The Immunology of Buruli Ulcer

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Buruli ulcer (BU) represents a unique human mycobacteriosis. Caused by *Mycobacterium ulcerans*, the disease spectrum is dominated by the activity of mycolactone, a dermanecrotic toxin that has shown the ability to interfere with the immune response. This poses an additional difficulty to the understanding of the immunological determinants for the outcome of infection, a fundamental step to develop better preventive or curative strategies. In this chapter, the immune response against *M. ulcerans* is reviewed, both with a series of clinical observations and experimental infection models and going through several other lines of evidence, including epidemiological and genetic studies. This holistic approach is expected to shed further light on the intriguing pathophysiology of this disease and help guide future research efforts.

1 Buruli Ulcer: The First Histological Observations of a Necrotic Track

Mycobacterium ulcerans has always been a distinct pathogen among mycobacteria. The classical clinical description of *M. ulcerans* infection, Buruli ulcer (BU), reports a lesion that usually begins as an indurated subcutaneous papule which slowly ulcerates, presenting as an ulcer with undermined edges [1–3]. Typical skin biopsies reveal epidermal destruction concurring with areas of hyperplasia and, in the deeper layers of the skin, a widespread panniculitis with many thrombotic capillaries and in some cases calcification of fat cells [1, 3–6]. In the necrotic core, a large number of bacteria are usually present in the extracellular space amidst apoptotic cell debris,

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sometimes surrounded by a belt of scattered neutrophils and macrophages with intracellular bacilli, as well as T cells and foci of B cells [1, 4, 7–10]. As such, suspicions early arose of BU pathology being dominated by an exotoxin that would diffuse and destroy the surrounding tissues [4, 11].

Indeed, purified in 1999, a polyketide-derived macrolide named mycolactone was shown to reproduce many of the histopathological hallmarks of BU disease, inducing extensive tissue necrosis and microvascular thrombosis, in a dose-dependent way [12, 13]. Specifically, mycolactone provokes apoptotic cell death by driving expression of pro-apoptotic proteins BCL2L11 (Bim) and Fas and inhibiting cell cycle progression [14–16]. Furthermore, this toxin has the remarkable ability to bind to the alpha subunit of the Sec61 translocon in the endoplasmatic reticulum (ER), impeding post-translational modifications, such as glycosylation, and translocation of newly synthesized membrane and secretory proteins into and across the ER membrane [17, 18]. Eventually, cytokines interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin (IL) 6 and cyclooxygenase (Cox) 2, as well as a variety of other secreted proteins, end up in the cytosol, being marked for proteasome degradation [17, 18]. Lastly, mycolactone also disturbs several pathways related to stress response, collagen biosynthesis and cytoskeleton dynamics, the latter mainly achieved by hyperactivation of the Wiskott–Aldrich syndrome protein (WASP) [19, 20].

The pleiotropic effects of mycolactone make *M. ulcerans* infection a unique paradigm of successful manipulation of the host immune orchestra. By inducing cellular death and preventing the generation and processing of crucial signaling molecules, the ability of the host to cope with the pathogen becomes severely crippled. Thus, what the actual role of the immune system is in BU, is a question of utmost importance to understand the disease.

2 Host Attempts to Control *Mycobacterium ulcerans* Infection

2.1 Local Immune Response

Due to its indolent course and various clinical presentations, characterizing the kinetics of the immune response against *M. ulcerans* in humans has always been a challenge. It is therefore not surprising that much of what is known about BU pathogenesis today came from studies resorting to animal models, which seem to present several resemblances to the human findings [1, 21–23].

Although understanding the very early stages of the contact of *M. ulcerans* with the host is still of extreme difficulty, it is currently thought that the pathogen depends on skin surface injuries or transmission vectors to penetrate the epidermal layer of the skin, highly rich in keratinocytes [24–26]. The importance of these cells for BU pathogenesis became evident when it was discovered that upon recognition of *M. ulcerans* through dectin-1, toll-like receptor (TLR) 2 and to a lesser extent TLR4, keratinocytes produced bactericidal reactive oxygen species and cathelicidin, as well as chemokines IL-8 and the monocyte chemoattractant protein (MCP) 1 [27].

