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Review

The role of low molecular weight thiols in *Mycobacterium tuberculosis*

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ABSTRACT

Low molecular weight (LMW) thiols are molecules with a functional sulfhydryl group that enable them to detoxify reactive oxygen species, reactive nitrogen species and other free radicals. Their roles range from their ability to modulate the immune system to their ability to prevent damage of biological molecules such as DNA and proteins by protecting against oxidative, nitrosative and acidic stress. LMW thiols are synthesized and found in both eukaryotes and prokaryotes. Due to their beneficial role to both eukaryotes and prokaryotes, their specific functions need to be elucidated, most especially in pathogenic prokaryotes such as *Mycobacterium tuberculosis* (*M.tb*), in order to provide a rationale for targeting their biosynthesis for drug development. Ergothioneine (ERG), mycothiol (MSH) and gamma-glutamylcysteine (GGC) are LMW thiols that have been shown to interplay to protect *M.tb* against cellular stress. Though ERG, MSH and GGC seem to have overlapping functions, studies are gradually revealing their unique physiological roles. Understanding their unique physiological role during the course of tuberculosis (TB) infection, would pave the way for the development of drugs that target their biosynthetic pathway. This review identifies the knowledge gap in the unique physiological roles of LMW thiols and proposes their mechanistic roles based on previous studies. In addition, it gives an update on identified inhibitors of their biosynthetic enzymes.

1. Introduction

Upon infection, the innate immune system is the first line of defence. It consists of inflammatory immune cells such as macrophages [1] that are able to phagocytose the pathogen, degrade it, secrete cytokines and chemokines to attract other immune cells (such as neutrophils) to the site of infection [2,3]. For macrophages to effectively degrade ingested pathogens, they have to undergo a maturation process whereby lysosomes fuse with phagosomes [4]. The resulting organelle (phagolysosome) contains hydrolases, antimicrobial peptides, and toxins. It is acidic and has a low level of nutrients such as irons [5,6]. During the process, protein recognition receptors are activated, leading to the recruitment of NADPH oxidase [7], and the generation of oxidative stress [8] in order to enhance the degradation of the pathogen. Neutrophils are amongst the first immune cells attracted to the site of infection, and are thought to be involved in the early clearance of pathogens, the activation of macrophages, and the induction of the adaptive immune system through secretion of pro-inflammatory cytokines. However, it remains unclear if neutrophils are able to directly kill mycobacteria [9–11]. Monocytes are recruited under the influence of cytokines, and are able to differentiate into macrophages that would phagocytose the pathogen [12]. In addition to macrophages, dendritic cells participate in the transition between the innate and adaptive immune defence. They phagocytose pathogens, migrate to the lymph nodes to present antigens on their surface to trigger the adaptive immune system consisting of T-lymphocytes [13]. Activated T-lymphocytes in turn trigger the activation of B-lymphocytes that secrete antigen-specific receptors known as antibodies. These together with defined array of cytokines and chemokines secreted by these immune cells are able to sustain both the adaptive and innate immune defence [14,15]. *Mycobacterium tuberculosis* (*M.tb*), is able to circumvent these well-structured immune cocktail of cells and defence barriers to cause

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disease (tuberculosis). For instance during the innate response, *M.tb* is able to prevent the maturation of the macrophages (required for an effective ingestion of the pathogen) [16]. It does so, by secreting molecules such as nucleoside diphosphate kinase [17], PtpA [18] and TlyA [19]. In addition, the glycolipid content of the cell envelop of *M.tb* can also enable it to circumvent the innate immune response by interfering with the programing of the phagosome maturation [20,21]. *M.tb* can also tear the phagolysosome membrane to escape to the cytosol by secreting specific proteins through the ESX-1 secretion system [22] that acts with the mycobacteria lipid phthicerol dimycocerosate to lyse the phagolysosome membrane. *M.tb* uses many other mechanisms to circumvent the innate immune response as discussed recently [23]. However, it can also manipulate the adaptive immune system to suit its survival by altering the differentiation of monocytes into macrophages [12], by interfering with the migration of dendritic cells to the lymph node [13], thereby preventing the recruitment of T lymphocytes and consequently B lymphocytes. It also affects the adaptive immune system through other mechanisms that remain to be elucidated. In addition to these various mechanisms and proteins that *M.tb* uses to circumvent the host immune system, low molecular weight thiols (LMW) thiols also contribute to the survival of *M.tb* under adverse conditions. Low molecular thiols are molecules with a sufhydryl reducing group, important to both the host and the invading pathogens during infection. Glutathione (GSH) for instance is synthesized by eukaryotes, some gram negative prokaryotes, but very few gram positive or pathogenic prokaryotes [24,25]. Glutathione is able to provide a vast range of biological functions to eukaryotes [26]. It is able to provide an effective protection against cellular oxidative stress and contribute to the regulation of biological events such as apoptosis, cell proliferation, transcription and others [26]. Glutathione is therefore involved in many disease pathologies including infectious diseases [26]. For instance GSH enhances the clearance of *M.tb* by immune cells during infection [27,28]. Pathogens on the other hand, synthesize other LMW thiols that maintain their vital biological processes and modulate their physiology to ensure their fitness under adverse conditions [24,29]. Ergothioneine (ERG), mycothiol (MSH) and gamma-glutamylcysteine (GGC) have been shown recently to interplay to protect *M.tb* against various cellular stress conditions [30]. Ergothioneine (2-mercapto histidine trimethyl betaine) first identified in the ergot fungus in 1909, is absorbed by plants through by their roots from soil microorganisms [31,32]. It is synthesized by fungi and actinomycetes but not in animals that ingest it from food [32–36]. Ergothioneine plays various protective roles in both eukaryotes [37–40] and prokaryotes [41–44].

MSH ((2R)-2-acetamido-N-[(2R,3R,4R,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-[(2R,3S,5R,6R)-2,3,4,5,6-pentahydroxycyclohexyl] oxyoxan-3-yl]-3-sulfanylpropanamide), is synthesized and abundant in streptomycetes and mycobacteria but it is not found in animals [45]. Mycothiol has been implicated in many biological processes that sustain the survival of mycobacteria [46–49]. In this literature review, we suggest the mechanistic roles of LMW thiols during tuberculosis infection based on published studies.

