# Buruli ulcer: reductive evolution enhances pathogenicity of *Mycobacterium ulcerans*

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Abstract | Buruli ulcer is an emerging human disease caused by infection with a slow-growing pathogen, *Mycobacterium ulcerans*, that produces mycolactone, a cytotoxin with immunomodulatory properties. The disease is associated with wetlands in certain tropical countries, and evidence for a role of insects in transmission of this pathogen is growing. Comparative genomic analysis has revealed that *M. ulcerans* arose from *Mycobacterium marinum*, a ubiquitous fast-growing aquatic species, by horizontal transfer of a virulence plasmid that carries a cluster of genes for mycolactone production, followed by reductive evolution. Here, the ecology, microbiology, evolutionary genomics and immunopathology of Buruli ulcer are reviewed.

Virulence plasmid
An autonomously replicating DNA element that is found in some pathogenic microorganisms and harbours genes that are essential for that microorganism to cause disease.

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The human skin disease that results from infection with *Mycobacterium ulcerans* is commonly known as Buruli ulcer (BU), but would have been called Bairnsdale ulcer if microbiological history had been strictly respected. In 1935, a series of unusual, painless ulcers in patients from a remote farming community in the Bairnsdale district of south-east Australia was reported[1]. Some 13 years later, Australian researchers discovered the aetiological agent of Bairnsdale ulcer, a previously unknown mycobacterium that they named *M. ulcerans*[2]. During the 1960s, many cases of infection with *M. ulcerans* were reported in Uganda, particularly in Buruli County (now known as the Nakasongola district), and thus the disease became more generally known as BU. Today, the disease is far more widespread in West and Central Africa, especially among impoverished rural communities, although other parts of the world are also affected. Thirty countries, mainly in the tropics, have reported cases of BU and in some settings, such as Ghana or Benin, BU is now more prevalent than leprosy[3].

During the past 10 years, there has been considerable progress in our understanding of the ecology, aetiology and microbiology of BU, which has led to better definition of risk factors and awareness of the potential role of insects in transmission of the disease. Comparative mycobacterial genomics has underpinned these advances and provided compelling evidence for the emergence of *M. ulcerans* as a pathogen through horizontal gene transfer of a virulence plasmid. Here, we review the current state of our knowledge and comment on prospects for disease control.

## Physiopathology and medical interventions

*M. ulcerans* produces mycolactone, a macrolide cytotoxin with immunosuppressive properties that is present in the extracellular matrix surrounding large clusters of *M. ulcerans* cells organized as a biofilm[4]. BU begins as a small, painless, raised skin papule, nodule, plaque or oedema. Osteomyelitis may occur in bone adjacent to a cutaneous lesion. Later, destruction of the subcutaneous adipose tissue leads to collapse of the epidermis and formation of a characteristic ulcer with undermined edges[5–7] (FIG. 1). Advanced lesions display massive tissue destruction and minimal inflammation, with extracellular microcolonies of *M. ulcerans* in the superficial necrotic areas[8]. Despite some anti-phagocytic activity of mycolactone, phagocytes can internalize *M. ulcerans* *in vitro*, although this culminates in host cell death[9,10]. In contrast to wild-type strains, mycolactone-deficient mutants generate inflammation in the guinea pig model[11], suggesting that production of mycolactone by intracellular bacteria can suppress innate inflammatory responses to infection *in vivo*.

The physiopathology of BU is closely associated with diffusion of the toxin from bacterial foci, as first suspected from histopathological studies of infected tissues[12]. Injection of mycolactone is sufficient to induce

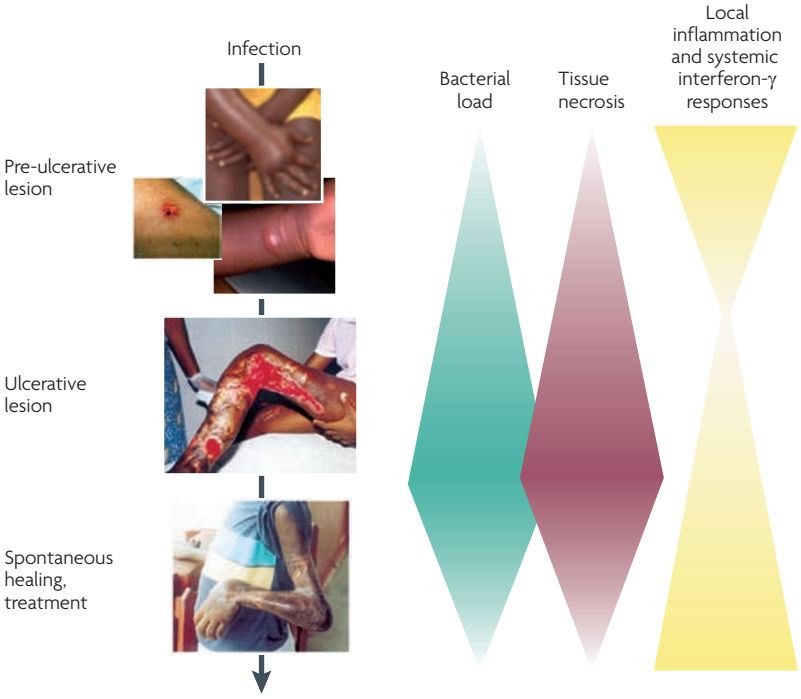



Figure 1 | **Disease progression.** The progression from the pre-ulcerative to the ulcerative and healing stages of Buruli ulcer disease is shown. The width of the green, red and yellow shapes denotes the extent of the progression for bacterial load, necrosis and inflammatory responses, respectively.

necrotic lesions in the skin of guinea pigs[13], by an apoptosis-dependent mechanism[14]. Consistent with the extensive tissue damage observed, mycolactone kills a range of mammalian cells[15], but the molecular basis of mycolactone-induced apoptosis nevertheless remains unclear. Indeed, studies that used mouse fibroblasts *in vitro* indicate that apoptosis follows cytopathic effects, including cytoskeletal rearrangements and cell-cycle arrest[13,14].

Although *M. ulcerans* displays no tropism for Schwann cells, nerve invasion occurs at the perineural and endoneural levels, causing degeneration[16]. Similar alterations are induced by injecting purified toxin into mice[17], suggesting that mycolactone diffuses into Schwann cells *in vivo* to cause the painlessness that characterizes the disease. A surprising feature of BU is the scarcity of secondary infections of these immunosuppressed open wounds, which raises the possibility that *M. ulcerans* produces secondary metabolites with anti-microbial activity (WHO provides a method for diagnosing secondary bacterial infection in *M. ulcerans* disease; see Further information).

BU is usually diagnosed on the basis of clinical findings, occasionally confirmed by microscopy, but to treat disease more effectively it is essential to develop novel, field-friendly diagnostic approaches to allow earlier identification of patients. The extensive cross-reactivity among mycobacterial antigens complicates the use of specific serological assays[18], although the unique ability of *M. ulcerans* to produce mycolactone makes this toxin an attractive candidate for the development of diagnostic tests. However, this hydrophobic molecule is not immunogenic, and attempts to raise anti-mycolactone antibodies have been unsuccessful.

