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## Sec61 blockade by mycolactone: a central mechanism in Buruli ulcer disease

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**Key words:** Sec61, mycolactone, Buruli ulcer, immunomodulation, stress

**Abbreviations:** AA, arachidonic acid; AGTR2, type 2 angiotensin II receptor; ATF4, Activating Transcription Factor 4; BU, Buruli ulcer; CHOP, C/EBP homologous protein; DC, dendritic cell; DRG, dorsal root ganglion; ER, endoplasmic reticulum; IL, interleukin; IFN, interferon, KCN4, Potassium voltage-gated channel subfamily A member 4; iNOS, nitric oxide synthase; ISR, integrated stress response; MHC, major histocompatibility complex; mTOR, mammalian target of rapamycin; PHA, phytohemagglutinin; SRP, signal peptide; TCR, T cell receptor; TMP, transmembrane protein; UPR, unfolded protein response; WASP, Wiskott-Aldrich syndrome protein.

## **Abstract**

Infection with *Mycobacterium ulcerans* results in a necrotizing skin disease known as a Buruli ulcer, the pathology of which is directly linked to the bacterial production of the toxin mycolactone. Recent studies have identified the protein translocation machinery of the endoplasmic reticulum (ER) membrane as the primary cellular target of mycolactone, and shown that the toxin binds to the core subunit of the Sec61 complex. Mycolactone binding strongly inhibits the capacity of the Sec61 translocon to transport newly synthesized membrane and secretory proteins into and across the ER membrane. Since the ER acts as the entry point for the mammalian secretory pathway, and hence regulates initial access to the entire endomembrane system, mycolactone treated cells have a reduced ability to produce a range of proteins including secretory cytokines and plasma membrane receptors. The global effect of this molecular blockade of protein translocation at the ER is that the host is unable to mount an effective immune response to the underlying mycobacterial infection. Prolonged exposure to mycolactone is normally cytotoxic, since it triggers stress responses activating the transcription factor ATF4 and ultimately inducing apoptosis.

## **A. BURULI ULCER, THE THIRD MOST COMMON MYCOBACTERIAL DISEASE**

### **A1. Epidemiology, transmission and clinical management of BU**

Buruli ulcer (BU) is a necrotizing skin disease caused by infection with *Mycobacterium ulcerans*, the third most prevalent mycobacterial disease after Tuberculosis and Leprosy (Demangel et al., 2009). Following its first clinical description by Sir Albert Cook in Uganda in 1897, BU was recognized in an increasing number of countries of Africa, South America and Western Pacific regions, prompting the World Health Organization (WHO) to declare BU as an emerging public health concern in 1998 (Wansbrough-Jones and Phillips, 2006). Today, BU is reported in 33 countries and considered by the WHO as one of the 17 neglected tropical diseases. Although its annual incidence has decreased from ~5000 to ~2000 cases since 2010, BU is likely underreported or unrecognized in most endemic areas. Epidemiological and genomic studies have revealed that *M. ulcerans* is associated with lentic environments (reviewed in (Zingue et al., 2018)), and suggest that the bacteria do not spread from human-to-human, but through displacements of reservoirs that remain to be discovered. The primary host of *M. ulcerans* is likely aquatic, and transmission of *M. ulcerans* from reservoirs to humans is currently believed to result from a combination of skin contamination with insect bites or puncture injuries (Wallace et al., 2017). BU typically starts as a painless subcutaneous nodule, oedema or plaque, enlarging over time. After weeks to months, the overlying epidermis opens to uncover indolent, necrotic lesions affecting cutaneous and subcutaneous tissues. Osteomyelitis may occur, as the result of an hematogenous seeding of bacteria from distant foci of infection (Walsh et al., 2008). The core of BU lesions, harboring clusters of extracellular bacilli, is typically devoid of inflammatory infiltrates (Guarner et al., 2003; Ruf et al., 2017). BU is efficiently treated by a combination of the antibiotics rifampicin and streptomycin administered daily for eight weeks, sometimes associated with surgical excision of lesional skin and skin grafting (Etuafu et al., 2005; Nienhuis et al., 2010). While rarely fatal, BU often results in permanent disfigurement and long-term disability.

