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Membrane Active Immunomodulator As a Novel Therapy for an Infectious Bacterial Disease, Buruli Ulcer

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Abstract. *Background/Aim: Mycobacterium ulcerans causes the necrotizing skin disease Buruli ulcer (BU), characterized by the formation of subcutaneous lesions and immunosuppression thought to be mediated by the virulence factor mycolactone. Since early BU lesions are typically painless, patients often seek standard oral antibiotic therapy at the advanced stages when the treatment is less effective. Given that currently there is no curative topical treatment for BU, our objective was to evaluate a plasma membrane fluidizer, diethyl azelate (DEA), as a potential novel topical therapy for BU. Materials and Methods: We evaluated the effects of DEA against bacterial extracts and live strains of M. ulcerans ATCC 35840 (mycolactone positive; M+) and ATCC 19423 (mycolactone negative; M–) by measuring cytokine levels in cultured cells and tissue extracts using multiplexed immunoassays and numbers of skin lesions as the endpoints. Results: In vitro, DEA counteracted immunosuppression induced by extract from the M+ strain in the 3-D human skin model (EpiDerm) and in human dendritic cells. In vivo, topical DEA reduced immunosuppressive activities of M+ and M- strains at all stages of BU, including advanced ulcers. DEA also diminished lesion formation and ulceration, accelerated healing of skin lesions and preserved normal immune responsiveness to pathogen-associated molecular pattern receptor agonists in blood of infected animals. Conclusion: The efficacy of DEA in BU models is linked to overcoming the immunosuppressive activity of virulence factors produced by M. ulcerans. Thanks to its pluripotent activity, DEA is a promising novel treatment for BU and possibly other pathogenic mycobacteria.*

Mycobacteriaceae are a large family of organisms with over 150 recognized species (1). Pathogenic mycobacteria invade and replicate in host macrophages and produce virulence factors to evade and suppress host immunity both locally and systemically (2-4).

The most common human pathogen of the group, *Mycobacterium tuberculosis*, is sometimes referred to as "the world's most successful pathogen" (5). Other human pathogens in the family are *M. leprae,* responsible for leprosy (6) and *M. ulcerans*, which causes a necrotizing skin disease known as Buruli ulcer (BU) (7).

BU is an emerging human disease affecting mainly children in tropical areas (7). It is endemic in sub-Saharan Africa but it has been reported worldwide, even in some non-tropical areas. BU starts with a papule, nodule, plaque, or edematous lesion that progresses to extensive skin ulceration. Surprisingly, even with extensive tissue destruction, the lesion is usually painless (8). Because of the nonspecific early symptoms of *M. ulcerans* infections and limited access to medical care in the predominantly rural regions where it occurs, patients usually seek treatment after the disease has reached an advanced stage, with consequent increased risk of permanent disabilities (9, 10).

M. ulcerans hosts a giant plasmid that encodes a macrolide lipid-like toxin called mycolactone. The plasmid-hosting feature is seemingly absent from other human pathogenic mycobacteria (11). Mycolactone is considered the key contributor to BU pathology, and at present is the only known virulence factor of *M. ulcerans* (12). Mycolactone is cytotoxic to mammalian cells at concentrations of >1 mg/ml (13-16), although cytotoxicity has been reported at levels as low as 2 ng/ml (17). At lower concentrations, mycolactone blocks the production of lipopolysaccharide (LPS)-dependent cytokines (18) and exerts potent local and systemic immunosuppression (19, 20).

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M. ulcerans infection affects the systemic metabolism of the host. Changes in blood sugar homeostasis were reported in infected patients (21). BU disease down-regulates blood levels of inflammatory mediators, acute-phase proteins, and markers of lipid metabolism. These effects may persist even after the elimination of bacteria with antibiotics (22). Insulin resistance has also been observed in other mycobacterial infections (23, 24).

The standard therapy for BU is the combination of oral rifampicin (10 mg/kg once daily) and clarithromycin (7.5 mg/kg twice daily) for 8 weeks. Improved oral therapies are under development (25) but curative topical treatments for BU do not exist. Delays in treatment often necessitate skin grafting (26). Patients with severe forms of BU or delayed therapy can be left with life-long disabilities and deformities (8). The disease can also be reactivated due to immunostimulation (27).

Drug resistance is on the rise in mycobacteria, including *M. ulcerans* (28-30), and is of a growing concern in BU (31). A paradoxical reaction to antibiotics occurs in ~10% of patients with BU who experience worsening of existing lesions or appearance of new lesions after the initiation of treatment (32). Given the limitations of current treatments for BU, better therapies are needed.

We have identified a novel family of immunomodulatory compounds that change the plasma membrane fluidity of host cells and consequently alter the activities of membrane-associated proteins, receptors and microbial toxins that interact with the plasma membrane. Esters of azelaic acid (azelates) are the first recognized agents of a new class of drugs that we have named membrane-active immunomodulators (33-35). The azelates counteracted detrimental effects of multiple pathogen-associated molecular pattern (PAMP) receptor agonists including LPS in cultured human cells and *in vivo*. A representative ester, diethyl azelate (DEA), preserved host immune function and diminished toxicity of virulence factors and bacterial pathogens such as anthrax toxin and methicillin-resistant *Staphylococcus aureus* in animal studies (34). DEA also significantly reduced markers of insulin resistance in humans (36).

The goal of this work was to evaluate the therapeutic potential of topical DEA for the treatment of BU. According to the US Food and Drug Administration, the key features of a drug candidate are its safety and efficacy, but its safety is cardinal (37). The safety profile of DEA is well-documented (33, 34, 36). To examine the efficacy of DEA against BU, we tested the hypothesis that DEA can prevent host immunosuppression by bacterial extracts and live *M. ulcerans* strains *in vitro* and *in vivo*. Having gleaned an early insight into the efficacy of DEA in BU disease models, we hereby propose that DEA is a potential novel treatment for BU.

Materials and Methods

Chemical synthesis. Unless specified otherwise, all chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). DEA was synthesized from azelaic acid and ethanol using acid-catalyzed esterification as described (36). Following solvent removal, the ester yielded was a clear, slightly yellow oil. DEA was purified by fractional distillation at reduced pressure and analyzed by mass spectrometry. The purity of the final product was >95% w/w.

Bacterial preparations. Two strains of *M. ulcerans*, ATCC35840 [mycolactone-positive (M+) and ATCC19423 (mycolactone-negative (M–)], were purchased from the American Type Culture Collection (Manassas, VA, USA). Bacterial stocks were reconstituted according to the supplier's protocols. The strains were maintained on Lowenstein–Jensen medium. Colonies from the medium were subcultured in Middlebrook 7H9 broth containing OADC growth supplement enrichment. To prepare bacterial lysates (further referred to as M+ or M– lysates), the bacterial suspensions were centrifuged at 10,000 × *g* for 30 min at room temperature and resuspended in 2 ml of phosphate-buffered saline (PBS), then disrupted by ultrasonication on ice in 30-s intervals with a 5-s pause between pulses for a total time of 5 min. PBS was then added to a final volume of 5 ml and the solution was clarified by sequential filtration through a 0.45 μm and 0.22 μm cellulose acetate syringe filters. Sterility was confirmed by streaking 10 ml of the preparation on a Middlebrook 7H10 agar-coated plate following incubation at 37˚C in an atmosphere with 5% CO_2. The yellow-colored filtrates were subdivided into small aliquots under minimum light exposure and stored frozen at –70˚C. For testing, the aliquots were thawed on ice under dimmed room light and used within ~1 h.

EpiDerm bioassays. The EpiDerm Skin Model was purchased from MatTek Corporation (Ashland, MA, USA) and the tissues were handled according to the supplier's instructions. Following an overnight equilibration in 6-well plates over 0.9 ml of maintenance medium in an incubator at 37˚C and 90% relative humidity, the medium was replaced and the tissues were incubated for 24 h with 50 μl of treatment solutions in propylene glycol or cell culture media (vehicle controls), with positive controls as specified for individual experiments. DEA was tested at different concentrations up to 25% w/v either alone or in combination with other agents as specified in the section *PAMP receptor agonists* below. For *in vitro* experiments, bacterial lysates were adjusted to the equivalent of 10^6 colony-forming units (CFU) per 50 μl. All treatments were performed in duplicate or triplicate. Upon completion of the experiment, culture media from the incubations (further referred to as conditioned media) were collected. Single tissue specimens from each group were dissected from the cell culture inserts using 8 mm dermal punches, fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin. Remaining tissue specimens were removed as described, placed into microtubes in 0.5 ml chilled lysis buffer (0.1% Triton X-100, 20 mM EDTA, 5 mM Tris pH 8, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride) and 1% each protease and phosphatase inhibitor cocktails from Sigma–Aldrich (St. Louis, MO, USA), disrupted on ice using an ultrasonic probe homogenizer and centrifuged at 4,500 × *g* at 4˚C for 10 min. Clear supernatants were transferred to fresh microtubes and immediately frozen at –70˚C for subsequent use in multiplexed immunoassays.

In vivo studies. Male and female Balb/c mice 8-10 weeks old, weighing approximately 25 g, were purchased from Taconic Farms (Hudson, NY, USA). Animals were acclimatized to laboratory surroundings for at least 72 h after delivery. Mice were housed in a temperature-controlled room (22-24˚C) with a 12-h light/dark cycle and were fed *ad libitum* with Purina 5000 rodent diet and purified water. Three to four mice per cage were maintained in open-top plastic cages with tops covered with stainless wire grid lids, and the cage floors were covered with wood chips which were changed twice weekly. Animal health and behavior were monitored twice daily. The environment including recommended temperature, light, housing and food was held constant to minimize suffering and distress. Veterinary service and consultation were available if needed. The protocol recommended euthanasia for all experimental animals showing debilitation of major morbidity, including loss of body weight greater than 20%.

Experimental protocols used throughout the studies were approved by the Institutional Animal Care and Use Committee at BTNS LLC (San Antonio, TX, USA) (protocol numbers BT-009-004 and BT-011-008). Upon completion of the experiments, the animals were euthanized by cervical dislocation since chemical means would interfere with cytokine level determination endpoints. All procedures were performed in compliance with the Animal Welfare Act Regulations (9 CFR 3) and the National Institutes of Health guidelines for the care and use of laboratory animals.

