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Mycolactone causes catastrophic Sec61-dependent loss of the endothelial glycocalyx and basement membrane: a new indirect mechanism driving tissue necrosis in *Mycobacterium ulcerans* infection

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Abstract

The drivers of tissue necrosis in *Mycobacterium ulcerans* infection (Buruli ulcer disease) have historically been ascribed solely to the directly cytotoxic action of the diffusible exotoxin, mycolactone. However, its role in the clinically-evident vascular component of disease aetiology remains poorly explained. We have now dissected mycolactone's effects on primary vascular endothelial cells *in vitro* and *in vivo*. We show that mycolactone-induced changes in endothelial morphology, adhesion, migration, and permeability are dependent on its action at the Sec61 translocon. Unbiased quantitative proteomics identified a profound effect on proteoglycans, driven by rapid loss of type II transmembrane proteins of the Golgi, including enzymes required for glycosaminoglycan (GAG) synthesis, combined with a reduction in the core proteins themselves. Loss of the glycocalyx is likely to be of particular mechanistic importance, since knockdown of galactosyltransferase II (beta-1,3-galactotransferase 6; B3Galt6), the GAG linker-building enzyme, phenocopied the permeability and phenotypic changes induced by mycolactone. Additionally, mycolactone depleted many secreted basement membrane components and microvascular basement membranes were disrupted *in vivo*. Remarkably, exogenous addition of laminin-511 reduced endothelial cell rounding, restored cell attachment and reversed the defective migration caused by mycolactone. Hence supplementing mycolactone-depleted extracellular matrix may be a future therapeutic avenue, to improve wound healing rates.

eLife assessment

The toxin mycolactone is produced by mycobacterium ulcerans which is responsible for the Buruli ulcer. The authors performed a **valuable** study showing the effects of mycolactone on blood vessels integrity. This **convincing** data provide new therapeutic targets to accelerate healing of Buruli ulcers.

Introduction

Buruli ulcer (BU) is a neglected tropical disease caused by subcutaneous infection with *Mycobacterium ulcerans,* characterised by development of large, painless plaques or open lesions, often associated with oedema. The disease is most common in West Africa but is also found in other tropical and subtropical regions including Australia. Although the lesion can be sterilised by a minimum two-month antimicrobial treatment course with rifampicin and clarithromycin, the wounds can take up to a year to heal and can lead to permanent disfigurement, especially when diagnosed late (1). The polyketide-derived toxin mycolactone, generated by *M. ulcerans*, is the critical driver of BU pathogenesis (2; 3). Continuous production of this virulence factor causes widespread coagulative necrosis and fibrin deposition in patient skin tissue, as it diffuses through tissue away from the infecting bacteria. Mycolactone is also responsible for the restricted immune response seen in BU. As well as showing long-term cytotoxicity to immune cells (2), mycolactone causes a rapid suppression of antigen presentation, co-stimulation and cytokine secretion at low doses (4; 5).

Many clinical features of BU can be attributed to the inhibitory action of mycolactone on the Sec61 translocon, (6; 7) the complex that translocates most membrane, secretory and organellar polypeptides into the endoplasmic reticulum (ER) (8). In co-translational translocation, nascent proteins are targeted to the ER surface by a signal peptide sequence at the N-terminus; the interaction between the signal peptide and the pore-forming protein of the translocon, Sec61α, opens a central channel that allows access to the ER lumen and a lateral gate through which transmembrane sequences can enter the membrane (9). Mycolactone docks to Sec61, preventing signal peptide engagement and locking the translocon in an inactive state with the lateral gate open but the channel blocked (10). The biogenesis of most secretory proteins and Type I and II membrane proteins is inhibited by mycolactone, while polytopic membrane proteins are largely unaffected (11; 12). Type III and tail-anchored proteins, which utilise alternative translocation pathways (13), are also resistant to mycolactone (7; 11; 14). The proteins whose translocation into the ER is blocked are synthesized in the cytosol where they are degraded by the proteosome (7) and selective autophagy (15; 16). Sec61 blockade induces an integrated stress response by activation of eIF2α kinases (12; 17) and an increase in autophagic flux (15) and without resolution the cells eventually undergo apoptosis (17; 18). The time from initial exposure to cell death varies between cell types, but for most human cells takes 3-5 days.

We have previously shown that endothelial cells are particularly sensitive to mycolactone. At low nanomolar concentrations, mycolactone depletes the anticoagulant receptor thrombomodulin (19) and junction proteins (20). It also increases the permeability of monolayers formed from endothelial cells derived from both vascular and lymphatic origin (20). While thrombomodulin depletion has also been observed in BU patient skin biopsies (19), this seems not to be the cause of the widespread fibrin deposition commonly seen within the skin tissue. Instead, this is linked to aberrant staining for the extrinsic clotting pathway initiator tissue factor (20). Tissue factor is normally located in the sub-endothelium where it is segregated from both the plasma proteins that drive coagulation and the surrounding dermal tissue (21). However, in BU patients, tissue factor was observed within the connective tissue distant from vessels and this spatially associated with fibrin deposition and early signs of necrosis (20). Our working model leading up to the current work was that mycolactone action at Sec61 in endothelial cells leads to vascular dysfunction and promotes the pathogenesis of BU. The current work seeks to explore the molecular mechanisms driving these events.

The integrity of the endothelium greatly depends on adequate production and maintenance of the extracellular matrix (ECM) (22), junctional complexes (23) and the glycocalyx, a highly charged coating of proteoglycans, glycolipids, glycoproteins and glycosaminoglycans (GAG) including heparan sulphate (HS), chondroitin sulphate (CS) and hyaluronic acid (24) covering the luminal side of the endothelium (25). The enzymatic glycosylation of heparan and chondroitin sulphate is initiated in the Golgi apparatus by transferase enzymes. This is also the site of the isomerisation and sulfation reactions needed to achieve the rich diversity of GAGs expressed at the cell surface (26). The glycocalyx acts as an exclusion zone for blood cells and controls interactions with platelets, blood clotting factors and immune cells as well as modulating fluid exchange and acting as a sensory system for the endothelial monolayer (26). On the basal side of the endothelium, is the basement membrane (BM), an ECM consisting of collagen type IV and laminins, crosslinked by perlecan, a HS proteoglycan, and/or nidogens (27). This sheet-like network forms a scaffold that interacts with integrins on the cell surface, controlling structural stability, cell adhesion and angiogenesis as well as preventing leukocyte extravasation (27; 28). Production of these complex structures, which preserve and regulate the barrier between blood and tissue, relies heavily on Sec61-dependent proteins.

In order to determine the molecular mechanisms driving mycolactone-induced endothelial cell dysfunction we have undertaken a detailed phenotypic and proteomic study of the changes it induces both *in vitro* and *in vivo*. Using primary human dermal microvascular endothelial cells (HDMEC), we found that, as well as increasing monolayer permeability, mycolactone caused rapid changes in endothelial cell morphology and migration, accompanied by loss of glycocalyx, adhesion and ECM proteins. Notably, structurally unrelated Sec61 inhibitors, Ipomoeassin F, and its derivatives induced comparable phenotypes in a similar time frame, highlighting the Sec61 dependency of ECM composition and function. We have dissected the roles of these different components in the response to mycolactone and found that loss of an enzyme critical for GAG biosynthesis phenocopied the changes seen in cell morphology and monolayer permeability. On the other hand, the effects on cell adhesion and migration were dependent on ECM interactions and could be ameliorated by application of exogenous laminin-511. Hence the current work presents a novel pathogenic mechanism in BU, driven by Sec61-dependent effects on endothelial cells.

Results

Sec61 blockade impacts endothelial cell morphology and adhesion

We recently observed that mycolactone induces morphological changes in primary endothelial cells *in vitro*, leading to a dose-dependent increase in monolayer permeability at 24 hours (20). To understand the longer-term effects of mycolactone, we performed time-lapse imaging of HDMECs exposed to mycolactone (Video 1) or solvent (DMSO) control (Video 2) every 30 minutes for 48 hours. As in previous observations, the cells began to take on an ‘elongated’ phenotype after 8 hours. The proportion of elongated cells increased with time (Fig 1A) and after 24 hours exposure, approximately half the cells (51.63±2.89%) had this phenotype. The average ratio of cell length to width doubled in 16 hours, and quadrupled after 24 hours exposure (Fig 1C). At 24 hours, a small proportion (9.73±4.01%) had acquired a rounded appearance (Fig 1B) similar to that reported for mycolactone exposure of fibroblasts (16) and epithelial cells (29). Notably, these cells retained the ability to reattach to the culture vessel (Video 1), in line with their continued viability in this time window (19). However, after this time their ability to re-adhere declined and the proportion

of detached cells steadily increased. Although the number of rounded cells increased between 24 and 48 hours, the elongated phenotype remained predominant at this time point (Fig 1B).

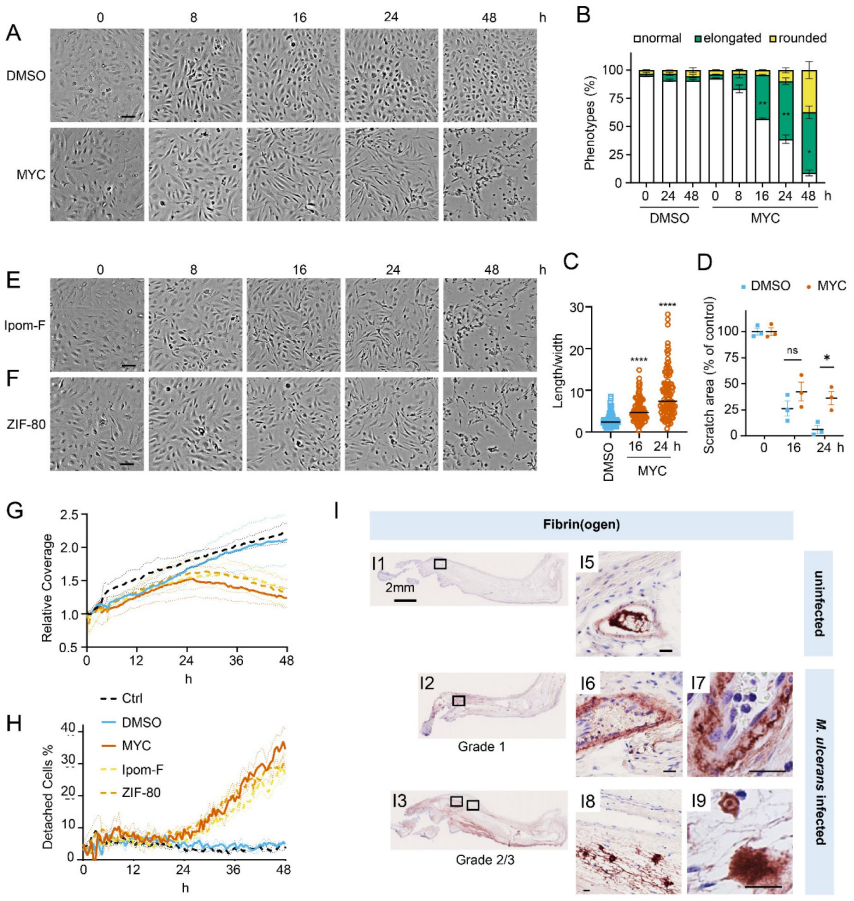

Figure 1.

Sec61 inhibition alters endothelial cell morphology and adhesion.

HDMECs were exposed to 10 ng/mL mycolactone (MYC), 0.02% DMSO, 400 nM Ipomoeassin F or 20 nM ZIF-80. **A-C**. Mycolactone-treated cells were imaged at indicated times in (**A**) and cell numbers of each phenotype (i.e. normal, elongated or rounded) were counted and presented as a percentage of total cell number per field in (**B**) (mean ± SEM of 3 independent experiments). (**C**) Length and width of each cell exposed to mycolactone for 16 and 24 hours or DMSO for 24 hours per image were measured and presented as a ratio. Data is representative of 3 independent experiments. ****, $p<0.0001$ (**D**) A scratch was introduced to a HDMEC monolayer prior to the treatment and visualised at 0, 16, 24 hours. The scratch area is presented as a percentage of the value obtained at 0 hour (mean ± SEM of 3 independent experiments) ns, not significant, *, $p<0.05$. Cells exposed to an alternative Sec61 inhibitor ipomoeassin F (IpomF) (**E**) or ZIF-80 (**F**) were imaged at indicated times. Images are representative of 3 independent experiments. Scale bar = 100 µm. **G-H**. Live cell imaging was performed with the zenCELL Owl incubator microscope every 30 minutes over 48 hours. Algorithms of cell coverage (**G**), detached cell numbers (**H**) per time point from 3 independent experiments are summarised as mean ± SEM. Data presented as cell coverage relative to the value obtained from initial time point (**G**) or a % of detached cells to total cell number (**H**) of each condition. **I.** Immunohistochemistry for fibrin(ogen) in the feet of C57BL/6 mice that received vehicle control (PBS) (I1, I5) or intradermal injection of 1 x10^5 colony forming units *M. ulcerans* at 21 (Grade 1; I2, I6-7) or 28 days (Grade 2/3;I3, I8-9) post-infection. Positive fibrin(ogen) staining is brown in colour, the haematoxylin counterstain is purple. Scale bars in I1-3: 2 mm; all others: 20 µm.

Next, we investigated how mycolactone affected HDMEC migration using scratch assays. While control cells were successfully able to close the scratch area within 24 hours, mycolactone-exposed cells displayed a gradual cessation in migration into the cell-free gap (Fig 1D). Thus, while at 16 hours similar numbers of cells had migrated into the scratch regardless of treatment, no further migration could be detected at 24 hours in the presence of mycolactone (Fig 1D). However, it should be noted that mycolactone has previously been reported to cause cell cycle arrest (2), which can be a confounding factor in such migration assays and may explain this finding.

