Selective suppression of dendritic cell functions by *Mycobacterium ulcerans* toxin mycolactone

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Mycolactone is a polyketide toxin produced by *Mycobacterium ulcerans* (*Mu*), the causative agent of the skin disease Buruli ulcer (BU). Surprisingly, infected tissues lack inflammatory infiltrates. Structural similarities between mycolactone and immunosuppressive agents led us to investigate the immunomodulatory properties of mycolactone on dendritic cells (DCs), the key initiators and regulators of immune responses. At noncytotoxic concentrations, phenotypic and functional maturation of both mouse and human DCs was inhibited by mycolactone. Notably, mycolactone blocked the emigration of mouse-skin DCs to draining lymph nodes, as well as their maturation in vivo. In human peripheral blood–derived DCs, mycolactone inhibited the ability to activate allogeneic T cell priming and to produce inflammatory molecules. Interestingly, production of the cytokines interleukin (IL) 12, tumor necrosis factor α, and IL-6 was only marginally affected, whereas production of the chemokines macrophage inflammatory protein (MIP) 1α, MIP-1β, regulated on activation, normal T cell expressed and secreted, interferon γ–inducible protein 10, and monocyte chemoattractant protein 1 was abolished at nanomolar concentrations. Importantly, mycolactone endogenously expressed by *Mu* mediated similar inhibitory effects on β-chemokine production by DCs. In accordance with the histopathological features of BUs, our results suggest that bacterial production of mycolactone may limit both the initiation of primary immune responses and the recruitment of inflammatory cells to the infection site. Moreover, they highlight a potential interest in mycolactone as a novel immunosuppressive agent.

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Abbreviations used: ADLN, auricular draining lymph node; BU, Buruli ulcer; iDC and mDC, immature and mature DC, respectively; IP, IFN-γ–inducible protein; MCP, monocyte chemoattractant protein; MFI, mean fluorescence intensity; MIP, macrophage inflammatory protein; *Mycobacterium ulcerans*, *Mu*; PGE_2, prostaglandin E_2; PI, propidium iodide; PKS, polyketide synthase; RANTES, regulated on activation, normal T cell expressed and secreted; TLR, Toll-like receptor.

Buruli ulcer (BU), caused by *Mycobacterium ulcerans* (*Mu*), is the third most common mycobacterial disease, after tuberculosis and leprosy, and represents an emerging global threat, with a markedly increased incidence in tropical countries over the last decade (1). Infection with *Mu* leads to destruction of both cutaneous and subcutaneous tissues. The pathology of BU is strongly associated with the production of mycolactone, because injection of purified toxin into the skin of guinea pigs is sufficient to provoke ulcers, and strains of *Mu* deficient for its biosynthesis do not cause disease (2).

The human immune response to *Mu* follows a complex scheme consisting of three phases. Lesions typically start as a single, painless, acid-fast positive subcutaneous nodule, edema, or plaque, enlarging over time. Histopathological analysis of human and animal skin biopsies (3–5), as well as quantitative measurement of cytokine mRNAs in human tissues (6, 7), have shown that nodules are the site of potent Th1 cell–oriented antimycobacterial inflammatory responses. As the disease progresses, ulcers eventually form and are characterized by extensive necrosis of subcutaneous tissues and dermal collagen (4). Surprisingly, in the face of considerable cellular necrosis, there is minimal inflammation. Moreover, suppression of Th 1 responses, as indicated by defective systemic production of IFN-γ, has been repeatedly reported in BU patients with active ulcers (7–12). Importantly, the defect in the IFN-γ response by T cells is not antigen specific and resolves during healing or after surgical excision of the lesions (11, 12), thus indicating

E. Coutanceau and J. Decalf contributed equally to this study.
The online version of this article contains supplemental material.

