone (1:1) to give the *title* compound **18** as a white solid (4.2 g, 78%), mp 88–89 °C [MALDI-Found $(M+Na)^+$: 472.9; $C_{22}H_{34}N_4O_4S$ requires 473.2]; δ_H (400 MHz, $CDCL_3$): 7.74–7.70 (2H, m), 7.66–7.61 (3H, m), 3.74 (2H, t, *J* 8.2 Hz), 2.36 (2H, t, *J* 7.5 Hz), 1.96 (2H, quintet, *J* 7.4 Hz), 1.66 (2H, quintet, *J* 7.4 Hz), 1.48 (2H, quintet, *J* 7.4 Hz), 1.30–1.26 (19H, m); δ_C (101 MHz, $CDCl_3$): 131.5, 129.7, 125.1, 56.1, 33.8, 29.5, 29.42, 29.4, 29.2, 29.17, 29.0, 28.9, 28.1, 24.7, 22.0; ν_{max}/cm^{-1}: 3467, 2921, 2851, 1694, 1597, 1466, 1359, 1152.

5.10. (*S*)-18-((1*R*,2*S*)-2-((19*R*,20*R*)-19-((*tert*-Butyldimethylsilyl)oxy)-20-(methoxycarbonyl)dotetracontyl)cyclopropyl)nonadecanoic acid (19)

Lithium *bis*(trimethylsilyl)amide (9.40 mL, 9.97 mmol, 1.06 M) was added dropwise to a stirred solution of ester **17** (2.95 g, 3.32 mmol) and sulfone **18** (1.79 g, 3.98 mmol) in dry THF (40 mL) under nitrogen at −15 °C. The mixture was stirred for 1 h at room temperature, then cooled to 0 °C and quenched with satd aq ammonium chloride (30 mL). The organic layer was extracted with petrol/ethyl acetate (10:1, 40 mL), and the aqueous layer was re-extracted with petrol/ethyl acetate (10:1, 2×20 mL). The combined organic layers were dried and evaporated; the product was purified by column chromatography eluting with 20:1 petrol/ethyl acetate to give (*E*/*Z*)-18-((1*R*,2*S*)-2-((19*R*,20*R*)-19-((*tert*-butyldimethylsilyl)-oxy)-20-(methoxycarbonyl)dotetracontyl)cyclopropyl)-nonadec-15-enoic acid (3.0 g, 80%) as a mixture in ratio (2.3:1); dipotassium azodicarboxylate (3.75 g, 19.5 mmol) was added to a stirred solution of the above alkene (2.9 g, 2.6 mmol) in THF (60 mL) and methanol (5 mL) at 5 °C. Glacial acetic acid (5 mL) in THF (5 mL) was added dropwise over 72 h. The mixture was poured in to satd aq sodium bicarbonate, then extracted with ethyl acetate (3×100 mL). The combined organic layers were dried and evaporated. The product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **19** as a semi-solid (2.6 g, 80%), α_D^{21}+4.1 (*c* 0.48 $CHCl_3$) {[MALDI-Found] $(M+Na)^+$: 1138.0530; $C_{72}H_{142}NaO_5Si$ requires 1138.0519}; δ_H (400 MHz, $CDCl_3$): 3.92 (1H, dt, *J* 4.8, 7.1 Hz), 3.66 (3H, s), 2.53 (1H, ddd, *J* 3.8, 7.2, 10.9 Hz), 2.36 (2H, t, *J* 7.5 Hz), 1.64 (3H, m), 1.57–1.03 (106H, br m), 0.94–0.83 (15H, m, including t at 0.87 with *J* 6.9 Hz), 0.73–0.62 (1H, m), 0.51–0.40 (1H, m), 0.26–0.07 (3H, m), 0.05 (3H, s), 0.02 (3H, s); δ_C (101 MHz, $CDCl_3$): 178.8, 175.2, 73.2, 68.2, 60.4, 51.6, 51.2, 38.7, 38.1, 37.4, 33.9, 33.7, 32.7, 31.9, 29.7, 29.6, 29.4, 29.3, 27.6, 27.5, 27.3, 25.8, 25.7, 23.7, 22.7, 21.0, 19.3, 19.2, 18.6, 18.0, 14.10, 14.0, 11.4, 10.5, 10.3, −4.4, −4.9; ν_{max}/cm^{-1}: 2924, 2853, 1743, 1711, 1464.

5.11. (*S*)-Eicosan-2-ol (21)

Bromoheptadecane (5.01 g, 15.6 mmol) was dissolved in dry THF (5 mL) and then added slowly to a stirred suspension of magnesium turnings (2.26 g, 94.1 mmol) in dry THF (8 mL) and warmed gently until the Grignard reagent started to form. The mixture was refluxed for 1 h. The Grignard reagent was added slowly to a stirred suspension of copper iodide (0.980 g, 5.17 mmol) in dry THF (40 mL) at −30 °C and stirred for 30 min. (*S*)-1-Epoxypropane (0.40 g, 6.89 mmol) in dry THF (5 mL) was added dropwise at −30 °C. The mixture allowed to reach room temperature and stirred for 16 h, then quenched with ammonium chloride and then extracted with ethyl acetate (3×100 mL). The combined organic layers were dried and evaporated to give the crude product; column chromatography eluting with petrol/ethyl acetate (10:1) gave (*S*)-eicosan-2-ol **21** (1.1 g, 53%). Mp 61–63 °C, α_D^{21}+3.8 (*c* 1.01, $CHCl_3$); δ_H (400 MHZ, $CDCl_3$): 3.80 (1H, sextet, *J* 6 Hz), 1.48 (2H, m), 1.38–1.25 (32H, br m), 1.2 (3H, d, *J* 6.2 Hz), 0.88 (3H, t, *J* 6.7 Hz); δ_C (101 MHz, $CDCl_3$): 68.2, 39.4, 31.9, 29.7, 29.66, 29.63, 29.6, 29.4, 25.8, 23.5, 22.5, 14.1; ν_{max}/cm^{-1}: 3386, 2924, 2853, 1464.

5.12. Methyl (*R*)-2-((*R*)-1-((*tert*-butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxoneonadecan-2-yl)cyclopropyl)nonadecyl)tetracosanoate (22)

Acid **19** (2.41 g, 2.16 mmol), (*S*)-eicosan-2-ol **21** (0.67 g, 2.22 mmol) and DMAP (0.39 g, 3.22 mmol) were dissolved in dry CH_2Cl_2 (15 mL) under nitrogen at room temperature. DCC (0.88 g, 4.3 mmol) in dry CH_2Cl_2 (7 mL) was added dropwise over 15 min and stirred at rt for 3 h, then diluted with DCM, filtered and evaporated. Column chromatography eluting with petrol/ethyl acetate (20:1) gave the *title* compound **22** as a colourless oil (2.4 g, 80%), α_D^{21}+6.1 (*c* 0.90, $CHCl_3$) [Found $(M+Na)^+$: 1418.3659; $C_{92}H_{182}NaO_5Si$ requires 1418.3654]; δ_H (400 MHz, $CDCl_3$): 4.91 (1H, sextet, *J* 6.2 Hz), 3.92 (1H, dt, *J* 4.8, 7.1 Hz), 3.66 (3H, s), 2.53 (1H, ddd, *J* 3.8, 7.1, 10.9 Hz), 2.27 (2H, t, *J* 7.5), 1.54–1.26 (142H, br m), 1.20 (3H, d, *J* 6.2 Hz), 0.91 (3H, d, *J* 6.8 Hz), 0.91–0.87 (15H, m, including two t at 0.87 with *J* 7 Hz), 0.71–0.63 (1H, m), 0.49–0.41 (1H, m), 0.22–0.08 (3H, m), 0.05 (3H, s), 0.02 (3H, s); δ_C (101 MHz, $CDCl_3$): 175.2, 173.6, 73.2, 70.7, 51.6, 51.2, 38.1, 37.4, 36.0, 34.8, 34.5, 33.7, 31.9, 30.1, 29.83, 29.8, 29.75, 29.73, 29.7, 29.6, 29.57, 29.52, 29.5, 29.4, 29.3, 29.2, 27.8, 27.5, 27.2, 26.2, 25.8, 25.4, 25.1, 23.8, 23.7, 22.7, 20.0, 19.7, 18.6, 18.0, 11.0, 10.5, −4.4, −4.9; ν_{max}/cm^{-1}: 2924, 2853, 1738, 1464.

5.13. (*R*)-2-((*R*)-1-Hydroxy-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxononadecan-2-yl)cyclo-propyl)nonadecyl)tetracosanoic acid (6a)

(a) Ester **22** (2.30 g, 1.79 mmol) was dissolved in dry THF (30 mL) in a dry polyethylene vial equipped with a rubber septum, followed by addition of pyridine (0.7 mL) at rt under nitrogen. The mixture was cooled to 10 °C, and then HF-pyridine complex as ~70% (4.0 mL) was added dropwise. The mixture was stirred at 43 °C for 17 h, then neutralized by pouring slowly into satd aq $NaHCO_3$ until no more CO_2 was liberated. The product was extracted with petrol/ethyl acetate (5:1, 3×30 mL), then the combined organic layers were dried and evaporated. Chromatography eluting with petrol/ethyl acetate (10:1) gave *methyl* (*R*)-2-((*R*)-1-*hydroxy*-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-*eicosan*-2-*yloxy*)-19-*oxononadecan*-2-*yl*)*cyclopropyl*)-*nonadecyl*)*tetracosanoate* as a colourless thick oil (1.5 g, 71%), α_D^{21}+7.3 (*c* 0.11, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1304.2782; $C_{86}H_{168}O_5Na$ requires 1304.2784]; δ_H (400 MHz, $CDCl_3$): 4.91 (1H, sextet, *J* 6.2 Hz), 3.71 (3H, s), 3.66 (1H, m), 2.53 (1H, m), 2.27 (2H, t, *J* 7.5 Hz), 1.54–1.26 (143H, br m), 1.20 (3H, d, *J* 6.2 Hz), 0.90 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.71–0.63

(1H, m), 0.49–0.41 (1H, m), 0.22–0.08 (3H, m); δ_C (101 MHz, $CDCl_3$):176.2, 173.6, 72.3, 70.7, 51.6, 51.2, 38.1, 37.4, 36.0, 34.8, 34.5, 33.7, 31.9, 30.1, 29.6, 29.5, 27.8, 27.5, 27.2, 26.1, 25.8, 25.4, 25.1, 23.8, 23.7, 22.7, 20.1, 19.7, 18.6, 18.0, 14.1, 10.5; ν_{max}/cm^{-1}: 3441, 2918, 2850, 1734, 1732, 1466.

(b) The above alcohol (1.42 g, 1.11 mmol) was dissolved in aq nBu_4NOH (64 mL, 5%) and refluxed at 100 °C for 24 h. The product was extracted with petroleum/ether (5:2, 3×75 mL), dried and evaporated. Chromatography eluting with petrol/ethyl acetate (5:1) gave the *title* compound **6a** as a white solid (0.84 g, 60%), α_D^{21}+7.6 (*c* 0.40, $CHCl_3$), mp 55–56 °C [MALDI-Found $(M+Na)^+$: 1290.2617; $C_{85}H_{166}O_5Na$ requires 1290.2627]; δ_H (400 MHz, $CDCl_3$): 4.91 (1H, sextet, *J* 6.2 Hz), 3.72 (1H, dt, *J* 4.8, 9.2 Hz), 2.46 (1H, dt, *J* 5.3, 9.0 Hz), 2.27 (2H, t, *J* 7.5 Hz), 1.81–0.95 (144H, br m), 1.20 (3H, d, *J* 6.2 Hz), 0.90 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.72–0.63 (1H, m), 0.50–0.41 (1H, m), 0.22–0.02 (3H, m); δ_C (101 MHz, $CDCl_3$): 179.6, 173.5, 72.1, 70.8, 50.8, 38.1, 37.4, 36.0, 35.5, 34.8, 34.5, 31.9, 30.1, 29.7, 29.54, 29.5, 29.43, 29.4, 29.3, 29.2, 27.32, 27.3, 26.1, 25.7, 25.4, 25.1, 22.7, 20.0, 19.7, 18.6, 14.1, 10.5; ν_{max}/cm^{-1}: 3432, 2918, 2850, 1721, 1712, 1470.

5.14. (*R*)-2-((*R*)-19-((1*S*,2*R*)-2-((*S*)-18-Carboxyoctadecan-2-yl)-cyclopropyl)-1-hydroxynonadecyl)tetracosanoic acid (23)

(a) (*S*)-18-((1*R*,2*S*)-2-((19*R*,20*R*)-19-((*tert*-Butyldimethylsilyl) oxy)-20-(methoxycarbonyl)dotetracontyl)-cyclopropyl)nonadecanoic acid (0.10 g, 0.09 mmol) was dissolved in dry THF (5 mL) in a dry polyethylene vial equipped with a rubber septum, and followed by addition of pyridine (0.1 mL) at rt under nitrogen. The mixture was cooled to 10 °C, and then HF-pyridine complex (~70%, 0.4 mL) was added dropwise. The mixture was stirred at 43 °C for 17 h, then poured slowly into satd aq sodium bicarbonate until no more CO_2 was liberated. The product was extracted with petrol/ethyl acetate (5:1, 3×10 mL), dried and evaporated; chromatography eluting with petrol/ethyl acetate (4:1) gave (*S*)-18-((1*R*,2*S*)-2-((19*R*,20*R*)-19-*Hydroxy*-20-(*methoxycarbonyl*)*dotetracontyl*)-*cyclopropyl*)*nona-decanoic acid* as a viscous oil (75 mg, 85%), α_D^{21}+7.8 (*c* 1.6, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1024.6; $C_{66}H_{128}NaO_5$ requires 1023.9]; δ_H (400 MHz, $CDCl_3$): 3.71 (3H, s), 3.68–3.65 (1H, m), 2.53 (1H, dt, *J* 4.7, 9.8 Hz), 2.36 (2H, t, *J* 7.5 Hz), 1.74–1.62 (4H, m), 1.55–1.26 (106 H, m), 0.91–0.86 (6H, d, *J* 5.4 Hz, including t with *J* 5.7 Hz), 0.69–0.62 (1H, m), 0.49–0.41 (1H, m), 0.22–0.08 (3H, m); δ_C (101 MHz, $CDCl_3$): 178.6, 176.3, 72.3, 60.4, 51.6, 51.0, 38.2, 37.4, 35.7, 34.5, 33.9, 31.9, 30.1, 29.76, 29.73, 29.7, 29.63, 29.6, 29.58, 29.54, 29.5, 29.47, 29.43, 29.4, 27.3, 29.1, 27.4, 27.2, 25.7, 24.7, 22.7, 21.1, 19.7, 18.6, 14.2; ν_{max}/cm^{-1}: 2917, 2849, 1733, 1701, 1464.

(b) The above acid (0.071 g, 0.072 mmol) was added to a stirred solution of THF (8 mL), water (1 mL) and MeOH (0.8 mL), followed by the addition of lithium hydroxide monohydrate (0.044 g, 1.073 mmol, 15 mol eq.). The mixture was heated at 45 °C for 16 h, then diluted with petrol/ethyl acetate (5:1, 4 mL), and acidified to pH 2 using satd aq $KHSO_4$. The aqueous layer was extracted with petrol/ethyl acetate (5:1, 3×5 mL), and the combined organic layers were dried and evaporated to give a crude product; chromatography eluting with petrol/ethyl acetate (1:3) gave the *title* compound **23** as a white solid (59 mg, 85%), mp 76–78 °C, α_D^{21}+5.8 (*c* 0.86, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1009.9520; $C_{65}H_{126}NaO_5$ requires 1009.9497]; δ_H (400 MHz, $CDCl_3$): 3.74–3.70 (1H, m), 2.53 (1H, dt, *J* 4.3, 9.8 Hz), 2.35 (2H, t, *J* 7.5 Hz), 1.77–1.62 (4H, m), 1.59–1.26 (107 H, m), 0.91–0.86 (6H, d, *J* 5.4 Hz, including t with *J* 5.7 Hz), 0.73–0.68 (1H, m), 0.50–0.44 (1H, m), 0.24–0.10 (3H, m); δ_C (101 MHz, $CDCl_3$): 180.1, 179.1, 72.2, 38.1, 37.5, 35.6, 34.5, 34.0, 31.9, 30.8, 29.7, 29.6, 29.5, 29.4, 29.1, 27.4, 27.3, 26.2, 25.7, 24.7, 22.7, 19.7, 18.6, 14.0, 10.5; ν_{max}/cm^{-1}: 2917, 2849, 1733, 1701, 1464.

5.15. (*R*)-2-((*R*)-1-((*tert*-Butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxo-nonadecan-2-yl)cyclopropyl)nonadecyl)tetracosanoic acid (24)

Imidazole (0.302 g, 4.507 mmol) was added to a stirred solution of acid **6a** (0.560 g, 0.448 mmol) in dry DMF (3.5 mL) and dry toluene (5 mL) at rt followed by the addition of TBDMSCl (0.675 g, 4.482 mmol) and DMAP (0.055 g, 0.448 mmol). The mixture was stirred at 70 °C for 18 h, then the solvent was removed under high vacuum and the residue was diluted with petrol/ethyl acetate (5:1, 30 mL) and water (20 mL). The organic layer separated and the aqueous layer was re-extracted with petrol/ethyl acetate (5:1, 2×20 mL). The combined organic layers were washed with water, dried and evaporated. The residue was dissolved in THF (8 mL), to this was added aq nBu_4NOH (4.5 mL, 4%). The mixture was stirred for 15 min at room temperature, and then diluted with water (5 mL) and petrol/ethyl acetate (2:1, 20 mL). The organic layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (2×20 mL). The combined organic layers were dried and evaporated to give a crude product; chromatography eluting with petrol/ethyl acetate (20:1) gave the *title* compound **24** as a syrup (0.53 g, 87%), α_D^{21}+23 (*c* 0.50, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1404.8; $C_{91}H_{180}NaO_5Si$ requires 1404.3]; δ_H (400 MHz, $CDCl_3$): 4.92 (1H, sextet, *J* 6.2 Hz), 3.83 (1H, m), 2.53 (1H, ddd, *J* 2.8, 6, 9.1 Hz), 2.27 (2H, t, *J* 7.4 Hz), 1.81–0.95 (143H, br m), 1.20 (3H, d, *J* 6.2 Hz), 0.993 (9H, s), 0.90 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.70–0.40 (1H, m), 0.48–0.41(1H, m), 0.15 (3H,s), 0.12 (3H, s), 0.22–0.06 (3H, m); δ_C (101 MHz, $CDCl_3$): 179.7, 173.6, 73.7, 70.7, 60.4, 50.1, 38.1, 37.4, 36.0, 35.5, 34.8, 34.5, 31.9, 30.1, 29.7, 29.5, 29.45, 29.4, 29.36, 29.3, 29.2, 27.3, 27.26, 26.1, 25.8, 25.4, 25.1, 22.7, 22.3, 20.0, 19.7, 18.6, 17.9, 14.1, 14.05, 10.5, −4.2, −4.9; ν_{max}/cm^{-1}: 3432, 2918, 2850, 1721, 1712, 1470.

5.16. 6-*O*-[(*R*)-2-((*R*)-1-Hydroxy-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxononadecan-2-yl)-cyclopropyl)nonadecyl)tetracosanoate]-α-D-glucopyranosyl-(1-1′)-6′-*O*-[(*R*)-2-((*R*)-1-hydroxy-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxononadecan-2-yl)cyclopropyl)nonadecyl)tetracosanoate]-α-D-glucopyranoside (29) and 6-*O*-[(*R*)-2-((*R*)-1-hydroxy)-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxononadecan-2-yl)*cyclopropyl*)nonadecyl)tetracosanoate]-α-D-glucopyranosyl-(1-1′)-α-D-glucopyranoside (31)

(i) 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (242 mg, 1.26 mmol) and DMAP (154 mg, 1.26 mmol) were added to a stirred solution of acid **24** (500 mg, 0.360 mmol), protected trehalose **25** (141 mg, 0.18 mmol)[67,70] and powdered 4 Å molecular sieves in dry CH_2Cl_2 (4 mL) at rt under nitrogen. The mixture was stirred for 5 days then three spatulas of silica gel was added and the solvent was evaporated under reduced pressure to give a residue; chromatography on silica eluting with petroleum ether/ethyl acetate (25:1) gave first compound **26** (0.33 g, 52%), α_D^{21}+21 (*c* 0.50, $CHCl_3$) [MALDI-Found $(M+Na)^+$ 3524.0; $C_{212}H_{426}NaO_{19}Si_8$ requires 3524.0]; δ_H (400 MHz, $CDCl_3$): 4.92 (2H, sextet, *J* 6.3 Hz), 4.85 (2H, d, *J* 3.0 Hz), 4.38 (2H, br, d, *J* 10.0 Hz), 4.04–3.98 (4H, m), 4.0–3.96 (2H, m) 3.93 (2H, m), 3.52 (2H, t, *J* 8.9 Hz), 3.38 (2H, dd, *J* 2.9, 9.3 Hz), 2.55 (2H, ddd, *J* 3.5, 4.8, 10.1 Hz), 2.26 (4H, t, *J* 7.5 Hz), 1.56–1.21 (284H, m), 1.20 (6H, d, *J* 6.2 Hz), 0.88 (18H, s), 0.90 (6H, d, *J* 6.8 Hz), 0.91–0.87 (12H, t, including two t with *J* 6.9 Hz), 0.72–0.65 (2H, m), 0.48–0.41 (2H, m), 0.22–0.06 (6H, m), 0.16 (18H, s), 0.145 (18H, s), 0.138 (18H, s), 0.062 (12H, s); δ_C (101 MHz, $CDCl_3$): 173.8, 173.6, 94.8, 73.5, 73.4, 72.8, 71.8, 70.7, 62.4, 60.4, 51.9, 41.3, 38.1, 37.4, 36.0, 34.5, 33.4, 31.9, 29.8, 29.5, 29.44, 29.42, 29.4, 29.3, 29.2, 27.3, 27.2, 26.2, 25.8, 25.4, 25.2, 22.7, 22.3, 20.0, 19.7, 18.6, 18.0, 14.3, 14.2, 14.1, 11.4, 10.5, 1.1, 1.0, 0.15, −4.5, −4.7; ν_{max}/cm^{-1}: 2924, 2854, 1733, 1465, 1375, 1254, 1215,

836, 760. The second fraction was glucopyranoside **27** (0.114 g, 32%), α_D^{21}+28 (*c* 0.50, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 2160.5; $C_{121}H_{248}O_{15}Si_7Na$ requires 2160.6]; δ_H (400 MHz, $CDCl_3$): 4.92 (2H, d, *J* 3 Hz), 4.91 (1H, sextet, *J* 6.2 Hz), 4.85 (1H, d, *J* 2.9 Hz), 4.36 (1H, dd, *J* 2, 11.6 Hz), 4.08 (1H, dd, *J* 4.0, 11.7 Hz), 4.04–3.98 (4H, m), 3.85 (1H, dt, *J* 3.2, 9.5 Hz), 3.69 (2H, m), 3.5 (2H, dt, *J* 4.6, 9.1 Hz), 3.41 (1H, dd, *J* 3.1, 9.3 Hz), 3.38 (1H, dd, *J* 2.8, 9.1 Hz), 2.55 (1H, ddd, *J* 3.4, 5.4, 10.3 Hz), 2.27 (2H, t, *J* 7.4 Hz), 1.73 (1H, br m), 1.68–1.21 (141H, m), 1.20 (3H, d, *J* 6.2 Hz), 0.88 (9H, s), 0.90 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.70–0.40 (1H, m), 0.48–0.41 (1H, m), 0.22–0.06 (3H, m), 0.17 (18H, s), 0.15 (18H, s), 0.14 (18H, s), 0.060 (3H, s), 0.052 (3H, s); δ_C (101 MHz, $CDCl_3$): 174.5, 173.6, 94.5, 94.4, 73.4, 73.3, 72.9, 72.8, 72.7, 72.0, 71.4, 70.7, 62.4, 61.7, 51.8, 38.1, 37.4, 36.0, 34.8, 34.4, 34.1, 33.4, 31.9, 30.1, 29.72, 29.7, 29.68, 29.65, 29.63, 29.6, 29.5, 29.45, 29.4, 28.1, 27.3, 26.3, 26.1, 25.8, 25.4, 25.1, 22.62, 22.6, 22.3, 20.0, 19.7, 18.7, 18.0, 14.1, 14.0, 10.5, 1.1, 1.05, 1.004, 1.0, 0.9, 0.8, 0.2, 0.03, −4.5, −4.7; ν_{max}/cm-1: 2924, 2853, 1743, 1464.9, 1251.6, 1163, 1099, 872, 839.

(ii) Tetrabutylammonium fluoride (0.334 mL, 0.334 mmol, 1M) was added to a stirred solution of glucopyranoside **26** (0.305 g, 0.087 mmol) in dry THF (25 mL) at 5 °C under nitrogen. The mixture was allowed to reach rt and stirred for 30 min, then the solvent was evaporated and the residue was purified by column chromatography eluting with $CHCl_3$/MeOH (10:1) to give glucopyranoside **28** as a viscous oil (0.19 g, 71%), α_D^{21}+26 (*c* 0.13, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 3091.8; $C_{194}H_{378}O_{19}Si_2Na$ requires 3091.8]; δ_H (400 MHz, $CDCl_3$+few drops of CD_3OD): 5.05 (2H, d, *J* 3.2 Hz), 4.92 (2H, sextet, *J* 6.3 Hz), 4.37 (2H, br, dd, *J* 3.5, 11.3 Hz), 4.21 (2H, br d, *J* 11.1 Hz), 3.93–3.85 (4H, m), 3.77 (2H, t, *J* 9 Hz), 3.51 (2H, dd, *J* 3.4, 9.4 Hz), 3.36 (2H, m), 3.30 (2H, t, *J* 9.4 Hz), 2.55 (2H, br m), 2.22 (4H, t, *J* 7.4 Hz), 1.61–1.24 (288H, m), 1.18 (6H, d, *J* 6.3 Hz), 0.88 (18H, s), 0.90 (6H, d, *J* 6.8 Hz), 0.89–0.87 (12H, t, including two t with *J* 6.9 Hz), 0.68–0.58 (2H, m), 0.49–0.38 (2H, m), 0.20–0.06 (6H, m), 0.02 (6H, s), 0.007 (6H, s); δ_C (101 MHz, $CDCl_3$+few drops of CD_3OD): 175.2, 173.8, 93.5, 73.2, 72.8, 70.8, 70.2, 70.1, 67.8, 62.9, 51.6, 50.2, 49.7, 49.5, 49.3, 49.1, 38.0, 37.3, 34.7, 34.4, 33.8, 32.0, 29.7, 29.6, 29.5, 29.42, 29.4, 29.3, 29.2, 26.3, 25.8, 25.4, 25.1, 22.7, 22.4, 20.0, 19.7, 18.6, 18.2, 14.0, 10.4, −4.6, −5.0; ν_{max}/cm^{-1}: 3421, 2922, 2853, 1732, 1728, 1465, 1375, 1253, 836, 721.

(iii) Tetrabutylammonium fluoride (0.144 mL, 0.144 mmol, 1M) was added to a stirred solution of glucopyranoside **27** (0.103 g, 0.0480 mmol) in dry THF (13 mL) at 5 °C under nitrogen. The mixture was allowed to reach rt and stirred for 20 min, then the solvent was evaporated and the residue was purified by column chromatography eluting with $CHCl_3$/MeOH (5:1) to give glucopyranoside **30** as a semi-solid (0.073 g, 89%), α_D^{21}+17 (*c* 0.78, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1728.3; $C_{103}H_{200}O_{15}SiNa$ requires 1728.4]; δ_H (400 MHz, $CDCl_3$+few drops of CD_3OD): 5.05 (2H, d, *J* 3.4 Hz), 4.85 (1H, sextet, *J* 6.2 Hz), 4.32–4.22 (2H, m), 3.92 (1H, br d, *J* 9.6 Hz), 3.86–3.78 (4H, m), 3.67 (1H, m), 3.5 (2H, dd, *J* 3.4, 9.7 Hz), 3.37–3.25 (3H, m), 2.52 (1H, ddd, *J* 3.4, 5.4, 10.3 Hz), 2.22 (2H, t, *J* 7.5 Hz), 1.58–1.50 (4H, m), 1.49–1.21 (145H, m), 1.16 (3H, d, *J* 6.3 Hz), 0.88 (9H, s), 0.7 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.66–0.57 (1H, m), 0.44–0.36 (1H, m), 0.10–0.03 (3H, m), 0.007 (3H, s), 0.015 (3H, s); δ_C (101 MHz, $CDCl_3$+few drops of CD_3OD): 175.1, 173.9, 93.5, 93.4, 73.2, 73.0, 72.6, 72.1, 71.5, 70.9, 70.7, 70.2, 69.9, 67.9, 62.4, 61.7, 51.6, 38.0, 37.3, 35.8, 34.7, 33.5, 31.8, 30.0, 29.72, 29.7, 29.6, 29.5, 29.4, 29.39, 29.3, 29.2, 29.0, 27.6, 26.9, 26.1, 25.6, 25.3, 25.0, 24.2, 22.62, 22.6, 19.8, 19.6, 18.5, 17.8, 14.6, 14.0, 10.4, −4.6, −5.0; ν_{max}/cm^{-1}: 3428, 2924, 2852, 1732, 1465, 1375, 1251, 835, 759.