To determine whether Sec61 inhibition by mycolactone was driving these abnormal phenotypes, we exposed endothelial cells to Ipomoeassin F or its more potent derivative, ZIF-80 (30). These are structurally distinct to mycolactone but inhibit Sec61α in a very similar manner since they compete for the same binding site (31; 32). Importantly, both compounds phenocopied the 'elongated' appearance preceding detachment in HDMEC within 24 hours (Fig 1E & F). Unbiased analysis of time-lapse data using zenCELL owl built-in algorithms allowed continuous estimation of cell coverage and detachment, although it could not be trained to recognise the elongated phenotype. As expected, cell coverage increased with time under control conditions, while the proportion of detached cells remained constant at approximately 5%. However, all three Sec61 inhibitors showed similar effects on both readouts (Fig 1 G & H). Interestingly, both measures remained similar to the control for approximately 24 hours, after which cell coverage declined with a corresponding increase in cell detachment. Taken together, this data strongly supports that these changes are driven by Sec61 inhibition and that endothelial cell homeostasis is dependent on adequate Sec61 function.

To establish the *in vivo* relevance of these findings, we performed fibrinogen immunostaining in the pre-ulcerative mouse footpad model of *M. ulcerans* infection (Fig 1I, S1). Fibrinogen is a high molecular weight (~330 kDa) plasma protein that is normally retained within the lumen of intact vessels and, indeed, in uninfected (vehicle control) mouse feet, fibrinogen was rarely detected, and then only within the vessel lumen (Fig 1I1 and 1I5). In contrast, at 21 days post infection (Grade 1 lesions; metatarsal thickness increase ~10%), fibrin(ogen) was seen within the blood vessel wall surrounding the endothelium (Fig 1I2, 1I6, 1I7). After 28 days (Grade 2/3 lesions, metatarsal thickness increase 50-100%) widespread fibrin(ogen) staining was seen outside blood vessels within the dermis, in foci consistent with its conversion to insoluble fibrin (Fig 1I8-9). The lack of signal in isotype control-stained tissue (FigS1B) confirms the specificity of staining. This penetration of fibrinogen between the endothelial monolayer lining the vessel, then through the vessel wall and conversion to fibrin by other components of the coagulation cascade within deeper tissue is consistent with the changes in endothelial cell morphology and monolayer integrity described here and previously (20) and demonstrates that the extravascular deposition of fibrin seen in patient biopsy samples is an early feature of infection.

Mycolactone predominantly targets proteins involved in glycosylation and adhesion

While proteomic studies of mycolactone action have been performed previously (6; 12; 14; 16), these have used whole cell lysates, leading to systematic limitations in detection of membrane and secreted proteins, due to their relatively low abundance compared to cytosolic proteins. Therefore, to understand the molecular mechanisms driving the pathogenic phenotypic changes in endothelial cells, we instead used a total membrane proteomics approach to enrich for the Sec61 substrates that are targeted by mycolactone.

We isolated total membrane fractions from HDMECs exposed to DMSO or mycolactone for 24 hours and analysed them by tandem mass tagging (TMT) multiplex LC/MS over biological triplicates (Fig 2A). A total of 6649 proteins were detected, of which 482 were significantly downregulated and 220 upregulated by mycolactone (> 2-Fold change, $p < 0.05$) (Fig 2B, Supplementary Files 2-5)). Among the total proteins discovered, 36.9% were trafficked via the secretory/endolysosomal pathways that primarily depend on the Sec61 translocon (Fig 2C). This group represented 84.6% of the downregulated but only 23.7% of the upregulated proteins. As predicted, membrane proteins were the most affected in the downregulated group, with little effect on cytoplasmic, cytoskeletal, mitochondrial or nuclear proteins. The downregulated fraction included previously published endothelial targets of mycolactone

including coagulation regulators thrombomodulin (TM), von Willebrand Factor (vWF), platelet endothelial cell adhesion molecule (CD31), endothelial protein C receptor and tissue factor pathway inhibitor (TFPI) and cell junction components tyrosine protein kinase receptor TIE1, angiopoietin-1 receptor (TEK), cadherin 5 (CDH5), junctional adhesion molecule 3 (JAM-3) and catenin β1 (19; 20) (Fig S2A), validating our dataset.

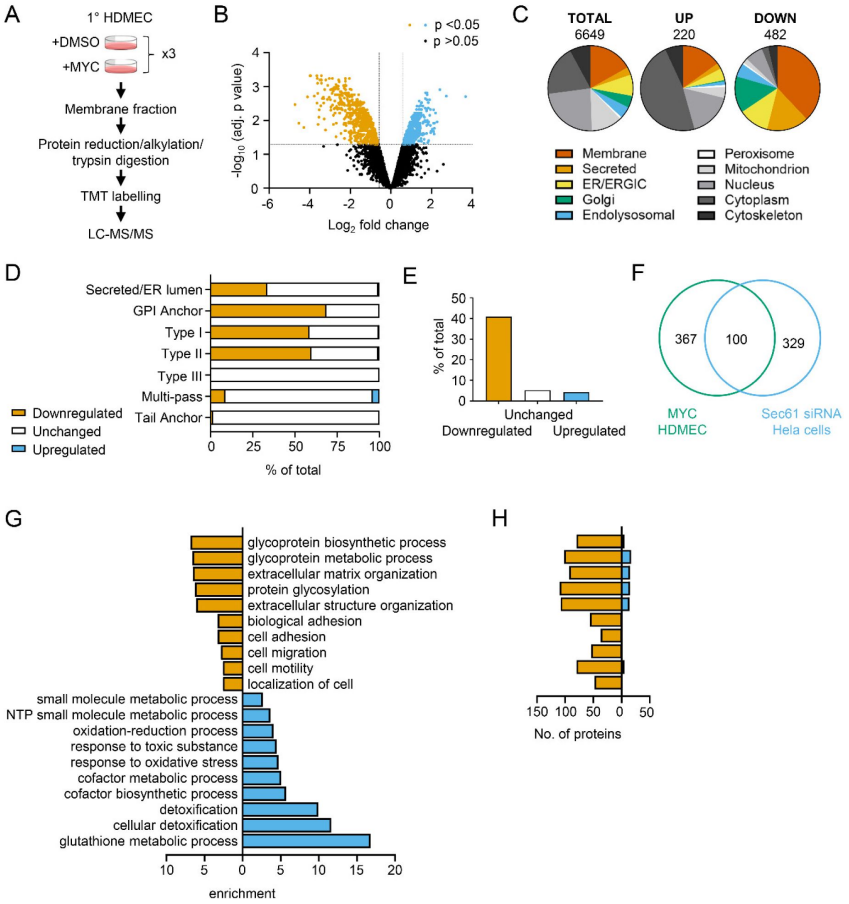

Figure 2.

Proteomic analysis reveals loss of proteins associated with glycosylation, adhesion and migration.

(**A**) Workflow for isolation and proteomic analysis of HDMEC membrane proteins. In 3 independent replicates, HDMEC were exposed to 10ng/ml mycolactone or DMSO for 24 hours, lysed by hypotonic lysis and membrane fractions enriched by differential centrifugation as described in Methods. Acetone precipitated proteins were reduced, alkylated and trypsinised then subjected to TMT labelling for quantitative proteomic analysis by LC-MS/MS. (**B**) Volcano Plot of differential expression between DMSO and mycolactone treated samples, plotting mean fold change against false discovery rate adjusted *p*-values; orange= downregulated, p<0.05; blue= upregulated, p<0.05; black=p>0.05 (**C**) Pie charts showing subcellular localisation of proteins in total, >2 fold upregulated or downregulated (p<0.05) fractions. (**D**) Quantitation of membrane or secreted proteins according to type: blue = upregulated; white = unchanged; orange = downregulated. (**E**) Percentage of downregulated, unchanged and upregulated multi-pass membrane proteins possessing a signal peptide. (**F**) Overlap between mycolactone downregulated endothelial membrane proteome and Sec61-dependent proteome. Venn diagram created using JVenn (41), showing overlap in significantly downregulated proteome between the dataset presented here and those obtained in Hela cells treated with siRNA for Sec61α (Nguyen et al., 2018). (**G**) Top significantly over-represented (p<0.05) GO groups in downregulated and upregulated data sets, compared to whole genome. Data generated with WebGestalt. (**H**) Quantitation of numbers of up and down regulated proteins in GO groups identified in (**G**).

As seen in previous proteomic studies and *in vitro* translocation assays (11; 12; 33; 34), mycolactone preferentially targeted secreted and single pass type I and type II membrane proteins in endothelial cells, with no effect on the EMC-dependent Type III proteins or the GET pathway-dependent tail-anchored proteins (Fig 2D). A small number (51 out of 606 detected) of multi-pass membrane proteins were also >2-fold downregulated by mycolactone (Supplementary File 6). This group was relatively enriched for signal peptide-bearing proteins (42% vs 4% amongst unchanged and upregulated multi-pass proteins) (Fig 2E). The rules governing sensitivity of this subgroup to mycolactone appear to be similar to those

reported for single pass type I proteins (12), with higher signal peptide hydrophobicity and a shorter distance between the signal peptide and first transmembrane domain being associated with increased resistance to the effects of mycolactone (Fig S2B and C). Of the remaining mycolactone-sensitive multi-pass proteins, 80% contained at least one long loop (>50aa) between transmembrane domains. Among the upregulated proteins, 88% of the integral membrane proteins were multi-pass membrane proteins, and only one of the predicted single pass proteins contained a signal peptide. Likewise, the four upregulated secreted proteins identified are all secreted by non-conventional pathways.

Overall, the data support the recently described model for the biogenesis of multi-pass proteins whereby the majority of multimembrane spanning proteins utilise an alternative translocon that includes Sec61 and the PAT, GEL and BOS complexes but, crucially, bypasses the lateral gate, instead relying on generation of a lipid-filled cavity on the opposite side of Sec61 (35; 36). Here, only those multi-pass proteins possessing a signal peptide or long internal loops require insertion into the membrane via the Sec61 channel, and therefore only these are sensitive to mycolactone.

Our membrane targeted approach identified a higher number of Sec61-dependent proteins in our control cells compared to previous studies (6; 12; 14) thus achieving our goal of wider capture of mycolactone-sensitive proteins. Moreover, when compared to siRNA-based Sec61α knockdown in Hela cells, despite the differences in cell type and methodology, 100 of the downregulated proteins were common to both datasets (Fig 2F) (37). While possession of a signal peptide or anchor appears to be crucial to mycolactone sensitivity (12), overall, no specific signal peptide sequence features were associated with downregulation. In keeping with this, there was very little overlap between mycolactone downregulated proteins and those lost following knockdown of translocon-associated proteins TRAPβ or knockout of Sec62/Sec63 (Fig S2D and S2E) (37; 38), which assist gating of the translocon by weak signal peptides. Thus, as suggested by analysis of the structure of the inhibited translocon (10), mycolactone acts via direct interaction with the Sec61α signal peptide binding site rather than through interference with accessory proteins.

Gene ontology (GO) analysis of mycolactone-upregulated proteins supported previous observations by ourselves and others of cellular stress responses, with significant enrichment of terms associated with oxidative stress and detoxification (Fig 2G) (12; 17; 39; 40). The upregulated proteins also included several proteins involved in the autophagy pathway, including SQSTM1/p62, which is involved in the cellular response to mycolactone (15) (Fig S2A). However, in the significantly downregulated fraction a distinct pattern emerged, with GO terms associated with glycosylation, matrix organisation, adhesion and cell migration showing the greatest over-representation compared to the whole genome. Within these GO groups, the vast majority of proteins detected in our proteome were downregulated by mycolactone (Fig 2H). Similar results were obtained when the downregulated proteins were compared to the total detected proteome (Fig S2F), showing this pattern was not an artefact resulting from membrane enrichment.

Mycolactone disproportionately targets Golgi-resident proteins involved in glycosylation and glycosaminoglycan chain synthesis leading to the loss of surface GAGs

The Golgi is the site of higher order protein glycosylation and GAG synthesis and, of the intracellular organelles, is the most affected by mycolactone (Fig 2C and S3A). The Golgi has a particularly high proportion of type II membrane proteins as the membrane anchor and sequences around it can act as a signal for Golgi retention (42) and nearly all of these Golgi-expressed type II membrane proteins were significantly downregulated by mycolactone (Fig

3A). Interestingly, type II Golgi proteins showed a higher degree of down-regulation by mycolactone than ER or plasma membrane localised type II proteins (Fig 3B). This suggests the signals that lead to Golgi localisation may make proteins more sensitive to Sec61 inhibition, although it is equally possible that Golgi proteins are turned over at a higher rate than those at other sites as depletion is generally at the turnover rate (19). The effect is not due to differences in transmembrane domain hydrophobicity, which shows little variation and has no impact on Type II protein levels in mycolactone-treated cells (Fig S3B).

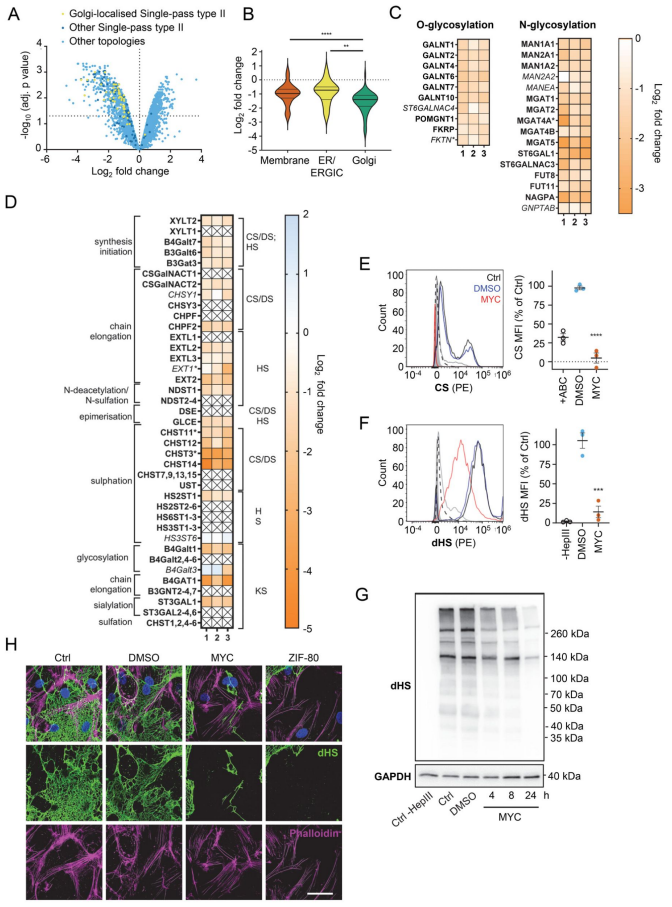

Figure 3.