Prior to experiments, the animals were randomized into treatment and control groups. Based on power calculations and to accommodate for possible losses, eight mice per group were used. For the short-term study, areas of ~1-2 cm^2 on animals' backs were shaved and suspensions of the live M+ or M– bacteria in conditioned medium in Middlebrook 7H9/OADC (10^8 CFU per 100 μl) were injected subcutaneously at a single site within the shaved area on day 1. Topical application of 100 μl DEA (10 g/kg) was initiated on day 2, and performed twice a day thereafter at the inoculation sites. This experiment comprised five groups: M– with and without DEA, M+ with and without DEA, and a control group injected with Middlebrook 7H9/OADC. We monitored the sizes of the induced granulomas in the animals as an indication of treatment effect. The following scoring system for subcutaneous nodules was used: 0: No palpable nodule; 1: barely palpable nodule <3 mm; 2: palpable nodule >3 mm; 3: visible nodule >5 mm; 4: large visible nodule >8 mm. Cumulative scores were calculated per group of eight animals. The animals were sacrificed on day 18 and exsanguinated for the preparation of plasma for multiplexed immunoassays. Skin from the area of bacterial inoculation was dissected and fixed in 10% phosphate-buffered formalin for immunohistochemistry.

For the experiment designed to evaluate the long-term effect of DEA treatment of *M. ulcerans* infections, suspensions of M+ in conditioned medium (equivalent of 10^9 CFU per 100 μl) were injected as above on day 1. Topical treatment with 100 μl DEA (10 g/kg) twice daily was initiated on day 2 (early), day 6 (intermediate), or day 37 (late). cumulative scores based on the total numbers of open lesions >2 mm in size were recorded for each group of eight animals. Animals were sacrificed on day 45.

Immunohistochemistry. Dissected murine skin tissues were fixed in 10% phosphate buffered formalin (Fisher Scientific, Pittsburgh, PA, USA) for 24 h and embedded in paraffin. Ten-micrometer tissue sections were stained with hematoxylin-eosin (HE), acid-fast (Ziehl–Neelsen) stain, and CD3/CD68 antibodies (all from Thermo Fisher Scientific, Waltham, MA, USA). Qualitative evaluation of the HE-stained tissues was carried out at 50× magnification using a Zeiss inverted microscope model 4717 (Zeiss, Dublin, CA, USA).

Blood processing. Whole peripheral blood was isolated from mice at sacrifice, collected into BD Vacutainer CPT Cell Preparation Tubes (Beckton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 1,500 × *g* at room temperature. The plasma layer was isolated according to the supplier's protocol.

Multiplexed immunoassays and cell viability assays. If not analyzed within 1 hour of sample collection, specimens were frozen at –70˚C. The specimens were subsequently thawed on ice and used in duplicate or triplicate when analyzed by multiplexed immunoassays using customized Procarta Cytokine, Chemokine and Growth Factors comprising Panel #1 (54 analytes) and Panel #2 (36 analytes), Human MMP 3Plex Panel, Human MMP 3Plex Panel, and Plexable Mouse Cytokine, Chemokine and Growth Factors (37 analytes); all from Affymetrix (Fremont, CA, USA). The specimens were tested at dilutions recommended by the supplier. The assays were performed according to the manufacturer's protocols. Pooled normal human plasma (SeraCare, Milford, MA, USA) was used as a positive control in all assays and the lysis buffer or cell culture media were used as negative controls. Multiplex immunoassays were performed using a Luminex 100 IS System (Luminex Corporation, Austin, TX, USA). Analyte concentrations were calculated from the standard curves using Bio-Plex Manager 4.1.1 (Bio-Rad Laboratories, Hercules, CA). CellTiter-Glo Luminescent Cell Viability (ATP) kits was purchased from Promega (Madison, WI, USA) and the assays were performed according to the supplier's instructions.

PAMP receptor agonist studies. PAMP receptor agonists from InvivoGen (San Diego, CA, USA) included the following: Human toll-like receptor (TLR1-9), dectin-1, and bacterial nucleotide-binding and oligomerization domain (NOD) agonists as described elsewhere (34). Murine whole blood was isolated at the time of sacrifice. The blood was diluted with an equal volume of RPMI-1640 medium containing 10% fetal bovine serum, then 140 μl aliquots were transferred into individual wells of 96-well plates and gently mixed in a horizontal shaker. The blood was stimulated for 24 hours with the cocktail of PAMP receptor agonists with DEA, and with DEA or the vehicle alone as controls, in a final volume of 250 μl. The plates were centrifuged at 800 × *g*, then clarified supernatants were harvested and stored at –80˚C for further analysis using multiplex immunoassays.

Dendritic cell stimulation. Plasmacytoid human dendritic cells were purchased from MatTek Corporation (number: DCP-100; Ashland, MA, USA) and handled according to the supplier's protocol. The cells were washed with RPMI 1640 supplemented with 10% fetal bovine serum, diluted to 40,000 cells per 250 μl and incubated with the M+ lysate (10^6 cells/50 μl) with LPS (50 μg/ml) and 0.5% DEA in an incubator at 37˚C and 90% relative humidity for 24 hours. The medium was then removed by centrifugation and the cells were lysed in chilled lysis buffer as described for EpiDerm specimens and subjected to multiplex immunoassays. Cytokine levels were normalized to the number of viable cells.

Statistical analysis. Power analysis was performed for all *in vitro* and *in vivo* studies that used multiplex immunoassays (31).

Statistical analysis was done using Student's *t*-statistic; values of $p<0.05$ were considered significant. The power calculations for *in vivo* studies were based on our multiple biomarker study in mice (30) whereby a power of 80% was achieved with four mice/group (NCSS PASS Version 08.0.8; NCSS Statistical Software, Kaysville, UT, USA) assuming a 5% significance level and two-tailed testing. The actual group sizes were increased to eight mice to compensate for possible losses during the experiment.

All samples/data were included in the analysis. Continuously distributed outcomes were summarized with the mean and standard deviation. Relative values were graphically represented with a heat map. All treatment groups were compared with regard to the mean using analysis of variance, and for each biomarker, pairwise comparisons between treatment groups were corrected for multiple testing using the Tukey method. For the analysis of the growth rates of *in vivo* lesions, the cumulative group pathology score instead of the individual pathology score was employed using a linear mixed-model fit using the lmer package in R (R Software, San Francisco, CA, USA). All statistical testing was two-sided with a nominal and experiment-wise significance level of 5% using SAS Version 9.2 (Cary, NC, USA) unless otherwise noted. R was used for graphics.

Results

DEA overcomes acute immunosuppression by the M+ lysate in human cells in vitro. In addition to antibacterial activities (34, 38), DEA is known to have a broad spectrum of immunomodulatory properties (34). In our studies, we used DEA against two strains of *M. ulcerans* that differed by their virulence factor repertoires. The M+ strain produces mycolactone A and B (17, 19) and the M– strain is considered mycolactone-negative (39). Nonetheless, these bacteria were isolated from a skin ulcer lesion and therefore are likely to produce some virulence factors (19, 40).

Given that bacterial preparations of *M. ulcerans* can exert acute immunosuppression in cultured mouse cells (41), we sought to determine whether DEA can protect human cells against immunosuppression induced by the M+ lysate in human cells. As the first model, we used the 3-dimensional EpiDerm reconstructed human epidermis that consists of normal human epidermal keratinocytes cultured as a multilayered and highly differentiated simulant and surrogate of human skin (42-44). The EpiDerm system exhibits *in vivo*-like morphological and growth characteristics, which are uniform and highly reproducible. EpiDerm is mitotically and metabolically active, and expresses markers of mature epidermis-specific differentiation.

In the past, we found that DEA was not cytotoxic in the EpiDerm model using formazan precipitation and ATP assays (34). ATP is often used as a surrogate endpoint to assess cellular proliferation, drug cytotoxicity (45) and response to skin irritants in keratinocytes (46).

In the present experiments, the EpiDerm tissues were treated topically for 24 hours with the M+ lysate, and with DEA and LPS or combinations thereof. LPS was used as a positive control as it normally triggers release of pro-inflammatory mediators (47) but *M. ulcerans* induces immunosuppression and renders tissues non-responsive to LPS stimulation (48).

ATP levels in the lysates from the M+ or LPS-treated tissues were slightly (albeit insignificantly) elevated compared to DEA alone, similar to other reports on LPS responses (46, 47). On the other hand, the levels of secreted ATP differed significantly ($p=0.03$) between M+ treated with LPS *versus* M+ treated with LPS and DEA, with ~10-fold increase in the latter group. Thus, DEA apparently abrogated the immunosuppression mediated by the M+ lysate and restored normal tissue responses to LPS based on ATP levels.

To further examine if DEA could restore normal immune response based on a multi-marker analysis, we treated EpiDerm tissue with the M+ lysate alone and in combination with 10% DEA for 24 hours. Culture medium (vehicle) was used as a negative control. The EpiDerm tissues were washed with PBS and stimulated with LPS (100 μg/ml) for 1 h. DEA alone did not significantly affect levels of any cytokine in the EpiDerm tissue and in the medium only, interleukin 1b (IL1B) was significantly increased (Figure 1A). In the presence of the M+ lysate, DEA significantly up-regulated interleukin 8 (IL8) and matrix metalloproteinase 8 (MMP8) and down-regulated leptin in the tissue (Figure 1B). In the conditioned media, DEA up-regulated IL8, IL1 receptor agonist (IL1RA) and macrophage migration inhibitory factor, and down-regulated leptin (Figure 1B).

These results indicated that DEA reversed the immuno-suppression caused by the M+ lysate in the human epidermis model. Inhibition of leptin by DEA is therapeutically relevant because mycobacteria can cause cells to increase production of leptin as a key element of their pathophysiology (47).

DEA prevents immunosuppression induced by M. ulcerans lysate in dendritic cells. As the second *in vitro* model, we used human plasmacytoid dendritic cells that are highly susceptible to *M. ulcerans* immunosuppression (14). The cells were first treated with the M+ lysate and DEA and then stimulated by LPS. Similar to the findings in the EpiDerm model, the intracellular and secreted ATP levels were not affected by DEA, consistent with its lack of cytotoxicity. In contrast, large differences were seen in the levels of secreted cytokines (Figure 1C). LPS alone did not induce significant changes in cytokine responses in dendritic cells exposed to the M+ lysate. When LPS and DEA were added, the treatment significantly down-regulated IL12p40, monocyte chemoattractant protein 1 (MCP1), tumor necrosis factor alpha (TNF), MMP1, MMP9, MMP12, and IL1RA. Based on these results, we conclude that DEA can reverse the immunosuppression in human dendritic cells caused by the M+ lysate.

