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*J Immunol* published online 30 December 2009
http://www.jimmunol.org/content/early/2009/12/30/jimmunol.0902854
Mycolactone Suppresses T Cell Responsiveness by Altering Both Early Signaling and Posttranslational Events

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Mycolactone is a diffusible lipid toxin produced by Mycobacterium ulcerans, the causative agent of a necrotizing skin disease referred to as Buruli ulcer. Intriguingly, patients with progressive lesions display a systemic suppression of Th1 responses that resolves on surgical excision of infected tissues. In this study, we examined the effects of mycolactone on the functional biology of T cells and identified two mechanisms by which mycolactone suppresses cell responsiveness to antigenic stimulation. At noncytotoxic concentrations, mycolactone blocked the activation-induced production of cytokines by a posttranscriptional, mammalian target of rapamycin, and cellular stress-independent mechanism. In addition, mycolactone triggered the lipid-raft association and activation of the Src-family kinase, Lck. Mycolactone-mediated hyperactivation of Lck resulted in the depletion of intracellular calcium stores and downregulation of the TCR, leading to impaired T cell responsiveness to stimulation. These biochemical alterations were not observed when T cells were exposed to other bacterial lipids, or to structurally related immunosuppressors. Mycolactone thus constitutes a novel type of T cell immunosuppressive agent, the potent activity of which may explain the defective cellular responses in Buruli ulcer patients. The Journal of Immunology, 2010, 184: 000–000.

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Received for publication August 27, 2009. Accepted for publication November 29, 2009.

This work was supported by the Agence Nationale de la Recherche (ANR-07-MIME-016-01), the Ligue Nationale contre le Cancer, and the financial support of the National Institute of the Allergy and Infectious Diseases, National Institutes of Health, contract N01-AI-25469 and Leprosy Research Support.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902854

Mycobacterium ulcerans is an emerging human pathogen causing an ulcerative skin disease called Buruli ulcer disease (BUD) (1). The extensive cutaneous and s.c. lesions provoked by BUD often lead to gross deformities and permanent disability. During the past 10 years, major advances have been accomplished in the control of BUD through the improvement of case recording and the definition of antibiotic therapies that are particularly effective at the early stages of the disease (2, 3). Despite these progresses, surgical excision of the lesions with skin grafting remains the only alternative for advanced ulcers. To minimize the morbidity and disability associated with BUD and to propose preventive approaches against M. ulcerans, it is essential to progress in our understanding of the pathogenesis of this disease (4). Several lines of evidence suggest that mycolactone, a macrolide toxin uniquely produced by M. ulcerans and essential for bacterial virulence, may contribute to the establishment of long-term infections by suppressing host immune defenses (5, 6). This notion is supported by the histopathological features of Buruli ulcers, which are characterized by a relative lack of inflammation regarding the extent of cellular necrosis (7).

Mycolactone is able to diffuse into and induce death of a variety of mammalian cells by an apoptosis-dependent mechanism (8, 9). In addition, the molecule displays a range of immunomodulatory effects at noncytotoxic concentrations on several types of APCs. There is complete inhibition of TNF production by monocytes and macrophages postinfection with M. ulcerans, or incubation with exogenous mycolactone (10–12). The TLR-induced production of multiple cytokines and chemokines is blocked at a posttranscriptional level in monocytes treated with mycolactone (13). In dendritic cells, mycolactone suppresses the TLR-induced production of inflammatory chemokines in a more selective manner, without major impact on their cytokine production (14). The molecular basis of these immunosuppressive effects has remained so far mysterious (1).

Previous work using crude preparations of mycolactone suggested that the immunomodulatory properties of mycolactone may extend to lymphocytes, as evidenced by their inhibitory action on the production of IL-2 by activated T cell lines (11). We have recently confirmed this finding on primary T cells using human peripheral blood CD4+ T lymphocytes and have shown that the effects of mycolactone are not restricted to IL-2, since it blocked the activation-induced production of IFN-γ, IL-4, IL-17, IL-10, TNF, IL-8, and MIP-1β (15). Several independent studies have reported defective systemic production of IFN-γ in patients with active ulcers (16–20). Our recent multiplex analysis of the immunological profile of BUD patients showed that the production of several other Th1, Th2, and Th17 cytokines was altered. These cellular response defects were independent of the activation stimulus. Moreover, they resolved after surgical excision of the lesions or antibiotic treatment, demonstrating their association with the presence of bacteria (15,
20–23). In experimentally infected animals, mycolactone has been shown to diffuse from cutaneous lesions and to gain access to the mononuclear cells of peripheral blood and lymphoid organs (24). Although direct assessments of mycolactone distribution in human patients have not been possible yet, because of the lack of detection reagents, the previously discussed observations collectively suggest that mycolactone interferes with T cells in vivo and impairs the development of cellular immunity in BUD patients.