Once mycolactone-producing strains of *M. ulcerans* reach the subcutaneous tissue, there is an influx, within the first hours, of neutrophils to the center of the lesion and of major histocompatibility complex (MHC) class II^+ cells to the periphery of the infection foci, followed by a sustained recruitment of macrophages for at least a week [22, 28]. During this stage, mycobacteria are phagocytosed and, with the contribution of the activity of its superoxide dismustase and catalase enzymes, transiently replicate within phagosomes [9, 29]. Upon replication, the pathogen is released to the extracellular space, accumulating in the newly formed necrotic areas where it perpetuates the cycle of mycolactone production, leading to the generation of acellular foci that continuously expand [9, 22]. Mycolactone further exacerbates this process by unbalancing the levels and activation state of thrombomodulin, protein C and other factors belonging to the coagulation cascade, favoring fibrin deposition and subsequent vessel thrombosis [30, 31]. As a consequence, the central necrotic area shows a high number of extracellular bacilli, surrounded by an infiltrate of neutrophils, macrophages and lymphocytes located in the interface between the necrotic area and healthy tissue [22]. With the inability of the immune system to reach the mycolactone producing-mycobacteria, the infection then progresses with the appearance of edema and ulceration of the epidermis, which can be potentiated by the coalescence of several foci of infection, if present [22].

Production of different congeners of mycolactone by different *M. ulcerans* lineages may alter the aggressiveness of the disease or even lead to distinct phenotypes, as it was initially observed in humans and later confirmed in mice [22, 23, 32–34]. In fact, in a proteomics study comparing a wild type (WT) mycolactone-producing strain with a mycolactone-negative strain isolated from a transposon library, several proteins related to virulence and stress response factors were found to be upregulated in the mycolactone-negative strain [35]. In practice, in the absence of mycolactone, as in the case of the natural mutant Mexican isolate 5114, infection with *M. ulcerans* resembles other mycobacterial infections [22, 23, 36]. Indeed, after an initial influx of neutrophils, well-formed granuloma-like structures, mostly composed of macrophages with epithelioid transformation and of lymphocytes, organize in the following days to weeks and surround the proliferating mycobacteria [12, 22, 23]. These inflammatory infiltrates eventually distribute homogeneously in the subcutaneous and muscle tissue, with no apparent necrosis or alterations occurring in the epidermal layer of the skin [12, 22, 23]. Surprisingly, the fact that mice are seemingly unable to eliminate the mycolactone-negative mutants for at least 12 months suggests that the bacteria use additional mycolactone-independent mechanisms to evade the immune system [22]. It should be noted that many of the classical mycobacterial virulence factors, such as the 6 kDa early secretory antigenic target (ESAT-6), the 10 kDa culture filtrate antigen (CFP-10) or alpha-crystallin-like protein (HspX) present in the Mexican ancestral lineage strain, are also highly immunogenic, thus indicating that this *M. ulcerans* lineage has evolved to survive regardless of their presence [37, 38].

These studies are unveiling how mycolactone shapes the cellular landscape of BU, but dose-response analyses reveal in more detail the high degree of pleiotropism presented by this toxin. Right from the inception of the immune response,

mycolactone is able to affect the phagocytic capacity of murine macrophages at high multiplicities of infection and to inhibit the production of a series of human and murine monocytic/macrophagic molecules, including the proinflammatory cytokine TNF-α [9, 12, 15, 28, 39, 40]. Moreover, it hinders the production of nitrous oxide species, assumed to be important to control the infection, according to in vitro experiments and a clinical trial with BU patients [41–43].

Mycolactone also possesses dose-dependent immunomodulatory effects on T cells and dendritic cells (DC). Besides suppressing DCs' maturation, migration and production of the chemokines macrophage inflammatory protein (MIP) 1α and MIP-1β, regulated on activation normal T cell expressed and secreted (RANTES), IFN-γ–inducible protein 10 kD and MCP-1, Sec61 blockade in DCs results in the suppression of direct and cross-presentation of synthetic peptides to $CD8^+$ T cells by downregulation of MHC class I and β2 microglobulin [44, 45]. As for T cells, mycolactone represses TNF-α activity over factor nuclear kappa B (NF-κB) and transcription and post translation of the T cell receptor, ultimately resulting in the blockage of IL-2 production, an essential cytokine for T cell proliferation [15, 46].