1.1. Low molecular weight thiols of M.tb

While glutathione (GSH) is rarely detected in pathogenic prokaryotes, ergothioneine (ERG) and mycothiol (MSH) are the major LMW thiols of mycobacteria [45]. Furthermore, gamma-glutamylcysteine (GGC), is an intermediate in ERG biosynthesis in mycobacteria [50] and GSH biosynthesis in other organisms [51]. Due to its rapid turnover during the biosynthesis of these LMW thiols, the physiological level of GGC is very low or undetectable by less sensitive quantification methods [41,45]. The low physiological level of GGC can also be explained by its high reactivity [52] that could be detrimental to the cells. However, lactic acid bacteria that cannot produce GSH such as *Leuconostoc kimchii* and *Leuconostoc mesenteroides,* produce GGC in large amounts [53]. Similarly, *M.tb* strains that have lost either the production of MSH or ERG produce a high physiological level of GGC [41,44]. In addition, under oxidative stress conditions *Leuconostoc kimchii* and *Leuconostoc mesenteroides* upregulate the expression of *gshA* (encoding for GshA required for GGC biosynthesis) [53]. Similarly, *M.tb* increases the production of GGC when treated with compounds that generate oxidative stress [54]. In addition, GshA (GSH1) was found to be essential for the *in vitro* growth of *Leishmania infantum* [55]. Heterozygous mutants of *Leishmania infantum* with one allele of *GSH1* inactivated, were sensitive to various cellular stress indicating the antioxidative role of GGC [55]. Furthermore, GGC was found to suppress lipid peroxidation and nitric oxide production in rats during drug administration [56]. Further studies elucidated the mechanism of the antioxidative role of GGC in mitochondria [57,58]. In view of these studies, further investigations in *M.tb* revealed that the absence of GGC aggravates the sensitivity of the ERG-deficient [41] and/or MSH-deficient mutants [30] *in vitro* and *ex vivo*. Therefore, these studies suggest that GGC plays a detoxification role in *M.tb* under adverse conditions.

LMW thiols have been detected both intracellularly and extracellularly. Extracellular LMW thiols could hamper exogenous toxins (before they penetrate and damage the cells), thereby, reducing their deleterious effects. *Saccharomyces cerevisiae* for instance, can export GSH in response to chronic exposure to arsenite, [59]. Similarly, when *M.tb* was treated with the antibiotic bacitracin, the level of extracellular thiols was high in the treated wild-type strain relative to the untreated wild-type strain [54]. Previous studies suggested that it is a mechanism used by microorganisms to reduce intracellular toxins [59]. This may explain why *M.tb* mutants unable to secrete LMW thiols were sensitive to bacitracin, as opposed to mutants that were able to secrete LMW thiols [54]. Furthermore, previous studies indicated that secretion of LMW thiols could be in an attempt to maintain homeostasis [60]. This was indicated when it was shown that mutants of *Saccharomyces cerevisiae* that accumulated a high intracellular concentration of GSH could secrete GSH as opposed to mutants that had lower levels of intracellular GSH [60]. Similarly, a high level of extracellular ERG was detected in *M.tb* mutants that produced a high intracellular level of ERG (Δ*mshA M.tb* mutant) and a high extracellular level of MSH was detected in *M.tb* mutants that produced a high intracellular level of MSH such as the complemented strain of the Δ*mshA* mutant, the Δ*egtA* mutant and the Δ*egtD* mutant [44,54]. Though secreted cysteine was detected and found to be high (relative to the wild-type) in the ERG-deficient mutant and the MSH-deficient mutant of *Streptomyces coelicolor* A3(2) [61], extracellular GGC has not been detected or reported [44,54,61]. This indicates that either GGC cannot be secreted, or it is rapidly consumed/oxidized when found extracellularly due to its high reactivity, or the quantification methods used in previous studies are not sensitive enough for the detection of extracellular GGC.

During phagocytosis of pathogens, toxins are generated to degrade the pathogen, therefore secretion of LMW thiols that are able to detoxify their environment, or at least alleviate the toxin before they penetrate the pathogen, could be another mechanism used by *M.tb* to circumvent the host immune system and cause disease.

1.2. Discrepancies around the detoxification roles of low molecular weight thiols in M.tb

In principle, LMW thiols should be able to detoxify both RNS (reactive nitrogen species) and ROS (reactive oxygen species). Therefore, the depletion of a LMW thiol in an organism would automatically aggravate its sensitivity to these stresses. However, previous investigations indicate that it is not necessarily the case [41,62–64]. The differences or discrepancy during *in vitro* investigation of the role of thiols using thiol-deficient mutants is linked to various factors. It is either due to the redundancy of the enzyme targeted in the pathway (enzyme compensated for by another homologous enzyme), an example is the mycothiol S-conjugate amidase (MCA) that compensates for MshB in MSH biosynthesis [65]. Another reason could be the overlapping