Treatment of BU remains complicated and generally requires both surgery, sometimes accompanied by skin grafts, and prolonged courses of antibiotics. WHO recommends that a combination of rifampicin (oral) and streptomycin (intramuscular) be administered daily for 8 weeks[19]. However, although results from this treatment are encouraging (4 weeks of treatment leads to culture negativity), most patients need to be hospitalized owing to the frequency, duration and route of antibiotic administration. New drugs are clearly needed to treat this disease and better biomarkers are required to monitor the therapeutic response of patients.

There is no vaccine against *M. ulcerans*, although significant, but short-lasting, protection against BU has been reported following immunization with the *Mycobacterium bovis* bacille Calmette–Guérin (BCG) vaccine[20]. Prospects for vaccine development include modified BCGs and subunit-based vaccines[21]. DNA vaccines that encode antigen 85A and the heat-shock protein Hsp65 have been shown to protect mice against *M. ulcerans* infection[22–25]. However, these vaccines remain less protective than BCG in mice, even when administered in DNA-prime–boost protocols. Comparative genomics could open new avenues for vaccine research and improved diagnostics.

## Epidemiology, risk factors and insect vectors

The epidemiology of BU is poorly understood and outbreaks are sporadic and unpredictable; however, proximity to, or contact with, slowly flowing or stagnant watercourses is a recognized risk factor[26–28]. Furthermore, disease is often confined to specific areas and has sometimes appeared following major environmental changes, such as deforestation, flooding, or the introduction of dams and irrigation systems[29]. In Benin, for example, the incidence of BU is tenfold greater in areas that have undergone environmental perturbation compared with control areas[29].

In these settings, *M. ulcerans* is associated with aquatic vegetation, such as plants and algae[30], whereas snails and other organisms that feed on this vegetation can serve as passive hosts[31]. Portaels and colleagues were the first to use PCR-based detection methods to investigate a role for insect vectors in transmission[32]. Other groups have performed similar environmental surveys to uncover likely niches of *M. ulcerans*. *M. ulcerans* from environmental specimens is notoriously difficult and unreliable to culture owing to the long generation time of the pathogen (>48 hours) and overgrowth by contaminants, including other mycobacteria. PCR is more reliable but is not foolproof, as one of the diagnostic PCR targets, insertion sequence (IS) *2404*, is not restricted to *M. ulcerans*. Other targets also reside on the virulence plasmid, and the plasmid can be transferred to other species[33]. Such PCR methods have been used to detect *M. ulcerans* in aquatic samples, including water, mud, plants, insects, molluscs and fish, from endemic areas.

**Schwann cell**
A cell that surrounds neurons, produces myelin in nerves in the peripheral nervous system and is the preferred niche for the leprosy bacillus, a relative of *Mycobacterium ulcerans*. Schwann cells were named after their discoverer, Theodor Schwann.

**Secondary infection**
An infection that occurs as a consequence of a weakened or perturbed immune response following a primary infection by a different pathogen.

**Insertion sequence**
A mobile DNA element that encodes at least one transposase which is flanked by short inverted repeats that permit the element to copy itself within or between chromosomes.

An important series of experiments performed by Marsollier and colleagues[34] showed that *M. ulcerans* is carried and multiplies in the water bug, *Naucoris cimicoides*. Using two different methods of infection, either feeding *N. cimicoides* with larvae containing *M. ulcerans* or directly injecting the bacteria, these investigators showed not only that the bacterium became established in *N. cimicoides* but also that the transmission of *M. ulcerans* to laboratory mice occurred through biting, leading to the appearance of necrotic lesions that were reminiscent of BU in humans[34].

Details are available of the trafficking of *M. ulcerans* through *N. cimicoides* in captivity[35,36]. The plasmatocytes in the coelomic cavity of the water bug phagocytose *M. ulcerans* and, as part of their circulatory process, transport the bacteria to the salivary glands where large-scale multiplication occurs[35]. Only toxin-producing *M. ulcerans* isolates can invade the salivary glands, and mycolactone is therefore key to both the early and long-term establishment of *M. ulcerans* in members of the Naucoridae family. Later, the raptorial legs of the insect are covered by biofilms that contain *M. ulcerans*, which could be important for transmission of the disease without biting[35,36].

*N. cimicoides* is a carnivorous organism that preys on other insects, snails and small fish, but it does not normally bite humans, which weakens the possibility that biting is the main route of infection of humans. However, in a recent serological survey of healthy individuals in a BU-endemic region, high titres of antibodies were found to *N. cimicoides* salivary proteins[37], indicating that humans can indeed be bitten. Importantly, immunization of mice with saliva from uninfected *N. cimicoides* conferred protection against subsequent infection with *M. ulcerans* from contaminated water bugs.

In a landmark study from Benin, *M. ulcerans* was isolated from the water strider (*Gerris* sp. from the aquatic order Hemiptera) in pure culture. Following isolation of the pathogen and infection of mice, the *M. ulcerans* strain was shown to be genetically and phenotypically identical to that isolated from patients living in the same region[38]. However, the water strider, like the Naucoridae, generally avoids humans.

During the past decade, there have been several outbreaks of BU disease, both in residents and visitors, in parts of south-east Australia, and this provided an ideal setting to study disease transmission. Using PCR, a strong association was found between *M. ulcerans* and mosquitoes, predominantly *Aedes camptorhynchus*[39]. The incidence of BU rose in the spring and summer, and was followed by a cluster of human cases in the autumn and winter months. From this survey, it was estimated that the incubation period for BU was 3–4 months[39]. Furthermore, a bimodal pattern was observed, in which peaks represented young children and the elderly; this was similar to the pattern observed in West Africa, with the exception that in south-east Australia the disease was less common in children[40,41]. One possible explanation for this difference lies in the more pronounced exposure to possible insect vectors, and the accompanying immunity to *M. ulcerans*, among the adult African population[37].

A parallel case-control study revealed that the risk of BU was halved in the group that took precautions against insect bites by applying insect repellent, wearing protective clothing or washing minor skin wounds[42]. This strongly suggests that *M. ulcerans* is transmitted to humans by mosquito bites but, in turn, raises questions as to how the mosquitoes themselves become infected. Such contamination could occur at the larval stage from the water where the mosquitoes breed or through blood meals from other infected hosts. The conclusions of an extensive case-control study in a BU-endemic region of Cameroon are also consistent with transmission through insect bites, as protection was observed if bed nets were used in the home[43]. However, a large temporal and spatial survey conducted across Ghana of 15 BU-endemic and 12 BU-non-endemic areas revealed no association between biting hemipterans and *M. ulcerans*[44]. From these and other epidemiological studies, there is little evidence for person-to-person transmission. In summary, although there is now a substantial body of evidence to indicate an association between *M. ulcerans* and different insects that share the same aquatic ecosystem, the actual means of transmission to humans remains elusive. Here, the availability of new molecular and immunological tools should help.