Although all of these mechanisms hinder cell-mediated immunity (CMI), none has been as extensively tested in in vivo models as IFN-γ, the hallmark cytokine of type 1 T helper (T_H1) responses. Indeed, mice infected with *M. ulcerans* strains of low and intermediate virulence produce increased levels of IFN-γ, which induce phagosome maturation and acidification, as well as nitrite production [43]. Likewise, IFN-$\gamma^{-/-}$ mice display a severe impairment in the growth control of lower virulence strains when compared with WT mice [43, 47]. Importantly, none of these phenomena are observed upon inoculation with highly virulent strains or high doses of mycolactone, which demonstrates the extent to which mycolactone can damage an otherwise protective immune response [43, 47].

Responses against *M. ulcerans* infection are therefore dependent on the amount/variant of mycolactone produced by the pathogen. While many authors recognize the importance of CMI, the accumulation of mycolactone disturbing a wide array of CMI processes and the occupation by the mycobacteria of the extracellular space for most of the infection period places several question marks on what is or would be the ideal protective immune response. As such, other degrees of evidence are needed to develop a more accurate picture.

2.2 Regional and Systemic Responses

Viable *M. ulcerans* bacilli can be seen in the draining lymph node (DLN) of mice very early after subcutaneous infection, where cells initiate an immune response, as measured by their production of IFN-γ upon ex-vivo stimulation [28]. However histological analyses of the DLN also reveal the typical pattern of cell depletion and/or necrosis, affecting in this case both the T and the B cell compartments [48]. Remarkably, albeit T cells show an impairment in their proliferative capacity to expand upon antigenic stimuli, they are deemed to be relatively resistant to cell death by mycolactone [46, 49]. In contrast, mycolactone interferes with murine T

cell expression of L-selectin (CD62-L), a necessary molecule to home these cells to the peripheral lymph nodes [49]. Whilst these findings in animal models of infection were so far not validated in humans, the indication that mycolactone can target cell homing mechanisms warrants further investigation [31, 49].

Both in humans and mice, *M. ulcerans* is occasionally found in internal organs, but probably due to its restrictive growth temperature, its viability there decreases over time and infection does not effectively establish [50–54]. Relevantly, mycolactone also disseminates systemically and, in spite of not causing major cell death or compromising immunity against other intracellular pathogens, it does alter the cytokine production of circulating cells [52, 54, 55]. As a result, some authors scrutinized the cytokine production pattern of peripheral blood cells from BU-infected patients, which did not lead to consistent results. Understandably, this can be attributed to differences in the genetic background of the study populations, the virulence of endemic *M. ulcerans* strains, the cells or stimuli applied in the assays used or even the power of the studies. IFN-γ, the most thoroughly analyzed cytokine until now, is the paradigm of this aspect, with some studies reporting opposite outcomes [56–63]. Nonetheless, the most consensual view until now is that BU is associated with an impaired T_H1 response, resulting in a lower proliferation of peripheral blood mononuclear cells (PBMCs) and production of IFN-γ upon stimulation [57, 60, 63, 64]. A deeper analysis of published studies further reveals that altough this response is not correlated with the stage of the lesion, it systematically returns in patients with healed lesions to the profile found in controls from endemic regions [59, 63]. Moreover, reports indicate that there is a correlation between the histological characteristics of a lesion and cytokine production, as patients who present well-formed granulomas stain more positively for IFN-γ, whereas patients without these structures tended to have more IL-10^+ cells [61, 65]. Interestingly, ex vivo IL-10 production by circulating leukocytes was found to increase during active disease in many of the studies, even if this was not statistically significant in some of them [56, 58–60, 62].

Consistent with some data in rodents, it was found that the production of chemokines such as IL-8, MIP-1β and MCP-1 was suppressed in BU patients throughout the different stages of the disease, strongly supporting the notion that *M. ulcerans* infection leads to a defect in immune cell recruitment in advanced stages of the infectious process [27, 44, 59]. A more recent analysis in humans with active BU confirmed the downregulation of MIP-1β and MCP-1 in the serum of patients, opening up a possibility to use certain chemokines as a molecular signature of the disease [31].