Topical DEA reduces signs of early BU disease in vivo. The *in vitro* experiments demonstrated that DEA preserved

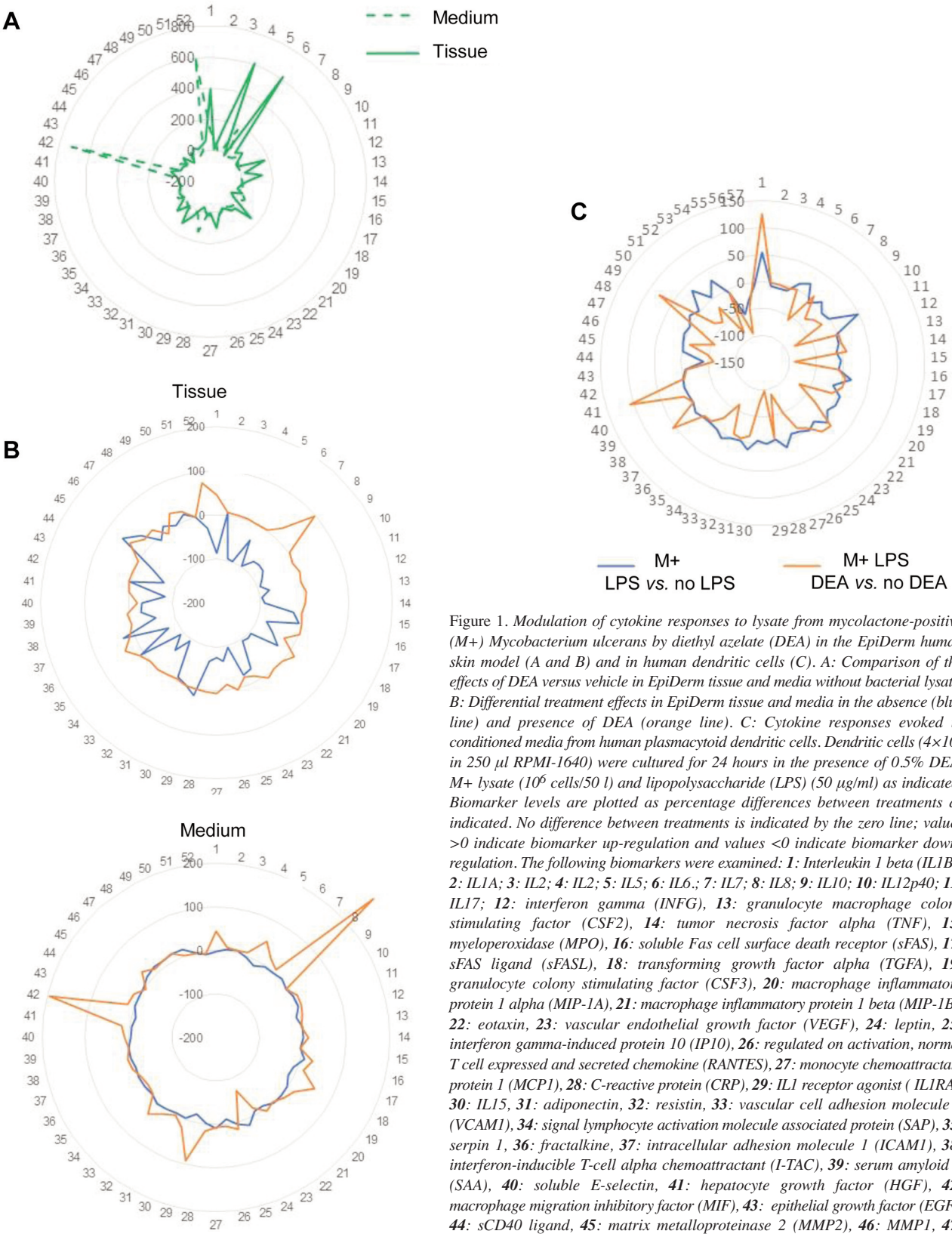

Figure 1. *Modulation of cytokine responses to lysate from mycolactone-positive (M+) Mycobacterium ulcerans by diethyl azelate (DEA) in the EpiDerm human skin model (A and B) and in human dendritic cells (C). A: Comparison of the effects of DEA versus vehicle in EpiDerm tissue and media without bacterial lysate. B: Differential treatment effects in EpiDerm tissue and media in the absence (blue line) and presence of DEA (orange line). C: Cytokine responses evoked in conditioned media from human plasmacytoid dendritic cells. Dendritic cells (4×10^5 in 250 μl RPMI-1640) were cultured for 24 hours in the presence of 0.5% DEA, M+ lysate (10^6 cells/50 l) and lipopolysaccharide (LPS) (50 μg/ml) as indicated. Biomarker levels are plotted as percentage differences between treatments as indicated. No difference between treatments is indicated by the zero line; values >0 indicate biomarker up-regulation and values <0 indicate biomarker down-regulation. The following biomarkers were examined:* ***1****: Interleukin 1 beta (IL1B);* ***2****: IL1A;* ***3****: IL2;* ***4****: IL2;* ***5****: IL5;* ***6****: IL6.;* ***7****: IL7;* ***8****: IL8;* ***9****: IL10;* ***10****: IL12p40;* ***11****: IL17;* ***12****: interferon gamma (INFG),* ***13****: granulocyte macrophage colony stimulating factor (CSF2),* ***14****: tumor necrosis factor alpha (TNF),* ***15****: myeloperoxidase (MPO),* ***16****: soluble Fas cell surface death receptor (sFAS),* ***17****: sFAS ligand (sFASL),* ***18****: transforming growth factor alpha (TGFA),* ***19****: granulocyte colony stimulating factor (CSF3),* ***20****: macrophage inflammatory protein 1 alpha (MIP-1A),* ***21****: macrophage inflammatory protein 1 beta (MIP-1B),* ***22****: eotaxin,* ***23****: vascular endothelial growth factor (VEGF),* ***24****: leptin,* ***25****: interferon gamma-induced protein 10 (IP10),* ***26****: regulated on activation, normal T cell expressed and secreted chemokine (RANTES),* ***27****: monocyte chemoattractant protein 1 (MCP1),* ***28****: C-reactive protein (CRP),* ***29****: IL1 receptor agonist (IL1RA),* ***30****: IL15,* ***31****: adiponectin,* ***32****: resistin,* ***33****: vascular cell adhesion molecule 1 (VCAM1),* ***34****: signal lymphocyte activation molecule associated protein (SAP),* ***35****: serpin 1,* ***36****: fractalkine,* ***37****: intracellular adhesion molecule 1 (ICAM1),* ***38****: interferon-inducible T-cell alpha chemoattractant (I-TAC),* ***39****: serum amyloid A (SAA),* ***40****: soluble E-selectin,* ***41****: hepatocyte growth factor (HGF),* ***42****: macrophage migration inhibitory factor (MIF),* ***43****: epithelial growth factor (EGF),* ***44****: sCD40 ligand,* ***45****: matrix metalloproteinase 2 (MMP2),* ***46****: MMP1,* ***47****: MMP3,* ***48****: MMP9,* ***49****: MMP13,* ***50****: MMP12,* ***51****: MMP7,* ***52****: MMP8.*

immune responses in human cells exposed to the M+ lysate. We further tested whether DEA curbed the signs of *M. ulcerans* infection in experimental animals. We also wished to determine whether the beneficial effects of DEA may be observed at the early stage of infection, based on the reported presence of mycolactone in blood mononuclear cells and lymph nodes of patients with BU weeks before ulcerative lesions were detectable (49, 50).

Mice were inoculated subcutaneously with live M– or M+ bacteria and treated with DEA as described above. The sizes of induced granulomas (subcutaneous nodules) in the animals were monitored as an indication of disease development. As shown in Figure 2A, mice inoculated with the M+ developed increasing numbers of nodules that followed a logarithmic growth curve over time. Fewer nodules were seen in the M– group. Topical DEA rapidly inhibited nodule growth in animals inoculated with either strain as evidenced by the numbers of nodules stabilized at the levels observed on day 3. Controls without bacterial injections had no visible adverse side-effects of DEA administration.

In the statistical analysis of the data, a linear mixed-effects model with a random intercept for strain was used since the graph indicated some effect from strain on the starting point. The fit was suboptimal because there were some outliers due to the bend that was detected in the best fit line for the untreated M+ group. Using the combined group pathology score as defined in the Materials and Methods, we found that the combination of M+ and the treatment led to a borderline significance in lowering the cumulative group pathology score compared to the same strain without the treatment (t=0.068). No significant treatment effect was observed for M–.

We used immunohistochemistry merely for a qualitative assessment of DEA effects. Skin biopsies stained for T-cells and macrophages showed multiple subcutaneous granulomas in the animals exposed to M+ alone and a lack thereof in the infected animals treated with DEA. Representative images of skin from animals injected with the M+ strain without or with DEA treatment (Figure 2B) indicate the reduction of subcutaneous granulomas in the latter group. The images of skin biopsies resembled those reported elsewhere (27, 51, 52).

DEA restores PAMP receptor signaling in infected animals. The M– lysate was modestly immunosuppressive *in vitro* and produced relatively few small subcutaneous nodules *in vivo*. To gain a further insight into the inter-strain differences, we compared the patterns of cytokines evoked in response to bacterial infection, treatment with DEA, and stimulation of the blood *ex vivo* by a cocktail of PAMP receptor agonists that were used to evaluate the functionality of PAMP receptor downstream signaling pathways on experiment day 18.

Whole peripheral blood was isolated from the control (*M. ulcerans*-naïve) mice and the animals inoculated with the M+ and M– strains, with or without topical DEA treatment. The blood was either analyzed directly (unstimulated) or was exposed *ex vivo* to a cocktail of PAMP receptor agonists (stimulated blood). Blood cytokine profiling quantified the levels of murine markers that closely matched analogous human markers. The results of this experiment are shown as differential heat maps in the controls (Figure 3) and in the animals exposed to the M+ and M– strains (Figure 4).

Specifically, Figure 3 shows the effect of PAMP receptor agonists on the levels of 35 blood markers in individual mice that were not exposed to the bacteria or DEA. Stimulation of the blood up-regulated most of the examined markers, as indicated by shades of green. The individual responses can be appreciated by examining the color patterns in the rows that contain data corresponding to individual cytokines in each animal. Biomarker patterns within each group were similar but clear differences were observed between the stimulated and unstimulated blood.

In contrast, the patterns of biomarkers in Figure 4, which presents the data on blood biomarkers in the animals exposed to bacteria, with or without DEA treatment or PAMP receptor agonist stimulation, are quite different from those of the controls.

The overall patterns of relative levels of blood biomarkers across different treatment groups in Figure 4 are summarized in Table I to allow for simple inter-group comparisons based on the global patterns of biomarkers affected by a given treatment in each animal. Up- or down-regulation of biomarkers was considered when the majority (>80%) of biomarkers in an animal were up- or down-regulated, respectively. Thus, the sums of the number of animals with up- and down-regulated patterns were always equal to eight, *i.e.* the total number of animals per group.