(iv) A dry polyethylene vial equipped with an acid-proof rubber septum was charged with glucopyranoside **28** (0.180 gm, 0.058 mmol) and pyridine (0.07 mL) in dry THF (20 mL) and stirred at rt under nitrogen. The mixture was cooled to 10 °C, and then HF-pyridine complex (~70%, 1.35 mL) was added dropwise. The mixture was stirred at 43 °C for 17 h, then diluted with THF (5 mL). The excess of the HF was neutralized with triethylamine (2 mL), and the solvent was evaporated under high vacuum; column chromatography eluting with $CHCl_3$/MeOH 10:1 gave the glucopyranoside **29** (0.095 g, 58%), α_D^{21}+31 (*c* 0.50, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 2863.6340; $C_{182}H_{350}NaO_{19}$ requires 2863.6314]; δ_H (400 MHz, $CDCl_3$+few drops of CD_3OD): 4.98 (2H, d, *J* 3.4 Hz), 4.87 (2H, sextet, *J* 6.4 Hz), 4.68 (2H, br d, *J* 10.8 Hz), 4.24 (2H, t, *J* 9.3 Hz), 3.93 (2H, dd, *J* 3.8, 10.9 Hz), 3.73 (2H, t, *J* 9.2 Hz), 3.66–3.62 (2H, m), 3.49 (2H, dd, *J* 3.7, 9.8 Hz), 3.19 (2H, t, *J* 9.5 Hz), 2.40 (2H, br m), 2.23 (4H, t, *J* 7.4 Hz), 1.58–1.53 (8H, m), 1.52–1.22 (284 H, m), 1.16 (6H, d, *J* 6.2 Hz), 0.86 (6H, d, *J* 6.8 Hz), 0.86–0.82 (12H, t, including two t with *J* 6.9 Hz), 0.66–0.59 (2H, m), 0.43–0.37 (2H, m), 0.18–0.04 (6H, m); δ_C (101 MHz, $CDCl_3$+few drops of CD_3OD): 175.7, 173.6, 95.2, 72.5, 71.1, 70.9, 70.7, 69.8, 64.7, 52.1, 38.0, 37.3, 34.7, 34.4, 33.8, 32.0, 29.7, 29.6, 29.5, 29.42, 29.4, 29.3, 29.2, 29.0, 27.2, 26.0, 25.3, 25.0, 22.7, 22.6, 19.9, 19.5, 18.5, 14.0, 10.4; ν_{max}/cm^{-1}: 3432, 2918, 2850, 1721, 1712, 1470.

(v) A dry polyethylene vial equipped with an acid-proof rubber septum was charged with glucopyranoside **30** (0.062 gm, 0.038 mmol) and pyridine (0.06 mL) in dry THF (10 mL) and stirred at rt under nitrogen. The mixture was cooled to 10 °C, and then HF-pyridine complex (~70%, 0.5 mL) was added dropwise. The mixture was stirred at 43 °C for 17 h then worked up and purified as above, eluting with $CHCl_3$/MeOH (5:1) to give the title glucopyranoside **31** as a white solid (0.035 mg, 58%), α_D^{21}+26 (*c* 1.12, $CHCl_3$), mp 116–117 °C [MALDI-Found $(M+Na)^+$: 1614.3698; $C_{97}H_{187}NaO_{15}$ requires 1614.3684]; δ_H (400 MHz, $CDCl_3$+few drops of CD_3OD): 5.08 (1H, d, *J* 2.8 Hz), 5.02 (1H, d, *J* 3.0 Hz), 4.88 (1H, sextet, *J* 6.3 Hz), 4.69 (1H, d, *J* 11.3 Hz), 4.22 (1H, t, *J* 9.1 Hz), 3.99–3.90 (2H, m), 3.88–3.78 (3H, m), 3.63–3.54 (3H, m), 3.5 (1H, dd, *J* 2.9, 10 Hz), 3.28 (1H, t, *J* 9.4 Hz), 3.21 (1H, t, *J* 9.4 Hz), 2.38 (1H, m), 2.23 (2H, t, *J* 7.5 Hz), 1.58–1.50 (4H, m), 1.49–1.21 (147H, m), 1.17 (3H, d, *J* 6.2 Hz), 0.86 (3H, d, *J* 6.8 Hz), 0.86–0.83 (6H, t, including two t with *J* 6.9 Hz), 0.66–0.59 (1H, m), 0.44–0.36 (1H, m), 0.18–0.04 (3H, m); δ_C (101 MHz, $CDCl_3$+few drops of CD_3OD): 175.4, 173.9, 94.32, 94.3, 74.4, 73.3, 72.5, 71.4, 71.3, 71.2, 70.7, 64.0, 62.9, 52.4, 38.0, 37.2, 35.7, 34.5, 34.3, 31.7, 30.1, 29.9, 29.5, 29.3, 29.2, 29.1, 28.1, 27.3, 25.3, 25.1, 24.9, 22.6, 19.8, 19.5, 18.7, 14.0, 10.3; ν_{max}/cm^{-1}: 3357, 2919, 2851, 1730, 1467, 1375, 759, 721.

5.17. 6-*O*-[(*R*)-2-((*R*)-1-Hydroxy-17-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-21-oxo-nonadecan-2-yl)-cyclopropyl)nonadecyl)tetracosanoate]-α-D-glucopyranosyl-(1-1′)-6′-*O*-[(R)-2-((*R*)-1-hydroxy-17-((1S,2R)-2-((S)-19-((*S*)-eicosan-2-yloxy)-21-oxononadecan-2-yl)cyclopropyl)nonadecyl)tetracosanoate]-α-D-glucopyranoside (32) and 6-*O*-[(*R*)-2-((*R*)-1-hydroxy-17-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-21-oxononadecan-2-yl)cyclo-propyl)nonadecyl)tetracosanoate]-α-D-glucopyranosyl-(1-1′)-α-D-gluco-pyranoside (33)

Compound **32**, a waxy colourless solid, was prepared as described in detail in the supplementary section. It showed α_D^{21}+34.2 (*c* 1.32, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 2863.6353, $C_{182}H_{350}NaO_{19}$ requires: 2863.6314]; δ_H ($CDCl_3$+few drops of CD_3OD): 4.98 (2H, d, *J* 3.4 Hz), 4.87 (2H, sext, *J* 6.2 Hz), 4.68 (2H, br d, *J* 10.9 Hz), 4.24 (2H, t, *J* 8.8 Hz), 3.93 (2H, m), 3.73 (2H, t, *J* 9.2 Hz), 3.66–3.62 (2H, m), 3.49 (2H, dd, *J* 3.4, 9.7 Hz), 3.19 (2H, t, *J* 9.5 Hz), 2.40 (2H, br m), 2.23 (4H, t, *J* 7.5 Hz), 1.58–1.53 (8H, m), 1.52–1.22 (284H, m), 1.16 (6H, d, *J* 6.2 Hz), 0.86 (6H, d, *J* 6.8 Hz), 0.86–0.82 (12H, br t, *J* 6.9 Hz), 0.66–0.59 (2H, m), 0.43–0.38 (2H, m), 0.18–0.05 (6H, m); δ_C ($CDCl_3$+few drops of CD_3OD): 175.5, 174.1, 94.9, 72.5, 71.8, 71.3, 71.0, 70.6, 69.8, 64.7, 52.1, 38.0, 37.3, 34.7, 34.4, 33.8, 31.9, 29.7, 29.6, 29.5, 29.42, 29.4, 29.3, 29.2, 29.0, 27.3, 26.0, 25.3, 25.0, 22.7, 22.6, 19.8, 19.5, 18.5, 14.0, 10.5; ν_{max}/cm^{-1}: 3371, 2918, 2850, 1732, 1467, 758, 723.

Compound **33**, a colourless waxy solid, showed α_D^{21}+33 (*c* 1.3, $CHCl_3$), mp 115–117 °C [MALDI-Found $(M+Na)^+$: 1614.3674, $C_{97}H_{187}NaO_{15}$ requires: 1614.3684]; δ_H ($CDCl_3$+few drops of CD_3OD): 5.07 (1H, d, *J* 3.1 Hz), 5.02 (1H, d, *J* 3.2 Hz), 4.88 (1H, sext, *J* 6.2 Hz), 4.69 (1H, d, *J* 11.3 Hz), 4.22 (1H, t, *J* 9.1 Hz), 3.99–3.90 (2H, m), 3.88–3.78 (3H, m), 3.63–3.54 (3H, m), 3.5 (1H, dd, *J* 2.9, 10 Hz), 3.28 (1H, t, *J* 9.4 Hz), 3.21 (1H, t, *J* 9.3 Hz), 2.38 (1H, m), 2.23 (2H, t, *J* 7.5 Hz), 1.58–1.50 (4H, m), 1.49–1.21 (147H, m), 1.17 (3H, d, *J* 6.2 Hz), 0.86 (3H, d, *J* 6.8 Hz), 0.86–0.83 (6H, br t, *J* 6.9 Hz), 0.67–0.59 (1H, m), 0.44–0.37 (1H, m), 0.18–0.04 (3H, m); δ_C ($CDCl_3$+few drops of CD_3OD): 175.5, 173.9, 94.3, 94.2, 74.5, 73.3, 72.5, 71.5, 71.3, 71.2, 70.6, 63.9, 62.9, 52.4, 38.0, 37.2, 35.7, 34.5, 34.4, 33.4, 31.7, 30.0, 29.9, 29.6, 29.3, 29.2, 29.1, 28.1, 27.3, 25.9, 25.2, 25.0, 24.9, 22.5, 19.8, 18.8, 18.4, 14.0, 10.3; ν_{max}/cm^{-1}: 3369, 2922, 2852, 1732, 1466, 1373, 759.

5.18. In vitro stimulation

BMDCs were generated as previously described.[72] Briefly, murine bone marrow from femur and tibia was flushed with PBS and red blood cells were lysed with Sigma's lysing buffer. After lysis, cells were cultured in a T75 flask 5% CO_2 at 37 °C in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Greiner), 10 ng/mL recombinant murine GM-CSF (Immunotools), 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/mL gentamycin (GIBCO) and non-essential amino acids (Thermo Fisher Scientific) for 7 days. Synthetic glycolipids were coated on flat bottomed culture plates (Greiner). BMDCs were harvested after 7 days and seeded in glycolipid-coated plates at a concentration of 10^6 cells/mL for 24 h. The level of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were evaluated by ELISA (eBiosciences).

5.19. In vivo assay

Water in oil in water emulsions (w/o/w) were prepared according to a previously described protocol.[73] Briefly, glycolipids were dissolved in 3.2% of Incomplete Freund's Adjuvant and vigorously vortexed. Next, PBS containing 0.2% of Tween 80 was added and the mixture was again vortexed. Groups of 4–5 mice were injected subcutaneously in both hind footpads with 25 μL of w/o/w containing 5 μg of glycolipids. Footpad swelling in individual mice was measured after 3 days with a caliper.

Acknowledgements

SGT wished to thank the Ministry of Higher Education in Kurdistan Region-Iraq and HMA the Government of Iraq for the award of PhD studentships. We wish to thank Dr. Paul Gates of Bristol University for carrying out accurate mass MALDI determinations. HGT holds a FRIA PhD fellowship. This research was partially funded by the Interuniversity Attraction Pole Programme IAP 7/32 and by TBVAC2020 under grant agreement no. H2020-PHC-643381.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2016.05.004.

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3.2 Inflammatory properties and adjuvant potential of synthetic glycolipids homologous to mycolate esters of the cell wall of *Mycobacterium tuberculosis*

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❖ Context

We have previously demonstrated that synthetic trehalose glycolipids are pro-inflammatory *in vitro* and *in vivo* and the inflammation induced *in vitro* by oxygenated TDM is higher than the one induced by alpha-TDM (chapter 3.1). In this chapter, we aimed to analyze in more detail the influence of the structure of glycolipids on their inflammatory potential and extend by analyzing their adjuvant properties in vivo. In this study, we did not focus only on trehalose based glycolipids (TDM and TMM) but also included glucose and arabinose monomycolates (GMM and AraMM). Indeed, the signaling pathway involved in the activation of cells by GMM and AraMM is not clearly elucidated. Thus, we were also interested in analyzing the pathway involved in the activation of cells by synthetic GMM and AraMM. To do that, the team of Prof. Mark Baird synthetized a series of 38 synthetic glycolipids that varies in term of the nature of sugar (trehalose, glucose or arabinose), the class (alpha-, methoxy-and keto-) and steochemistry (cis- and trans-) of MA binds to these sugars.

❖ Results

Our results demonstrate that synthetic GMM and AraMM, like TDM and TMM, activate bone-marrow derived dendritic cells in terms of the production of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β and IL-12) and reactive oxygen species, upregulation of costimulatory molecules and activation of the inflammasome NLRP3 by mechanism dependent on Mincle. TDM induces more pro-inflammatory cytokines than the corresponding TMM and GMM. AraMM is the weakest inducer of pro-inflammatory cytokines. In addition, the classes of mycolic acids bound to sugar appear to slightly influence the activation of BMDCs. Indeed, cis-methoxy-TDM is more inflammatory than cis-keto and cis-alpha. However, there is no difference in terms of local inflammation induced and Th1/Th17 responses between cis-methoxy and cis-alpha TDM in an OVA immunization model in C57BL/6 mice. Finally, our results showed that GMM and TMM, like TDM, can promote Th1/Th17 responses in vivo in a murine model.

❖ Conclusion

In this study, we showed that the class of MA bound to sugar weakly modulates the inflammatory potential of glycolipids in vitro. We also identified novel ligands of Mincle, namely GMM and AraMM. These data suggest that in addition to hexose esters Mincle can also recognize pentose esters, even if AraMM only modesty activates BMDCs and does not induce adaptive immune response in vivo. However, this compound was found to be a stronger activator of the inflammasome after LPS priming. It would be of great interest to analyze the effect of combining AraMM with a TLR agonist *in vivo*. Finally, we demonstrated that GMM can be used as adjuvant to promote Th1 and Th17 responses and that further analysis of the potential of GMM as novel adjuvant for subunit vaccines is warranted.

Research Article

Journal of Innate Immunity

J Innate Immun
DOI: 10.1159/000450955

Received: February 22, 2016
Accepted after revision: September 21, 2016
Published online: November 18, 2016

Inflammatory Properties and Adjuvant Potential of Synthetic Glycolipids Homologous to Mycolate Esters of the Cell Wall of *Mycobacterium tuberculosis*

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Keywords

Mycobacterium tuberculosis · Glycolipids · Trehalose dimycolate · Trehalose monomycolate · Glucose monomycolate · Arabinose monomycolate · Inflammasome · Adjuvant

Abstract

The cell wall of mycobacteria is characterised by glycolipids composed of different classes of mycolic acids (MAs; alpha-, keto-, and methoxy-) and sugars (trehalose, glucose, and arabinose). Studies using mutant *Mtb* strains have shown that the structure of MAs influences the inflammatory potential of these glycolipids. As mutant *Mtb* strains possess a complex mixture of glycolipids, we analysed the inflammatory potential of single classes of mycolate esters of the *Mtb* cell wall using 38 different synthetic analogues. Our results show that synthetic trehalose dimycolate (TDM) and trehalose, glucose, and arabinose monomycolates (TMM, GMM, and AraMM) activate bone marrow-derived dendritic cells in terms of the production of pro-inflammatory cytokines (IL-6 and TNF-α) and reactive oxygen species, upregulation of co-stimulatory molecules, and activation of NLRP3 inflammasome by a mechanism dependent on Mincle. These findings demonstrate that Mincle receptor can also recognise pentose esters and seem to contradict the hypothesis that production of GMM is an escape mechanism used by pathogenic mycobacteria to avoid recognition by the innate immune system. Finally, our experiments indicate that TMM and GMM, as well as TDM, can promote Th1 and Th17 responses in mice in an OVA immunisation model, and that further analysis of their potential as novel adjuvants for subunit vaccines is warranted.

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Introduction

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis, belongs to the single genus *Mycobacterium* of the Mycobacteriaceae family. Bacteria from this family are characterised by the presence in their cell wall of high molecular weight C-60 to C-90 2-alkyl-3-hydroxy fatty acids called mycolic acids (MAs). MAs are the major lipids constituting the mycobacterial cell wall [1, 2], present as free MAs [3] or esterified to different sugars. They are predominantly bound to the cell wall with arabinogalactan as penta-arabinose tetramycolates, but they are also present as non-bound solvent extractable esters, such as trehalose mono- and dimycolate (TMM and TDM), glucose monomycolate (GMM) and glycerol monomycolate [1, 2].

Among these components, trehalose 6,6′-dimycolate (TDM) – also known as cord factor – is the major and most studied mycolate ester. Indeed, in mouse models TDM can mimic certain aspects related to *Mtb* infection, including the production of pro-inflammatory cytokines [4, 5], the induction of granuloma and procoagulant activity [6–8]. In addition, TDM can delay phagolysosome fusion [9], is involved in tissue damage and necrosis, and possesses adjuvant properties [10]. However, the natural TDM used in these different studies was isolated from mycobacteria and consisted of a complex mixture of structurally related compounds composed of mycolates from different classes. Indeed, based on the functional group(s) present on their meromycolate chain, MAs are subdivided in different classes. The principal classes are the alpha-, keto-, and methoxy-MAs. These classes differ considerably between mycobacterial species and strains [11], and appear to influence their virulence. For example, the attenuated vaccine strain *M. bovis* BCG-Pasteur lacks methoxy-MAs [12] and saprophytic mycobacteria such as *M. smegmatis* fail to produce oxygenated (keto- and methoxy-) MAs [2]. In contrast, highly pathogenic mycobacteria such as *Mtb* and *M. leprae* produce oxygenated MAs. Genetic inactivation of enzymatic pathways involved in the synthesis of oxygenated MAs resulted in in vivo-attenuated *Mtb* strains [13, 14]. Therefore, the use of synthetic analogues can be useful to discriminate the contribution of different types of TDM or of mycolate esters in general to the pathogenicity of mycobacterial infection and to the inflammatory potential of these cell wall components. Indeed, the contribution of other mycolate esters found in the cell wall of *Mtb* such as TMM, GMM, and arabinose mycolates (AraMMs) in the activation of innate immune cells following mycobacterial infection is poorly defined.

TMM is known as a key precursor for the biosynthesis of TDM and has been reported to have some similarity with TDM in terms of inflammatory potential [4, 15, 16]. GMM has been demonstrated to be an antigen that is presented by CD1b molecules to T cells [17], but the activation of innate immune cells by GMM from *Mtb* is controversial. Indeed, it was reported that Mincle (macrophage inducible C-type lectin receptor) does not recognise GMM obtained by trehalase treatment of TDM [18], but that GMM of corynebacteria (C-32 mycolates in corynebacteria vs. C-60 to C-90 mycolates in mycobacteria) binds to Mincle and activates cells [19]. Mincle and MCL (macrophage C-type lectin) have been shown to be essential receptors for the recognition of TDM [18, 20] and TMM [21], and hence crucial for the stimulation of cord factor-induced innate immune responses. On the other hand, the arabino-mycolate ester (a complex mixture of mono-AraMMs, tetra-arabinose tetra-mycolates, penta-arabinose tetra-mycolates, and hexa-arabinose tetra-mycolates) obtained by acid hydrolysis of the BCG cell wall skeleton was reported to induce the production of TNF-α by mechanisms dependent on the TLR-2/MyD88 pathway [22]. Finally, it has been shown that trehalose dibehenate (TDB), a short-acyl-chain structural analogue of TDM, activates the intracellular multiprotein complex called the NLRP3 inflammasome [23] and that this activation is essential for the Th17 responses induced by TDB [24]. For the moment, it is still unknown whether TMM, GMM, and arabinose mycolates can activate the inflammasome as well.

To address the relationship between the structure and the inflammatory power of mycolate esters and the signalling pathways involved, in this study we assayed 38 single synthetic mycolate ester isomers of high purity and known stereochemistry [25–28]. The mycolate esters tested here vary in terms of their carbohydrate moiety (trehalose, glucose, or arabinose) and in terms of the number, nature, and class of MAs bound to it. The selected mycolate esters are composed of MAs that are representative of the major classes found in the cell wall of *Mtb*, namely alpha-, methoxy-, and keto-mycolates of either the *cis* or *trans* configuration and with different chain lengths. In addition, synthetic mycolate esters composed of alpha-mycolates containing an alkene group and homologous to those found in non-pathogenic mycobacteria (such as *M. smegmatis*) were also tested here for comparison purposes. Using murine bone marrow-derived dendritic cells (BMDCs), we examined in vitro the ability

of these compounds to induce the production of pro-inflammatory cytokines (TNF-α, IL-6, IL-12, and IL-1β) and we identified the pathways involved in their inflammatory potential. We also confirmed their in vivo inflammatory potential and tested their adjuvant capacity to promote adaptive immune responses in an ovalbumin vaccination model.

Material and Methods

Mice

C57BL/6$^{wt/wt}$, MyD88$^{-/-}$, and MALT1$^{-/-}$ mice were bred and kept at the experimental animal facilities of WIV-ISP (Ukkel site, Brussels), complying with the Belgian legislation that transposes European Directive 2009/41/EC, repealing Directive 90/219/EC (EC, 2009). For some experiments, female C57BL/6 mice aged 6–8 weeks were purchased from Janvier Labs. Breeding pairs of MyD88$^{-/-}$ mice were kindly provided by C. Desmet (Cellular and Molecular Physiology, GIGA-Research, ULg, Belgium). MALT1$^{-/-}$ mice were as described [29]. Bones (femurs and tibia) of Mincle$^{-/-}$, FcRγ$^{-/-}$, and NLRP3$^{-/-}$ mice were provided by R. Lang (University Clinics Erlangen, Germany) and B. Ryffel (CNRS, Orleans, France).

Preparation of Glycolipid-Coated Plates

Stocks of synthetic glycolipids (synthetised at Bangor University) [25–28, 30, 31], TDB (Invivogen), and natural TDM from *Mtb* (Adipogen) were solubilised in chloroform-methanol solution (9:1) at 5 mg/mL. The different glycolipids were then dissolved in isopropanol (ISO) and coated on flat-bottomed culture plates (Greiner). After evaporation of the solvent, the plates were used directly or stored at –20°C. The final concentrations are indicated in the figure legends and Results section. For the experiments performed at equimolar concentrations, the molarity of natural TDM (a mix) was calculated using an estimated molecular weight of 2,754 g/mol. A possible negative effect of the tested glycolipids on cell viability was tested by Alamar blue assay and no increased mortality was observed under the test conditions.

Generation of BMDCs

BMDCs were generated as previously described [32]. Briefly, murine bone marrow from the femur and tibia was flushed with PBS and red blood cells were lysed with Sigma's lysing buffer. After lysis, cells were cultured (5% CO_2 at 37°C) in a T75 flask in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10 ng/mL recombinant murine GM-CSF (Immunotools), 5×10^{-5} M 2-mercaptoethanol, 100 μg/mL gentamycin (GIBCO), 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids (Thermo Fisher Scientific), and 10% foetal calf serum (FCS; Greiner). Cells were differentiated for 6–7 days and the medium was replaced every 3 days. Ninety per cent of CD11c-positive cells were obtained after differentiation.

In vitro Stimulation with Glycolipids and Analysis of Samples

BMDCs were harvested after 7 days using a cell scraper, counted and seeded in glycolipid-coated plates at a concentration of 10^6 cells/mL for different time periods. Culture supernatants were collected after 24 h and the content of pro-inflammatory cytokines TNF-α, IL-12p40, IL-1β, and IL-6 was determined by ELISA (eBiosciences).

The expression of costimulatory molecules was analysed after 48 h of incubation with the different compounds. Cells were labelled with anti-mouse CD80, anti-mouse CD86, and anti-mouse major histocompatibility complex class II (MHC-II) monoclonal antibodies (eBiosciences). Fluorescence was analysed using a FACSCalibur flow cytometer and CELL-QUEST software (BD Biosciences).

HEK-Blue mMincle Stimulation

HEK (human embryonic kidney)-blue mMincle cells (Invivogen) stably transfected with murine C-type lectin receptor *mincle* gene, as well as the genes of the Mincle-NF-κB signalling pathway and secreted embryonic alkaline phosphatase (SEAP) under the control of NF-κB activation, were used to analyse the activation of the Mincle receptor by the compounds. HEK-blue mMincle cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose and supplemented with 10% FCS, selective antibiotics, and 2 mM glutamine. Cells were passed in a T75 flask when 70–80% confluence was reached. For the stimulation, cells were seeded in 96-well plates at a density of 5×10^4 cells/well for 24 h. Quanti-blue substrate (Invivogen) was used for the detection of SEAP in the supernatant according to the manufacturer's instructions.

Inflammasome Assays

BMDCs at 1×10^6 cells/mL were primed for 3 h with 1 μg/mL of *Escherichia coli* K12 ultra-pure LPS (Invivogen) prior to stimulation with glycolipids (on coated plates) and controls. Cells were stimulated in triplicate with 5 mM ATP (for 1 h) or 10 μg/mL of MA esters (for 5 h). For the inhibition studies, glibenclamide (50 μM; Sigma), ebselen (10 μM; Sigma), cytochalasin D (1 μM; Sigma), CA-074 methyl ester (10 μM; Sigma), and Z-VAD-FMK (10 μM; Invivogen) were added 1 h prior to glycolipid stimulation. The effect of these inhibitors on cell viability was tested by Alamar blue or MTT assay and no increased mortality was observed under the test conditions. IL-1β cytokine production in cell-free supernatant was evaluated by ELISA (eBiosciences).

For the detection of activated caspase-1 in supernatants, cells were stimulated in FCS-free medium. Triplicate samples were pooled and the proteins were concentrated by methanol-chloroform precipitation. Briefly, 500 μL of supernatant was mixed with 500 μL of methanol and 125 μL of chloroform, vortexed and centrifuged. The aqueous phase was collected, washed with methanol, mixed and centrifuged. The supernatant was discarded and the pellet was dried, reconstituted in SDS sample buffer (75 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.01% bromophenol blue and 10% glycerol) and heated for 5 min at 95°C. Proteins were separated by electrophoresis for 1.5 h at 100 V in 15% Tris-Glycine Gel and transferred to a nitrocellulose membrane using a semi-dry system (Amersham Bioscience). Three per cent BSA and TBS-Tween 20 were used as blocking and washing buffer, respectively. Monoclonal mouse anti-caspase-1 p20 Casper-1 (Adipogen) was used as the primary antibody and polyclonal goat anti-mouse IgG HRP (R&D Systems) as a secondary antibody. The revelation was done by applying ECL substrate (GE Healthcare Life Sciences) on the membrane and pictures were taken using an ImageQuant LAS 4,000 device.

Reactive Oxygen Species Assay

Reactive oxygen species (ROS) were analysed with cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay according to the manufacturer's protocol (Thermo Fisher Scientific). Briefly, BMDCs (10^6 cells/mL) were pre-incubated for 30 min in PBS to allow the dye to enter the cells, washed and seeded in 24-well coated plates for 4 h. ROS levels were analysed by flow cytometry. The results were analysed using a FACSCalibur flow cytometer and CELL-QUEST software (BD Biosciences).

Footpad Immunisation with Ovalbumin

Water in oil in water (w/o/w) emulsions were prepared by minor modification of a previously described protocol [33]. Briefly, glycolipids were dissolved in 30% incomplete Freund's adjuvant (IFA) and vortexed vigorously. Next, 0.1 M PBS was added and vortexed again vigorously to make a water in oil emulsion, and finally PBS containing 0.2% of Tween 80 and the required amount of ovalbumin was added to the water in oil preparation to make a w/o/w emulsion. Groups of 4–5 mice were injected subcutaneously in both hind footpads with 25 μL of the emulsion containing 30% IFA, 5 μg of glycolipids, and 25 μg of ovalbumin (Invivogen). Footpad swelling in individual mice was measured with a caliper before and at several time points after injection.

Analysis of Local Footpad Inflammation

One week after the administration of the w/o/w samples, the mice were sacrificed and the tissues of the hind footpads were collected and digested using collagenase and DNAse (Sigma). Cells were collected and strained with a 100-μm nylon cell strainer (BD Biosciences). Total RNA was isolated from footpad cells with Trireagent (Sigma-Aldrich) according to the manufacturer's instructions. cDNA was prepared using the GoScript Reverse Transcriptase system (Promega). qRT-PCR was performed on Stratagene 3000p using the GoTaq qPCR Master mix (Promega) according to the manufacturer's instructions. The forward and reverse primers used are described below. GAPDH was used as the reference housekeeping gene for the normalisation. Additionally, footpad cells were incubated with Ly6G-Ly6C PE and viability dye V450 (eBiosciences), and the fluorescence was analysed with a FACSverse device.

Primers

CXCL1 forward: 5′-CCGAAGTCATAGCCACACTCAA-3′; CXCL1 reverse: 5′-GCAGTCTGTCTTCTTTCTCCGTTAC-3′; IL-1β forward: 5′-TTGACGGACCCCAAAAGAT-3′; IL-1β reverse: 5′-AGCTGGATGCTCTCATCAGG-3′; IL-12p35 forward: 5′-CCTCGGCATCCAGC-3′; IL-12p35 reverse: 5′-TCTGGCCGTCCTCA-3′; TNF-α forward: 5′-CATCTTCTCAAAATTCGAGTGACAA-3′; TNF-α reverse: 5′-TGGGAGTAGACAAGGTACAACCC-3′; GAPDH forward: 5′-TCGGCCTTGACTGTGCCGTT-3′; GAPDH reverse: 5′-TCCCAGCCTCGTCCCGTAGAC-3′.