More extensive multi-analyte profiling of serum proteins in BU patients and endemic controls revealed that, although the disease does not influence significantly the leukocyte composition of the peripheral blood, it impacts an even wider array of circulating molecules [31, 66]. Indeed, several of these analytes contribute to acute phase reaction, metabolism, coagulation and tissue remodeling, with some of them already having been implicated in healing speed [31, 66]. Specifically regarding metabolic factors, *M. ulcerans* not only interferes with energy-generation, but also with peptide, lipid and nucleotide pathways [66]. Even though many of these alterations seem to be in line with the expected response of the host to infectious

processes, others, as the proposed impairment of the tricarboxylic acid (TCA) cycle, could be the result of a direct effect of mycolactone [66, 67]. In reality, among TCA cycle intermediates, patients with BU have decreased citrate levels, whose relevance in other infectious diseases is associated with generating prostaglandins, nitric oxide and other antimicrobial peptides [66, 67].

All of these studies provide indications that the repercussions of *M. ulcerans* are more widespread than initially thought. While the disease focus is in the subcutaneous tissue, the ability of mycolactone to exert its actions beyond this local site most certainly contributes to the establishment of a permissive environment for the growth of the pathogen. This further hints on many other aspects of infection, such as immune cell metabolism, which are likely determinants for the way host cells interact with the pathogen.

3 Diagnostics Research Make it Evident: The Triggering of Cellular and Humoral Arms

Early attempts to find a better BU diagnostic method culminated in the development of burulin, a whole cell lysate of *M. ulcerans* that induced a delayed-type hypersensitive response in individuals from BU-endemic regions, more noticeable in people with already healed lesions [68–70]. Unfortunately, patients presented an equally strong reaction to tuberculin, consequently making the test of little usefulness in regions where tuberculosis is also prevalent or where the majority of the population is vaccinated with Bacillus Calmette-Guérin (BCG) [69, 70]. Furthermore, PBMCs from BU patients were later shown to proliferate very little and produce only small amounts of IFN-γ upon stimulation with live or dead *M. ulcerans* when compared to healthy individuals with a positive tuberculin test [64]. In contrast, a significant proportion of the evaluated patients possessed *M. ulcerans*-specific antibodies, which contrary to the cellular responses, can be detected in significant amounts throughout the different stages of the disease [64, 69]. Interestingly, incubation of serum from healthy endemic family controls with an *M. ulcerans* culture filtrate also revealed a notable amount of specific immunoglobulin (Ig) G, but no IgM, implying that this latter class of Igs has more potential for the identification of new BU cases [71]. In accordance, most epidemiological studies managed to screen populations in contact with *M. ulcerans* with moderate success, by focusing on detecting IgG against the 18 kDa small heat shock protein (shsp), a constitutive *M. ulcerans* cell wall protein involved in biofilm production [72–75]. Screenings of *M. ulcerans* whole protein lysates also identified the 65 kDa heat shock protein (Hsp65) as a potential candidate for a serological approach to determine active BU disease [73]. However, this protein has homologs in other mycobacterial species, thus prompting further analysis on the specificity of such antibodies [77].

All in all, in addition to reinforcing a role for CMI in BU, the search for novel diagnostic methods has put some visibility on the presence of antibodies in the

context of *M. ulcerans* exposure and infection. Regarding each of these aspects, it remains to be established why not all exposed individuals develop the disease and how antibodies contribute to the control of *M. ulcerans* infections.

4 Antibiotic Treatment in the Aid of the Host Immune Response

Antibiotic regimens based on the conjoint use of rifampicin with another drug have been used with success in the treatment of BU, rapidly killing *M. ulcerans* and restoring a vast array of processes needed for tissue homeostasis [78, 79]. Probably the most critical achievement of these drugs is to break the chain of mycolactone production, which allows mycolactone to wane from the system [80]. Indeed, direct correlations between mycolactone concentration and the time needed to heal BU lesions were established, suggesting this is a crucial factor for the immune response to re-establish [80]. Following this idea, both in humans and experimentally infected mice, treatment is associated with restoration of systemic levels of several inflammatory molecules, including IL-4, IL-7, granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as the chemokines IL-8 and MCP-1 [59, 81]. Simultaneously, in contrast to the typical absence of local inflammatory infiltrates seen in the central area of active untreated BU lesions, patients undergoing chemotherapy display very diverse organizations of cellular immune infiltrates, including classical granulomas, diffuse infiltrates or dense lymphocyte aggregations [79, 82, 83]. While this likely corresponds to different stages of the healing process, it is not clear if these might also represent inter-individual differences among BU patients [79, 83, 84]. Nevertheless, it is perceivable amidst the different histological observations in both humans and experimentally infected mice under treatment that phagocytes are needed throughout the process for uptaking bacteria and cellular debris, paving the way for wound healing [79, 83–85]. In mice, the regional preservation of DLN structures is noticeable, including germinal centers, a pattern not common to untreated groups, possibly due to the persistence of viable mycolactone-producing bacteria in the organ [85].