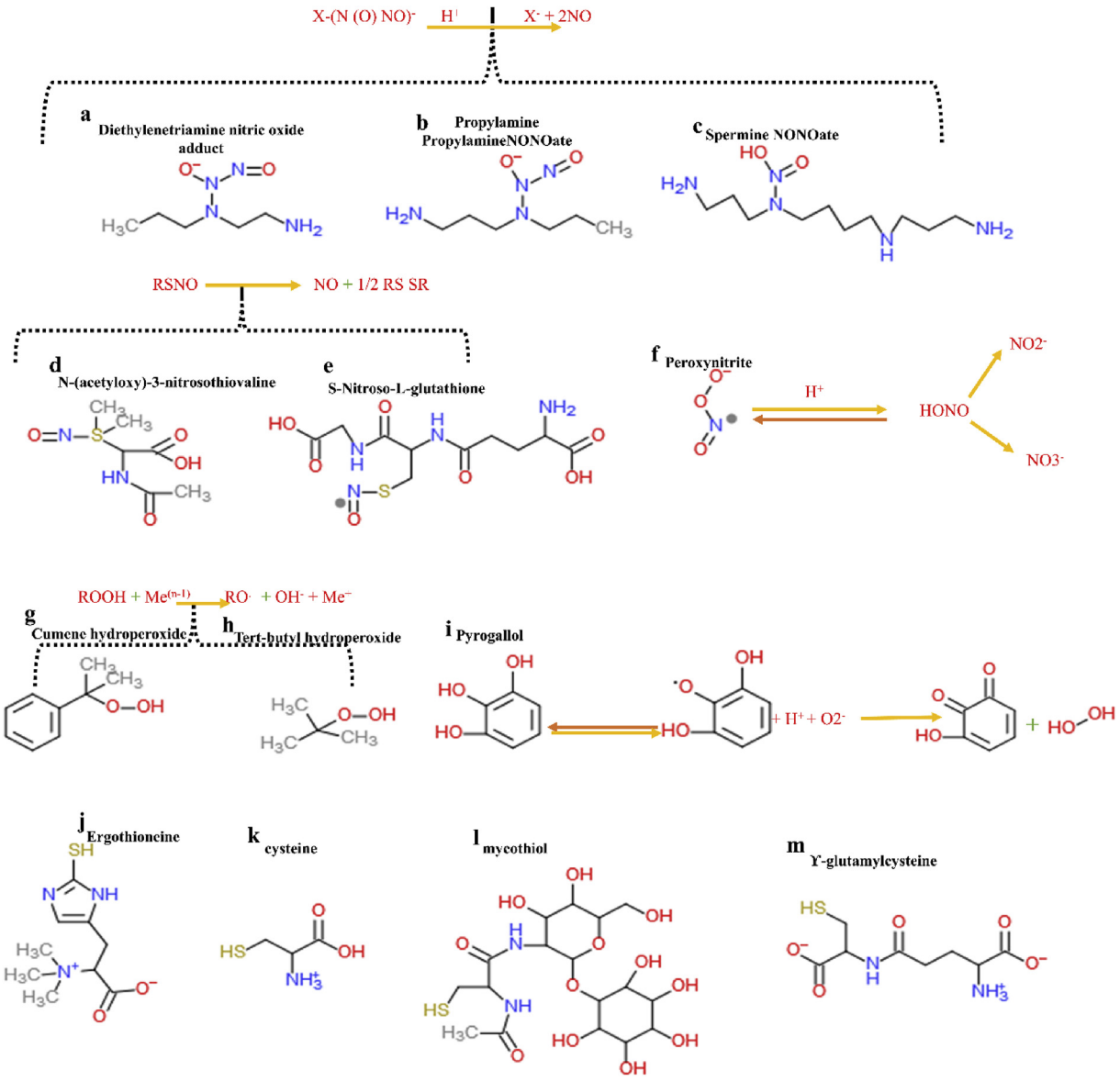

Fig. 1. Structural difference between LMW thiols, ROS and NOS donors. Compounds **a**, **b** and **c** are nitric oxides donor known as Noates, and able to generate nitroxative stress through the same mechanism as displayed in the equation above their structure. Compounds **d** and **e** are RNS donors that generate nitrosative stress through a different mechanism as displayed in the equation. Compound **f** can be converted to nitrate or nitrite which can lead nitrosative stress. Compounds **g** and **h** are ROS donors that generate oxidative stress through the same mechanism as displayed in the equation. Compound **i** is a ROS donor that act by autoxidizing to generate superoxide and hydrogen peroxide. Compound **j** (ergothioneine) has a unique imidazole ring and a tri-methylated Nitrogen atom, different in structure to other LMW thiols such as compound **k** (cysteine), or compound **l** (mycothiol) which has many free hydroxyl groups as opposed to ERG or cysteine. Compound **m** (gamma-glutamylcysteine) is another LMW thiols that is unique in the sense that it has two carboxyl groups. The scientific names of the compounds displayed in this figures are. **a**: (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate (Diethylenetriamine nitric oxide adduct). **b**: (Z)-1-[N-(3-aminopropyl)-N-(n-propyl)amino]diazen-1-ium-1,2-diolate (Propylamine PropylamineNONOate). **c**: (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio) butyl]-amino] diazen-1-ium-1,2-diolate (Spermine NONOate). **d:** N-(acetyloxy)-3-nitrosothiovaline (SNAP). **e:** N-(N-L-γ-glutamyl-S-nitroso-L-cysteinyl)-glycine (S-Nitroso-L-glutathione). **f:** Peroxynitrite. **g:** 2-hydroperoxypropan-2-ylbenzene (Cumene hydroperoxide). **h:** 2-hydroperoxy-2-methylpropane (*Tert*-butyl hydroperoxide). **i:** 1,2,3-Trihydroxybenzene pyrogallic acid (Pyrogallol). **j:** 3-(2-Sulfanylidene-1,3-dihydroimidazol-4-yl)-2-(trimethylazaniumyl)propanoate (Ergothioneine). **k**: cysteine. **l**: (2R)-2-acetamido-N-[(2R,3R,4R,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-[(2R,3S,5R,6R)-2,3,4,5,6-pentahydroxycyclohexyl]oxyoxan-3-yl]-3-sulfanylpropanamide (mycothiol). **m**: Ɣ-glutamylcysteine.

function of LMW thiols, where one thiol would overcompensate for the absence of the other and consequently protects the generated mutants against toxins [30,41,62]. For instance it was shown that the level of GGC and MSH was elevated in the ERG-deficient mutants, while the level of ERG and GGC was elevated in the MSH-deficient *M.tb* mutants [41]. However, when two or more LMW thiols were depleted simultaneously, the sensitivity of *M.tb* to ROS and RNS became severe [30]. The discrepancy could also be associated with the difference in the chemical structure (Fig. 1) of each LMW thiol and donors used to generate oxidative and nitrosative stress since that will influence greatly their interaction and reactivity. This is supported by previous studies that demonstrated that the reactivity of various LMW thiols to the same oxidant could differ [66]. More information on the properties of redox-active molecules [67,68], and the chemical property of ERG [69] can be found in the recent reviews.

1.3. Proposed mechanistic role of ERG during tuberculosis infection

In spite of the discrepancies discussed above, it has been established that MSH is able to protect mycobacteria against ROS, RNS, antibiotics (except isoniazid (INH) and ethiomamide (ETH)) and other toxins [43,70,71]. However, it is not essential for the survival of *M.tb in vivo* [72]. Interestingly, ERG-deficient *M.tb* mutants can grow on solid cultures without catalase (catalase is able to detoxify ROS [73]) as opposed to MSH-deficient mutants [41,43,72], indicating the significance of MSH over ERG in the detoxification of ROS under *in vitro* conditions. Therefore, if mycobacteria are able to grow without catalase in the absence of ERG, while they can't in the absence of MSH, it is possible that they rely mainly on MSH to detoxify *in vitro* ROS, while ERG ROS detoxification role could occur as secondary compensatory mechanism [30,62], or under extremely toxic conditions [54], or ERG is not required to detoxify the ROS generated under the tested *in vitro* conditions. This is supported by previous studies showing that ERG reacts very slowly with superoxide and hydrogen peroxide and can't prevent microsomal lipid peroxidation in the presence of iron ions (ferric and ferrous), however it is able to scavenge hydroxyl radicals and inhibit iron or copper ion-dependent generation of oxidative stress [74]. The growth of a MSH-deficient mutant has been investigated over a period of 8 weeks in both immunocompetent and immunocompromised mice [72]. A marginal decrease in the growth of the MSH-deficient mutant in the immunocompetent mice after 3 weeks was observed. However, no significant growth defect was observed at later time points [72]. In addition, it had no growth defect in the immunocompromised mice over the period of 8 weeks [72]. Indicating that, though MSH could be essential for the protection of mycobacteria against ROS *in vitro* [41,43,72], it is either compensated for *in vivo*, and/or the ROS that MSH is able to detoxify *in vitro*, were not encountered during the tested *in vivo* conditions. Furthermore, ERG-deficient mutants have a growth defect after 5 weeks in immunocompetent mice [43]. The mechanistic role of ERG during infection requires more investigation. It is not clear if the attenuation of the ERG-deficient mutants *in vivo* is due solely to the anti-oxidative role of ERG, or other mechanisms associated with the physiological role of ERG. The first line of defence during TB infection is the phagocytosis of mycobacteria by the mature phagosome. During which mycobacteria encounter acidic, oxidative and nitrosative stress [75], however, a proportion of *M.tb* is able to escape this first line of defence [18,76]. It is possible that the role of ERG during infection goes beyond the protection of *M.tb* against the first line of defence (innate immunity), explaining the inability of the ERG-deficient mutants to survive not only *ex-vivo*, but also *in vivo* [43]. Some studies indicated the role of ERG during nutrient starvation and oxygen depletion. It was shown that ERG accumulated from the late logarithmic phase in *M.tb* [42], while the ERG-deficient *M.tb* mutants have a growth deficiency during nutrient starvation [42] and during the late logarithmic phase of growth [41]. Furthermore, it was shown that nutrient depletion is a mechanism that enhances phagocytosis of pathogens [77]. In addition, genome wide transcriptomic studies of *M.tb* during infection revealed transcriptomic profiles depicting adaptation to low pH, oxygen depletion, iron limitation, nitrosative stress and nutrient depletion [78–81]. Furthermore, it was shown that ERG is also synthesized by the strictly anaerobic bacterium *Chlorobium limicola* under anoxic conditions (absence of oxygen), indicating that ERG has an oxygen independent role [82] in this organism. Therefore, these studies indicate that ERG may be required for the long term survival under nutrient depletion of mycobacteria which may explain the growth defect observed after 4 days in macrophages [43] but not earlier [42]. It is also plausible that ERG enables *M.tb* to survive within nutrient deprived environments *in vivo* (Fig. 2) explaining their growth defect *in vivo* [43]. In addition, as opposed to other LMW thiols, it was indicated that ERG is not consumed and can accumulate in eukaryotic cells throughout their lifespan [83,84], further supporting the possible role of ERG at a later stage of infection. If the secretion of ERG occurs *ex-vivo* and *in vivo* as was previously shown *in vitro* [54,62], therefore, in view of the immune-regulatory properties of ERG [85,86], it may interfere with the recruitment and the effectiveness of the adaptive immune system that depends mainly on the cocktail of cytokines and chemokines secreted by the immune cells of the innate immune system [14,15] (Fig. 2), further explaining the growth defect of ERG-deficient mutants *in vivo* [43]. In addition, granuloma are aggregates of immune cells that create an enclosed environment that should restrict the multiplication and spreading of *M.tb*, occurring in both active and latent TB [87,88]. Therefore, the ability of granuloma to prevent dissemination of TB determines the disease outcome [89]. In view of the immune regulatory properties of ERG [85,86], it is also possible that ERG enables survival of *M.tb* within the granuloma by modulating the formation and activation of the immune cells constituting the granuloma (Fig. 2). Another possible mechanistic role of ERG during infection lies in its ability to scavenge specific free radicals [74]. Since the generation of free radicals is a mechanism used by macrophages to degrade phagocytosed mycobacteria [90], it is possible ERG is able to alleviate both extracellular toxin (in case secretion occurs as well *in vivo*) and intracellular toxins (Fig. 2).