A visual assessment of Figure 4 shows definitive effects of stimulation on the patterns of biomarkers in the M+ and M– treatment groups. Note that without PAMP receptor agonist stimulation, the number of animals with up- and down-regulation of biomarkers were identical, suggesting a lack of responsiveness to PAMP receptor agonist stimulation, which is consistent with immunosuppression by the live M+ and M– strains. Interestingly, the patterns of blood biomarkers in both the M+ and M– animals treated with DEA showed variable but quite different patterns of responses to stimulation with the PAMP receptor agonists. This suggests that both M+ and M– were immunosuppressive and DEA was able to overcome that effect.

The variation in the levels of individual markers can be further appreciated by examining the percentage differences in the levels of blood biomarkers under both *M. ulcerans* strains. As shown in Figure 5A, DEA treatment of the M+-infected animals without agonist stimulation significantly up-regulated eotaxin, colony-stimulating factor (CSF2), IL1A, IL12p40, chemokine (C-X-C motif) ligand 1, also

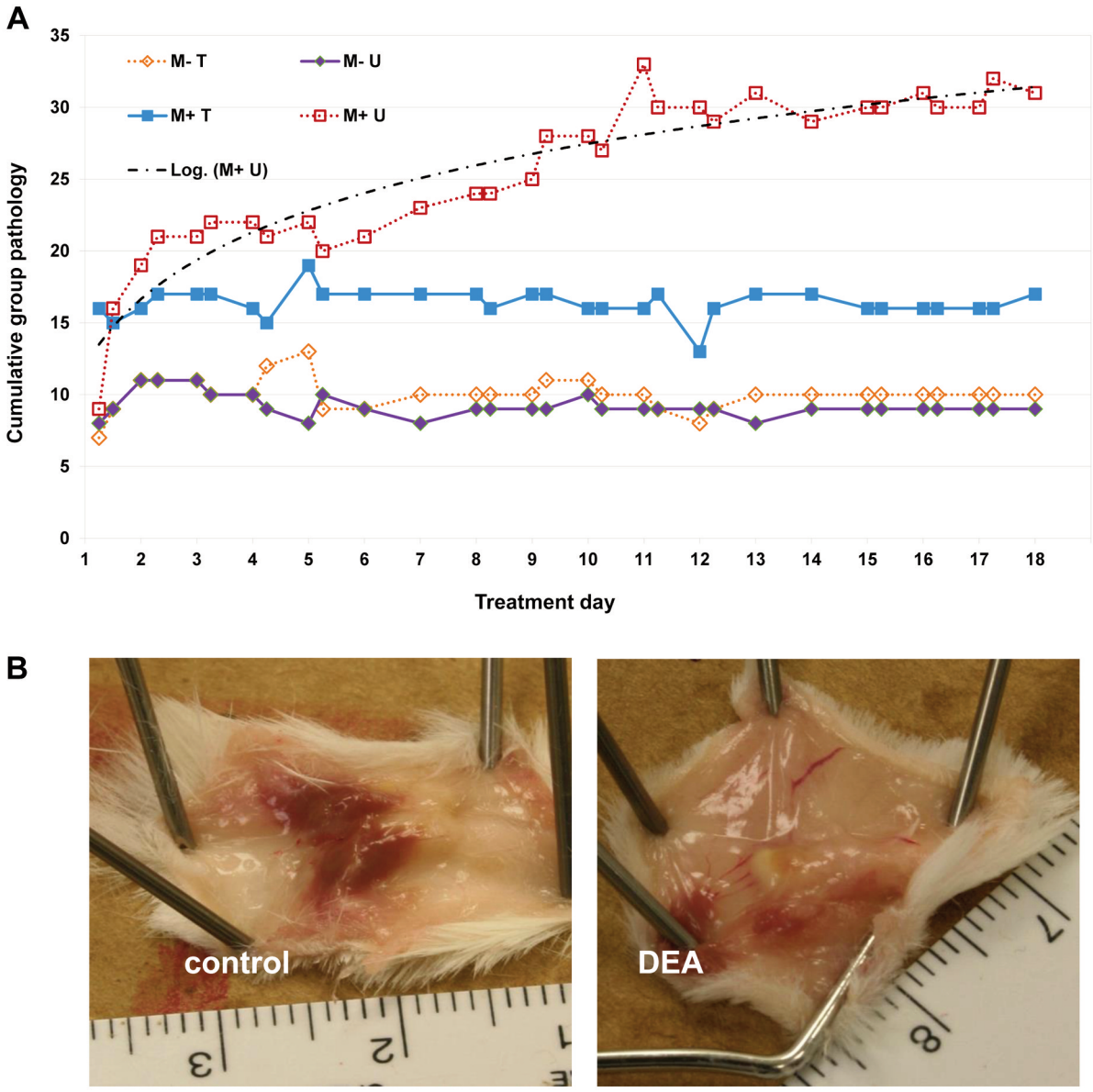

Figure 2. *Immunomodulatory activities of diethyl azelate (DEA) against Mycobacterium ulcerans in vivo. A: Prevention of subcutaneous nodule formation. Live mycolactone-positive (M+) or mycolactone-negative (M–) M. ulcerans (both at 10^9 colony-forming units in 100 µl) were inoculated subcutaneously in four male and four female Balb/c mice per group. Diethyl azelate (DEA) at 10 g/kg was applied topically twice a day starting on day 2. Average cumulative pathology scores based on the size and number of subcutaneous nodules in the untreated (U) and DEA-treated (T) groups (n=8 each) are shown as a function of time. The mean standard errors were under 20%. B: Representative images of skin from animals (n=8 per group) injected with M+ M. ulcerans without (left panel) or with DEA treatment (right panel).*

known as keratinocytes-derived chemokine (KC), MCP1, MCP3, interferon inducible protein 1 alpha (IP1A), and macrophage inflammatory protein 2 alpha (MIP2A). In the blood of animals treated with DEA and stimulated with PAMP receptor agonists *ex vivo*, there was significant up-regulation of eotaxin, IL1a, CXCL1, MIP1a and MIP2. In comparison, DEA treatment without stimulation of the M– infected mice (Figure 5B) had a modest yet significant inhibitory effect on most markers (blue line). DEA treatment of the stimulated samples (red line) significantly increased levels of LPS-induced chemokine (LIX), MCP1, IL10, IL17, MCP3, eotaxin, IL12p70, IL3 and IL5 and reduced those of Regulated on activation, normal T cell expressed and secreted chemokine (RANTES), soluble

Table I. *Summary of the effects of diethyl azelate (DEA) treatment on blood biomarkers in vivo. Overall patterns of biomarker expression were examined in each of eight animals per group, whereby up-regulation or down-regulation was assigned when >80% of the number of markers were affected in each animal in the group (as represented by rows in Figure 4), with the total number added up to eight in each group. Stimulated: Treated with pathogen-associated molecular pattern receptor agonists.*

	Unstimulated				Stimulated			
	DEA		No DEA		DEA		No DEA	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
M+	4	4	2	6	0	8	2	6
M–	8	0	6	2	2	6	6	2

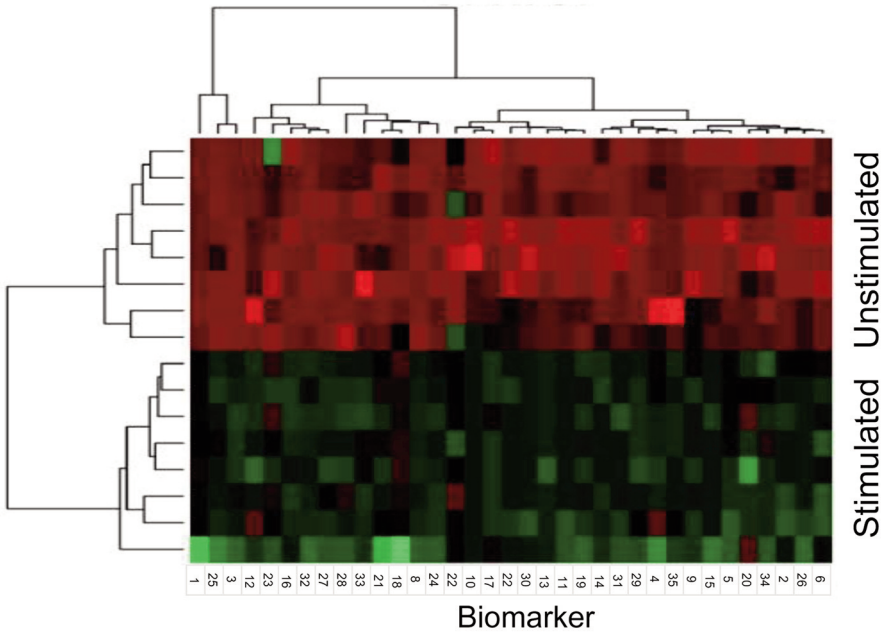

Figure 3. *Effects of pathogen-associated molecular pattern receptor agonists on relative levels of blood biomarkers in control animals not exposed to mycolactone-positive (M+) or mycolactone-negative (M–) strains of Mycobacterium ulcerans. Whole peripheral blood was collected on day 18 as described in the Materials and Methods. All groups consisted of four male and four female mice. Biomarkers were quantitated in whole blood that was either unstimulated or treated with pathogen-associated molecular pattern receptor agonists (stimulated) as described in the Materials and Methods. Heat maps indicate down-regulation and up-regulation of individual biomarkers with shades of red and green, respectively. The following biomarkers were examined:* ***1****: Adipokine;* ***2****: B-lymphocyte chemoattractant;* ***3****: eotaxin;* ***4****: granulocyte colony-stimulating factor;* ***5****: granulocyte-macrophage colony-stimulating factor;* ***6****: interferon gamma;* ***7****: interleukin 10 (IL10);* ***8****: IL12p40;* ***9****: IL23p70;* ***10****: IL13;* ***11****: IL17;* ***12****: IL1A;* ***13****: IL1B;* ***14****: IL2;* ***15****: IL21;* ***16****: IL23;* ***17****: IL3;* ***18****: IL4;* ***19****: IL5;* ***20****: IL6;* ***21****: IL9;* ***22****: interferon gamma-induced protein 10 (IP10),* ***23****: keratinocyte chemoattractant (KC),* ***24****: leptin,* ***25****: leukemia inhibitory factor (LIF),* ***26****: chemokine (C-X-C motif) ligand 5 (LIX),* ***27****: monocyte chemoattractant protein 1 (MCP1),* ***28****: monocyte-chemotactic protein 3 (MCP3),* ***29****: macrophage migration inhibitory factor 1 alpha (MIF1A),* ***30****: macrophage inflammatory protein 2 (MIP2),* ***31****: regulated on activation, normal T cell expressed and secreted chemokine (RANTES),* ***32****: soluble receptor activator of nuclear factor kappa-B ligand (sRANKL),* ***33****: transforming growth factor beta (TGFB),* ***34****: tumor necrosis factor alpha (TNF),* ***35****: vascular endothelial growth factor (VEGF).*

Receptor activator of nuclear factor kappa-B ligand (RANKL), and transforming growth factor beta (TGFB). Importantly, control experiments (Figure 5C) showed an unremarkable effect of DEA *versus* vehicle in the animals not infected with *M. ulcerans*.