In the current study, we have investigated in detail the impact and molecular basis of mycolactone action in human T cells. We show that mycolactone is a potent suppressor of lymphocyte effector functions that acts in at least two ways: by prompting the dysregulation of intracellular signaling pathways coupled to TCR activation, and by inhibiting the activation-induced production of cytokines. The dramatic consequences of mycolactone exposure on the capacity of T cells to respond to stimulation may help explain the abnormal cellular immune responses in BUD patients. Moreover, they highlight the potential of this natural macrolide as a T cell immunosuppressive agent.

Materials and Methods
Mycolactone preparation
Mycolactone was isolated from exponentially growing cultures of M. ulcerans 1615 (ATCC 35840), as previously described (6). The concentration and purity of the preparations were determined by means of HPLC-MS/MS (24). Purified mycolactone solutions were kept at −20°C, protected from light.

Reagents
The anti–PLC-γ1 mAb B-6-I-4 was from Upstate Biotechnology (Lake Placid, NY) and the anti-Lck Ab from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Fyn Ab, anti–phospho-ZAP-70 (Y319) Ab, anti–ZAP-70 mAb 99F2, anti-LAT Ab, anti–phospho-PLC-γ1 (Y783) Ab, anti–phospho-ERK (T202/Y204) Ab, anti–ERK Ab, anti–phospho-JNK (T183/Y185) Ab, anti–NK Ab, anti–phospho-p38 (T180/Y182) Ab, anti–p38 Ab, and anti–phospho-Src (Y416) Ab were from Cell Signaling Technology (Beverley, MA). This latter Ab was used to detect both Lck and Fyn activated forms. FITC-conjugated anti-human CD3 (clone SK7; BD Biosciences, San Jose, CA) and PE-conjugated anti-CD28 Abs (both at 10 μg/ml) were from Cell Signaling Technology (Beverley, MA). The specificity of the staining (data not shown). Samples were analyzed on the basis of the total amount of proteins per lane.

Animals
Six-wk-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in a BSL-3 animal facility at the Institut Pasteur, in full compliance with French and European regulations and guidelines on experiments with live animals.

RNA extraction and RT-PCR
Total cellular RNA was extracted from Jurkat E6.1 cells (referred to as Jurkat cells) using the Qiagen RNeasy Mini Kit and then digested with TURBO DNase (Ambion, Austin, TX) for 15 min at 37°C. First-strand cDNA was synthesized from 2 μg total RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The expression of IL-2 transcripts was quantified by real-time RT-PCR using the TaqMan Gene Expression Assays probe (Assay ID Hs00174114_m1, Applied Biosystems). Amplification experiments were performed in triplicate, from 30 ng cDNA template in a final volume of 20 μl in a 96-well PCR plate. Amplification conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C with the ABI 7300 Sequence Detection System (Applied Biosystems). Results were normalized with the 2−ΔΔCt method using 18S as a reference (TaqMan Gene Expression Assay/Eukaryotic 18S rRNA, Assay ID Hs009999901_s1).

Cell cultures
Human and mouse PBL were isolated from whole blood by sedimentation on a Ficoll-Hypaque gradient (GE Healthcare) or Mammal Lympholyte (Cedarlane Laboratories, Burlington, Ontario, Canada), respectively, followed by adherence to plastic. PBL and Jurkat cells were cultured in RPMI 1640 medium (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated FCS (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich), 100 IU penicillin/ml, and 100 μg streptomycin/ml.