In view of these studies, we propose the following mechanistic role of ERG in *M.tb* during infection. It enables *M.tb* to survive during nutrient starvation within the macrophage, and within the granuloma (Fig. 2). It enables *M.tb* to modulate and/or interfere with the effective recruitment, maturation, activation of the immune cells of the innate and adaptive immune system, by modulating the cytokines production by activated macrophages and dendritic cells (Fig. 2). It is also able to scavenge specific free radicals released against *M.tb* during phagocytosis, or in the toxic granuloma. Furthermore, in view of its immune-regulatory ability, ERG can also modulate the formation immune cells constituting the granuloma to suit the survival of *M.tb* (Fig. 2). However, it remains to be demonstrated exactly how does ERG enable *M.tb* survival during nutrient starvation, how it modulates the immune system to suit the survival of *M.tb*, what are the *in vivo* free radicals scavenged by ERG, and how *M.tb* uses ERG to detoxify its environment in order to suit its survival.

1.4. Low molecular weight thiols and acidic stress

Macrophages are able to engulf *M.tb* into their phagosomes, however to ensure efficient killing of *M.tb*, these phagosomes must fuse with lysosomes. The resulting organelle has a very low pH that is suitable for the activity of hydrolases, ROS and RNS against the intracellular mycobacteria [90–92]. *M. tuberculosis* is resistant to acidic stress [93], and is able to survive through the phagosome/lysosome maturation and acidification [76,94], indicating that it has a protective/defence mechanism against acidification. Through some transcriptomic studies, validated by quantification of soluble and membrane proteins during the adaptation of *C. glutamicum* to pH fluctuation, it was demonstrated that there is a functional link between pH acclimatization, oxidative stress, iron homeostasis, and metabolic alterations [95]. In addition, it was indicated that acidic stress can induce oxidative stress in *Bacillus subtilis* [96] and *Corynebacterium glutamicum* [95]. Moreover, exposure to any other form of cellular stress such as antibiotics, heavy metals, xenobiotics, heat, high salt, can induce secondary oxidative stress [97,98]. Furthermore, ROS can indirectly regulate intracellular pH by targeting proteins and biochemical pathways that are involved in maintaining the pH (pHi) homeostasis [99,100]. Therefore, LMW thiols may protect against acidic stress by scavenging ROS that are able to affect proteins and pathways that regulate pHi homeostasis (Fig. 3). They may also protect against acidic stress by *S*-thiolation of molecules (form mixed disulfides with protein thiols) that are labile under acidic stress (Fig. 3). Protein *S*-thiolation is a post-translational modification of proteins by thiols that act by protecting the cysteine residues of enzymes in order to prevent irreversible over-oxidation to sulfonic acid [101,102]. This phenomenon has been described and discussed recently

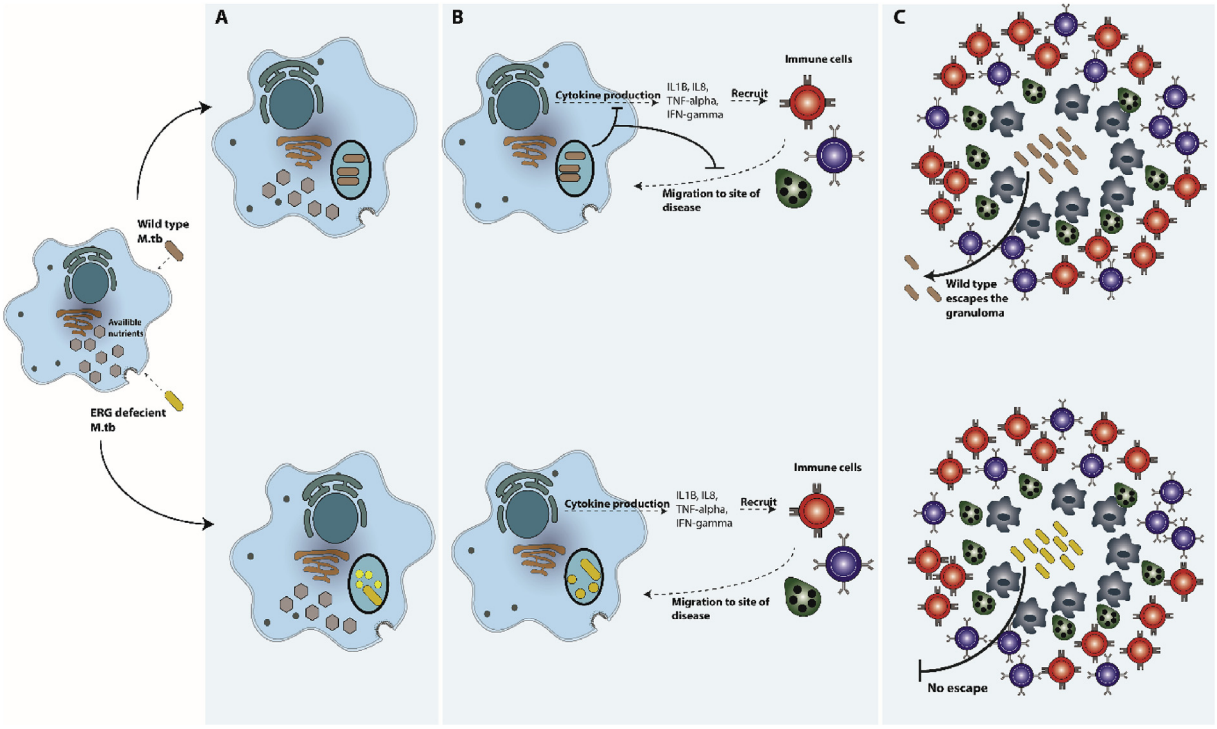

Fig. 2. Suggested general role of ERG during infection. **a)** Nutrient starvation has been indicated in both macrophages and the granuloma. Therefore in view of the role of ERG in the growth of *M.tb* in nutrient deprived media *in vitro*, ERG may also enable *M.tb* to survive in the nutrient deprived environment of the granuloma and the macrophages. **b)** During infection, macrophages phagocytose *M.tb* and secrete cytokines and chemokines to induce other innate immune cells and also to activate the adaptive immune system. However ERG secreted by *M.tb* can modulate the production of cytokines thereby interfering with this process to suit the survival of *M.tb*. **d)** In addition, in view of its immune-regulatory property, ERG is able to modulate the formation of immune cells constituting the granuloma to suits the survival of *M.tb*.