### **A2. Genetics, chemistry and biodistribution of mycolactone**

*M. ulcerans* is unique amongst human pathogens in its capacity to produce a diffusible toxin called mycolactone (George et al., 1999). The biosynthesis of mycolactone is permitted by giant polyketide synthases, whose genes are harbored by a megaplasmid (Stinear et al., 2004). *M. ulcerans* strains of different geographical origins, or genetically related mycobacteria, produce variants of a canonical mycolactone structure, corresponding to a 12-membered lactone ring substituted with two polyketide-derived chains (Figure 1; reviewed in (Gehring and Altmann, 2017; Saint-Auret et al., 2017)). Mycolactone is central to the pathogenesis of BU. Its production is required for bacterial virulence, and injection of purified mycolactone in the dermis of rodent models is sufficient to induce BU-like lesions (George et al., 1999). While *M. ulcerans* bacteria rarely disseminate beyond the skin, mycolactone has a body-wide distribution. Its distinctive mass spectrometric signature was detected in peripheral blood cells, spleen, liver and kidneys of mice experimentally infected with *M. ulcerans* (Hong et al., 2008). In patients with progressive disease, structurally intact mycolactone was detected in ulcer exudates, healthy skin around ulcers and serum (Sarfo et al., 2011, 2014). Notably, mycolactone's presence was detected in perilesional skin several weeks after completion of antibiotic therapy (Sarfo et al., 2011, 2014), indicating a slow elimination rate. Alterations in the systemic production of IFN- $\gamma$ , that are resolved after surgical excision of the lesions, have also been reported in several immunological studies of BU patients (Gooding et al., 2001; Phillips et al., 2009; Prevot et al., 2004; Westenbrink et al., 2005; Yeboah-Manu et al., 2006). Notably, defective production of IFN- $\gamma$  in *ex vivo* stimulation assays of whole blood was observed with both antigen-specific (Gooding et al., 2001; Prevot et al., 2004) and non-specific (Phillips et al., 2009; Westenbrink et al., 2005; Yeboah-Manu et al., 2006) activation stimuli (such as the T-cell mitogen PHA). Multi-analyte profiling of PHA-stimulated whole blood culture supernatants revealed that in fact, most T cell-derived cytokines were suppressed during disease progression (Phillips et al., 2009). Moreover, patients with BU displayed a distinctive proteomic signature

in their serum, marked by a downregulation of multiple mediators of inflammation (Phillips et al., 2009, 2014). The immunosuppressive signature of BU persisted weeks after completion of antibiotic therapy in treated individuals (Phillips et al., 2009, 2014), thus correlating with the continued presence of mycolactone.

### **A3. Mycolactone: more than just a cytotoxin**

The contribution of mycolactone to each manifestation of BU disease, including skin necrosis associated with a relative lack of inflammatory infiltrates and pain, and defective cellular responses at the systemic level, have been the subject of intensive research over the past decades. The following section provides an overview of the main findings.

The cellular mechanism(s) of mycolactone-induced skin ulceration were first investigated by monitoring the cytopathic effects of mycolactone on cultured keratinocytes, fibroblasts, epithelial and endothelial cells. Fluorescently-labelled mycolactones penetrated cultured fibroblasts in a non-saturable and non-competitive manner, compatible with passive diffusion across the plasma membrane, to localize in the cytosol (Chany et al., 2011; Guenin-Mace et al., 2015; Snyder and Small, 2003). According to recent studies using computer simulations or lipid monolayers (Lopez et al., 2018; Nitenberg et al., 2018), the passage of mycolactone across cellular membranes may nevertheless alter their dynamic properties, and cause mechanical and physical perturbations (see also Section B3). Short-term (4-16h) exposure to mycolactone induced rapid alterations in the actin cytoskeleton of HeLa cells, coinciding with a defective capacity of the cells to establish adhesive contacts and migrate directionally in wound-healing assays *in vitro* (Guenin-Mace et al., 2013). In all skin cells studied, longer treatments (>48h) induced cell retraction followed by detachment and apoptosis, albeit with slight differences in time-to-death across cell types (Bieri et al., 2017; Dangy et al., 2016; Gama et al., 2014; George et al., 2000; Guenin-Mace et al., 2013; Ogbechi et al., 2015; Snyder and Small, 2003). In human dermal microvascular endothelial cells, mycolactone treatment also resulted in the depletion of the blood coagulation regulator thrombomodulin from the cell surface (Ogbechi et al., 2015). When mycolactone was injected intradermally into mouse ears, it caused major alterations in the architecture of the epidermis (Guenin-Mace et al., 2013). Collectively, these studies thus suggested that mycolactone provokes BU formation by a combination of cell death in dermis and subcutaneous tissues, remodelling of the epidermis, loss of healing potential and coagulation control.

Foxwell and co-workers were the first to demonstrate that mycolactone also displays intrinsic immunomodulatory properties. In their seminal 1999 paper, the authors showed that mycolactone prevents the lipopolysaccharide-induced release of the cytokines TNF and IL-10 by human monocytes, and the production of IL-2 by activated T lymphocytes, under conditions that do not alter cell viability (Pahlevan et al., 1999). Subsequent work by others showed that non-cytotoxic treatments with mycolactone impair the phenotypic and functional maturation of dendritic cells (DCs), resulting in a reduced ability to activate T cells and produce inflammatory chemokines in response to stimulation (Coutanceau et al., 2007). Mycolactone was also found to decrease DC expression of MHC class I and II, in a dose-dependent manner that affected both direct and indirect antigen presentation (Grotzke et al., 2017). In monocytes and macrophages, mycolactone prevented the activation-induced production of cytokines and chemokines post-transcriptionally, and irrespective of the activation stimulus (Hall et al., 2014; Simmonds et al., 2009). In resting T cells, it downregulated the basal expression of the T Cell Receptor (TCR) and homing receptor L-selectin (CD62L), leading to altered responsiveness to TCR stimulation and an impaired capacity to reach peripheral lymph nodes *in vivo* (Boulkroun et al., 2010; Guenin-Mace et al., 2011, 2015). Mycolactone also limited the capacity of T cells to produce cytokines in response to activation stimuli that bypass the TCR, in spite of a robust induction of cytokine mRNAs (Boulkroun et al., 2010). It is important to note that primary macrophages, DCs were susceptible to prolonged (>48h) treatment with mycolactone (>10nM) (Coutanceau et al., 2007; Guenin-Mace et al., 2015). In contrast, the