The immune break caused by mycolactone, in several reports described as paradoxical reactions in BU patients, has frequently been mistaken for treatment failure [86–89]. In fact, patients under standard regimens of antibiotherapy may display temporary clinical deterioration with local signs of increasing inflammation, ulceration and even development of new lesions [86, 87, 90]. These reactions appear to be due to the presence of large amounts of antigens, that once mycolactone starts to wane from the system, elicit a potent inflammatory response [86, 87]. In accordance, many of these paradoxical reactions appear to be related with age, duration of treatment, rapidly progressing forms of the disease such as edema, or even incomplete surgical lesion excisions that leave mycobacteria in surrounding tissues [86–88, 91, 92]. However, attempts to find biomarkers that could help to predict

paradoxical reactions, such as neopterin, a catabolic product released by macrophages upon IFN-γ stimulation, have so far failed [93]. While this makes it undeniably more difficult to prevent such phenomena, patients' symptoms have been successfully managed with corticosteroid adjunctive therapy [94, 95]. In a similar way, BU patients co-infected with human immunodeficiency virus (HIV) have benefited from such an approach, since they develop a comparable immune reconstitution syndrome once treatment with antiretroviral therapy starts [96, 97]. Although evidence is still relatively limited, HIV infected patients tend to generally develop BU more frequently and with worse clinical outcomes when compared to the general population, in spite of no direct correlation with the degree of immunosuppression having been established [96, 98–100]. On the other hand, immunomodulation with corticosteroids has been suggested to favor activation of latent *M. ulcerans* infection [101]. Moreover, these immunosuppressive drugs prolonged the time needed to completely resolve infection during antibiotherapy in mice, an effect attributed primarily to the disturbance of immune cell recruitment to the site of infection [101, 102].

As a whole, this reinforces the notion that CMI is critical for the control of BU. In this line, innate effector cells emerge as one of the most, if not the main responsible determinant for containing bacterial proliferation at the site of lesion. Still, to what degree their interaction with mycolactone-compromised T and B cells influences their activity and consequently the outcome of BU treatment is yet to be clarified.

5 Epidemiological Clues and the Search for Novel Resistance and Susceptibility Markers

More than ever, there has been an effort in integrating epidemiological and pathophysiological observations in a more comprehensive understanding of diseases [103]. The demography of BU has been however a source of debate, as efforts to identify risk populations have not always been conclusive [104]. Interestingly though, large observational studies appear to converge in some aspects: children under 15 years and the elderly are at a higher risk of contracting the disease, with the latter group also presenting more frequently severe phenotypes [92, 105–111]; and gender distribution of cases varies according to age group, with males tending to predominate in the younger ages and females in the adulthood [105, 107, 108, 110–113]. While these differences could be a product of behavioral exposure, they could too indicate an underlying physiological basis, which would be in agreement with the ascertained roles of age and sex in numerous infectious diseases, including other mycobacterioses [114, 115].

The incidence of tuberculosis in humans is also higher in children and older adults, albeit the clinical features tend to be distinct, probably as a consequence of the likewise distinct underlying immune response [116]. Nevertheless, in both these populations, *Mycobacterium tuberculosis* growth and active disease is predicted to be favored by an unbalance in the percentage of IFN-γ^+ $CD4^+$ T lymphocytes recruited to the infection site [117–120]. These findings were further replicated in

aged murine models of tuberculosis infection and ultimately match the postulated importance of IFN-γ in BU, which if not totally ablated by mycolactone, confers some degree of protection against *M. ulcerans* [43, 47, 115, 121]. Therefore, taken all of this into account, stratification by age of the cellular and molecular profile of BU patients could provide a critical avenue to obtain new information on the determinants of susceptibility *vs* protective immune responses, both collectively as well as in different age groups.

