Pleiotropic molecular effects of the *Mycobacterium ulcerans* virulence factor mycolactone underlying the cell death and immunosuppression seen in Buruli ulcer

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Abstract

Mycolactone is a polyketide macrolide lipid-like secondary metabolite synthesised by *Mycobacterium ulcerans*, the causative agent of Buruli ulcer (BU), and is the only virulence factor for this pathogen identified to date. Prolonged exposure to high concentrations of mycolactone is cytotoxic to diverse mammalian cells (albeit with varying efficiency), whereas at lower doses it has a spectrum of immunosuppressive activities. Combined, these pleiotropic properties have a powerful influence on local and systemic cellular function that should explain the pathophysiology of BU disease. The past decade has seen significant advances in our understanding of the molecular mechanisms underlying these effects in a range of different cell types. This review will focus on the current state of our knowledge of mycolactone function, its molecular and cellular targets, seeking to identify commonalities between the different functional and cellular systems. Since mycolactone influences fundamental cellular processes (cell division, cell death, and inflammation), getting to the root of how mycolactone achieves this could have profound impact on our understanding of eukaryotic cell biology.

Introduction

BU is a chronic necrotising skin ulcer caused by infection with the environmental opportunistic pathogen, *Mycobacterium ulcerans*. While the mechanism by which infection is acquired from the environment is still controversial (person to person transmission does not occur except in one reported case of mechanical skin breakage [biting]), what is clear is that the production of mycolactone by the bacilli is essential to the pathophysiology of the disease. Mycolactone has been shown to cause the two determining features of the ulcers - the cellular cytotoxicity and the pronounced immunosuppression - which act together to drive the progressive ulceration and persistent infections seen in patients. There are many excellent reviews of BU disease pathogenesis [1, 2], treatment [3] and transmission [4] as well as *M. ulcerans* microbiology [5, 6] in the literature - this review will therefore focus specifically on the cellular actions of mycolactone on eukaryotic (host) cells, and recent advances in our understanding of the molecular mechanism by which mycolactone mediates these effects.

Mycolactone

The existence of a factor that contributed to the virulence of *M. ulcerans* was postulated as long ago as 1965 [7], but was first assumed to be a secreted protein toxin. Sterile filtrates of liquid cultures were shown to induce rounding up, detachment and death of L929 fibroblasts in culture [8], and also to inhibit the proliferation of T cells and phagocytosis by murine macrophages [9]. The cytotoxic activity and an ability to suppress cytokine production was subsequently shown to reside in the alcohol soluble lipids (ASLs) extracted from *M. ulcerans* culture supernatants [10, 11]; but it wasn't until 1999 that mycolactone was isolated to purity by Pamela Small's group [12]. In contrast to initial

expectations, it is now clear that mycolactone largely resides in the extracellular matrix (biofilm) that forms around growing bacterial colonies/clusters [13].

Mycolactone is a polyketide lactone (Figure 1), consisting of a 12-membered lactone core and two polyketide side chains [12]. As such, it is lipid-like in its characteristics and highly hydrophobic. The most virulent strains of *M. ulcerans* makes a 3:2 ratio of mycolactone A/B (Z-/E- isomers of the C4-C5 bond in the long polyketide side chains) and most research into the biological functions of mycolactone has focussed on this. Experimental investigation has been facilitated in recent years by the availability of synthetic mycolactone [14] with which to carry out biological assays. So far, the biological activity of the biological synthetic compound appears to be identical to that of the purified natural A/B form ([15] and Hall and Simmonds unpublished). However, a number of naturally occurring variants of mycolactone are produced by clinical isolates from geographically diverse areas as well as other non-pathogenic mycolactone-producing mycobacteria (MPM) (Figure 1). Indeed most strains of *M. ulcerans* make a range of mycolactone-like molecules [16]. Each of these mycolactones contain the lactone ring and vary by the length of the side chain, and the location of hydroxyl groups and double bonds within it and they all have differing abilities to induce cellular cytotoxicity and suppress cytokine production (see below, and reviewed in [17, 18]). This implies that the biological function is conferred largely by the long polyketide side chain, and this was confirmed in a recent study where synthetic mycolactones A/B, C and F had differing LC₅₀ values, and loss of this side chain renders the compound effectively inert [15]. On the other hand, modifications of the short polyketide chain had a less profound influence over cytotoxic activity [15].