viability of human primary T cells and polymorphonuclear neutrophils treated under the same conditions was minimally affected (Guenin-Mace et al., 2015), showing that certain cell types resist mycolactone toxicity. Recent work indicated that in sensory neurons, Schwann cells and microglia, nanomolar concentrations of mycolactone similarly prevent the activation-induced production of pro-inflammatory cytokines (Isaac et al., 2017). Further, the systemic administration of mycolactone protected mice against chronic skin inflammation and rheumatoid arthritis (Guenin-Mace et al., 2015), whilst intrathecal injection of mycolactone in rats downregulated the basal production of inflammatory cytokines in the spinal cord (Isaac et al., 2017).

BU lesions typically show axonal degeneration and disruption of nerve fibers (Zavattaro et al., 2012). In mice, neural pathology associated with hypoesthesia was induced by infection with *M. ulcerans*, or injection of purified mycolactone (En et al., 2008; Goto et al., 2006). Short-term exposure to mycolactone (24h, 100 nM) induced significant neurite degeneration in rat and human primary dorsal root ganglion (DRG) sensory neurons (Anand et al., 2016). Longer treatments (>48h) induced massive mortality of primary DRGs in two studies (Anand et al., 2016; Isaac et al., 2017), although a third study reported a minimal loss of viability following exposure to mycolactone doses of up to 70 $\mu$ M (Song et al., 2017). Prolonged (>48h) exposure to nanomolar concentrations of mycolactone also caused significant mortality in Schwann cells and microglia (Isaac et al., 2017). Whether mycolactone reaches the central nervous system *in vivo* is unknown. However, with cytotoxic concentrations of mycolactone matching with its estimated level in BU lesions (Sarfo et al., 2014), these cellular studies support the view that BU-associated analgesia may be due, at least partially, to its cytopathic effects on peripheral nerves. It is noteworthy that infection with *M. ulcerans*, or injection of low doses (5 $\mu$ g) mycolactone, can induce local hypoesthesia in the absence of nerve destruction (Marion et al., 2014). Moreover, systemic administration of mycolactone (2 $\mu$ g) limited the development of inflammatory pain in mouse footpads (Guenin-Mace et al., 2015). Therefore, mycolactone likely reduces BU-associated pain by multiple mechanisms besides cytotoxicity, as discussed further in Section B.

## **B. MYCOLACTONE BLOCKS PROTEIN TRANSLOCATION AT THE ENDOPLASMIC RETICULUM**

Since mycolactone alone recapitulates the effects of an *M. ulcerans* infection (George et al., 1999; Sarfo et al., 2016), the compound could be exploited to identify physiologically relevant molecular targets. One such target is the Sec61 translocon, a membrane embedded protein complex responsible for the translocation of newly synthesized polypeptides into the endoplasmic reticulum (ER), and hence the eukaryotic secretory pathway (Lang et al., 2017).

### **B1. Mycolactone acts at the Sec61 complex**

The first indication that mycolactone selectively inhibited protein translocation across the ER membrane, and that it did so by inhibiting the heterotrimeric Sec61 complex, was the finding that the toxin blocked the translocation of model secretory and membrane proteins (Hall et al., 2014). Hence, mycolactone strongly inhibited the translocation of precursor proteins that use the well-defined signal recognition particle (SRP)-dependent co-translational route into the ER ((Hall et al., 2014); see Figure 2, Co-). At a global level, the mycolactone treatment of cells led to a selective reduction in the production of N-glycosylated membrane and secretory proteins (Hall et al., 2014), consistent with the effects of other inhibitors of protein translocation at the ER ((Cross et al., 2009); Figure 2). Furthermore, non-translocated forms of specific precursor proteins could be stabilized in cell culture models by using proteasome inhibitors (Hall et al., 2014), suggesting that the non-translocated forms of these hydrophobic precursor proteins are normally degraded via cytosolic quality control pathways (Casson et al., 2016).