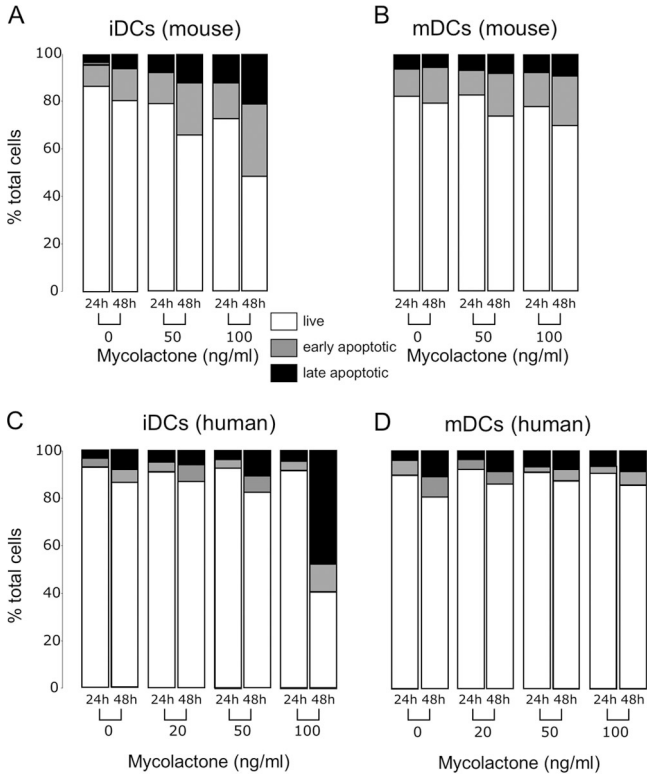

Figure 1. DCs survive exposure to mycolactone doses <50 ng/ml. (A and B) Induction of apoptosis in mouse bone marrow–derived DCs after exposure to mycolactone. The analysis was performed on $CD11c^+$-gated cells incubated with mycolactone for 24 or 48 h concomitantly with 10 ng/ml LPS (iDCs) or after 24 h of stimulation with 10 ng/ml LPS (mDCs). (C and D) Induction of apoptosis in human peripheral blood–derived DCs incubated with mycolactone for 24 or 48 h concomitantly (iDCs) or after 48 h of stimulation with TNF-α/PGE_2 (mDCs). Annexin V^+/PI^- cells were identified as early apoptotic cells, Annexin V^+/PI^+ cells were identified as late apoptotic cells, and Annexin V^-/PI^- cells were identified as live cells. Data are mean percentages and are representative of three independent experiments.

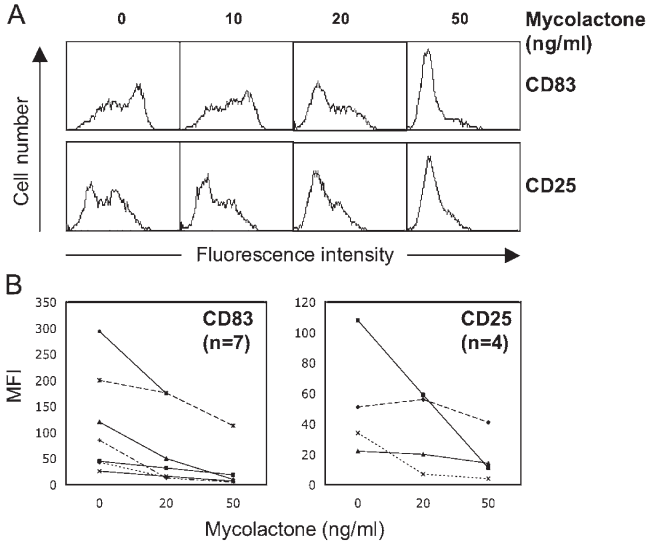

Figure 2. Mycolactone blocks DC maturation. (A) Representative histograms of CD83 and CD25 expression on human iDCs after stimulation by TNF-α/PGE_2 in the presence of increasing doses of mycolactone for 48 h. (B) MFI of CD83 and CD25 expression on iDCs after stimulation by TNF-α/PGE_2 for 48 h in the presence of mycolactone. Each line corresponds to one of the *n* donors.

that the presence of *Mu* is critical for local and systemic suppression of cellular responses.

Intriguingly, the structure of mycolactone reveals a macrocyclic polyketide, typical of a large class of natural products produced by actinomycetes. The structure of mycolactone shares similar features with the macrocyclic triene rapamycin from *Streptomyces*, the macrolide lactone FK506, and the soil fungi metabolite cyclosporine A, all potent immunosuppressive drugs altering the functional immunobiology of lymphocytes and DCs (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070234/DC1). DCs are key players in the initiation of cellular responses and the regulatory processes leading to impaired cellular immunity. On the basis of the structural resemblances of mycolactone with these immunosuppressors, and from the observation that BU patients with active ulcers display cellular anergy, we postulated that mycolactone may exert immunomodulatory properties on DCs. The present study utilizes both mouse and human primary DCs and demonstrates that mycolactone is indeed a potent suppressor of the capacity of DCs to prime cellular immune responses and produce the chemotactic signals critical for the initiation of an inflammatory response. Our results provide novel information into the pathogenesis of *Mu* infection and further characterize a potentially useful immunosuppressive agent.

RESULTS

Effect of mycolactone on DC viability

Given the data that mycolactone induces cell death in several cell types, it was initially important to assess the impact of the toxin on DC viability. The induction of apoptosis in immature and mature DC (iDC and mDC, respectively) preparations after exposure to mycolactone for 24–48 h was determined by phosphatidylserine exposure (Annexin V staining) and loss of membrane integrity (propidium iodide [PI] staining). In both mouse and human DCs, we observed a dose-dependent increase in the percentage of apoptotic iDCs (Fig. 1, A and C). Induction of apoptosis nevertheless remained marginal with mycolactone doses <50 ng/ml. Interestingly, iDCs of both mouse and human origin were more sensitive to mycolactone-induced apoptosis than mDCs (Fig. 1, B and D). This was the case when LPS or TNF was used as a maturation stimulus. Of note, only low levels of necrotic cells were observed in the cell cultures (unpublished data).