## Genetically similar, phenotypically distinct

Despite their contrasting phenotypes, *M. ulcerans* and *M. marinum* have almost identical genome sequences (FIG. 2a). *M. ulcerans* replicates slowly and produces no photochromogenic pigments. By contrast, *M. marinum* doubles every 6–11 hours and produces no mycolactone, but is photochromogenic and has a dichotomous lifestyle: *M. marinum* lives in diverse aquatic niches as well as a range of different intracellular environments, from free-living aquatic amoebae and fish to macrophages from both frogs and humans[45]. In aquatic species, *M. marinum* causes a disseminated granulomatous disease that resembles dermal infection with *Mycobacterium tuberculosis*[46]. In humans, however, it provokes relatively minor granulomatous skin lesions, usually on the cooler extremities of the body.

Early phylogenetic work that applied multilocus sequence analysis (MLSA) revealed a clear delineation between strains of *M. ulcerans* and *M. marinum*, and indicated that all *M. ulcerans* strains have evolved from a common *M. marinum* progenitor[47–50]. MLSA also highlighted phylogeographical clonality among *M. ulcerans* strains (FIG. 2b), in which isolates from several African countries, South East Asia and both northern and south-eastern Australia belonged to three distinct genotypes, whereas strains from Japan and China were represented by two closely related genotypes and strains from Mexico and Suriname were represented by distinct genotypes[50,51]. These data have been corroborated and refined by microarray analyses[52,53]. Structural differences in mycolactone are also observed in strains of different genotype. Interestingly, the *M. ulcerans* isolates from South East Asia and Australia are more closely related to the African genotype than to strains from elsewhere. The lack of sequence diversity among

Raptorial legs
The two powerful front legs of some insect species that have evolved to catch and hold prey.

Photochromogenic pigment
A distinctive, bright yellow carotenoid pigment that is produced owing to exposure to light.

Dichotomous lifestyle
The ability to live in two different environments; for example, the ability to inhabit diverse aquatic ecosystems as an extracellular bacterium as well as different intracellular niches, such as within amoebae or host macrophages.

Granulomatous disease
The pathology associated with the distinctive, organized cellular immune response following mycobacterial infection.

Multilocus sequence analysis
A system for studying the population structure of bacterial populations by comparing the DNA sequences from a set of conserved genes among different strains.