Endothelial glycosaminoglycan chain synthesis is blocked by mycolactone.

HDMECs exposed to 10 ng/mL of mycolactone (MYC) or 0.02% DMSO for 24 hours or indicated times were subjected to proteomic analysis (**A-D**), surface immunostaining (**E-F, H**) or immunoblotting (**G**). (**A**) Volcano Plot of differential expression between DMSO and MYC treated samples, plotting mean fold change against false discovery rate adjusted *p*-values. Pale blue=total detected proteins; dark blue=Type II membrane proteins; yellow=Golgi-localised Type II membrane proteins. (**B**) Violin plot showing fold change in protein levels for Type II membrane proteins grouped according to subcellular location. ns, not significant; **, $p < 0.01$; ****, $p < 0.0001$. (**C**) Heat map showing fold change in Golgi-localised O- and N-glycosylation enzymes in mycolactone exposed HDMEC. Dual-colour coding is shown., only one unique peptide detected in asterisks, and significantly downregulated ($p < 0.05$) or not ($p \geq 0.05$) in bold or *Italic* respectively. (**D**) Genes in GAG biosynthesis categorised according to function and side chains of chondroitin sulphate/ dermatan sulphate (CS/DS), heparan sulphate (HS) or keratan sulphate (KS). Heatmap showing Log2 fold change of these genes in response to mycolactone in three independent experiments. Dual-colour coding is shown. Genes undetected are indicated as crossed, only one unique peptide detected in asterisks, and significantly downregulated ($p < 0.05$) or not ($p \geq 0.05$) in bold or *Italic* respectively (**E-F**) Cells were treated with or without chondroitinase ABC (ABC) or heparinase III (HepIII), immunostained with anti-chondroitin sulphate (CS), anti-Δ-heparan sulphate (dHS) antibodies or the isotype controls for flow cytometry analysis. Histogram plot for single cell population of CS (E) and dHS (F) and the respective mean fluorescence intensity (MFI) are shown. Unstained untreated cells filled grey; isotype control of untreated cells, dashed line in black; Untreated cells incubated with chondroitinase ABC prior to CS staining or without HepIII prior to dHS staining, grey line; untreated cells with CS-PE or dHS-PE, black line; cells exposed to DMSO stained with antibodies, blue line; cells exposed to MYC stained with antibodies, red line. MFI is presented as a % of untreated control (mean ± SEM of 3 independent experiments). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (**G**) Cells were lysed, treated with heparinase III and analysed by immunoblotting. HS neoepitopes were visualised with anti-Δ-heparan sulphate (dHS) antibody with the approximate migration of molecular weight markers in kDa. GAPDH as loading control. Images are representative of 3 independent experiments. (**H**) Cells were incubated with HepIII, fixed and immunostained with anti-dHS antibody (green), permeabilised and labelled with TRITC-conjugated phalloidin (magenta). Nuclei were stained with DAPI (blue). Images are representative of 2 independent experiments. Scale bar = 50 μm.

Detailed analysis of our dataset revealed that targeting of Golgi-localised proteins by mycolactone leads to significantly decreased abundance of multiple enzymes involved in both higher order N- and O-linked glycosylation (Fig 3C). However, the biggest impact is seen in GAG production, with the majority of the enzymes involved in GAG synthesis lost in mycolactone treated HDMECs (Fig 3D and S3C). All of the 23 proteins in the GAG biosynthetic pathway detected in our analysis are type II membrane proteins and 19 (82%) of these were downregulated by mycolactone (Fig 3D), affecting every step of glycosaminoglycan production (Fig S3C). Three of the mycolactone-targeted proteins were involved in initial steps of keratan sulphate formation, four in common synthesis initiation of chondroitin sulphate (CS), dermatan sulphate (DS) and heparan sulphate (HS), six in chain elongation of CS/DS and HS (two and four, respectively), and six in epimerisation or sulfation processes that enhance the structural diversity of CS/DS or HS (Fig 3D and S3C).

Given the importance of GAGs to endothelial function and the dramatic loss in GAG biosynthetic enzymes induced by mycolactone, we evaluated surface levels of the predominant endothelial GAGs, HS, and CS, using flow cytometry on HDMECs exposed to mycolactone for 24 hours. As a control, chondrointinase ABC was used to remove surface CS, resulting in fluorescence levels 60% lower than untreated cells. Remarkably, CS fluorescence intensity was even lower in cells exposed to mycolactone (Fig 3E). Similarly, using an antibody specific for a neoepitope of HS generated by heparinase III digestion, dHS, disrupted surface HS expression was observed in mycolactone-exposed cells (14.11±7.40% vs. DMSO solvent control 105.30±9.79%, $P = 0.0002$, Fig 3F). In addition, HS-containing proteoglycans were detected by immunoblot using the anti-dHS antibody. Heparinase III digestion revealed an abundance and diversity of heparan sulphate containing proteins present in untreated or DMSO-exposed HDMECs that decreased progressively with mycolactone exposure (Fig 3G). By immunofluorescence, HS forms a mesh-like network around and between cells in untreated and DMSO solvent controls (Fig 3H). However, in HDMECs exposed to mycolactone, or ZIF-80, the HS-positive network was disrupted within 20 hours (Fig 3H). Collectively, this data confirms that Sec61 inhibition by mycolactone profoundly impairs the ability of endothelial cells to synthesise GAG chains.

Loss of galactosyltransferase II drives changes in endothelial cell morphology and monolayer permeability

We reasoned that mycolactone-dependant depletion of any enzyme involved in the early stages of GAG biosynthesis would, on its own, be sufficient to explain the loss of HS and CS. Therefore, we validated its effect on the GAG linker building enzyme galactosyltransferase II (B3Galt6) by immunofluorescence. Endothelial B3Galt6 colocalised with the Golgi marker Giantin in a perinuclear region in untreated cells and was unchanged in those exposed to the solvent control (0.02% DMSO) (Fig 4A). B3Galt6 expression levels remained normal in HDMECs exposed to mycolactone for 6 hours but a clear reduction was seen after 12 hours (Fig 4A). Notably, ZIF-80 reduced B3Galt6 expression in a similar manner (Fig S4A).

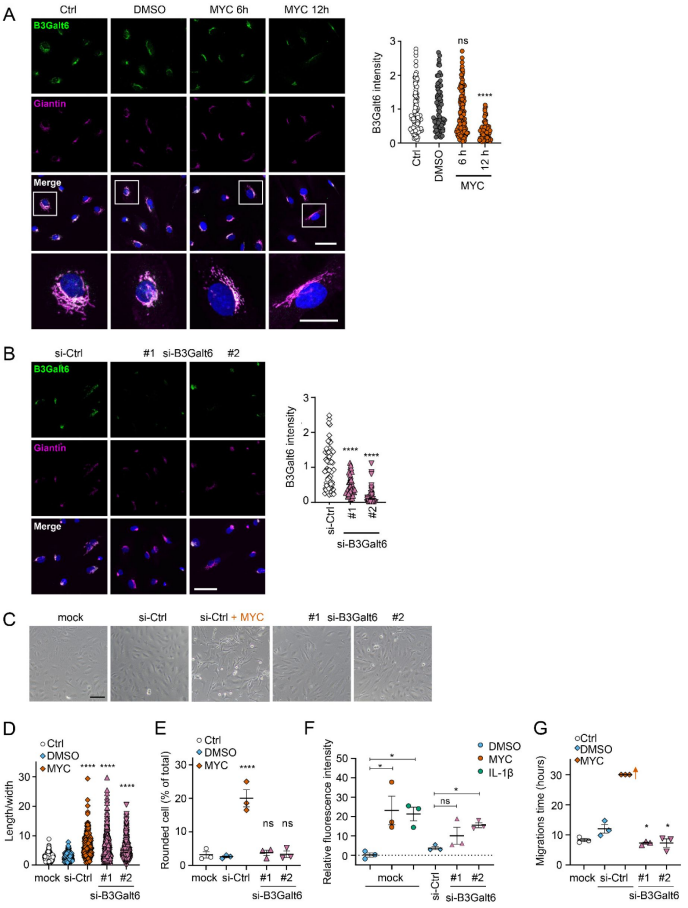

Figure 4.

Loss of B3Galt6 affects endothelial cell morphology and monolayer permeability.

A. HDMECs exposed to 10 ng/mL mycolactone (MYC) or 0.02% DMSO for indicated times. **B-G**. HUVECs transfected with si-B3Galt6 or si-negative control (ctrl) oligos for 48 hours. (**A-B**) Cells were fixed, permeabilised and immunostained with anti-B3Galt6 and anti-giantin antibodies. B3Galt6 (green) and the Golgi apparatus (magenta) were visualised and nuclei stained with DAPI (blue). Scale bar = 50 μm (20 μm in the crop panels of A). Corrected total cell fluorescence of B3Galt6 in Golgi apparatus per cell was measured and presented as a value normalised to the mean value obtained from untreated control of each experiment. More than 30 cells per condition were measured per experiment. Images and quantification are representative of 3 independent experiments. (**C**) HUVECS exposed to 10 ng/mL mycolactone (MYC) or 0.02% DMSO for 24 hours one day post-transfection were imaged by an inverted microscope. (**D**) Length and width of each cell presented as a ratio. At least 100 cells were measured for each treatment. Values are representative of 3 independent experiments. (**E**) Rounded cell number per image presented as a % of total cell number per condition (values represent the mean ± SEM of 3 independent experiments). (**F**) Permeability of transfected HUVEC monolayers on inserts with 1 μm pores treated with 100 ng/mL IL-1β, 10 ng/mL mycolactone (MYC) or 0.02% DMSO for 24 hours was quantified. Fluorescence intensity of FITC-dextran in the receiver wells was measured and presented as a % where 100% is the value obtained from transwell lacking a cell monolayer, and 0% is untreated control wells (mean ± SEM of 3 independent experiments). (**G**) HUVECs were transfected with si-B3Galt6 or si-negative control (si-ctrl) oligos. A scratch was introduced to the monolayer prior to the treatment (10 ng/mL mycolactone (MYC) or DMSO) and live cell imaging was performed with the zenCELL Owl incubator microscope every 15 minutes for 30 hours. Migration time in hours (hrs) to reform the monolayer is presented as mean ± SEM (n = 3); wells with no visible monolayer at the end point were given a maximum value = 30. ns, not significant; *, $P < 0.05$; ****, $P < 0.0001$.

In order to investigate whether loss of B3Galt6 was sufficient to induce the phenotypic changes we saw after mycolactone exposure, we knocked down B3Galt6 in HUVECs using siRNA. The reduction in B3Galt6 protein expression compared to cells transfected with non-targeting si-control RNA (Fig 4B) was comparable to that caused by mycolactone (~80%). B3Galt6 siRNA-treated cells demonstrated a similar elongated appearance (Fig 4C) and image analysis confirmed a significant increase in the ratio of cell length to width in HUVECs transfected with si-B3Galt6 RNA (Fig 4D). However, knockdown of B3Galt6 did not recapitulate the cell rounding phenotype (Fig 4E).

We next investigated the potential contribution of B3Galt6 loss to the previously observed mycolactone-induced increase in HDMEC and human dermal lymphatic endothelial cell monolayer permeability (20). Exposure of mock-transfected HUVEC monolayers to 10 ng/mL mycolactone for 24 hours increased permeability to 23.13±7.38%, an effect comparable to

100 ng/mL IL-1β (21.30±3.48%) (Fig 4E). B3Galt6 knockdown in HUVECs also led to a rise in monolayer permeability (10.08±4.37% and 15.47±1.27% of the values seen in empty wells, P = 0.2371 and 0.0367, for two different oligonucleotides, Fig 4F). Interestingly, B3Galt6 knockdown did not reduce the rate of HUVEC migration in scratch assays (Fig 4G); in fact the cells exhibited a slightly increased healing rate compared to controls.

Mycolactone rapidly depletes endothelial surface proteoglycans

Since loss of GAGs did not explain all the phenotypes observed, we considered the so-called core proteins to which GAGs synthesised in the Golgi are covalently linked to form the proteoglycans. These can be secretory, plasma membrane or GPI-anchored proteins, all of which require the Sec61 translocon for their biogenesis. Our proteome revealed that seven HS, CS, and/or DS-carrying proteoglycans were significantly down-regulated after 24 hours mycolactone exposure (Fig 5A).

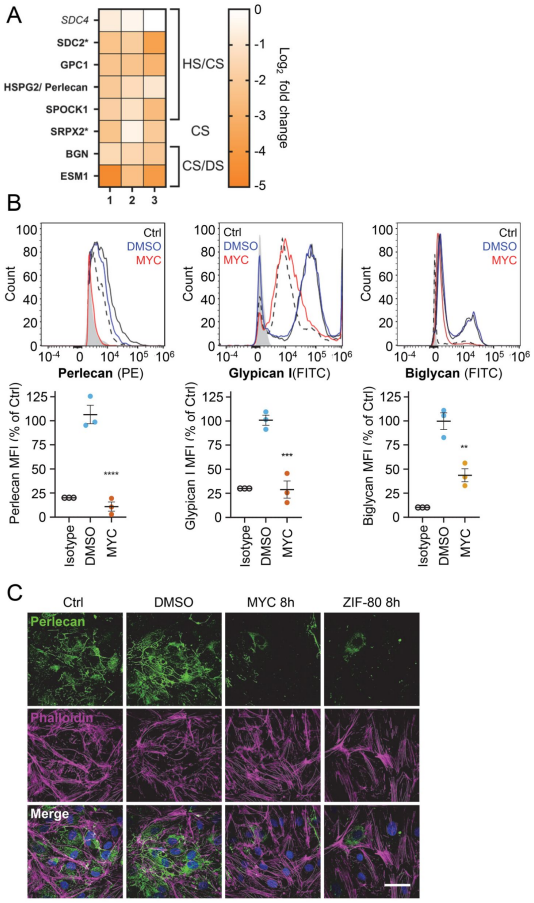

Figure 5.

Mycolactone causes a rapid loss of multiple proteoglycans.

