The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 15 2009

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J Immunol (2009) 182 (4): 2194–2202.
https://doi.org/10.4049/jimmunol.0802294

Mycolactone Inhibits Monocyte Cytokine Production by a Posttranscriptional Mechanism[1]

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The virulence and immunosuppressive activity of *Mycobacterium ulcerans* is attributed to mycolactone, a macrolide toxin synthesized by the bacteria. We have explored the consequence and mechanism of mycolactone pretreatment of primary human monocytes activated by a wide range of TLR ligands. The production of cytokines (TNF, IL-1β, IL-6, IL-10, and IFN-γ-inducible protein-10), chemokines (IL-8), and intracellular effector molecules (exemplified by cyclooxygenase-2) was found to be powerfully and dose dependently inhibited by mycolactone, irrespective of the stimulating ligand. However, mycolactone had no effect on the activation of signaling pathways that are known to be important in inducing these genes, including the MAPK and NF-κB pathways. Unexpectedly, LPS-dependent transcription of TNF, IL-6, and cyclooxygenase-2 mRNA was found not to be inhibited, implying that mycolactone has a novel mechanism of action and must function posttranscriptionally. We propose that mycolactone mediates its effects by inhibiting the translation of a specific subset of proteins in primary human monocytes. This mechanism is distinct from rapamycin, another naturally occurring immunosuppressive lactone. The current findings also suggest that monocyte-derived cytokine transcript and protein levels may not correlate in Buruli ulcer lesions, and urge caution in the interpretation of RT-PCR data obtained from patient biopsy samples. ***The Journal of Immunology,*** **2009, 182: 2194–2202.**

Buruli ulcer (BU)[5] is the third most common mycobacterial disease in the immunocompetent after tuberculosis and leprosy (1, 2). It is caused by cutaneous infection with *Mycobacterium ulcerans* and is characterized by progressive necrotic skin ulcers containing extracellular bacterial microcolonies of acid-fast bacilli. The first sign of infection is the presence of a preulcerative nodule, which can progress to either ulcerative or edematous forms. Despite the presence of high bacterial loads in the clinical lesions, there is a striking lack of pain and inflammation. Nodules and small ulcerated BU lesions can now be treated successfully with antibiotics, but larger (category III, >15 cm in diameter) lesions often require surgical resection and, in severe cases, amputation. Because the disease is most frequently seen in developing countries of Africa, Asia, and, less commonly, Latin America, identification and treatment of cases can be problematic. The pathogenesis of BU is now known to require the synthesis by *M. ulcerans* of a macrolide toxin known as mycolactone (3–6). This persists in the extramicrobial milieu and presumably diffuses through infected tissue in advance of progressing infection (7). Mycolactone rapidly enters cells and it is found in the cytoplasm, but excluded from the plasma membrane and nucleus (8). It causes cell cycle arrest, rounding up, and apoptosis/necrosis of transformed cell lines in vitro, and causes the necrosis of adipocytes and fibroblasts in vivo.

Because of the poor inflammatory reaction in BU, much research has focused on the way mycolactone modulates the immune response. Several groups have recently shown that, like other mycobacterial infections, BU may undergo an early infectious phase in which the bacteria are phagocytosed and then proliferate inside macrophages (9, 10). It has been postulated that when mycolactone production by the intracellular organisms reaches a critical point, this results in apoptosis of the macrophage releasing the bacilli and associated mycolactone, which then exerts its effects on the surrounding tissue (7, 10).

Mycolactone has also been shown to be powerfully immunosuppressive at the molecular level. Research in this laboratory was the first to show that a crude preparation of mycolactone could strongly inhibit the production of IL-2 from T cells (11). Moreover, mycolactone inhibited TNF-dependent activation of the transcription factor NF-κB in T cell lines (11). Preliminary studies in primary human monocytes also indicated that TNF-α and IL-10 cytokine production in response to LPS was also attenuated (11). Subsequently, it was shown by other laboratories that murine bone marrow-derived macrophages secrete dramatically less TNF when infected with wild-type, mycolactone-producing, *M. ulcerans* strains compared with mycolactone-negative strains, and that this

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Received for publication July 30, 2008. Accepted for publication December 1, 2008.

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[1] This work was supported by the Arthritis Research Council, Kennedy Institute of Rheumatology Trustees, and Union Bank of Switzerland Optimus Foundation, and also the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and the World Health Organization.

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[4] Deceased.

[5] Abbreviations used in this paper: BU, Buruli ulcer; ActD, actinomycin D; CHX, cycloheximide; Cox, cyclooxygenase; Ct, cycle threshold; eIF, eukaryotic initiation factor; EtOH, ethanol; FWB, FACS wash buffer; IP-10, IFN-γ-inducible protein-10; Met/Cys, methionine and cysteine; mTORC1, mammalian target of rapamycin complex 1; poly(I:C), polyinosinic-polycytidylic acid; PSI, proteasome inhibitor; qRT-PCR, quantitative RT-PCR.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802294

was mycolactone dependent (12). Furthermore, mycolactone can inhibit maturation of dendritic cells and selectively suppress the production of cytokines and chemokines from these cells in response to LPS and polyinosinic-polycytidylic acid (poly(I:C)) (13).

It is still unclear precisely how these in vitro findings relate to cutaneous infections of *M. ulcerans*. Histopathological examination of excised tissue from lesions shows that there can be a cellular immune response to infection, including the presence of CD68-positive cells (14, 15). Also, experimental *M. ulcerans* infection of guinea pigs shows infiltration of mononuclear cells at the edge of the region of coagulative necrosis, distant from the localization of the colonies of bacteria (7). How such cells can be present without inflammation is one of the unresolved issues in BU. In most infections the first line of defense against invading pathogens are the TLRs that activate the innate immune response (16). The TNF produced by TLR-dependent activation of monocytes and macrophages is known to be a key driver of the subsequent inflammation in vivo. We have therefore investigated in depth the consequence and mechanism of mycolactone treatment of primary human monocytes. We found that mycolactone can specifically inhibit the production of a subset of inflammatory mediators without affecting gene transcription. This suggests a novel posttranscriptional mechanism by which mycolactone suppresses the innate immune response.

Materials and Methods

Reagents

RPMI 1640 cell culture medium was from Cambrex, and FBS was from PAA. The ligands used to stimulate the different TLRs were LPS from *Escherichia coli* (InvivoGen), poly(I:C), Pam_3Cys-Ser$(Lys)_4$.3HCl (Pam_3Cys), MALP-2, flagellin, and R848 (all from Alexis Biochemicals). Control inhibitors were as follows; the proteasome inhibitor (PSI) was from Calbiochem; cycloheximide (CHX), actinomycin D (ActD), and brefeldin A were from Sigma-Aldrich; and rapamycin and LY294002 were from Cell Signaling Technology. All reagents were tested for endotoxin contamination using the *limulus* amebocyte lysate assay from Cambrex (17) and found to have <0.1 U/ml LPS.