Mycolactone inhibits DC maturation

We next examined whether the maturation of DCs is influenced by exposure to mycolactone. Only mycolactone doses <50 ng/ml were considered, and PI^+ cells were excluded

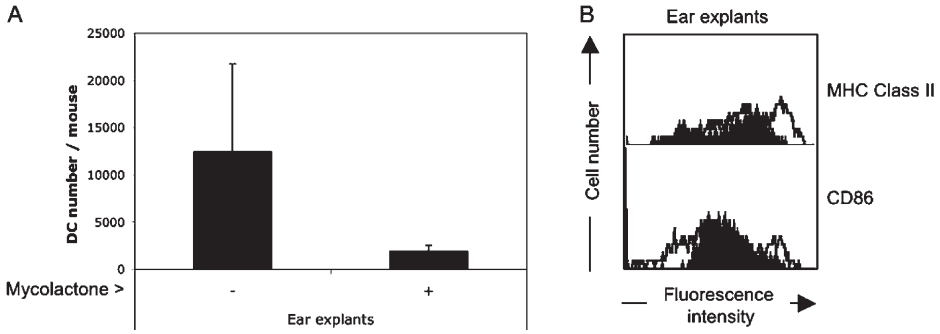

Figure 3. Mycolactone inhibits functional maturation of DCs in vivo. (A) Mice were injected intradermally into the ear with TNF-α alone (−) or TNF-α + mycolactone (+). After 4 h, mice were killed. Ear explants were placed in an overnight culture, and the number of DCs in the crawl-out population was determined. Data are means and SD of $CD11c^+$ PI^- cell numbers from pooled skin explant cultures of two independent experiments. (B) Histograms of MHC class II and CD86 expression on DCs ($CD11c^+$ PI^-) from skin explant cultures of mice injected with TNF-α alone (white) or TNF-α + mycolactone (black). Data are representative of pooled skin explant cultures from two independent experiments.

from the flow cytometric analysis. A strong inhibitory effect was seen in human iDCs matured by TNF-α/prostaglandin E_2 (PGE_2), as demonstrated by the failure to up-regulate CD83 and CD25 (Fig. 2 A). Although the mean fluorescence intensity (MFI) for CD83 and CD25 expression varied among donors, we observed a reproducible inhibition of maturation when iDCs were exposed to mycolactone (Fig. 2 B). To better quantify the inhibitory effect of mycolactone, the percentage of inhibition was calculated for several DC maturation markers (CD83, CD25, CD80, and CD40; Table I). Data from iDCs exposed to maturation stimuli for 24 and 48 h are shown. CD83 was the most affected marker, with a significant reduction in surface expression 24 h after treatment in the presence of 20 ng/ml mycolactone. CD25 expression was also markedly altered, whereas the expression of CD80 and CD40 was only marginally modified. Similarly, in mouse iDCs stimulated with LPS in the presence of mycolactone, we observed a dose-dependent inhibition of CD86 and MHC class II surface expression (unpublished data). To determine if this effect was reversible, we washed out the mycolactone after 24 h and returned them to culture wells containing maturation stimuli. In both the mouse and human culture systems, inhibition of maturation persisted after mycolactone was removed from the culture medium (unpublished data).

Mycolactone inhibits functional maturation and migration programs in DCs

To evaluate mycolactone effects on DC migration, we used an in vivo migration model in which TNF-α is injected intradermally into the ear, which triggers a massive trafficking of DCs into the auricular draining lymph node (ADLN) (13). DC migration was quantified by sacrificing the mice after 4 h and assessing the number and maturation state of DCs emigrating from the skin. When mycolactone was coadministered with TNF-α, we observed a striking reduction in the proportion of migratory DCs (Fig. 3 A). In addition, we observed a reduced CD86 and MHC class II surface expression on DCs that crawled out of the skin, as well as on DCs that reached ADLNs in the conditions under which mice had been injected with mycolactone (Fig. 3 B and not depicted). These data indicated that mycolactone impairs the capacity of DCs to undergo maturation and up-regulate the machinery required for effective migration to the draining lymph node.

We also observed an inhibition of functional maturation in human DCs. This was shown by assaying for the priming of allogeneic T cells in a mixed lymphocyte reaction. Strikingly, exposure of DCs to 10 ng/ml mycolactone during maturation resulted in >70% inhibition of their allostimulatory capacity (Fig. 4). Collectively, the data in Figs. 2–4 demonstrate that exposure of DC to noncytotoxic doses of mycolactone results in the inhibition of both phenotypic and functional maturation of DCs.

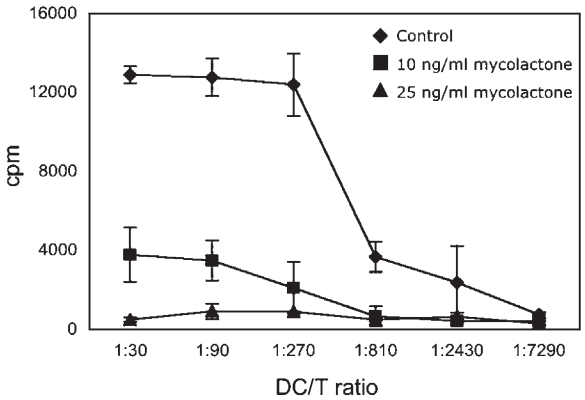

Figure 4. Mycolactone reduces the allostimulatory capacity of human DCs. The proliferation of peripheral blood lymphocytes after incubation with allogeneic mycolactone-treated DCs is shown. DCs were matured in vitro with TNF-α/PGE_2 in the presence of 0, 10, or 25 ng/ml mycolactone for 48 h and extensively washed before addition to the lymphocytes. T cell proliferation was measured after 6 d of co-culture. Data are means and SD of [^{3}H]thymidine incorporation measured on triplicate wells and are representative of five independent experiments.