Mycolactone production is due to the giant (174Kb) plasmid pMUM [19] that is present in all MPM. There is still a paucity of experimental data relating to the timing, regulation and extent of mycolactone production for *M. ulcerans* bacilli. The *mls* genes encoded in pMUM have been reported to have a strong sigA type promoter that is constitutively expressed during planktonic monoculture under laboratory conditions [20]. But how this relates to expression in its environmental niche (itself not clearly defined) or during different stages of clinical ulcers is not known. What is clear, however, is that during antibiotic treatment of patients with advanced ulcers, a non-inflammatory immunohistochemistry reverts to a granulomatous pattern more similar to that seen in other mycobacterial infections (such as *M. tuberculosis*, *M. leprae* and *M. marinum* M), possibly suggesting that rifampicin/streptomycin are able to switch off mycolactone production as part of the mycobacteriocidal activity [21]. On the other hand, mycolactone has been successfully isolated from ulcer exudates of patients who have completed a sterilising course of antibiotic therapy [22] and also persists in experimental infections [23].

It is assumed that mycolactone accesses mammalian cells by means of passive diffusion due to its hydrophobic nature. Bodipy-labelled mycolactone has been detected in the cytosol of cells within 2 minutes of exposure, but interestingly is excluded from the nucleus [24, 25]. Other *in vivo* experiments have demonstrated that mycolactone can diffuse away from the site of injection and can access all bodily compartments with the exception of the brain [26]. In a patient study, mycolactone could be detected in the serum of 3 out of 6 BU patients prior to starting antibiotic therapy [22]. Thus it seems that mycolactone can have wide-reaching systemic, yet not universal, effects.

Cytotoxic activity

At high doses (usually considered to be above 1µg/ml, around 1.3µM) and/or prolonged exposure times (>24-48 hours), mycolactone is generally cytotoxic to mammalian cells. The most sensitive cells identified to date are L929 murine fibroblasts and these are frequently used as reporter cells. DNA content analysis showed that mycolactone induces cell cycle arrest at G1/G0 [12]. The cells also undergo cytoskeletal rearrangements and become apoptotic. Interestingly, different types of cells show different susceptibilities to mycolactone-induced cytotoxicity. For instance, purified human

monocytes [27] show no significant reduction in viability after 24 hours, whereas immature dendritic cells (DCs) are relatively more sensitive after 48 hours [28]. Even prolonged (>24 hour) exposure of PBMCs [29], mature DCs [28], Jurkat T cells [29] and myeloid cell lines, such as J774 (human macrophage-like) [30], RAW264.7 (murine macrophage; Hall and Simmonds, unpublished) and CLC (adherent carp monocytic) cells [31] causes only minimal cytotoxicity, growth inhibition or apoptosis.

It is also interesting to consider cytotoxicity in the context of infected skin, and different groups have now investigated the sensitivity of myocytes, keratinocytes, adipocytes, fibroblasts and Schwann cells to mycolactone [32-35]. All of these cells can be killed by mycolactone, although adipocytes in particular seem to require extended exposure times even to see small levels of necrosis [35]. The neurotoxicity has been a suggested mechanism for the painlessness of BU ulcers although the connection between cell cycle arrest and toxicity is unclear [33]. Even though the keratinocytes at the basal layer of epidermis are rapidly dividing in human skin and are reportedly sensitive to mycolactone, the epidermis is often observed to be undergoing hyperplasia during BU [36]. On the other hand, relatively resistant adipocytes do not divide beyond puberty but are killed in BU, providing the classic "ghost-cell" appearance of coagulative necrosis in histopathology indicating that they must be susceptible to mycolactone's effects, either directly or indirectly. How these different *in vitro* and *in vivo* phenomena are related is a highly interesting question, and the mechanism of cell death as a consequence of mycolactone exposure in infections appears to be more complex than simply a matter of cell cycle arrest.

In fact, recent work by Caroline Demangel's group has shed light on a mechanism of mycolactone induced cell death [37]. Here, low doses of mycolactone were shown to cause cells to rapidly form filopodia; clusters of actin bundles associated with wound healing and cell-cell interactions. This was found to be due to mycolactone-dependent enhancement of actin polymerisation following its binding to the GTPase domain of the Wiskott-Aldrich syndrome Protein (WASP) family of proteins. The inappropriate activation of WASP and relocalisation of the actin nucleating complex Arp2/3 are thought to explain the well characterised cytoskeletal rearrangement in mycolactone treated cells. These were associated with loss of cell adhesion and e-cadherin-dependent tight junctions which could be partially restored by the addition of the WASP inhibitor, wiskostatin. Indeed e-cadherin itself appears to be down regulated by mycolactone ([37] Table 1). It therefore appears that, at least *in vitro*, cell death is related to anoikis, i.e. apoptosis due to detachment.