IL-2 induction by mycolactone
Human T cells were inoculated in 96-well plates (5 × 103 cells per well) in the presence of increasing doses of mycolactone for 6 h (Jurkat) or 24 h (PBL), and then stimulated for 24 h with 50 ng/ml PMA and 1 μg/ml calcium Iono, or with 10 μg/ml anti-CD3 and anti-CD28 Abs. The release of IL-2 into culture supernatants was assessed by ELISA, using the Duoset kit from R&D Systems (Minneapolis, MN), according to the manufacturer’s protocol.

IL-2 intracytoplasmic staining assay
Sixteen hours after s.c. injection of 50 μg mycolactone, blood aliquots from C57BL/6 mice (n = 6) were pooled and a PBL-enriched fraction isolated by sedimentation over Mammal Lympholyte. Spleens were harvested and homogenized independently by sieving through 200-μm mesh, and spleenocytes were then resuspended in culture medium. PBL and spleen cells were plated in 96-well plates at 5 × 103 cells per well in the presence of anti-CD3 and anti-CD28 Abs (both at 10 μg/ml), 50 ng/ml PMA, and 1 μg/ml calcium Iono, or in the absence of stimuli at 37°C. Two hours later, brefeldin A (10 μg/ml, Sigma-Aldrich) was added to the cells. After 6 h of stimulation, cells were fixed with 4% (w/v) paraformaldehyde 20 min at room temperature and permeabilized in 0.1% BSA, 0.5% saponin in PBS. Cells were then stained with APC-conjugated anti-mouse IL-2 and FITC-conjugated anti-CD4 (both from BD Biosciences). Controls performed in the absence of anti-IL-2 Ab, or by preincubation of anti-mouse IL-2 Ab with 0.25 μg IL-2 were performed, to confirm the specificity of the staining (data not shown). Samples were analyzed on a FACS calibur (Becton-Dickinson, San Jose, CA).

EMSA
Nuclear extracts were prepared with the nuclear extraction kit (Panomics, Ozyrne, Montigny-Le-Bretonneux, France). Lamin B1, as detected by Western blot, was used as an internal standard for purity and concentration determinations. Nuclear extracts were analyzed with the EMSA “gel shift” kit using the NE-PER nuclear extract kits (Pierce) and NF-κB (AY1030) probes (all from Panomics), according to the manufacturer’s protocol. The specificity of binding was assessed by preincubating cell lysates with a 50× excess of unlabeled probe.
Calcium measurements

Jurkat cells (2 × 10⁶) were washed once and loaded with 1 μM fura2-AM (Molecular Probes, Eugene, OR) in a 50 mM HEPES buffer pH 7.2 supplemented with 120 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM KCl, 1 mM NaHPO₄, and 1 mg/ml glucose. Fluorescence of the cellular suspension was measured in quartz cuvettes thermostatically controlled at 37°C with a Perkin-Elmer LS-5B luminescence spectrometer (Perkin-Elmer, Waltham, MA). The cell suspension was excited alternatively at 340 and 380 nm and the fluorescence measured at 510 nm. The intracellular calcium response to stimulation was studied by measuring cell fluorescence after addition of 2.5 μM ionomycin in the presence of 20 mM EGTA.

In vitro kinase assay

The assays were performed in standard reactions containing 375 μM Src-kinase substrate peptide (Upstate Biotechnology) in 100 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 5 mM MnCl₂, 0.3 mM Na-orthovanadate, 1.2 mM DTT, 25 μM ATP, 0.5 μCi (γ³²P) ATP (GE Healthcare). When specified, mycolactone was added at a final concentration of 10 μM. The reactions were initiated by addition of the immunoprecipitated kinases, and allowed to proceed for 30 min at 30°C. [³²P] incorporation was measured by spotting 20 μl of each reaction mixture onto a 2 × 2-cm square of Whatman P81 phosphocellulose filter paper. Filters were thoroughly washed in 1%orthophosphoric acid, air-dried, then submitted to autoradiography and phosphorimaging (Typhoon 9400, GE Healthcare).