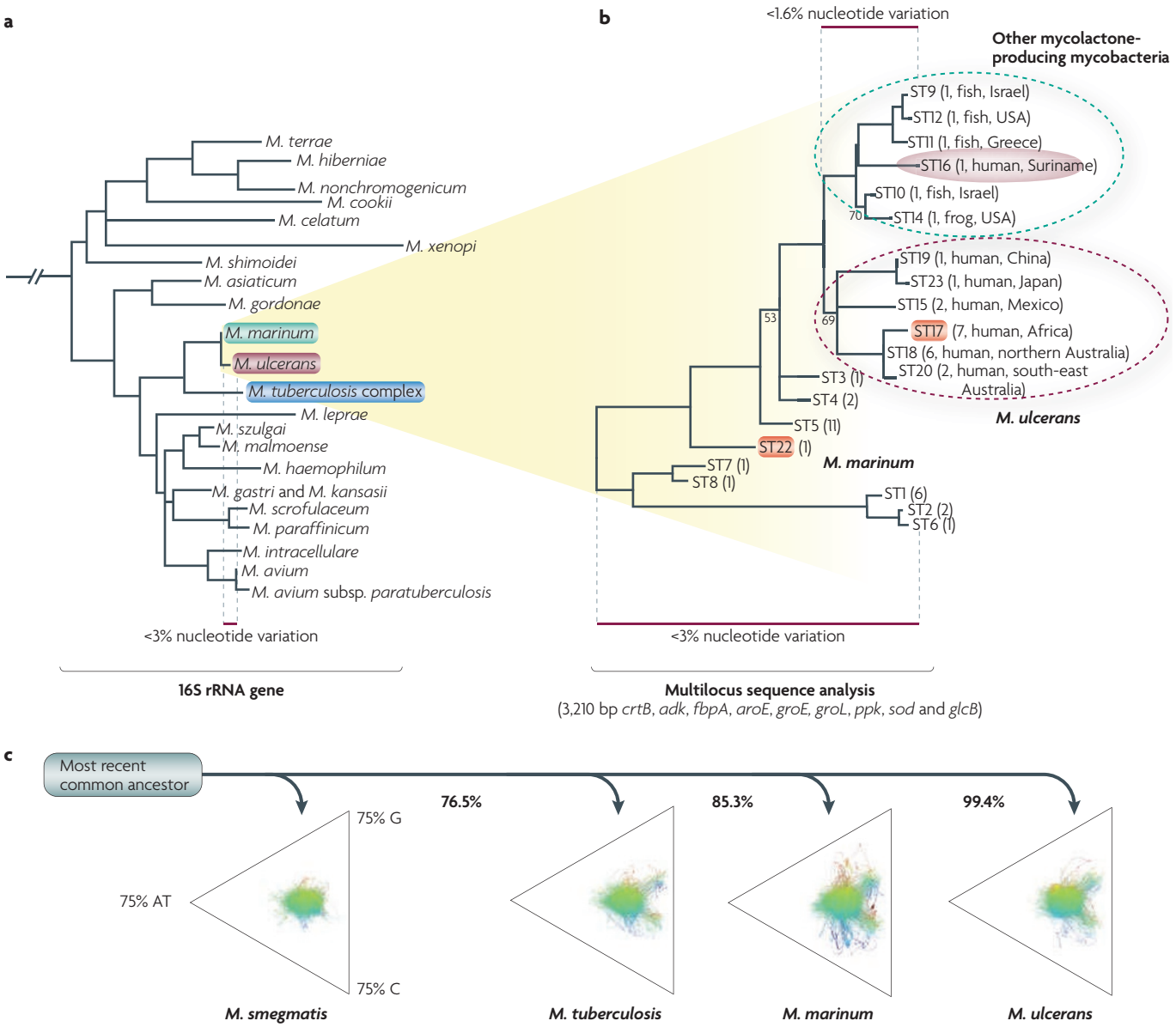



Figure 2 | **Phylogenetic analysis and evolutionary scenario for *Mycobacterium ulcerans*, *Mycobacterium marinum* and *Mycobacterium tuberculosis* inferred from genomics. a** | 16S rRNA phylogeny of the slow-growing mycobacteria, showing the relationship of *M. ulcerans* (highlighted in red) and *M. marinum* (highlighted in green) to *M. tuberculosis* (highlighted in blue) and other mycobacterial pathogens[101]. The purple line indicates defined clusters, such as *M. marinum*, *M. ulcerans* and the mycolactone-producing mycobacteria. **b** | The evolutionary history of *M. marinum*, *M. ulcerans* (indicated by a dashed red oval) and other mycolactone-producing mycobacteria (indicated by a dashed green oval) was inferred using the neighbour-joining method[102] from multilocus sequence data[33]. The bootstrap consensus tree was inferred from 1,000 replicates and was assumed to represent the evolutionary history of the taxa analysed[103]. Branches that correspond to partitions reproduced in less than 50% of the replicates were collapsed. The tree is drawn to scale, and branch lengths represent the evolutionary distances used to infer the phylogenetic tree. The first, second and third codon positions were included, and a total of 3,210 positions were included in the final data set. Phylogenetic analyses were conducted in MEGA4 (molecular evolutionary genetics analysis 4)[104]. Complete genome sequences were available for strains of the sequence types (STs) highlighted in red; the number of genome sequences available and their phylogenetic and geographical origin are indicated in brackets. The strain isolated from humans is indicated in pink. The yellow triangle shows that part **b** is an expansion of a section of the tree shown in part **a**. **c** | Whole-genome DNA-composition analyses of four mycobacterial genomes generated with gene spaghetti, a method for visualizing DNA base composition variation and the use of codons in a genome. The gradient of colours reflects the AT skew (T – A)/(T + A), from 60% T-rich (dark red) to 60% A-rich (dark blue). The florid gene-spaghetti patterns for *M. ulcerans*, *M. marinum and M. tuberculosis* reflect the important contribution of lateral gene transfer to the evolution of these species compared with *M. smegmatis*. The average percentage amino-acid identity between each species is also indicated (in bold) for a core set of 1,072 orthologous coding DNA sequences that were identified by whole genome comparisons between these species.

Table 1 | **Genomic comparisons of *Mycobacterium marinum* and *Mycobacterium ulcerans***

| | *M. marinum* M | *M. ulcerans* Agy99 |
|---|---|---|
| **Genome size and arrangement** | • Circular chromosome; 6,637 kb<br>• Circular mercury resistance plasmid (pMM23; 23 kb) | • Circular chromosome; 5,632 kb<br>• Circular mycolactone-associated plasmid (pMUM001; 174 kb) |
| **Number of genes** | 5,424 | 4,160 |
| **Number of pseudogenes** | 65 | 771 |
| **Number of insertion sequences (ISs)** | 7 ISs; *Myma01* (7 copies), *Myma02* (7 copies), *Myma03* (4 copies), *Myma04* (5 copies) and *Myma05*, *Myma06* and *Myma07* (2 copies of each) | IS*2404* (213 copies) and IS*2606* (91 copies) |
| **Number of PE and PPE genes** | 175 PE genes and 106 PPE genes | 69 PE genes and 46 PPE genes |
| **Number of ESX secretion systems** | 5 | 3 |
| **Number of ESX effectors** | 18 *espA* paralogues and 31 *esx* paralogues | 2 *espA* paralogues and 14 *esx* paralogues |
| **Number of phospholipase C genes** | Seven phospholipase C paralogues (*plcB*, *plcB1*, *plcB2*, *plcB3*, *plcB4*, *plcB5* and *plcB6*) | One gene that encodes phospholipase C (*plcB*); others have become pseudogenized or lost by DNA deletion |
| **Number of lipoproteins** | 88 genes that encode lipoproteins | 77 genes that encode lipoproteins; *lipY* has been lost by deletion |
| **Phenolic glycolipids** | Mycoside M | Phenolphthiodiolone lipid backbone cannot be glycosylated as the glycosyl transferase (MUL_1998) has been pseudogenized |
| **Mycolactone** | Not produced | Mycolactone A and B |

the African isolates suggests that this *M. ulcerans* clone spread recently throughout Africa. The availability of complete genome sequences from an African epidemic strain of *M. ulcerans* (strain Agy99, isolated from Ghana in 1999) and a strain of clinical *M. marinum* (strain M) confirmed this evolutionary scenario, highlighting how horizontal gene transfer and reductive evolution have remodelled an *M. marinum* progenitor into *M. ulcerans* (FIG. 2c).

*In silico* genomic comparisons of *M. ulcerans* with *M. marinum* confirmed the close genetic relationship between these species, as they shared more than 4,000 orthologous and syntenic protein-coding DNA sequences (CDSs) (TABLE 1) and had an average sequence identity of 98.3%. This analysis also revealed that *M. ulcerans* had lost over 1.1 Mb of DNA owing to deletions (TABLE 1), whereas 168 kb had been acquired by *M. marinum*, mostly in the form of 10 prophages. Also evident were many chromosome rearrangements that were facilitated, at least in part, by the high number of IS*2404* (213 copies) and IS*2606* (91 copies), which disrupt >110 genes[54]. All *M. ulcerans* strains tested have 11 chromosomal CDSs that seem to be specific to this bacterium and might, in conjunction with mycolactone, contribute to the pathology associated with BU. These genes, and their products, could also be used to develop new diagnostic tests[54].

*M. ulcerans* has thus evolved through lateral gene transfer and reductive evolution, acquisition of the virulence plasmid pMUM001 (the role of which is discussed below), massive expansion of IS*2404* and IS*2606*, extensive pseudogene formation, genome rearrangements and gene deletion. Like *Yersinia pestis*[55] and *Bordetella pertussis*[56], *M. ulcerans* has all the characteristics of a bacterium that has recently passed through an evolutionary bottleneck and is adapting to a new niche environment. The challenge remains to find that niche, and in this respect, genomics may hold some clues.

## Loss or gain of virulence and immunogenicity?

The trend that has emerged from reductive evolution in *M. ulcerans* is the loss of many virulence factors and immunogens compared with its *M. marinum* progenitor (TABLE 1) and the gain of an immunosuppressive cytotoxin (FIG. 3). Most noteworthy is the drastic reduction in the cell surface proteins PE and PPE from 281 in *M. marinum* to 115 in *M. ulcerans*, accompanied by depletion of the related ESX secretion systems and their effector proteins. PE and PPE proteins have characteristic amino-terminal domains and biased amino-acid content, as they are particularly rich in glycine and alanine. They are restricted to mycobacteria, where they are found in the cell envelope, but their precise function is unknown. The ESX loci, which encode type VII secretion systems[57], are required to export members of the ESAT-6 (6 kDa early secretory antigenic target) protein family and specific effectors, such as EspA (ESX-1 secretion-associated protein A)[58]. In at least one case, ESX-1, they are major contributors to mycobacterial virulence.

A few examples merit comment. In *M. marinum*, LipY is an immunodominant PPE protein with triacylglycerol hydrolase activity[59], yet its gene has been deleted by *M. ulcerans*. In another example, the ESX secretome

Syntenic protein-coding DNA sequence
An orthologous protein-coding DNA sequence that shares the same genomic arrangement and order in two or more strains of a particular species.