Analysis of Adaptive Immune Responses

One week after the subcutaneous administration of the different w/o/w preparations, mice were sacrificed, popliteal and inguinal lymph nodes were removed and passed through a 100-μm nylon cell strainer (BD Biosciences). Cells were counted and stimulated in RPMI medium supplemented with 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 10% FCS in round-bottomed 96-well plates with 5 μg/mL of ovalbumin, culture medium as a negative control and Concanavalin A (4 μg/mL; Sigma) as a positive control. Cell-free culture supernatants were harvested after 24 and 72 h of incubation at 37°C, 5% CO_2. Levels of IL-2 were measured in 24-h supernatants by ELISA (eBiosciences). Levels of IFN-γ and IL-17A were determined in 72-h supernatants by ELISA (BD Pharmingen and eBiosciences, respectively).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). For the statistical analysis of the in vitro experiments, data from 3–4 independent experiments were pooled and tested for a Gaussian distribution with the D'Agostino Pearson test followed by pairwise comparisons performed by the Mann-Whitney test. For the analysis of the in vivo experiments, pairwise comparisons were performed by Mann-Whitney test. For all analyses, $p < 0.05$ was considered statistically significant.

Results

Synthetic TDMs and TMMs Are Inflammatory

To study the influence of the class of mycolates and the number of mycolate chains on the inflammatory potential of trehalose mycolate esters, 17 TDMs and 13 TMMs were synthetised (Table 1, TDM and TMM columns) [25–28, 30, 31]. These compounds vary in terms of their MA class (alpha-, methoxy-, keto-), the configuration of their cyclopropanation (*cis*- vs. *trans*-), and the number of MAs bound to trehalose (dimycolates vs. monomycolates). BMDCs were incubated for 24 h with the different synthetic TMMs, TDMs, evaporated ISO as a negative control, trehalose-6,6-dibehenate (TDB), or TDM isolated from *Mtb* (TDM natural mix) as positive controls. The production of the pro-inflammatory cytokines IL-6 and TNF-α was evaluated by ELISA in culture supernatants and the results obtained for 10 TDMs and 9 TMMs at equimolar concentrations are shown in Figure 1, while results obtained at 10 μg/mL for 16 TDMs and 11 TMMs are shown in online supplementary Table S1 (see www.karger.com/doi/10.1159/000450955 for all online suppl. material). All the tested synthetic TMMs and TDMs stimulated the production of TNF-α and IL-6. Interestingly, when dose-response analysis was performed with pairs of TMMs and TDMs composed of the same mycolate moiety, we observed that the number of mycolate chains bound to trehalose influenced the level of TNF-α and IL-6 produced. Indeed, as shown in Figure 1a and b, at equimolar concentrations the *cis*-methoxy TMM KB51 (monomycolate) induced TNF-α and IL-6 levels that were 2- to 3-fold lower than those achieved by stimulation with the corresponding *cis*-methoxy TDM KB52 (dimycolate).

Table 1. Structure, class, code names, and reference to synthesis of the tested synthetic mycolate ester

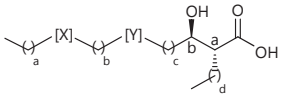
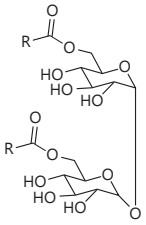
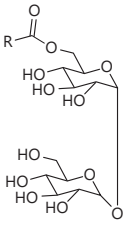
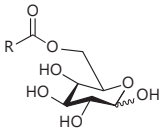
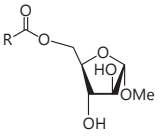
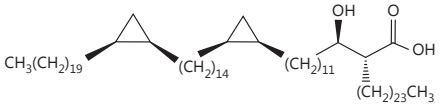
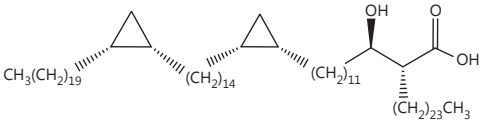
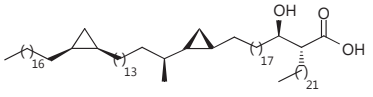
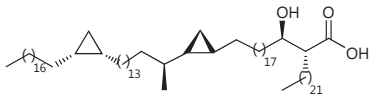
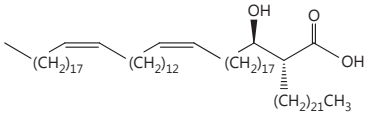
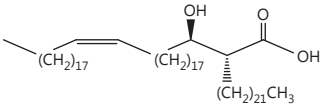
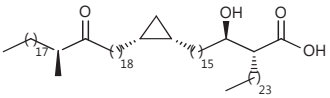
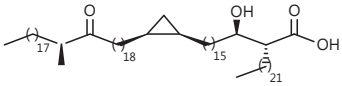
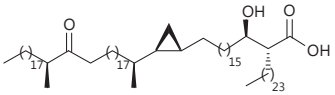
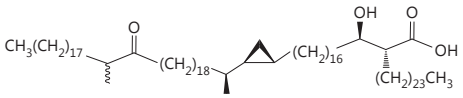
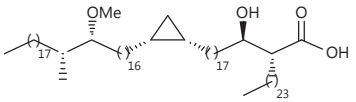
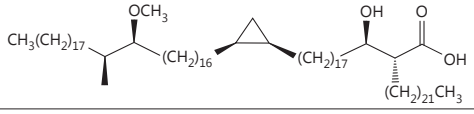
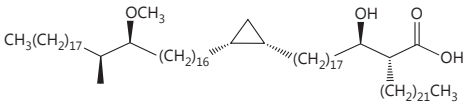
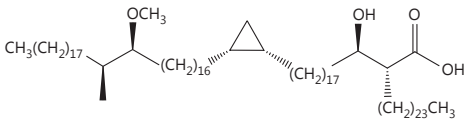
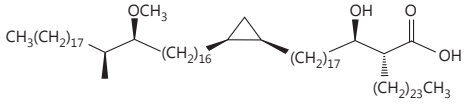
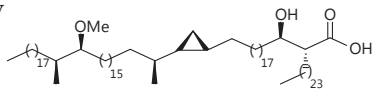
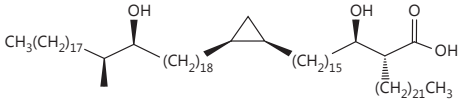
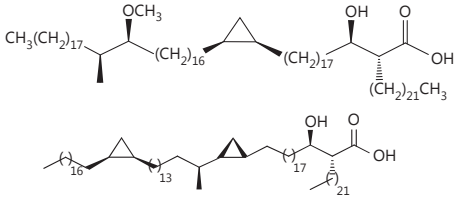
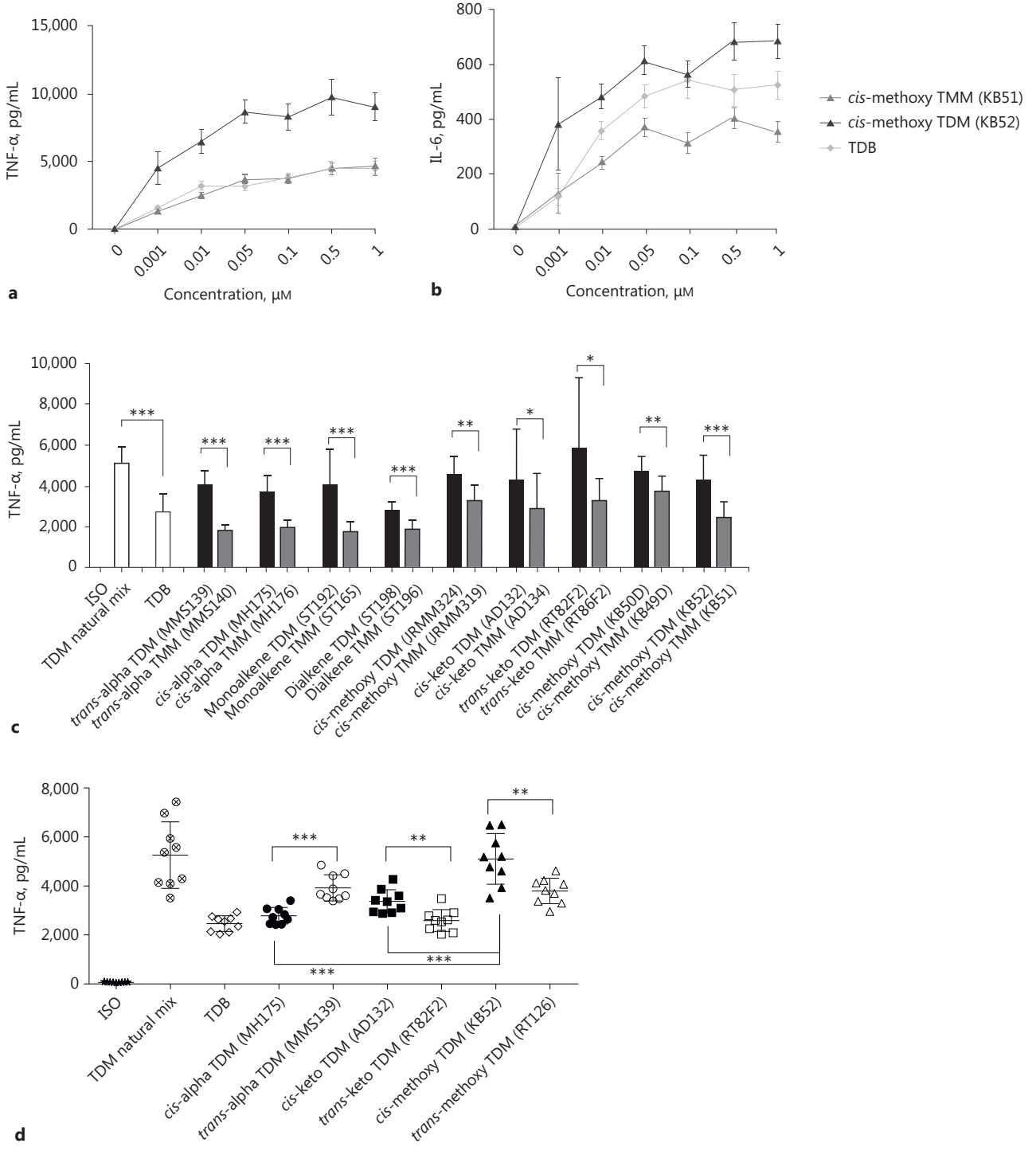
Class of mycolic acid	Structure of mycolic acid	TDM	TMM	GMM	AraMM
					
Alpha mycolic acids					
α		JRMM337 [27]	JRMM336 [27]		
α		MH175 [27]	MH176 [27]	SMP74 [26]	
α		MMS144 [28]	MMS146 [28]		
α		MMS139 [28]	MMS140 [28]		
α-diene		ST198 [27]	ST196 [27]		
α-alkene		ST192 [27]	ST165 [27]		
Keto mycolic acids					
cis-keto		AD132 [27]	AD134 [27]		
cis-keto			RT136 [27]		
cis-keto		RT137 [31]			
trans-keto		RT82 [31]	RT86 [31]		

Table 1 (continued)

Class of mycolic acid	Structure of mycolic acid	TDM	TMM	GMM	AraMM
trans-keto				SMP75 [26]	MOD23 [25]
Methoxy mycolic acids					
cis-methoxy		AD104 [27]	AD118 [27]		
cis-methoxy		KB52 [31]	KB51 [31]	SMP73 [26]	MOD16 [25]
cis-methoxy		KB50 [31]	KB49 [31]	SMP70 [26]	
cis-methoxy		KB91 [27]		SMP71 [26]	
cis-methoxy		JRMM324 [27]	JRMM319 [27]		MOD18 [25]
trans-methoxy		RT126 [27]			
Hydroxy mycolic acids					
Hydroxy		RT141 [27]			
Mixed mycolic acids					
Mixed methoxy and α-mycolates		KB55 [31]			

TNF-α levels induced by KB51 were comparable to those induced by TDB, while IL-6 levels induced by KB51 were lower than those obtained by TDB. This influence of the number of mycolate chains on the intensity of the inflammatory response was observed for a total of 9 TMM/TDM pairs representative of the different classes and configurations of mycolates present in mycobacteria (Fig. 1c). Indeed, statistically significant differences in the levels of TNF-α (Fig. 1c) and IL-6 (data not shown) were observed when comparing the data obtained for a given TDM with

the data obtained for the corresponding TMM (same mycolate moiety). In addition, the majority of the tested TDM compounds induced significantly more TNF-α compared to TDB (statistical analysis not shown). Only the dialkene TDM (ST198) and the *cis*-keto TDM (AD132) induced TNF-α levels comparable to those induced by TDB (Fig. 1c). Concerning the influence of the class of MAs on the levels of inflammatory responses induced by



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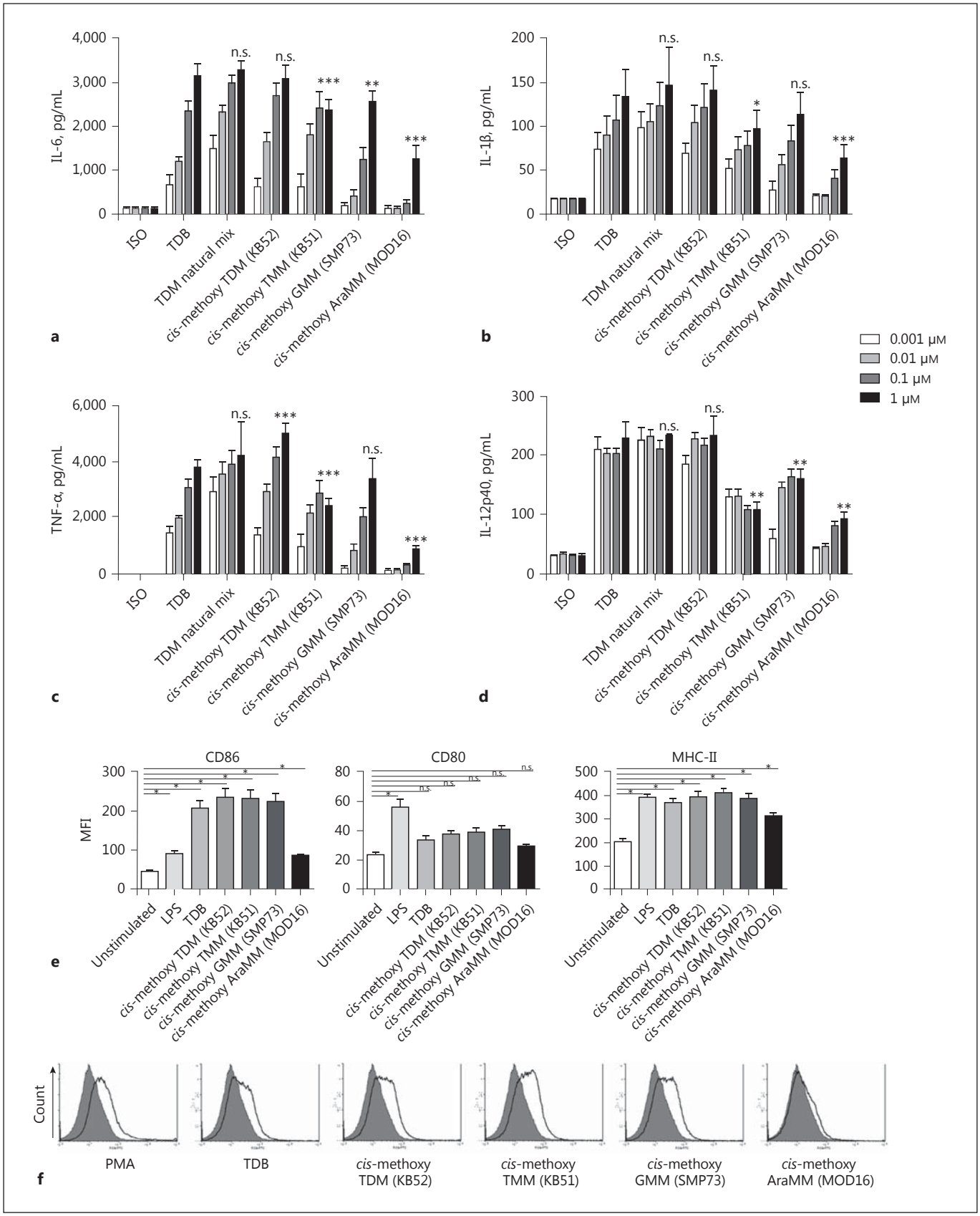
TDM, 8 synthetic TDMs representative of the different classes and configurations of mycolates present in mycobacteria were selected and a dose-response analysis was performed. All the tested synthetic compounds induced significant TNF-α production already at the lowest 0.001 μM concentration tested and a response plateau was observed at 0.1–1 μM (online suppl. Fig. S1). To compare the responses induced with TDMs from the same class but of different configurations (*cis*- vs. *trans*-), data obtained in 3 independent experiments at 1 μM with alpha, keto, or methoxy TDMs of *cis*- or *trans*-configuration were pooled and the differences were analysed statistically (Fig. 1d). Results indicated that the tested *trans*-alpha TDM (MMS139) was more inflammatory than the tested *cis*-alpha TDM (MH175), while for the oxygenated mycolates *cis*- compounds were more inflammatory than *trans*- compounds (Fig. 1d). When the results obtained for *cis*- compounds are statistically compared, *cis*-methoxy-compound (KB52) induces higher TNF-α levels compared to *cis*-alpha TDM (MH175) or *cis*-keto TDM (AD132).

Synthetic AraMMs and GMMs Are Inflammatory

We next assessed the importance of the sugar moiety on the inflammatory potential of synthetic glycolipids. For that purpose, levels of pro-inflammatory cytokines produced by a *cis*-methoxy mycolate bound to trehalose (TDM-KB52 or TMM-KB51), or to glucose (GMM-SMP73) or arabinose (AraMM-MOD16) were evaluated. As shown in Figure 2a–d, significant TNF-α, IL-6, IL-12p40, and IL-1β levels were measured in BMDC culture supernatants with all the tested compounds after 24 h of incubation. At the highest concentration tested (1 μM), TDB induced significantly more IL-6, IL-1β, TNF-α, and IL-12p40 than *cis*-methoxy AraMM-MOD16 and TMM-KB51, whereas GMM-SMP73 – compared to TDB – induced comparable levels of IL-1β and TNF-α but less IL-12p40 and IL-6. Finally, TDM-KB52 induced similar levels of IL-6, IL-1β, and IL-12p40 but higher TNF-α levels as compared to levels achieved by TDB. Overall, these data indicate that AraMM-MOD16 is the weakest inducer of pro-inflammatory cytokines in comparison to TDM-KB52, TMM-KB51, and GMM-SMP73. In an additional experiment, we also compared the inflammatory potential of 5 synthetic GMMs and 3 AraMMs composed of mycolates from different classes and configuration (see online suppl. Fig. S2). Notably, all the tested GMMs induced TNF-α levels at least comparable to those obtained with TDB (see online suppl. Fig. S2). In contrast, the TNF-α levels induced with the 3 different synthetic AraMMs were lower than those induced with TDB or the tested GMMs. Thus, AraMMs are the weakest inducers of pro-inflammatory cytokines and TDMs the strongest inducers, while TMMs and GMMs induce responses comparable in magnitude to those observed after stimulation with TDB.

As a second read-out for the activation of BMDCs, we measured the surface expression of the costimulatory molecules CD86 and CD80, MHC-II molecules, and production of ROS induced by incubation with the synthetic glycolipids. Expression of CD86, CD80, and MHC-II was increased after 48 h of incubation with TDM-KB52, TMM-KB51, GMM-SMP73, AraMM-MOD16, and LPS and TDB controls as compared to non-stimulated BMDCs (Fig. 2e). AraMM-MOD16 (and LPS) stimulated CD86 expression to a lesser extent than the other mycolate esters. Expression of CD80 and MHC-II was increased by all 4 compounds to the same level as by TDB. TDM-KB52, TMM-KB51, and GMM-SMP73 all significantly induced the production of intracellular ROS, while

Fig. 1. Synthetic TMM and TDM esters stimulate the in vitro production of TNF-α and IL-6 by BMDCs. **a**, **b** BMDCs derived from C57BL/6 mice were stimulated for 24 h in triplicate cultures with increasing concentrations of plate-coated synthetic *cis*-methoxy TMM-KB51, *cis*-methoxy TDM-KB52, or TDB. The supernatants were harvested from separate wells and the volume of pro-inflammatory cytokines (TNF-α and IL-6) was determined by sandwich ELISA. Results are expressed as the mean amount of cytokines ± SD of 3 independent experiments. **c** Separate BMDC cultures derived from C57BL/6 mice were stimulated for 24 h in triplicate with 9 pairs of synthetic TDM versus corresponding TMM esters at 0.1 μM, with evaporated ISO as a negative control and TDB or natural TDM at the same concentration as positive controls. The supernatants were harvested and the amount of TNF-α was determined by sandwich ELISA. Results are expressed as the mean amount of TNF-α ± SD and data of 4 representative experiments have been pooled. Statistical analysis of results obtained for the TDM/TMM pairs was performed by the Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **d** Separate BMDC cultures derived from C57BL/6 mice were stimulated for 24 h in triplicate with single TDMs representative of the different classes and configurations of mycolates present in mycobacteria at 1 μM, with evaporated ISO as a negative control and TDB or natural TDM at the same concentration as positive controls. The supernatants were harvested and the amount of TNF-α was determined by sandwich ELISA. Results are expressed as the mean amount of TNF-α ± SD and data of 3 representative experiments have been pooled. Statistical analysis of the results of the different groups was performed by the Mann-Whitney test. ** $p < 0.01$ and *** $p < 0.001$.



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the weakest response was again observed for the synthetic AraMM tested (Fig. 2f).

Synthetic AraMMs and GMMs Activate BMDCs by Mechanisms Dependent on the C-Type Lectin Receptor Mincle

Several receptors have been reported to interact with mycolate esters. For example, recognition of natural arabinose mycolate esters has been described to be dependent on MyD88 and TLR-2 [22], while the C-type lectin receptor Mincle and MCL receptor have been associated with recognition of cord factor, its structural analogue TDB, TMM, and GMM from corynebacteria [18–21, 34]. In order to identify the signalling pathways involved in the inflammatory responses observed with the synthetic compounds, BMDCs were generated from C57BL/6 WT, Myd88$^{-/-}$, Mincle$^{-/-}$, FcRγ$^{-/-}$, and MALT1$^{-/-}$ mice. MALT1 and FcRγ are 2 proteins involved in the Mincle-NF-κB activation pathway. As shown in Figure 3a, TNF-α production induced by LPS was dependent on Myd88 but was independent of Myd88 for the synthetic TDM-KB52, TMM-KB51, GMM-SMP73, TDB, and natural TDM mix, and also for AraMM-MOD16, in contrast to what was reported for natural AraMM isolated from BCG [22]. TNF-α production induced by LPS was, as expected, independent of Mincle, FcRγ, and MALT1, while complete inhibition of TNF-α production was observed in BMDCs from Mincle$^{-/-}$, FcRγ$^{-/-}$, and MALT1$^{-/-}$ mice after stimulation with synthetic TDM-KB52, TMM-KB51, GMM-SMP73, TDB, natural TDM mix and with AraMM-MOD16 (Fig. 3b, c). Similar results were obtained when piceatannol was used as an inhibitor of spleen tyrosine kinase, a protein involved in NF-κB activation by Mincle (data not shown). In addition, using the HEK-blue murine Mincle reporter cell line (Invivogen), we confirmed that synthetic glucose and arabinose monomycolates are Mincle agonists (Fig. 3d). Comparable levels of NF-κB-inducible SEAP were induced by TDM-KB52, TMM-KB51, GMM-SMP73, and TDB at concentrations as low as 0.001 μM, while 100-fold-higher concentrations of AraMM-MOD16 were needed to stimulate comparable SEAP levels, suggesting a weaker interaction with Mincle. Similar results were obtained with all the tested synthetic TDMs, TMMs, GMMs, and AraMMs (see online suppl. Fig. S3 for the TDM results).

In summary, these results indicate that synthetic GMMs and AraMMs activate BMDCs by mechanisms that are independent of the Myd88 pathway but dependent on the Mincle-FcRγ-Syk MALT1 pathway, demonstrating that the C-type lectin Mincle receptor can also recognise MAs from mycobacteria bound to glucose and arabinose.

GMMs and AraMMs Activate the NLRP3 Inflammasome

It was previously shown that the production of IL-1β induced by natural TDM (mix) and TDB is associated with activation of the NLRP3 inflammasome [23]. The inflammasome is a multiprotein platform which mediates the maturation of caspase-1. Mature caspase-1 cleaves pro-IL-1β and pro-IL-18, and induces the secretion of mature IL-1β and IL-18 [35]. The activation of the inflammasome by TDB is dependent on Mincle and has been shown to be essential for its ability to induce IL-17 responses [24]. As we found that synthetic GMMs and AraMMs also bind to Mincle and induce the production

Fig. 2. Synthetic GMMs and AraMMs activate BMDCs. **a–d** BMDC cultures from C57BL/6 mice were stimulated for 24 h in triplicate with escalating concentrations of plate-coated synthetic mycolate esters composed of the same *cis*-methoxy mycolate bound twice to trehalose (TDM-KB52) or once to trehalose (TMM-KB51), glucose (GMM-SMP73), arabinose (AraMM-MOD16), or controls (natural TDM mix, TDB, or evaporated ISO). The supernatants were harvested and the volumes of pro-inflammatory cytokines (IL-6, **a**; IL-1β, **b**; TNF-α, **c**; IL-12p40, **d**) were determined by ELISA. Results are expressed as the mean amount of cytokines ± SD (n = 4 independent experiments). Statistical analysis on the results obtained for synthetic compounds and natural TDM mix compared to TDB was performed by the Mann-Whitney test. n.s., not significant. ** $p < 0.01$, and *** $p < 0.001$. **e** Upregulation of costimulatory molecules (CD86 and CD80) and MHC-II was evaluated by flow cytometric analysis after 48 h of stimulation with 10 μg/mL of synthetic mycolate esters composed of the same *cis*-methoxy mycolate bound twice to trehalose (TDM-KB52) or once to trehalose (TMM-KB51), glucose (GMM-SMP73), arabinose (AraMM-MOD16), or controls (unstimulated, LPS 100 ng/mL, or TDB 10 μg/mL). Results are the mean ± SD and representative of at least 3 independent experiments. Statistical analysis on results obtained for synthetic compounds, TDB, or LPS compared to unstimulated cells was performed by the Mann-Whitney test: n.s., not significant. * $p < 0.05$. **f** BMDCs were treated for 30 min with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and stimulated for 4 h with 10 μg/mL of synthetic mycolate esters composed of the same *cis*-methoxy mycolate bound twice to trehalose (TDM-KB52) or once to trehalose (TMM-KB51), glucose (GMM-SMP73), arabinose (AraMM-MOD16), or controls (unstimulated, PMA 100 ng/mL, or TDB 10 μg/mL). The production of ROS by the tested compound (black line) was measured by flow cytometry and compared to unstimulated cells (grey shading).