Detergent-resistant membrane microdomain preparation

Jurkat T cells (2–5 × 10⁶) were sonicated gently (four 5-s bursts in 0.5 ml ice-cold TNE buffer [25 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM EDTA]) supplemented with a mixture of protease and phosphatase inhibitor (EDTA-free inhibitor mixture, Roche, Meylan, France), 1 mM PMSF, and 1 mM Na-orthovanadate. After centrifugation at 800 g for 10 min, the postnuclear supernatant was incubated with Triton X-100 at a final concentration of 0.4%, followed by sequential with 30% and 5% sucrose. After centrifugation at 38,000 rpm for 1 h at 4°C, lysates were then mixed with 80% sucrose and overlaid sequentially with 1.8 M sucrose. Confocal microscopy and quantitative image analysis

Confocal microscopy was carried out on a Zeiss LSM510 (Zeiss, Oberkochen, Germany) using a 63× objective. Image deconvolution was performed using the Huygens Deconvolution Software (Scientific Volume Imaging BV, Hilversum, The Netherlands).

Protein phosphorylation multiplex analysis

Changes in the phosphorylation of tyrosine residues on Lck, CD3ε, LAT, and Erk1/2 were measured by LumineX, using reagents provided in the BeadLyte 7-plex human TCR signaling kit (Millipore, Bedford, MA). Working solutions of the various compounds to assess were prepared in TBS at 10 or 100 μg/ml, then diluted 1/1000 in cell cultures and incubated for 16 h at 37°C. The impact of Src-family kinase inhibition was assessed by incubating control wells with 10 μM PP2 for 2 h before the end of treatment. Cell lysates were prepared as described previously and diluted to a final concentration of 1 μg/μl. Cell lysis (25 μl) was incubated with Ab-linked beads overnight at 4°C, washed twice with wash solution, and incubated for 1 h with biotinylated secondary Abs. A final incubation of 30 min with streptavidin-PE preceded the acquisition on the LumineX 100IS, and the analysis of fluorescence intensities with the OmniViz program.

Results

Mycolactone blocks TCR- and PMA/Iono-induced production of IL-2 by T cells

We have shown previously that s.c. injected mycolactone gains access to the mononuclear cells present in the peripheral blood and spleens of mice (24). To evaluate the impact of mycolactone diffusion on the functional properties of host lymphocytes, C57BL/6 mice were injected with mycolactone via the s.c. route and 24 h later, the capacity of PBL and splenocytes to produce IL-2 on stimulation was assessed ex vivo. The IL-2 response of PBL to CD3/CD28 or PMA/Iono stimulation was markedly reduced in mycolactone-injected mice (Fig. 1A and data not shown). This was also the case for spleen lymphocytes, when either CD3/CD28 or PMA/Iono were used as activation stimuli (Fig. 1B).

This inhibitory role was further examined in vitro, by measuring the activation-induced production of IL-2 by mouse or human PBL treated with mycolactone. A dose-dependent inhibition of cytokine production was observed irrespective of the stimulating agent, with >75% suppression of IL-2 production in the presence of only 16 ng/ml (20 nM) of mycolactone (Fig. 1C). Notably, mycolactone exerted comparable inhibition on the activation-induced production of IL-2 by Jurkat cells (Supplemental Fig. 1). In both cell systems, mycolactone displayed biological activity with only marginal induction of cell apoptosis (Fig. 1D, Supplemental Fig. 1). These results demonstrate that mycolactone...
displays direct immunosuppressive effects on T cells, that we investigated in the Jurkat cell model.

*Mycolactone alters the activation-induced production of IL-2 at several levels*

We examined whether mycolactone altered the expression of IL-2 at the transcriptional level. Using RT-PCR, we observed a reduction in transcription of the IL-2 gene in Jurkat cells exposed to mycolactone for 16 h prior to stimulation via CD3/CD28 (Fig. 2A). In contrast, the PMA/Iono-induced transcription of IL-2 was augmented by mycolactone, with enhanced production of IL-2 transcript after only 1 h of exposure to mycolactone (Fig. 2A).