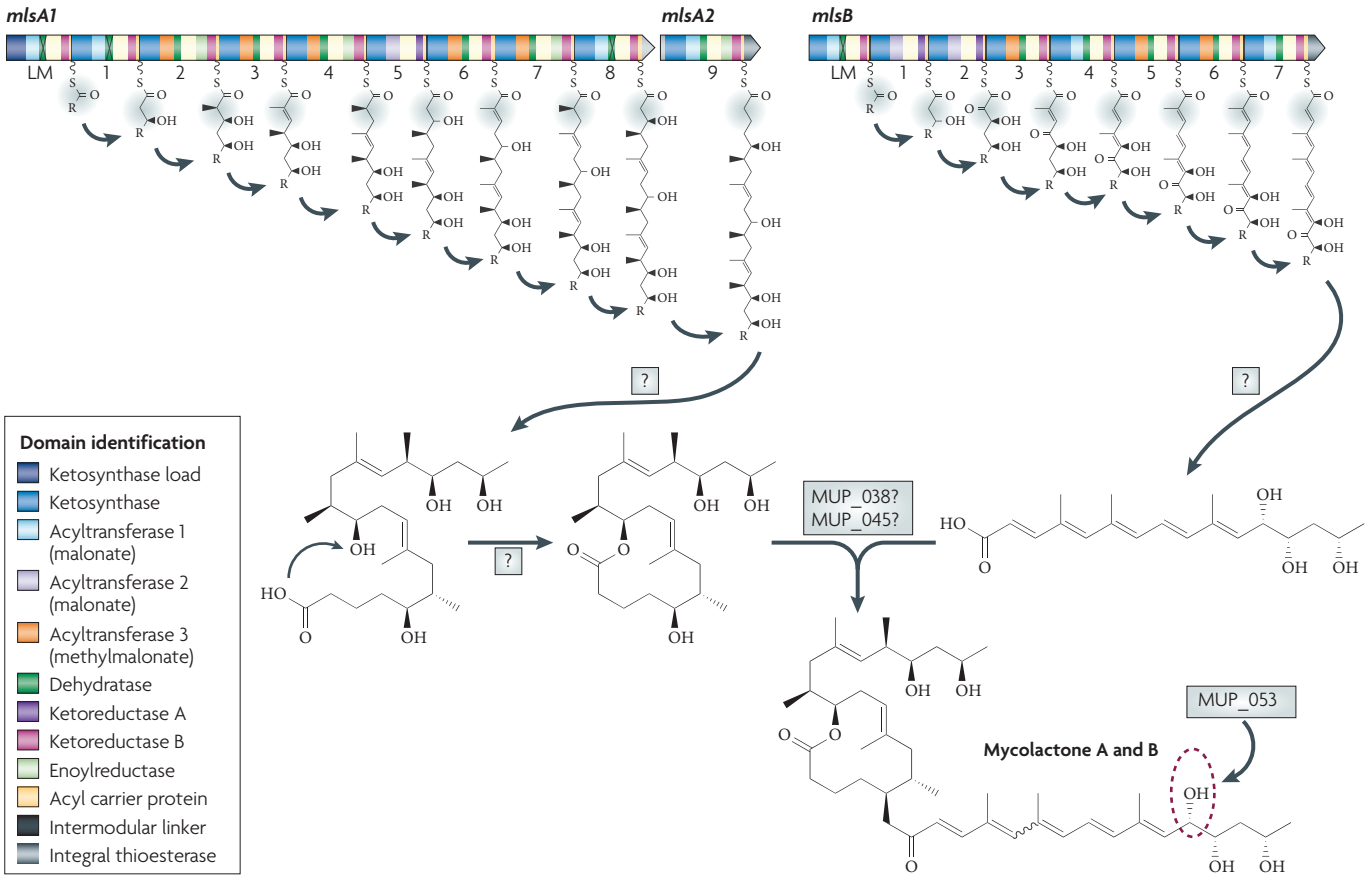



Figure 3 | **Proposed pathway for the biosynthesis of mycolactone A and B.** The *mls* cluster and accessory gene arrangement of pMUM001, as well as the domain and module organization of the mycolactone polyketide synthase (PKS) genes, are shown. The different domains of each of the three Mls proteins (MlsA1, MlsA2 and MlsB) are represented by the coloured blocks described in the key. Module arrangements are depicted below each protein. The module responsible for a particular chain extension and the modified substrate are shown tethered to the acyl carrier protein domain of each extension module. Key steps in the biosynthesis pathway are shown and the enzyme involved is indicated if known. A question mark indicates that the enzymes involved have not yet been identified or in the case of MUP_038 and MUP_045 have not been definitively proven. The dashed red circle indicates that the hydroxyl group is catalysed by MUP_053. LM, loading module.

was depleted from five systems to three[54]. Consequently, the protein substrates these systems export — including potent T-cell antigens, such as ESAT-6, which have important roles in granuloma formation and other aspects of pathogenesis[58], and the EspA effector proteins[60], which have been reduced from 18 paralogues in *M. marinum* to two in *M. ulcerans* — will be missing or less abundant. A recent study examined a wide range of *M. ulcerans* strains for disruption to the ESX loci, and found that although some strains exhibit the same pattern as *M. ulcerans* Agy99, others have acquired independent deletions or loss-of-function mutations in these regions. This suggests that loss of ESX loci provides a selective advantage for mycolactone producers[61] or, alternatively, that gain of a powerful cytotoxin renders these virulence factors superfluous.

Phenolic glycolipids (PGLs) are abundant cell-wall components, antigens and major virulence factors for several mycobacterial pathogens and can modulate innate immunity[62]. In *M. marinum*, PGLs are synthesized by glycosylation of a highly apolar and abundant polyketide-derived methyl-branched lipid intermediate called phenolphthiodiolone. *M. ulcerans* also synthesizes prodigious quantities of phenolphthiodiolone but cannot make PGL, as the gene for the glycosyl transferase that adds the rhamnosyl moiety (locus tag MUL_1998) is inactive[54,63]. These observations are summarized in TABLE 1.

Other gene losses from *M. ulcerans* give some clues as to its habitat. For example, phytoene dehydrogenase, encoded by *crtI*, is an essential enzyme for the biosynthesis of light-inducible carotenoid pigments by *M. marinum*. These pigments protect the bacterium from damage following exposure to sunlight[64]. *M. ulcerans* has an identical pigment locus to *M. marinum* but its *crtI* gene has a premature stop codon, suggesting that its pigments are not required for survival, presumably because the bacterium is not exposed to sunlight. Another observation from the *M. ulcerans* genome is that the pathways for anaerobic respiration have been

**Polyketide**
A polymer made up of ketide monomers: organic acids with keto groups, such as malonic acid, that are commonly produced as secondary metabolites by certain bacteria and fungi.

lost, indicating that the bacterium might occupy an aerobic or microaerophilic environment. Together, these data provide a profile of a bacterium that has adapted to a dark and aerobic environment where slow growth, decreased ESX-mediated virulence and production of mycolactone provide an advantage for survival. This profile also suggests that we may need to think more broadly and look more widely than aquatic invertebrates to find the true reservoir of this pathogen.