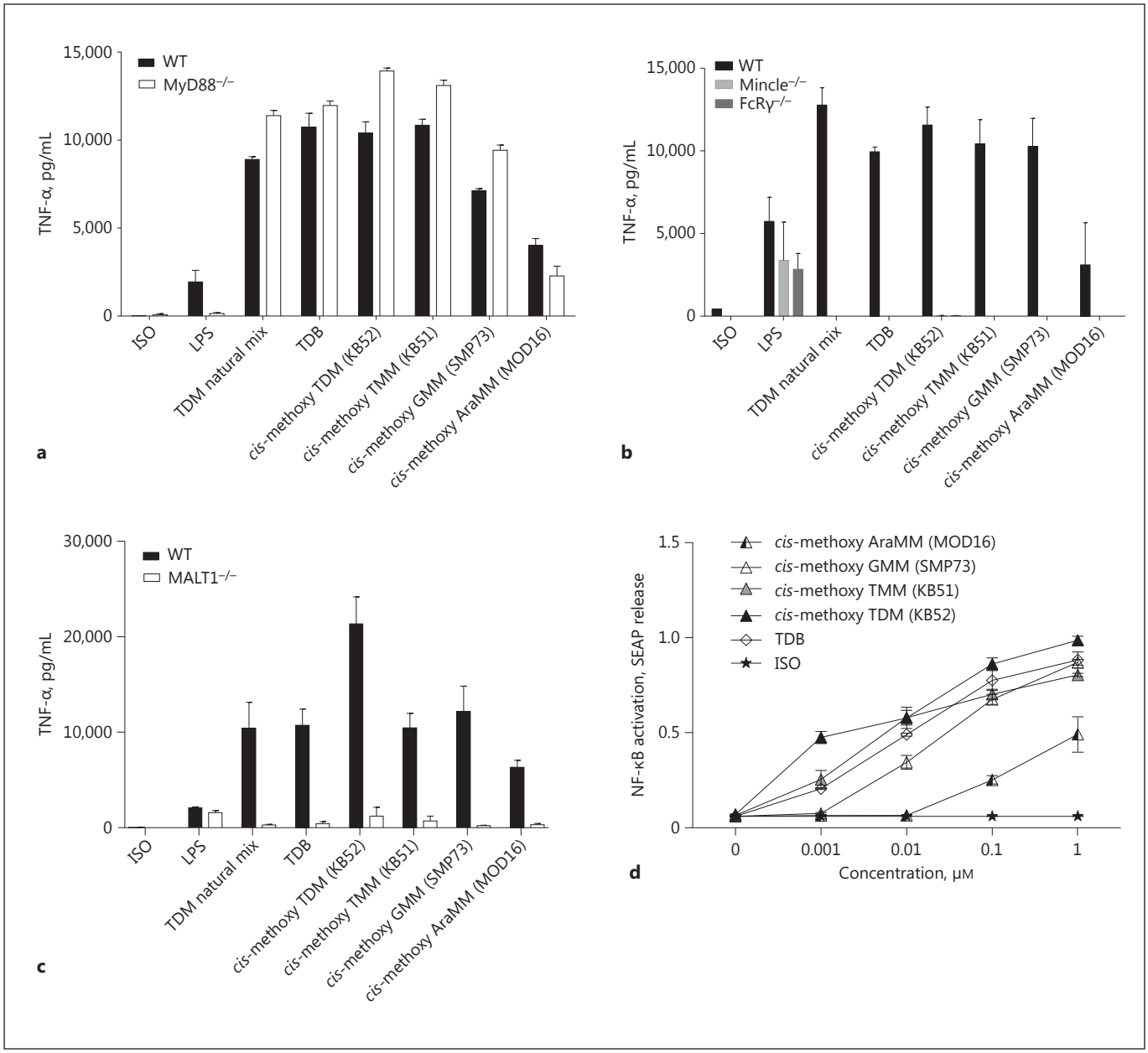
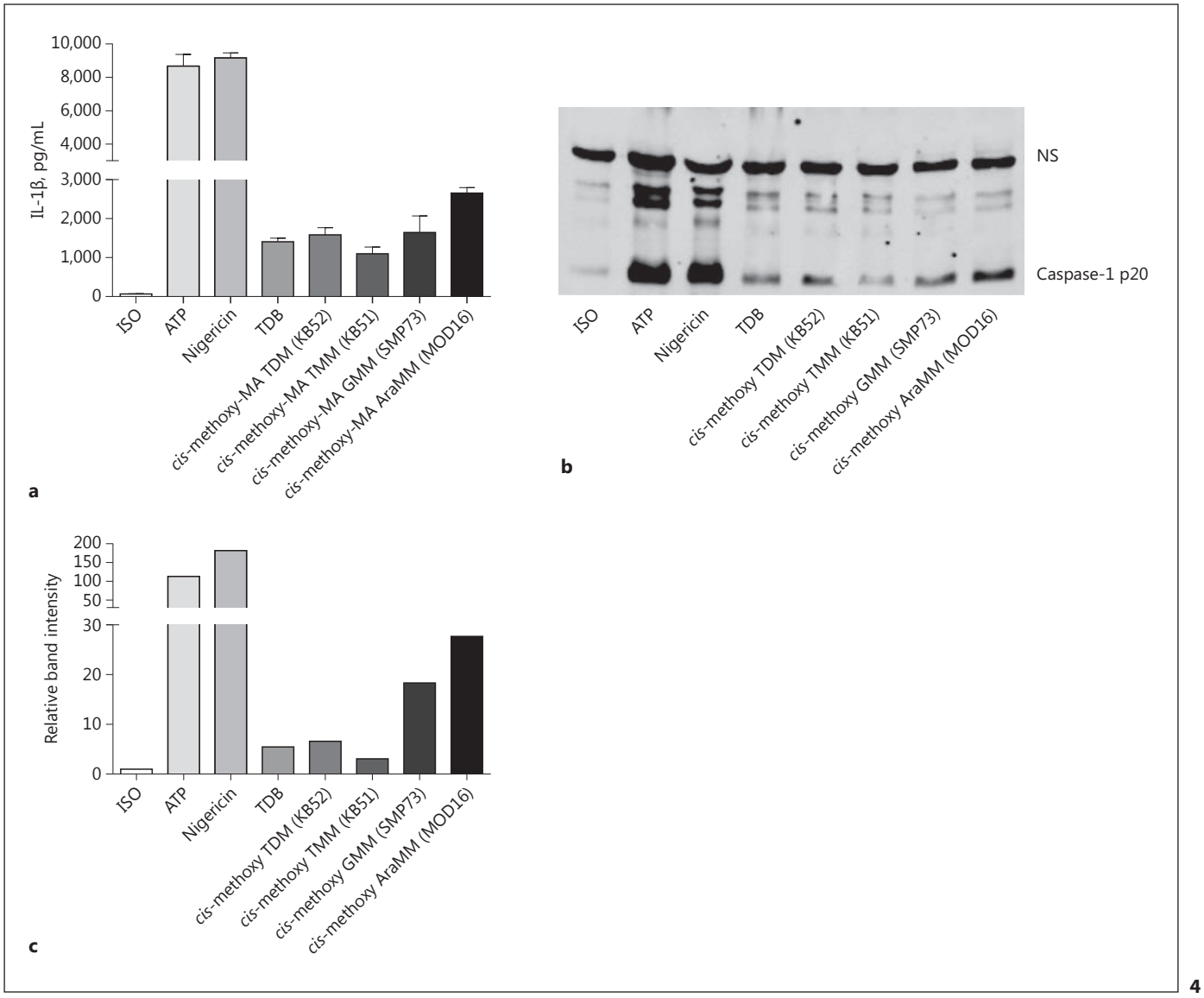


Fig. 3. Synthetic GMMs and AraMMs activate BMDCs by Mincle-dependent mechanisms. **a–c** BMDCs from C57BL/6 WT, MyD88$^{-/-}$, FcRγ$^{-/-}$, Mincle$^{-/-}$, and MALT1$^{-/-}$ mice were stimulated in triplicate with 10 μg/mL of different synthetic mycolate esters, TDB, natural TDM mix, or negative control (evaporated ISO) for 24 h. The supernatants were analysed for their content in pro-inflammatory cytokines (TNF-α) by ELISA. Results are expressed as the mean volume of TNF-α ± SD and are representative of at least 4 independent experiments. **d** HEK293 reporter cells expressing murine Mincle were used to evaluate binding to the Mincle receptor. SEAP release after NF-κB activation was evaluated by colorimetric assay using Quantie-blue substrate and by measuring the OD at 655 nm.

of ROS species, we next analysed whether these compounds could also activate the inflammasome. A selected series of 4 mycolate esters composed of the same *cis*-methoxy mycolate were tested, i.e. TDM-KB52, TMM-KB51, GMM-SMP73, and AraMM-MOD16. BMDCs were primed with LPS 3 h prior to incubation for 5 h with the tested compounds and controls. As shown in Figure 4a, synthetic *cis*-methoxy TDM-KB52, TMM-KB51, GMM-



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SMP73, and AraMM-MOD16 all stimulated IL-1β secretion by LPS-primed BMDCs to levels comparable to those achieved by natural TDM and TDB. Because the secretion of IL-1β is dependent on the maturation of caspase-1, we also analysed the secretion of the active caspase-1 p20 subunit in the supernatant and found that stimulation of LPS-primed BMDCs by TDM-KB52, TMM-KB51, GMM-SMP73, or AraMM-MOD16 was sufficient to induce caspase-1 maturation (Fig. 4b, c). Blocking of caspase activity with the pan-caspase-inhibitor Z-VAD-FMK strongly reduced the secretion of IL-1β (Fig. 4d), while, as expected, levels of secreted TNF-α were only marginally reduced following Z-VAD-FMK addition (Fig. 4e). Overall, these results indicate that synthetic TMM, GMM, and AraMM can also induce a caspase-1/inflammasome-dependent secretion of IL-1β in BMDCs. Using BMDCs derived from NLRP3$^{-/-}$ KO and caspase-1/11$^{-/-}$ mice, we observed that IL-1β induction by the tested compounds was completely abrogated in the absence of NLRP3 and significantly reduced in the absence of caspase-1/11$^{-/-}$ (Fig. 4f), demonstrating that synthetic TMM, GMM, and AraMM induce the production of IL-1β by NLRP3-dependent mechanisms (Fig. 4f). The fact that only levels of IL-1β but not of TNF-α were affected by the genetic deletion of NLRP3 or caspase-1/11 confirms that these deletions only impact inflammasome

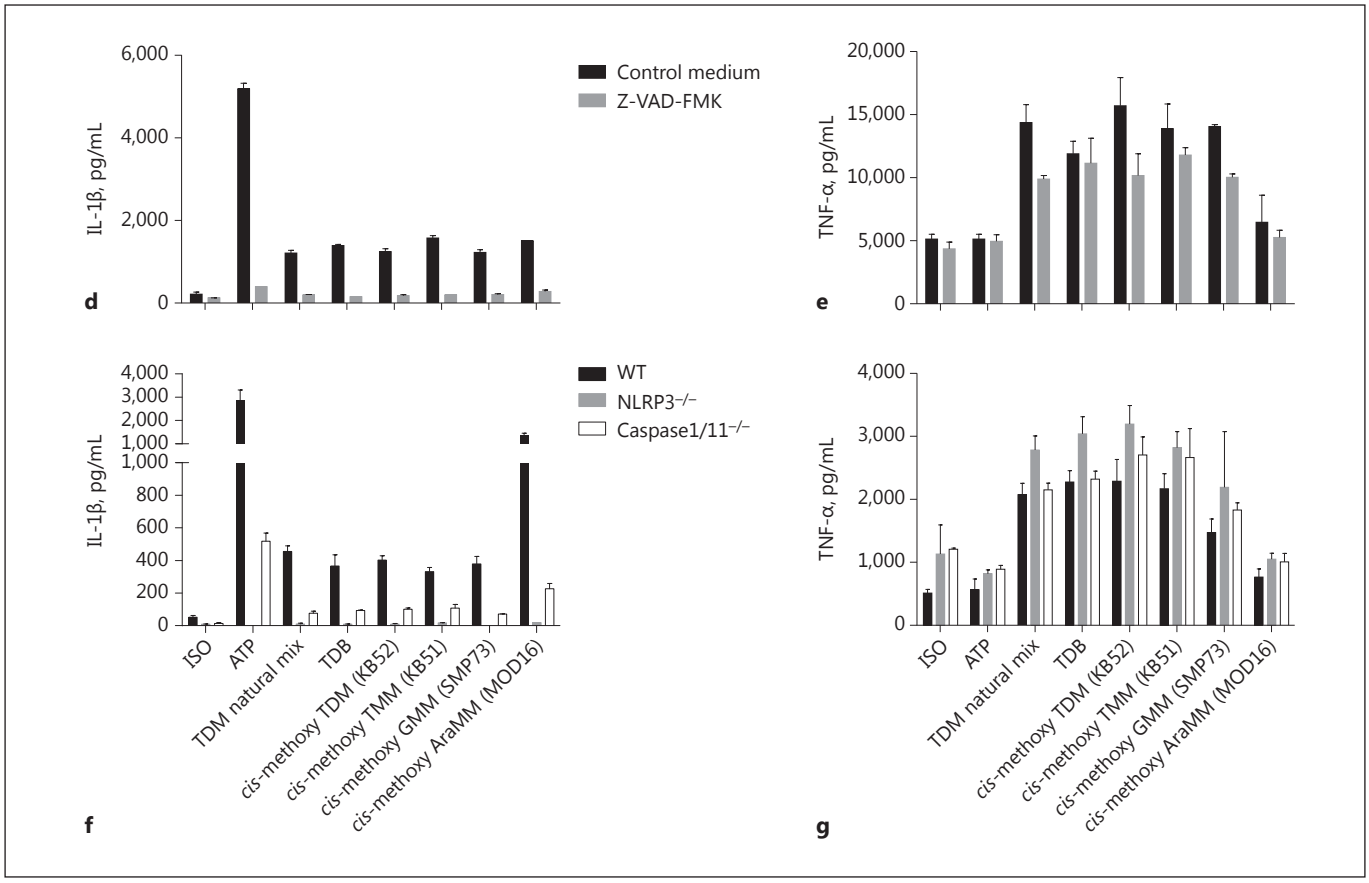

Fig. 4. Synthetic glucose and arabinose mycolate esters activate the NLRP3 inflammasome. **a** BMDCs prepared from C57BL/6 mice were primed for 3 h with 1 μg/mL of ultrapure LPS and then stimulated for 5 h with 10 μg/mL of synthetic mycolate esters, with evaporated ISO as a negative control and TDB or natural TDM mix at the same concentration as positive controls. The supernatants were harvested and the amount of IL-1β in the supernatants was determined by ELISA. **b** The secretion of active caspase-1 p20 was detected by Western blot in the supernatant after methanol-chloroform precipitation. NS, non-specific band. **c** Quantification of caspase-1 p20 in the supernatant. The relative intensity of caspase-1 p20 was determined relative to a non-specific band used as a loading control in the supernatant. **d**, **e** BMDCs from C57BL/6 mice were primed with 1 μg/mL of ultrapure LPS for 2 h, incubated with 10 μM of Z-VAD-FMK or control medium for 1 h and stimulated with 10 μg/mL of synthetic mycolate esters and controls for 5 h. Secretion of IL-1β and TNF-α was determined by ELISA in the supernatants. **f**, **g** BMDCs were prepared from WT, NLRP3$^{-/-}$, and caspase-1/11$^{-/-}$ C57BL/6 mice and primed for 3 h with 1 μg/mL of ultrapure LPS before stimulation for 5 h with 10 μg/mL of synthetic mycolate esters and controls. The amount of IL-1β and TNF-α in the supernatants was determined by ELISA. Results represent the mean ± SD of triplicate cultures and are representative of at least 2 independent experiments.

activation (Fig. 4g). As ROS production, potassium efflux, and cathepsin B activity are involved in inflammasome activation by TDB [23], we analysed whether similar mechanisms are also involved in BMDC activation by synthetic mycolate esters. To this end, production of IL-1β following incubation of BMDCs with ROS scavenger (Ebselen) and synthetic TDM-KB52, TMM-KB51, GMM-SMP73, and AraMM-MOD16 was measured. A significant reduction in IL-1β secretion was observed (see online suppl. Fig. S4A) following incubation with the different compounds in the presence of Ebselen. The inhibition of IL-1β secretion was also observed when Glibenclamide was used as an inhibitor of potassium efflux pump or when a culture medium rich in potassium was used to inhibit potassium efflux (see online suppl. Fig. S4B). Finally, blocking cathepsin B activity with the pharmacological inhibitor CA-074 and blocking actin polymerisation with cytochalasin D significantly reduced the production of IL-1β by TDB and synthetic TDM-KB52, TMM-KB51, GMM-SMP73, and AraMM-MOD16 my-

colate esters without affecting the level of IL-1β induced with the positive control ATP (see online suppl. Fig. S4C, D). In summary, these data indicate that ROS production, potassium efflux, cathepsin B activity, and phagocytosis are mechanisms involved in IL-1β induction by synthetic mycolate esters.

Synthetic Mycolate Esters Demonstrate Inflammatory and Adjuvant Properties in vivo

Our in vitro results indicated that synthetic TDMs and TMMs, GMMs, and to a lesser extent AraMMs had an inflammatory potential. To confirm these findings in vivo and to explore their possible use as novel adjuvants for subunit vaccines, w/o/w emulsions composed of IFA as a vehicle, synthetic mycolate esters, and ovalbumin as a test antigen were prepared and injected once in the 2 hind footpads of C57BL/6 mice. Footpad swelling was monitored for 1 week as a read-out of local inflammation; on day 7 we analysed the type of cells recruited in the footpad and the upregulation of proinflammatory chemokines and cytokines in these cells. Finally, OVA-specific immune responses were analysed by stimulating cells isolated from the lymph nodes draining the injection site 1 week after injection. Results obtained after injection of w/o/w emulsions composed of 30% IFA and *cis*-methoxy TDM-KB52, TMM-KB51, GMM-SMP73, or AraMM-MOD16 (10 μg/mouse) and ovalbumin (50 μg/mouse) are shown in Figures 5 and 6. Responses were compared with those obtained with a vehicle control (30% IFA + ovalbumin) or TDB w/o/w emulsions (30% IFA, TDB 10 μg/mouse and ovalbumin 50 μg/mouse). As shown in Figure 5a, neither AraMM-MOD16 nor 30% IFA vehicle control induced any footpad swelling, while TDM-KB52, TMM-KB51, and GMM-SMP73 induced a footpad swelling of 1–2 mm over 7 days, comparable to that observed for a w/o/w emulsion with TDB. Interestingly, GMM-SMP73 induced a significantly weaker footpad swelling than TDB. Cells recruited to the site of injection were analysed by flow cytometry. The analysis of cells recruited to the footpad showed a high infiltration of granulocytes (Ly6C+ and Ly6G+) for TDB, natural TDM, TDM-KB52, TMM-KB51, and GMM-SPM73, but not for AraMM-MOD16 (Fig. 5b). qRT-PCR analysis confirmed that all the positive compounds (in terms of footpad swelling and granulocyte infiltration) also induced an increased expression of pro-inflammatory cytokines (IL-12p35, IL-1β, and TNF-α) and chemokines (CXCL1) as compared to vehicle control and AraMM-MOD16 (Fig. 5c–f). No significant difference was observed between TDB, natural TDM, TDM-KB52, TMM-KB51, and GMM-SMP73. The draining lymph node cell number increased 2-fold in animals injected with these 5 compounds, while the cell number was comparable in lymph nodes from animals injected with vehicle control or the AraMM-MOD16 group (Fig. 6a). Production of OVA-specific IL-2, IFN-γ, and IL-17A was measured in draining lymph node cell cultures stimulated in vitro with ovalbumin. Formulation with AraMM-MOD16 stimulated low levels of OVA-specific IL-2, IFN-γ, and no IL-17A production, whereas formulation with TDM-KB52, TMM-KB51, and GMM-SMP73 induced elevated levels of these cytokines, comparable to those achieved by TDB (Fig. 6b–d).

Our analysis of the in vitro inflammatory properties of the synthetic mycolate esters had shown that the class of mycolate bound to a given sugar exerted only a small influence on the magnitude of the inflammatory response (Fig. 1). Nevertheless, there was a trend of the tested synthetic methoxy TDM to induce more TNF-α than TDB or the alpha- and keto-compounds. We therefore compared the in vivo responses of w/o/w emulsions composed of *cis*-alpha TDM-MH175 or TMM-MH176 with responses obtained with w/o/w emulsions composed of *cis*-methoxy TDM-KB52 or TMM-KB51. No differences between the *cis*-alpha and *cis*-methoxy mycolate esters could be observed in terms of footpad swelling and OVA-specific IFN-γ and IL-17A responses (see online suppl. Fig. S5).

Discussion

The cell wall of mycobacteria is characterised by components that interact with different arms of the immune system in the case of infection. Several of these components have been associated with virulence and the capacity to modulate host immune responses. In this work, we were interested in analysing the role of esters of MAs using pure synthetic compounds. MAs are high molecular weight C-60 to C-90 2-alkyl-3-hydroxy fatty acids typical of mycobacteria. Different classes of MAs are present in mycobacteria, mainly alpha-, keto-, and methoxy-MAs, but also alkene and diene MAs. In addition, MAs can exist in different isomeric forms (e.g. *cis*- and *trans*-stereochemistry). In the mycobacterial cell wall, MAs are present as free MAs or esterified to different sugars (trehalose, glucose, or arabinose). TDM or cord factor is probably the most abundant and best studied of the non-cell wall-bound mycolate esters, and several studies have reported that changing the class of MA in TDM influences its inflammatory properties. Indeed, TDM from the *Mtb ΔcmaA2* mutant, lacking *trans*-cyclopropanation of

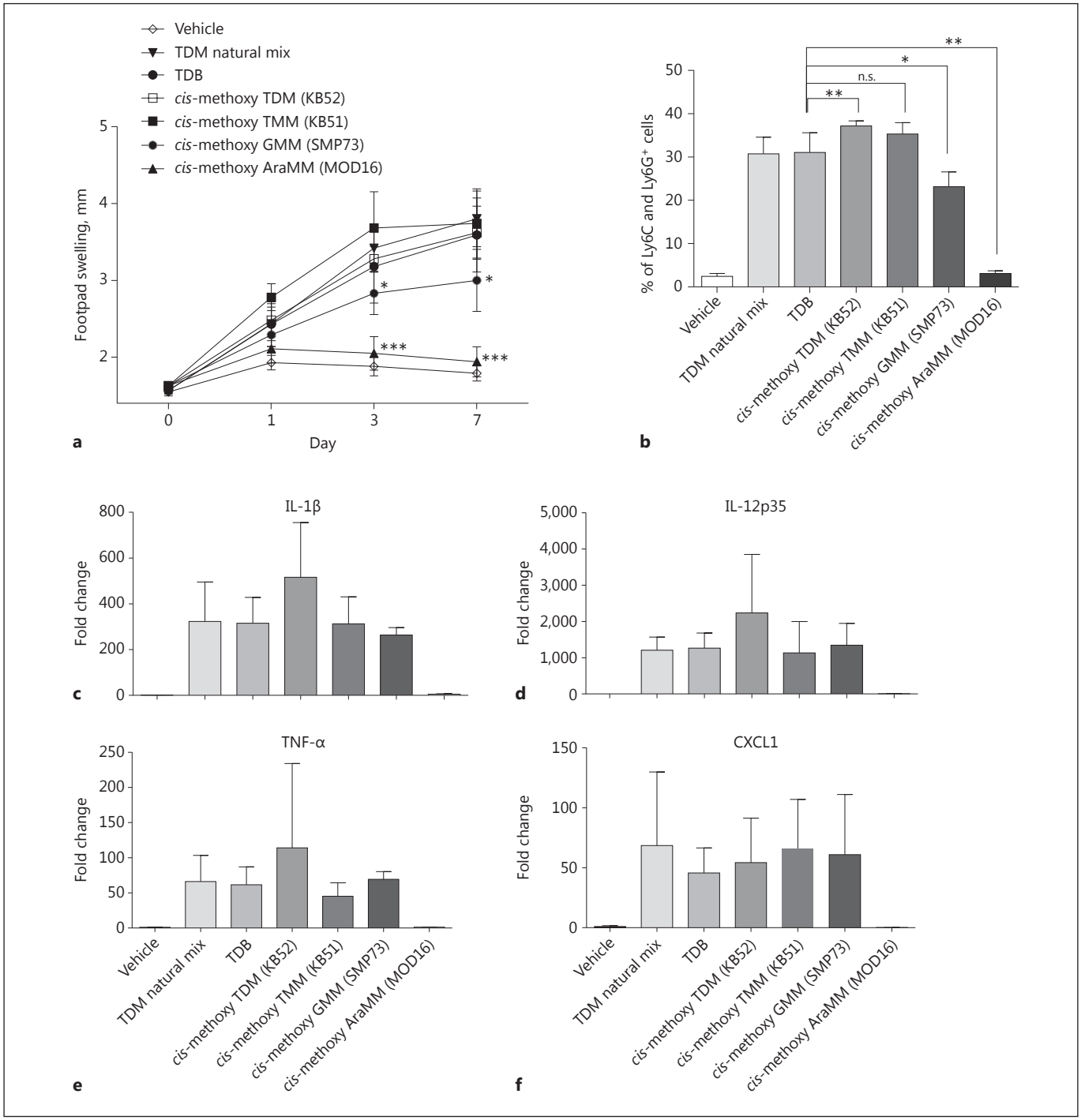

Fig. 5. In vivo inflammatory properties of synthetic mycolate esters. C57BL/6 mice were injected subcutaneously in the 2 hind footpads with w/o/w emulsions composed of 30% IFA, 10 μg/mouse of specified synthetic mycolate esters, and 50 μg/mouse of ovalbumin. In the emulsion of the vehicle control group no glycolipid was present. In the TDB control group 10 μg/mouse of TDB replaced the synthetic glycolipids. **a** Mean footpad size (mm) of 5 mice tested individually was measured with a caliper on the day of injection and 1, 3, and 7 days after injection. **b** The percentage of granulocytes (Ly6G+ and Ly6C+) was determined in cells isolated from the footpad by flow cytometry 7 days after administration. **c–f** The relative expressions of cytokines and chemokines were determined by qRT-PCR in the footpad cells. Fold changes were determined in comparison to vehicle. Footpad swelling and the percentage of granulocytes induced by synthetic mycolate esters were statistically analysed in comparison to TDB using the Mann-Whitney test. n.s., not significant. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

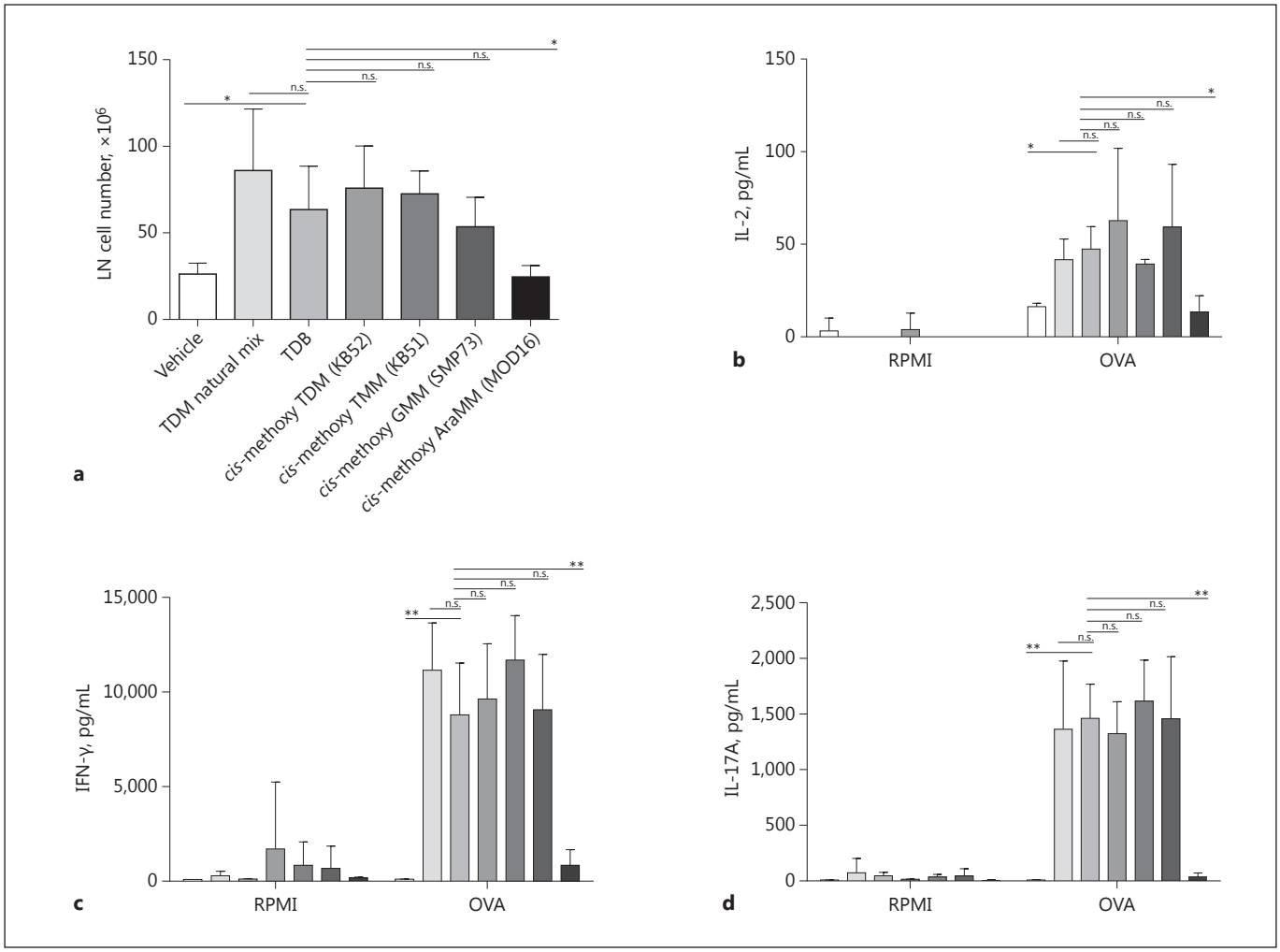

Fig. 6. In vivo adjuvant potential of synthetic mycolate esters in an experimental OVA model. **a** C57BL/6 mice were injected subcutaneously in the 2 hind footpads with w/o/w emulsions composed of 30% IFA, 10 μg/mouse of specified synthetic mycolate esters, and 50 μg/mouse of ovalbumin. In the emulsion of the vehicle control group no glycolipid was present. In the TDB control group 10 μg/mouse of TDB replaced the synthetic glycolipids. Mice were sacrificed 7 days after immunisation and cells from pooled popliteal and inguinal lymph nodes were collected, counted (**a**) and stimulated with culture medium (RPMI) or 5 μg/mL of ovalbumin (OVA) for 24 or 72 h. Supernatants were harvested and the concentrations of IL-2 (**b**), IFN-γ (**c**), and IL-17A (**d**) were determined by ELISA. Results are means ± SD of 5 mice tested individually and representative of at least 4 independent experiments. n.s., not significant. * $p < 0.05$, and ** $p < 0.01$ as determined by the Mann-Whitney test.