HDMECs exposed to 10 ng/mL mycolactone (MYC), 0.02% DMSO or 20 nM ZIF-80 for 24 hours or indicated times. (**A**) Heatmap showing representative data for genes encoding proteoglycans. Dual-colour coding for log2 fold change in response to MYC is shown. Possible attached glycosaminoglycan chains such as heparan sulphate (HS), chondroitin sulphate (CS) or dermatan sulphate (DS) Candidates with one unique peptide detected indicated with asterisks, significantly downregulated ($p < 0.05$) or not ($p \geq 0.05$) in bold or *Italic* respectively. (**B**) Cells were harvested for flow cytometry analysis. Histogram plots for single cell population of perlecan, glypican-1 and biglycan. Unstained untreated cells, filled grey; isotype control of untreated cells, dashed black line. untreated cells stained with antibodies, black line; cells exposed to DMSO stained with antibodies, blue line; cells exposed to MYC stained with antibodies, red line. MFI is presented as a % of untreated control (mean ± SEM of 3 independent experiments). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (**C**) Cells were fixed and immunostained with anti-perlecan antibody (green), permeabilised and labelled with TRITC-conjugated phalloidin (magenta). Nuclei were stained with DAPI (blue). Images are representative of 3 independent experiments. Scale bar = 50 μm.

Using flow cytometry, we validated the changes in abundance of three cell surface proteoglycans; perlecan (HSPG2; secreted, HS/CS), glypican-1 (GPC1; GPI-anchored, HS/CS) and biglycan (BGN; secreted, CS/DS). Syndecan-2, a membrane-bound protein for which only one unique peptide was found in the proteome, could not be detected by flow cytometry. The most profound effects were seen for perlecan and glypican-1 (detection at 10.8±4.8% and 28.8±9.0% of untreated control, Fig 5B), while biglycan was partly reduced (43.7±6.8% of untreated control). As the turnover rate of HS proteoglycans is rapid ($t_{1/2}$= 3-4 hours in granulosa and 6.9 hours in macrophages (43; 44)), we explored the rate of perlecan and

glypican-1 loss at early time points in HUVECs. A ~50% reduction in perlecan was evident after only 2 hours mycolactone treatment, reaching significance at 6 hours. Depletion of glypican-1 was slower, evident at 6 hours and reaching significance at 24 hours (Fig S5A).

Immunofluorescence staining of HDMECs showed abundant perlecan staining in control cells, particularly around intercellular junctions, but the staining rapidly decreased in response to mycolactone, with reduced expression detectable after 8 hours (Fig 5C). HDMECs exposed to ZIF-80 for 8 hours displayed similarly limited perlecan-positive junctional staining (Fig 5C). The parallel loss of GAGs and the proteoglycans that bear them means that the glycocalyx is severely disrupted by mycolactone.

Mycolactone depletes endothelial basement membrane components and their ligands

Taken together, our results so far show that mycolactone profoundly depletes the endothelial glycocalyx, due to the loss of both GAG and proteoglycan biosynthesis following Sec61 inhibition. However, while loss of GAG production affected permeability, it had less impact on adhesion and migration. We therefore next focused on the downregulated proteins in our dataset with GO classifications linked to these processes. Numerous adhesion molecules and basement membrane components were downregulated by mycolactone, including nidogen 1 (NID1), laminins and collagens (Fig 6A). Although the abundance of major BM component collagen IV was not significantly influenced by mycolactone, perhaps indicating a slow turnover rate, several ER-localised and/or secreted enzymes involved in collagen biosynthesis (Table S3), were reduced as previously reported in murine fibroblasts (16). Laminins are the other key constituent glycoproteins of the BM and important binding partners for endothelial cell integrins. Our proteomic data suggested multiple laminins are affected by mycolactone. Laminin α4 and α5 are both common to all types of vessel wall, but α4 has a slightly higher turnover rate (45). By immunofluorescence staining, laminin α4 was seen in perinuclear regions within cells and in the network bridging intercellular junctions between endothelial cells in untreated and DMSO control HDMEC (Fig 6B). After 16 hours of exposure to mycolactone, the perinuclear staining was absent and the laminin-positive network between cells had become disconnected (Fig 6B). The same striking decrease was also seen in HDMECs exposed to ZIF-80 (Fig 6B).

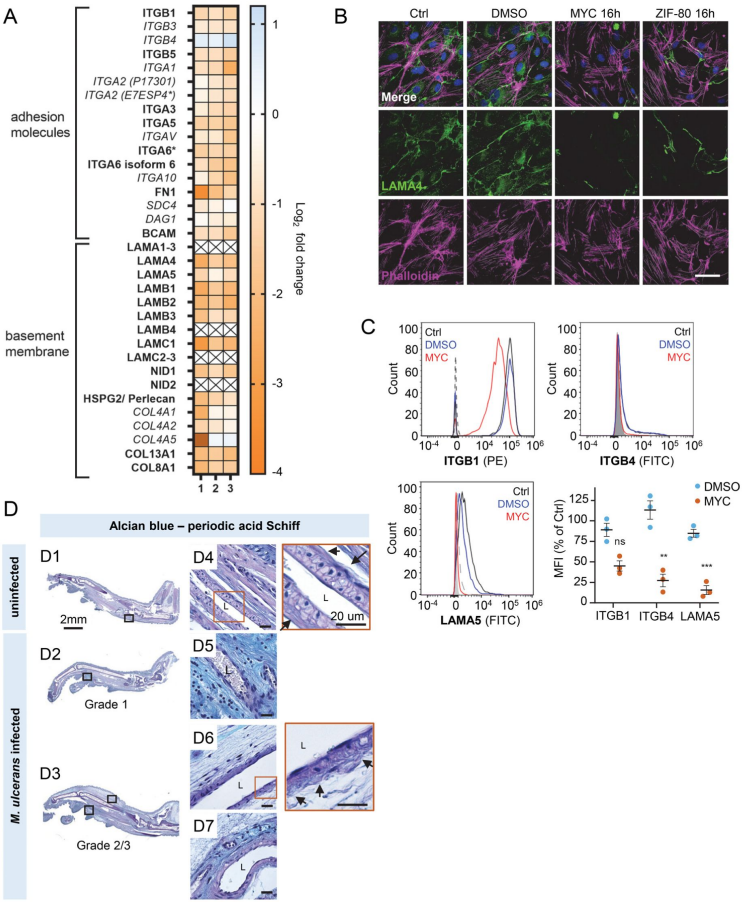

Figure 6.

Mycolactone interferes with the interaction between endothelial surface receptors and the basement membrane.

HDMECs exposed to 10 ng/mL mycolactone (MYC), 0.02% DMSO or 20 nM ZIF-80 for 24 hours or indicated times. (**A**) Heatmap showing representative data for genes encoding junctional or adhesion molecules, basement membrane components and proteins involved in platelet adhesion. Dual-colour coding for log2 fold change in response to MYC is shown. Candidate with one unique peptide detected is indicated with asterisks, significantly down-regulated ($p < 0.05$) or not ($p \geq 0.05$) in bold or *Italic* respectively. (**B**) Cells were fixed and immunostained with anti-laminin α4 antibody (green), permeabilised and labelled with TRITC-conjugated phalloidin (magenta). Nuclei were stained with DAPI (blue). Images are representative of 2 independent experiments. Scale bar = 50 μm. (**C**) Cells were harvested for flow cytometry analysis. Histogram plots for single cell population of integrin β1, integrin β4 and laminin α5. Unstained, untreated cells, filled grey; isotype control of untreated cells, dashed black line. untreated cells stained with antibodies, black line; cells exposed to DMSO stained with antibodies, blue line; cells exposed to MYC stained with antibodies, red line. MFI is presented as a % of untreated control (mean ± SEM of 3 independent experiments). ns, not significant; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (**D**) Alcian blue-periodic acid Schiff stain in the feet of C57BL/6 mice that received vehicle control (PBS) (D1, D4) or intradermal injection of $1*10^5$ colony forming units *M. ulcerans* at 21 (Grade 1; D2, D5) or 28 days (Grade 2/3;I3, D3, D6-7) post-infection. Neutral glycans are indicated by purple staining and acidic glycans by light blue Blood vessel lumens are indicated by an "L". Scale bars in D1-3: 2 mm; all others: 20 μm.

The effect of mycolactone on the abundance of the laminin binding integrin β subunits β1 and β4 and laminin α5 in HDMEC were determined by flow cytometry (Fig 6D). After 24 hours, they were reduced to 45.0±6.2%, 27.3±7.7% and 15.6±5.4% respectively of control levels (Fig 6C). In addition, the loss of expression of the basement membrane component fibronectin and cell surface integrin α5 were validated using immunoblot analysis; fibronectin levels decreased very rapidly showing >75% depletion after 4 hours exposure to mycolactone ($p<0.01$) (Fig S6A) whilst the level of integrin α5 decreased more slowly, reaching ~50% of control levels at 24h ($p<0.01$; Fig S6B).

To determine whether the basement membrane was disrupted *in vivo*, we stained the tissue sections from *M. ulcerans*-infected mice with the alcian blue-periodic acid Schiff (AB-PAS) method. In mouse feet receiving the vehicle control (Fig 6D1), the dermis contained neutral glycans (purple staining) and the vasculature displayed an intact vessel basement membrane (Fig 6D4, insert). At early stages of infection (Grade 1; Fig 6D2), immune cell infiltration could be seen in these regions in proximity to mycobacterial clusters (Fig S1A)

and the surrounding dermal tissue had become more acidic (blue staining) (Fig 6D5). At later stages of infection, when the metatarsal area was more swollen (Fig 6D3) and the dermis showed marked oedema and the fibrous architecture was disrupted (Fig 6D6-7), there was an overall reduction in the intensity of staining around the vasculature and the vessel basement membranes were irregular (Fig 6D6, insert).

Exogenous laminin-α5 ameliorates mycolactone-driven cell detachment and impaired migration

Since laminins are secreted proteins, which are then deposited to form cell-associated extracellular matrix, we wondered whether exogenous provision of these molecules might protect mycolactone-exposed cells. We therefore coated tissue culture plates with laminin-111, -411 or -511, complexes that contain laminin β1γ1 in combination with laminins α1, α4 or α5 respectively. As expected (46), primary HDMECs efficiently re-attached to laminin-511-coated culture vessels, with very little reattachment to uncoated vessels (P = 0.0020, Fig S7A). Re-attachment laminin-411 or the non-endothelial specific laminin-111 was also observed albeit to a lesser extent (P = 0.1226 and 0.3365 compared to the uncoated wells, respectively). We then quantified the re-attachment of endothelial cells that had been pre-exposed to mycolactone for 24 hours compared to controls (Fig 7A). Remarkably, mycolactone-exposed cells re-adhered to specifically to laminin-511-(but not 411- or 111-) coated vessels with the same efficiency as controls (Fig 7A).

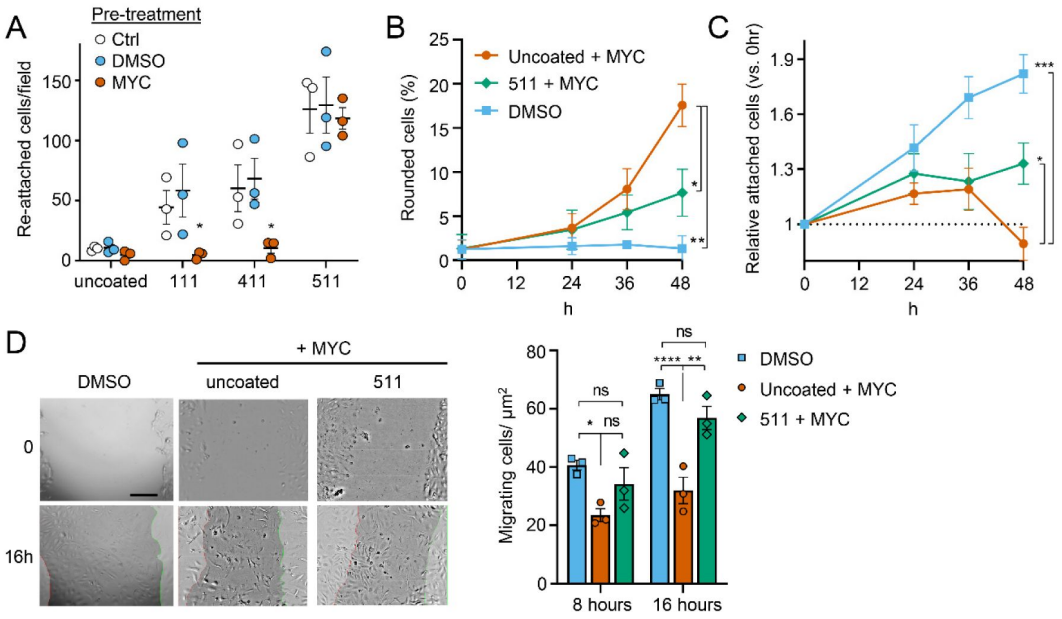

Figure 7.

Laminin α5 ameliorates mycolactone-driven endothelial cell detachment and impaired migration.

Endothelial cells exposed to 10 ng/mL mycolactone (MYC) or 0.02% DMSO for 24 hours or indicated times. **A**. Treated HDMECs were harvested, mixed with or without anti-integrin β1 blocking antibody and layered to laminin-511, 411, 111 or uncoated plates. After an hour, unbound cells were washed away and attached cells were imaged and cell numbers per field are presented as mean ± SEM of 3 independent experiments. **B-D**. Endothelial cells seeded onto laminin-511 or uncoated plates were exposed to mycolactone (MYC) or DMSO. (**B-C**) HDMECs

were imaged every 30 minutes over 48 hours. Rounded or attached cells per condition were counted at 0, 24, 36, 48 hours. Data are presented as a % of total cell number of each condition (**B**) or normalised to the attached cell number counted at 0 hours (**C**) (mean ± SEM of 3 independent experiments). (**D**) A scratch was introduced to a HUVEC monolayer prior to treatment. The wounded area was imaged every 15 minutes for 24 hours. Cells migrating into the original scratch area were counted at 0, 8 and 16 hours. Data are presented as cell count per scratch area (mean ± SEM of 3 independent experiments). ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

We then investigated whether exogenous laminin-511 could ameliorate the cell rounding, attachment or migration phenotypes observed in response to mycolactone using time-lapse imaging of HDMECs. On uncoated wells, mycolactone caused the expected phenotypic changes (Fig 8B-C), and remarkably, exogenous laminin α5 significantly reduced mycolactone-driven cell rounding, even after 48 hours (7.7±1.5% vs. 17.6±2.4%, $P = 0.0194$, Fig 8B). Similarly, while the relative number of attached cells did not increase steadily with time as for the DMSO control (Fig 8C), laminin-511 coating prevented the decrease in attached cells seen between 36 and 48 hours in uncoated wells ($P = 0.0156$). These effects were absent in laminin-411 and -111-coated wells (Fig S7B-C). Laminin coating did not impact HDMEC survival in the presence or absence of mycolactone at 48 hours (Fig S7D), although as mentioned before, cell death due to mycolactone is minimal prior to 72 hours (19).