Hall et al. showed that the inhibition of Sec61-dependent protein translocation by mycolactone can be studied in the absence of other cellular effects by using cell-free protein synthesis performed in the presence of ER derived membrane vesicles (Hall et al., 2014). This approach has helped to define the range of protein substrates affected by mycolactone and establish at what stage during the translocation of nascent polypeptides across the ER membrane it exerts its effects. In this way, it was shown that although mycolactone strongly inhibits the co-translational translocation of typical mammalian secretory proteins, it is less effective at blocking the post-translational translocation of the more unusual short secretory proteins ((McKenna et al., 2016); see Figure 2, Post-). For short secretory proteins, both the length of the precursor and the precise composition of its ER targeting signal influenced the effectiveness of the mycolactone blockade (McKenna et al., 2016). This behavior of short secretory proteins suggests that the toxin does not simply close the Sec61 channel to all subsequent polypeptide translocation. Alternatively, in the case of proteins that are delivered to the Sec61 complex via a post-translational mechanism (Figure 2), other factors, such as the actions of the ER luminal chaperone BiP, may be able to mitigate the effects of mycolactone (Hassdenteufel et al., 2018). By using established techniques, including cross-linking, to study different stages of the ER translocation process in this cell-free system (Cross et al., 2009), the point of the pathway at which mycolactone acts was identified ((McKenna et al., 2016); see also Figure 2). It was found that the binding of SRP to the hydrophobic ER targeting signal as it emerges from the ribosomal exit tunnel is unaffected, as is the delivery of the resulting targeting complex to the ER membrane embedded SRP receptor and the subsequent engagement of the nascent polypeptide with the Sec61 complex (Figure 2, see stages 1 to 3). However, in the presence of mycolactone the ability of these nascent polypeptides to proceed to the next step of the ER translocation process (Figure 2, see stage 4) is either efficiently prevented (co-translational substrates) or variably reduced (post-translational substrates) (McKenna et al., 2016).

The Sec61 $\alpha$  subunit of the heterotrimeric Sec61 complex is a multi-spanning integral membrane protein that forms the core of the regulated channel through which the majority of newly synthesized membrane and secretory proteins access both the lipid bilayer and lumen of the ER ((Lang et al., 2017), Figure 2). Sec61 $\alpha$  is the target for several small molecule inhibitors, including cotransin, decatransin and apratoxin, that bind directly to it (Junne et al., 2015; Mackinnon et al., 2014; Paatero et al., 2016). Mycolactone treatment alters the conformation of Sec61 $\alpha$  as judged by protease sensitivity (McKenna et al., 2016), and mycolactone efficiently displaces cotransin (CT7) from its previously defined binding site on Sec61 $\alpha$  (Baron et al., 2016), both indicative of a direct interaction. Definitive evidence that mycolactone binds to Sec61 $\alpha$  came from the finding that a single amino acid change to the protein that reverses the inhibitory effect of cotransin by disrupting its binding, also negates the effects of mycolactone, and hence cells that express the R66G mutant of Sec61 $\alpha$  are resistant to the toxin (Baron et al., 2016).

## **B2. Mycolactone: potent yet selective**

In cell-free systems, the co-translational translocation of a range of precursor proteins that are synthesized with a hydrophobic ER targeting signal was inhibited by mycolactone (Baron et al., 2016; Hall et al., 2014; McKenna et al., 2016). These inhibitory effects of mycolactone extended to both fully translocated soluble proteins and integral membrane proteins and can affect precursors with both cleavable and non-cleavable ER targeting signals (Figure 3). Single-spanning membrane proteins that integrate via the Sec61 complex may be classified as either type I, II or III transmembrane proteins, according to the presence of a cleavable targeting signal and the location of N-terminus of the mature polypeptide relative to the ER membrane ((Goder and Spiess, 2001); Figure 3). Using these criteria, the translocation of soluble secretory proteins and ER resident chaperones, and the integration of type I and type II membrane proteins were all strongly inhibited by mycolactone ((Baron et al., 2016; Hall et al., 2014; McKenna et al., 2016); Figure 3). To date, only a handful of multi-spanning membrane proteins have been studied using this cell-free system, but one of these model substrates was completely refractive to the toxin (Baron et al., 2016).

The substrate specific actions of mycolactone were further underlined by a complete absence of any effect on the Sec61 dependent insertion of type III membrane proteins (McKenna et al., 2017) that have a single transmembrane span, which also acts as the ER targeting signal (Figure 3). Furthermore, the effectiveness with which mycolactone blocks the insertion of a type I membrane protein can be influenced by both the distance between its N-terminal signal peptide (Figure 3, SP) and the properties of its transmembrane spanning domain ((McKenna et al., 2017), Figure 3, TMD). Thus, exactly how a nascent precursor protein engages the Sec61 complex (Figure 2, stage 4) is probably an important factor in determining the effectiveness with which mycolactone blocks its subsequent translocation (McKenna et al., 2017). The profiling of mycolactone's signature in the proteomes of CD4<sup>+</sup> T lymphocytes, DCs and DRG neurons (Baron et al., 2016; Grotzke et al., 2017; Morel et al., 2018), broadly supports this model for the effects of mycolactone on different classes of single-spanning membrane proteins ((McKenna et al., 2017), Figure 3). The global effects of mycolactone on the biogenesis of multi-spanning membrane proteins are more complex, and further cell-free studies of such substrates should help reveal how they arise from the inhibition of the Sec61 complex.