![Figure 2A](http://www.jimmunol.org/)

**FIGURE 2.** Mycolactone blocks the activation-induced production of IL-2 at several levels. A, IL-2 mRNA expression in Jurkat cells treated with 100 ng/ml mycolactone or not (control [Ctrl]), then stimulated with anti-CD3 and anti-CD28 Abs for 6 h, PMA/Iono for 3 h, or left unstimulated. Data are mean ± SD of triplicates, compared with paired Student t tests (*p ≤ 0.05). B, Effect of mycolactone on the binding activity of NF-κB, AP-1, tandem repeats of NFAT/AP-1, or the whole IL-2 promoter region. Transfected cells were incubated with mycolactone for 24 h or not (Ctrl), then activated for 6 h. Data are mean luminescence intensities ± SD of quadruplicates, compared by ANOVA with Tukey posttest (**p ≤ 0.001, ***p ≤ 0.01, *p ≤ 0.05; NS: not statistically different). C, Binding activity of NFAT and AP-1 in Jurkat cells incubated with 100 ng/ml mycolactone or not (Ctrl), stimulated with PMA/Iono for 1 h, as evidenced by EMSA. As specificity controls, lysates of PMA/Iono-stimulated cells were preincubated with 50X unlabeled probe. Data are representative of three independent experiments.

The expression of the IL-2 gene is under the control of a promoter containing binding sites for several transcription factors, including the NFAT, NF-κB, and AP-1 (25). To evaluate the effect of mycolactone on transcription mediated by these factors, Jurkat cells were transiently transfected with reporter plasmids encoding the luciferase gene under the control of NF-κB, AP-1, NFAT/AP-1 binding sites or the whole IL-2 promoter region. When Jurkat cells were treated with mycolactone for 16 h, expression of the luciferase gene by any of these constructs after cell activation via CD3/CD28 was dose-dependently inhibited (Fig. 2B). No such inhibitory effect was observed when mycolactone-treated Jurkat cells were stimulated...
with PMA/Iono. In this case, even though the production of IL-2 protein was completely abolished, mycolactone augmented the PMA/Iono-induced activation of NFAT, AP-1, and IL-2 promoter, whereas NF-κB remained unchanged (Fig. 2B). Intriguingly, in the absence of stimulation mycolactone induced substantial nuclear localization of both NFAT and AP-1 as evidenced by an EMSA, whereas the basal activity of NF-κB was not modulated (Fig. 2C and data not shown). The augmentation of NFAT and AP-1 binding activity was detectable after 1 h of treatment with mycolactone, and increased over time. It did not result in an enhanced transcription of IL-2, as IL-2 mRNA were undetectable in unstimulated, mycolactone-exposed T cells (data not shown). Collectively, these results thus indicate that mycolactone alters the activation-induced production of IL-2 in two ways: by suppressing the TCR-induced transcription of the IL-2 gene, and by blocking the translation of IL-2 message at a posttranscriptional level.

**IL-2 production arrest is not the result of a stress response or mammalian target of Rapamycin complex 1 inhibition**

Like its structural analog Rapa, we postulated that mycolactone may block the synthesis of IL-2 in T cells by modulating the activity of the mammalian target of Rapamycin complex 1 (mTORC1) (26, 27). However, in contrast to Rapa, mycolactone did not alter the constitutive activation of mTORC1 in Jurkat cells, or the TCR-induced activation of mTORC1 in PBL, as evidenced by phosphorylation of mTORC1 substrates 70S6 kinase and the eukaryotic initiation factor 4E-binding protein 1 4E-BP1 (Fig. 3A). Downmodulation of protein synthesis may occur in eukaryotic cells as a general response to exogenous stressors, via the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) (28). Whereas phosphorylation of eIF2α was efficiently induced by heat shock in Jurkat cells, no evidence of integrated stress response to mycolactone could be demonstrated (Fig. 3B).

**Stimulation of MAPK activity and elevated cytoplasmic calcium in mycolactone-treated cells**

Having shown that mycolactone increases the nuclear localization and DNA binding activity of NFAT and AP-1 in T cells, we explored potential upstream events that may lead to these alterations. The nuclear translocation of AP-1 and NFAT requires the activation of different TCR-coupled signaling pathways, involving the MAP kinases and the calcium-dependent phosphatase calcineurin, respectively (25). In Jurkat cells, mycolactone induced a marked augmentation of the phosphorylation of the p44/42 MAPK (Erk1/2), whereas it had only a minor effect on the activation state of JNK and p38 (Fig. 4A). With regards to NFAT activation we observed an increase in the cytoplasmic level of intracellular Ca²⁺ of unstimulated, mycolactone-treated cells (Fig. 4B). This alteration correlated with an impaired capacity of mycolactone-exposed cells to mobilize their intracellular calcium in response to ionomycin stimulation in the absence of external calcium, showing that intracellular calcium stores were partially depleted by mycolactone (Fig. 4B). The impact of mycolactone on Ca²⁺ release-activated Ca²⁺ (CRAC) currents was examined by use of thapsigargin (TG). In ethanol-treated control cells, TG caused a sustained elevation of cytosolic calcium, indicative of an influx of external calcium via the CRAC channels. The TG-induced elevation of calcium declined more rapidly in mycolactone-exposed cells, suggesting that the activation of CRAC currents was limited by mycolactone (Supplemental Fig. 2).