[67]. This may explain why the MSH-deficient *M.tb* [47] and *C. glutamicum* [101] mutants are sensitive to acidic stress, a phenotype that was reversed upon chemical and genetic complementation [101], implicating MSH in the protection against acidic stress. This may explain why the chemical probe AC2P36 that selectively kills *M.tb* at acidic pH acts by depleting cellular LMW thiol [103]. Furthermore, it was shown that methylglyoxal S-conjugate of GSH is able to activate the potassium efflux system which consists of proteins KefB and KefC that are able to ensure pHi homeostasis through the trafficking of Na^+ and K^+ ions [104]. Indicating, the indirect implication of GSH in the protection of bacteria against acid stress [105–111]. However, most prokaryotes such as *M.tb* do not synthesize GSH. Nevertheless, the ability of GSH to

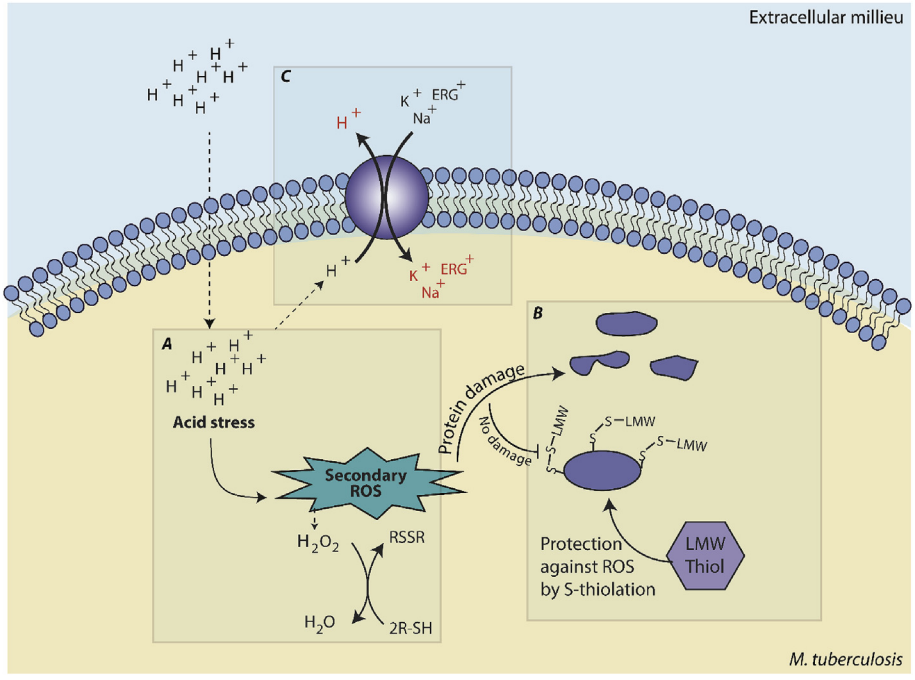

Fig. 3. Suggested mechanistic role of LMW thiols during acidic stress a) Acidic stress can indirectly induce oxidative stress (secondary oxidative stress), therefore LMW thiols indirectly protect against acidic stress in this case by counteracting the oxidative stress generated as a result of acidic stress. (b) Acidic stress affects the stability of proteins, as protonation of amino acids residues proteins during acidic stress would automatically affect their hydrogen bonds and consequently the tertiary structure/stability of the protein, in addition secondary oxidative stress as a result of acidic stress can also damage proteins. However, LMW thiols can protect protein through a process known as S-thiolation and prevent the damaging effect of acidic stress. (c) Intracellular pH homeostasis which is required to prevent acidic stress, is regulated through transport of cations across the membrane. LMW thiols such as ERG can be positively charged and co-transported across the membrane thereby participating in pH homeostasis.

protect against acidic stress suggests that other LMW thiols may play a similar function. *M. tuberculosis* is able to synthesize GGC which is different to GSH by the absence of the glycine moiety, which may explain their demonstrated overlapping functions [58], though it still remains to be investigated if GGC can indirectly maintain pH homeostasis in bacteria and *M.tb* similar to GSH in eukaryotes. As for ERG, there is no evidence yet of its implication in pHi homeostasis, however, if this depends on the trafficking of cations such as Na^+ and K^+ across the membrane as shown with GSH [104], it is more likely that ERG is involved, since it is co-transported with Na^+ via the eukaryote OCTN1 transporter [84,112]. This is supported by studies showing that the transport of ERG is also pH dependent, implicating ERG in the maintenance of pHi homeostasis [84,112]. It could also be attributed to its redox potential (−60 mV) which is higher (therefore has a higher tendency of becoming positively charged) than the redox potential of most LMW thiols [113].

Therefore, it is possible that LMW thiols protect *M.tb* against acidic stress through three possible mechanisms. LMW thiols would be able to scavenge free radicals generated in process known as secondary oxidative stress which is a secondary effect of acidic stress [95,96] (Fig. 3). Furthermore, acidic stress affects the stability of proteins, as protonation of amino acids residues in proteins during acidic stress would automatically affect their hydrogen bonds and consequently the tertiary structure of the protein [102]. Proteins can also be damaged by ROS generated as a result of secondary oxidative stress [95]. However, they can be protected by a process known as *S-thiolation* by LMW thiols [101] (Fig. 3). Finally, pH homeostasis is maintained by co-transport of cations and protons across the membrane. Positively charged LMW thiols such as ERG are co-transported across the membrane with sodium ions, they can therefore participate in the equilibration of pH homeostasis during the process, thereby protecting *M.tb* against acidic stress (Fig. 3).

1.5. Low molecular weight thiols, lipid metabolism and inflammation

Inflammation is a process during which activated phagocytic cells such as neutrophils and macrophages produce large amounts of reactive oxygen (ROS), reactive nitrogen (RNS) and chlorine species. The released ROS and RNS are able to kill invading agents and modulate the production of key cytokines and chemokines that in turn modulate the inflammatory response and vice versa [114]. Therefore, the production of ROS and RNS by macrophages is regulated by specific cytokines and chemokines and vice versa [115] (Fig. 4) (reciprocal event).