Importantly, these global proteomic analyses also highlighted alterations beyond those on the Sec61 substrates detailed above, most-likely resulting from the cascading effects of the protein translocation blockade (Baron et al., 2016; Grotzke et al., 2017; Morel et al., 2018) and from cellular stress responses that are described below in Section C. These effects have particularly important consequences for the immune control of *M. ulcerans* infection (Baron et al., 2016; Grotzke et al., 2017); and all clinical manifestations of BU (as outlined in Section C). Taken together (Baron et al., 2016; Hall et al., 2014; McKenna et al., 2016, 2017), these detailed molecular studies suggest that the efficiency with which mycolactone inhibits the biogenesis of individual membrane and secretory protein precursors is dependent upon the precise nature by which they employ the Sec61 translocation complex (Figures 2 and 3). This process is unexpectedly complex (Devaraneni et al., 2011; Hassdenteufel et al., 2018; Watson et al., 2013) and may also be influenced by a number of accessory components that are beyond the scope of this review (see (Lang et al., 2017) for further details).

### **B3. Mycolactone perturbs the lipid bilayer**

Mycolactone was originally isolated from acetone-soluble lipids prepared from *M. ulcerans*, indicative of its lipophilic properties ((George et al., 1999); see Figure 1), and recent biophysical and computational studies confirm that mycolactone binds to a range of artificial phospholipid membranes (Lopez et al., 2018; Nitenberg et al., 2018). Interestingly, the inclusion of cholesterol in these model systems promotes the insertion of mycolactone into the lipid phase leading to potential membrane destabilization (Lopez et al., 2018; Nitenberg et al., 2018). In the case of the cholesterol rich plasma membrane (Jacquemyn et al., 2017), these findings have clear implications for the mechanism by which mycolactone enters the cytosol and thereby accesses its intracellular targets, including the Sec61 complex. Likewise, toxin induced changes to the plasma membrane bilayer (Lopez et al., 2018; Nitenberg et al., 2018) might contribute to its cellular effects. Cholesterol is inhibitory to co-translocation protein translocation via the Sec61 complex (Kalies and Romisch, 2015), and hence its level in the ER membrane is comparatively low (Jacquemyn et al., 2017). Given that a single point mutation in the Sec61 $\alpha$  subunit confers broad resistance to mycolactone (Baron et al., 2016), the potential significance of any mycolactone-mediated disruption of the phospholipid component of the ER membrane to its strong inhibitory effects on protein translocation remains unclear.

## **C. MYCOLACTONE-MEDIATED SEC61 BLOCKADE AND CLINICAL MANIFESTATIONS OF BU**



Expression of a toxin resistant mutant of Sec61 $\alpha$  abolished the inhibitory effect of mycolactone on the homing potential and effector functions of immune cells (Baron et al., 2016), demonstrating that the Sec61 complex is the host receptor mediating the diverse immunomodulatory effects of mycolactone. Production of IFN- $\gamma$  by T cells, and IFN- $\gamma$  induced expression of nitric oxide synthase (iNOS) in infected macrophages are essential parameters of host resistance to mycobacterial infection (Bieri et al., 2016; Flynn and Chan, 2001). By blocking the production of IFN- $\gamma$  and the IFN- $\gamma$  receptor, mycolactone-mediated inhibition of Sec61 not only prevents the autocrine activation of IFN-responsive genes in lymphocytes, but also hampers the capacity of macrophages to produce iNOS in response to IFN- $\gamma$  stimulation (Baron et al., 2016). Hence, the protein translocation blockade detailed above (Section B) provides a molecular explanation for how mycolactone impairs the development of protective immune responses in patients that are infected with *M. ulcerans* (Figure 4).

In contrast, a Sec61 blockade is unlikely to explain the rapid effects of mycolactone on actin polymerization and cell adhesion described in Section A3. In cell-free assays of actin polymerization, mycolactone mimicked endogenous GTPase CDC42 in the activation of Wiskott-Aldrich syndrome proteins WASP and N-WASP (Guenin-Mace et al., 2013). A significant co-localization of mycolactone and active WASP was consistently observed 1h after the treatment of HeLa cells (Guenin-Mace et al., 2013). On this basis, a fraction of mycolactone may bind to cytosolic WASP/N-WASP following its diffusion through the plasma membrane, leading to the uncontrolled assembly of actin and defective cell-matrix adhesion. Although this mechanism may not be central to BU pathogenesis, we speculate that it may synergize with Sec61-dependent alterations in skin integrity (Figure 4).