The DEA treatment of mice infected with either the M+ or M– strain affected many of the cytokines which are characteristic of the immunosuppressive signature of BU (5, 12, 17). Taken together, the results suggest that DEA treatment *in vivo* can overcome immunosuppression induced by *M. ulcerans*.

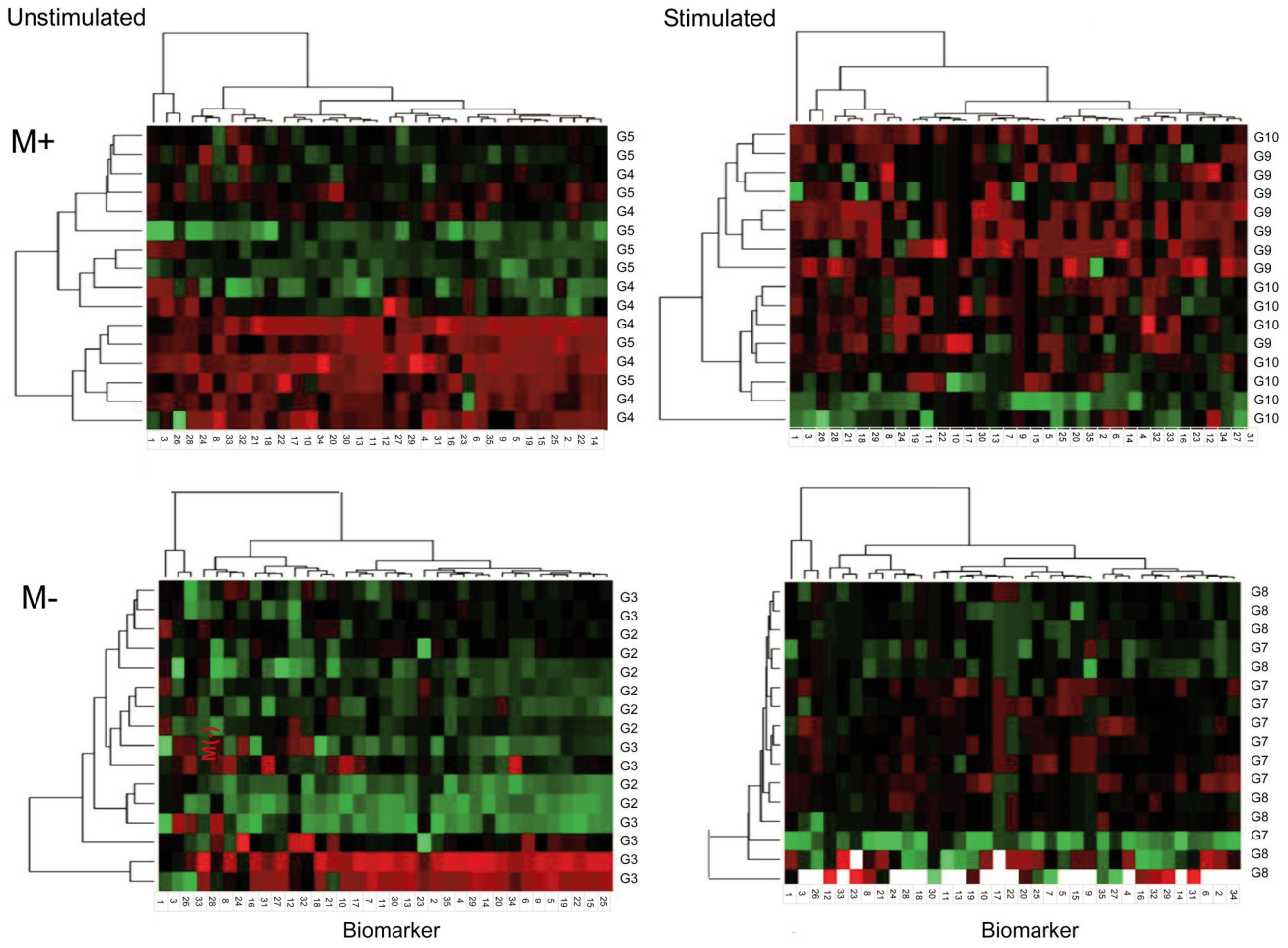

Figure 4. *Effects of diethyl azelate (DEA) on the relative levels of blood biomarker in animals exposed to mycolactone-positive (M+) or -negative (M–) Mycobacterium ulcerans. Whole peripheral blood was isolated on day 18 as described in the Materials and Methods. All groups consisted of four male and four female mice. Biomarkers were quantitated in whole blood that was either unstimulated or stimulated with pathogen-associated molecular pattern receptor agonists and DEA. Heat maps indicate down-regulation and up-regulation of individual biomarkers with shades of red and green, respectively. The treatments were as follows: G2-5: unstimulated; G7-10: stimulated. DEA was tested in G2, G4, G7 and G9. The biomarkers were as listed in the legend to Figure 3.*

Topical DEA efficacy in advanced BU disease in vivo. The standard antibiotic therapy for BU is usually effective but the adherence is often challenging for the patients (7). Advanced disease is more difficult to treat and may require surgical intervention (26). Our goal was to evaluate the efficacy of topical DEA in mouse models that simulated early, intermediate and late stages of BU disease.

The mice were inoculated with the more aggressive live M+ bacteria and DEA treatment was started either at the time of early nodule formation (early; day 2), at the stage of early open lesion formation (intermediate; day 6), or at the stage of advanced disease (late; day 37). As shown in Figure 6, at all stages there was a highly significant reduction in the total numbers of open lesions and in the time that open lesions were present. Between day 32 and day 36 (*i.e.*, before the DEA treatment was started in the late treatment group, which in this time frame served as an untreated control prior to the DEA exposure), significant differences were seen between the untreated *versus* intermediate-treated (p=0.0007), the untreated *versus* early-treated (p=0.004), and the intermediate-treated *versus* early-treated (p=0.002) mice.

When the total number of lesion-days (the sum of the lesions across the time period) were analyzed, there was no statistical difference between the early (estimated lesion-days: 82.25) *versus* the intermediate start of DEA treatment (estimated lesion-days: 100.5). However, the late treatment was significantly better than the early treatment with an estimated lesion-days of 149.5 (p=0.01399).

Importantly, topical DEA reduced the apparent BU burden at all stages of the disease even when the treatment was

initiated at the advanced stage. The experiment also showed that the murine model mimicked the clinical scenario (54) and demonstrated an early efficacy of DEA in the murine model of BU disease.

Discussion

Strengths and limitations of the study. We have demonstrated that DEA treatment prevented immunosuppression in a human epidermis model *in vitro* and in human plasmacytoid dendritic cells challenged *ex vivo* with the M+ lysate. Furthermore, mice inoculated subcutaneously with the live M+ or M– bacterial strains developed subcutaneous nodules at the site of inoculation. In both cases, DEA delayed the onset of ulcer formation, reduced the sizes and numbers of skin lesions, preserved systemic immune responses and accelerated ulcer healing. Topical DEA was well tolerated in the animals.

In some cases, our approaches differed from the conventional methodology used in BU research. Since this might be viewed as a potential limitation, clarifications are warranted. Firstly, we used bacterial lysates from *M. ulcerans* strains (nominally M+ and M–) but we have not characterized their active components. Such a characterization might be useful in development of a targeted therapy for BU. However, DEA does not to our knowledge have a particular molecular target. The locus of pharmacological action of DEA appears to be the plasma membrane itself (34). Given this unique feature of DEA, we focused on using DEA to broadly modulate the pathological effects of *M. ulcerans* in the host.

Secondly, the EpiDerm system was neither used nor validated before as a model for testing BU drugs. Although as far as we are aware we are the first to employ EpiDerm in BU studies, we have shown before that EpiDerm is immunologically active and produces a vast repertoire of cytokines (34). The present work demonstrated that the *in vitro* response of EpiDerm to immunosuppression by bacterial lysates and the mitigation of this process by DEA resembled the *in vivo* effects of DEA against *M. ulcerans* infections, similar to another report (53).

Thirdly, when considering animal models of BU, we opted not to use footpad injections known to cause excessive discomfort to the animals (8). Our alternative protocol of subcutaneous dorsal injections of mice with the live bacteria might be viewed as unconventional for BU. Nonetheless, the dorsal administration was well-tolerated and caused the formation of open lesions over time, comparable to the outcomes in conventional models of BU (54).

Last but not least, despite the limited scope of the work in preclinical models of BU, we have demonstrated the efficacy of topical DEA, a broad-spectrum immunomodulator that is not an antibiotic and whose mode of action is quite different from the current BU therapies.