Mycolactone-treated DCs fail to produce chemoattractant molecules

In addition to the IFN-γ response defect in *Mu*-infected patients, the ulcers are characterized by an abnormally low amount of inflammation. We therefore evaluated the effect of mycolactone on iDC biology, as tissue DCs play an important role in the initiation of an inflammatory response and the attraction of innate effector cells to the site of infection. We performed a broad screen of inflammatory cytokines and chemokines produced by DCs after exposure to Toll-like receptor (TLR) ligands. As shown in Fig. 5 (A and B), mycolactone treatment only moderately affected the capacity of mouse or human DCs to produce IL-12 upon stimulation with LPS or poly I:C, TLR-4, and TLR-3 ligands, respectively. Similarly, little effect was seen on TNF-α and IL-6 production in LPS- or poly I:C–stimulated human DCs (Fig. 5, D and E). In mouse DCs stimulated with LPS, mRNA expression for IL-6 was not substantially modified (Fig. 5 C). Inhibition of IL-1α and IL-1β mRNA expression was observed in LPS-stimulated mouse iDCs, but no comparable suppression of IL-1β production could be detected in LPS- or poly I:C–stimulated human DCs (unpublished data). Therefore, exposure of iDCs to mycolactone during stimulation does not critically modify their expression of inflammatory cytokines.

In contrast, the production of macrophage inflammatory protein (MIP) 1α, MIP-1β, regulated on activation, normal T cell expressed and secreted (RANTES), IFN-γ–inducible protein (IP) 10, and monocyte chemoattractant protein (MCP) 1 was markedly reduced by a 24-h exposure of human iDCs to 25 ng/ml mycolactone (Fig. 6). This was evident in both LPS- and poly I:C–treated iDCs. However, this inhibitory effect was not observed with the neutrophil chemoattractant IL-8, the expression level of which was not modified by mycolactone treatment in LPS- or poly I:C–stimulated human iDCs (Fig. 6). To evaluate whether mycolactone endogenously expressed by *Mu* could mediate similar effects, we infected iDCs with WT *Mu* and compared it with a mycolactone-deficient mutant (*mup045*). Interestingly, all of the proinflammatory effects of adding the WT *Mu* were

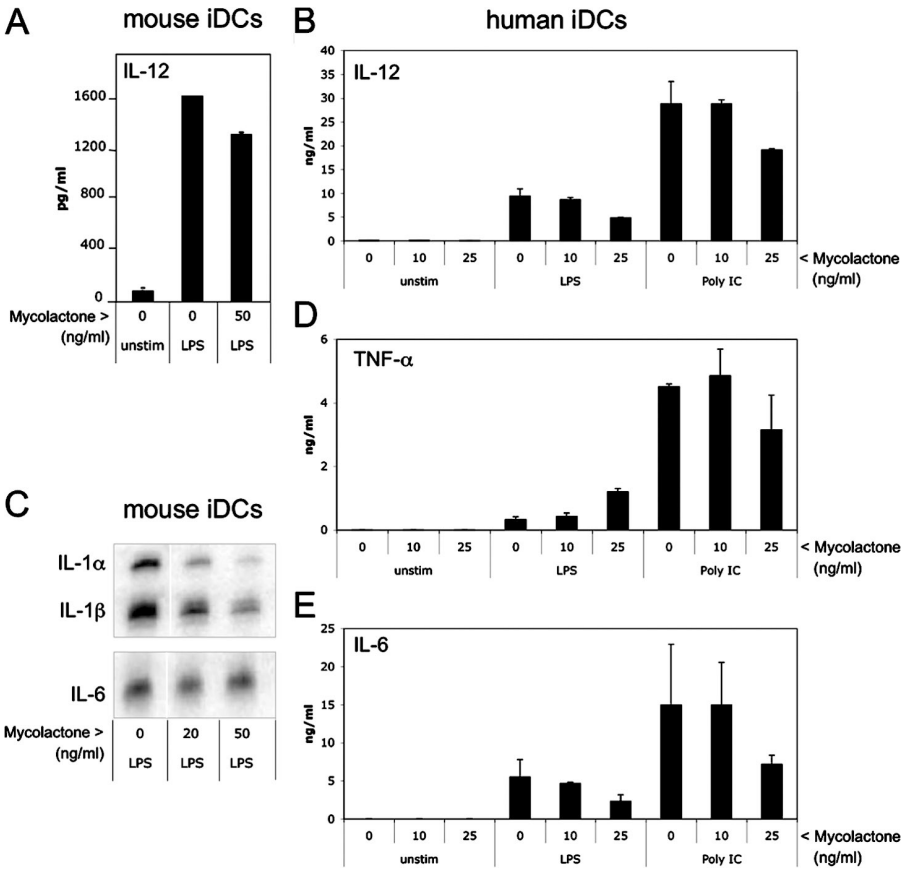

Figure 5. Mycolactone-treated DCs only moderately alter the expression of inflammatory cytokines by iDCs. (A) Concentration of IL-12 (p40 subunit) in culture supernatants from mouse iDCs stimulated with LPS in the presence of 0 or 50 ng/ml mycolactone for 48 h or left unstimulated (unstim). (C) IL-1α, IL-1β, and IL-6 mRNA expression is shown for mouse iDCs stimulated with LPS for 48 h in the presence of increasing doses of mycolactone. (B, D, and E) Production of IL-12 (p70), TNF-α, and IL-6 is shown for human peripheral blood–derived iDCs stimulated with LPS or poly I:C in the presence of increasing doses of mycolactone for 24 h. Data are means and SD and are representative of two independent experiments.