On the other hand, low molecular weight thiols synthesized by mycobacteria are also able to modulate the production of cytokines. For instance, ERG was able to attenuate mesenteric ischemia reperfusion injury in the intestine of rats that were supplemented with ERG (10 mg/kg/d) prior to intestinal ischemia [116]. Ergothioneine was able to do so, by suppressing the production of pro-inflammatory cytokines (TNF-α, IL-1β), the activity of tissues malondialdehide, and myeloperoxidase and by enhancing the production of the tissue heat shock protein 70. Furthermore, treatment with 20 mM ERG of mouse bone marrow derived macrophages (BMDM) for 24 h, revealed that under standard conditions, ERG had not significant effect on the level of pro-inflammatory cytokines. However, under inflammatory conditions stimulated by toll-like receptors agonists, the level of pro-inflammatory cytokines such as IL-6, IL-12, IL-1β increased while the level of IL-10 decreased in macrophages pre-treated with ERG. In addition, ERG was able to induce Th17 shift in $CD4^+$ T cells [85].

On the other hand, lipids are also able to modulate the inflammatory response via cytokines and chemokines production [117–119]. Four MHC-1 proteins (CD1a, CD1b, CD1c and CD1d) are able to present lipid antigens to T-cells at the surface of antigen-presenting cells (APCs) [120]. *Mycobacterium tuberculosis* has a complex lipid bilayer in its cell wall [121]. CD1d is able to bind to various *M.tb* lipids and induce T-cell to produce cytokines such as IFN-γ and TNF-α [122–126]. Trehalose-6,6′-dimycolate (TDM) is a mycobacterial cell wall glycolipid that is able to bind to mincle (specific macrophage receptor) to induce the production of nitric oxide, cytokines (TNF, IL-6, MIP-2 or CXCL2) and the formation of granulomas in mice [127]. Therefore, the modulation of the lipid components of mycobacteria membrane is a mechanism that could protect mycobacteria against exogenous attack, preventing penetration of toxins [54], and a mechanism that regulates the host immune response to suit the survival of mycobacteria [128].

Furthermore, there are established links between LMW thiols and mycobacterial membrane lipid biosynthesis. The enzyme IpsA, is a LacI-type transcriptional regulator that is able to trigger myo-inositol formation by activating ino1 (encodes inositol phosphate synthase). It is essential both for MSH production and the synthesis of inositol-derived lipids of the cell wall, since myo-inositol is the building block of MSH and a precursor of phosphatidylinositol (a component of the plasma

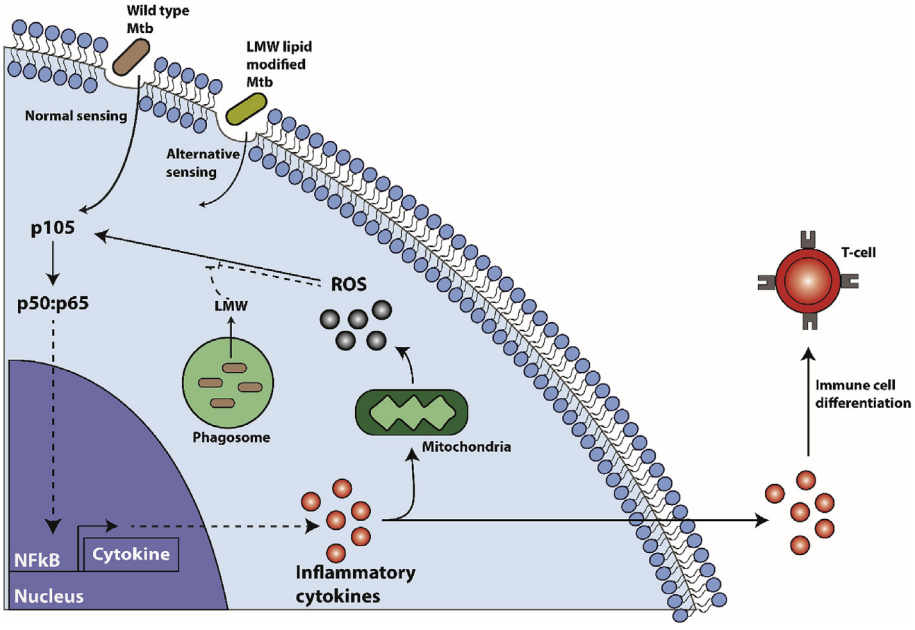

Fig. 4. Illustration of the interplay between LMW Thiols, ROS, cytokines production by macrophages and mycobacteria lipid layer. The production of cytokines regulate the production of ROS, RNS and vice versa. However, LMW thiols can indirectly affect the production of cytokines by detoxifying the ROS and RNS, consequently affecting the effective differentiation of immune cells and the effective phagocytosis of M.tb (suiting the survival of M.tb). Furthermore, LMW can directly modulate the production of cytokines and consequently affect the effective differentiation of immune cells and the effective phagocytosis of M.tb (suiting the survival of M.tb) as shown in Fig. 2b. In addition, under normal circumstances, APC can bind to specific lipid antigen on the membrane of mycobacteria in order to activate the production of cytokines, which in turn modulate the differentiation of T-cells, however LMW thiols may affect the lipid composition/structure of mycobacteria membrane and thereby indirectly affecting the adaptive immune system.

membrane) and derived lipids [129]. This may explain why *M. smegmatis* MSH-deficient mutants display a defective biofilm layer [130], since free mycolic acids from the mycobacterial membrane are key factors for the formation of biofilms by mycobacteria [131]. Furthermore, WhiB3 is a cytoplasmic redox sensor that is able to regulate virulence lipids anabolism [132] and ERG production [43]. In addition, the expression of *whiB3* is subverted by the inactivation of MSH production [133]. The homologous enzymes [41] IscS (cysteine desulfurase) and EgtE (C-S lyase) are also implicated in both the production of LMW thiols and the modulation of lipid synthesis in bacteria. The IscS enzyme of *E.coli* was shown to enhance the steady state biosynthesis of ERG in the presence of the sulfurtransferase of *Chlorobium limicola* (EanB), cysteine, and N-α-trimethylhistidine [82] *in vitro*. Furthermore, it was shown that EgtE is able to catalyse the last step of ERG biosynthesis [50]. The EgtE-deficient *M.tb* mutant that is unable to produce extracellular LMW thiols [54] and the IscS-deficient *M.tb* mutant that has a reduced level of ERG [41] have an altered membrane lipid profile [54,134].These studies therefore indicate an interplay between the production of LMW thiols and mycobacteria membrane lipid build-up.

In view of these studies, we suggest the following mechanism. Since the secretion of cytokines by immune cells enhance the production of ROS and RNS to degrade the pathogen, while the produced ROS and RNS can in turn modulate the production of cytokines [114,115]. Low molecular thiols synthesized and/or secreted by *M.tb* would modulate the production of cytokines indirectly by scavenging/detoxifying ROS and RNS (Fig. 4). In addition, as shown with ERG [85,116], it is possible that LMW thiols can directly modulate the production of cytokines to suit the survival of *M.tb* in macrophages (Fig. 4). Furthermore, in view of studies that demonstrate the role of mycobacterial membrane lipid on the regulation of cytokines productions, and the studies that indicate a role of LMW thiols on the membrane of mycobacterial lipid membrane [41,43,50,54,129–133], we suggest that LMW thiols could also modulate the production of cytokines to suit the survival of *M.tb* indirectly by affecting the lipid build-up of the pathogen membrane (Fig. 4). However these mechanistic roles of LMW thiols remain to be validated.