Mycolactone was purified from intact *M. ulcerans* strain 1615 using well-established protocols, as described (4). MU 1615 is a member of the classical highly virulent group of *M. ulcerans*. The major mycolactone produced by MU 1615 is a 3:2 mixture of two stereoisomers (18) designated mycolactone A and mycolactone B. This is the type of mycolactone made by all classical (African) isolates and has the highest cytopathic activity (8). Mycolactone purity was validated by mass spectrometry that showed mycolactone A/B in the expected ratio.

Preparation of primary human monocytes, cell culture, and activation

Primary human monocytes were isolated from single-donor plateletpheresis residues purchased from the North London Blood Transfusion Service. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation before elutriation and culture, as previously described (19, 20). For cytokine/chemokine assays, monocytes (>80% pure by FACS) were plated at 1×10^5 cells/well in 96-well plates, and attached cells were preincubated at 37°C with inhibitors for 1 h and then stimulated with different TLR ligands, i.e., 10 ng/ml LPS, 10 ng/ml Pam_3Cys, 10 ng/ml MALP-2, 10 ng/ml flagellin, 10 μg/ml poly(I:C), or 1 μg/ml R848 for 22 h. Supernatants were collected ensuring that at least 50 μl remained, in order not to disturb loosely attached monocytes. Cell viability was examined by a MTT assay (Sigma-Aldrich) (21). For intracellular assays, short stimulations (4 h or less) were performed in 1.5-ml Eppendorf tubes and cells were harvested by centrifugation.

To examine secreted vs nonsecreted IL-6 production following LPS stimulation, monocytes were plated and stimulated with LPS, as described above. However, following a 3-h incubation (at which point monocytes are still attached to the surface of the wells), supernatants were harvested and cells were lysed with 100 μl of chloramphenicol acetyltransferase lysis buffer (10 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40) containing 1× protease inhibitor mixture (Sigma-Aldrich), frozen at −80°C, and subjected to two freeze/thaw cycles.

Cytokine immunoassays

Sandwich ELISAs were used to measure cytokine/chemokine levels in cell supernatants, using standard techniques. Paired Abs were used for TNF, IL-6, IL-8, IL-10, and CXCL10 (referred to in this study as IFN-γ-inducible protein-10 (IP-10; BD Biosciences) and IL-1β (R&D Systems)). Protein standards were from PeproTech. To determine the levels of IL-6 in cell lysates, the same Abs as for supernatants were used. In this study, the standards were diluted in chloramphenicol acetyltransferase lysis buffer, and the lysates were assayed undiluted.

Western immunoblotting

Monocytes were either used fresh or rested in an incubator overnight in a loosely capped tube at a density of 1×10^6 cells/ml, then used at $1–2 \times 10^6$ cells/point. Cells were treated as required and lysed in 1% Triton X-100 lysis buffer (10 mM Tris (pH 7.6) 150 mM NaCl, 1 mM EDTA containing 1% Triton X-100, 1× protease inhibitor mixture (Sigma-Aldrich), 5 mM NaF, and 1 mM $NaVO_3$). In some cases, cells were lysed with 1× Laemli loading buffer and sonicated. Cell lysates were separated on 7 or 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes using standard techniques. Immunoblotting was performed using Abs to cyclooxygenase (Cox-2; Alexis Biochemicals), IκBα (Santa Cruz Biotechnology), phospho-p42/p44 (ERK) Thr^{202}/Tyr^{204}, phospho-p54 (SAPK/JNK) Thr^{183}/Tyr^{185}, phospho-p38 MAPK Thr^{180}/Tyr^{182}, phospho-p70 S6 kinase Thr^{389}, phospho-Akt Ser^{473}, phospho-eukaryotic initiation factor (eIF)4E Ser^{209}, phospho-eIF2α Ser^{51} or total eIF2α (all Cell Signaling Technology), or total p38 MAPK (22) (gift from J. Saklatvala, Kennedy Institute of Rheumatology, London, U.K.). In all cases, the blot was developed using a HRP-conjugated anti-rabbit secondary Ab and ECL detection reagents (GE Healthcare).

EMSAs

Nuclear extracts and EMSAs were performed essentially as described previously (23). Briefly, 2 μg of nuclear extracts from primary human monocytes was incubated with 5×10^4 cpm [γ^{32}P]ATP-labeled double-stranded NF-κB consensus probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega) for 30 min at room temperature in a total volume of 10 μl. In some cases, excess unlabeled competitor DNA was added to control samples. The reactions were then run on a 5% nondenaturing polyacrylamide gel, dried, and exposed to Hyperfilm MP at −70°C.

Quantitative RT-PCR (qRT-PCR)

Levels of different endogenous mRNA transcripts were quantified by one-step real-time RT-PCR. Total RNA was extracted from 5×10^5 monocytes (>90% pure by FACS) using the total RNA (InvivoGen) or RNeasy blood (Qiagen) kits, according to the manufacturer's protocols. Real-time qRT-PCR was performed on a Rotor-Gene 6000 (Corbett Life Science) using the Superscript III platinum one-step RT-PCR kit (Invitrogen) and Assay-On-Demand premixed Taqman probe master mixes (Applied Biosystems). Each RNA sample was run in triplicate, and relative gene expression was calculated using the $\Delta\Delta$Ct method (24), using GAPDH as the comparator.

Flow cytometry

The presence of intracellular TNF was detected by flow cytometry after permeabilization and staining with cytokine-specific Abs. Freshly elutriated monocytes (>80% by FACS) were preincubated with 10 μg/ml brefeldin A and/or 50 ng/ml mycolactone for 1 h and then stimulated with 10 ng/ml LPS for 4 h. Cells were harvested by centrifugation at $1000 \times g$ for 3 min, washed once with PBS, fixed for 20 min on ice with Cytofix Fixation Buffer (BD Biosciences), and then washed twice with FACS wash buffer (FWB; 2% human serum, 0.1% NaN_3 in PBS). The following day, cells were permeabilized in FWB containing 0.1% saponin for 30 min on ice, and then allophycocyanin-conjugated mouse anti-TNF Ab (Clone Mab11) or mouse IgG1 K isotype control (both eBioscience) was added to a final concentration of 5 μg/ml for 1 h. Stained cells were washed three times with saponin containing FWB, and the final resuspension was in FWB. Flow cytometry was performed on a FACSCanto flow cytometer (BD Biosciences), and data were analyzed using FlowJo software. Monocytes were gated on their forward and side scatter characteristics.