Mycolactone was shown to activate Type 2 angiotensin II receptors (AGTR2) in neurons, leading to phospholipase A2-mediated arachidonic acid (AA) liberation, the generation of prostaglandin E2 from AA by cyclooxygenase-1, and subsequent activation of KCNQ4 potassium channels. The resulting hyperpolarization of neurons was proposed to mediate the analgesic properties of mycolactone (Marion et al., 2014). In support of this model, the hypoesthesia that is associated with skin lesions in mice infected with *M. ulcerans*, or injected with mycolactone, was reduced by local administration of an AGTR2 blocker (Marion et al., 2014). Based on the current model described in Section B, it is possible that the biogenesis of the multi-spanning membrane protein AGTR2 may be resistant to the effects of mycolactone on protein translocation. Nevertheless, the data presented in Section A3 strongly suggest that the Sec61-dependent anti-inflammatory activity of mycolactone on the immune and nervous systems, and toxicity in neurons, also contribute to BU-associated analgesia (Figure 4).

By transducing cells with the mycolactone-resistant R66G mutant of Sec61, it was shown that mycolactone's toxicity strictly depends on its binding to Sec61 (Baron et al., 2016). Ogbechi et al. found that the mycolactone-dependent inhibition of Sec61-mediated protein translocation in both RAW264.7 and HeLa cells induces the integrated stress response (ISR) via cytosolic sensors that are linked to the activity of the ATF4 transcription factor (Ogbechi et al., 2018). Whilst the ISR provides some degree of protection against the effects of mycolactone, prolonged exposure to the toxin results in programmed cell death via increases in the level of the pro-apoptotic factor Bim (Ogbechi et al., 2018). Interestingly in this study the translational activation of ATF4 was observed in the absence of an unfolded protein response (UPR) (Ogbechi et al., 2018) that is indicative of ER stress (Oakes and Papa, 2015). In contrast, we observed that DCs display clear hallmarks of ER stress-specific activation signals within hours of mycolactone treatment (Morel et al., 2018), consistent with a broad-ranging blockade of protein translocation (Grotzke et al., 2017). However, mycolactone-driven ER stress in DCs differed from a conventional UPR since there was a downregulation of BiP (Morel et al., 2018), a master regulator of the UPR that is induced by canonical ER stress but that relies on mycolactone-sensitive, Sec61 dependent, translocation to access the ER lumen ((Baron et al., 2016), Figure 3). In practice, whether mycolactone-driven ATF4 induction results from the ISR, the UPR, or a combination of these stress responses, may well depend on cell type. Importantly, both studies conclude that a sustained, mycolactone-mediated, Sec61 blockade triggers cellular stress responses that eventually induce apoptosis via the ATF4/CHOP/Bim signaling pathway (Morel et

al., 2018; Ogbechi et al., 2018). The ability of mycolactone to induce apoptosis via Bim may be further enhanced as a consequence of its inhibition of the mTOR signaling pathway (Bieri et al., 2017).

## D. CONCLUSIONS

Recent years have witnessed tremendous progresses in our understanding of the molecular mechanisms underpinning mycolactone biology, and therefore BU pathogenesis. In addition to highlighting the critical importance of Sec61 activity for immune cell function, migration and communication, the substrate selectivity of mycolactone inhibition reveals clear gaps in our understanding of membrane protein integration (McKenna et al., 2017; Morel et al., 2018). Further, studies using mycolactone have identified a novel mechanism of immunomodulation that has been evolved by *M. ulcerans*, and there is hope that this might be exploited therapeutically in order to limit inflammatory disorders. Hence, in mouse models of human diseases, systematically delivered mycolactone was effective in limiting skin inflammation and inflammatory pain (Guenin-Mace et al., 2015). There is also hope that small molecule inhibitors of protein translocation at the ER, including mycolactone, may provide a starting point for the development of new therapeutic agents such as novel anticancer drugs (Van Puyenbroeck and Vermeire, 2018).

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## CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

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## FIGURE LEGENDS

**Figure 1: Structure of *M. ulcerans*-derived mycolactone stereoisomers A/B.** The red line indicates the region where A and B differ (from (Kishi, 2011)).

**Figure 2: Mycolactone inhibits Sec61-dependent protein translocation.** The majority of secretory and membrane protein precursors are delivered to the cytosolic face of the endoplasmic reticulum as ribosome bound nascent chains (Co-). This co-translational delivery pathway starts with the binding of the signal recognition particle (SRP) to a hydrophobic signal sequence (Stage 1). The binding of SRP to its cognate receptor, SR (Stage 2) facilitates the transfer of the ribosome nascent chain complex to the Sec61 membrane translocation complex (Stage 3). Mycolactone appears to act by inhibiting the subsequent events of nascent chain engagement with (Stage 4) and membrane insertion by the Sec61 complex (Stage 5). For precursors that are delivered to the ER post-translationally (Post-), only proteins that require the Sec61 translocon are targets for mycolactone. Hence, the translocation of some short secretory proteins (SSPs) is inhibited, but the membrane insertion of tail-anchored (TA) proteins is unaffected.