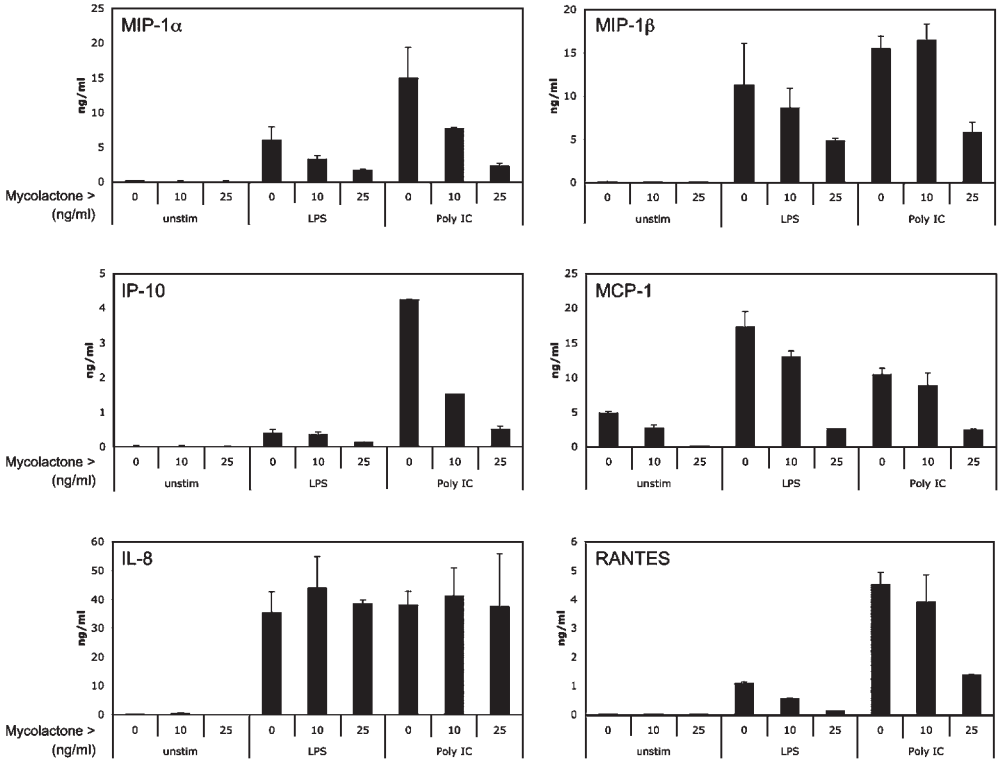

Figure 6. Mycolactone considerably affects the expression of inflammatory chemokines by iDCs. The expression of MIP-1α, MIP-1β, RANTES, IP-10, MCP-1, and IL-8 chemokines is shown for human monocyte-derived iDCs, or TNF-α/PGE_2–matured mDCs, after stimulation with LPS or poly I:C in the presence of increasing doses of mycolactone for 24 h. Data are means and SD and are representative of two independent experiments.

masked by the presence of endogenous mycolactone, whereas *mup045* was capable of inducing a moderate cytokine and chemokine response (unpublished data). In addition, we added LPS to the *Mu*-infected DC cultures and found that endogenous mycolactone was indeed capable of inhibiting the production of the β-chemokines MIP-1α, MIP-1β, and RANTES, as well as MCP-1 and IP-10 (Fig. 7 and not depicted). As a control, we added back exogenous mycolactone to the *mup045*-infected DC cultures. Based on these studies, we conclude that the immunosuppressive effects of mycolactone account for the abnormal inflammatory response in BUs.

DISCUSSION

Until recently, it was assumed that *Mu* was the only *Mycobacterium* species producing mycolactone. In fact, other mycolactone-producing mycobacteria have been isolated from fish and frogs presenting ulcerative diseases (14–17). All these species contain versions of the *Mu* virulence plasmid, produce mycolactones, and show genetic relatedness to *Mu* and *M. marinum*, a *Mycobacterium* species causing localized skin granulomas in humans and an acute lethal pathology in fish (18). On the basis of a genetic analysis, Yip et al. (18) suggested that mycolactone-producing mycobacteria may have evolved from a common *M. marinum* progenitor to form two

Table I. Inhibition of CD83, CD25, CD80, and CD40 expression in human peripheral blood–derived DCs after stimulation with TNF-α/PGE_2 in the presence of mycolactone

	iDC + TNF-α/PGE_2			
Treatment duration	24 h		48 h	
Mycolactone dose (ng/ml)	20	50	20	50
CD83	43*	77*	53*	67*
CD25	28	34*	33	59*
CD80	1	31	5	24
CD40	4	5	19	37

MFIs of mycolactone-treated DCs were compared with the MFI of control DCs using the Wilcoxon nonparametric test. Mean inhibition percentages calculated on *n* independent experiments ($n = 3–6$) are shown. *, $P < 0.07$.