## Mycolactone production

A major finding from the *M. ulcerans* genome project was the virulence plasmid pMUM001 (REFS 65,66), which carries a cluster of three large genes (*mlsA1*, *mlsA2* and *mlsB*, which are 51 kb, 7.6 kb and 43 kb in size, respectively) that encode type I polyketide synthases (PKSs) (FIG. 3). Like type I fatty acid synthases, type I PKSs contain the multiple enzymatic activities that are required for one round of chain extension and modification in a single polypeptide. Detailed analysis of their predicted module and domain structure strongly suggested that these PKSs produce mycolactone, and this was subsequently confirmed by transposon mutagenesis[66].

Together, MlsA1 and MlsA2 constitute a loading module and nine extension modules that synthesize the macrolactone core and upper side chain, whereas MlsB, with its loading module and seven extension modules, produces the acyl side chain. The proposed biosynthesis pathway for mycolactone is shown in FIG. 3, and highlights the sequential incorporation and modification of either acetate or propionate subunits at each extension module. pMUM001 also harbours three additional CDSs that encode putative auxiliary enzymes for mycolactone synthesis (FIG. 3). Cyp140A7 is a cytochrome P450 hydroxylase that probably hydroxlates C-12′ of the mycolactone side chain[67–69]. Experimental evidence is lacking for the remaining two enzymes, a type II thioesterase (locus tag MUP_038) and a FabH-like ketosynthase (locus tag MUP_045), but they may play parts in chain termination and transfer of the mycolactone acyl side chain to the core[15] (FIG. 3).

There are only a few reports of biochemical studies of the mycolactone PKS. The ketoreductase domains have been expressed and their enzymatic function confirmed[70]. A similar study with the integral thioesterase domains of MlsA2 and MlsB[71] (FIG. 3), which may release the mature polyketide chain, revealed little activity, suggesting that these domains may be inactive in the Mls complex[71]. A proteomic investigation identified MlsA1, MlsB, Cyp140A7 and MUP_045 in association with both the cytoplasmic and membrane fraction, indicating that synthesis occurs close to the membrane, which could facilitate mycolactone export[72] (FIG. 3).

Different strains of *M. ulcerans,* and close relatives from fish and frogs, produce at least five structurally distinct mycolactones, named mycolactone A/B, C, D, E and F[73–77]. *M. ulcerans* strains from Africa, Malaysia and Japan produce mycolactone A/B, Australian strains produce mycolactone C, Chinese strains produce mycolactone D and *Mycobacterium liflandii* and *Mycobacterium pseudoshottsii* produce the unique mycolactones E and F, respectively[15]. The mycolactone type produced by South American strains is unknown. Immunosuppressive and cytotoxic activity measurements revealed a convenient 'alphabetical' gradient in which mycolactone A/B is the most potent and mycolactone F is the least potent[67]. Interestingly, all mycolactones have a conserved core structure, and any variation occurs in the length, methyl branching, oxidation state and stereochemistry of the acyl side chain[15].

## Mycolactone-producer evolution

Mycolactone-producing mycobacteria have been recovered from fish and frogs around the world, but so far have not been associated with human disease. Based on minor phenotypic differences, they were given species names, such as *M. pseudoshottsii, M. liflandii* and *M. marinum*[15,49,69,73,78]. However, thorough phylogenetic comparisons (FIG. 2b) have shown that all mycolactone producers are closely related to *M. ulcerans* and have evolved from a common *M. marinum* progenitor to form a genetically cohesive group among a more diverse assemblage of *M. marinum* strains[49]. Like *M. ulcerans*, the fish and frog strains have a virulence plasmid and multiple copies of IS*2404*, but the pattern of DNA deletion and pseudogene accumulation seems to be different: only those strains that cause BU had reduced genomes. Comparisons of plasmid and chromosomal gene sequences show that plasmid acquisition, and the subsequent ability to produce mycolactone, was probably the key driver of speciation. As these mycolactone producers then radiated around the world, ongoing evolution produced at least two genetically distinct ecotypes that can be broadly divided into those that typically cause disease in ectotherms, such as fish and frogs, and those that typically cause disease in endotherms, such as humans.

## Molecular target of mycolactone

Despite efforts from many research groups, the molecular target of mycolactone and the mechanism used by the toxin to suppress immune cell functions remain mysterious. The structure of mycolactone resembles that of the immunosuppressive agents FK506 and rapamycin (BOX 1). However, mycolactone has different effects on dendritic cell (DC) and T-cell immunobiology, suggesting that the toxin might bind to a different receptor and interfere with distinct signalling pathways. A fluorescent derivative of mycolactone and a $^{14}$C-labelled form of the toxin were both found to accumulate in a time- and dose-dependent manner in the cytoplasm of treated cells[79] (C.D., unpublished observations), which supports the idea that mycolactone diffuses passively into target cells to interact with a cytosolic receptor. So far, structure–function studies have been limited to natural mycolactones. Strategies for the generation of additional mycolactone variants are required to investigate further the molecular mechanism of mycolactone action. In addition, detoxified variants of mycolactone might compete with mycolactone for receptor binding, and could therefore constitute valuable functional inhibitors of toxin. These could eventually become novel anti-BU therapeutics.

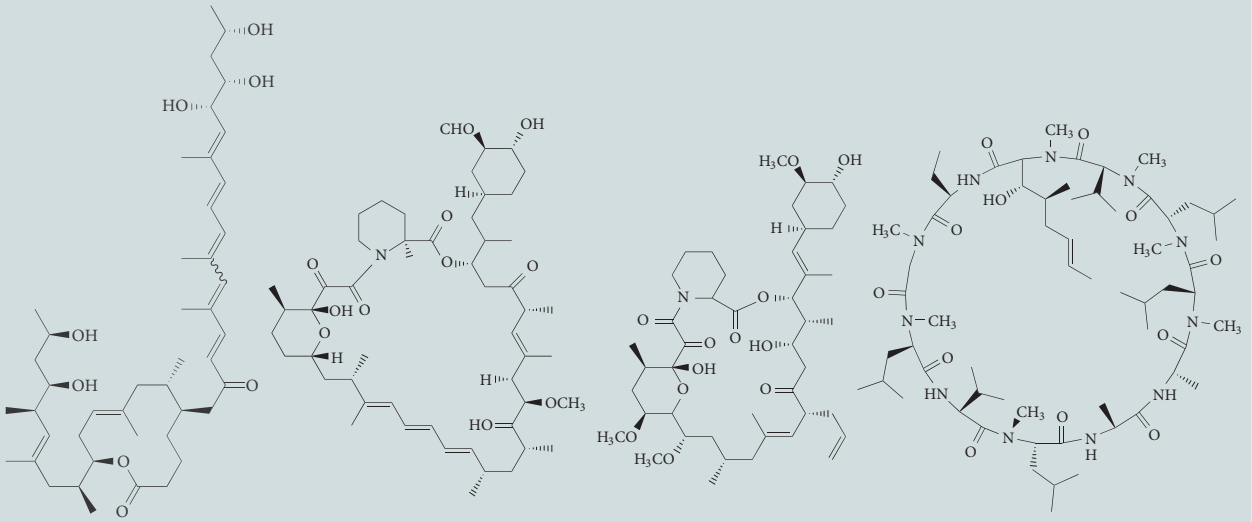
Inflammatory infiltrate
White blood cells that leave the blood to infiltrate inflamed connective tissues.