MAs, is hyperinflammatory for macrophages compared to TDM extracted from wild-type *Mtb* [36]. Likewise, the cord factor isolated from *Mtb ΔmmaA4,* lacking oxygenated MA classes, induces in vitro more TNF-α and IL-12 in macrophages than TDM from wild-type *Mtb* [4]. On the other hand, TDM isolated from *Mtb ΔpcaA* (an enzyme required for α-mycolate cyclopropanation) is hypoinflammatory in macrophages [5]. In this study, we analysed the inflammatory power of 17 pure synthetic TDMs along with 13 TMMs, 5 GMMs, and 3 AraMMs. Our results only partially confirm the data previously obtained using these mutant *Mtb* strains [4, 5, 36]. Indeed, the monoalkene TDM and particularly the dialkene TDM, respectively lacking 1 and 2 cyclopropanes, show a lower inflammatory potential, confirming the findings of Rao et al. [5]. However, we found that the class of mycolate (alpha- vs. keto- vs. methoxy-) and the *cis*- versus *trans*-conformation only poorly influences the intensity of the inflammatory responses induced by these compounds. *Cis*-methoxy-mycolate esters seem to be more

inflammatory compared to *cis*-alpha- and *cis*-keto-mycolate esters in vitro, but this observation was not confirmed in vivo. It is important to mention that the naturally isolated TDM preparations from *Mtb* mutants were composed of a complex mixture containing diverse mycolates in terms of class and chain length (C-60 to C-90), while the synthetic compounds tested in our study were of a particular class and generally restricted in chain length from C-74 to C-84. Synthetic compounds with chain lengths varying in a wider range between C-60 and C-90 need to be tested to make definitive conclusions. Moreover, Ostrop et al. [37] have shown that natural TDM induces a different profile of pro-inflammatory cytokines and chemokines compared to TDB in primary human APCs. These results suggest a potential role of chain length or presence of chemical groups in the activation of primary human cells. To elucidate this, it would be of great interest to compare the inflammatory potential of the synthetic compounds of our study in primary human APCs.

In addition, with this study we were able to demonstrate that besides TDMs and TMMs, GMMs and AraMMs are also inflammatory mycolate esters. Their ability to induce inflammatory responses is linked to their interaction with the C-type lectin receptor Mincle. Using BMDCs of Mincle$^{-/-}$ mice and human reporter cells expressing murine Mincle, we showed that long-chain mycobacterial GMMs – similarly to corynebacterial GMMs – can indeed interact with Mincle [19]. These results are different from those previously reported by Ishikawa et al. [18], who showed that GMM isolated by trehalase treatment of natural TDM does not interact with mouse Mincle receptor. This difference in results is possibly linked to the use of products of enzymatic treatment as compared to the use of pure synthetic compounds in our study and in the study by van der Peet et al. [19]. In addition, Matsunaga et al. [38], reported that, shortly after infection, mycobacteria have a downregulated production of TDM and an upregulated production of GMM. This reciprocal regulation is caused by competitive substrate selection by antigen 85A. The results reported by Matsunaga et al. [38], coupled to the results of Ishikawa et al. [18], led to the hypothesis that upregulation of GMM production is an escape mechanism of pathogenic mycobacteria to avoid recognition by the innate immune system. Our results suggest that even if in vivo a downregulation of TDM and an upregulation of GMM occur, innate immune cells remain able to recognise GMM via Mincle.

Regarding AraMMs, our results differ from what was described for natural AraMM purified from *M. bovis* BCG, for which a dependence on TLR-2/Myd88 was reported [22]. However, in the report by Miyauchi [22], arabino-mycolate esters obtained by acid hydrolysis of BCG cell wall skeleton were used, which consist of a complex mixture of mono-arabinose monomycolates, tetra-arabinose tetra-mycolates, penta-arabinose tetra-mycolates and hexa-arabinose tetra-mycolate. The relative proportion of these different compounds in the preparation was not quantified; we can therefore not exclude that mono-arabinose monomycolates signal via Mincle while the other arabinose mycolates would signal via TLR-2/MyD88. It is noteworthy that, to our knowledge, this is the first study demonstrating that the C-type receptor Mincle can recognise pentose-based glycolipids, even if the intensity of the induced downstream signal was lower than the signal induced by trehalose- and glucose-based glycolipids.

In addition, our study demonstrates that, like TDMs, TMMs, and GMMs, AraMMs also induce the activation of the NLRP3 inflammasome. It should be noted that, even if the tested synthetic AraMMs were not as inflammatory as the synthetic TDMs, TMMs, and GMMs in the in vitro and in vivo experiments performed, in an in vitro model involving an LPS priming of BMDCs, a level of IL-1β secretion was observed for all the tested AraMMs comparable to the levels observed for synthetic TDMs, TMMs, and GMMs. This indicates that synthetic AraMMs have a potential comparable to the other synthetic mycolate esters to induce NLRP3 inflammasome activation and, hence, pro-IL-1β processing. It is tempting to speculate that combining synthetic AraMM with a TLR-agonist could lead to the development of adjuvant formulations with Th1 and Th17 promoting potential. Indeed, the TLR agonist could trigger the production of pro-Th1 cytokines along with pro-IL-1β (similar to the LPS priming in our experimental set-up) and synthetic AraMM could lead to pro-IL-1β maturation by NLRP3 activation resulting in the induction of Th17 responses [24, 39].

The in vivo analysis of the tested synthetic mycolate esters in IFA formulations with ovalbumin confirmed the inflammatory properties of synthetic TDMs, TMMs, and GMMs as measured by footpad swelling following subcutaneous administration. No difference in terms of footpad swelling or OVA-specific T cell responses could be observed when comparing the *cis*-alpha TDM-MH175 or TMM-MH176 compounds to the *cis*-methoxy TDM-KB52 or TMM-KB51 compounds, indicating that also in vivo the class of mycolate does not influence the inflammatory power of mycolate esters. Interestingly, Huber et al. [40] recently reported that trehalose monoesters with

short acyl chains (C12, 14, 16, 18, 20) induce significantly lower Th1 and Th17 responses compared to trehalose diester analogues. These results demonstrated that the adjuvant property of simple fatty acids requires 2 acyl chains. In contrast to that study, we observed comparable levels of Th1 and Th17 immune responses between TDM-KB52 and TMM-KB51, or between TDM-MH175 and TMM-MH176, suggesting that, with a longer acyl chain, TMMs can also trigger strong immune responses in vivo. In contrast to what was reported by Shenderov et al. [39], purified peptidoglycan was not needed and formulation of these synthetic mycolate esters in IFA was sufficient in our hands to trigger antigen-specific Th17 responses along with Th1 responses. Differences in formulation and read-outs could account for this discrepancy. Our results indicate that synthetic TDMs, TMMs, and GMMs should be further evaluated as novel adjuvants for subunit vaccines to be used against infectious diseases for which Th1 and Th17 CD4+ T cell responses correlate with protection. However, it is known that natural TDM extracted from *Mtb* can be toxic in vivo [41]. Nevertheless, this toxicity is linked to the nature of the acyl chain bound to trehalose. Indeed, TDM from *Rhodococcus* sp. 4306 with a shorter chain length (C34–38) induces lower granulomatogenicity compared to TDM from *Mtb* [15]. In addition, using natural TDMs extracted from different mycobacteria species that vary in terms of the classes and proportions of mycolates present in their cell wall, Fujita et al. [42] showed that the classes of MA bound to trehalose can influence the toxicity of the molecule. Thus, toxicity of the synthetic compounds with an adjuvant potential described in this study should be assessed in vivo to down-select non-toxic compounds.

GMMs from mycobacteria are also protective non-protein antigens recognised by CD1b-restricted T cells. Hence, given this dual ability of GMMs to stimulate both innate and adaptive responses, it is tempting to speculate that administration of IFA formulations of synthetic GMMs with no additional adjuvant could stimulate protective immune responses. In future experiments it will be of interest to evaluate such formulations in animal models such as guinea pigs or human group 1 CD1 transgenic mice [43]. In addition, as already analysed for TMM [21] and in order to simplify the synthesis process, it would be interesting to determine the minimal carbon chain length necessary for the adjuvant potential of GMM and for its antigenic potential in tuberculosis vaccines. More importantly, this study raises the interesting possibility that GMM through Mincle and CD1b may contribute to both innate (induction of pro-inflammatory cytokines) and acquired (induction of Th1/Th17-CD1b-restricted T cell responses) immunity against mycobacteria. This fact provides an interesting possibility in the tuberculosis vaccine field to target both classical and non-classical T cell responses by combining synthetic GMM with a protective antigen.

Acknowledgements

Hermann Giresse Tima holds a FRIA PhD fellowship. This research was partially funded by the Interuniversity Attraction Pole Programme IAP 7/32 and by TBVAC2020 under grant agreement No. H2020-PHC-643381. MOM thanks the Ministry of Higher Education and Scientific Research, Kurdistan Region, Iraq, for the award of a PhD studentship. Roland Lang received support from the Deutsche Forschungsgemeinschaft (SFB 796/B6). We also thank Fabienne Jurion and Christophe van den Poel for technical support, and Virginie Maghe for advice concerning statistical analysis of the data.

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3.3 Evaluation of glucose and arabinose monomycolates from *Mycobacterium tuberculosis* as adjuvants for subunit vaccines.

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❖ Context

Adjuvants are molecules, compounds or macromolecular complexes that enhance the strength and the duration of the immune responses induced to a co-administered antigen. Adjuvants can be classified as delivery systems and immuno-stimulatory molecules. Delivery systems consist of vehicle used to efficiently transport or present antigens and immuno-stimulatory molecules. There are very few adjuvant licensed for humans that can induce robust cell mediated immune responses necessary for the control of infectious diseases such as TB. In the previous chapter, we demonstrated that GMM and AraMM are pro-inflammatory compounds. We observed that AraMM is a stronger activator of the NLRP3 inflammasome in vitro following priming of BMDCs with LPS. Thus, given the ability of AraMM to strongly induce NLRP3 inflammasome activation, vaccination with formulations in which synthetic AraMM is combined with TLR-agonists could result in a new vaccine formulation able to promote both Th1 and Th17 responses. In addition, our results showed that GMM can be used as adjuvant to generate Th1 and Th17 responses. In this study, we have analyzed the adjuvant potential of sub-unit vaccine formulations composed of different glycolipids including short and long chain GMMs and AraMM alone or in combination with a TLR agonist, MPLA. We also investigated the influence of the formulation (water in oil emulsion vs liposome) on the immune responses induced.

❖ Results

Our results show that short acyl chain GMM activates BMDCs by mechanisms dependent on Mincle. Further, we demonstrated that short acyl chain GMM formulated in emulsion with OVA triggers the induction of Th1 and Th17 immune responses in an OVA immunization model in C57BL/6 mice. In addition, our data indicate that AraMM combined with MPLA increase its adjuvant effect. Indeed, when AraMM is formulated in emulsion with MPLA, it induces Th1 and Th17 immune responses at a level comparable to those induced by TDB positive control. Finally, we compared immune responses induced by GMM and AraMM formulated in cationic liposomes (DDA) or emulsion with a protective mycobacterial recombinant protein (PPE44). We observed that DDA liposome formulation does not induce sustained local inflammation in comparison to emulsion formulation. Furthermore, our data showed that administration of GMM in both formulations had the same tendency and the levels of PPE44-specific IL-2, IFN-γ and IL-17A were similar to those observed for the TDB groups. Interestingly, AraMM formulated in DDA, without additional immuno-stimulatory compound, also induced the production of IL-2 and IFN-γ at levels comparable to those induced by TDB in DDA liposomes or w/o/w emulsion. Indicating that DDA can potentiate the immune responses induced by AraMM *in vivo*. However, no IL-17A responses were generated by administration of the DDA-AraMM formulation.

❖ Conclusion

Altogether, these data demonstrate that DDA-GMM and DDA-AraMM like DDA-TDB can be used as adjuvants to generate cell mediated immune responses without inducing significant local inflammation.

Title: Evaluation of glucose and arabinose monomycolates from *Mycobacterium tuberculosis* as adjuvants for subunit vaccines.

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Abstract:

The cell wall of *M. tuberculosis* contains immuno-stimulatory glycolipids, such as trehalose dimycolates, trehalose monomycolates, glucose monomycolates and arabinose mycolates. In the present study, we have analyzed the adjuvant potential of different glycolipids including short chain glucose and arabinose monomycolate formulated in combination or not with the Toll Like Receptor 4 (TLR4) agonist, monophosphoryl lipid A (MPLA). We also investigated the influence of the formulation (water in oil emulsion vs liposome) on the immune responses induced. Our results indicate/show that a synthetic analogue of GMM with a shorter C42 acyl chain possesses similar adjuvant potential as long acyl chain (C82) *cis*-methoxy GMM (SMP73). In addition, increased responses were observed by combining MPLA with AraMM as compared to responses induced by formulation with the individual compounds. Finally, we show that synthetic arabinose monomycolate (AraMM) and GMM formulated in cationic liposomes with a protein antigen can be used as adjuvants to promote antigen-specific humoral and cell-mediated immune responses without inducing sustained inflammation at the site of injection.

Introduction:

Vaccines represent one of most relevant advances in the healthcare history. They have allowed the effective control of several diseases such as polio, smallpox, measles, diphtheria, tetanus and rabies [1]. Nevertheless, there is still an urgent need for vaccines against several infectious agents such as *Mycobacterium tuberculosis*, human immunodeficiency virus (HIV), hepatitis C virus (HCV), Ebola virus, *Plasmodium falciparum* and dengue virus among others. Subunit vaccines represent one category of vaccines currently in development against these infections. This type of vaccines consists of recombinant proteins or synthetic peptides, DNA or RNA formulated in an appropriate delivery system and/or adjuvant. Adjuvants are molecules, compounds or macromolecular complexes that enhance the strength and the duration of the immune responses induced to a co-administrated antigens. Delivery systems consist of vehicle used to efficiently transport or present antigens and adjuvants [2]. Some molecules such as aluminium hydroxide (Alum) or virosomes can be both adjuvants and delivery systems [3].

In contrast to first generation subunit vaccines that used one immuno-stimulatory compound or delivery system (Alum-based vaccines), the new strategy in the subunit vaccine field is to combine several adjuvants and delivery systems. This combination aims to act synergistically to increase or modulate the immune responses induced [2,3]. For example, encapsulation of TLR9 agonist CpG and antigen in polymeric micro-particles significantly increased the levels of antigen-specific cytotoxic T cell activity induced by the immunization as compared to administration of a formulation with non-encapsulated CpG [4]. Another example is the adjuvant system AS04. Indeed, addition of monophosphoryl lipid A to Alum in AS04 containing Human papilloma virus (HPV) antigens has been reported to significantly increase the titers of anti-HPV antibodies in mice and humans compared to the same vaccine formulated in Alum alone [5,6]. MF59 is a licensed water in oil adjuvant that is known to induce Th2 immune responses [7]. It has been reported that addition of TLR ligands such as CpG to MF59 based influenza vaccine does not increase antibody titers in mice compared to administration of influenza vaccine in MF59 alone. However, the addition of this TLR ligand induces a shift to a Th1 profile of immune responses [8]. In addition, safety concerns are a major barrier that has restricted the development of several experimental adjuvants since Alum [9]. Delivery systems can reduce the toxicity of an adjuvant, for example by limiting or reducing systemic exposure

and inflammation. Indeed, Smirnov and co-workers reported that the addition of a C18 acyl chain to an imidazoquinoline (TLR7 and/or TLR8 agonist) and formulation in liposome reduces systemic exposure to imidazoquinoline [10]. Liposomes were also demonstrated to reduce the toxicity induced by MPL and saponin [3].

There are very few adjuvants licensed for use in humans that can induce robust cell-mediated immune (CMI) responses [11], responses which are crucial for the control of intracellular infections and for cancer therapeutic vaccines. Among these licensed adjuvants, none can effectively induce both Th1 and Th17 immune responses, known to play an important role in the control of infectious diseases such as tuberculosis (TB) [12]. Thus, development of adjuvants for human use inducing both Th1 and Th17 responses are needed.

The cell wall of *M. tuberculosis* is rich of compounds with immuno-stimulatory potential that are relevant for the development of adjuvants for subunit vaccines [13]. These compounds include mycolate esters (trehalose dimycolate (TDM), trehalose monomycolate (TMM), glucose monomycolate (GMM), arabinose mycolates. Trehalose dibehenate (TDB) formulated in dimethyldioctadecylammonium (DDA) liposomes is currently investigated in clinical trials for adjuvant purposes [14]. Known as CAF01, this adjuvant has been shown to induce long-lived Th1 and Th17 immune responses [15].

We have previously reported that synthetic GMM formulated in water in oil emulsion induces strong ovalbumin-specific Th1 and Th17 responses at levels comparable to those achieved by TDB [16]. By using reporter cell systems, Van der Peet et *al.,* have shown that GMM composed of mycolates with a shorter acyl chain length (C34) can still bind to Mincle receptor [17]. However, it was to our knowledge not yet reported whether GMMs with such a short acyl chain can favour induction of cell-mediated immune responses *in vivo*. Regarding synthetic AraMMs, we have previously shown that this class of compounds can induce *in vitro* a modest production of pro-inflammatory cytokines but induces a strong activation of the NLRP3 inflammasome by a mechanism dependent on the Mincle pathway [16]. In an ovalbumin vaccination murine model, AraMM formulations did not induce significant ovalbumin-specific immune responses. Nevertheless, given the ability of AraMMs to strongly induce NLRP3 inflammasome activation, vaccination with formulations in which synthetic AraMMs are combined with TLR-agonists could result in a vaccine formulation able to promote both Th1 and Th17 responses.

In this study, we have analyzed the adjuvant potential of sub-unit vaccine formulations composed of different glycolipids including short and long acyl chain GMMs and AraMMs alone or in combination with a TLR agonist, MPLA. We also investigated the influence of the formulation (water in oil emulsion vs liposome) on the immune responses induced.

Results

Short acyl chain GMM activates BMDCs by mechanisms dependent on Mincle and induces Th1 and Th17 responses

Adjuvants are defined as compounds capable of activating innate immunity by inducing cytokine and chemokine production by dendritic cells (DCs) and participate to the induction of antigen-specific immune responses [18]. We have previously reported that synthetic long acyl chain *cis*-methoxy GMM (SMP73) formulated in a water in oil emulsion induces strong ovalbumin-specific Th1 and Th17 responses at levels comparable to those achieved by a TDB formulation [16]. In order to analyze whether the reduction of the acyl chain length of GMM can have an impact on the inflammatory and adjuvant potential of this class of compounds, a short acyl chain GMM (KB177) was synthetized and its activity compared to the one of GMM-SMP73. As represented in Figure 1A, KB177 and SMP73 possess respectively an acyl group composed of 42 and 82 carbon atoms (Fig.1A). A comparative analysis of the immuno-stimulatory properties of these two synthetic GMMs was subsequently performed *in vitro* using bone marrow derived dendritic cells (BMDCs). As shown in Figure 1B and 1C, 24h incubation of BMDCs with KB177, SMP73 or TDB as a positive control resulted in strong TNF-alpha and IL-6 production. However, the level of TNF-α induced by short chain GMM (KB177) was significantly lower than the level produced by long chain *cis*-methoxy GMM (SMP73), suggesting that the mycolate chain length on GMM can have an impact on the TNF-α response induced. In contrast, no significant differences in the level of IL-6 induced were observed. It was reported that synthetic short chain GMM homologous to GMM from corynebacteria, structurally similar to the KB177 compound tested in this study, can bind to Mincle receptor [17]. Therefore, using a reporter cell line, we tested the interaction of KB177 with Mincle (Fig. 1D). Our data confirmed the observation of Van der Peet and co-workers that short acyl chain GMM can bind to Mincle. However, at lower concentration, dose-response curves seem to suggest that the interaction of short chain GMM (KB177) with Mincle is weaker than the one of long acyl chain GMM (SMP73).

As we previously reported, footpad immunization of mice with long chain *cis*-methoxy GMM (SMP73) in a water in oil in water (w/o/w) IFA emulsion containing ovalbumin as a test antigen,

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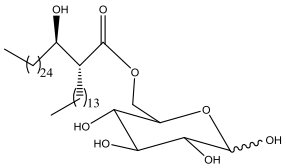

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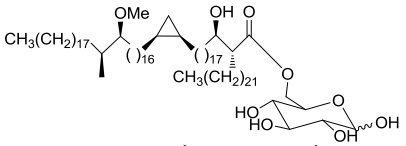

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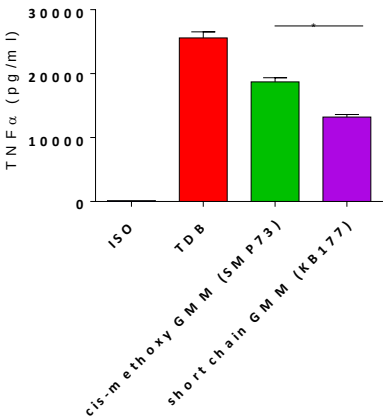

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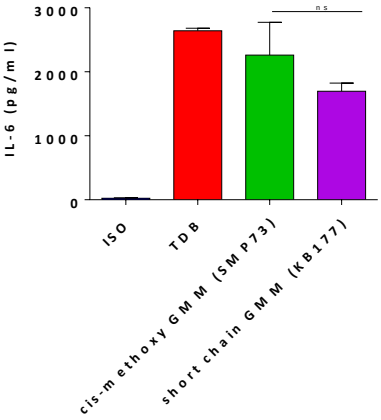

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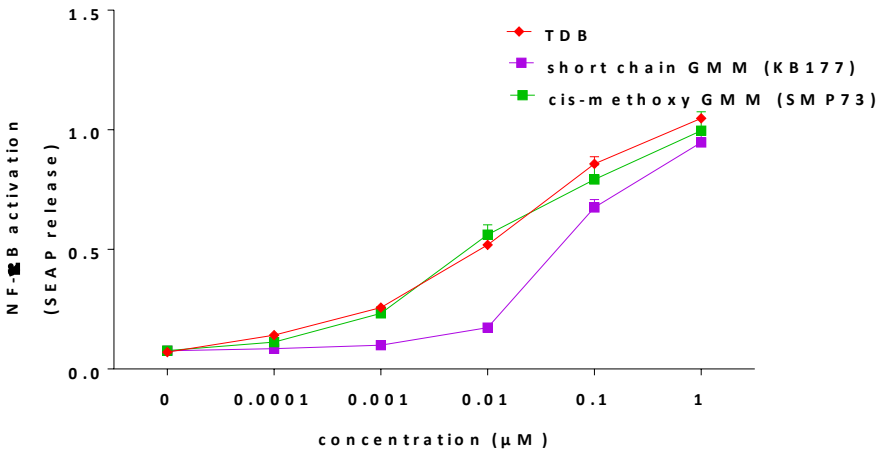

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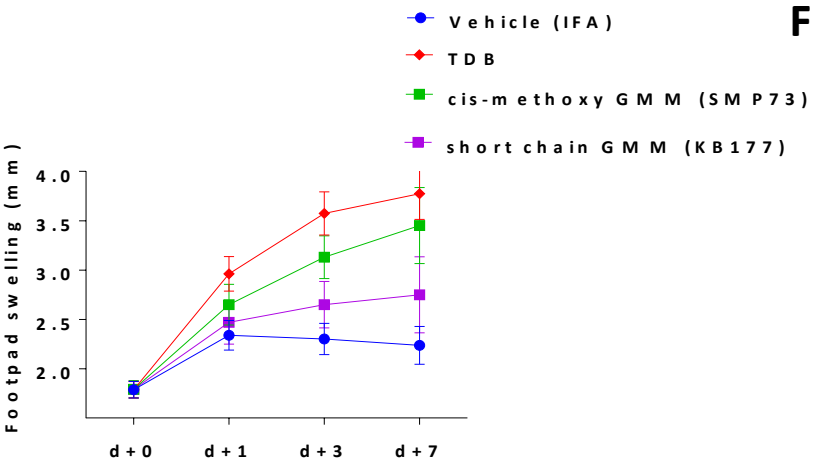

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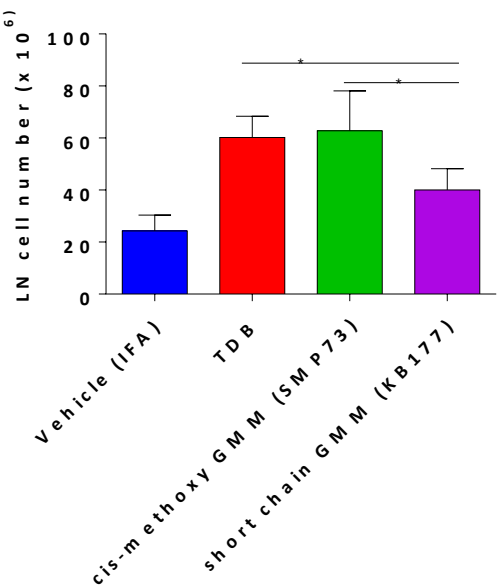


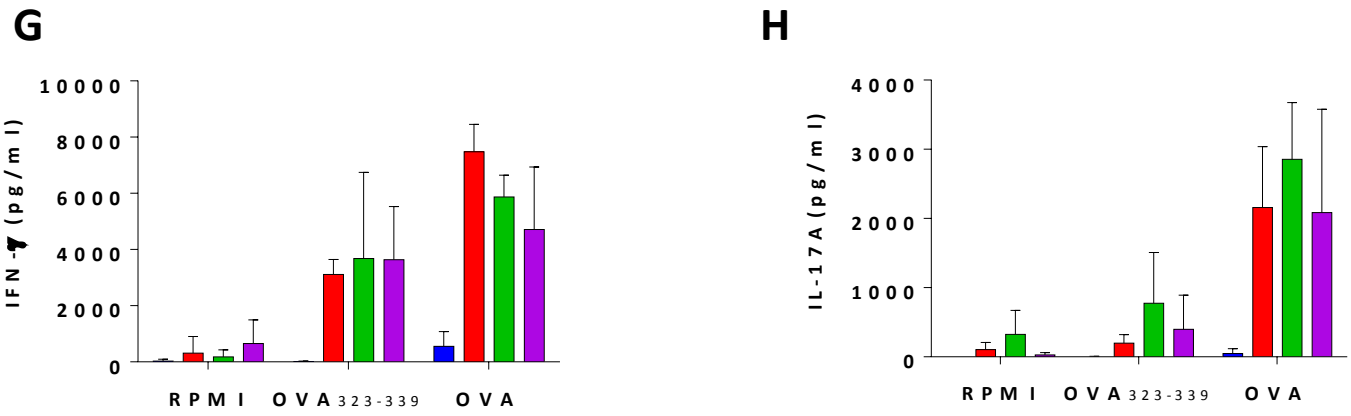


Figure 1: Short chain GMM activates BMDCs by a mechanism dependent on Mincle and induces Th1 and Th17 responses.

A: Structures of short chain GMM (KB177) and long chain *cis*-methoxy GMM (SMP73).

B-C: BMDCs derived from C57BL/6 mice were stimulated for 24 h in triplicate cultures with 1mM of plate-coated synthetic long chain *cis*-methoxy GMM (SMP73) (green), short chain GMM (KB177) (purple) or TDB (red). The supernatants were harvested from separate wells and the amount of pro-inflammatory cytokines (TNF-α and IL-6) was determined by sandwich ELISA. Results are expressed as mean pg/ml of cytokines ± SD of two independent experiments: IL-6 (B) or TNF-α (C).

D: HEK293 reporter cell expressing murine Mincle were used to evaluate the binding to Mincle receptor. SEAP release after NF-κB activation was evaluated by colorimetric assay using Quantie-blue substrate and by measuring the OD at 655 nm. Short chain GMM (purple square, TDB (red diamonds) and long chain *cis*-methoxy GMM (green squares).

E-H: C57BL/6 mice were injected s.c in the two hind footpads with w/o/w emulsions composed of 30% Incomplete Freund's Adjuvant, 10µg/mouse of specified synthetic mycolate esters and 50 µg/mouse of ovalbumin. In the emulsion of the vehicle control group no glycolipid was present, in the TDB control group 10 µg/mouse of TDB replaced the synthetic glycolipids. Mean footpad size (mm) of five mice tested individually was measured with a caliper on the day of injection and 1, 3 and 7 days after injection (**E**). Mice were sacrificed 7 day post immunization and cells from pooled popliteal and inguinal lymph nodes were collected, counted (**F**) and stimulated with culture medium (RPMI), 10µg/ml of peptide $OVA_{323-339}$ or with 5µg/ml of ovalbumin (OVA) for 72h. Supernatants were harvested and the concentration of IFN-γ and IL-17A (**G** and **H**) was determined by ELISA.

Results are means ± SD of five mice tested individually and representative of three independent experiments. ns= not significant, *, **, ***= statistically significant, as determined by Mann-Whitney test, $p<0.05$, $p<0.01$ or $p<0.001$.

can induce footpad swelling as well as OVA-specific Th1 and Th17 immune responses. Using a similar experimental protocol, we investigated the inflammatory and adjuvant properties of short chain GMM *in vivo*. C57BL/6 mice were immunized once in the two hind footpads with an IFA emulsion composed of recombinant OVA with or without synthetic SMP73, KB177 or TDB. Footpad swelling was measured for one week as a read-out of local inflammation (Fig. 1E) and the obtained data show that formulation with KB177 is inducing a milder local inflammation as compared to inflammation induced by formulations with SMP73 or TDB. In addition, the draining lymph node cell number increases significantly in these two groups in comparison to vehicle and KB177 (Fig. 1F). OVA-specific IFN-γ and IL-17A were analyzed in the culture supernatant of draining lymph nodes stimulated with medium, $OVA_{323-339}$ I-A^b restricted $CD4^+$ peptide or recombinant OVA. As shown in Fig. 1G and 1H, OVA-specific IFN-γ and IL-17A levels were greatly increased and significantly higher than the levels measured in control mice immunized with IFA emulsion without glycolipids. In contrast to local inflammation, there was no difference in terms of OVA-specific IFN-γ and IL-17A responses induced by vaccination with an IFA formulation containing short chain GMM (KB177) as compared to long chain GMM (SMP73).