Immunosuppressive activity

One of the most striking features of BU histopathology sections is abundant foci of extracellular acid-fast bacilli in the absence of an inflammatory infiltrate. Instead, mononuclear cells are found the periphery of the infection but not close to the bacilli, suggesting the signals required to access the infection are missing [26, 38, 39]. It is now generally accepted that mycolactone is the immunosuppressive factor. Since this immunosuppression is so central to the body's inability to clear the infection (although it should be noted that this is not always the case - some patients do spontaneously heal and this is associated with a delayed-type hypersensitivity response [40]) much research attention has focussed on this area in the last decade. Immunosuppressive doses of mycolactone are generally lower than cytotoxic doses on the same cell type, and are certainly faster acting. For instance, primary human monocytes begin to produce TNF within 40 minutes of exposure to an inflammatory agent and mycolactone effectively obliterates this at a tenth of the cytotoxic dose [27].

Mycolactone can suppress both innate and adaptive immune responses in patients and in experimentally infected mice. Mycolactone is also able to suppress innate responses by restricting many of the functions of tissue resident macrophages. Like other mycobacterial infections, *M. ulcerans* can be phagocytosed by macrophages in the early stages of infection [41], presumably escaping digestion by preventing phagolysosome formation similarly to *M. tuberculosis*. Over short

time periods and at low MOIs it can proliferate inside murine macrophages, until mycolactone causes the apoptosis/necrosis of the macrophages and the release of the bacilli into the intercellular space [39]. Despite this, it has been shown *in vitro* that wild type strains of *M. ulcerans* are not phagocytosed as effectively as mycolactone-negative strains and exogenous mycolactone has the ability to complement this activity [41, 42].

One of the other key events that would normally occur upon phagocytosis of a microbe is activation by pathogen-associated molecular patterns (PAMPs) - including stimulation of Toll-like receptors [TLRs] and the NLRP3 inflammasome - resulting in intracellular signalling cascades that result in a rapid, profound and controlled inflammatory response, including the production of cytokines and chemokines to recruit other inflammatory cells to the site of infection. However, cells exposed to mycolactone-producing strains of *M. ulcerans* secrete dramatically less TNF than those encountering mycolactone-negative strains [43]. Purified mycolactone profoundly inhibits the ability of monocytes, macrophages, T cells and DCs to do this [10, 27, 43, 44]. Furthermore, the production of other cytokines and chemokines, (including IL-6, IL-8, IL-10 and IP-10) is also dramatically reduced in both primary human monocytes and macrophages, as is expression of the pro-inflammatory mediator cyclooxygenase-2 (COX-2) (Table 1; [27] and Hall and Simmonds, unpublished). Failure to produce cytokines and chemokines at the site of infection is considered a likely explanation for the lack of an inflammatory infiltrate close to bacilli, with the peripheral infiltrate indicating the limit of biologically active doses of mycolactone [2]. However, the technical expertise to measure or detect such low levels of mycolactone *in situ* is still lacking.

In addition to inhibiting macrophage function, mycolactone also inhibits DC maturation, as determined by the maturation marker CD83 (see Table 1), and a lack of the expected upregulation of the costimulatory molecules CD80 and CD40 in response to PAMPs such as lipopolysaccharide (LPS) [28]. In addition, DC chemokine production is suppressed, principally affecting IP-10, MCP-1 and MIP-1 α [28]. This may explain the anergy that has been reported in patients, where their T cells no longer respond to mycobacterial antigens after exposure [45], and contribute to the reduced Th1/Th2/Th17 responses seen in whole blood-restimulations [44]. In addition to inhibiting the costimulatory and migratory function of DCs, and therefore indirectly affecting T cell activation, T cells themselves are also directly sensitive. Low doses of mycolactone directly block the mitogen- and CD3/CD28-dependent production of IL-2, IL-4, IL-8, IL-10, IL-17, IFN γ and MIP-1 β from CD4 $^{+}$ T cells [44]. Furthermore, lymphocyte homing to peripheral lymph nodes is inhibited due to a down-regulation of L-selectin and LFA-1 on the surface of T cells [46].

Mechanism underlying mycolactone-dependent inhibition of protein production.