For migration, we performed a scratch assay on HUVECs in wells coated or not with laminins prior to mycolactone exposure. Monitoring cell migration using time-lapse imaging revealed that control HUVECs took less than 16 hours to close a 600-800 μm gap (Video 3). By contrast, the leading edge of wounded HUVEC monolayers exposed to mycolactone gradually stopped migrating into the cell-free region after ~7 hours; at this point the cells began to migrate randomly before undergoing the previously described morphological changes (Video 4). However, strikingly, in HUVEC monolayers plated onto laminin-511, cells continued to migrate into the gap in the presence of mycolactone, with a leading edge still evident after 16 hours (Video 5). Cell counts per unit scratch area at 8 and 16 hours showed that cells plated onto laminin-511 were able to migrate back at a rate comparable to that seen in monolayers exposed to DMSO in uncoated wells (Fig 7D, $P = 0.286$). We did not see these same effects on laminin-411 and -111-coated wells (Video 6 and 7) where migration rates remained significantly lower than the control ($P = 0.0054$ and 0.0003, respectively, Fig S7E).

This ability of laminin α5 to reverse or diminish the impact of mycolactone on endothelial cell adhesion, morphology and migration highlights the contribution of the loss of basement membrane proteins to the phenotypic changes induced by mycolactone and presents an unanticipated potential for use in wound care in Buruli ulcer skin lesions, although such therapies are currently in their infancy (47).

Discussion

Until recently, the pathogenesis of BU was thought to rely on two factors; immunosuppression due to the action of mycolactone on innate and adaptive immunity, and direct cytotoxic action of mycolactone on the cells present within the subcutis leading to cell death and necrosis. Our findings provide further evidence supporting a third and vital pathway to tissue necrosis; the induction of endothelial dysfunction that drives an indirect

mechanism leading to tissue necrosis via the breakdown of vessel integrity and fibrin-driven ischemia within tissue.

The current work reaffirms the critical role that Sec61 inhibition plays in the virulence mechanism of mycolactone. In this post-transcriptional, co-translational mechanism responsible for changes in protein abundance, proteins are made in the wrong cellular compartment (the cytoplasm) and degraded by the ubiquitin-proteasome system or removed by autophagy (7; 15). During the current studies we tried, without success, to express examples of our library of SEC61A1 mutants that confer resistance to mycolactone (10; 17) in primary endothelial cells. This suggests that the endothelium is particularly sensitive to functional perturbation of the Sec61 translocon and perhaps explains why these cells are so exquisitely sensitive to the compound. As an alternative approach, we tested two analogues of the structurally unrelated Sec61 inhibitor Ipomoeassin F that was first isolated as a natural product of the “Morning Glory” flower (31). Across multiple readouts, this induced comparable phenotypes to mycolactone, in the same time frame, including changes in morphology, loss of GAGs, matrisome proteins required for their synthesis, proteoglycan core proteins and basement membrane proteins. Hence, we are confident that the primary target of mycolactone in endothelial cells is Sec61, as it has already been shown in immune cells (6; 7; 14), fibroblasts (15) and epithelial cells (17). Since epithelial cells showed similar effects on migration to those we report here, these effects probably depend on its action on the Sec61 translocon rather than other previously proposed mechanisms such as WASP activation (29).

Although the Sec61 translocon is thought to be required for the biosynthesis of approximately 30% of the proteome, mycolactone only inhibits production of specific subsets of proteins that traffic through the ER. As their depletion rate depends on protein turnover, inhibitory effects cannot be predicted *a priori*. Therefore, we performed quantitative proteomic analysis of total membrane fractions of primary endothelial cells to identify as many of the targets of mycolactone as possible, since “whole cell” approaches can bias against membrane proteins, particularly insoluble ones. Indeed, this approach was successful, more than doubling the number of detected proteins classified as membrane, secretory, ER/ERGIC, Golgi or endolysosomal compared to previous studies (12). Significant depletion of our previously discovered targets by candidate gene approaches (including loss of CDH5, TIE-1, TEK, JAM-C, CD31, vWF, TFPI and TM (19; 20), and induction of SQSTM1/p62 (15)) validates this data set. The pattern of protein topologies affected by mycolactone reflected that seen in *in vitro* translocation assays and whole cell proteome analysis (6; 11; 12; 16; 34), further supporting mycolactone selectivity towards secreted, Type I and Type II single pass membrane proteins, with few multi-pass proteins and no Type III or tail-anchored membrane proteins showing any reduction in expression.

GO analysis confirmed induction of cytoplasmic/oxidative stress responses (17; 39; 48) amongst >220 up-regulated processes in mycolactone-exposed endothelial cells. However, in this work we focussed on the >480 down-regulated proteins, which represented a striking inadequacy in components of glycoprotein biosynthesis and metabolism and ECM organization, many of which we have validated individually. Taking together the cellular compartment analysis and our understanding of mycolactone’s cellular target, we were able to correctly hypothesise that the effects on endothelial cell function were exacerbated by loss of Golgi-localised Type II transmembrane enzymes involved in GAG (CS, DS and HS) production. Up to now, the impact of mycolactone on Golgi function has been underappreciated, but the wider ranging effect on protein glycosylation may explain why mycolactone has such a strong effect on glycosylated protein production irrespective of topology (7). It also suggests that the effects of mycolactone may be even more far-reaching than expected, as even molecules resistant to the Sec61 blockade at the protein level may be functionally affected due to the loss of glycosylation.

Since GAG biosynthesis is a sequential process, and the endothelial glycocalyx is essential to maintain monolayer permeability (49), we reasoned that loss of one of the GAG linker-building enzymes common to CS, DS and HS could be sufficient to explain the mycolactone-induced phenotype. This was supported by siRNA-mediated knockdown of B3Galt6, which transfers galactose to substrates such as galactose-beta-1,4-xylose, i.e. the third step in this process. B3Galt6 knockdown phenocopied the elongated appearance seen in primary endothelial cells exposed to mycolactone. The intermediate phenotype seen in some experiments suggests that depletion of junctional molecules by mycolactone (20), also plays an important contributory role. In the context of BU, it is interesting to note that children born with 'linkeropathies', who have a reduced ability to synthesise GAG linker regions (50; 51), display phenotypes such as skin fragility and delayed wound healing (52) that are similar to antibiotic-treated *M. ulcerans* infections. As well as increasing permeability, the loss of the glycocalyx could exacerbate the inhibition of leukocyte homing caused by mycolactone (4). Notably other viral and bacterial pathogens promote colonisation by degrading the endothelial glycocalyx [(53)–(55)], however here the mechanism is via inducing the production of heparanase and other proteinases.

Importantly, it is not only the GAGs of the apical glycocalyx that are depleted by mycolactone. Many proteoglycan core proteins are also lost. The secretory protein perlecan is notable for being a component of the glycocalyx as well as the BM and was profoundly and rapidly lost from the surface of primary endothelial cells following mycolactone exposure. Other BM components, particularly laminins and their cellular receptors, were also found to be depleted. Excitingly, providing an exogenous coating of laminin α5-containing laminin-511 complex to tissue culture wells protected endothelial cells from mycolactone-driven changes, improving adhesion, and reversing the migration defect. We have not been able to ascribe this to the retention of a specific adhesion molecule, and instead postulate that rescue could be via residual expression of a wide variety of laminin α5 receptors. This is supported by previous work showing that laminin α5 is more promiscuous that laminin α4 (46).

Adequate adhesion to the BM is critical for endothelial cell proliferation, migration, morphogenesis and survival (22). Furthermore, loss of perlecan and laminin α4, or reduced binding to fibronectin, disturbs the structural integrity and maturation of microvessels [(56)–(58)], Finally, laminin α5 not only guides tissue patterning (59) and development (60) but also maintains vascular homeostasis by stabilising endothelial cell tight junctions (28). Therefore, it is perhaps not surprising that we found the BM to be disturbed in *M. ulcerans* infected footpads. Moreover, this was seen in more advanced infections where fibrin deposition was also present within tissue, due to disturbance of the boundary between damaged vessels and dermal connective tissue. It is possible that these effects are exacerbated by IL-1β *in vivo*; this Sec61-independent pro-inflammatory cytokine has been shown to be induced in macrophages by mycolactone and *M. ulcerans* (61; 62) and is known to have profound effects on endothelial cell function, including the downregulation of anticoagulant and junctional proteins, induction of vascular permeability and upregulation of BM degrading proteinases (63). There is considerable overlap in the endothelial cell responses to IL-1β and mycolactone, although the former's effects are mediated predominately at the transcriptional level. An additive effect of mycolactone has been shown for some of these phenotypes *in vitro* (19; 20) although the *in vivo* situation is likely more complex (62)

In summary, this study identifies loss/disruption of the endothelial glycocalyx and BM as a critical molecular process in the pathogenesis of Buruli ulcer. Since these changes occur prior to mycolactone-driven apoptosis [(17)–(19)], they provide further support for our working model whereby mycolactone builds a hyper-coagulative environment alongside disruption of the endothelial monolayer and BM integrity. We propose that this leads to

leakage of high molecular weight plasma proteins into the connective tissue where they activate the coagulation cascade leading to fibrin deposition and tissue ischemia. The detection of extravascular fibrinogen at early stages of infection prior to widespread tissue damage and necrosis provides further evidence that endothelial dysfunction could be a driver of disease progression. Rethinking of BU as a vascular disease may ultimately lead to improved therapies that support better wound healing, alongside antibiotic treatment. However, it should be remembered that tissue repair requires a controlled progression through a series of different stages (64); following injury, under normal circumstances, platelet accumulation in a fibrin and fibronectin rich matrix is followed by an inflammation phase (65). Therefore, ameliorating the coagulative features with anticoagulants alongside the standard antimycobacterial drugs may be of most value in the initial stages of treatment, while bioactive dressings containing laminin-derived peptides might be more useful to promote healing at later stages. In this context, laminin-derived bioactive peptides have recently been proposed as a treatment for defective tissue repair (47) and indeed, accelerate re-epithelialisation in wounds of diabetic animals (66; 67), suggesting this novel approach may be an effective complement to current therapies and could alleviate the long wound healing times experienced by BU patients.

Materials and methods

Mycolactone and other translocation inhibitors

For all experiments, we used synthetic mycolactone A/B (68), which was generously donated by Prof. Yoshito Kishi (Harvard University). Ipomoeassin F and ZIF-80 (Compound 2 in ref 30) were synthesised by Wei Shi. All compounds were diluted from stock solutions in DMSO and were used at the minimal inhibitory concentration, which was 10 ng/ml (~13 nM) mycolactone (15), 400nM Ipomoessin F (69) and 20nM ZIF-80 (30). To control for potential impact of the DMSO solvent on cell function, DMSO diluted equivalently was used; typically this was 0.02%.

Cell culture and treatment

Juvenile, single donor human microvascular endothelial cells (HDMEC) and human umbilical vein endothelial cells (HUVEC) (Promocell) were cultured in hVEGF containing Endothelial cell growth medium 2 (Promocell) at 37°C and 5% CO_2. Cells were routinely seeded at a concentration of 1 x $10^4/cm^2$ in $25cm^2$ or $75cm^2$ flasks for no more than 15 population doublings. Where used, laminin-511, -411 or -111 (BioLamina, Sweden) were coated on the surface of uncoated 96-well tissue culture plates at 5 µg/mL in PBS at 4°C overnight, then washed with medium prior to further experiment.

Time-lapse imaging of live cells

For time-lapse monitoring, endothelial cells were imaged every 30 minutes using a zenCELL Owl incubator microscope (innoME GmbH, supplied by LabLogic UK) for 48 hours. Time-lapse videos were generated with zencell-owl software (version 3.3, innoME GmbH), and analysed using their built-in algorithms of relative cell coverage, proportion of detached cells, and total cell numbers. In some cases, images of cells from certain time points were further analysed in Image J (v1.52n) to cell count of rounded cells per field, and/or the proportion and length/width ratio of elongated cells

Scratch assay

Endothelial cells were grown to confluency in 24 well plates then single lines were scratched into the monolayer using a p20 pipette tip. Healing of HDMECs was monitored by imaging at various time points up to 24 hours. Each assay was carried out in triplicate wells. Wounded HUVECs were monitored every 15 minutes by zenCELL Owl microscope (innoME GmbH) for up to 30 hours.

Mycobacterium ulcerans mouse footpad infection model

Mycobacterium ulcerans strain Mu_1082 was cultivated on Middlebrook 7H11 agar (Merck) supplemented with 0.2% glycerol (ThermoFisher Scientific) and 10% OADC (ThermoFisher Scientific). Several days before inoculation, bacteria were scraped from the plate and resuspended in 10ml 7H9 medium (Becton Dickinson) containing 0.5% glycerol, 10% OADC and 0.2% Tween-80 (Merck) and incubated shaking with 3µm glass beads for 3 days at 31°C. To prepare the inoculum, cultures were allowed to stand for 10min (to allow clumps to settle) then 1ml culture was centrifuged at 13,000 x g for 2min. The supernatant was removed, and the pellet resuspended in Dulbecco's PBS (Fisher Scientific). After measuring the OD600, 3.33 x 10^7 bacteria were pelleted and resuspended in 10ml PBS, to give an inoculum of ~10^5 cfu/footpad in a volume of 30µl.