1.6. Drug targets in the biosynthesis of low molecular weight thiols

The biosynthesis of MSH involves five enzymes, MshA, MshA2, MshB, MshC, and MshD (Table 1). MshA is an N-acetylglucosamine transferase that catalyses the formation of N-acetylglucosaminylinositol phosphate when 1L-inositol-1-phosphate reacts with UDP-N-acetylglucosamine (UDP-GlcNAc) (a simplified illustration of the pathway has been described [44]). It is encoded by *mshA* (*Rv0486* in H37Rv *M.tb, MSMEG_0924 in M. smegmatis)*. The gene *mshA* is not essential for the *in vitro* growth of *M.tb* [41,72], however it is essential for MSH biosynthesis [41,72,135]. Mycothiol biosynthesis is an attractive drug target since eukaryotes do not synthesize MSH. UDP-(5F)-GlcNAc is a competitive (versus UDP-GlcNAc) inhibitor of the MshA of *Corynebacterium glutamicum* (CgMshA), it is not known if it can also inhibit the MshA of *M.tb* though it is more likely since it competes with the substrate used by both enzymes (UDP-GlcNAc) [136].

A phosphatase named MshA2 is thought to dephosphorylate the product of the reaction of MshA to yield *N*-acetylglucosaminylinositol (GlcNAc-Ins) [70]. However, this phosphatase has not yet been identified [70].

The enzyme MshB deacetylates the product of MshA2 (GlcNAc-Ins) to form glucosaminylinositol (GlcN-Ins). MshB is encoded by *mshB* (*Rv1170* in H37Rv *M.tb*, *MSMEG_5117* in *M. smegmatis*) and is not essential for MSH biosynthesis as low levels of MSH are detected in mutants deficient in MshB [135,137]. MshB shares high homology with the mycothiol S-conjugate amidase Rv1082 (MCA) [65]. A low level of MSH is present in the MshB-deficient mutant [137] (Table 1). Thus, it was more reasonable to target both enzymes (MshB and MCA) in a therapeutic approach. Therefore, a library that was biased to include inhibitors that were structurally similar to previously identified inhibitors of MCA was used to identify compounds that can inhibit both MshB and MCA [138]. One sulphonamide (E)-N-((2R,3R,4R,5S,6R)-2-(Cyclohexylthio)-4,5-dihydroxy-6-(hydroxymethyl)-tetrahydro-2H-pyran-3-yl)-4-phenylbut-1-ene-1-sulfonamide) was found to highly inhibit both MshB (~80% inhibition) and MCA (~70% inhibition) while an heterocyclic compound, (5-(4-Chlorophenyl)-N-((2R,3R,4R,5S,6R)-2-(cyclohexylthio)-tetrahydro-4,5-dihydroxy-6-(hydroxymethyl)-2H-pyran-3-yl)furan-2-carboxamide) was found to inhibit almost 100% of the activity of MshB and about 80% of the activity of MCA, at 200 μM [138].

The enzyme MshC ligates cysteine to the product of MshB (GlcN-Ins) to produce cysteinylglucosaminylinositol (Cys-GlcN-Ins). MshC encoded by *mshC* (*Rv2130c* in H37Rv *M.tb*, *MSMEG_4193* in *M. smegmatis*) is essential for MSH biosynthesis since *M. bovis* [139] and *M. smegmatis* [135] deficient in MshC can't produce MSH. However, MshC is essential for the *in vitro* growth of *M.tb* [46]. The MSH-deficient *M.tb* mutants have been successfully generated [41,72], therefore, the MshC-deficient *M.tb* mutants are not obtainable probably because of another unknown

Table 1
Characterization and inhibition of the biosynthetic enzymes of LMW thiols.

Enzymes	Homologs (≥50%)	Roles in thiols production	Inhibitors	Kinetic of inhibitors
MshA	None	Essential for MSH biosynthesis [72,135]	UDP-(5F)-GlcNAc [136]	Km = 1.4 ± 0.2 μM
MshA2	Unknown	Unknown	Unknown	
MshB	Mca (Rv1082) [65]	Required but not essential for MSH biosynthesis [135,137]	Sulphonamide	80% inhibition
			Amide	50% inhibition
			Heterocycle [138]	100% inhibition at 200 μM
MshC	None	Essential for MSH production [135,139]	NTF1836 [140,141] and dequalinium derivatives [142]	IC_{50} ~100 μM IC_{50} ~24 μM
MshD	None	Essential for MSH biosynthesis [135,143,144]	Not determined	
EgtA	None	Essential for GGC production, required for ERG biosynthesis [41]	MSO (partial)	10% inhibition at 1 μM
			BSO (complete) [146]	20% inhibition at 10 μM
				75% inhibition at 1 μM
				95% inhibition at 10 μM
EgtB	None	Essential for ERG biosynthesis [41]	Not determined	
EgtC	Rv1061 [41]	Required but not essential for ERG biosynthesis [41]	Not determined	
EgtD	None	Essential for ERG biosynthesis [41]	Chloroquine [54]	100%inhibition 10 mM
			methyl-histidine	Ki = 5.4 ± 1.6
			chloro-histidine	Ki = 6.2 ± 1.5
			bromo-histidine [132]	Ki = 8.2 ± 2.4
EgtE	Csd (Rv1464) and IscS (Rv3025) [41]	Not essential for ERG biosynthesis [41]	Not determined	

additional physiological role of MshC (Table 1). The double essentiality of MshC (for MSH production and for the viability of *M.tb*) prompted investigations into potential inhibitors of MshC. NTF1836 is able to inhibit the activity of MshC (IC_{50} ~ 100 μM) and the growth of *M. smegmatis* after incubation for 8 h at 40 μM [140]. NTF1836 was able to inhibit in a dose dependent manner the growth of actively growing *M.tb*, starting from 20 μM (with a 97% loss in viability at NTF1836 > 50 μM) [141]. It was also able to inhibit the growth of clinical isolates of *M.tb* (95% inhibition at ~110 μM), the growth of non-replicating *M.tb* in a model of dormancy (99% loss in viability at ~90 μM) and the production of MSH [141]. Using a different screening method, dequalinium chloride was found to be an ATP-competitive inhibitor of MshC (IC_{50} ~24 μM) [142] and was also able to inhibit the growth of *M.tb* both under aerobic (1.2 μg/mL) and anaerobic growth conditions (0.3 μg/mL) [142].

MshD is an acetyltransferase that acetylates the product of MshC (Cys-GlcN-Ins) to produce MSH. It is encoded by *mshD* (*Rv0819* in H37Rv *M.tb*, *MSMEG_5754*). More than 95% of MSH is lost in the MshD-deficient mutant [135,143,144]. Deletion of *mshD*, results in the accumulation of the product of MshC, cysteinylglucosaminylinositol that constitutes a potential low molecular weight thiol with detoxification abilities [144]. However, though the Δ*mshD* mutant may be protected by this metabolite, it is not efficient enough to ensure its survival within macrophages [135,145]. No inhibitor of MshD has been identified to date.