It is predicted that the proteins affected by mycolactone may share an underlying mechanism of production that could explain their co-regulation. The gene regulatory networks of many inflammatory mediators, including cytokines, chemokines and some PAMP-induced costimulatory molecules have many components in common, making them attractive targets for investigation. Transcriptional regulation by the NF κ B and IRF transcription factors is the most widely studied example, as well as mRNA stabilisation mediated by p38 MAPK and AU-rich elements (AREs) in the 3' UTRs of inflammatory genes [47, 48]. Furthermore, some cytokines such as TNF α are translationally derepressed by LPS by a mechanism that is also partially dependent on AREs but is still not yet fully understood [49]. Production of mature TNF α and IL-1 β are additionally dependent on cleavage of pro-proteins by TACE (tumour necrosis factor- α -converting enzyme) and caspase-1 (in the NLRP3 inflammasome), respectively [50, 51].

Early work on the mechanism of suppression by mycolactone was carried out in Jurkat T cells using ASLs and focussed on the suppression of IL-2 production [10]. In this cell line, mycolactone strongly inhibited the activation of NF κ B following TNF α stimulation, which led to the initial assumption that this was a generalisable mechanism, even though these authors found no effect on NF κ B activation

in other different model systems (PMA-induced in Jurkat cells, IL-1 induced in HeLa cells and LPS induced in RAW264.7 cells) [10]. Indeed the effect of mycolactone on steady state IL-2 mRNA levels turns out to be dependent on the stimulus, being substantially reduced during the CD3/CD28 response and enhanced for PMA/ionomycin [29]. More recently transcriptional profiling of primary human T cells found no significant changes in mRNA level [46]. The situation is similar in myeloid cells since a detailed investigation of the effect of mycolactone on the LPS-dependent upregulation of TNF α , IL-6 and COX-2 showed that while protein production is completely ablated by low immunosuppressive (non-cytotoxic) doses of mycolactone, the steady state induced mRNA levels are not affected or are again enhanced in the case of TNF α [27]. This is true for primary human monocytes [27], RAW264.7 cells [41], primary human macrophages and HeLa cells (Hall and Simmonds unpublished). Thus it seems that, with the exception of the transformed Jurkat T cell line, transcriptional regulation cannot explain the immunosuppression seen in mycolactone-exposed cells.

A detailed investigation in primary human monocytes was the first to propose a post-transcriptional mechanism for mycolactone function, probably at the level of translation [27]. Analysis of the signalling pathways revealed that neither MAPK or NF κ B signalling was affected in these cells, and neither did the proteins appear to be trapped inside the cells. The enhanced or unaffected transcription would also seem to rule out an effect on mRNA stability - indeed even inhibitors such as SB203080, which targets p38 MAPK, are only cause partial inhibition of TNF production [52], in contrast to the complete block seen following mycolactone exposure. While it is clear that mycolactone is acting post-transcriptionally, mycolactone is not a general inhibitor of protein production in monocytes, since it does not suppress total protein synthesis. Total incorporation of ³⁵S-labelled Met/Cys is not altered by the presence of mycolactone [27] and neither are general pathways controlling translation: mycolactone does not affect phosphorylation of the mammalian target of rapamycin complex 1 [MTORC1] targets eIF4E or p70s6 kinase or the endoplasmic reticulum stress response marker eIF2 α [27]. This finding was later confirmed in T cells [29]. Thus it seems that mycolactone is selectively targeting a subset of genes for control. One obvious candidate for mediating such gene-selective control would be microRNAs but there is little evidence to support this. A small yet significant decrease in let-7b levels has been observed in T cells exposed to mycolactone. Surprisingly, inhibition of let-7b caused a decrease in L-selectin mRNA and overexpression increased it, suggesting that L-selectin is not a direct target of let-7b (since the opposite pattern would have been expected). However the findings were not confirmed at the protein level. Our group is currently investigating translational and post-translational mechanisms of protein production inhibition.

Conclusion

Taken together, the loss of protein production by key players in the immune response and cellular adhesion would appear to be driving the immunosuppression and tissue necrosis in BU (Table 1). Indeed, when all available literature are surveyed, only three proteins could be identified that are upregulated by mycolactone (Table 2); the chemokine receptor CCR7 [46] and two muscle-specific E3 ubiquitin ligases (MuRF-1 and Atrogin-1)[34]. Of course there are many proteins which escape mycolactone-dependent regulation (notably constitutive expression of actin, CD28, p38 MAPK and eIF2 α and also the LPS-dependent resynthesis of I κ B α , Table 2) since total cellular protein production rates are not suppressed [27]. However, it is currently unclear how the suppression of constitutively expressed proteins such as e-cadherin and CD3 fits with our current models of mycolactone function. While the recent finding of a direct interaction between mycolactone and WASP is exciting, it would not appear to explain the inhibition of cytokine production since wiskostatin treatment does not reverse the action of mycolactone as expected (Hall & Simmonds, in preparation). Instead, the cellular compartments of the sensitive and insensitive proteins may be informative when considering a unifying mechanism for this fascinating pleiotropic molecule.