All *in vivo* procedures were approved by the local ethics committee and UK Home office and met relevant animal welfare and biosafety regulatory standards. In this publication we present new histological analysis of archived material from eight to nine-week-old C57BL/6J female mice (Charles River, UK), which had been inoculated intradermally with 30 µl of the bacterial suspension or vehicle control (PBS) to the left hind footpad. Mice were maintained under specific pathogen-free conditions at a temperature of 20–24 °C and humidity of 45 to 65% in individually HEPA filtered cages. The mice had free access to water and a standard balanced diet, standard bedding and enrichments including a tunnel and nesting material. Infected mice were housed separately from uninfected mice, and blinding was not possible as the infection is clearly visible. Infection grade was assessed daily according to the method of Converse (70), where Grade 1 showed swelling of the metatarsal area (<50% increase compared to normal), Grade 2 showed greater swelling (50-150%) and Grade 3 had swelling further up the leg, visualised at the hock. Mice were killed by a schedule 1 method (cervical dislocation). The whole foot was then removed and fixed by immersion in 10% neutral buffered formalin for at least 24 hours.

Histological analysis of murine foot samples

Fixed murine feet were decalcified using the EDTA-based Osteosoft solution (Merck) and then embedded in paraffin for histological analysis.by Ziehl-Neelsen stain, Alcian blue-periodic acid Schiff stain, and immunohistochemistry (IHC) for fibrin(ogen). For IHC staining, 5-µm tissue sections on polylysine-coated slides were deparaffinised, endogenous peroxidase quenched, epitope unmasked with heated IHC citrate buffer (pH 6.0) (Merck) and blocked with 5% bovine serum albumin. The tissue sections were incubated with anti-fibrinogen antibody (A0080, DAKO) or matched isotype control overnight at 4°C. Staining was then performed with biotinylated horse anti-rabbit IgG (Vector Laboratories) and VECTASTAIN Elite ABC kit and ImmPACT NovaRED peroxidase substrate and further counterstained with Harris Haematoxylin (ThermoFisher Scientific). Whole slide images were captured using the NanoZoomer slide scanner (Hamamatsu Photonics) and analysed using ImageScope software (Leica Biosystems) and ndp2.view software (Hamamatsu). Some

photographs were taken with Micropix microscope camera (acquisition software Cytocam) attached to a Yenway CX40 laboratory microscope (Micropix).

Membrane protein preparation

HDMEC (1 x 10^7 cells) were seeded onto 15cm dishes (Corning) and grown to 90% confluency then exposed to solvent carrier DMSO or 10ng/ml mycolactone for 24 hours. Cells were washed four times in PBS and once in lysis buffer (10mM Tris pH 7.5, 250mM Sucrose, protease inhibitor cocktail). Cells were incubated for 20min on ice in 10ml lysis buffer then lysed in by 20 strokes dounce homogenisation. Lysates were centrifuged at 1,000x*g* for 10min at 4°C then the post-nuclear supernatant was centrifuged at 100,000x*g* for 1 hour at 4°C. Pellets were resuspended in 110 µl lysis buffer. Protein concentration was determined by BCA assay and 50 µg aliquots were subjected to acetone precipitation. Triplicate samples were prepared from 3 independent assays.

Proteomics

Acetone precipitated proteins were reduced, alkylated and digested with trypsin before 9-plex isobaric TMT labelling according to the manufacturer's protocol (https://www.thermofisher.com/document-connect/document-connect.html? url= https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0016969_2162457_TMT10plex_UG.pdf). Labelled peptides were separated by high pH reverse phase liquid chromatography, collecting 20 fractions which were then lyophilised, desalted and analysed by LC-MS/MS. TMT labelled samples were analysed by the SPS-MP3 method using an Orbitrap Lumos mass spectrometer. Spectra were searched using the Mascot search engine version (Matrix Science) and analysed using the Proteome Discovery platform. (Version 2.2ThermoFisher Scientific). NA values and low confidence proteins were removed, and data was normalised using each channel median. Differential expression analysis was carried out using Limma. Adjusted p values were calculated by the Benjamini-Hochberg method. UniProt and the Human Protein Atlas (https://www.proteinatlas.org) were used to determine protein location and characteristics. Over-representation of GO groups was assesses using Webgestalt (www.webgestalt.org(http://www.webgestalt.org/)) (ref). Signal peptide ΔG values were obtained via the ΔG Prediction Server V1.0 (https://dgpred.cbr.su.se). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (71) partner repository with the dataset identifier PXD037489.

siRNA transfection

Transfections of siRNA (assay ID#112321, #112322 for B3GALT6 or Silencer negative control No.1 siRNA AM4611) diluted to give a final concentration of 50 nM in Opti-MEM (Gibco) were performed on HUVECs at 30-40% confluency using Escort IV transfection reagent (L3287, Merck). After 48 hours, transfectants were subjected to the respective *in vitro* assays.

Flow cytometry

Flow cytometry was carried out according to standard methods as described in (19) using an Attune NxT flow cytometer (ThermoFisher Scientific). Cells were detached with non-enzymatic cell dissociation solution (Merck) or briefly (for Itgb4 staining only) trypsinised with 0.04% trypsin/ 0.03% EDTA (PromoCell). For surface GAG detection, cells were treated with 1mU of heparinase III (EC4.2.2.8 from *Flavobacterium heparinum*) to expose neo-epitope of heparan sulphate or with chondroitinase ABC (EC 4.2.2.4 from *Proteus vulgaris*) (AMS Biotechnology) for 1h at 37°C prior to the staining procedures. Antibodies were Δ-HS (F69-

3G10, AMS Biotechnology), CS (CS56, Merck), perlecan (7B5, ThermoFisher Scientific), glypican-1 (AF4519), integrin β4/ CD104 (clone 439-9B, eBioscience), integrin β1/ CD29 (P4C10, NBP2-36561), syndecan-2 (MAB2965), biglycan (AF2667), laminin α5 (NBP2-42391) from Biotechne. Isotype control mouse IgG1 (P3.6.2.8.1; 14-4714-81 from Invitrogen), mouse IgG2b (MG2B00), goat IgG (AB-108-C from R&D), rat IgG2b (14-4031-81), mouse IgM (PFR-03) and fluorophore-conjugated secondary antibodies goat anti-mouse IgG PE (12-4010-82), donkey anti-goat IgG FITC (A16000) and anti-rat IgG FITC (31629) were from ThermoFisher Scientific. The main population was gated by forward and side scatter plot of untreated cells using FlowJo (v9); among this, single cell population of 10^4 cells per condition was subjected to analysis. Mean fluorescence intensity was determined and presented as % relative to untreated control.

Immunoblotting

Immunoblotting was carried out according to standard methods as described in (19). Endothelial cells were lysed either in RIPA buffer (where protein content-equalised post-nuclear fractions were used) or directly in 'gel sample buffer' (with sonication to degrade genomic DNA). Immunoblotting of commercial pre-cast gels (BioRad) used either Immobilon PVDF membranes (Merck) or nitrocellulose membrane (GE Healthcare). Antibodies used in this study were: Δ-HS (F69-3G10, AMS Biotechnology); anti-fibronectin (AB1945, Merck); anti-integrin α5 (sc-166665); anti-rabbit-HRP (GE Healthcare, NA934V), anti-mouse-HRP (GE Healthcare, NA931V). To visualise HS neoepitope, protein lysate was digested with 1 mU of heparinase III (EC4.2.2.8 from *Flavobacterium heparinum*) prior to SDS-PAGE.

Immunofluorescence

Immunofluorescent imaging was carried out according to standard methods as described in (15). Cells were fixed with 4% paraformaldehyde in PBS. For visualising intracellular markers, cells were permeabilised wit 0.25% Nonidet P-40 alternative in NETGEL buffer (150 mM NaCl, 5mM EDTA, 50 mM Tris-Cl, pH 7.4, 0.05% Nonidet P-40 alternative, 0.25% gelatin and 0.02% sodium azide). Antibodies used in this study were: B3Galt6 (H00126792-B01P, Biotechne), Giantin (ab80864, abcam), Laminin α4 (AF7340, Biotechne), Δ-HS (F69-3G10, AMS Biotechnology), perlecan (7B5, ThermoFisher Scientific), TRITC-conjugated phalloidin (FAK100, Merck), Alexa Fluor 594 goat anti-rabbit (A11012), Alexa Fluor 488 donkey anti-mouse (A21202) and Alexa Fluor 488 donkey anti-sheep (A11015) from Invitrogen/ThermoFisher Scientific. For B3Galt6 intensity in Golgi apparatus, the region of interest per cell was defined by giantin-positive staining using ImageJ selection tools. The integrated density of B3Galt6 fluorescence of selected regions and background reading were then measured and the difference between the two numbers were corrected total cell fluorescence.

Vascular permeability assay

Endothelial cells were seeded on hanging cell culture inserts containing 1 μm pores with a polyethylene terephthalate membrane (Falcon). Treatment as above or with 100 ng/mL IL-1β (Gibco) were applied to both the insert and receiver wells. After indicated time, fluorescein isothiocyanate (FITC)-conjugated dextran (70 kDa, Millipore) was applied to each insert for 20 minutes. The fluorescence intensity of the solution in the receiver wells was then assessed by a fluorescent plate reader (FLUOstar Omega, BMG Labtech) with excitation/ emission wavelength at 485/ 530 nm. Fluorescence intensity was normalised to untreated control wells with an intact monolayer of endothelial cells and expressed as a % of subtracted value obtained from wells where no cells were seeded to the insert.

Adhesion assay

HDMECs were harvested, incubated with anti-integrin β1 (clone P4C10, NBP2-36561, Biotechne) or isotype control mouse IgG1 (P3.6.2.8.1; 14-4714-81 from Invitrogen) for 5 minutes, then 1.5 x 10^4 cells were added to the wells of a 96 well plate that had been coated or not with different laminins as described above. After 1 hour, each well was washed three times with serum free medium and attached cells were imaged with a digital microscope camera (Micropix) attached to an AE31E inverted microscope (Motic). The cell count per image was determined using ImageJ.

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We are extremely grateful to Prof Yoshito Kishi (Harvard University, USA) for the gift of synthetic mycolactone A/B. We dedicate this paper to the memory of this tremendous scientist who did some much to advance research into the pathogenesis of Buruli ulcer. We thank Prof Richard Phillips (Kumasi Centre for Collaborative Research in Tropical Medicine) who provided the *M. ulcerans* strain used in this work. We thank Katherine Walker and Ella May of the University of Surrey's Veterinary Pathology Centre for their assistance with tissue processing and staining of murine samples. This work is supported by a Wellcome Trust Investigator Award in Science to Prof Rachel Simmonds (WT202843/Z/16/Z). Chemical synthesis of Ipomoeassin F and ZIF-80 was supported by an AREA grant GM116032 from the National Institute of General Medical Sciences of the National Institutes of Health (NIH) to Prof Wei Q Shi.

Additional Files

Supplementary File 1

Supplementary figures (found at the end of this PDF).

Supplementary File 2

HDMEC membrane fraction raw peptide data.

Supplementary File 3

Normalised differential expression analysis for membrane fractions of HDMEC incubated for 24 hours with DMSO vs Mycolactone

Supplementary File 4

HDMEC membrane fraction proteins significantly downregulated by mycolactone.

Supplementary File 5

HDMEC membrane fraction proteins significantly upregulated by mycolactone.

Video 1. HDMECs were exposed to 10 ng/mL mycolactone. Live cell imaging was performed with using the zenCELL Owl incubator microscope every 30 minutes for 48 hours. Time-lapse videos (representative of 3 independent experiments) were generated with zencell-owl software. Time stamp and scale bar as indicated.

Video 2. HDMECs were exposed to 0.02% DMSO. Live cell imaging was performed with using the zenCELL Owl incubator microscope every 30 minutes for 48 hours. Time-lapse videos (representative of 3 independent experiments) were generated with zencell-owl software. Time stamp and scale bar as indicated.

Video 3. A scratch was introduced to HUVEC monolayer exposed to 0.02% DMSO and live cell imaging was performed with the zenCELL Owl incubator microscope every 15 min for 23 hours. Time-lapse videos (representative of 3 independent experiments) were generated with zencell-owl software. Time stamp and scale bar as indicated.

Video 4. A scratch was introduced to HUVEC monolayer exposed to 10 ng/mL mycolactone and live cell imaging was performed with the zenCELL Owl incubator microscope every 15 min for 23 hours. Time-lapse videos (representative of 3 independent experiments) were generated with zencell-owl software. Time stamp and scale bar as indicated.

Video 5. HUVECs were seeded on to laminin-511 coated well. The following day, a scratch was introduced to the monolayer and the cells were exposed to 10 ng/mL mycolactone. Live cell imaging was then performed with the zenCELL Owl incubator microscope every 15 min for 23 hours. Time-lapse videos (representative of 3 independent experiments) were generated with zencell-owl software. Time stamp and scale bar as indicated.

Video 6. HUVECs were seeded on to laminin-411 coated well. The following day, a scratch was introduced to the monolayer and the cells were exposed to 10 ng/mL mycolactone. Live cell imaging was then performed with the zenCELL Owl incubator microscope every 15 min for 23 hours. Time-lapse videos (representative of 3 independent experiments) were generated with zencell-owl software. Time stamp and scale bar as indicated.

Video 7. HUVECs were seeded on to laminin-111 coated well. The following day, a scratch was introduced to the monolayer and the cells were exposed to 10 ng/mL mycolactone. Live cell imaging was then performed with the zenCELL Owl incubator microscope every 15 min for 23 hours. Time-lapse videos (representative of 3 independent experiments) were generated with zencell-owl software. Time stamp and scale bar as indicated.

Supplementary Figures

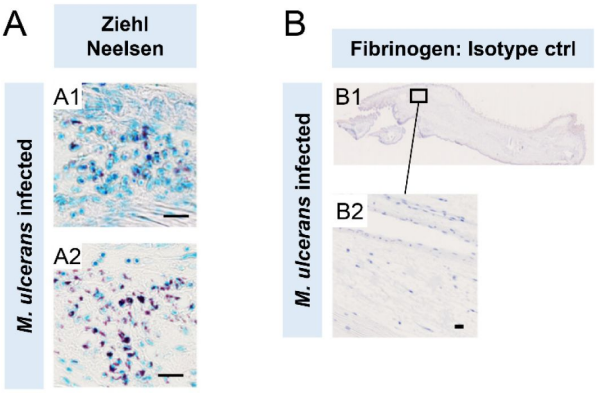

Figure S1.

Profile of Buruli ulcer mouse footpad model.