**Figure 3: The effects of mycolactone on different categories of Sec61 clients.** The Sec61 complex is capable of mediating the full translocation of both soluble secretory and ER resident proteins into the ER lumen (Lang et al., 2017). Likewise, it can facilitate the membrane insertion of a range of integral membrane proteins with different combinations of signal sequences and transmembrane topologies, typically denoted Type I, Type II and Type III (Goder and Spiess, 2001). In contrast, tail-anchored (TA) membrane proteins do not require the Sec61 complex for membrane insertion (Johnson et al., 2013). The effects of mycolactone on these different classes of proteins, as determined using both cell-free translation systems (CFT) and cell culture studies (Baron et al., 2016; Hall et al., 2014; McKenna et al., 2016, 2017; Morel et al., 2018), are summarized above. The N-terminal signal peptides (SP) found on secretory and Type I membrane proteins are cleaved after the precursor is targeted to the ER. The transmembrane domains (TMD) found on Type II and Type III proteins act as signal-anchor sequences and are not removed from the mature proteins.

**Figure 4:** Parallels between BU hallmarks and the biological effects of mycolactone-mediated Sec61 blockade.

Figure 1

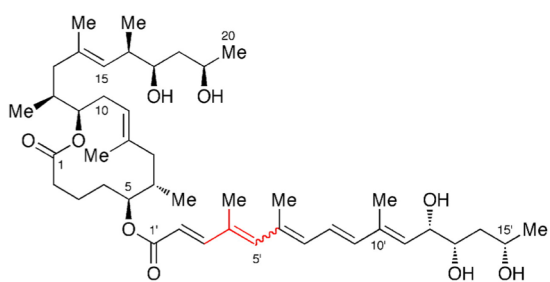
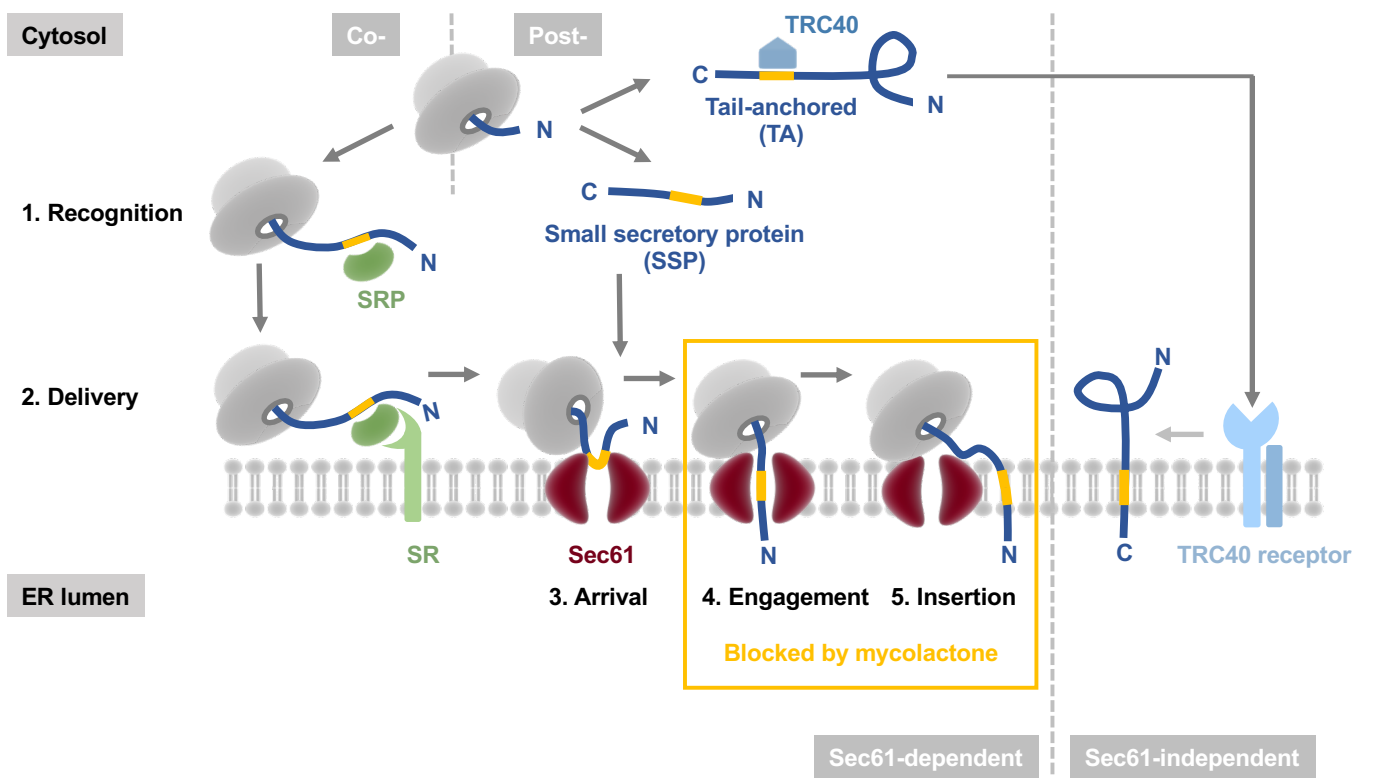
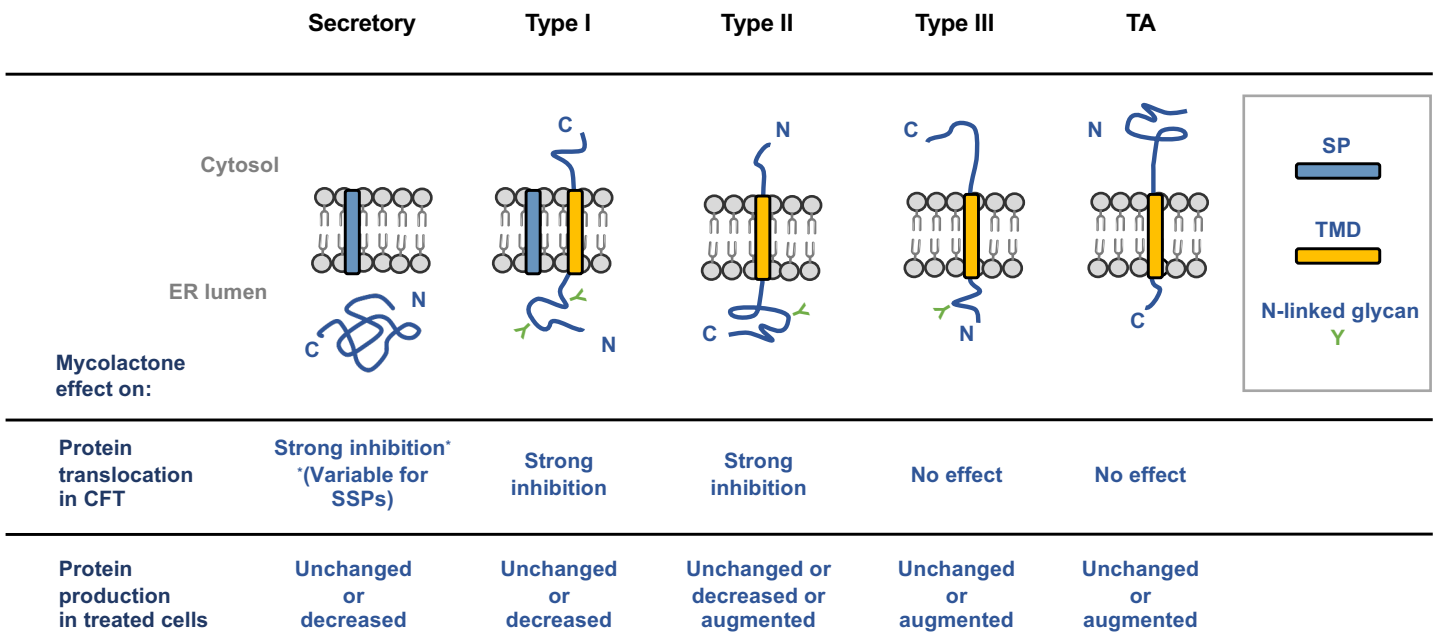