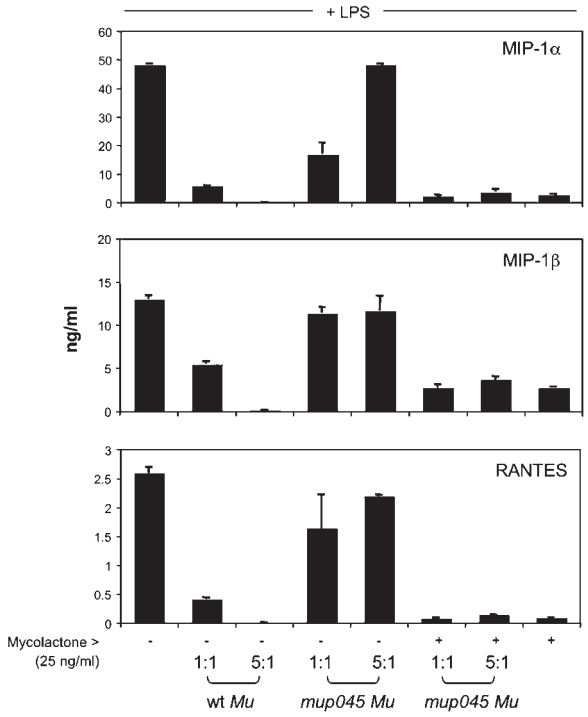

Figure 7. Endogenous production of mycolactone blocks the expression of inflammatory chemokines by *Mu*–infected iDCs. The expression of MIP-1α, MIP-1β, and RANTES is shown for human monocyte-derived iDCs after infection with WT *Mu* or a mycolactone-deficient mutant (*mup045*), using an multiplicity of infection of 1:1 or 5:1 acid-fast bacilli/cell, in the presence of 10 μg/ml LPS. Controls include medium only, iDCs stimulated with LPS in the presence of 25 ng/ml mycolactone, and iDCs infected with *mup045* in the presence of 25 ng/ml mycolactone. Data are means and SD and are representative of four independent experiments.

ecotypes causing disease in either ectotherms or endotherms, such as humans. The expression of mycolactone-like compounds nevertheless remains restricted to these particular species, and no such complex polyketides have thus far been isolated from other pathogenic mycobacteria.

Polyketides are a diverse class of secondary metabolites produced by polyketide synthases (PKSs). Mycobacteria express a remarkable arrays of PKSs, which generate multiple cell wall–associated lipids (19). At the interface with the host immune system, some of these envelope components have been shown to be important virulence factors and immune modulators. For example, in *M. tuberculosis*, glycosylation of the PKS products phenol phtiodiolones leads to the generation of phenolic glycolipids, which are potent inhibitors of proinflammatory cytokine release by monocyte-derived macrophages (20). With respect to mycolactone, the genetic basis of its biosynthesis has been recently elucidated with the isolation of a 174-kb plasmid encoding three modular PKSs (21). Interestingly, mycolactone PKSs present highly unusual features in terms of size and structure, and mycolactone is the first example of a complex polyketide isolated from pathogenic mycobacteria. In fact, the macrocyclic polyketide structure of mycolactone shares similarities with that of complex polyketides typically produced by filamentous soil actinomycetes such as *Streptomyces*. Strikingly, analysis of the chromosomal sequence reveals that numerous PKS genes are inactivated in *Mu*, suggesting that energy and PKS-specific substrates are preferentially allocated to mycolactone biosynthesis in this species (22). In the present study, we demonstrate that this original polyketide has the unique capacity to modulate DC functions in a selective manner.

The ulcerative properties of *Mu* have been attributed to the cytopathic action of mycolactone, which is able to cause growth arrest in cultured fibroblasts, as well as cell death via apoptosis in fibroblast and macrophage cell models after 3–5 d of exposure (23). Cell susceptibility to mycolactone activity nevertheless varies with the cell type. For example, *Mu* lipid extracts showed no cytostatic activity on Jurkat T cells, whereas they markedly suppressed the cell-cycle progression and viability of a mouse pre–B cell line (24). In the present study, we found that the susceptibility of DCs to mycolactone cytotoxicity was higher during the immature state. Collectively, these observations suggest that the molecular target of mycolactone is ubiquitous in eukaryotic cells but may be differentially expressed, depending on the cell type or the cell activation state.

Although the mechanism by which mycolactone acts as a cytotoxic agent is intriguing, we have focused on the immunosuppressive effects of nontoxic doses. We found that mycolactone strongly affects the maturation of both mouse and human DCs. In particular, we show a failure to up-regulate the phenotypic markers of the mature DCs CD83 and CD25, and to a lower extent CD80 and CD40. This maturation defect was not reversed by removal of mycolactone from cell-culture medium, suggesting that mycolactone durably affects DC maturation. In addition, we demonstrate that mycolactone-treated DCs show a reduced allostimulatory capacity and that they are inhibited in their ability to cross-present antigens to T cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20070234/DC1). That said, other features of maturing DCs remained intact. In particular, phagocytic activity of DCs, as measured by the quantity of intracellular fluorescent beads after 4 h, was not reduced by coincubation of the cells with 10–100 ng/ml mycolactone (unpublished data). The fact that mycolactone limits the migratory properties of DCs, as well as their ability to mature and activate antigen-specific T cells in vivo (Fig. S3), thus further suggests that mycolactone suppresses the capacity of DCs to prime cellular immune responses. These findings may help explain the global defect in IFN-γ production by T cells in BU patients and the fact that once BU lesions are excised, there is a restoration of Th1 cellular responses (12).