## Suppression of immune cell functions

Although the basis of mycolactone cytotoxicity remains obscure, our understanding of the mechanism of mycolactone-induced immunosuppression has progressed. However, drawing conclusions on the type of immune response that is mounted in humans against *M. ulcerans* infection is rendered difficult by apparently contradictory results. *In vitro* studies show unambiguously that non-toxic doses of mycolactone are immunosuppressive on professional antigen-presenting cells. The most striking finding is the complete inhibition of tumour necrosis factor (TNF) production by monocytes and macrophages following infection with *M. ulcerans* or incubation with exogenous mycolactone[9,25,80]. Notably, bacterial production of mycolactone was also found to suppress the capacity of DCs to prime cellular responses and produce chemotactic signals that are crucial for inflammatory responses[81] (FIG. 4). This selective effect of mycolactone on the ability of immature DCs to secrete chemoattractants for monocytes and T helper 1 ($T_H1$) lymphocytes is in accordance with the histopathology of BU, and supports the idea that local production of mycolactone in the skin prevents the trafficking of inflammatory cells to the ulcerative lesion.

However, these results seem to contradict quantitative studies of intralesional mRNA which indicate that the innate immune system is activated at the site of BU lesions, as shown by high mRNA levels for the cytokines interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-6, IL-10, IL-12, IL-15 and TNF, and the chemokine IL-8 (REFS 7,82,83). There are several possible, non-exclusive explanations for this discrepancy between *in vitro* and *in vivo* observations. Results from mouse studies[84] indicate that the lack of inflammatory infiltrates in BU is caused by the continuous destruction of inflammatory cells, rather than local immunosuppression. Alternatively, mycolactone or some other *M. ulcerans* component, may suppress the expression of inflammatory cytokines

Box 1 | **Parallels between mycolactone and other immunosuppressors**



| | Mycolactone A and B | Rapamycin | FK506 | Cyclosporin A |
|---|---|---|---|---|
| **Origin** | *Mycobacterium ulcerans* | *S. hygroscopius* | *S. tsukubaensis* | *Tolypocladium inflatum* |
| **Molecular target** | ? | FKBP1A | FKBP1A | Cyclophilin (immunophilin) |
| **Mode of action** | ? | mTOR inhibition | Calcineurin inhibition | Calcineurin inhibition |
| **Effect on DCs** | ↓ Co-stimulatory molecules<br>↓ IL-6 and IL-12<br>No effect on TNF or IL-8<br>↓ β-chemokines | ↓ Co-stimulatory molecules<br>↓ IL-12 | ↓ Co-stimulatory molecules<br>↓ IL-6, IL-12, IL-8 and TNF<br>↓ β-chemokines | ↓ Co-stimulatory molecules<br>↓ IL-6, IL-12 and TNF |
| **Effects on LTs** | ↓ IL-2 and IFN-γ<br>Variable cytostatic effect | ↓ Protein synthesis<br>Cell-cycle arrest | ↓ IL-2 and IFN-γ | ↓ IL-2, IFN-γ and IL-4 |

Intriguingly, the structure of mycolactone is related to that of a family of natural products produced by actinomycetes[15]. In particular, it shares similar features with rapamycin, a macrocyclic triene produced by *Streptomyces hygroscopicus* and FK506, a macrolide lactone from *Streptomyces tsukubaensis* (see the figure). The molecular structure of the soil fungi metabolite cyclosporin A is more distant, but has a similar size and a similar proportion of hydrophobic groups[81]. FK506, rapamycin and cyclosporin A are all potent immunosuppressive drugs that alter the functional biology of lymphocytes and dendritic cells[99]. FK506 and rapamycin bind the same intracellular receptor, FKBP1A, although the resulting complex targets a different molecule. By contrast, FK506 and cyclosporin A bind different targets, but they both act by inhibiting calcineurin activity and produce similar biological effects. Similarly to mycolactone, FK506 modulates the production of β-chemokines. However, FK506 blocks dendritic cell production of inflammatory cytokines that are not altered by mycolactone, suggesting that mycolactone binds a different receptor and interferes with another signalling pathway that remains to be identified[100]. IFN, interferon; IL, interleukin; mTOR, mammalian target of rapamycin; TNF, tumour necrosis factor.