Ergothioneine biosynthesis in mycobacteria involves five enzymes [50], EgtA, EgtB, EgtC, EgtD and EgtE, all encoded by genes clustered in the same operon. The enzyme EgtA is a synthase encoded by *egtA* (*Rv3704c* in H37Rv and *MSMEG_6250* in *M. smegmatis*) that catalyses the formation of GGC from glutamate and cysteine [50,146]. It is not essential for ERG biosynthesis as low levels of ERG are detected in mutants deficient in EgtA [41,61]. However, these mutants are sensitive to oxidative stress and nitrosative stress and have an *ex-vivo* and *in vivo* growth defect [41,43]. In view of the compelling evidence of the detoxification role of GGC in both eukaryotes and prokaryotes [41,53,56–58,147], and the role of ERG in the *in vivo* survival of mycobacteria [43] and their survival during nutrient starvation [42], EgtA is an attractive drug target since it is essential for GGC biosynthesis and required for in ERG biosynthesis. The enzyme EgtA is partially inhibited by L-methionine-S,R-sulphoximine (MSO) and strongly inhibited by D,L-buthionine-S,R-sulphoximine (BSO) [146]. However more specific inhibitors of EgtA are needed, since BSO can also inhibit GSH biosynthesis in eukaryotes [148] where it is required for an effective clearance of *M.tb* during infection [27,28].

EgtD is a SAM (S-adenosylmethionine)-dependent methyltransferase that is able to methylate histidine to yield hercynine (N-α-trimethylhistidine) during ERG biosynthesis [50]. It is encoded by *egtD* (*Rv3701c* in H37Rv, *MSMEG_6247* in *M. smegmatis*). Though, *M.tb* has many other SAM-dependent methyltransferases (such as Rv2622, Rv0560, Rv2966c and others), EgtD is essential for ERG biosynthesis [41–43,62]. This is because though EgtD has the typical SAM-binding pocket found in all SAM-dependent enzymes of both eukaryotes and prokaryotes [149,150]; it has a unique substrate-binding domain, specific only to histidine or mono-, di-methylhistidine [150]. Therefore, EgtD is also an attractive drug target. Chloroquine an anti-malarial drug was found to inhibit the activity of EgtD [54], however it also inhibits the human histamine methyltransferase (HNMT) [54,151], owing its side effects during patient administration [152]. This is because Chloroquine is an allosteric inhibitor of SAM-dependant enzymes. The inhibitory effect of Chloroquine on EgtD is higher than its effect on HNMT, since HNMT activity was partially inhibited by the same concentration that inhibited completely the activity of EgtD [54]. Further kinetics studies are required to determine if the inhibitory effect of chloroquine on EgtD would outweigh its effect on HNMT. Another approach would be to target the Histidine substrate-binding site of EgtD, since it is unique as opposed to the SAM binding site. In that avenue, derivatives of histidine such as, methyl-histidine (inhibitory constant Ki = 5.4 ± 1.6), chloro-histidine (Ki = 6.2 ± 1.5) and bromo-histidine (Ki = 8.2 ± 2.4) were demonstrated to have a strong inhibitory effect on the activity of EgtD [153].

The enzyme EgtB is a non-heme iron enzyme (ironbound holoenzyme) that catalyses the oxygen-dependent C-S (carbon-sulfur) formation between the reaction product of EgtD (Hercynine) and the reaction product of EgtA (GGC) to yield hercynine sulfoxide-GGC [50]. It is encoded by *egtB* (*Rv3703c* in H37Rv, *MSMEG_6249* in *M. smegmatis*) and is essential for ERG biosynthesis. The enzyme EgtB is found in almost all pathogens that synthesize ERG, either as an independent enzyme or fused to EgtD. For instance Egt1 that appears to be a fusion of EgtB and EgtD, catalyses the first step of ERG biosynthesis in most fungal species [154,155]. Hercynine, the product of EgtD and the substrate of EgtB, can inhibit the activity of EgtD when in excess, this serves as a negative feedback regulatory mechanism of ERG levels [153]. This may explain why the enzyme EgtB is essential for ERG biosynthesis [41], since hercynine is more likely to accumulate in the absence of EgtB [50]. In addition, NCBI protein blast search revealed no highly homologous enzyme of EgtB in *M.tb* that could compensate for the absence of EgtB in the Δ*egtB M.tb* mutant [41]. However, EgtB has a homologous domain with DinB proteins found across many species including eukaryotes. Nevertheless, most single-domain DinB proteins are zinc-dependent thiol *S*-transferases [156] or maleylpyruvate isomerases [157] indicating that the iron- and O2-dependency of EgtB may be unique [158]. No inhibitor of EgtB has been reported yet. However, the accumulation of GGC in the Δ*egtB* mutant (though it is ERG-deficient) compromises the sensitivity of this mutant to cellular stress [41]. In addition, the ability of EgtB to use cysteine as a substrate in the absence of GGC (which may explain the detectable level of ERG in the Δ*egtA* mutant [41,50,61]), indicates that it would be more efficient to target both EgtB and EgtA in a therapeutic approach.

The enzyme EgtC is a glutamine amidohydrolase that catalyses the cleavage of the gamma-glutamyl tail of the reaction product of EgtB [50]. It is encoded by *egtC* (*Rv3702c* in H37Rv *M.tb*, *MSMEG_6248* in *M. smegmatis*). It is required for ERG biosynthesis though detectable levels of ERG are reported in the Δ*egtC* mutant; probably resulting from a partial compensation by Rv1061, a homologous enzyme [41]. No inhibitors of EgtC have yet been reported but structural analyses of EgtC complexed to its substrate or its product glutamate reveal active-site residues that determine the substrate specificity of EgtC [159]. This information would facilitate design of drugs that target EgtC.

The enzyme EgtE is a PLP (pyrodoxal-5′-phosphate)-mediated C-S lyase that catalyses the last step of ERG biosynthesis. It is able to cleave pyruvate and ammonia from the reaction product of EgtC to yield ERG in the presence of a reducing buffer [50,160]. It is encoded by *egtE* (*Rv3700c* in H37Rv, *MSMEG_6246* in *M. smegmatis*) and is not essential for ERG biosynthesis, since the level of ERG is not significantly altered in mutants deficient in EgtE [41]. This is either because the production of ERG is non-enzymatically induced in the presence of PLP [160,161] and/or EgtE is homologous to Csd (Rv1464) and IscS (Rv3025) which may have overlapping functions, since mutants deficient in IscS produced less ERG than their wild-type strain [41]. Inhibitors of EgtE have not yet been reported, however, in spite of the non-essential role of EgtE on ERG biosynthesis, it is an attractive drug target since studies indicate the role of EgtE in the lipid build-up of mycobacterial membrane affecting the permeability of anti-tuberculosis drugs [54].

2. Concluding remarks

Low molecular weight thiols are generally known to protect against oxidative and nitrosative stress. However, studies are gradually revealing that their role goes beyond that. In addition, though there are few overlaps in their function, each LMW thiol remains unique and plays a major role during infection. Therefore, to better understand TB disease pathology, and to enable a rational design of drugs targeting the

biosynthesis of LMW thiols, it is essential to understand their specific mechanistic roles during infection.

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