Table 1 Proteins shown to be down-regulated by mycolactone

Protein downregulated	Cell Type	Function	Ref
<i>Membrane proteins</i>			
CD3	T cell	TCR signal transduction	[29]
CD25	DC	IL-2 receptor α chain, DC maturation marker	[28]
CD40 (partial)	DC	Co-stimulatory protein	[28]
CD62-L	T cell	Adhesion, T cell homing	[46]
CD80 (partial)	DC	Co-stimulatory protein	[28]
CD83	DC	Dendritic maturation marker	[28]
CD86	DC	Co-stimulatory protein	[28]
e-Cadherin	HeLa	Adhesion molecule	[37]
LFA-1	T cell	Adhesion, T cell homing	[46]
MHCII	DC	Antigen presentation molecule	[28]
T cell receptor	T cell	Antigen recognition	[29]
<i>Cytokines</i>			
IL-1 β (partial)	Mo M ϕ DC	Cytokine (proinflammatory)	[27, 28]
IL-2	T cell	Cytokine (T cell proliferation and differentiation)	[10, 26, 29, 44]
IL-4	T cell	Cytokine (Th2 responses)	[44]
IL-6	M ϕ Mo DC	Cytokine (proinflammatory)	[27, 28]
IL-10	T cell Mo M ϕ	Cytokine (Anti-inflammatory, Th2 responses)	[10, 27]
IL 12 (partial)	DC	Cytokine	[28]
IL- 17	T cell	Cytokine (proinflammatory)	[44]
Interferon γ	T cell	Cytokine (proinflammatory)	[44]
TNF α	T cell Mo M ϕ	Cytokine (proinflammatory)	[10, 27, 43, 44]
<i>Chemokines</i>			
IL-8	T cell Mo M ϕ	Chemokine	[27, 44]
IP-10	Mo M ϕ DC	Chemokine (reporter for IFN α/β production)	[27, 28]
MCP-1	DC	Chemokine	[28]
MIP1 α	DC	Activates granulocytes, chemokine	[28]
MIP1 β	T cell	Activates granulocytes, chemokine	[44]
RANTES	DC	Chemokine	[28]
<i>Intracellular</i>			
COX-2	Mo M ϕ	Prostaglandin synthesis	[27]

Abbreviations: CD, cluster of differentiation; COX-2, cyclooxygenase-2; DC, dendritic cell; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MIP-1 α , macrophage inflammatory protein-1 α ; Mo, monocytes; M ϕ , macrophage RANTES, regulated upon activation normal T cell expressed and presumably secreted; TNF α , tumour necrosis factor α

Table 2 Proteins known to be unaffected or up-regulated by mycolactone

Protein unaffected	Cell type	Function	Ref
Actin	Mo, HeLa	Cytoskeleton	[27, 37]
CD28	T cell	Costimulatory receptor	[29]
eIF2 α	T cell, Mo	Translation initiation	[27, 29]
fyn	T cell	Signalling molecule	[29]
I κ B α	Mo	Signalling molecule (inhibition of NF κ B)	[27]
LAT	T cell	Signalling molecule	[29]
lck	T cell	Signalling molecule	[29]
p34 Arp2/3	HeLa	Cytoskeletal regulator	[37]
p38 MAPK	T cell, Mo	Signalling molecule	[27, 29]
PLC γ 1	T cell	Signalling molecule	[29]
N-WASP	HeLa	Cytoskeletal regulator	[37]
ZAP70	T cell	Signalling molecule	[29]
Protein upregulated	Cell Type	Function	Ref
CCR7	T cell	Chemokine receptor	[29]
MuRF-1	Muscle	Muscle-specific E3 ubiquitin ligase	[34]
Atrogin-1	Muscle	Muscle-specific E3 ubiquitin ligase	[34]

Abbreviations: CD, cluster of differentiation; DC, dendritic cell; Mo, monocytes; M ϕ , macrophage

Figure Legends

Figure 1 The structure of naturally occurring mycolactones

The complete structure of mycolactone A/B is shown, which exists in nature as 3:2 ratio of Z-/E-isomers of the C4-C5 bond in the long polyketide side chains. Since it is this long chain that varies between geographically diverse clinical isolates (mycolactones A-D) and other non-pathogenic MPM (mycolactones E, F and S1, S2), these chains alone are shown. Data from [12, 17, 53-55]

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