C57BL/6 mice receiving 1*10^5 colony forming units *M. ulcerans* Mu_1082 strain injected intradermally into the footpad were sacrificed at 21or 28 days. Injected feet were fixed, decalcified and embedded in paraffin. (**A**) Sections stained for acid fast bacilli with Ziehl Neelson stain from mice infected for 21 days (A1) or 28 days (A2). At 21 days immune cell infiltration as well as apparently intracellular bacteria can be clearly seen in proximity to mycobacterial clusters (**B**) *M. ulcerans* mouse feet infected stained with isotype control antibody. Scale bars: 20 μm.

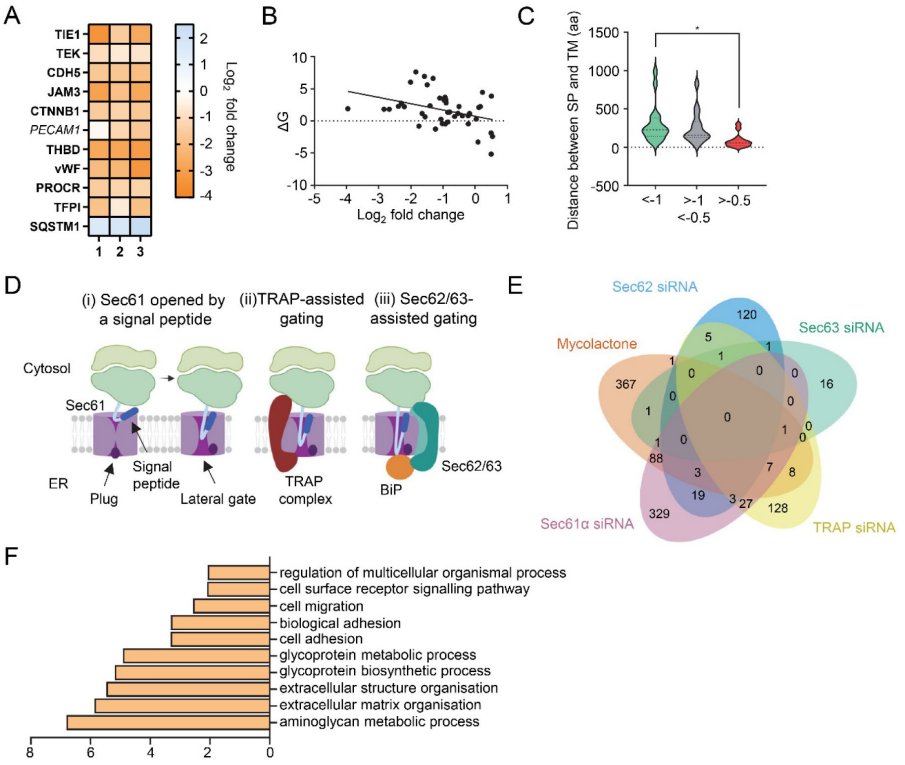

Figure S2.

Proteomic analysis reveals loss of proteins associated with glycosylation, adhesion and migration.

(**A**) Heat map showing fold change detected in this dataset for previously validated endothelial cell mycolactone targets (Ogbechi et al, 2015, Hsieh et al 2022). Dual-colour coding is shown., significantly downregulated ($p < 0.05$) or not ($p \geq 0.05$) in bold or *Italic* respectively. (**B**) Significant association between multipass protein membrane protein signal peptide (SP) ΔG values and level of downregulation by mycolactone ($p<0.05$). Membrane protein type and SP sequence based on Uniprot data, ΔG quantified using ΔG Prediction Server V1.0 (https://dgpred.cbr.su.se). (**C**). Impact of distance between signal peptide and first transmembrane domain on susceptibility of multipass proteins to downregulation by mycolactone. (**D**) Model depicting assisted and unassisted channel opening by signal peptides. (i) Binding of a nascent chain signal peptide to Sec61α opens a lateral gate into the membrane and causes a shift in the position of the plug domain, allowing access to the ER lumen.(ii) TRAP increases translocation of proteins whose signal peptides bear a high GP content. TRAP is a heterotetrameric complex which interacts with the ribosome on the cytosolic side of the ER membrane and with Sec61α on the luminal side, binding to a hinge region between the N- and C-terminal halves of the protein facilitating channel opening (12) (iii) The Sec62/63 complex is involved in post-translational translocation but can also assist opening of the channel for proteins with signal peptides that gate slowly due to the presence relatively long but less hydrophobic “H-regions” and lower carboxy terminal polarity

(3). This complex also interacts with the ribosome and the translocon and may also interact directly with the nascent peptide chain (4). In addition, Sec63 recruits BiP to the translocon to further assist channel opening on the luminal side (3). (**E**) Overlap between mycolactone-downregulated endothelial membrane proteome and translocon-dependent proteome. Venn diagram created using JVenn (5) showing overlap in significantly downregulated proteome between the dataset presented here and those obtained in Hela cells treated with siRNA for Sec61α and translocon associated protein TRAP or HEK293 cells with Sec62 or Sec623 knocked out (13). (**F**) Top significantly over-represented (p<0.05) Gene Ontology groups in upregulated data set, compared to whole genome. Data generated with WebGestalt.

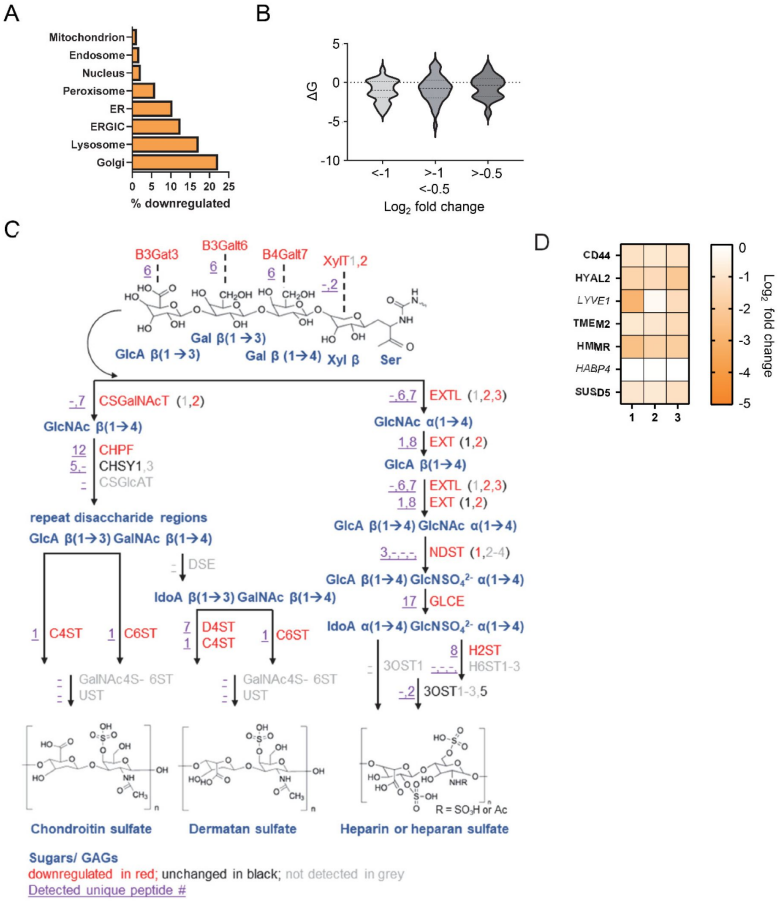

Figure S3.

Mycolactone targets Golgi proteins involved in glycosaminoglycan chain synthesis initiation.

HDMECs exposed to 10 ng/mL mycolactone (MYC) or 0.02% DMSO for 24 hours. **(A)** Downregulated intracellular proteins according to subcellular location, presented as percentage of total. **(B)** Impact of membrane anchor ΔG values on fold change in expression induced by mycolactone for all identified Type II membrane proteins. (**C**) Cartoon representing mycolactone-downregulated steps of GAG enzymatic synthesis. Each intermediate product is shown in blue and alongside with its responsible enzyme(s). Candidates significantly downregulated ($p < 0.05$) by mycolactone are shown in red, unchanged in black and undetected in grey. Unique peptide numbers detected in proteome are in purple (underlined).

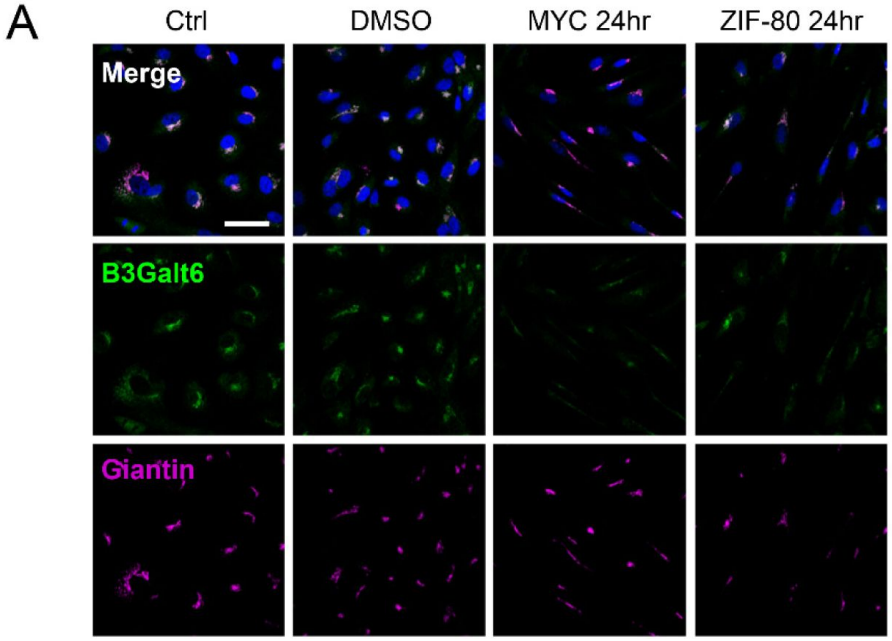

Figure S4.

Sec61 blockade suppresses B3Galt6 expression in endothelial cells.

(**A**) HDMECs exposed to 10 ng/mL of mycolactone (MYC), 0.02% DMSO, 20 nM ZIF-80 or remained untreated for 24 hours were fixed, permeabilised and immunostained with anti-B3Galt6 and anti-giantin antibodies and nuclei stained with DAPI. Images are representative of 2 independent experiments. Scale bar = 50 µm.

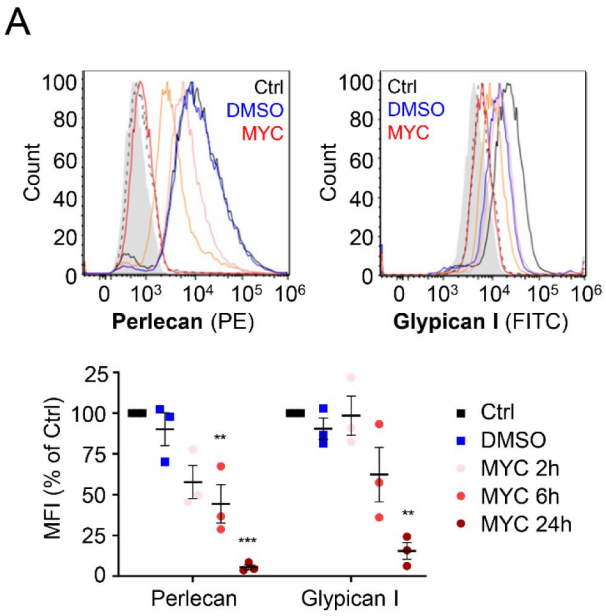

Figure S5.

Mycolactone's effect on endothelial surface proteoglycans.

HUVECs exposed to 10 ng/mL of mycolactone (MYC), 0.02% DMSO or remained untreated for indicated times. Cells were harvested for flow cytometry analysis. Histogram plots for single cell population of perlecan and glypican-1. Unstained, untreated cells, filled grey; isotype control of untreated cells, dashed black line. untreated cells stained with antibodies, black line; cells exposed to DMSO stained with antibodies, blue line; cells exposed to MYC for 2, 6, and 24 hours stained with antibodies, pink, orange and red line, respectively. MFI is presented as a % of untreated control (mean ± SEM of 3 independent experiments). ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.

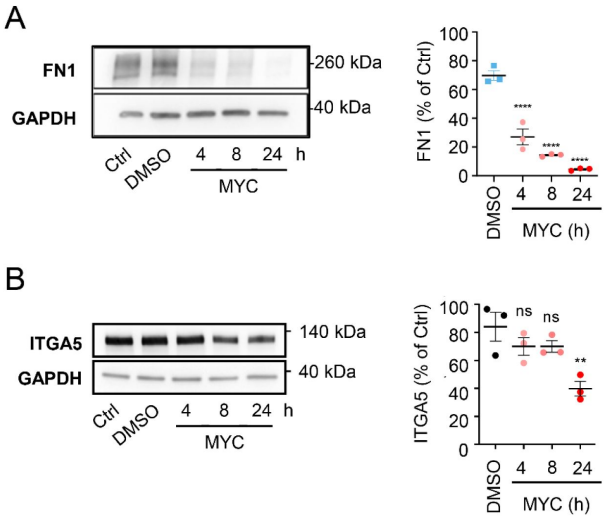

Figure S6.

Mycolactone impacts endothelial cell adhesion molecules.

HDMECs exposed to 10 ng/mL of mycolactone (MYC), 0.02% DMSO or remained untreated for indicated times. Cells were lysed and subjected to immunoblotting with anti-fibronectin (**A**) and anti-integrin α5 (**B**) antibodies. Each immunoblot intensity was normalised according to GAPDH and untreated controls. Data from 3 independent experiments are presented (mean ± SEM). ns, not significant; **, $P < 0.01$; ****, $P < 0.0001$.