We also explored the effect of mycolactone on the ability of DCs to secrete proinflammatory molecules. Surprisingly, we observed a selective effect on inducible chemokines. In particular, mycolactone blocked the production of the monocyte and lymphocyte chemoattractants MIP-1α, MIP-1β, IP-10, RANTES, and MCP-1. MIP-1α, MIP-1β, and

RANTES are ligands for CC chemokine receptor 5, which is specifically expressed by iDCs and Th1 T cells (25, 26). Defective production of these inflammatory chemokines is therefore likely to interfere with both the innate and adaptive immune responses to *Mu* infection. Specifically, decreased β-chemokine production by mycolactone-exposed DCs may limit the recruitment and activation of iDC precursors to the inflammation site. In addition, defective production of these CC chemokine receptor 5 ligands, as well as the CXC chemokine receptor 3 ligand IP-10, is likely to interfere with the homing capacity of IFN-γ–secreting T cells to infected tissues. This hypothesis is strongly supported by the observation that, in contrast to DCs infected with *mup045*, DCs infected with the WT strain failed to produce the chemokines MIP-1α, MIP-1β, and RANTES. In accordance with the histopathological features of BUs, bacterial production of mycolactone may therefore be responsible for suppressing the innate immune responses of iDCs, thus preventing the trafficking of inflammatory cells to the ulcerative lesion.

The exogenous addition of mycolactone to *mup045*-infected DCs resulted in a strong reduction of their β-chemokine response to infection, further demonstrating the critical importance of the toxin in this process. Interestingly, the inhibition of β-chemokine production by exogenous mycolactone in *mup045*-infected DCs was incomplete, particularly in the case of MIP-1β, suggesting that mycolactone may regulate the expression of other immunosuppressive agents.

Suppression of these inflammatory chemokines was nearly complete with minimal doses of mycolactone, whereas the expression of the inflammatory cytokines TNF-α, IL-12, and IL-6 was only marginally modified. Accordingly, IL-12, IL-1β, and TNF-α mRNA were detected at considerable levels in lesions from BU patients and were not substantially modulated between the nodular and ulcerative stages of the disease (6), suggesting that mycolactone has little impact on the level of expression of these cytokines in vivo. Th 1 cellular responses, as measured by IFN-γ production, are vigorous during the nodular stage of BU and become defective at later stages of the disease. From our results, we can propose that, in contrast to inflammatory cytokines, defective inflammatory chemokines may constitute a hallmark of early *Mu* infection, as well as a sensitive indicator of BU progression from the nodular to the ulcerative stage.

Given the structural similarity between mycolactone and the immunosuppressive drugs FK506 and rapamycin (Fig. S1), it is interesting to examine their respective activities. FK506 and rapamycin are structural analogues binding the same intracellular receptor, FKBP12, even though the resulting complex targets a different molecule. Although FK506 and rapamycin are used clinically to suppress activated T cells, recent data suggest that they also affect DC differentiation and function (27, 28). Interestingly, FK506 has been shown to modulate the production of β-chemokines (29, 30). However, FK506 blocks the production of inflammatory cytokines such as IL-12 and TNF-α (27), as well as IL-8 (31, 32), all molecules that are not altered in mycolactone-treated DCs. Our results therefore suggest that mycolactone binds a different molecular target than FK506 or rapamycin. Further work will be required to map the precise mechanism underlying mycolactone immunosuppressive activity. In conclusion, we demonstrate in this study that mycolactone interferes with DC biology in a unique manner, which helps account for the pathologic features of BU. Moreover, we show that mycolactone inhibits DC maturation with a selective effect on the chemotactic signals critical for the initiation of an inflammatory response. This specificity of action defines a novel class of potentially useful immunosuppressive agents.

MATERIALS AND METHODS

Bacterial strains. Mu 1615 (Trudeau Mycobacterial Culture Collection strain; American Type Culture Collection) was originally isolated in Malaysia from human skin biopsies. The *mup045* mutant (MU1615::Tn118) possesses a transposon insertion inside the *mup045* gene, which results in its inability to produce mycolactone (3, 21). The Mu 1615 and *mup045* mutant strains were provided by P.L. Small (University of Tennessee, Knoxville, TN). The bacteria were grown on agar plates (7H9; DIFCO) supplemented with 10% oleic acid–albumin–dextrose (Becton Dickinson) at 31°C. For infection studies, bacteria from exponentially growing cultures were harvested and resuspended in a DC culture medium before homogenization by filtration through a syringe needle and enumeration by the Ziehl-Neelsen procedure. Bacteria were added to DC cultures at a multiplicity of infection of 1:1 or 5:1, and plates were centrifuged for 2 min at 700 rpm before incubation at 37°C.

Animals. 6-wk-old C57BL/6JIco and OT2 female mice were purchased from Charles Rivers Laboratories. Animals were maintained under specific pathogen-free conditions and used at 8–12 wk of age. All animal work was approved by institutional animal experimentation committees.