Metabolic labeling

Overall translation rates in monocytes were assessed by metabolic labeling with [^{35}S]methionine and cysteine (Met/Cys). Monocytes were washed twice in metabolic labeling medium (Met/Cys-free RPMI 1640 containing 5% dialyzed FBS and 2 mM L-glutamine (all Sigma-Aldrich)) and starved

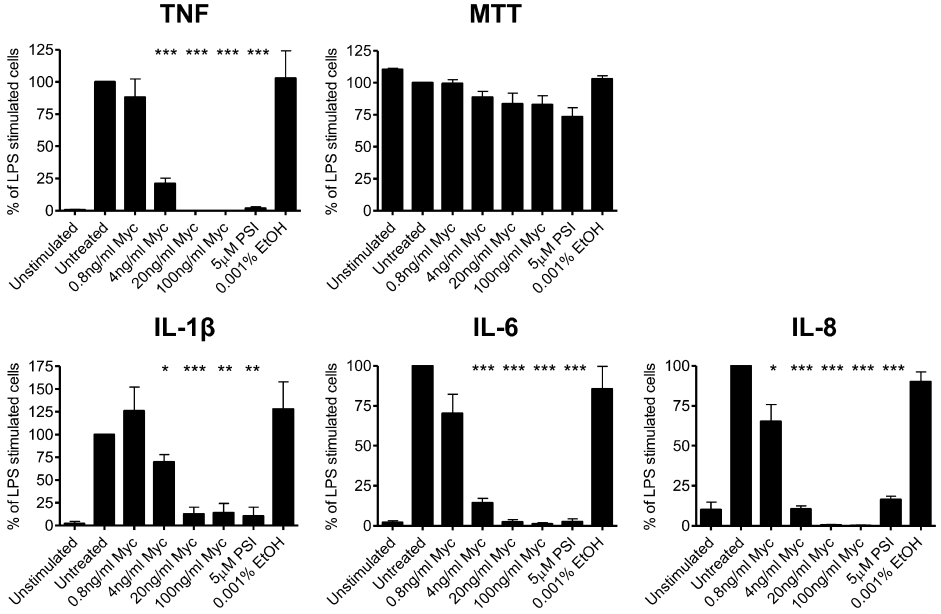

FIGURE 1. Inhibition of LPS-dependent cytokine production by mycolactone. Freshly elutriated primary human monocytes were treated with the indicated inhibitors or controls for 1 h, followed by LPS stimulation for 22 h. Supernatants were harvested, and the viability of the cells was assessed by a MTT assay. Cytokine concentrations in supernatants were assessed by ELISA. Experiments were performed in triplicate in three to five donors and are presented as mean ± SEM normalized to that of LPS-stimulated cells. Unstimulated, no LPS treatment; Untreated, no inhibitors but LPS treated; Myc, mycolactone; EtOH, absolute ethanol diluted to the same extent as that found in 100 ng/ml mycolactone; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$. Statistical significance was assessed using paired Student's *t* test.

overnight at 1×10^6 cells/ml in a loosely capped tube at 37°C. Cells were then pretreated with inhibitors and stimulated with LPS, as before. Two hours following LPS stimulation, 25 μCi of Promix [^{35}S]Met/Cys (Amersham) was added. Labeling continued for 4 h, after which time cells were washed twice with ice-cold PBS and then lysed in 1% Triton X-100 lysis buffer. After removal of insoluble material by centrifugation, the incorporation of [^{35}S]Met/Cys in this cytosolic fraction was assessed by scintillation in a Wallac 1410 liquid scintillation counter and is expressed as decays per minute for the whole sample.

Statistics

Statistical significance was assessed, where appropriate, using paired Student's *t* test using Graphpad Prism v.4.

Results

Mycolactone inhibits TLR-dependent cytokine production from primary human monocytes

In our previous study (11), we found that partially purified mycolactone dose dependently inhibited LPS-induced TNF and IL-10 production from primary human monocytes. We have now confirmed this finding with purified mycolactone, and extended it to show dose-dependent ablation of IL-6 and IL-8 (Fig. 1). IL-1β production was also greatly reduced, but even at 100 ng/ml mycolactone, it could not be completely abolished. As a positive control for inhibition of NF-κB-dependent genes, PSI, which prevents IκBα degradation and subsequent NF-κB activation, was used. This strongly inhibited production of the cytokines, as expected. Purified mycolactone is dissolved in ethanol (EtOH) because it is a hydrophobic lipid-like molecule. To rule out an influence of EtOH on cytokine production, this was included in the experiments at a concentration of 0.001% (representing the same EtOH dilution as 100 ng/ml mycolactone). This had no discernable effect.

In this system, mycolactone was not cytotoxic to monocytes as assessed by MTT assays (Fig. 1). There was a small, but reproducible reduction in viability that was not statistically significant, but this was trivial compared with the dramatic loss in cytokine production by these same cells. Treatment of monocytes with 100 ng/ml mycolactone had a lesser effect on viability than the PSI, and these cells still secreted moderate amounts of IL-8 (Fig. 1). As previously published (11), mycolactone inhibited the attachment and formation of LPS-dependent clumps of monocytes on the surface of the tissue culture plastic.

LPS is a ligand for TLR4, which is one of the two TLRs that are generally considered to be stimulated by mycobacteria. However, the TLR(s) that recognizes *M. ulcerans* has not been specifically established. We therefore examined the effect of mycolactone on cytokine and chemokine production following stimulation with a broad range of other TLR ligands: poly(I:C) (TLR3), Pam_3Cys (TLR1/2), MALP-2 (TLR2/6), flagellin (TLR5), and R848 (TLR8). Cytokine production in response to all TLR ligands tested was ablated by 20 ng/ml mycolactone (Table I). We found that for any cytokine produced in response to a particular ligand, it could be efficiently inhibited by mycolactone. This suggested that mycolactone acts to inhibit a pathway that is common to all of these TLRs. In these assays, IP-10 was used as a secondary readout for

Table I. *Inhibition of cytokine production by mycolactone*[a]

	TNF		IL-1β		IL-6		IL-8		IL-10		IP-10	
	ng/ml	% Inhibition	ng/ml	% Inhibition	ng/ml	% Inhibition	ng/ml	% Inhibition	ng/ml	% Inhibition	ng/ml	% Inhibition
LPS	4.64	>95	2.97	78.4	63.8	>96.7	210	100	0.12	>84.1	NI	
Poly(I:C)	NI		NI		NI		NI		NI		0.46	>83.8
Pam_3Cys	5.31	>97.8	0.65	46.7	42.0	97.2	134	100	NI		NI	
MALP-2	2.95	>74.6	0.18	47.0	28.2	92	98.3	100	NI		NI	
Flagellin	1.05	>95	0.13	30.5	8.43	>91.1	90.5	100	NI		NI	
R848	20.7	>98.8	2.45	71.8	75.8	>99.3	216	100	0.15	>90.9	NI	

[a] The mean induction of each mediator by that ligand, over all donors, is presented as ng/ml. Mean percentage of inhibition by 20 ng/ml mycolactone is also presented. In many cases, the level was reduced to below the detection limit in that ELISA. n = 3–8 donors; NI, none induced (below the detection limit of the ELISA; 13–41 pg/ml).