Combining a TLR4 ligand with arabinose monomycolate can increase its adjuvant effect

A new strategy in the design of adjuvants for subunit vaccines is the combination of several adjuvants that activate different pathways in order to synergistically enhance or modulate the immune responses induced. We have previously shown that AraMM formulated in w/o/w emulsion with OVA as a test antigen does not induce substantial innate and adaptive immune responses *in vivo* [16]. However, this class of compounds induces *in vitro* a moderate activation of BMDCs in terms of production of pro-inflammatory cytokines and a robust activation of the NLRP3 inflammasome. The production of pro-inflammatory cytokines was lower than the one induced by synthetic trehalose and glucose-based mycolate esters, while NLRP3 inflammasome activation was comparable with all the tested classes of compounds [16]. Monophosphoryl lipid A (MPLA) is a structural analogue of LPS but with a more acceptable safety profile [19]. In order to evaluate whether combination of Mincle agonist (AraMM) and TLR4 agonist (MPLA) can synergistically enhance immune responses, we immunized mice with w/o/w emulsion containing recombinant OVA with or without *cis*-methoxy AraMM (MOD16) and with or without MPLA. Our data showed that except for the TDB group, none of the

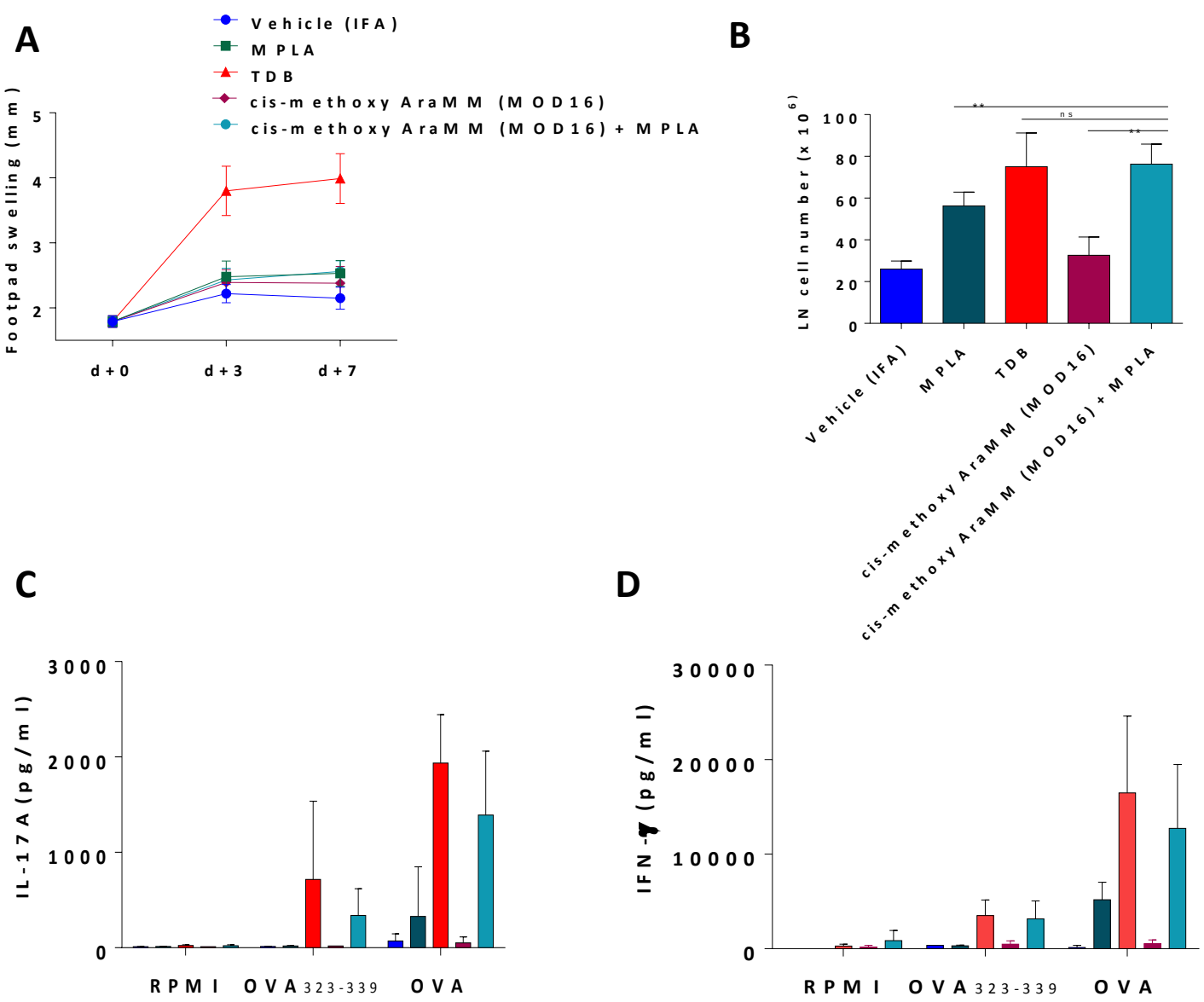


Figure 2: Synergistic effect between Mincle and TLR agonists

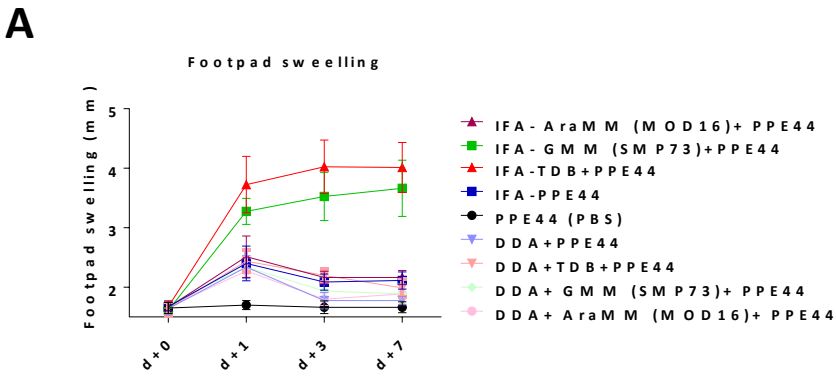
A: C57BL/6 mice were injected s.c in the two hind footpads with w/o/w emulsions composed of 30% Incomplete Freund's Adjuvant, 10µg/mouse of specified synthetic mycolate esters and 50 µg/mouse of ovalbumin. In the group with MPLA, 20µg of MPLA was added in the w/o/w emulsion. In the emulsion of the vehicle control group no glycolipid was present, in the TDB control group 10µg/mouse of TDB replaced the synthetic glycolipids. Footpad swelling of 5-6 mice tested individually was measured with caliper the day of the injection and 3 and 7 days after injection (**A**). Mice were sacrificed 7 day post immunization and cells from pooled popliteal and inguinal lymph nodes were collected, counted (**B**) and stimulated with culture medium (RPMI), 10µg/ml of peptide $OVA_{323-339}$ or with 5µg/ml of ovalbumin (OVA) for 72h. Supernatants were harvested and the concentration of IFN-γ and IL-17A (**C** and **D**) was determined by ELISA. Results are means ± SD of 5-6 mice tested individually and representative of two independent experiments. ns= not significant, *, **, ***= statistically significant, as determined by Mann-Whitney test, $p<0.05$, $p<0.01$ or $p<0.001$.

formulations tested induced footpad swelling over 7 days. Interestingly, when cells recruited to the draining (popliteal and inguinal) lymph nodes were counted, we observed that administration of the formulations composed of MPLA or *cis*-methoxy AraMM (MOD16) combined with MPLA induced at least a two fold increase in recruited cells as compared to the vehicle control group or the *cis*-methoxy AraMM (MOD16) group. In addition, combination of MPLA and *cis*-methoxy AraMM significantly enhanced the production of OVA-specific IL-17A and IFN-γ in lymph node cells stimulated with recombinant OVA or $OVA_{323-339}$ peptide. The IL-17A and IFN-γ levels induced by the MOD16 + MPLA formulation were comparable to the ones observed for the TDB group. Immunization with MPLA but not MOD16 alone resulted in generation of IFN-γ responses, while significant IL-17A responses were absent in both groups.

GMM and AraMM formulated in DDA liposomes induce antigen-specific Th1, Th17 and antibody responses without induction of sustained local inflammation

Immunization with IFA emulsion containing glycolipids induces a strong local inflammation characterized by a strong footpad swelling (2 mm) and neutrophil recruitment [16]. As IFA formulations are obviously too reactogenic (Figure 1E and 2A) and as it was reported that formulation of immuno-stimulatory compounds in liposomes could reduce their toxicity [2], we formulated synthetic glycolipids in DDA liposomes and compared the responses induced to those observed after administration of w/o/w emulsions.

The model antigen tested in this part of the work was recombinant PPE44, a protective mycobacterial antigen that could be included in TB subunit vaccine [20]. Using the same protocol of immunization as previously described [16], we observed as expected that w/o/w emulsions containing TDB or *cis*-methoxy GMM (SMP73) induce a strong footpad swelling while vehicle and w/o/w emulsion with *cis*-methoxy AraMM (MOD16) did only induce a moderate and transient inflammation at day 1 post-immunization. Interestingly, all the DDA liposome formulations tested also only induced a moderate and transient footpad swelling at day 1 (Fig.3A). This swelling at the site of injection resolved at day 3 after vaccination. In addition, footpad swelling in the w/o/w TDB and GMM groups correlated with an important recruitment of neutrophils and cells in the lymph nodes (Fig.3B and 3C). These data indicate that formulation in DDA liposomes can significantly reduce local inflammation induced by synthetic mycolate esters.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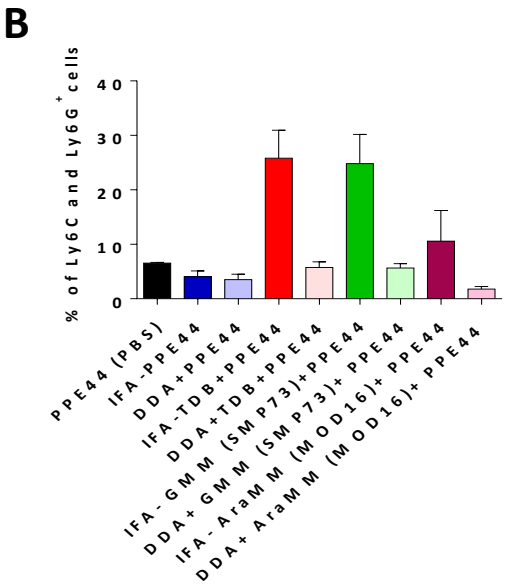
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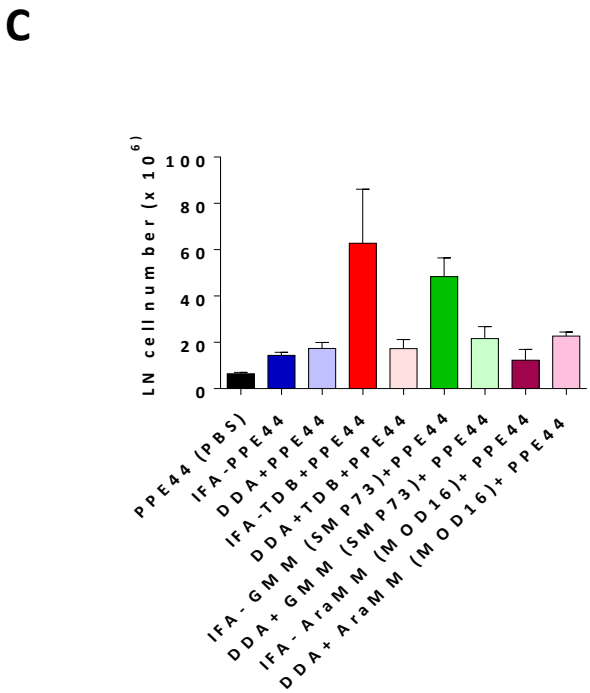
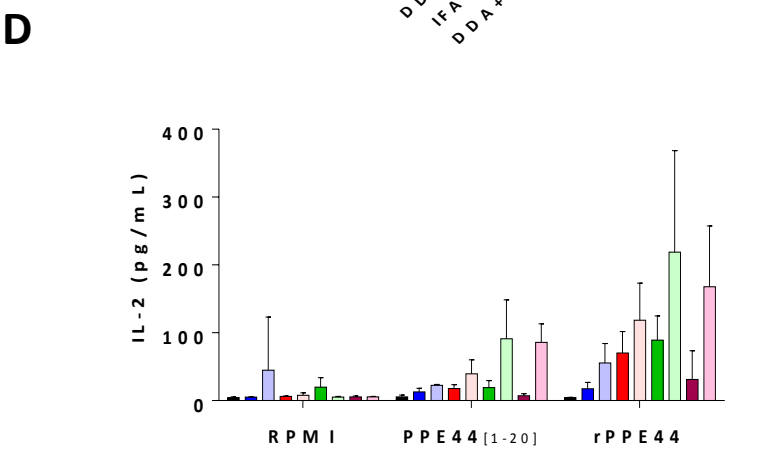
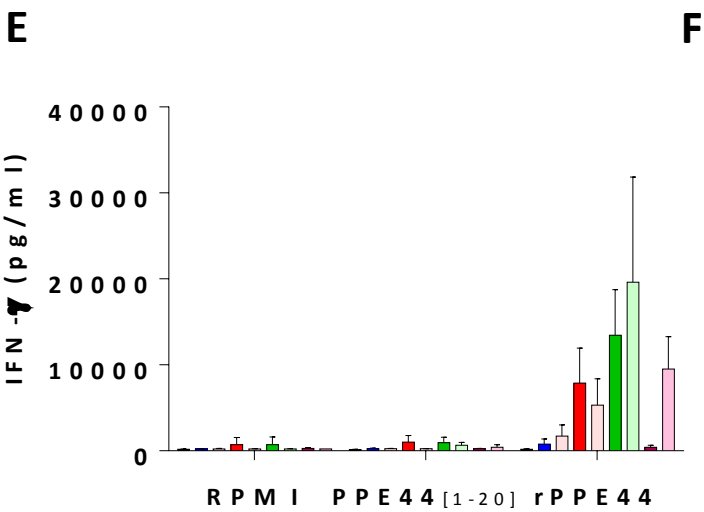
DDA+TDB+PPE44
IFA-GMM (SMP73)+PPE44
DDA+GMM (SMP73)+ PPE44
IFA-AraMM (MOD16)+ PPE44
DDA+AraMM (MOD16)+ PPE44


C
LN cellnumber (x 10^6)
100
80
60
40
20
0
PPE44 (PBS)
IFA-PPE44
DDA+PPE44
IFA-TDB+PPE44
DDA+TDB+PPE44
IFA-GMM (SMP73)+PPE44
DDA+GMM (SMP73)+ PPE44
IFA-AraMM (MOD16)+ PPE44
DDA+AraMM (MOD16)+ PPE44


D
IL-2 (pg/mL)
400
300
200
100
0
RPMI
PPE44[1-20]
rPPE44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IFN-γ (pg/ml)
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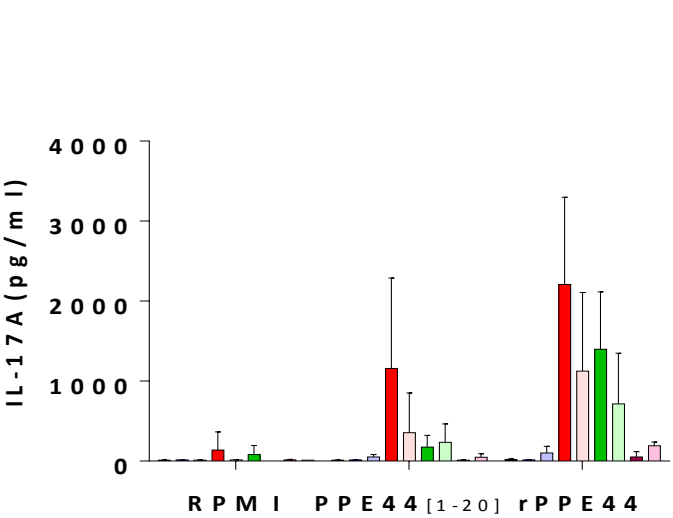
30000
20000
10000
0
RPMI
PPE44[1-20]
rPPE44


F
IL-17A (pg/ml)
4000
3000
2000
1000
0
RPMI
PPE44[1-20]
rPPE44

G

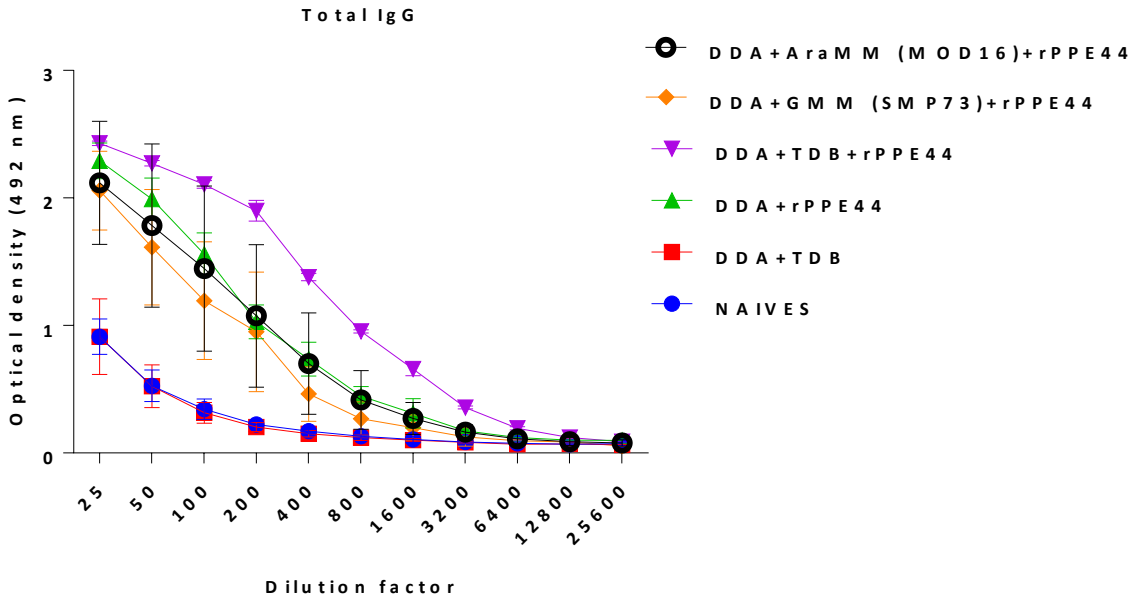


Figure 3: Mycolate monoesters formulated in DDA liposomes induce Th1 and Th17 responses without substantial inflammation.

Groups of 4-5 C57BL/6 mice were injected s.c in the two hind footpads with w/o/w emulsions composed of 30% Incomplete Freund's Adjuvant, 10µg/mouse of specified synthetic mycolate esters and 4µg/mouse of recombinant PPE44 or with DDA liposomes composed of 125µg/mouse of DDA, 25µg/mouse of glycolipids and 4µg/mouse of recombinant PPE44.

A: Mean footpad size (mm) of five mice tested individually was measured with a caliper on the day of injection and 1, 3 and 7 days after injection.

B: Percentage of granulocytes (Ly6G+ and Ly6C+) was determined in cells isolated from footpad by flow cytometry 7 days post administration.

C-**F**: Mice were sacrificed 7 day post immunization and cells from pooled popliteal and inguinal lymph nodes were collected, counted (**C**) and stimulated with culture medium (RPMI), 10µg/ml of peptide $PPE44_{1-20}$ or with 5µg/ml of PPE44 for 24h and 72h. Supernatants were harvested and the concentration of IL-2 (**D**), IFN-γ and IL-17A (**E** and **F**) was determined by ELISA.

H: Anti-PPE44 IgG-isotype antibodies were measured by ELISA in serum harvested three weeks after the last immunization. Optical density levels of serial two-fold serum dilutions are presented as the mean ± standard error of the mean.

Results are means ± SD of 5 mice tested individually from one preliminary experiment. ns= not significant, *, **, ***= statistically significant, as determined by Mann-Whitney test, $p<0.05$, $p<0.01$ or $p<0.001$

For the evaluation of the vaccine induced PPE44-specific cellular immune responses, we analyzed the production of IL-2, IL-17A and IFN-γ in the supernatants of cultures of cells isolated from draining lymph nodes stimulated with medium, recombinant PPE44 or the immunodominant $PPE44_{1-20}$ peptide. Confirming results reported for the H1 fusion protein composed of Ag85B and ESAT-6 as antigens [14,15] , TDB-DDA induced a strong production of PPE44-specific IL-2, IFN-γ and IL-17A. The levels of cytokines induced were comparable to those induce by w/o/w-TDB. Administration of *cis*-methoxy GMM (SMP73) in both formulations had the same tendency and the levels of PPE44-specific IL-2, IFN-γ and IL-17A were similar to those observed for the TDB groups. Interestingly, AraMM formulated in DDA, without additional immuno-stimulatory compound, also induced the production of IL-2 and IFN-γ at levels comparable to those induced by TDB in DDA liposomes or w/o/w emulsion. Indicating that DDA can potentiate the immune responses induced by AraMM *in vivo.* Nevertheless, no IL-17A responses were generated by administration of the DDA-AraMM formulation. In order to analyze humoral immune response induced by PPE44 formulated with GMM or AraMM in DDA, mice were subcutaneously vaccinated twice with an interval of at least three weeks. Anti-PPE44 IgG levels were analyzed in the sera three weeks after the last immunization. As expected, in mice vaccinated with DDA-TDB in the absence of recombinant protein, the PPE44-specific antibody levels were comparable to the ones of negative control mice. PPE44-specific levels of total IgG were observed in all the groups vaccinated with formulations in which recombinant PPE44 was present. However, significantly higher antibody titers as compared to mice vaccinated with DDA+PPE44 were observed only in the DDA-TDB-PPE4 group. In contrast to the Th1 and Th17 responses, GMM and AraMM formulations did not lead to increased antibody levels as compared to the one observed in the DDA+rPPE44 group.

Altogether, these data demonstrate that DDA-GMM and DDA-AraMM like DDA-TDB can be used as adjuvants to generate antibody and cell mediated immune responses without inducing significant local inflammation.

Inflammasome activation by DDA and glycolipids is necessary for the adjuvant properties of DDA/glycolipid liposomes

In this last part of the work, we were interested in the elucidation of the mechanisms involved in the induction of antigen-specific responses observed *in vivo* when recombinant antigen is

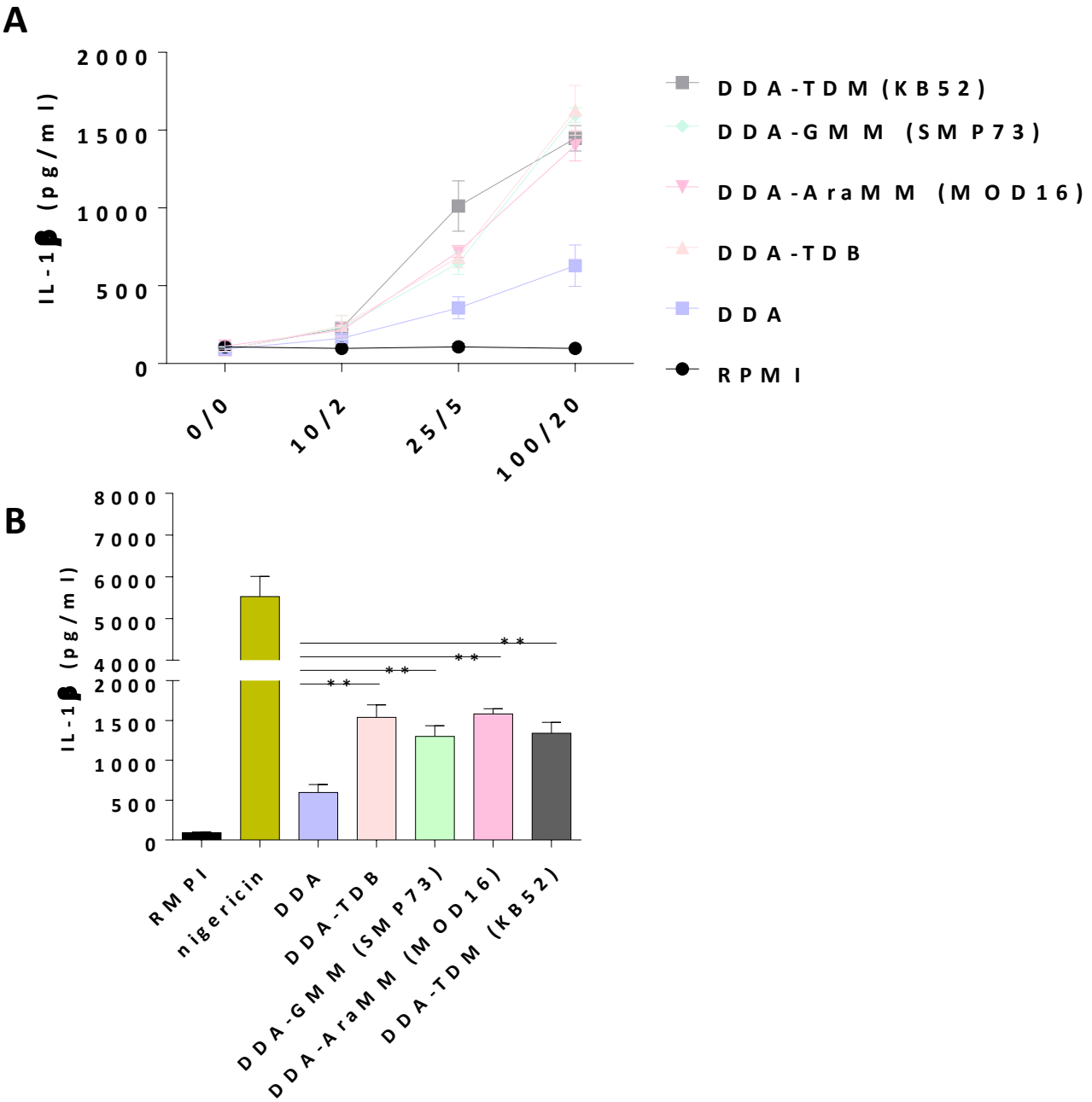

A
2000
1500
1000
500
0
IL-1β (pg/ml)
0/0
10/2
25/5
100/20
DDA-TDM (KB52)
DDA-GMM (SMP73)
DDA-AraMM (MOD16)
DDA-TDB
DDA
RPMI
B
8000
7000
6000
5000
4000
2000
1500
1000
500
0
IL-1β (pg/ml)
**
**
**
**
RMPI
nigericin
DDA
DDA-TDB
DDA-GMM (SMP73)
DDA-AraMM (MOD16)
DDA-TDM (KB52)
C
WT
MyD88-/-
IL-1R-/-
2000
1500
1000
500
0
IFN-γ (pg/ml)
RPMI
OVA323-339
OVA
D
WT
MyD88-/-
IL-1R-/-
400
300
200
100
0
IL-17A (pg/ml)
RPMI
OVA323-339
OVA

Figure 4: Inflammasome activation by DDA and glycolipids are necessary for the adjuvanticity of DDA/glycolipids liposomes.

A-B: BMDCs prepared from C57BL/6 mice were primed for 3 h with 1µg/mL of ultrapure LPS and stimulated next for 5 h with (**A**) indicated amount in (µg/ml) of DDA/synthetic mycolate esters (ratio 5/1) or **(B**) 100/20 µg/ml of DDA/synthetic mycolate esters, with RPMI as negative control and TDB at the same concentration as positive control. The supernatants were harvested and the amount of IL-1β in the supernatants was determined by ELISA.

C-D: Groups of 4-5 C57BL/6 mice were injected s.c in the two hind footpads with DDA liposomes composed of 125µg/mouse of DDA and 4µg/mouse of ovalbumin. Mice were sacrificed 7 day post immunization and cells from pooled popliteal and inguinal lymph nodes were collected, counted and stimulated with culture medium (RPMI), 10µg/ml of peptide $OVA_{323-339}$ or with 5µg/ml of OVA for 72h. Supernatants were harvested and the concentration of IFN-γ and IL-17A was determined by ELISA.

formulated with AraMM in DDA. It was already reported that DDA functions by triggering a depot effect at the site of injection and by significantly increasing antigen uptake and presentation by DCs [21,22]. In addition, we have previously reported that AraMM activates NLRP3 inflammasome and induces a strong production of IL-1β *in vitro*. We hypothesized that DDA may influence the activation of inflammasome by AraMM. This was first tested *in vitro* using BMDCs primed with LPS and stimulated with DDA alone or combined with the different synthetic glycolipids. Interestingly, DDA alone induced a significant dose-dependent production of IL-1β by primed-BMDCs (Fig.4A). Combination of DDA with glycolipids increased about two-fold the levels of IL-1β produced (Fig.4B). These data suggest that DDA can activate the inflammasome and that this activation is enhanced in the presence of mycolate esters. It was already reported by Desel and co-workers that the induction of Th17 responses induced by DDA-TDB is dependent on the activation of the inflammasome [23]. However, it is not known whether the adjuvant potential of DDA by itself also depends on inflammasome activation *in vivo*. Therefore, we analyzed here whether IL-1β is involved in the adjuvant potential of DDA *in vivo*. For that purpose, WT, MyD88$^{-/-}$ and IL-1R$^{-/-}$ C57BL/6 mice were immunized once in the two hind footpads with DDA containing recombinant OVA. After 1 week, the mice were sacrificed and OVA-specific immune responses were analyzed after stimulation of cells isolated from the draining lymph nodes with RPMI, recombinant OVA or $OVA_{323-339}$ peptide. OVA-specific IFN-γ and IL-17A were induced in mice vaccinated with DDA-OVA liposomes, but these responses were completely abrogated in MyD88$^{-/-}$ and IL-1R$^{-/-}$ mice (Fig 4.C and 4D, preliminary data). In addition, the DDA-OVA induced responses were lower than those induced by DDA-glycolipids. Altogether, these data suggest that DDA and glycolipids may synergistically activate the inflammasome.