Parallel to age, the influence of sex over biological processes such as the immune response can be observed in a broad range of conditions, from infectious diseases to vaccination [122]. Even though some differences in the immune system of both genders are already established at birth, the effects become much more pronounced after puberty [122, 123]. This suggests that differences in exposure to environmental reservoirs of *M. ulcerans* explain better the higher incidence of BU among male pre-pubertal children than sex-related biological differences. After puberty however, sex hormones start to exert a powerful influence over several pathways of both the innate and acquired immune system arms resulting, for instance, in a bias towards T_H2 cells in females and T_H1 cells in males [122]. Strikingly, both male humans and mice appear to be more vulnerable to mycobacterial infections than their female counterparts, an observation that has been attributed to the effects of testosterone on, among others, macrophage motility [124, 125]. However, considering that BU pathogenesis and clinical features are mainly attributed to the activities of mycolactone, any effect of sex hormones in the immune response should pass unnoticeable unless it is in relationship with the mechanism of action of the toxin. In this line, estrogen has been shown to be able to activate neural WASP through a phosphorylation cascade induced by focal adhesion kinase, which could then render the cells more prone to the actions of mycolactone [20, 126]. In the end, this might be just one of several explanations for the seemingly increased susceptibility of adult women to the disease than men [127, 128].

Another debatable topic in BU is disease transmission. Although not the main focus of this chapter, some epidemiological records and experimental laboratory results point to environmental exposure as the main source for BU acquisition, namely through contact of injured skin with water bodies and through insect bites [25, 26, 113, 129–133]. However, some work in Africa, Asia and Australia additionally raised the possibility of host genetics playing a major part in BU development [92, 134–137]. Even though no evidence apart from anecdotal reports supports human-to-human transmission, the odds ratio (OR) of acquiring BU was determined as five times higher in people with family history of BU [25, 137–139]. Curiously, when this relationship was explored more thoroughly, strong associations with grandparent BU history but not contemporaneous family members were found [137]. In parallel, BU WHO category III lesions were recently suggested to constitute distinct clinical entities from categories I and II. When compared to both milder categories, they showed no dependency on time to develop and take considerably longer to heal [89, 109, 140]. Altogether, this strongly implies that additional host-intrinsic factors influence BU outcome, a lead followed by some studies

evaluating the role of single nucleotide polymorphisms (SNPs) in genes related to the immune function (Table 1). For instance, confirmation of the critical importance of nitric oxide (NO) and IFN-γ to BU pathogenesis was achieved, as SNPs leading to lower promoter activity of *iNOS* and *IFNG* were associated with higher susceptibility to the disease [141].

However, genetic analyses are also helping to unravel the key function of other undisclosed molecular players of the immune system. Actually, the first ever human gene to be associated with BU was the solute carrier family 11, member 1 (*SLC11A1* or *NRAMP1*), whose main function is to transport divalent cations, including Zn^{2+}, Fe^{2+} and Mn^{2+}, to the late endosomal/lysosomal compartment [142, 143]. Since lysosomes eventually fuse with the phagosomes, where *M. ulcerans* resides transiently, this provides an important clue for the importance of the intramacrophage phase for the containment of *M. ulcerans* infection [9, 44, 143]. Accordingly, in a Beninese cohort of 208 patients, SNPs in autophagy-related genes E3 ubiquitin-protein ligase (*PARK2*) and autophagy-related protein 16-1 (*ATG16L1*) were associated with susceptibility to disease acquisition and phenotype aggressiveness [144]. In the same line, two different polymorphisms in the gene encoding the nucleotide-binding oligomerization domain-containing 2 (NOD2) protein, which is involved in bacterial recognition, were also associated with disease phenotype [144]. In fact, in other infections with intracellular pathogens including some species of mycobacteria, NOD2, PARK2 and the autophagy process have been described to help to control bacterial load and to improve host survival [145, 146]. Overall, this underlines a prominent role for xenophagy, the recognition and targeting of bacteria to autophagosomes, in the control of *M. ulcerans* infection, hence requiring further research (Table 1).

Table 1 Summary of genes significantly associated with BU disease susceptibility and or phenotype severity

First author and year	Country (Continent)	Population (n cases)	Gene	SNP rs# number	Association	Reported adjusted OR (95% CI)
Stienstra et al. (2006)	Ghana (Africa)	182	*SLC11A1 (NRAMP1)*	rs17235409	Disease susceptibility	2.89 (1.41–5.91)
Capela et al. (2016)	Benin (Africa)	208	*PARK2*	rs1333955	Disease susceptibility	1.43 (1.00–2.06)
			NOD2	rs9302752	WHO lesion category	2.23 (1.14–4.37)
				rs2066842	WHO lesion category	12.7 (0.60–269)
			ATG16L1	rs2241880	Lesion ulceration	0.35 (0.13–0.90)
Bibert et al. (2017)	Ghana (Africa)	96	*iNOS*	rs9282799	Disease susceptibility	1.99 (1.22–3.26)
			IFNG	rs2069705	Disease susceptibility	1.56 (1.14–1.99)