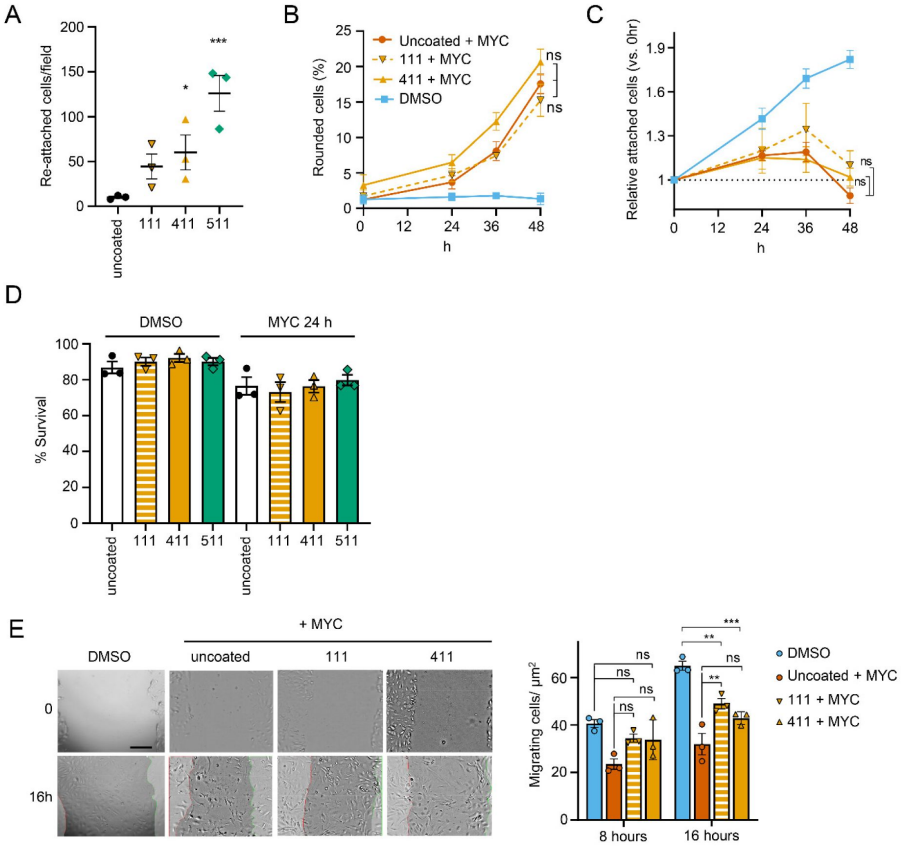

Figure S7.

Endothelial cell adhesion to various laminin isoforms.

(A) HDMECs were harvested and layered to laminin-511, 411, 111 or uncoated wells for one hour. Non-adherent cells were washed away and attached cells per field were counted. mean ± SEM (n = 3). **(B)** HDMECs seeded onto different laminin isoforms were untreated or exposed to 0.02% DMSO or 10 ng/mL mycolactone (MYC) for 48 hrs. Their viability was assayed using CellEvent detection kit as described in (Ogbechi et al, 2018). The number of live cells (negative for both active caspase 3/7 and PI) in three fields was determined and expressed as a proportion of total cells (Mean ± SEM, $n = 3$ independent experiments). ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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Reviewer #1 (Public Review):

The authors have investigated the effect of the toxin mycolactone produced by mycobacterium ulcerans on the endothelium. Mycobacterium ulcerans is involved in Buruli ulcer classified as a neglected disease by WHO. This disease has dramatic consequences on the microcirculation causing important cutaneous lesions. The authors have previously demonstrated that endothelial cells are especially sensitive to mycolactone. The present study brings more insight into the mechanism involved in mycolactone-induced endothelial cells defect and thus in microcirculatory dysfunction. The authors showed that mycolactone directly affected the synthesis of proteoglycans at the level of the golgi with a major consequence on the quality of the glycocalyx and thus on the endothelial function and structure. Importantly, the authors show that blockade of the enzyme involve in this synthesis (galactosyltransferase II) phenocopied the effects of mycolactone. The effect of mycolactone on the endothelium was confirmed in vivo. Finally, the authors showed that exogenous laminin-511 reversed the effects of mycolactone, thus opening an important therapeutic perspective for the treatment of wound healing in patients suffering Buruli ulcer and presenting lesions.

Reviewer #2 (Public Review):

The authors dissected the effects of mycolacton on endothelial cell biology and vessel integrity. The study follows up on previous work by the same group, which highlighted alterations in vascular permeability and coagulation in patients with Buruli ulcer. It provides a mechanistic explanation for these clinical observations, and suggests that blockade of Sec61 in endothelial cells contributes to tissue necrosis and slow wound healing.

Overall, the generated data support their conclusions and I only have two major criticisms:

- Replicating the effects of mycolactone on endothelial parameters with Ipomoeassin F (or its derivative ZIF-80) does not demonstrate that these effects are due to Sec61 blockade. This would require genetic proof, using for example endothelial cells expressing Sec61A mutants that confer resistance to mycolactone blockade. The authors claimed in the Discussion that they could not express such mutants in primary endothelial cells, but did they try expressing mutants in HUVEC cell lines? Without such genetic evidence all statements claiming a causative link between the observed effects on endothelial parameters and Sec61 blockade should be removed or rephrased. The same applies to speculations on the role of Sec61 in epithelial migration defects in discussion. Data corresponding to Ipomoeassin F and ZIF-80 do not add important information, and may be removed or shown as supplemental information.
- While statistical analysis is done and P values are provided, no information is given on the statistical tests used, neither in methods nor results. This must be corrected, to evaluate the repeatability and reproducibility of their data.

Reviewer #3 (Public Review):

Buruli ulcer is a severe skin infection in humans that is caused by a bacterium, Mycobacterium ulcerans. The main clinical sign is a massive tissue necrosis subsequent to an edema stage. The main virulence factor called mycolactone is a polyketide with a lactone core and a long alkyl chain that is released within vesicles by the bacterium. Mycolactone was already shown to account for several disease phenotypes characteristic of Buruli ulcer,

for instance tissue necrosis, host immune response modulation and local analgesia. A large number of cellular pathways in various cell types was reported to be impacted by mycolactone. Among those, the Sec61 translocon involved in the transport of certain proteins to the endoplasmic reticulum was first identified by the authors of the study and is currently the most consensual target. Mycolactone disruption of Sec61 function was then shown to directly impact on cell apoptosis in macrophages, limited immune responses by T-cells and increased autophagy in dermal endothelial cells and fibroblasts. In their manuscript, Tzung-Harn Hsieh and their collaborators investigated the Sec61- dependent role of mycolactone on morphology, adhesion and migration of primary human dermal microvascular endothelial cells (HDMEC). They used a combination of sugar and proteomic studies on a live image-based phenotypic assay on HDMEC to characterize the effect of mycolactone. First, they showed that upon incubation of monolayer of HDMEC with mycolactone at low dose (10 ng/mL) for 24h, the cells become elongated before rounding and eventually detached from the culture dish at 48h. Next, mycolactone was probed on a scratch assay and migration of the cells ceased upon a 24h incubation. The same effect as mycolactone on these two assays was observed for two other Sec61 inhibitors Ipomoeassin F and ZIF-80. Then, the authors resorted to the widely established mouse footpad model of M. ulcerans infection to evidence fibrinogen accumulation outside the blood vessel within the endothelium at 28 days post-infection, correlating with severe endothelial cell morphology changes.

To dissect the molecular pathways involved in these phenotypes, the authors performed an HDMEC membrane protein analysis and showed a decrease in the numbers of proteins involved in glycosylation and adhesion. As protein glycosylation mainly occurs in the Golgi apparatus, a deeper analysis revealed that enzymes involved in glycosaminoglycan (GAG) synthesis were lost in mycolactone treated HDMEC. A combination of immunofluorescence and flow cytometry approaches confirmed the impact of mycolactone on the ability of endothelial cells to synthesize GAG chains. The mycolactone effect on cell elongation was phenocopied by knock-down of galactosyltransferase II (B3Galt6) involved in GAG biosynthesis. A second extensive analysis of the endothelial basement membrane component and their ligands identified multiple laminins affected by mycolactone. Using similar functional studies as for GAG, the impact of mycolactone on cell rounding and migration could be reversed by the addition of laminin α5.

The major strengths of the study relies on a combination of cleverly designed phenotypic assays and in-depth cleverly designed membrane proteomic studies and follow-up analysis. The results really support the conclusions. Congratulations!
The discussion takes into account the current state of the art, which has mostly been established by the authors of the present manuscript.

Author Response

Reviewer #2 (Public Review):

The authors dissected the effects of mycolacton on endothelial cell biology and vessel integrity. The study follows up on previous work by the same group, which highlighted alterations in vascular permeability and coagulation in patients with Buruli ulcer. It provides a mechanistic explanation for these clinical observations, and suggests that blockade of Sec61 in endothelial cells contributes to tissue necrosis and slow wound healing. Overall, the generated data support their conclusions and I only have two major criticisms:

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- *While statistical analysis is done and P values are provided, no information is given on the statistical tests used, neither in methods nor results. This must be corrected, to evaluate the repeatability and reproducibility of their data.*

We respectfully but fundamentally disagree with the comments regarding the Sec61 dependence of the effects that we observed. We showed that loss of glycocalyx and basement membrane components underpinned the phenotypic changes in endothelial cells (morphological changes, loss of adhesion, increased permeability, and reduced ability to repair scratch wounds). We demonstrated that we could phenocopy permeability increases and elongation phenotype by knocking down the type II membrane protein B3Galt6, and reverse the adhesion defect by exogenous provision of the secreted laminin-511 heterotrimer.

Our conclusion that mycolactone mediates these effects via Sec61 inhibition is not based solely on the use of alternative inhibitors but is built on several pillars of evidence:

First, the proteomics data conforms entirely to predictions based on the topology of affected vs. non-effected proteins, and agrees with independently published proteomic datasets from T lymphocytes, dendritic cells and sensory neurons (ref.12), as well as biochemical studies performed using in vitro translocation assays (ref.11,34). Furthermore, the pattern of membrane protein down regulation observed in our experiments fits perfectly with established models of protein translocation mechanisms, particularly with respect to the lack of effect on specific topologies of multipass membrane proteins, tail anchored- and type III membrane proteins (ref.34-36).

Second, since Sec61 very highly conserved amongst mammals and is found in all nucleated cells, it is hard to conceptualise a framework in which mycolactone targets Sec61 in some cells and not others, as this reviewer suggests might be the case for epithelial cells [noting that the work being referred to (ref.29) predates our 2014 work showing that mycolactone is a Sec61 inhibitor (ref.7)]. Indeed, mycolactone has been shown to target Sec61 in multiple independent approaches including forward genetic screens involving random mutagenesis and CRISPR/Cas9 (ref.10, PMID: 35939511). Genetic evidence has previously been provided for the Sec61 dependence of mycolactone effects in epithelial cells (ref.10,17). We have unpublished genetic evidence that the rounding and detachment of epithelial cells due to mycolactone is reduced when resistance mutations are over expressed, and will consider including this in the next version of the manuscript.

Third, given this weight of evidence, one would be hard-pressed to provide an alternative explanation for the specific down-regulation of glycosaminoglycan-synthesising enzymes and adhesion/basement membrane molecules while most cytosolic and non-Sec61 dependent membrane proteins are unchanged or upregulated. However, seeking to be as rigorous as possible we have here shown that a completely independent Sec61 inhibitor produces the same phenotype at the gross and molecular level. Ipomoeassin F (Ipom-F) is a glycolipid, not a polyketide lactone, yet they both compete for binding with cotransin in Sec61α (ref.6). There is significant overlap in the cellular responses to mycolactone and Ipom-F, including the induction of the integrated stress response (ref.17, PMID: 34079010), which we observed

again in the current data, providing further evidence that this approach is useful when genetic approaches are technically unattainable.

Therefore, we are confident the effects seen on endothelial cells are Sec61-dependent. We are happy to provide more detail on our lengthy attempts at over-expressing mycolactone resistant SEC61A1 genes in HUVECs; primary endothelial cells derived from the umbilical vein. We are highly experienced in this area, and have previously stably expressed these proteins in epithelial cell lines, reproducing the resistance profile (ref.10,17). Notably though, these cells do not have normal 'fitness' in the absence of challenge. Since endothelial cells (and endothelial cell lines; PMID: 12560236) are extremely hard to transfect with plasmids, with efficiency routinely 5-10% (including in our hands), we developed a lentivirus system. We were eventually (after multiple attempts using different protocols) able to transduce primary HUVECs with constructs expressing GFP (at an efficiency of about 10-20%) and select/expand these under puromycin selection. Never-the-less, we never recovered any cells that expressed the flag-tagged SEC61A1 wild type or SEC61A1 carrying the resistance mutant D60G. We also attempted to select D60G-transduced cells with mycolactone epimers, an approach that can help the cells compete against non-transduced cells in culture flasks (ref.10). We concluded that primary endothelial cells are unable to tolerate the expression of additional Sec61α, and this was incompatible with survival.

It's also important to note that most endothelial cell specialists would agree that endothelial cell lines are not good models of endothelial behaviour. We tested the HMEC-1 cell line, but found it did not express prototypical endothelial marker vWF in the expected way. Therefore we focussed our efforts on primary endothelial cells. Should we be able to overcome the dual challenge of the necessity to work in primary cells, and the difficulty of over-expressing Sec61, we will update this paper at a later date with this data, and will also expand the above arguments.

We apologise for the embarrassing oversight of not including information about the statistical analyses we used, which of course we will correct in full in the revised version. However, we would like to provide this information to readers of the current version of the manuscript. All data were analysed using GraphPad Prism Version 9.4.1:

Figure 1: one-way ANOVA with Dunnett's (panel A) or Tukey's (panel B) correction for multiple comparisons

Figure 2 supplement: one-way ANOVA with Tukey's correction for multiple comparisons (analysed panel)

Figure 3: one-way ANOVA with Tukey's (panel B) or Dunnett's (panel E&F) correction for multiple comparisons

Figure 4: one-way ANOVA with Dunnett's correction for multiple comparisons (all analysed panels)

Figure 5 and supplement: one-way ANOVA with Dunnett's correction for multiple comparisons (all analysed panels)

Figure 6: one-way ANOVA with Dunnett's correction for multiple comparisons (analysed panel)

Figure 6 supplement: one-way ANOVA with Dunnett's correction for multiple comparisons (all analysed panels)

Figure 7: two-way ANOVA with Tukey's correction for multiple comparisons (all analysed panels; panels B&C also included the Geisser Greenhouse correction for sphericity)

Figure 7 supplement: Panels A&D used a repeated measures one-way ANOVA with Dunnett's correction for multiple comparisons (panel D also included the Geisser Greenhouse correction for sphericity). Panels B,C&E used a two-way ANOVA with Tukey's correction for multiple comparisons (panels B&C also included the Geisser Greenhouse correction for sphericity)