Discussion:

Subunit vaccines composed of highly purified recombinant antigens or synthetic peptides are generally considered as safe alternatives to traditional live attenuated or inactivated whole cell vaccines. However, antigens or synthetic peptides are poorly immunogenic and need to be formulated in adjuvants and in appropriate delivery systems in order to achieve generation of sustained adaptive responses [2]. There are very few adjuvants licensed for human use that can promote cell-mediated immune responses, which are necessary in the immune protection against intracellular pathogens and cancer [3]. An interesting strategy in the development of subunit vaccine is the combination of multiple pattern recognition receptor (PRR) agonists that activate several downstream pathways [24]. For example, recent data have reported a synergistic effect of combining TLR2/TLR3/TLR9 [25] or CD40/TLR3/TLR4/TLR7 [26] agonists. These combinations are generally coupled with the use of an appropriate delivery system that can ensure co-localization of antigen and adjuvant to the same antigen-presenting cells.

The cell wall of *M. tuberculosis* contains immuno-stimulatory glycolipids that can potentially be used as adjuvants. These glycolipids comprise among others TDMs, TMMs, GMMs and AraMMs. In this study, we have explored different aspects in the design of subunit vaccines composed of recombinant protein antigens formulated with glycolipids, specifically based on synthetic GMMs and AraMMs. These aspects include the reduction of the chain length of GMM, the combination of AraMM with MPLA and the use of liposome formulations instead of emulsions. Our results show that a synthetic analogue of GMM composed of a C42 mycolate, a structure similar to that found in mycolates from corynebacteria, possesses similar adjuvant capacity *in vivo* in comparison to a long chain *cis*-methoxy GMM (SMP73). In addition, C42 GMM was less inflammatory *in vitro* and *in vivo*, as indicated by a significantly lower production of TNF-α and a reduction of footpad swelling in comparison to long C82 chain *cis*-methoxy GMM (SMP73) and TDB. Interestingly, Van der Peet and colleagues reported that glucose mono-behenate (C22) is not able to activate Mincle reporter cells and consequently is not immuno-stimulatory [17]. Altogether, these data suggest that the structure of the acyl chain of GMM is critical for its immuno-stimulatory property. When comparing the structure of short chain GMM (C42) and glucose monobehenate (GMB), it is important to note that short chain GMM (KB177) is composed of a meromycolate chain of C25 and an alpha

chain of C14 while GMB is composed only of an alpha chain of C22 and no meromycolate chain. This suggests that for glucose-based glycolipids, presence of a meromycolate chain is indispensable for sustained Mincle recognition when reducing the chain length of the acyl group. Van Der Peet et al., recently investigated the lipid structure required for the activation of Mincle by glucose esters [27]. They have found that GMB activates reporter cells at higher concentration (1000 times compared to TDM). Additionally, they found that the chain length of the α-alkyl side chain of the lipid influences the level of activation of glucose esters. Indeed, glucose with an α-pentyl derivative signals much more weakly through Mincle compared to glucose mono-coryonomycobacteria (GMCM), which possesses an α-chain composed of 14 carbon atoms. While a glucose monoester with an α-chain composed of 23 carbon atoms signals to a similar extent through Mincle as α-chain C14 GMCM. Altogether, these data suggest that an alpha-alkyl side chain of C14 is the minimal size of the alpha-alkyl side chain for optimal signaling through Mincle and adjuvant potential of glucose-based glycolipids.

As previously mentioned, the combination of a delivery system such as emulsion and one or more immuno-stimulatory molecules is a promising approach for the rational design of adjuvants for subunit vaccines. In this study, we analyzed the synergistic effect of combining a C-type lectin receptor agonist (AraMM) and a TLR agonist (MPLA) in emulsion. Indeed, we have previously demonstrated that AraMM can activate BMDCs, in terms of production of pro-inflammatory cytokines and inflammasome activation, by mechanism dependent on C-type lectin receptor Mincle. However, this compound appears to be inactive *in vivo* when formulated in w/o/w emulsion. Here, we showed that adding MPLA to emulsion-containing AraMM significantly increases the level of Th1 and Th17 immune responses induced. These data demonstrated a synergistic effect of coupling a C-type lectin receptor agonist with a TLR agonist. MPLA is a less toxic structural analogue of LPS recognized by TLR4. MPLA recognition triggers the activation of two different signaling pathways that are MyD88 and TRIF dependent. Signaling pathway downstream of Mincle receptor is dependent on the spleen tyrosine kinase (Syk). Altogether, these data suggest that combination of these two immuno-stimulatory molecules will lead to the activation of three different signaling pathways which can synergistically enhance immune responses induced. Considering that AraMM was able to strongly activate the inflammasome in LPS-primed BMDCs, we hypothesize that in MPLA/AraMM formulation MPLA will prime APCs and that AraMM would strongly activate the

inflammasome *in vivo.* However, it is very important to bear in mind that combining several PRR agonists can also lead to antagonistic effect. Indeed, negative cross-regulation between PRRs has been reported. For example, Simmons and colleagues demonstrated that TLR2 agonists inhibit the production of type I IFN and cross-presentation processing induced by TLR9 [28]. In addition, numerous reports demonstrated that Syk can suppress TLR responses by inducing the degradation of MyD88 and TRIF [29–31]. In addition, it was recently report by Greco et *al.,* that Mincle negatively regulates TLR-4 activation induced by LPS [32]. However, in the latter study co-activation of Mincle and TLR-4 resulted only in the reduction of inflammatory responses induced by LPS *in vitro.* Thus, in the context of adjuvant development, we can speculate that if co-activation of Mincle and TLR-4 will similarly also result in reduced inflammatory responses this should result in a control of the inflammation induced by vaccination without affecting adjuvant property. In addition, several reports suggest that TLR activation leads to surface expression of Mincle receptor and by this way could contribute to the increase of Mincle responses induced by AraMM [33–35]. Synergistic effects of combining Mincle and TLR receptor have also been reported by P. Andersen's group that demonstrated that a TLR9 agonist CpG ODN 1826 acts synergistically with DDA-glycerol monomycolate (MMG) and enhances Th1 and Th17 responses induced by DDA- MMG alone [36].

Finally, in this study we demonstrated that DDA liposomes can be used to formulate GMM and AraMM. This formulation induces anti-PPE44 specific antibodies and PPE44-specific Th1 and Th17 immune responses without induction of a strong local inflammation. These data suggest that formulation of glycolipids in DDA liposomes reduces the toxic effect induced by mycolate esters. Indeed, Hunter et *al.,* showed that TDM formulated in the presence of oil forms a monolayer structure that induces granulomas and necrosis [37]. Strong neutrophil recruitment induced by emulsion formulation can explain the reported necrosis. In addition, Wook-Bin Lee and colleagues demonstrated that neutrophils express Mincle and can recognize TDM [35]. This recognition of TDM by neutrophils appears to be important at the early stage of the induction of granuloma by w/o/w emulsion containing TDM [35]. Given the fact that DDA liposomes preferentially target APCs [21], it is tempting to speculate that formulation of glycolipids in DDA liposomes will specifically present mycolate esters in a form that avoids recognition by neutrophils or recruitment of neutrophils and by this way avoids the exacerbation of the immune responses. Given the fact that TDM has been considered for

decades to be toxic for human use [38] and the fact that the less toxic analogue TDB induces a different profile of cytokines and chemokines in human APCs [39], we could imagine that the use of TDM in DDA may induce a different profile of adaptive response *in vivo* that can be more protective than TDB without side effect.

Interestingly, when formulated in DDA, AraMM induces antigen-specific Th1 responses similar to those induced by DDA-TDB or DDA-GMM. This result suggests that DDA can enhance immune responses induced by AraMM. Several hypothesis can explain this observation. First, AraMM may be presented to APCs in a more optimal manner when formulated in DDA liposomes. Second, by inducing a depot effect at the site of injection, DDA liposomes might maintain AraMM longer at the site of injection and this may enhance APCs activation. Finally, as we showed in this paper, this may be due to a synergistic effect of DDA and AraMM on inflammasome activation. Inflammasome is a multiprotein complex involved in the maturation of IL-1β and IL-18 [40]. IL-1β plays a role in the polarization of Th17 response [41] and IL-18 is involved in the generation of Th1 response [42]. It was already known that the Th17 response induced by DDA-TDB is related to the activation of the inflammasome [23]. However, in that report data obtained with DDA alone were not described and therefore the possible synergistic effect of DDA liposome and TDB was not investigated. To our knowledge, our study is the first to demonstrate a possible synergistic effect of DDA liposomes and immuno-stimulatory molecules such as glycolipids on the activation of inflammasome. Our results also demonstrate that DDA-AraMM induces only a marginal Th17 response. IL-1β, IL-6 and TGF-β cytokines are known to drive Th17 polarization [41]. Therefore, a weak expression of IL-6 and TGF-β can explain the marginal IL-17 response induced. These data are preliminary and need to be repeated before a final conclusion can be drawn. If confirmed, expression of these 3 cytokines at the injection site and in the draining lymph nodes will be analyzed. We also need to investigate if DDA induces the activation of the inflammasome *in vivo.*

In summary, this study explores several strategies, which can lead to the design of more rational adjuvants able to favor induction of humoral and cell-mediated immune responses.

Materials & methods

Mice

C57BL/6 $^{wt/wt}$, MyD88$^{-/-}$ and IL-1R$^{-/-}$ mice were bred and kept at the experimental animal facilities of WIV-ISP (Ukkel site, Brussels), complying with the Belgian legislation that transposes European Directive 2009/41/EC, repealing Directive 90/219/EC (EC, 2009). Breeding pairs of MyD88$^{-/-}$ mice were kindly provided by C. Desmet (Cellular and Molecular Physiology, GIGA-Research, ULg, Belgium). IL-1R$^{-/-}$ mice were provided by E. Muraille (University of Namur, Belgium).

Preparation of glycolipid coated plates

Stocks of synthetic short chain GMM (KB177), long chain *cis*-methoxy GMM (SMP73), *cis*-methoxy AraMM (MOD16) (synthetized at Bangor University [25–28,30,31]) and TDB (Invivogen) were solubilized in chloroform-methanol solution (9:1) at 5mg/mL. The different glycolipids were then dissolved in isopropanol and coated on flat bottomed culture plates (Greiner). After evaporation of the solvent, the plates were used directly or stored at -20°C. Final concentrations are indicated in the figure legends and results section. A possible negative effect of the tested glycolipids on cell viability was tested by Alamar blue assay and no increased mortality was observed under the test conditions (data not shown).

Generation of BMDCs

BMDCs were generated as previously described [32]. Briefly, murine bone marrow from femur and tibia was flushed with PBS and red blood cells were lysed with Sigma's lysing buffer. After lysis, cells were cultured (5% CO2 at 37°C) in T75 flask in DMEM medium (Life Technologies, Carlsbad, CA, USA) supplemented with 20 ng/mL recombinant murine GM-CSF (Immunotools), 5×10^{-5} M 2-mercaptoethanol, 100 µg/mL gentamycin (GIBCO), 2mM glutamine, 1 mM sodium pyruvate, non-essential amino acids (Thermo Fisher Scientific) and 10% fetal calf serum (FCS, Greiner). Cells were differentiated for 6-7 days and the medium was replaced every 3 days. 90 % of CD11c positive cells were obtained after differentiation.

HEK-blue mMincle stimulation

Human embryonic kidney (HEK)-blue mMincle cells (Invivogen) stably transfected with murine C-type lectin receptor mincle gene, as well as the genes of Mincle-NF-κB signalling pathway

and secreted alkaline phosphatase (SEAP) under the control of NF-κB activation, were used to analyze the activation of Mincle receptor by the compounds. HEK-blue mMincle cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4.5g/L glucose and supplemented with 10% FCS, selective antibiotics and 2mM glutamine. Cells were passed in T75 flask when 70-80% confluence was reached. For the stimulation, cells were seeded in 96 well plates at the density of 5.104 cells/ well for 24 h. Quanti-blue substrate (Invivogen) was used for the detection of SEAP in the supernatant according to the manufacturer's instructions.

Preparation of water in oil in water emulsion and liposomes

The protocol for the water in oil in water emulsions (w/o/w) was based on the one by Yarkoni & Rapp in 1977 with some small changes [43]. Briefly, glycolipids were dissolved in 30% of Incomplete Freund's Adjuvant (IFA) and vortexed vigorously. Next, we added 0.1M of PBS and vortexed vigorously to obtain water in oil in water emulsion. Finally, we added and vortexed again PBS containing 0.2% of Tween 80 containing the needed amount of protein (OVA or recombinant PPE44).

DDA liposomes were prepared using the dry-heat method. Briefly, DDA and glycolipids were dissolved in chloroform-methanol (9:1, v/v) and mixed at a ratio of 5:1 (DDA/glycolipids). After evaporation of organic solvent, sterile PBS was added and heated at 60°C for 20 minutes with vortexing every 2 minutes. Next, the preparation was cooled at room temperature before adding the equal amount of PBS containing recombinant proteins (PPE44 or OVA).

Inflammasome assays

BMDCs at 1.10^6 cells/ mL were primed for 3 h with 1µg/mL of E. coli K12 ultra-pure LPS (Invivogen) prior to stimulation with coated glycolipids, DDA, DDA/glycolipids and controls. Cells were stimulated in triplicate with 5mM ATP (for 1h), glycolipids, DDA or DDA/glycolipids (for 5h). IL-1β cytokine production in cell-free supernatant was evaluated by ELISA (eBiosciences).

Footpad immunization

Groups of 4-6 mice were injected subcutaneously in both hind footpads with 25 µl of the emulsion containing 30% IFA, 5µg of glycolipids and 25µg of ovalbumin (Invivogen) or

liposome containing 125 µg of DDA, 25µg of glycolipids and 25 µg of ovalbumin. For the experiment with recombinant PPE44, we used 4 µg of protein. Footpad swelling in individual mice was measured with a caliper before and at several time points after injection.

Analysis of local inflammation

One week after administration of the w/o/w or liposomes, the mice were sacrificed and the tissues of the hind footpads were collected and digested using collagenase and DNAse (Sigma). Cells were collected and strained with a 100 -µm nylon cell strainer (BD). Footpad cells were incubated with Ly6G-Ly6C PE and viability dye V450 (eBiosciences) and the fluorescence was analysed with a FACSverse device.

Analysis of adaptive immune responses

One week after subcutaneous administration of the different w/o/w preparations, mice were sacrificed and popliteal and inguinal lymph nodes were removed and passed through a 100 -µm nylon cell strainer (BD). Cells were counted and stimulated in RPMI medium supplemented with 5×10^{-5} M 2-mercaptoethanol, antibiotics and 10% FCS in round-bottom 96 well plates with 10µg/ml of peptides ($OVA_{323-339}$, $PPE44_{1-20}$), 5µg/mL of recombinant proteins (OVA and PPE44), culture medium as a negative control and Concanavalin A (Sigma) (4µg/mL) as a positive control. Cell free culture supernatants were harvested after 24h and 72h of incubation at 37°C-5% CO2. Levels of IL-2 were measured in 24h supernatants by ELISA (eBiosciences). Levels of TNF-α, IFN-γ and IL-17A were determined in 72h supernatants by ELISA (respectively BD Pharmingen and eBiosciences).

Analysis of antibody level in sera

Sera of vaccinated mice were collected three weeks after the last immunisation. Levels of specific antibodies in individual sera were determined by ELISA. For that purpose, 96-well plates were coated overnight with the specific recombinant protein at 500 ng/well in borate buffer. Adsorption sites were saturated during 1 h with 5% skimmed milk in PBS. Serial two-fold dilutions of sera were added for 2 h. Then, a peroxidase-labelled secondary rat anti-murine IgG (LO-MK-1 for IgG, purchased at Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium) was added for 1.5 h. Plates were developed following the same protocol used for cytokine detection, as previously described.

Statistical analyses

Statistical analyses were performed using Graphpad Prism 6 software (Graphpad Software, La Jolla, CA, USA). For the statistical analysis, data were tested for Gaussian distribution with D'Agostino Pearson test followed by pairwise comparisons performed by Mann-Whitney test. For all analyses, $p<0.05$ was considered as statistically significant.

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4. DISCUSSION

DISCUSSION:

Tuberculosis (TB) is an infectious disease caused by bacteria belonging to the *Mycobacterium tuberculosis complex* (MTBC*).* MTBC regroups closely related mycobacterial species that cause TB in both humans and animals. Despite the development of antibiotics and of a vaccine, TB continues to be one of the leading causes of morbidity and mortality throughout the world. Indeed, TB is the second most common cause of death from a single pathogen after HIV. Considerable progress regarding the diagnostic of TB has been achieved in the last few years and new molecular and rapid diagnostic tests have been developed, however conventional tests such as sputum smear microscopy and culture remain in use in the field as the new molecular tests are intended for use in laboratories with dedicated infrastructure and experienced staff and this is not always the case in most of the laboratories in countries with a high TB incidence [456]. Regarding treatment, there are effective drug treatments available since 1940s. The current treatment recommended by the World Health Organization consists of 6 months regimen of four first line drugs (isoniazid, rifampicin, ethambutol and pyrazinamide). However, the emergency of drug resistance poses a problem since the past years and several anti-TB drugs are currently in clinical development. A more effective vaccine against pulmonary TB, which represents the source of disease transmission, is also needed. The existing BCG vaccine shows an extremely variable protection (from 0 to 80%) against this form of TB in adults and adolescents. The global plan against TB is to eliminate TB as a public health problem in 2050 (defined as <1 case per million people per year). To achieve this ambitious goal, better and cheaper diagnostics, better antibiotics and a better vaccine are needed. To do that, a better understanding of mechanisms related to *M. tuberculosis* pathogenicity is needed. In this study, we analysed the host-pathogen interaction by assessing the impact of the fine structures of glycolipids homologous to those found on the cell wall of *M. tuberculosis* in the modulation of the host immune response. We also took advantage of the inflammatory potential of these glycolipids and analyzed whether these compounds can be used as adjuvant for subunit vaccine.

4.1 Host-pathogen interaction

4.1.1 Mycolate esters as modulator of immune responses

The cell wall of *M.tuberculosis* is repleted of lipids with biological activity. Among these lipids, mycolate esters that are present as non-bound extractable glycolipids, more particularly TDM, have been known for decades to be important pathogenesis associated components. It has been reported that modification of the composition of TDM results in diverse inflammatory profiles suggesting that each class of compound of this complex mixture of TDMs differentially contributes to pathogenesis. However, these TDMs purified from the cell wall of mutant strains of *M. tuberculosis* remain a complex mixture of molecules. Hence, the use of *M. tuberculosis* mutants does not allow to precisely determine the impact of the different classes of TDM in the inflammatory power of mycobacteria. In the present study, using 17 pure synthetic TDMs that represent the majority of classes of TDM present in *M. tuberculosis*, we analyzed the relationship between the fine chemical structure of TDM and innate immune activation. Our results only partially confirmed data reported with mutated strains. Actually, we showed that alpha'-monoalkene TDM and alpha-diene TDM, both without cyclopropanation, induce less pro-inflammatory cytokines compared to alpha-TDM with cyclopropanation, suggesting that cyclopropanation is involved in the activation of innate immune cells. These results are in agreement with the findings of Rao and colleagues [171]. However, only *cis*-cyclopropanation has been reported to regulate positively the activation of macrophages. Indeed, in 2006 Rao et *al.* reported that *trans*-cyclopropanation of MAs on TDM suppresses the activation of macrophages. They generated *M. tuberculosis* strain deficient for *cmaA2*, an enzyme involved in the synthesis of *trans*-cyclopropanation on oxygenated MAs. This *cmaA2* null mutant was hypervirulent *in vitro* and *in vivo.* Further, TDM of this mutant induces 5 fold more TNF-α compared to TDM from WT strain of *M. tuberculosis* [171]. Interestingly, our results showed that *trans*-oxygenated MA TDMs induce less TNF-α compared to *cis*-oxygenated MAs TDMs, even if the differences are not substantial. This observation suggests that *trans*-cyclopropanation may regulate negatively the activation of BMDCs. To confirm this hypothesis, it would be interesting to compare the inflammatory potential of TDM composed of oxygenated MA with or without cyclopropanation.

Nevertheless, given the fact that cyclopropanation is absent in nonpathogenic mycobacteria, it can be speculated from our data and the data obtained with mutant strains that cyclopropanation of MAs in *M. tuberculosis* modulates positively or negatively the activation of the innate immune cells.

In addition to cyclopropanation, oxygenation of MAs has been reported to be related to the pathogenesis of *M. tuberculosis* [170,457]. As mentioned, Dao and colleagues demonstrated that TDM from the *mmaA4* null mutant, a mutant defective in the production of oxygenated MAs, produces more IL-12p40 and TNF-α compared to TDM from WT *M. tuberculosis*. Additionally, they showed that WT TDM inhibits the production of IL-12p40 and not TNF-α induced by TDM from mutant Δ*mmaA4*, suggesting that oxygenated-MA TDMs inhibit the production of IL-12p40 (a cytokine involved in the generation of Th1 protective immune response). We did not observe a similar difference of IL-12p40 production between *cis*-methoxy TDM, TDB or natural TDM. On the contrary synthetic *cis*-methoxy TDM induced even more IL-12p40 than TDB, suggesting that the oxygenation of *cis*-methoxy TDM does not inhibit production of IL-12p40. Furthermore, we observed that *cis*-alpha TDM and *cis*-methoxy TDM induce similar levels of Th1 immune responses *in vivo,* suggesting that the classes of MAs on TDM do not impact on the induction of specific immune responses *in vivo*. The discrepancy between our *in vitro* data and the data of Dao and colleagues can be explained by the fact that we used different cell types. Indeed, Dao et *al.* also reported that BMDCs from BALB/c mice infected with Δ*mmaA4* or WT strains produce the same level of IL-12p40, indicating that the effect of this mutation on the production of IL-12p40 is selective for macrophages (from BALB/c mice). This type of cell type-selective effect has been reported for other infections such as leishmaniosis or toxoplasmosis [458]. However, Dao et *al.* did not test *in vivo* the immune response induced by TDM from mutant Δ*mmaA4.* They also found that Δ*mmaA4* mutant strain produces epoxy-MA. We cannot exclude that the effect observed with this mutant was due to the presence of epoxy-MA. To make a definitive conclusion, it would be interesting to comparatively investigate the inflammatory potential of the synthetic mycolate esters (with an epoxy-MA TDM) in bone marrow derived macrophages and dendritic cells of BALB/c and C57BL/6 mice.

Relevance of these studies

The analysis of the impact of the structure of mycolate esters on the immune activation is relevant for two reasons: the development of new drugs and the development of attenuated strains. Indeed, in *M. tuberculosis*, there are 8 S-adenosylmethionine (SAM)-dependent methyltransferases. 6 of these enzymes have been reported to participate in mycolic acid modification (MmaA1 to 4, PcaA and CmaA2). MmaA4 is involved in the synthesis of oxygenated mycolates. MmaA3 is implicated in the synthesis of methoxymycolates by adding a methyl group to hydroxyl group. MmaA1 and CmaA2 induce *trans*- cyclopropanation of oxygenated MAs. MmaA2 and PcaA add cyclopropane rings to the distal and proximal positions of the alpha mycolate respectively. Thus, identifying chemical groups involved in the inhibition of innate immune cell activation could allow to specifically target enzymes involved in the generation of this group with antibiotics. One interesting example is Thiacetazone (TAC). TAC is an anti-tubercular and bacteriostatic drug used in combination with isoniazid in Africa and South America [459]. TAC and its structural analogues (SRI-224 and SRI-286) have been shown to inhibit the cyclopropanation of MAs on the cell wall of *M. tuberculosis* and *M. marinum* [460]. Later, it was reported that MmaA4 is also necessary for the TAC susceptibility of *M. tuberculosis* [461]. Barkan and co-workers have developed strains of *M. tuberculosis* that do not possess any cyclopropanation (MGM1990) and any cyclopropanation and oxygenated MAs (MGM1991) [462]. They showed that mutant with no cyclopropanation (MGM1990) is highly attenuated throughout the infection. In addition, MGM1990 showed a hyper-inflammatory phenotype characterized by a higher production of unspecific IFN-γ and IL-17A. Interestingly, the MGM1991 mutant defective in cyclopropanation and oxygenation did not show this hyper-inflammatory phenotype (MGM1991), suggesting that the hyper-inflammatory phenotype observed is dependent on the unsaturated oxygenated mycolates. Further, in our lab, we have demonstrated that the mutant MGM1991 is highly attenuated and showed a better protection in a mouse *M. tuberculosis* infection model than BCG [463].

Mycolic acids are also found in the cell wall esterified to other sugars than trehalose such as glucose or arabinose. While glucose monomycolate (GMM) is found naturally in the cell wall of pathogenic mycobacteria, arabinose mycolates are components of the cell wall skeleton (CWS). CWS is a high molecular complex composed of mycolic acids, arabinogalactan and peptidoglycan [464]. CWS of *M. bovis* BCG Tokyo 172 has been shown to possess inflammatory and antitumor properties in preclinical and clinical studies [464–467]. Further, it has been reported

that innate activation and antitumor activities of the CWS is dependent on TLR2[468]. In addition, Miyauchi and co-workers identified arabinose mycolates as component of CWS that activate macrophages by mechanisms dependent on TLR2. In our study, we showed that arabinose monomycolate induces the production of pro-inflammatory cytokines on BMDCs even if the level achieved was significantly lower than that induced by trehalose and glucose-based mycolate esters. This observation was also reported by Miyauchi et *al.* who showed that TDM is 10 times more active than arabinose mycolates. However, in our study, we found that the activation of BMDCs by synthetic arabinose monomycolate is independent of the MyD88 pathway but dependent on the Mincle pathway. This difference in the results could be explained by the fact that arabinose mycolates of Miyauchi and colleagues are a complex mixture of mono-arabinose mono-mycolate, penta-arabinose tetra-mycolate and hexa-arabinose tetra-mycolate. We cannot exclude that penta-arabinose tetra-mycolate and hexa-arabinose tetra-mycolate could bind TLR2 while mono-arabinose mono-mycolate is recognized by Mincle. Similar discrepant results were also obtained with TDM. Indeed, it was first reported that TDM is recognized by TLR2, CD14 and the scavenger receptor MARCO [469]. Later several studies demonstrated that the induction of the production of cytokines, the granulomatogenic and the adjuvant properties of TDM were totally dependent on Mincle pathway [156,232,233,237,453].

GMM is a glycolipid present in pathogenic mycobacteria *in vivo*. Here, we demonstrated that short chain and long chain GMM are recognized by Mincle. This study is in agreement with the findings of Van der Peet [241] and disagree with the data of Ishikawa et *al.* who showed that long chain GMM obtained by trehalase treatment of TDM is not recognized by Mincle [232]. Given the fact that we used synthetic compounds with defined structure, we hypothesize that this discrepancy can be due to the specificity of the enzyme. Indeed, Ishikawa and colleagues did not characterize products resulting from the enzymatic reaction. We could speculate that if trehalase cuts the ester bond between trehalose and MA, and considering that trehalose or MA do not activate Mincle, this could explain the result of Ishikawa. Of course, this is speculative and needs to be clarified to make a definitive conclusion.

4.1.2 Structure required for Mincle activation

In this study, we demonstrated that in addition to the recognition of hexose monosaccharides or disaccharides, Mincle is able to recognize pentose monosaccharides. Indeed, we showed, by using BMDCs from Mincle, MALT-1 and FcRγ KO mice and reporter cells, that AraMM is recognized by Mincle even if the activation of cells induced by AraMM is lower than the one induced by the other tested glycolipids. These results suggest a lower binding of Mincle to AraMM compared to GMM, TMM, TDM and TDB. Recently, Decout and co-workers also reported that synthetic AraMM is recognized by Mincle [470]. In addition, we showed that TDM induces more pro-inflammatory cytokines compared to TMM *in vitro* while *in vivo* both induce similar levels of cytokines and chemokines as well as mixed Th1/Th17 responses. These data suggest that with longer chain length fatty acid (MA), the number of fatty acid chains bound to the sugar or the type of sugar (trehalose vs glucose) do not influence the adjuvant potential *in vivo.* However, Huber and co-workers recently reported that with short length fatty acid (C20 to C12), trehalose monoesters induced lower level of G-CSF and NO as well as Th1/Th17 immune responses than the trehalose diesters. They found a good correlation between the binding of these glycolipids to the recombinant Mincle-Fc fusion protein and the production of G-CSF and NO, suggesting that the interaction with Mincle influences the activation of APC and consequently the induction of adaptive immune responses [471]. Recently, the crystal structures of bovine and human Mincle have been reported by two laboratories [472,473]. They showed that in the carbon recognition domain (CRD) of Mincle, there are two sugar binding sites and a hydrophobic pocket region. One of the two glucose residues of trehalose binds to one sugar binding site by a Ca^{2+} dependent manner whereas the second glucose binds to the other sugar binding site. The binding to the second site enhances the affinity of trehalose compared to glucose by 36 fold. In addition, the interaction of the acyl chain with the hydrophobic pocket of Mincle, dedicated for the recognition of the acyl chain, enhances the affinity of TDM compared to trehalose by 56 fold [474]. Furthermore, it has also been reported that the affinity of trehalose diesters is superior compared to the affinity of trehalose monoesters [475]. These data corroborated our *in vitro* data and the data of Huber et *al* [471]. However, *in vivo* our data differ from the data of Huber et *al.* Indeed, we reported that TDM and TMM have the same adjuvant potential while Huber and colleagues showed that the adjuvant capacity of trehalose diesters is superior from that of trehalose monoesters. It is important to keep in mind that the carbon chain length was very different (C74 to C80 in our

study and C12 to C20 in the Huber et *al*. study). Rambaruth and co-workers demonstrated that the acyl chain length influences the affinity of trehalose esters for mouse Mincle [473]. In this study, they used structural analogues of TDM with very short chain length (from 3 to 12 carbon atoms) simple fatty acids. We suggest that with longer fatty acid chains the affinity of TMM can significantly increase to a level sufficient to induce priming of strong adaptive immune response (comparable to the level induced by TDM). This hypothesis is supported by our *in vitro* data. Indeed, even if TDM is superior in inducing the secretion of pro-inflammatory cytokines compared to the corresponding TMM, TMM induced substantial level of cytokines in contrast to trehalose monoesters with shorter chain which were negative or induced very low level of NO or cytokine. In addition, we did not do a dose response curve *in vivo*. It is not excluded that at lower concentration the adjuvant potential of TDM will be superior to the one of TMM.