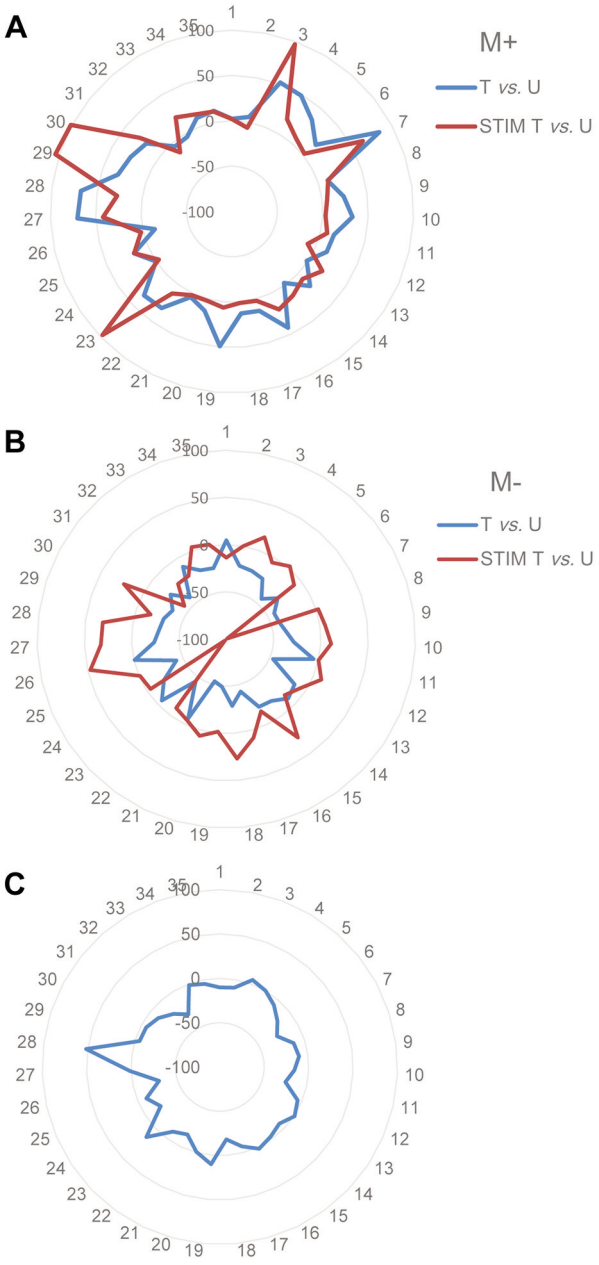

Figure 5. *Effects of diethyl azelate (DEA) on biomarker levels in blood from animals exposed to live mycolactone-positive (M+); (A) or mycolactone-negative Mycobacterium ulcerans (M–); (B), and controls without any bacterial treatment (C). All groups consisted of four male and four female mice. The animals were either untreated (U) or received topical treatment of 10 g/kg DEA (T). Whole peripheral blood was collected on day 18. In (A) and (B), biomarkers were quantitated in whole blood that was either unstimulated or treated with a cocktail of pathogen-associated molecular pattern receptor (PAMP) agonists (stimulated; STIM) as described in the Materials and Methods. The data are shown as percentage differences in the levels of secreted markers in untreated (U) groups and groups treated topically with 10 g/kg DEA (T) with/without stimulation (STIM). In (C), the comparison was made between DEA and vehicle in the absence of bacterial infection or PAMP receptor agonist stimulation. The biomarkers were as listed in the legend to Figure 3.*

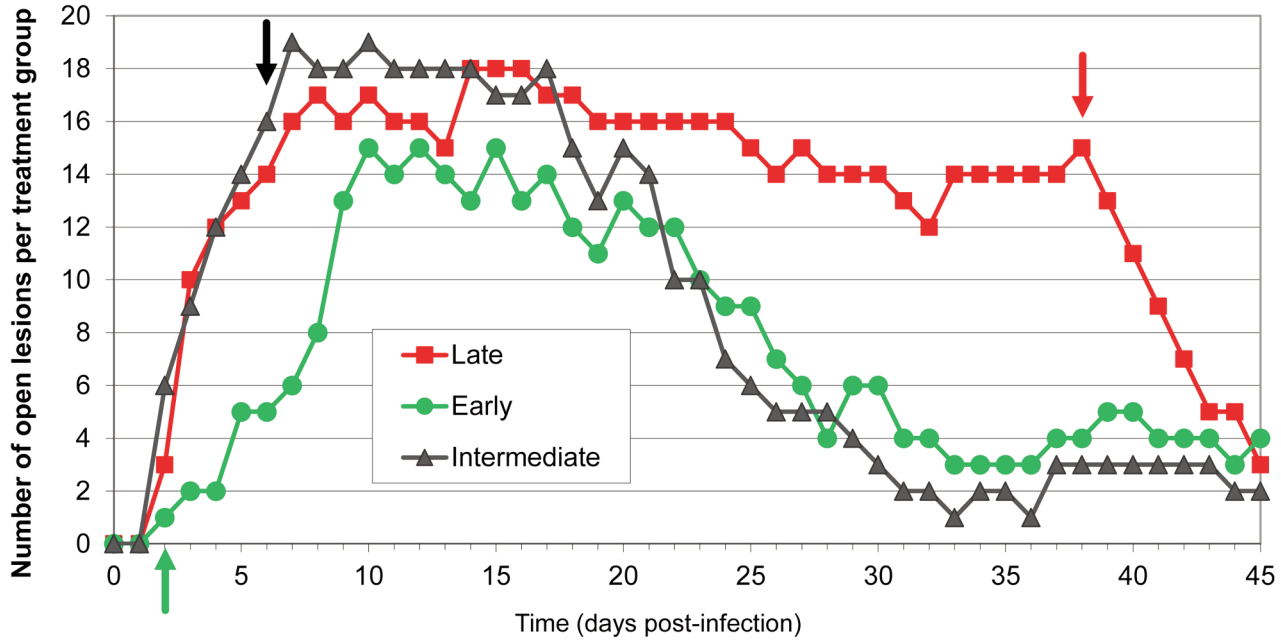

Figure 6. *Efficacy of topical diethyl azelate (DEA) in advanced Buruli ulcer disease in vivo. Live mycolactone-positive (M+) Mycobacterium ulcerans (10^9 colony-forming units in 50 μl) were inoculated subcutaneously in Balb/c mice (four males and four females) per group. Topical DEA (10 g/kg) was applied twice a day starting on day 2 (early), day 6 (intermediate), or day 37 (late) as indicated by the arrows. The mean standard errors were under 20%. Cumulative pathology scores based on the number of open lesions per each group are shown as a function of time.*

Pluripotent immunomodulator DEA can fight BU disease on many levels. The key feature of BU disease is immunosuppression of the host responses by *M. ulcerans*. Patients with active BU fail to produce cytokines on stimulation with mitogenic agents (2). This effect is consistent with an apparent immunosuppressive activity of mycolactone, which inhibits cytokine production *in vivo* (16).

Paradoxically, as shown in infected tissues from mice and patients with BU, *M. ulcerans* can also lead to production of the powerful pro-inflammatory cytokine IL1B in a process mediated by TLR2 that targets NOD, leucine-rich domain protein and pyrin domain-containing protein 3 (NLRP3/1) inflammasome, with simultaneous inhibition of other inflammatory cytokines (55). The coexistence of the immunosuppression and inflammation due to *M. ulcerans* may be due to its different effects on local, regional and systemic immunosuppression (56).

Mycobacteriaceae have evolved mechanisms to evade immune surveillance and facilitate their survival in the host, including suppression of TLR signaling (57). The innate immune system appears to play a role in defense against infections with *M. ulcerans* (58) since immune cells expressing pattern recognition receptors such as TLRs can detect invading pathogens and contain the disease. This mechanism is likely to play a role in spontaneous healing of BU (59) and *M. ulcerans*-specific immune responses in exposed but otherwise healthy individuals (58). Thus, drugs that modulate host responses may be useful in BU disease. Thanks to its ability to modulate PAMP receptor-induced responses in cultured human cells and *in vivo* (34, 35), DEA holds such a therapeutic potential for BU.

Additional benefits of DEA may be realized at the plasma membrane. Mycolactone has been shown to disturb lipid organization in biological membranes, with potential resultant effects on cell functions and signaling pathways (60, 61). Being a membrane fluidizer, DEA may directly counteract interaction of mycolactone with the plasma membrane and thus modulate aberrant cytokine signaling (34, 35).

Oral antibiotics are presently the primary treatment for BU. Our approach is different because DEA, despite its antibacterial activities (34), does not act solely or primarily as an antibiotic. The effective dose of DEA in our studies was lower than the dose responsible for its *in-vitro* growth-suppressive effect. Specifically, mice received a daily topical application of 0.2 ml of DEA. Given an average mouse blood volume of 2 ml (62) and assuming that the entire DEA ends up in blood, the maximum concentration will be 10% DEA. Yet DEA is very rapidly metabolized *in vivo* (33) thus its levels in blood are expected to be much lower. We propose that DEA does not kill the bacteria but instead interferes with

the bacterial immunosuppressant, mycolactone, which is responsible for host immunosuppression.

The *in vivo* effects of topical DEA are reminiscent of the host responses induced by antibiotics, which can also restore immunoreactivity in BU (51). Nonetheless, antibiotics reduce the levels of virulence factor(s) by destroying bacteria, not by direct mediation of immunotoxicity (63). The advantages of DEA over oral antibiotics include the topical activity of DEA, its economical synthesis from readily available precursors, and its chemical and thermal stability. In our work, DEA has shown no signs of toxicity *in vitro* or *in vivo* (33-35).

DEA may be useful in overcoming BU-associated insulin resistance that can linger even after antibiotic therapy (21, 23). We envision a future parallel use of both topical and oral DEA to combat BU. The proposed use of oral DEA is supported by the report on benefits of a ketogenic diet in BU disease and the observation that dietary β-hydroxybutyrate, which diffused to skin, reduced the mycobacterial load in mice (55). Since β-hydroxybutyrate has implications for the treatment of insulin resistance (64) and because DEA can modulate insulin resistance in humans (36), the use of topical and oral DEA might be advantageous in BU disease.

As a side note, although not presented here, our preliminary mass spectral analysis of the bacterial lysates suggested the presence of mycolactone A/B in the M+ strain and of a possible edaxadiene analog in the M– strain. Remarkably, the presence of edaxadiene has only been reported to date in *M. tuberculosis* (65). Our early findings support the notion presented elsewhere that *M. ulcerans* might produce virulence factors other than mycolactone (66) and future experiments may address that intriguing possibility.

Conclusion

In a drug development process, *in vitro* models are indispensable first steps in studying new drugs but a better proof of concept comes from *in vivo* studies, which are closer to the intended use of the drug in humans. Our *in vitro* data but especially the *in vivo* data, suggest that DEA has potential as a novel therapy for BU. With a favorable safety profile and efficacy demonstrated in laboratory and human studies, DEA holds promise for augmenting the current BU drug armamentarium.

Conflicts of Interest

EI and RTS are the founders, owners and officers of New Frontier Labs. This research was funded by New Frontier Labs.

Authors' Contributions

Conceptualization, EI and RTS; methodology, EI and RTS; software, EI, RTS, CL; validation, EI and RTS; formal analysis, EI, RTS, CL; investigation, EI and RTS; resources, EI and RTS; data curation, EI and RTS; writing- original draft preparation, EI and RTS; writing- review and editing, EI, RTS, CL; visualization, EI, RTS, CL; supervision, EI and RTS; project administration, EI and RTS; funding acquisition, EI and RTS.