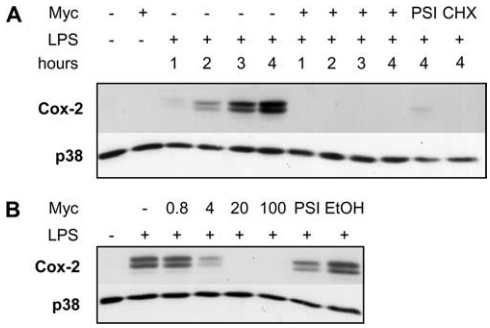

FIGURE 2. Inhibition of LPS-dependent Cox-2 synthesis by mycolactone. Primary human monocytes were untreated (−) or treated with mycolactone at 50 ng/ml (*A*) or at the indicated concentrations (*B*), 5 μM PSI, 10 μg/ml CHX, or 0.001% EtOH for 1 h, and then stimulated with 10 ng/ml LPS for the indicated time (*A*) or 3 h (*B*). Cell lysates were subjected to Western blotting for Cox-2 with total p38 as a loading control. The experiment was performed in monocytes from three different human donors with comparable results.

type I IFN production by the MyD88-independent pathway following poly(I:C) stimulation. IP-10 production following IFN-β stimulation of monocytes is also inhibited by mycolactone (data not shown). However, similarly to LPS, IL-1β induced by other TLR ligands could not be suppressed to the same extent as the other mediators (Table I).

Mycolactone prevents LPS-dependent up-regulation of Cox-2

Because mycolactone strongly inhibited the production of TLR-dependent secreted proteins, we were interested to discover its effect on the production of a LPS-dependent cytosolic protein such as Cox-2. The typical LPS-dependent up-regulation of Cox-2 could not be detected in cells that had been pretreated with 50 ng/ml mycolactone (Fig. 2*A*). This effect was also found to be dose dependent (Fig. 2*B*), and no Cox-2 protein could be detected at 20 ng/ml mycolactone.

The effect of mycolactone on LPS-dependent signaling events

The data seemed to indicate that mycolactone could be specifically inhibiting one of the signaling pathways that are activated following exposure of cells to each of the TLR ligands. First, the effect of mycolactone on the MAPK family members ERK, JNK, and p38 was investigated. These were rapidly and transiently phosphorylated following LPS stimulation of monocytes (Fig. 3*A*). Mycolactone itself does not result in phosphorylation of these proteins (data not shown), and neither did it alter the kinetics or extent of phosphorylation in cells that had been preincubated with 50 ng/ml mycolactone (Fig. 3*A*).

To examine whether mycolactone was exerting its effect by inhibiting the activation of NF-κB, similarly to the Jurkat T cell line (11), EMSAs were performed on nuclear proteins extracted from cells pretreated with mycolactone and stimulated with LPS, as well as Western blots for IκBα degradation and resynthesis. As expected, low levels of NF-κB dimers were observed by EMSA in resting cells, probably due to nuclear shuttling, but these were strongly up-regulated in response to LPS (Fig. 3*B*). The specificity of the interaction with the NF-κB consensus oligonucleotides was confirmed by adding an excess of cold, unlabeled oligonucleotide, which effectively competed for NF-κB binding. However, we found that mycolactone was not able to inhibit the translocation of NF-κB dimers to the nucleus of primary human monocytes (Fig. 3*B*). Furthermore, the kinetics of IκBα degradation and resynthesis were unaffected in cells that had been preincubated with 50 ng/ml mycolactone (Fig. 3*C*). Taken together, this strongly suggests that mycolactone does not affect activation of the NF-κB pathway in monocytes.