Mycolactone preparation. Mycolactone purification was performed as previously described (2). The purity of the preparation was controlled by mass spectrometry analysis, as previously described (33).

Generation of DCs from mouse bone marrow. Bone marrow–derived DCs were essentially generated by the procedure described by Inaba et al. (34). In brief, total bone marrow was recovered from the femurs of C57/BL6 mice, and cell suspensions were stained with a cocktail of M5/114 (anti–MHC class II), RA3-6B2 (anti-B220), HO2.2 (anti-CD8), GK1.5 (anti-CD4), and RB6-8C5 (anti–Ly-6G) antibodies, followed by magnetic bead negative selection. The remaining cells were cultured in RPMI 1640 supplemented with 10% FCS, 50 μM 2-ME, 2.5 ng/ml recombinant mouse GM-CSF and 10 ng/ml recombinant mouse IL-4.

Generation of DCs from human peripheral blood. PBMCs were isolated from whole blood by sedimentation over a Ficoll-Hypaque gradient (GE Healthcare). T cell–enriched and T cell–depleted fractions were prepared by adherence to plastic in 1% single donor plasma. iDCs were prepared from the T cell–depleted fraction by culturing cells in the presence of 1,000 U/ml GM-CSF (Berlex) and 500–1,000 U/ml IL-4 (R&D Systems) for 6 d. Cultured cells consisted of >75% $CD14^-$ $CD83^-$ $HLA\text{-}DR^+$ DCs, with contaminating cells being B cells and dying myeloid cells. To generate mDCs, cultures were stimulated on day 6 with 50 ng/ml TNF-α (Qbiogene) and 10 mM PGE_2 (Sigma-Aldrich) for 36–48 h, as previously described (23). At that time, cells were >85% $CD14^-$ $CD83^+$ $HLA\text{-}DR^{hi}$ DCs.

DC cytokine production analysis. The production of IL-12 by mouse DCs was measured in culture supernatants after a 48-h stimulation with 10 μg/ml LPS (Sigma-Aldrich) in the presence of mycolactone, using an ELISA assay based on the detection of p40 subunit (Biolegend). Expression of cytokine

mRNA by mycolactone-exposed mouse DCs was also measured after a 48-h stimulation with 10 μg/ml LPS, using the multiprobe RNase protection assay (BD Biosciences). In brief, a ^{32}P-labeled mcK-2b template was prepared. Total mRNA, isolated from DCs using RNAzol (Cinna/TEL-TEST), was then hybridized with the probes and digested with RNase. Protected probes were analyzed by migration on polyacrylamide gel (Bio-Rad Laboratories), according to the manufacturer's protocols. Templates for the housekeeping genes L32 and GAPDH were used to normalize the total RNA content of the samples.

Cytokine and chemokine release by monocyte-derived DCs was measured by Luminex (12-plex kit; Biosource International). 30,000–45,000 iDCs (TNF-α/PGE_2–matured DCs) were stimulated with 10 μg/ml LPS or poly I:C (Sigma-Aldrich) in 200 μl RPMI 1640 with 1% human serum for 20 h, in the presence of the doses of mycolactone indicated in the figures. Alternatively, 100,000 iDCs were infected with *Mu* (WT or *mup045*) and incubated for 20 h in the presence of 10 μg/ml LPS. 50 μl of supernatants or standard were incubated with antibody-linked beads for 2 h, washed twice, and incubated for 1 h with biotinylated secondary antibodies. A final incubation of 30 min with streptavidin-PE was performed before data acquisition (100 events minimum per bead) on a 100 IS apparatus (Luminex). Values above or below the standard curves were replaced by the lowest or highest concentrations measured.

Allogeneic mixed leukocyte reaction. 2×10^5 purified T cells per well were plated with mDCs exposed to the doses of mycolactone indicated in the figures in RPMI 1640 with 5% pooled human serum (Labquip). Co-cultures were incubated for 4–5 d at 37°C and pulsed with 1 μCi (0.037 MBq) [^{3}H]thymidine during the last 16 h of culture. [^{3}H]thymidine incorporation was measured by means of a liquid scintillation counter (Micro96; Skatron).

Skin explant cultures and preparation of ADLN cell suspensions. C57BL/6 mice ($n = 5$) were injected intradermally in the ear with 50 ng TNF-α (R&D Systems), with or without 50 μg mycolactone. 4 h later, animals were killed, and ears were surgically removed. Dorsal and ventral halves were split and layered on RPMI 1640 with 10% FCS, 100 mM Hepes, and 100 μg/ml penicillin. After 24 h at 37°C, ear skin explant migrating cells were pooled and filtered through a 70-μm nylon mesh before antibody staining. ADLN cells were dissociated from the matrix by a 25-min incubation at 37°C with 1 mg/ml collagenase D and 40 μg/ml DNase I (Roche Diagnostics) in RPMI 1640. Cells were collected and washed in PBS before FACS analysis.

Online supplemental material. Fig. S1 shows the structures of mycolactone, cyclosporin A, rapamycin, and FK506. Fig. S2 depicts in vitro cross-presentation studies. Fig. S3 shows that mycolactone inhibits the capacity of DCs to prime lymphocytes in vivo. The percentage of proliferating $CFSE^+$ cells was calculated as previously described (35). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070234/DC1.

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