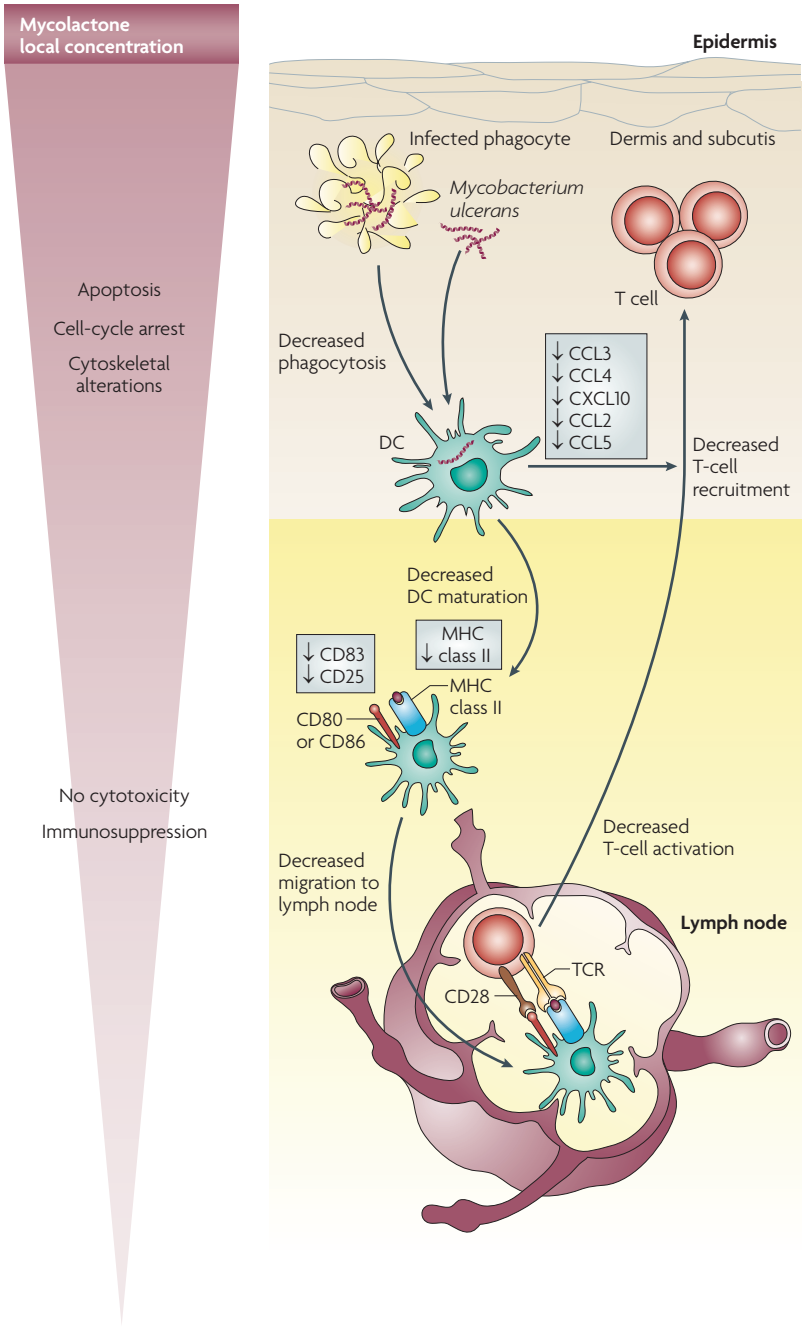



Figure 4 | **Model of the cytotoxic and immunosuppressive actions of mycolactone *in vivo*.** The triangle illustrates the gradient of mycolactone concentration from infectious foci in the skin to internal tissues, and the associated biological effects. The multiple immunosuppressive properties of mycolactone on dendritic cells (DCs) are shown, and their consequences on the initiation of primary immune responses and recruitment of inflammatory cells to the site of infection indicated[81]. CCL, C-C motif chemokine; CXCL10, C-X-C motif chemokine 10; MHC, major histocompatibility complex, TCR, T-cell receptor.

at a post-transcriptional level, by inhibiting protein translation or secretion. It will therefore be essential in the future to find ways of quantifying mycolactone in infected tissues, measure the local production of chemoattractants and further dissect the molecular mechanisms of mycolactone immunosuppression.

Several independent studies have reported modulation of the systemic IFN-γ responses in patients with BU using restimulation assays of peripheral blood mononuclear cells *ex vivo*[83,85–90]. One of these studies reported an inverse ratio of IFN-γ versus IL-10 in patients with ulcerative disease compared with subjects at the nodular stage[83]. Although the IFN-γ responses of patients with BU were not always significantly different to those of healthy controls, they were lower at the early stages compared with the ulcerative or healing stages[88,89,91]. Cases of disseminated disease have been reported in patients with AIDS, and there is an increased prevalence of HIV-1 or HIV-2 in patients with BU. Importantly, the systemic suppression of IFN-γ responses in patients with BU is not specific for mycobacterial antigens, and resolves after surgical excision of the lesion[90]. In mice, cellular response defects occur following infection with wild-type *M. ulcerans*, but not with a mycolactone-deficient mutant[95]. Although there is no definitive evidence that mycolactone circulates in humans, it seems likely that the toxin is responsible for this systemic immunosuppression. In support of this idea, we found that subcutaneously delivered mycolactone diffuses into the peripheral blood of mice, and accumulates in internal organs with a tropism for lymphoid organs[95]. Furthermore, mycolactone is a potent suppressor of IL-2 and IFN-γ production by human T cells *in vitro*[96] (C.D., unpublished observations).

The generation of appropriate IFN-γ responses is crucial for protective immunity against most mycobacterial infections. In BU, spontaneous healing often coincides with conversion to a positive delayed hypersensitivity against *M. ulcerans* antigens, suggesting that $T_H1$ cellular immune responses are beneficial and contribute to the eradication of bacilli[97]. Interestingly, antibiotic treatment of BU induces vigorous inflammation in different compartments of the skin[98], indicating that patients can mount $T_H1$ responses. During the disease, the generation of cellular immunity can thus be suppressed by mycolactone in two ways (FIG. 4): first, at the site of infection, where the mycolactone concentration is cytotoxic, by killing resident DCs and inflammatory infiltrates; and second, at the systemic level, by reducing the ability of DCs and T cells to respond to stimulation without any major impact on their viability. In this model, withholding the immunosuppression imposed by mycolactone using inhibitors of its biosynthesis, or ablating its biological activity *in vivo*, would be sufficient to trigger the development of cellular immunity and allow the host immune system to control the infection.

## Concluding remarks

Although there has been impressive recent progress in our exploration of the role of aquatic insects in the transmission of BU, our understanding of the precise mechanisms that occur remains incomplete. The coming years will reveal whether insects truly act as disease vectors or if they have simply been incriminated by their association with *M. ulcerans*. The highly sensitive molecular tools now available for tracking the BU bacillus will find increasing application and, like other genome-derived

approaches, help to pinpoint the environmental source of infection. The history of BU provides a cautionary tale for other emerging diseases, as human intervention in the environment has clearly favoured emergence of the disease through the creation of new niches and habitats both for *M. ulcerans* and the aquatic insects within which it resides. Acquisition of the virulence plasmid by an ancestral *M. marinum* species though horizontal gene transfer was the main driver for disease emergence in humans and probably also for the infection of lower life forms. Unravelling the immunosuppressive pathways induced by mycolactone in mammalian cells will certainly be a profitable area of research, and improved understanding of the BU structure–activity relationship may enable the dissociation of cytotoxicity from immunosuppression. In turn, it would be satisfying if a version of a once disfiguring toxin could be engineered to afford therapeutic benefits similar to those of rapamycin to humans.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### DATABASES
**Entrez Gene:** http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
*crtI* | *mlsA1* | *mlsA2* | *mlsB* | MUL_1998
**Entrez Genome Project:** http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
*Bordetella pertussis* | *Mycobacterium bovis* bacille Calmette–Guérin | *Mycobacterium liflandii* | *Mycobacterium tuberculosis* | *Mycobacterium ulcerans* | *Yersinia pestis*
**UniProtKB:** http://www.uniprot.org
IFN-γ | IL-1β | IL-2 | IL-6 | IL-8 | IL-10 | IL-15 | LipY

### FURTHER INFORMATION
**Caroline Demangel's homepage:** http://www.pasteur.fr/recherche/unites/Pmi
**Stewart T. Cole's homepage:** http://cole-lab.epfl.ch/
**Timothy P. Stinear's homepage:** http://www.med.monash.edu.au/microbiology/research/stinear.html
**BuruList World-Wide Web Server:** http://genolist.pasteur.fr/BuruList
**WHO (secondary bacterial infection in M. ulcerans disease):** http://www.who.int/buruli/information/diagnosis/en/index6.html

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