References

1 King HC, Khera-Butler T, James P, Oakley BB, Erenso G, Aseffa A, Knight R, Wellington EM and Courtenay O: Environmental reservoirs of pathogenic mycobacteria across the Ethiopian biogeographical landscape. PLoS One *12(3)*: e0173811, 2017. PMID: 28333945. DOI: 10.1371/journal.pone.0173811

2 Phillips R, Sarfo FS, Guenin-Macé L, Decalf J, Wansbrough-Jones M, Albert ML and Demangel C: Immunosuppressive signature of cutaneous Mycobacterium ulcerans infection in the peripheral blood of patients with buruli ulcer disease. J Infect Dis *200(11)*: 1675-1684, 2009. PMID: 19863437. DOI: 10.1086/646615

3 Weiner J 3rd, Parida SK, Maertzdorf J, Black GF, Repsilber D, Telaar A, Mohney RP, Arndt-Sullivan C, Ganoza CA, Faé KC, Walzl G and Kaufmann SH: Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. PLoS One *7(7)*: e40221, 2012. PMID: 22844400. DOI: 10.1371/journal.pone.0040221

4 Raghuvanshi S, Sharma P, Singh S, Van Kaer L and Das G: Mycobacterium tuberculosis evades host immunity by recruiting mesenchymal stem cells. Proc Natl Acad Sci USA *107(50)*: 21653-21658, 2010. PMID: 21135221. DOI: 10.1073/pnas.1007967107

5 Hingley-Wilson SM, Sambandamurthy VK and Jacobs WR Jr: Survival perspectives from the world's most successful pathogen, Mycobacterium tuberculosis. Nat Immunol *4(10)*: 949-955, 2003. PMID: 14515128. DOI: 10.1038/ni981

6 Masaki T, Qu J, Cholewa-Waclaw J, Burr K, Raaum R and Rambukkana A: Reprogramming adult Schwann cells to stem cell-like cells by leprosy bacilli promotes dissemination of infection. Cell *152(1-2)*: 51-67, 2013. PMID: 23332746. DOI: 10.1016/j.cell.2012.12.014

7 Johnson PD, Stinear T, Small PL, Pluschke G, Merritt RW, Portaels F, Huygen K, Hayman JA and Asiedu K: Buruli ulcer (M. ulcerans infection): new insights, new hope for disease control. PLoS Med *2(4)*: e108, 2005. PMID: 15839744. DOI: 10.1371/journal.pmed.0020108

8 Yotsu RR, Suzuki K, Simmonds RE, Bedimo R, Ablordey A, Yeboah-Manu D, Phillips R and Asiedu K: Buruli ulcer: a review of the current knowledge. Curr Trop Med Rep *5(4)*: 247-256, 2018. PMID: 30460172. DOI: 10.1007/s40475-018-0166-2

9 Mulder AA, Boerma RP, Barogui Y, Zinsou C, Johnson RC, Gbovi J, van der Werf TS and Stienstra Y: Healthcare seeking behaviour for Buruli ulcer in Benin: a model to capture therapy choice of patients and healthy community members. Trans R Soc Trop Med Hyg *102(9)*: 912-920, 2008. PMID: 18617207. DOI: 10.1016/j.trstmh.2008.05.026

10 Klis S, Kingma R, Tuah W, Stienstra Y and van der Werf TS: Compliance with antimicrobial therapy for buruli ulcer. Antimicrob Agents Chemother *58(10)*: 6340, 2014. PMID: 25225342. DOI: 10.1128/AAC.03763-14

11 Nakanaga K, Yotsu RR, Hoshino Y, Suzuki K, Makino M and Ishii N: Buruli ulcer and mycolactone-producing mycobacteria.

Jpn J Infect Dis *66(2)*: 83-88, 2013. PMID: 23514902. DOI: 10.7883/yoken.66.83

12 Hall B and Simmonds R: Pleiotropic molecular effects of the Mycobacterium ulcerans virulence factor mycolactone underlying the cell death and immunosuppression seen in Buruli ulcer. Biochem Soc Trans *42(1)*: 177-183, 2014. PMID: 24450648. DOI: 10.1042/BST20130133

13 Boulkroun S, Guenin-Macé L, Thoulouze MI, Monot M, Merckx A, Langsley G, Bismuth G, Di Bartolo V and Demangel C: Mycolactone suppresses T cell responsiveness by altering both early signaling and posttranslational events. J Immunol *184(3)*: 1436-1444, 2010. PMID: 20042571. DOI: 10.4049/jimmunol.0902854

14 Coutanceau E, Decalf J, Martino A, Babon A, Winter N, Cole ST, Albert ML and Demangel C: Selective suppression of dendritic cell functions by Mycobacterium ulcerans toxin mycolactone. J Exp Med *204(6)*: 1395-1403, 2007. PMID: 17517970. DOI: 10.1084/jem.20070234

15 Guenin-Macé L, Veyron-Churlet R, Thoulouze MI, Romet-Lemonne G, Hong H, Leadlay PF, Danckaert A, Ruf MT, Mostowy S, Zurzolo C, Bousso P, Chrétien F, Carlier MF and Demangel C: Mycolactone activation of Wiskott-Aldrich syndrome proteins underpins Buruli ulcer formation. J Clin Invest *123(4)*: 1501-1512, 2013. PMID: 23549080. DOI: 10.1172/JCI66576

16 Simmonds RE, Lali FV, Smallie T, Small PL and Foxwell BM: Mycolactone inhibits monocyte cytokine production by a posttranscriptional mechanism. J Immunol *182(4)*: 2194-2202, 2009. PMID: 19201873. DOI: 10.4049/jimmunol.0802294

17 Daniel AK, Lee RE, Portaels F and Small PL: Analysis of Mycobacterium species for the presence of a macrolide toxin, mycolactone. Infect Immun *72(1)*: 123-132, 2004. PMID: 14688089. DOI: 10.1128/IAI.72.1.123-132.2004

18 Hall BS, Hill K, McKenna M, Ogbechi J, High S, Willis AE and Simmonds RE: The pathogenic mechanism of the Mycobacterium ulcerans virulence factor, mycolactone, depends on blockade of protein translocation into the ER. PLoS Pathog *10(4)*: e1004061, 2014. PMID: 24699819. DOI: 10.1371/journal.ppat.1004061

19 Yip MJ, Porter JL, Fyfe JA, Lavender CJ, Portaels F, Rhodes M, Kator H, Colorni A, Jenkin GA and Stinear T: Evolution of Mycobacterium ulcerans and other mycolactone-producing mycobacteria from a common Mycobacterium marinum progenitor. J Bacteriol *189(5)*: 2021-2029, 2007. PMID: 17172337. DOI: 10.1128/JB.01442-06

20 Gooding TM, Kemp AS, Robins-Browne RM, Smith M and Johnson PD: Acquired T-helper 1 lymphocyte anergy following infection with Mycobacterium ulcerans. Clin Infect Dis *36(8)*: 1076-1077, 2003. PMID: 12684923. DOI: 10.1086/368315

21 Niang F, Sarfo FS, Frimpong M, Guenin-Macé L, Wansbrough-Jones M, Stinear T, Phillips RO and Demangel C: Metabolomic profiles delineate mycolactone signature in Buruli ulcer disease. Sci Rep *5*: 17693, 2015. PMID: 26634444. DOI: 10.1038/srep17693

22 Phillips RO, Sarfo FS, Landier J, Oldenburg R, Frimpong M, Wansbrough-Jones M, Abass K, Thompson W, Forson M, Fontanet A, Niang F and Demangel C: Combined inflammatory and metabolic defects reflected by reduced serum protein levels in patients with Buruli ulcer disease. PLoS Negl Trop Dis *8(4)*: e2786, 2014. PMID: 24722524. DOI: 10.1371/journal.pntd.0002786

23 Podell BK, Ackart DF, Kirk NM, Eck SP, Bell C and Basaraba RJ: Non-diabetic hyperglycemia exacerbates disease severity in Mycobacterium tuberculosis infected guinea pigs. PLoS One *7(10)*: e46824, 2012. PMID: 23056469. DOI: 10.1371/journal.pone.0046824

24 Mao F, Chen T, Zhao Y, Zhang C, Bai B, Zhao S, Xu Z and Shi C: Insulin resistance: a potential marker and risk factor for active tuberculosis? Med Hypotheses *77(1)*: 66-68, 2011. PMID: 21459520. DOI: 10.1016/j.mehy.2011.03.025

25 Johnson PDR: Buruli ulcer: cured by 8 weeks of oral antibiotics? Lancet *395(10232)*: 1231-1232, 2020. PMID: 32171421. DOI: 10.1016/S0140-6736(20)30478-5

26 Weiss MG: Stigma and the social burden of neglected tropical diseases. PLoS Negl Trop Dis *2(5)*: e237, 2008. PMID: 18478049. DOI: 10.1371/journal.pntd.0000237

27 Schütte D and Pluschke G: Immunosuppression and treatment-associated inflammatory response in patients with Mycobacterium ulcerans infection (Buruli ulcer). Expert Opin Biol Ther *9(2)*: 187-200, 2009. PMID: 19236249. DOI: 10.1517/14712590802631854

28 Johnson R, Streicher EM, Louw GE, Warren RM, van Helden PD and Victor TC: Drug resistance in Mycobacterium tuberculosis. Curr Issues Mol Biol *8(2)*: 97-111, 2006. PMID: 16878362.