**Mycolactone activates the Src-family kinase Lck signaling cascade in T cells**

We reasoned that a mechanism by which mycolactone causes enhanced Erk1/2 phosphorylation and calcium elevation may be a common event coupled to T cell activation. To test this hypothesis, a panel of tyrosine and serine/threonine kinases that are activated during TCR stimulation was screened. We observed that mycolactone treatment resulted in the enhanced phosphorylation of Src-family kinases in the catalytic loop (Fig. 5A). This effect was specific to Lck, as when we immunoprecipitated the two main Src-family kinases of T lymphocytes (Lck and Fyn) from cells exposed to mycolactone, only Lck was hyperphosphorylated (Fig. 5B). Enhanced phosphorylation of Lck in mycolactone-treated cells correlated with an increased enzyme activity, as demonstrated by an in vitro kinase assay using immunoprecipitated Lck and a Src-specific substrate peptide (Fig. 5C). Importantly, addition of mycolactone to immunoprecipitated Lck in vitro did not affect its kinase activity, suggesting that mycolactone does not activate Lck by direct interaction.

Phosphorylation of Lck is an early event of the TCR signaling cascade triggering the phosphorylation of ITAMs on the TCR/CD3 complex (29). Phosphorylated ITAMs create binding sites for the protein kinase ZAP-70, leading to its recruitment and activation. ZAP-70 phosphorylates the transmembrane adaptor protein, LAT, which recruits a broad range of signaling molecules leading to PLC-γ1 and MAPK activation. PLC-γ1 then initiates the release of calcium from intracellular stores. We found that ZAP-70, LAT, and PLC-γ1 were hyperphosphorylated in mycolactone-exposed T cells, whereas their protein levels were unaffected (Fig. 5A). In contrast, mycolactone did not interfere with the activity of other protein kinases, such as Akt.

To determine the contribution of Lck activation to all the previously described biochemical events, we used the selective inhibitor of Src-family kinases PP2. The addition of PP2 to mycolactone-exposed cells abrogated the phosphorylation of Lck without altering the basal level of the protein (Fig. 5D and data not shown). Similarly, mycolactone-induced phosphorylation of ZAP-70, LAT, PLC-γ1 and Erk1/2 was inhibited by PP2. PP2 also abolished the elevation of cytoplasmic calcium in Jurkat cells exposed to mycolactone (Fig. 5E). Furthermore, no increase in the cytoplasmic level of calcium could be induced by mycolactone in the Lck-deficient Jurkat cell variant JCaM1, demonstrating the critical importance of Lck in the biological activity of mycolactone (Fig. 5E).
Mycolactone-treated T cells downregulate TCR expression

In addition to triggering downstream cascades, Lck activity regulates the expression of the TCR/CD3 complex (30). In Jurkat cells exposed to mycolactone for 16 h, the expression of the TCR\(\gamma\)-chain was dramatically reduced (Fig. 6A). Surface expression of the CD3 complex, as measured by flow cytometry was also strongly downregulated. This inhibitory effect was selective, as the level of expression of CD28 was hardly modified by mycolactone treatment (Fig. 6B). Inhibition of TCR expression was dependent on the activation of Src-family kinases, as PP2 treatment restored both TCR\(\gamma\) and CD3 expression levels in mycolactone-treated cells (Fig. 6C). Downregulation of CD3, but not CD28, was similarly observed in PBL incubated with mycolactone for 4 d (Fig. 6D).