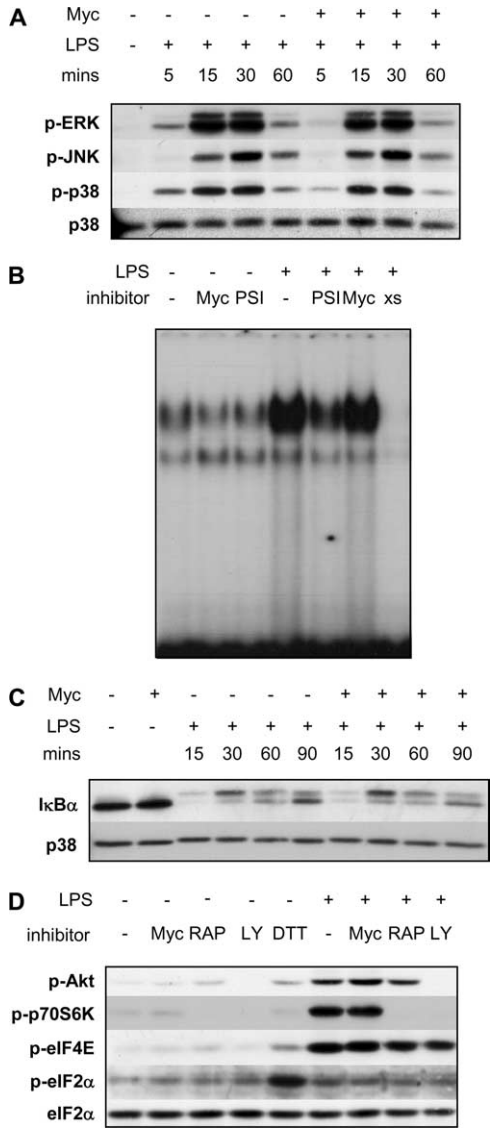

FIGURE 3. Mycolactone does not affect the MAPK, NF-κB, or mTORC1 signaling pathways. *A*, Primary human monocytes were untreated (−) or treated with 50 ng/ml mycolactone for 1 h, and then stimulated with 10 ng/ml LPS for the indicated times. Cell lysates were subjected to Western blotting for phospho-ERK, phospho-JNK, and phospho-p38 MAPK, as well as total p38 MAPK as a loading control. *B*, Primary human monocytes were untreated (−) or treated with either 50 ng/ml mycolactone or 5 μM PSI for 1 h, and then stimulated with 10 ng/ml LPS for 2 h. Nuclear proteins were extracted and subjected to EMSA using ^{32}P-labeled NF-κB consensus oligonucleotides, as described in *Materials and Methods*. In one case, excess cold unlabeled NF-κB consensus was added (xs). *C*, Primary human monocytes were untreated (−) or treated with 50 ng/ml mycolactone for 1 h, and then stimulated with 10 ng/ml LPS for the indicated times. Cell lysates were subjected to Western blotting for IκBα and total p38 as a loading control. *D*, Primary human monocytes were treated with either 50 ng/ml mycolactone (Myc), 10 nM rapamycin (RAP), 10 μM LY294002 (LY), or 10 mM DTT for 1 h or pretreated for 1 h and then stimulated with 10 ng/ml LPS for 1 h. Cell lysates were subjected to Western blotting for phospho-Akt, phospho-p70 S6 kinase, phospho-eIF2α, or total eIF2α as a loading control. In each case, the experiment was performed in monocytes from three different human donors with comparable results.

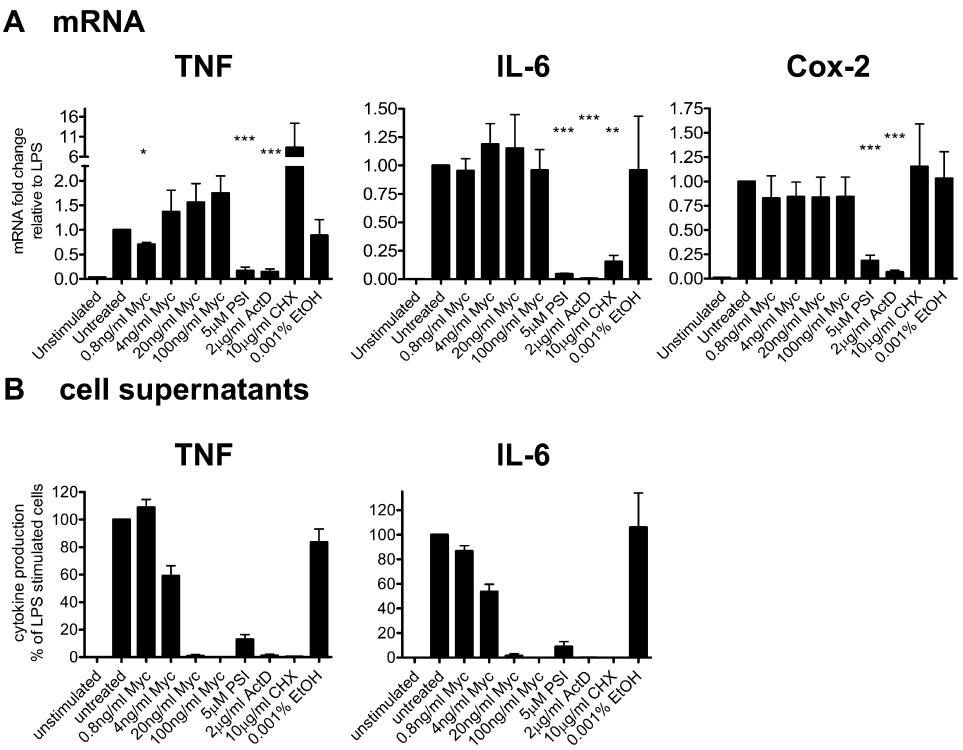

FIGURE 4. Mycolactone does not affect transcription of the genes for TNF, IL-6, or Cox-2. Primary human monocytes were untreated or treated with the indicated concentrations of mycolactone (Myc), PSI, ActD, CHX, or EtOH for 1 h, and then left unstimulated or stimulated with 10 ng/ml LPS for 2 h. *A*, Total RNA was used as a template in qRT-PCR gene expression assays for TNF, IL-6, or Cox-2, performed in triplicate. Relative mRNA fold change was calculated using the ΔΔCt method. All results are presented as mean ± SEM for $n = 3$–5 donors. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$. Statistical significance was assessed using paired Student's t test. *B*, Supernatants taken from the same experiments were subjected to ELISA for TNF and IL-6 to determine cytokine concentrations for each donor.

Mycolactone is a macrolide with structural similarities to rapamycin. Like mycolactone, rapamycin is an immunosuppressive compound that exerts its effects independently of the NF-κB and MAPK pathways (25). In light of the above findings, we investigated whether mycolactone could be acting by modulating the rapamycin-sensitive complex: mammalian target of rapamycin complex 1 (mTORC1). LPS strongly induces the phosphorylation of p70 S6 kinase 1 h after stimulation (Fig. 3*D*). Rapamycin inhibited this, as expected; however, mycolactone had no effect, suggesting that it does not inhibit mTORC1 function.

Other LPS-dependent signaling events that might be modulated by mycolactone were also examined. Mycolactone is known to contain polyketide side chains that resemble lipid; therefore, the effect of mycolactone on lipid signaling in monocytes was examined. The PI3K-dependent activation of Akt was inhibited by LY294002, as expected, but, again, not by mycolactone (Fig. 3*D*), suggesting that it does not mediate its effects by targeting this pathway. Also, because mycolactone is cytotoxic, the possibility that stress-induced pathways might be modulated was investigated. However, stress-induced phosphorylation of eIF2α, exemplified in this study by treatment with DTT to induce the unfolded protein response, was not activated by mycolactone in primary human monocytes (Fig. 3*D*). Neither was LPS-dependent activation of eIF4E (Fig. 3*D*).

Mycolactone has no influence on steady-state TNF, IL-6, or Cox-2 mRNA transcript levels following LPS stimulation

Using qRT-PCR, the influence of mycolactone over LPS-dependent transcription of TNF, IL-6, and Cox-2 was investigated (Fig. 4). Two hours after stimulation, transcription of these genes was up-regulated by LPS ~30-, 500-, and 100-fold, respectively. The transcriptional inhibitor ActD and NF-κB inhibitor PSI were effective inhibitors of transcription in these experiments, as expected. In addition, the general inhibitor of translation CHX had different effects on the different transcripts; it had no effect on Cox-2 transcription, inhibited IL-6 transcription, and superinduced TNF transcription.