Regarding GMM, the structure required for the binding to Mincle is different. Indeed, it was reported by using a cell reporter system that glucose monobehenate (GMB) is less active than Glucose monocoryonomycolate (GMCM). Here, we confirmed the fact that short chain GMM is recognized by Mincle and extended these finding by demonstrating that this compound possesses adjuvant potential and can induce Th1/Th17 to the level comparable to that induced by GMM, TDB and TDM. The fact that GMCM is much more active than GMB is very particular for Mincle ligand. In fact, it has been shown that the substitution of MAs with shorter fatty acid chains such a behenic acid do not impact the signalling through Mincle. It is for example the case with TDB, trehalose monobehenate and glycerol monobehenate (GroMB) [235,243]. This observation suggests that in the case of glucose, the meromycolate chain of the acyl group is indispensable for the recognition of the compound by Mincle. Van Der Peet et *al.* recently investigated the lipid structure required for the activation of Mincle by glucose esters [476]. They have found that GMB activates reporter cells at higher concentration (1000 times compared to TDM). Interestingly, they found that the chain length of the α-chain of the lipid influences the level of activation of glucose esters. Indeed, a glucose with an α-pentyl derivative signals much more weakly through Mincle compared GMCM, which possesses a α-chain with 14 carbon atoms. In addition, glucose monoester with a C23 derivative signals similarly through Mincle in comparison with GMCM. Glucose ester with a C14 α-chain seems to be enough for optimal signalling through Mincle. More recently, Decout

and colleagues also analysed the structure of glycolipids required for the recognition by Mincle[470]. They have found that the 3-hydroxy group, found in MAs and coryno MAs, is dispensable for the binding to Mincle. In addition, they confirmed that the α-chain is indispensable for the recognition of glucose esters by Mincle. Indeed, they observed that glucose esterified with 2-tetradecyloctadecanoic acid (GC14C18) is recognized by humanMincle-Fc and showed a strong activity, comparable to those induced by GMCM. In contrast, 3-hydroxyoctadecanoic acid (G3OHC18) that lacks α-chain is not recognized by humanMincle-Fc. By using molecular dynamic stimulations, they have demonstrated that in addition to the known 3 sites necessary for the recognition of glycolipids by Mincle, an additional short loop stretch formed by amino acid residues L172 and V173 may be necessary for the recognition of glucose esters. Indeed, they have found that the C14 α-alkyl chain of GMCM interacts with amino acid residues L172 and V173 and this interaction induces conformational modifications of the receptor. In contrast, G3OHC18 does not induce this conformational change and as a result the alkyl chain is not properly inserted into the hydrophobic pocket and remains at the surface. According to their data and the studies of the crystallography structure of the receptor as well as the structure-function relationship analysis, the authors discussed the fact that there are four binding sites (two interacting with hydrophilic head of the molecule and two interacting with the alkyl chain) on Mincle receptor and at least three need to be engaged to induce the activation of the receptor. However, as mentioned, we observed that *in vitro* TDM is more inflammatory than TMM and GMM. Thus, these data do not explain this observation. It may be possible that other receptors such as MCL could play a role in a strengthening of the response to Mincle. Indeed, it has been reported that Mincle and MCL form heterodimer [245].

Given that in nature the chain length of the α-chain discriminates pathogenic and non-pathogenic actinomycete species, the data from Van Der Peet suggest that Mincle could provide the capacity to survey and discriminate pathogenic and non-pathogenic actinomycetes. Interestingly, we found that glucose ester with shorter meromycolate chain and shorter α-chain induced significantly lower TNF-α and footpad swelling but similar Th1/Th17 responses compared to GMM. Altogether, these data reveal the structure requirement for GMM necessary to interact with Mincle and to induce strong adaptive immune response with lower reactogenicity.

4.1.3 Mycolate esters and inflammasome

In this work, we showed that TDM, TMM, GMM and AraMM activate the NLRP3 inflammasome. The inflammasome is a multiprotein complex that leads to the maturation and release of IL-1β and IL-18. This complex is important for the control of *M. tuberculosis* given that IL-1R KO, IL-1β KO and IL-18 KO are much more susceptible to *M. tuberculosis* infection than WT mice [477–479]. IL-1β and IL-18 participate to the protective response to TB by inducing protective immune responses. Indeed, it has been shown that IL-1β plays a role in the generation of Th17 immune response [480] while IL-18 is involved in the amplification of Th1 responses [481,482]. The contribution of mycolate esters in the activation of the inflammasome by *M. tuberculosis* appears to be negligible *in vitro*. Indeed, it has been reported that the activation of NLRP3 inflammasome by *M. tuberculosis* in BMDCs and BMDMs is dependent of the ESX-1 system and cytosolic escape of mycobacteria [483], suggesting that the mycobacteria need to be in the cytosol to activate NLRP3 inflammasome. However, we have observed that the cell membrane receptor Mincle is necessary for the activation of the inflammasome by mycolate esters (data not shown). The same observation has been reported with TDB by Desel et *al* [237]. It is important to bear in mind that these studies have been done with mycobacterial strains cultivated in 7H9 medium and this medium, differently from Sauton medium, is not known to favor development of an important layer of cord factor on the surface of the mycobacteria.

Even if inflammasome dependent cytokines are necessary for the control of *M. tuberculosis* infection, NLRP3 inflammasome appears not to play an important role. Indeed, mice deficient for NLRP3, ASC or caspase-1 are not more susceptible than WT mice. These data suggest that *in vivo* IL-1β and IL-18 are cleaved by other enzymes such as cathepsins and elastases. These enzymes highly expressed by neutrophils have been shown to induce the maturation of pro-IL-1β and IL-18 by mechanisms independent of the inflammasome [484].

4.1.4 Interaction with Mincle and relevance for the mycobacteria

Here, we demonstrated that several compounds found on the surface of mycobacteria can bind to Mincle and activate Mincle-Syk-CARD9-MALT-1 pathway and the inflammasome. This activation leads to the production of pro-inflammatory cytokines, chemokines, antibodies as

well as Th1 and Th17 immune responses. From this point of view, Mincle interaction seems to favor host's response to *M. tuberculosis.* However, the role of Mincle in infection is much more complex. Indeed, Mincle has been reported to have a dual function as immuno-activator and regulator of the immune response. There is increased evidence in the literature suggesting that Mincle can modulate innate and adaptive immune responses. Devi and colleagues reported that *Helicobacter pylori* induces anti-inflammatory cytokines trough binding to Mincle. Indeed, knockdown of Mincle in human macrophages results in the upregulation of pro-inflammatory cytokines and the down-regulation of anti-inflammatory cytokines [485]. Regarding mycobacteria, Zhang and co-workers showed that neutrophils produce no or weak pro-inflammatory cytokines following stimulation with a single agonist of PRRs while stimulation with several agonists that activate a Syk dependent and MyD88 dependent pathways or with whole mycobacteria induce the production of pro- and anti-inflammatory cytokines. Further, they demonstrated that Mincle and TLR2 stimulation on neutrophils leads to the production of substantial amounts of IL-10 that participate to the reduction of lung inflammation *in vivo* [486]*.* Recently, Patin and colleagues demonstrated that stimulation of Mincle and TLR2 respectively by TDM and TLR2 agonist (PAM_3CSK4) triggers macrophage production of IL-10 [487]. This produced IL-10 subsequently modulates the production of IL-12p40 secretion in an autocrine manner. In addition, they showed that Mincle is essential for macrophage IL-10 response to BCG. Owing to the complexity of interaction between mycobacterial PAMPs and innate immune cells, a co-activation of Mincle and TLR2 pathways for example by TDM and the lipoproteins is most likely to occur *in vivo.* Thus, these data demonstrate a mechanism by which Mincle may modulate innate immune responses to mycobacteria through counter-regulation of IL-12 by IL-10. It remains to be determined whether the production of IL-10 through co-stimulation is a mechanism of the mycobacteria to escape the immune system or participate in the control of excessive inflammation that can induce tissue destruction. Recently, the role of Mincle in the regulation of inflammation has been demonstrated by Lee and co-workers [238]. In addition to this production of IL-10, Mincle has also been demonstrated to inhibit immune response induced by TLR4 agonist. This inhibition appears to be independent of IL-10 production but dependent on A20 and ABIN3 [488]. Indeed, if Mincle is deleted a decreased production of A20 and ABIN3 is observed. It was recently demonstrated that Leishmania inhibits activation and migration of DCs to the lymph

node by mechanisms dependent on Mincle. In addition, the authors showed that Leishmania shifts Mincle to an inhibitory FcRγ/SHP1 axis in DC.Mincle can inhibit

In this study, we identified several ligands of Mincle that may modulate immune response by these described mechanisms.

4.2 Antigen property of glucose monomycolate

Besides being a Mincle ligand, GMM is also known to be a protective non-protein antigen recognized by CD1b restricted T cells [147]. CD1b belongs to the family of group 1 CD1 molecules. These molecules are specialized in the presentation of lipid, glycolipid and lipopeptide antigens. CD1 molecules have a low level of polymorphism in their antigen-binding domain, making them very attractive for the development of subunit vaccine that would be given to a widely diverse human population. Most of the strategies used in the development of subunit vaccines against TB are focused on protein-antigen while increased evidence suggest a protective role also of lipid antigens. Indeed, people exposed to *M. tuberculosis* show a higher frequency of group 1 CD1-restricted T cells [148,165]. In addition, it has been reported that human group 1 transgenic mice infected with *M. tuberculosis* or vaccinated with lipid antigens showed a robust group 1 CD1-restricted *M. tuberculosis* lipid specific T cell response [344]. Furthermore, Zhao and colleagues demonstrated by using transgenic mice expressing human group 1 CD1 molecules that lipid-specific CD1b molecules are protective during *M. tuberculosis* infection [346]. Interestingly, Seshadri and colleagues analyzed the lipid and protein antigen responses in patients with *M. tuberculosis* infection compared to non-infected individuals. They found that GMM is the immunodominant lipid antigen. This lipid antigen is recognized by polyfunctional CD4 T^+ cells that express CD40L, IFN-γ, IL-2 and TNF-α. In addition, they showed a poor correlation between CD4 T^+ cell responses to lipids and CD4 T^+ cell responses to proteins, suggesting a non-redundant role of T cell response to mycobacterial lipids and protein antigens. Thus, the use of synthetic GMMs, investigated in this study, as lipid antigen in combination with *M. tuberculosis* protein-based subunit vaccines may lead to the development of better protective vaccines.

Regarding the structure required for the antigenic presentation of GMM by CD1b molecules, Moody and co-workers reported that long chain GMM (C80) is better presented to CD1b-

restricted T cells than the same glycolipid with shorter chain (C32). Additionally, they demonstrated that varying the meromycolate chain and the α-chain of GMM influences the activation of CD1b-restricted T cells. They showed that this is due to the fact that GMM (C80) is processed in the late endosome while GMM with shorter chain is not. Furthermore, GMM (C80) is preferentially presented by DCs while GMM (C32) is presented by non-professional APCs such as B cells or thymocytes. These non-professional APCs lack prominent endosomal presentation pathway [489]. These data showed that lipid chain length influences the antigenic presentation of GMM. To the best of our knowledge, it is not known whether the classes of MAs bound to glucose can have an impact on the antigenic presentation of GMM.

4.3 Mycolate esters as adjuvants

In this study, we tested the adjuvant potential of a series of mycolate esters that varies in terms of degree of acylation (TDM vs TMM), the sugar moiety (trehalose vs glucose vs arabinose) in two different formulations (emulsion vs liposome). Our results showed that TDM, TMM and GMM induced similar level of Th1 and Th17 responses in both emulsion and liposome formulations whereas AraMM induces Th1 and antibody responses only when formulated in DDA liposomes. We also found that mycolate esters in DDA liposomes are less reactogenic compared to emulsion formulations as shown by a lower recruitment of inflammatory cells such as neutrophils at the site of injection. These findings corroborate previous results that demonstrated that liposome formulations can reduce the toxicity of immune-stimulatory compounds. There are few adjuvants approved for human use which can induce CMI responses. However, there are promising candidates in clinical trials able to induce this type of response. Among these compounds, to the best of our knowledge, only DDA-TDB (CAF01) adjuvant is able to induce Th1 as well as Th17 responses. There is increased evidence in the literature supporting the protective role of Th17 $CD4^+$ T cells against infectious diseases [490–495]. For example, IL-17 secreted by Th17 $CD4^+$ T cells has been shown to be important for the recruitment of Th1 responses in the lung. Th17 $CD4^+$ T cells can directly induce protection against *M. tuberculosis* infection [318,496]. These data demonstrate the interest of targeting the Mincle dependent pathway for the development of adjuvants. In addition, it is reported that the combination of several immune-stimulatory compounds that can activate different pathways may act synergistically to induce better responses. This is the approach used by

GlaxoSmithKline in the development of Adjuvant System and by Staten Serum Institute in the development of cationic adjuvant formulation (CAF) adjuvant. Thus, the combination of Mincle adjuvant with TLR agonist may lead to induction of better protective responses. Nordly and colleagues showed that incorporation of MPLA in CAF01 adjuvant (DDA/TDB) induces strong $CD8^+$ T cell responses without disturbing humoral responses [497]. Similar results have been shown with combined Mincle agonist (GroMM), DDA and TLR3 agonist (Poly I:C) [498]. However, as previously discussed, it is important to bear in mind that activation of several pathways may lead to antagonistic effect as well. Thus, caution is needed when combining immuno-stimulatory compounds in adjuvant formulations.

In conclusion, mycolate esters analyzed in this study have an interesting potential for development of novel adjuvants. Nevertheless, TDM has been reported to be toxic and this toxicity seems to depend on lipid-lipid interactions with the host cell membrane. Indeed, TDM has been shown to influence cell membrane integrity and stability, leading to acyl chain disorder, alteration of membrane curvature and disruption of lipid microdomains. The authors suggested that this is due to the integration of long chain fatty acid (MAs) into phospholipid bilayer and this may account for the TDM induced inhibition of phago-lysosomal fusion [499,500]. Further, Hunter and co-workers reported that interaction of TDM with host lipids induced necrosis in host tissues[159]. The mechanism is not clearly understood but it appears to depend on the conformation of TDM in monolayer structure [153]. However, it is not known whether TDM formulated in DDA liposomes is still toxic for the host cells.

The use of the complex synthetic compounds analysed in this study in subunit vaccines can be debated, especially due to the cost of their production and the fact that TDB seems to work. However, we believe that reducing the chain length may have an impact on the quality of the induced responses. For example, TDM and TDB have been shown to induce different types of responses in different innate immune cells in human [239]. It is tempting to speculate that testing synthetic cord factor analogues with different structures is an attractive strategy to develop new adjuvant for human use and may allow to specifically activate a type of APC and hence adjuvant characteristics. Thus, it would be of great interest to analyse the activation of human APCs by the synthetic compounds used in this study. Regarding GMM, we believe that the use of this class of compounds in subunit vaccine against TB may have a real advantage. Indeed,

as previously discussed, GMM is also a lipid antigen presented in the context of CD1b molecule and has adjuvant properties. Thus the use of this compound in combination with a protective protein antigen may lead to a better protection by a synergistic activation of classical and non-classical T cell responses.

4.4 Limitation of adjuvant development

In this study, we applied the most widely used tools for preclinical evaluation and mechanistic studies of adjuvants. Namely, analysis of the activation of APC *in vitro* and of the induced immunity in experimental rodent models. Of course, these approaches have some limitations. Some discordance in the responses induced in rodents compared to humans has been reported [363]. There are also difference in the expression patterns of PRRs between humans and rodents [501]. In addition, the experimental conditions that we used do not reflect exactly the practice of human vaccination. For example, we have analyzed the subcutaneous route of administration, which is not the typical route of administration of human vaccines. It is indeed known that the route of administration can impact the type of immune responses induced, for example by activating different types of APCs. Indeed, dermal DCs and Langerhans cells have been reported to play distinct roles in the adaptive immunity [502]. Other factors such as the dose of antigens, the type of antigens used also influence the predictive results in humans. Finally, an important consideration when selecting an adjuvant for clinical studies is the choice of a model than can be predictive for responses in humans. Non-human primates (NHP) are an alternative for the evaluation of the potency of a novel adjuvant. The major problem for using NHP is the high cost of animals and the availability, along with ethical issues.

4.5 Conclusion and perspectives

In the present study, we investigated the inflammatory and adjuvant potential of synthetic mycolate esters homologous of those found in the cell wall of *M. tuberculosis.* These mycolate esters vary in terms of sugar (trehalose, glucose and arabinose) and lipid (alpha-, methoxy-, keto-, wax-ester MAs) moieties. TDM, TMM, GMM and AraMM were found to be potent activators of mouse BMDCs *in vitro.* This activation was shown to be dependent on the Mincle pathway. Our results demonstrated that the classes of MAs bound to sugar do not consistently impact the level of pro-inflammatory and adjuvant responses induced. Rather, the nature of

the sugar and the number of acyl chains bound to it seem to dictate the affinity of the glycolipid for the Mincle receptor *in vitro. In vivo,* we observed that TDM, TMM and GMM induce similar immune responses characterized by a production of antigen-specific IFN-γ and IL-17A. The immune responses induced by AraMM were found to be dependent on the type of formulation. Indeed, AraMM in emulsion does not induce substantial levels of Th1 responses while AraMM in DDA liposome induces Th1 immune response. This can be explained by the intrinsic adjuvant potential of DDA liposomes. This study increases our knowledge on host-pathogen interactions during *M. tuberculosis* infection by identifying receptors involved in the recognition of several PAMPs. Furthermore, we showed that the activation of this receptor by these mycolate esters triggers the induction of protective Th1 and Th17 immune responses. However, mycobacteria are composed of several PAMPs which can auto-regulate their function and by this way modify the course of infection. For example, by varying the balance between pro- and anti-inflammatory cytokines.

Many aspects of these complex relationships between the host and the mycobacteria have been elucidated but many others remain elusive. Among others aspects, the pathway and molecular mechanisms involved in the exhaustion of T cells during chronic infection are not fully understood. Indeed, during chronic infection such as tuberculosis, it has been reported that overexposure of specific antigen to T cell may decline their function [503–505]. These exhausted T cells showed a different phenotype characterized by the expression of multiple inhibitor factors (such as PD-1, Tim-3, LAG-3), a decrease in cytokine production (IFN-γ, IL-2, TNF-α) and an increase of anti-inflammatory cytokine production (IL-10 and TGF-β) [503,506–510]. Thus, it is of great interest to identify molecules for immunotherapy that can prevent or avert and rescue the function of these exhausted T cells during *M. tuberculosis* infection. A second aspect that is intensively studied is the early clearance of *M. tuberculosis*. This phenomenon is defined as the eradication of the mycobacteria before the development of adaptive immune responses. This phenomenon can explain why some individuals are naturally resistant to *M. tuberculosis* infection. Indeed, numerous reports including the one of Houk and co-workers showed that some individuals repeatedly exposed to *Mtb* as a consequence of prolonged close-contact with pulmonary TB patients, did not convert to a positive skin test reaction [511,512]. These data indicate that these individuals have a protective innate immunity without the involvement of adaptive immune responses. One explanation of these observations is the

epigenetic reprogramming of innate immune cells such as monocytes, macrophages and NK cells. In fact, several recent findings demonstrated that innate immune cells could have a sort of memory following exposure to microorganisms or vaccines and can respond stronger following a second exposure to microbial molecules [512]. This phenomenon is known as trained immunity. Thus, understanding in detail molecular and immunological mechanisms of trained immunity could lead to the development of new strategies to control TB, by for example improving early clearance by trained innate immune cells. Finally, one interesting area explored to better control *M. tuberculosis* infection is immuno-metabolism. Indeed, upon infection, the activation of immune responses is coupled by a switch in the bioenergetics pathway required for the production of antimicrobial and inflammatory molecules [513,514]. This effect is known as Warburg effect. Warburg effect is controlled by the transcription factor hypoxia inducible factor-1α (HIF-1) [515,516]. Elks and colleagues have demonstrated that stabilization of HIF-1α pharmacologically or genetically in zebrafish following infection with *M. marinum* reduced bacterial burden at the early stage of infection [517]. Several studies reviewed by Shi et *al.* [513] have shown that the modulation of the Warbug effect is one mechanism used by *M. tuberculosis* to survive and persist in the host. Therefore, enhancing antimicrobial and pro-inflammatory function by targeting host cell metabolism could be an interesting avenue to develop novel therapeutic strategies. This can be achieved by a better understanding of immuno-metabolism in TB.

These different tracks could lead to the development of better strategies to control *M. tuberculosis* infection.

An interesting characteristic of mycobacteria is the wide presence of immuno-stimulatory molecules in their cell wall. This property has been used for decades for example in complete Freund's adjuvant. In the present study, we showed that glycolipids of the cell wall of *M. tuberculosis* can promote antibodies, Th1 and Th17 responses. We believe that glucose esters with short or long chain are of great interest in the area of adjuvant for subunit vaccine either alone or in combination with several others PAMPs. Regarding the use of short chain GMM as adjuvant, the group of Nigou demonstrated that based on the analysis of the structure required for the binding to Mincle, it is possible to rationally design more structurally simple glucose esters to be used as adjuvants [470]. The next step of our study will be to investigate the

adjuvant potential of GMM, by characterising the phenotype of T cell responses induced (the poly-functionality of T cells) and the isotype of antibody responses induced. It would also be of great interest to compare the inflammatory immune responses induced by GMM and TDB in human APCs (DCs and macrophages). Regarding the antigenic properties of GMM, it is not known whether synthetic GMMs can activate CD1b-restricted T cells and whether the classes of mycolate bound to glucose can impact this activation. These experiments have to be done in order to select the best candidate for challenge models. For that purpose, we plan to evaluate the efficacy of GMM-based subunit vaccine in combination with a protective antigen (for example PPE44) in an experimental tuberculosis challenge model in mice and in guinea-pigs.

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6. ABBREVIATIONS

AG: Arabinogalactan

Ag: Antigen

AIM2: Absent in melanoma 2

APC: Antigen presenting cells

AraMM: Arabinose monomycolate

ATP: Adenosine triphosphate

BCG: Bacillus of Calmette-Guérin

BMDCs: Bone marrow derived dendritic cells

BMDMs: Bone marrow derived macrophages

CAF01: Cationic adjuvant formulation 01

CCL: Chemokine (C-C motif) ligand

CD: Cluster of differentiation

CFP10: culture filtrate protein 10

CFU: colony forming unit

cGAMP: Cyclic guanosine mono-phosphate-AMP.

cGAS: Cyclic guanosine mono-phosphate-AMP synthase

CLRs: C-type lectin receptors

CR: Complement receptor

DCs: Dendritic cells

DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3 grabbing non integrin

DDA: dimethyldioctadecylammonium

Dectin-1: Dendritic cell-associated C-type lectin 1

Dectin-2: Dendritic cell-associated C-type lectin 2

DNA: deoxy-ribo nucleic acid

ELISA: enzyme-linked immunosorbent assay

ELISPOT: Enzyme-linked immunospot

ESAT-6: Early secreted antigenic target 6 kDa

FARES: fonds des affections respiratoires

FcRγ: Fc receptor gamma

GLA: Glucopyranosyl lipid adjuvant

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GMM: Glucose monomycolate

GroMM: Glycerol monomycolate

GTP: Guanosine triphosphate

HIV: Human immunodeficiency virus

HLA: Human leucocyte antigen

hsp: Heat shock protein

IFN: Interferon

IGRA: interferon-gamma release assay

IL: Interleukin

iNOS: inducible nitric oxide synthase

IRFs: Interferon regulatory factors

KO: Knock-out

LAM: Lipoarabinomannan

LM: Lipomannan

LPS: Lipopolysaccharides

LRR: Leucine rich repeats

LTBI: Latently tuberculosis infected

MAIT: Mucosal-associated invariant T cells

MAPK: Mitogen-activated protein kinase

MARCO: Macrophage receptor with collagenous structure

MAs: Mycolic acids

MCL: Macrophage inducible receptor

MDP: Muramyl dipeptide

MDR-TB: Multi-drug resistant tuberculosis

MHC: Major histocompatibility complex

Mincle: Macrophage inducible C-type lectin

MPLA: monophosphoryl lipid

MPTR: major polymorphic tandem repeat sequence

MR: Mannose receptor

MTBC: *Mycobacterium tuberculosis* complex

MyD88: Myeloid Differentiation primary response gene 88

NFAT: Nuclear factor of activated T-cells

NF-κB: Nuclear factor-kappa B

NKC: natural killer complex

NLRP: Nucleotide-binding oligomerization domain (NOD) like receptor protein

NLRs: Nod like receptors

NO: Nitric oxide

NOD: Nucleotide-binding oligomerization domain

PAMPs: Pathogen associated molecular patterns

PAS: para-aminosalicylic acid

PBMCs: Peripheral blood mononuclear cell

PDIM: Phthiocerol dimycocerosates

PGL: phenolic glycolipids

PGRS: polymorphic G+C rich sequence

PI3P: Phosphatidyl-inositol-3-phosphate

PIMs: Phosphatidyl-*myo*-inositol mannosides

PRRs: Pattern recognition receptors

RD: Region of difference

RLRs: Retinoid acid-inducible gene I like receptors

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SL: Sulpholipids

SR: Scavenger receptor

TAP-1: Transporter associated with Antigen Processing 1

TAT: Twin arginine translocation

TB: Tuberculosis

TDB: Trehalose dibehenate

TDM: Trehalose dimycolate

TGF-β: Transforming growth factor beta

TLRs: Toll like receptors

TMM: Trehalose monomycolate

TNF: Tumor necrosis factor

Treg: Regulatory T cells

TRIF: TIR-domain-containing adapter-inducing interferon-β

WHO: World health organization

WT: Wild type

XDR-TB: Extensively drug resistant tuberculosis

7. SUMMARY

Tuberculosis remains among the most deadly health threats to humankind. This poverty-related disease, caused by bacteria of the *Mycobacterium tuberculosis* complex, kills each year more than 1.5 million people. It is estimated that a further 2 billion individuals are latently infected with *M. tuberculosis.* Most of them will never develop any clinical symptoms, although 5 to 10% of these latently infected individuals are at risk to develop TB in their lifetime. During the last decades the situation worsened because of deteriorating socio-economic conditions, the increased incidence of drug-resistant *M.tuberculosis* strains and the co-infection with HIV (a major risk factor for development of TB). Currently only one vaccine is available against TB, the Bacille of Calmette and Guérin (BCG). However, its efficacy is extremely variable against the contagious form of TB - pulmonary TB - in adults and adolescents (ranging from 0 to 80%). Therefore, to control and eliminate TB, a better vaccine, efficient drug treatments and more rapid and cheaper diagnostic techniques are needed.

In this PhD thesis we have attempted to provide additional information to reach this goal. Indeed, we investigated the inflammatory and adjuvant potential of mycolate esters homologous of those found in the cell wall of *M. tuberculosis.* These mycolate esters vary in terms of sugar (trehalose, glucose and arabinose) and lipid (alpha-, methoxy-, keto-, wax-ester MAs) moieties. TDM, TMM, GMM and AraMM were found to be potent activators of BMDCs *in vitro.* This activation was shown to be dependent on the Mincle pathway. Our results demonstrated that the classes of MAs bound to sugar do not consistently impact the level of pro-inflammatory and adjuvant responses induced. Rather, the nature of the sugar and the number of acyl chains bound to it seem to dictate the affinity of the glycolipid for Mincle receptor *in vitro. In vivo,* we observed that TDM, TMM and GMM induce similar immune responses characterized by a production of antigen-specific IFN-γ and IL-17A. The immune responses induced by AraMM are dependent on the type of formulation. Indeed, AraMM in emulsion does not induce substantial level of Th1 while AraMM in DDA liposome induces Th1 immune response. This study increases our knowledge on host-pathogen interactions during *M. tuberculosis* infection by identifying the receptor involved in the recognition of several PAMPs. Furthermore, we showed that the activation of this receptor by these mycolate esters triggers the induction of protective Th1 and Th17 immune responses.