Recruitment of Lck to plasma membrane lipid rafts in mycolactone-exposed T cells

As the phosphorylation of Lck critically depends on its association with lipid rafts, we examined whether mycolactone recruits Lck to these detergent-resistant membrane microdomains (DRMs). Analysis of mycolactone-exposed T cells by confocal microscopy revealed that in resting cells, Lck distributes at the cortical level and in an intracellular compartment, in close proximity to the raft-associated glycosphingolipid GM1 but with only marginal colocalization (Fig. 7A). Strikingly, after only 30 min of exposure to mycolactone, all Lck colocalized with GM1 at the plasma membrane. This finding was confirmed by density gradient fractionation of Jurkat T cells, after lysis with Triton X-100. The global distribution of
GM1 was not affected by a 16 h treatment with 100 ng/ml mycolactone (Fig. 7B). However, we noted a significant accumulation of DRM-associated Lck in mycolactone-exposed T cells, compared with untreated controls (Fig. 7B). Lck concentrated in the lipid rafts of mycolactone-treated cells in the activated form, as demonstrated by phosphorylation of the activating residue Tyr394. In contrast, the levels of Fyn or the transmembrane adaptor Cbp/PAG were not modified by toxin treatment (Fig. 7B and data not shown).

Mycolactone differs from other lipophilic molecules by its stimulatory activity on the Lck signaling cascade

The data presented in Figs. 4–6 show that mycolactone-induced activation of Lck, as witnessed by its action on TCR surface expression and intracellular calcium stores, impairs profoundly the capacity of T cells to respond to stimulation. We next examined to what extent the stimulatory effect of mycolactone on Lck is specific to this lipid. We used a multiplex approach allowing us to detect simultaneously changes in the tyrosine phosphorylation status of Lck, CD3ε, LAT, and Erk1/2. In accordance with our previous observations, cell exposure to mycolactone triggered the phosphorylation of Lck, CD3ε, LAT, and Erk1/2 (Fig. 8). These effects were abolished in the presence of PP2 (data not shown). In contrast, equivalent concentrations of E. coli LPS, M. smegmatis lipooarabinomannans (LAMs), or M. leprae phenolic glycolipid (PGL) did not induce significant phosphorylation of these proteins. This was also the case for Rapa and cyclosporin A, two natural immunosuppressors sharing structural homology with mycolactone, thus establishing the functional specificity of mycolactone.

Discussion

The pathogenesis of Buruli ulcers is thought to be essentially mediated by the production of mycolactone at the site of infection (6). The extensive tissue necrosis and minimal inflammation in Buruli ulcers constitute the hallmarks of these lesions, and reflect the cytocidal and immunosuppressive properties of this original macrolide (1). Mycolactone is able to diffuse rapidly within target cells, as shown by the cytosolic accumulation of fluorescent derivatives (31). Mycolactone then triggers diverse cytopathic effects, including cytoskeletal rearrangements and cell cycle arrest, eventually culminating in apoptotic/necrotic cell death (9). The manifestations and levels of mycolactone cytotoxicity vary extensively among cell types, suggesting that the molecular target of mycolactone may be differentially expressed or have different functions in different cells. At noncytostatic and cytotoxic concentrations, mycolactone displays immunomodulatory properties on human primary monocytes and dendritic cells, indicating that it may limit the initiation of innate immune responses in vivo (13, 14). In this study, we show that mycolactone also impacts the generation of T cell-derived cytokines and thereby potentially impacts adaptive immune responses.
Mycolactone were able to suppress IL-2 production by T cells, in response to mycolactone addition to the cells, suppression of IL-2 production was induced more rapidly and this concentration of mycolactone did not affect TCR signaling cascade by mycolactone results in downregulation of the Lck signaling cascade.