29 Cambau E, Perani E, Guillemin I, Jamet P and Ji B: Multidrug-resistance to dapsone, rifampicin, and ofloxacin in Mycobacterium leprae. Lancet *349(9045)*: 103-104, 1997. PMID: 8996430. DOI: 10.1016/S0140-6736(05)60888-4

30 Owusu E, Newman MJ, Kotey NK, Akumwena A and Bannerman E: Susceptibility profiles of *Mycobacterium ulcerans* isolates to streptomycin and rifampicin in two districts of the eastern region of Ghana. Int J Microbiol *2016*: 8304524, 2016. PMID: 28070190. DOI: 10.1155/2016/8304524

31 Beissner M, Awua-Boateng NY, Thompson W, Nienhuis WA, Klutse E, Agbenorku P, Nitschke J, Herbinger KH, Siegmund V, Fleischmann E, Adjei O, Fleischer B, van der Werf TS, Loscher T and Bretzel G: A genotypic approach for detection, identification, and characterization of drug resistance in Mycobacterium ulcerans in clinical samples and isolates from Ghana. Am J Trop Med Hyg *83(5)*: 1059-1065, 2010. PMID: 21036838. DOI: 10.4269/ajtmh.2010.10-0263

32 Frimpong M, Agbavor B, Duah MS, Loglo A, Sarpong FN, Boakye-Appiah J, Abass KM, Dongyele M, Amofa G, Tuah W, Frempong M, Amoako YA, Wansbrough-Jones M and Phillips RO: Paradoxical reactions in Buruli ulcer after initiation of antibiotic therapy: Relationship to bacterial load. PLoS Negl Trop Dis *13(8)*: e0007689, 2019. PMID: 31449522. DOI: 10.1371/journal.pntd.0007689

33 Izbicka E and Streeper R: Azelaic acid esters as pluripotent immunomodulatory molecules: nutritional supplements or drugs. Nutraceuticals *1(1)*: 42-53, 2021. DOI: 10.3390/nutraceuticals 1010006

34 Izbicka E, Streeper RT and Louden C: Adaptive membrane fluidity modulation: a feedback regulated homeostatic system and target for pharmacological intervention. In Vivo *35(6)*: 3073-3095, 2021. PMID: 34697139. DOI: 10.21873/invivo.12603

35 Izbicka E and Streeper RT: Adaptive membrane fluidity modulation: a feedback regulated homeostatic system hiding in plain sight. In Vivo *35(6)*: 2991-3000, 2021. PMID: 34697130. DOI: 10.21873/invivo.12594

36 Streeper RT, Louden C and Izbicka E: Oral azelaic acid ester decreases markers of insulin resistance in overweight human male subjects. In Vivo *34(3)*: 1173-1186, 2020. PMID: 32354907. DOI: 10.21873/invivo.11890

37 Ross JS, Dzara K and Downing NS: Efficacy and safety concerns are important reasons why the FDA requires multiple reviews before approval of new drugs. Health Aff (Millwood) *34(4)*: 681-688, 2015. PMID: 25847652. DOI: 10.1377/hlthaff.2014.1160

38 Charnock C, Brudeli B and Klaveness J: Evaluation of the antibacterial efficacy of diesters of azelaic acid. Eur J Pharm Sci *21(5)*: 589-596, 2004. PMID: 15066659. DOI: 10.1016/j.ejps.2003.12.006

39 MacCallum P and Tolhurst JC: A new mycobacterial infection in man. J Pathol Bacteriol *60(1)*: 93-122, 1948. PMID: 18876541.

40 Tsukamura M, Kaneda K, Imaeda T and Mikoshiba H: [A taxonomic study on a mycobacterium which caused a skin ulcer in a Japanese girl and resembled Mycobacterium ulcerans]. Kekkaku *64(11)*: 691-697, 1989. PMID: 2593460.

41 Pimsler M, Sponsler TA and Meyers WM: Immunosuppressive properties of the soluble toxin from Mycobacterium ulcerans. J Infect Dis *157(3)*: 577-580, 1988. PMID: 2963867. DOI: 10.1093/infdis/157.3.577

42 Kejlová K, Labský J, Jírová D and Bendová H: Hydrophilic polymers – biocompatibility testing *in vitro*. Toxicol In Vitro *19(7)*: 957-962, 2005. PMID: 16081240. DOI: 10.1016/j.tiv.2005.06.032

43 Hayden P, Bachelor M, Ayehunie S, Letasiova S, Kaluzhny Y, Klausner M and Kandáro H: Application of MatTek in vitro reconstructed human skin models for safety, efficacy screening, and basic preclinical research. Appl In Vitro Toxicol *1(3)*, 2015. DOI: 10.1089/aivt.2015.0012

44 McKim JM Jr, Keller DJ 3rd and Gorski JR: An in vitro method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing. Cutan Ocul Toxicol *31(4)*: 292-305, 2012. PMID: 22494060. DOI: 10.3109/15569527.2012.667031

45 Crouch SP, Kozlowski R, Slater KJ and Fletcher J: The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods *160(1)*: 81-88, 1993. PMID: 7680699. DOI: 10.1016/0022-1759(93)90011-u

46 Mizumoto N, Mummert ME, Shalhevet D and Takashima A: Keratinocyte ATP release assay for testing skin-irritating potentials of structurally diverse chemicals. J Invest Dermatol *121(5)*: 1066-1072, 2003. PMID: 14708608. DOI: 10.1046/j.1523-1747.2003.12558.x

47 Wieland CW, Florquin S, Chan ED, Leemans JC, Weijer S, Verbon A, Fantuzzi G and van der Poll T: Pulmonary Mycobacterium tuberculosis infection in leptin-deficient ob/ob mice. Int Immunol *17(11)*: 1399-1408, 2005. PMID: 16141243. DOI: 10.1093/intimm/dxh317

48 Pahlevan AA, Wright DJ, Andrews C, George KM, Small PL and Foxwell BM: The inhibitory action of Mycobacterium ulcerans soluble factor on monocyte/T cell cytokine production and NF-kappa B function. J Immunol *163(7)*: 3928-3935, 1999. PMID: 10490994.

49 Sarfo FS, Le Chevalier F, Aka N, Phillips RO, Amoako Y, Boneca IG, Lenormand P, Dosso M, Wansbrough-Jones M, Veyron-Churlet R, Guenin-Macé L and Demangel C: Mycolactone diffuses into the peripheral blood of Buruli ulcer patients – implications for diagnosis and disease monitoring. PLoS Negl Trop Dis *5(7)*: e1237, 2011. PMID: 21811642. DOI: 10.1371/journal.pntd.0001237

50 Sarfo FS, Phillips R, Wansbrough-Jones M and Simmonds RE: Recent advances: role of mycolactone in the pathogenesis and monitoring of Mycobacterium ulcerans infection/Buruli ulcer disease. Cell Microbiol *18(1)*: 17-29, 2016. PMID: 26572803. DOI: 10.1111/cmi.12547

51 Schütte D, Um-Boock A, Mensah-Quainoo E, Itin P, Schmid P and Pluschke G: Development of highly organized lymphoid structures in Buruli ulcer lesions after treatment with rifampicin and streptomycin. PLoS Negl Trop Dis *1(1)*: e2, 2007. PMID: 17989779. DOI: 10.1371/journal.pntd.0000002

52 Kiszewski AE, Becerril E, Aguilar LD, Kader IT, Myers W, Portaels F and Hernàndez Pando R: The local immune response in ulcerative lesions of Buruli disease. Clin Exp Immunol *143(3)*: 445-451, 2006. PMID: 16487243. DOI: 10.1111/j.1365-2249.2006.03020.x

53 Scherr N, Bieri R, Thomas SS, Chauffour A, Kalia NP, Schneide P, Ruf MT, Lamelas A, Manimekalai MSS, Grüber G, Ishii N, Suzuki K, Tanner M, Moraski GC, Miller MJ, Witschel M, Jarlier V, Pluschke G and Pethe K: Targeting the Mycobacterium ulcerans cytochrome bc_1:aa_3 for the treatment of Buruli ulcer. Nat Commun *9(1)*: 5370, 2018. PMID: 30560872. DOI: 10.1038/s41467-018-07804-8

54 Bolz M and Ruf MT: Buruli ulcer in animals and experimental infection models. In: Buruli ulcer: Mycobacterium ulcerans disease. Pluschke G and Roltgen K (eds.). Cham, Switzerland, pp. 159-181, 2019.

55 Foulon M, Robbe-Saule M, Esnault L, Malloci M, Mery A, Saint-André JP, Croue A, Kempf M, Homedan C, Marion E and Marsollier L: Ketogenic diet impairment of Mycobacterium ulcerans growth and toxin production and enhancement of host response to infection in an experimental mouse model. J Infect Dis *224(11)*: 1973-1983, 2021. PMID: 33944942. DOI: 10.1093/infdis/jiab236

56 Fraga AG, Cruz A, Martins TG, Torrado E, Saraiva M, Pereira DR, Meyers WM, Portaels F, Silva MT, Castro AG and Pedrosa J: Mycobacterium ulcerans triggers T-cell immunity followed by local and regional but not systemic immunosuppression. Infect Immun *79(1)*: 421-430, 2011. PMID: 20974825. DOI: 10.1128/IAI.00820-10

57 Ubol S, Chareonsirisuthigul T, Kasisith J and Klungthong C: Clinical isolates of dengue virus with distinctive susceptibility to nitric oxide radical induce differential gene responses in THP-1 cells. Virology *376(2)*: 290-296, 2008. PMID: 18455750. DOI: 10.1016/j.virol.2008.03.030

58 Röltgen K and Pluschke G: Buruli ulcer: The efficacy of innate immune defense may be a key determinant for the outcome of infection with *Mycobacterium ulcerans*. Front Microbiol *11*: 1018, 2020. PMID: 32523571. DOI: 10.3389/fmicb.2020.01018

59 Marion E, Chauty A, Kempf M, Le Corre Y, Delneste Y, Croue A and Marsollier L: Clinical features of spontaneous partial healing during Mycobacterium ulcerans infection. Open Forum Infect Dis *3(1)*: ofw013, 2016. PMID: 26925431. DOI: 10.1093/ofid/ofw013

60 Tello Rubio B, Bugault F, Baudon B, Raynal B, Brûlé S, Morel JD, Saint-Auret S, Blanchard N, Demangel C and Guenin-Macé L: Molecular mechanisms underpinning the circulation and cellular uptake of *Mycobacterium ulcerans* toxin mycolactone. Front Pharmacol *12*: 733496, 2021. PMID: 34603049. DOI: 10.3389/fphar.2021.733496

61 Nitenberg M, Bénarouche A, Maniti O, Marion E, Marsollier L, Géan J, Dufourc EJ, Cavalier JF, Canaan S and Girard-Egrot AP:

The potent effect of mycolactone on lipid membranes. PLoS Pathog *14(1)*: e1006814, 2018. PMID: 29320578. DOI: 10.1371/journal.ppat.1006814

62 Riches AC, Sharp JG, Thomas DB and Smith SV: Blood volume determination in the mouse. J Physiol *228(2)*: 279-284, 1973. PMID: 4687099. DOI: 10.1113/jphysiol.1973.sp010086

63 Kapoor G, Saigal S and Elongavan A: Action and resistance mechanisms of antibiotics: A guide for clinicians. J Anaesthesiol Clin Pharmacol *33(3)*: 300-305, 2017. PMID: 29109626. DOI: 10.4103/joacp.JOACP_349_15

64 Newman JC and Verdin E: β-hydroxybutyrate: much more than a metabolite. Diabetes Res Clin Pract *106(2)*: 173-181, 2014. PMID: 25193333. DOI: 10.1016/j.diabres.2014.08.009

65 Mann FM, Xu M, Chen X, Fulton DB, Russell DG and Peters RJ: Edaxadiene: a new bioactive diterpene from Mycobacterium tuberculosis. J Am Chem Soc *131(48)*: 17526-17527, 2009. PMID: 19583202. DOI: 10.1021/ja9019287

66 George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R and Small PL: Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. Science *283(5403)*: 854-857, 1999. PMID: 9933171. DOI: 10.1126/science.283.5403.854

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