In contrast, mycolactone did not reduce transcription of TNF, IL-6, or Cox-2 over a range of doses up to 100 ng/ml, more than sufficient to ablate protein production (Fig. 4*A*). For TNF, our data even suggest a slightly increased level of transcript. ELISAs on supernatants from these same cells showed that production and secretion of TNF and IL-6 were inhibited at 2 h to a similar extent as in the overnight cultures (Fig. 4*B*). This strongly suggests that the function of mycolactone in monocytes is to inhibit LPS-responsive genes at a posttranscriptional level.

Mycolactone inhibits production of a subset of proteins in monocytes

To investigate the mechanism of mycolactone's action, additional experiments were performed. The effect on protein production could be by an inhibition of protein translation, protein stability, or even secretion. To examine a possible effect on secretion, we used two different techniques. First, parallel ELISAs were performed for cytokines present in the supernatants and lysates of primary human monocytes. IL-6 could be easily detected in the lysates of LPS-treated cells, presumably representing translated IL-6 transiting through the endoplasmic reticulum (~9% of the total detected; Fig. 5*A*). In contrast, TNF, which is induced to less than tenth of the level of IL-6 following LPS stimulation of monocytes (Table I), could not be detected in the lysates in the absence of brefeldin A (data not shown). Because the intracellular fraction of TNF was below the detection limit of the ELISA (23 pg/ml), the experiment focused on IL-6 production. Pretreatment of monocytes with brefeldin A, an inhibitor of protein secretion, resulted in an absence of IL-6 in the supernatants, but an increase in intracellular IL-6, as expected (Fig. 5*A*). In contrast, mycolactone dose dependently inhibited both secreted and unsecreted IL-6, as did PSI and CHX (Fig. 5*A*). This shows that mycolactone acts to inhibit IL-6 production rather than secretion.

We also examined a potential role in TNF secretion by means of flow cytometry for intracellular TNF. Similar to our findings in cell lysates, TNF could not be detected in LPS-stimulated monocytes in the absence of brefeldin A (Fig. 5*Bii*), whereas LPS induced a