We identify two molecular mechanisms by which mycolactone limits T cell responsiveness to stimulation. One of them involves a posttranscriptional, mTORC1- and stress-independent suppression of activation-induced IL-2 production. This effect is induced independently of TCR stimulation and is likely to extend beyond IL-2, as mycolactone also blocked the PMA/Iono-induced production of a number of cytokines and chemokines by human peripheral blood CD4+ T lymphocytes. Another consequence of mycolactone diffusion into T cells was the hyperactivation of Lck, a Src-family kinase primarily expressed by T lymphocytes. Our in vitro findings suggest that both inhibitory processes occur in T cells, with different dose responses and kinetics. In contrast to Lck-mediated down-modulation of CD3, which required 4 d of exposure to mycolactone in primary T cells, posttranscriptional suppression of IL-2 production was induced more rapidly and could be observed when mycolactone was added to the cells concomitantly with stimulating agents. Inhibition of IL-2 synthesis required lower concentrations of mycolactone, as 10 ng/ml mycolactone were able to suppress IL-2 production by >2-fold, whereas this concentration of mycolactone did not affect TCR levels (Figs. 1C, 6D). As inhibition of TCR-induced signals and IL-2 protein expression cannot be studied independently, it is difficult to establish their individual contribution to mycolactone-induced T cell unresponsiveness. The fact that Src-family kinases are activated in PBL of mice injected with mycolactone nevertheless shows that the hyperactivating activity of mycolactone on Lck is relevant in vivo, and suggests that the downstream cascade of events we describe in vitro contributes to limit TCR-mediated signals (Supplemental Fig. 3).

Mycolactone induced major changes in the partition of Lck between intracellular and plasma membrane compartments, promoting the concentration of Lck within plasma membrane lipid rafts after only 30 min of exposure. These data thus suggest that mycolactone may achieve Lck activation by promoting its transport to DRMs. Mycolactone-provoked accumulation of Lck in plasma membrane rafts may alter the balance with its regulators Csk and CD45, leading to kinase activation by autophosphorylation. Although the mechanism governing Lck redistribution is intriguing, we have focused this study on its consequences on T cell functional biology. We show that mycolactone-mediated activation of Lck alters the capacity of T cells to respond to stimulation in several ways. First, activation of the Lck signaling cascade by mycolactone results in downregulation of the TCR/CD3 complex, thereby impairing T cell responsiveness to anti-CD3/CD28 cross-linking. Second, mycolactone-induced activation of PLC-γ1 leads to intracellular Ca2+ store depletion and failure to mobilize Ca2+ appropriately in response to Iono stimulation. Both effects may account for the impaired IL-2 transcription in mycolactone-treated cells, as previously reported in various T cell anergy models (32). Increased basal Lck activity, or altered intracellular Ca2+ concentrations have been described in anergic human Th2 or Th1 clones, respectively (33, 34). Autoreactive B cells rendered anergic by the continuous BCR engagement by a cross-reactive Ag display an increased intracellular Ca2+, dependent on Src-kinase activity (35). Hence, we propose that mycolactone-mediated constitutive activation of Lck leads to functional anergy in T cells by mimicking persistent TCR stimulation in the absence of costimulatory signal. In accordance with this hypothesis, mycolactone-exposed T cells still failed to produce IL-2 24 h after mycolactone was removed from cell culture supernatant (Supplemental Fig. 4). These findings may explain the systemic suppression of Th1 cellular responses in BUD patients and the fact that defects in T cell responses are not confined to mycobacterial Ags and extend to various Ags and stimuli.

It is not clear at this stage if the effects of mycolactone on Lck kinase activity and on IL-2 synthesis are connected. However, our results do allow us to conclude that the mechanism used by
mycolactone to inhibit T cell responsiveness to stimulation is potent and distinct from that of known immunosuppressive compounds. As far as we know, this is the first example of T cell immunosuppression involving the hyperactivation of Lck. In humans, the Src-family of kinases comprises nine members that are either ubiquitously expressed or restricted to specific tissues or cell lineages (36). Although Lck was the principal target of mycolactone in T cells, the stimulatory activity of mycolactone may apply to other kinases of the Src-family expressed by other cell types, such as dendritic cells, and help explain the highly variable effects of mycolactone. One future challenge will be to further dissect the effects of mycolactone in immune and nonhematopoietic cells, which we believe underlie the pathology of M. ulcerans infection.

Acknowledgments

We are grateful to Clotilde Randriamampita for technical help with the intracellular calcium measurements and to the Center for Immunology at the Institute Pasteur for support in conducting the Luminex study. We thank the Colorado State University for providing M. leprae PGL-1, and M. smegmatis LAM, received as part of the National Institutes of Health National Institute of Allergy and Infectious Diseases contract HHSN266200400091C, entitled “Tuberculosis Vaccine Testing and Research Materials,” which was awarded to Colorado State University.

Disclosures

The authors have no financial conflicts of interest.

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