Figure 2



**Figure 3**



**Figure 4**

<b>Hallmarks of BU</b>	<b>Established and predicted <i>in vivo</i> consequences of mycolactone-mediated Sec61 blockade</b>
<b>Skin ulceration Coagulative necrosis</b>	<ul style="list-style-type: none"><li>• Pro-apoptotic in keratinocytes, endothelial cells and fibroblasts</li><li>• Depletion of thrombomodulin from dermal endothelial cells</li><li>• Ulceration of the skin following <i>in situ</i> injection of mycolactone in rodent models</li></ul>
<b>Local analgesia</b>	<ul style="list-style-type: none"><li>• Pro-apoptotic in neurons, Schwann cells and microglia</li><li>• Altered cytokine production by neurons, Schwann cells and microglia</li><li>• Inhibition of inflammatory pain and neuro-inflammation in mycolactone-injected rodents</li></ul>
<b>Defects in local inflammation and systemic cellular immune responses</b>	<ul style="list-style-type: none"><li>• Pro-apoptotic in macrophages, DCs and B cells</li><li>• Altered activation-induced production of cytokines/chemokines by all immune cell subsets</li><li>• Altered DC maturation, defective direct and indirect antigen presentation by DCs</li><li>• Altered T cells migration, altered IFN-<math>\gamma</math> signaling in T cells and macrophages</li><li>• Inhibition of systemic inflammation in mycolactone-injected mice</li></ul>