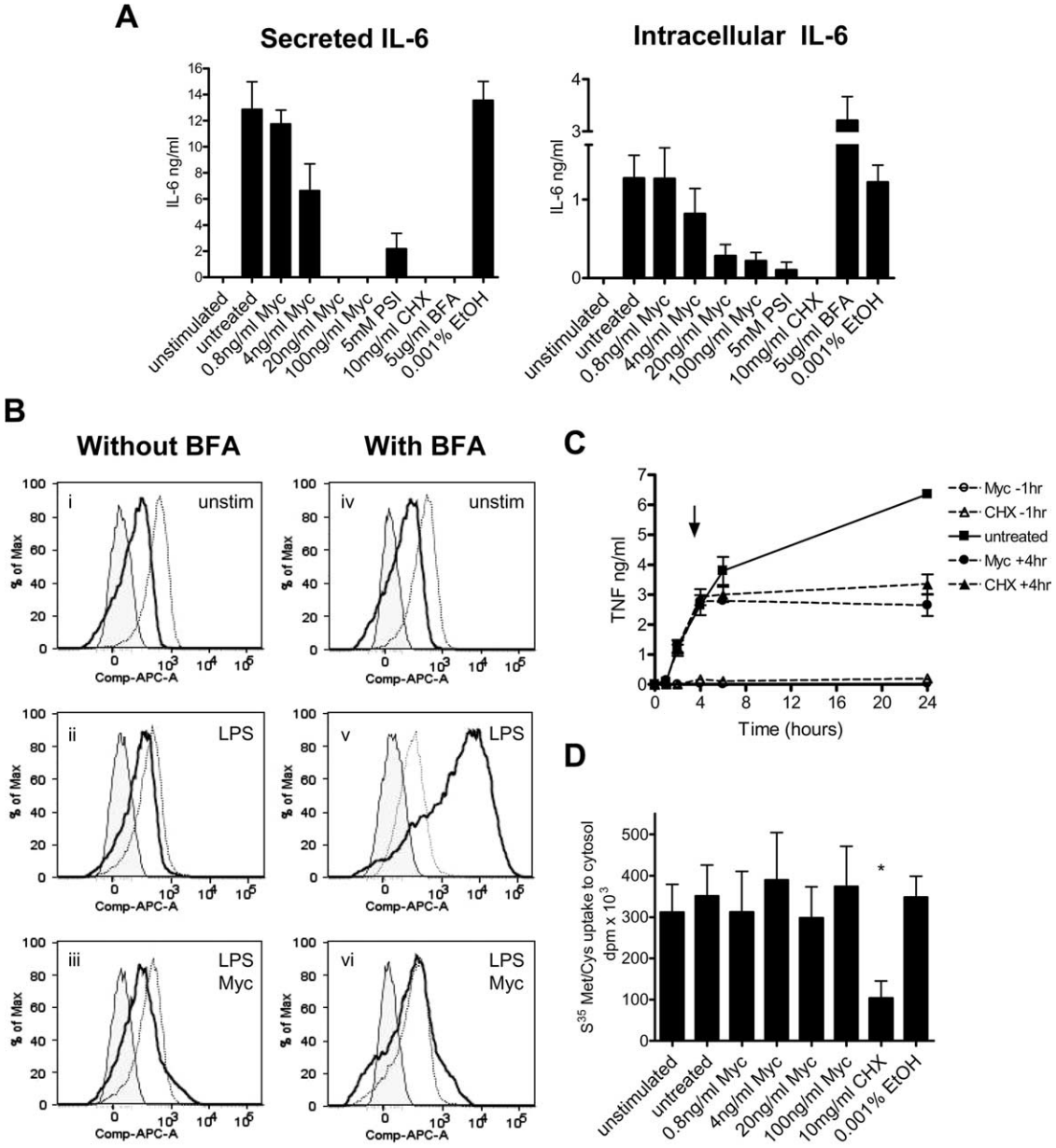

FIGURE 5. Mycolactone inhibits protein production in monocytes. *A*, Freshly elutriated primary human monocytes were pretreated with the indicated inhibitors or controls for 1 h, followed by LPS stimulation for 3 h. Supernatants were harvested cells lysed directly in the plate, as described in *Materials and Methods*. Cytokine concentrations in supernatants and lysates were assessed by ELISA and are presented as mean ± SEM, $n = 3$. *B*, Freshly elutriated monocytes were pretreated with mycolactone (Myc) and/or brefeldin A for 1 h, and then stimulated with 10 ng/ml LPS for 4 h. TNF was detected by intracellular FACS staining using an allophycocyanin-conjugated anti-TNF mouse mAb or a mouse IgG1 isotype control. Cells only, gray tinted area; isotype control, dotted line; anti-TNF, heavy black line. The experiment was performed in monocytes from three different human donors with comparable results. *C*, Primary human monocytes were treated with 50 ng/ml mycolactone (Myc) or 10 μg/ml CHX, either 1 h before 10 ng/ml LPS addition (−1 h) or 4 h after (+4 h; see arrow), in triplicate. Small, 50-μl aliquots of supernatant were taken at various timepoints after LPS additions, as indicated. The concentration of TNF in supernatants was assessed by ELISA and is presented as mean ± SD. *D*, Primary human monocytes were starved of Met/Cys overnight, then pretreated with the indicated inhibitors or controls for 1 h, and stimulated with LPS stimulation for 6 h, the last 4 h of which were in the presence of 25 μCi of [^{35}S]Met/Cys. Cytosolic ^{35}S uptake was assessed by scintillation and presented as mean ± SEM for 1×10^6 cells, $n = 3$. Statistical significance was assessed by paired Student's *t* test; *, $p < 0.05$. Unstimulated, no LPS treatment; Untreated, no inhibitors, but LPS treated; Myc, mycolactone; EtOH, absolute ethanol diluted to the same extent as that found in 100 ng/ml mycolactone, i.e., 0.001%.

large shift in fluorescence in brefeldin A-treated cells (Fig. 5*Bv*), as expected. We found that mycolactone on its own did not increase LPS-dependent intracellular TNF (Fig. 5*Biii*), and when it was added to brefeldin A-treated cells, intracellular TNF was strongly inhibited (Fig. 5*Bvi*). This shows that mycolactone inhibits TNF production rather than secretion.

To test a possible effect on protein stability, either 50 ng/ml mycolactone or 10 μg/ml CHX was added to monocyte cell cultures either 1 h before LPS stimulation or 4 h afterward. Aliquots of the supernatant were removed at different timepoints, and TNF (Fig. 5*C*) and IL-6 (data not shown) were assessed by ELISA. In untreated cells, this resulted in an accumulation of TNF and IL-6 in the supernatants. As expected, pretreatment with either mycolactone or CHX efficiently inhibited the production of both TNF and IL-6. Addition of either compound at 4 h was found to prevent any further increase in the levels of both TNF and IL-6 in the

supernatants, implying that mycolactone acts after the major signaling events have occurred in these cells and in a manner that rapidly prevents further protein production. However, TNF and IL-6 levels did not drop, suggesting that protein stability was unaffected.

Finally, the possibility that mycolactone was a general inhibitor of translation was examined by metabolic labeling. The amount of ^{35}S-labeled Met/Cys in the cytosol of cells pretreated with mycolactone and then stimulated with LPS did not differ from that of untreated cells or unstimulated cells (Fig. 5*D*). This is in stark contrast to CHX, which strongly inhibited protein synthesis, as expected. This suggests that mycolactone acts to inhibit the production of only a subset of proteins in primary human monocytes.

Discussion

The molecular events in BU that cause CD68-positive cells to be recruited close to sites of bacterial colonies without overt inflammation are currently unclear. To understand how these two, apparently contradictory, observations can be reconciled, it is important to understand the effect of mycolactone in these cells. We have studied in depth the effect of mycolactone on primary human monocytes as an in vitro model of cells arriving at the site of *M. ulcerans* infection that are exposed to mycolactone. Concentrations of mycolactone as low as 20 ng/ml were found to be sufficient to result in a profound reduction of the TLR-dependent expression of secreted cytokines (TNF, IL-1β, IL-6, IL-10, and IP-10), chemokines (IL-8), and the cytosolic proinflammatory mediator Cox-2 in these cells. This concentration is in close agreement with those used by other groups to examine mycolactone's immunomodulatory activity (7, 12, 13).

When we looked further into the mechanism behind the mycolactone-dependent inhibition of protein production in monocytes, we found that transcripts encoding the genes for three of the inhibited proteins were not ablated in line with the protein. Mycolactone at concentrations up to 100 ng/ml did not reduce the LPS-dependent steady-state levels of TNF, IL-6, and Cox-2 transcripts. This is the first investigation of the effect of mycolactone on TNF transcript levels by a qRT-PCR approach. Coutanceau et al. (9) previously used an RNase protection assay approach to examine transcript levels in RAW246.7 cells activated with LPS/IFN-γ. In this study, reduced TNF transcripts in cells treated with high doses of mycolactone were noted and were thought to explain the reduced TNF production in cells experimentally infected with *M. ulcerans*. However, this required extremely high doses (500 ng/ml), 25 times higher than the dose required for complete abolition of TNF production in our study (9).

The current findings implicate a posttranscriptional mechanism for mycolactone action in these cells, and several additional lines of evidence support this contention. First, mycolactone did not influence the key signaling pathways that are activated during LPS-dependent gene induction. In particular, activation of the NF-κB and MAPK pathways was unaffected. Neither were the PI3K or mammalian target of rapamycin signaling pathways suppressed (Fig. 3). Second, mycolactone was able to halt cytokine production by preactivated monocytes (Fig. 5). Third, mycolactone was similarly inhibitory against all the stimuli tested, which signal via widely different pathways and to different target genes, and this is strongly suggestive of a mechanism that is both shared and occurs after target gene activation.

Posttranscriptional inhibition of protein production could target a number of cellular processes that are required for normal protein production. However, some of these can be ruled out using the current data, as follows: 1) Regulated splicing efficiency is not a likely mechanism because we used probes specific for the mature mRNAs. Furthermore, mycolactone and CHX differed in their effect on the examined transcripts. We found that CHX superinduced TNF steady-state transcript levels as expected due to an effect on splicing efficiency (26), whereas mycolactone had no effect (Fig. 4). 2) The current data do not support a model by which mycolactone enhances mRNA degradation. This would be expected to result in a decrease in transcript levels, which was not observed in the monocytes (Fig. 4). Furthermore, Fig. 5*B* shows that the effect of mycolactone, just like that of CHX, was immediate. An effect on message stability would be expected to have a delayed kinetic in this experiment. 3) A potential effect on the secretion of cytokines was investigated directly. Levels of intracellular IL-6 (presumably in the process of being secreted; Fig. 5*A*) and brefeldin A-dependent intracellular TNF (Fig. 5*B*) were inhibited by mycolactone. In addition, mycolactone alone did not boost intracellular cytokine levels; thus, mycolactone does not act on secretory pathways. 4) Finally, because the levels of TNF in supernatants plateaued rather than fell when mycolactone was added after LPS stimulation (Fig. 5*C*), this would seem to preclude an effect on protein stability. We therefore currently propose a model in which mycolactone inhibits translation of these transcripts.

It is important to note that mycolactone did not inhibit all protein synthesis; incorporation of radiolabeled amino acids, which reflects overall de novo protein synthesis, was not altered by mycolactone (Fig. 5*D*). At a more specific level, mycolactone was unable to completely halt IL-1β production in response to any TLR ligand (Table I). Production of IL-1β differs from (most) other cytokines in that it is strongly influenced by the activity of the NALP3 inflammasome. This processes previously synthesized pro-IL-1β into the mature form that can be detected by the ELISA. Although this has not been examined directly, the finding that mycolactone only partially inhibits IL-1β suggests that mycolactone does not inhibit the activity of the inflammasome. Other intracellular mediators also escaped inhibition by mycolactone. LPS-dependent resynthesis of IκBα was not affected (Fig. 3*C*). Furthermore, it can also be inferred that production of IκBξ is normal in these cells, because this is an absolute requirement for IL-6 transcription (27) and this was not affected by mycolactone (Fig. 4*A*).

Mycolactone has some structural similarities to rapamycin, which is another naturally occurring immunomodulatory lactone. However, the mechanisms of action of these two molecules do not coincide. Rapamycin inhibits mTORC1 by its interaction with FK506-binding protein 12 and subsequent phosphorylation of p70 S6 kinase and eIF4E-binding protein. Mycolactone inhibits neither of these functions (Fig. 3*D* and data not shown), and also, despite its potential to down-regulate translation, rapamycin does not inhibit TNF production by primary human monocytes (25).

The current data suggest that mycolactone is acting in a very specific way to inhibit translation of a subset of effector molecules that are normally produced in response to infection. The precise mechanism of inhibition in these cells does not seem to be related to induction of eIF2α phosphorylation or inhibition of LPS-dependent phosphorylation of eIF4E (Fig. 3*D*), which are known to inhibit translation by altering the efficiency of the initiation step of translation (28). The mechanism is also clearly different from that which we previously described in the Jurkat T cell line in response to TNF stimulation (11). In this study, mycolactone suppressed activation of the transcription factor NF-κB, a key pathway that results in expression of proinflammatory and antiapoptotic genes. This is presumably sufficient to explain the loss of IL-2 production in these cells and, because mycolactone also induces apoptosis and necrosis in cell lines, this was attractive as a general mechanism for mycolactone's activity.

However, the current study strongly suggests that mycolactone may act by different mechanisms in different cell types. This is not surprising because it is now well known that the immunomodulatory signaling pathways differ significantly between transformed cell lines and primary myeloid cells (29), a finding that may be greatly influenced by the fact that the latter do not proliferate. There are also profound differences in immune cell signaling pathways between human and murine cells (30) and between primary human cells of different origin (31). It may be that mycolactone's mechanism of action and molecular target(s), which until now have remained elusive, will have to be determined for each of the cell types involved in the pathogenesis of BU. Indeed, mycolactone has been previously shown to result in the rapid cessation of all protein synthesis in L929 fibroblasts (3), in stark contrast to our findings in primary human monocytes (Fig. 5*C*). This difference is probably explained by differences in mycolactone cytotoxicity between the two types of cell: L929 cells enter cell cycle arrest, leading to cell death. In contrast, primary human monocytes are not proliferating and more resistant to mycolactone's cytotoxic effects (Fig. 1).

Irrespective of the underlying molecular mechanism, these findings have important implications for studying the immune response to BU. Several groups have used qRT-PCR for cytokine transcripts as markers for immune system activation and found that such transcripts are present in regions that also have higher levels of cellular infiltration (32–34). However, our findings would argue that this mRNA might be functionally inert and unable to produce inflammatory mediators if these transcripts have been derived from monocytes exposed to mycolactone. Clearly, this will relate to the distance between the cells and the bacilli as well as the (as yet unknown) distance that mycolactone can diffuse through tissue. It will also be important to determine whether mycolactone exhibits translational control in other cell types present in BU and how these interact to regulate the cytokine milieu. Only then will clear interpretation of RT-PCR data of patient biopsy samples be possible.

The pathogenesis of BU involves responses of many different cell types to *M. ulcerans* and mycolactone. Fibroblasts, adipocytes, skeletal muscle, endothelial cells, and keratinocytes are all present in the affected structures. In addition, different types of cellular infiltration have been observed, including T cells, macrophages, dendritic cells, and, in some cases, neutrophils. Although TNF is produced predominantly by monocytes/macrophages, other chemokines like IL-8 can be produced by many of these cell types, including dendritic cells. Coutanceau et al. (13) have reported that IL-8 production by dendritic cells is not affected by mycolactone, suggesting that IL-8 may indeed be present in BU lesions from sources other than monocytes in which IL-8 production is inhibited. It is important to emphasize that mycolactone seems to act in a cell- and gene-specific manner, and it will be important to delineate its activity in all involved cells.

Several authors have now shown that there is Th1/Th2 skewing in the circulating T cells of patients with BU, and that this changes as the ulcer advances through nodular to ulcerative stages of disease (33, 35–40). In this study, the ex vivo responses of PBMCs or whole blood to mycobacterial extracts or purified mycobacterial Ags are studied, and have been shown to be capable of secreting both IFN-γ and IL-10, presumably by the T cells in the mixed cultures. We did not address secondary (adaptive) responses to processed Ags or the direct effect of mycolactone on T cell-derived cytokines in our study; instead, we studied the innate immune response in monocytes. It would be premature to hypothesize a direct link between inhibition of cytokine translation by mycolactone in monocytes and the cellular response to *M. ulcerans* infection and tissue necrosis. However, recently mycolactone was shown to be present in the PBMCs of BU patients (41).

BU has been designated a neglected tropical disease by the World Health Organization, and there is currently a great need for improved diagnostics and treatments. Identification of the molecular target(s) of mycolactone will clearly be of great importance in understanding the development of the disease and providing novel pathways for pharmacological intervention. A shift in focus away from the classical signaling pathways of immune cell activation toward translational control is a significant step in that understanding.

Acknowledgments

We are grateful to Dr. Pam Small (University of Knoxville) for the generous gift of purified mycolactone and to Dr. Jerry Saklatvala (Kennedy Institute of Rheumatology, Imperial College London) for the total p38 Ab. We are also grateful to Dr. David Wright for helpful discussions and for critical reading of the manuscript. This paper is dedicated to the life and work of Brian Foxwell.

Disclosures

The authors have no financial conflict of interest.

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