Given the contribution of many genes to a clinical phenotype, gene association analyses can also present some interpretation challenges: single studies often lack the necessary power to control for all interactions, and genetic polymorphisms can not only vary in frequency, but additionally have different functional consequences among different populations [147]. A good example is the lack of association of *PARK2* with susceptibility to BU, as recently described in Ghana [141]. Although seemingly conflicting with previous results, the study populations were different and the tested polymorphisms were not the same, thus making direct data comparison difficult. Taken as a whole nonetheless, these population studies hold potential for a better understanding of host immune responses against BU, due to their ability to suggest, identify, or confirm potentially relevant targets for patient stratification or even disease treatment that could provide indispensable for future public health interventions.

6 (Un)successful Preventive Approaches

As of now, the only available vaccine targeting mycobacterial diseases is the BCG vaccine, a live attenuated bovine tuberculosis bacillus (*Mycobacterium bovis*) widely deployed against tuberculosis, but under an intense scrutiny for its unsatisfactory results [148]. In the case of BU, even though BCG appears to decrease the incidence of osteomyelitis forms, the incidence of new BU cases remains mostly unaltered, reinforcing the urge for better alternatives [149–155].

Efforts to develop a specific vaccine against *M. ulcerans* started several decades ago. Initial immunization studies in mice with different live mycobacterial species, including BCG and *M. ulcerans*, looked very promising in preventing the development of footpad swelling upon later challenge with *M. ulcerans* [156]. However, more recent experimental data clarified that even if a booster dose of the vaccine is administered or if the dose of *M. ulcerans* challenge is lowered, mice are only able to contain the infection for a short period of time, which is in accordance with observations in humans [48, 157, 158]. Nevertheless, these data provide important hints on the determinants of a protective immune response, some of which in agreement with what is seen during *M. ulcerans* clearance upon antibiotic treatment. Indeed, BCG-immunized mice display visible local cellular infiltrates together with a more rapid and sustained increase of IFN-γ and TNF-α levels and, in later time points, of the chemotactic MIP-2 and of inducible nitric oxide synthase (iNOS)$^+$ cells [48]. Concomitantly, a prolonged presence of CD4$^+$ T cells producing IFN-γ is evident in murine DLN, indicating that the trafficking of the bacteria to the DLN and subsequent presentation to the T cell compartment is temporarily being achieved [48]. Interestingly, immunization with a mycolactone-negative *M. ulcerans* strain appeared to be less effective than BCG in attracting cells to the site of the lesion and in inducing iNOS$^+$ cell accumulation [48]. On the other hand, in a study employing dewaxed *M. ulcerans* one month before infection, a strong generation of IgG against the bacilli was observed, with very little bacilli present in footpad

sections and no phenotype development [159]. However, this study only evaluated protection for 28 days, and thus it remains to be investigated whether protection is long-lasting. Moreover, it is unclear if protective immunogens could have been removed during the dewaxing process, in spite of a general lack of protection detected in immunization interventions employing surface proteins, such as the 18 kDa shsp and the 27 kDa laminar binding protein [32, 160, 161]. Still, these results add to others confirming the extreme hydrophobicity of the *M. ulcerans* cell wall, which cumulatively with mycolactone represents a virulence factor and aids in shielding the pathogen from immune surveillance mechanisms, consequently adding another layer of complexity to the development of preventive strategies [162].

Other immunization alternatives have thus been tested, such as vaccination with the mycolyl-transferase antigen 85A (Ag85A), a major secreted member of a family of proteins with important cell wall synthetic activity in several mycobacterial species, including *M. ulcerans* [163, 164]. Whether in the form of a plasmid DNA vaccine or as a recombinant protein, vaccination strategies with Ag85A conferred some degree of protection by eliciting a cellular response with high levels of IFN-γ, IL-2 and Ag85-specific IgGs, but, similarly to BCG, eventually failed to deter disease development [163–166]. Likewise, a vaccine employing DNA plasmids of polyketide synthase domains of *Mycobacterium leprae* Hsp65, previously tested in a murine model of tuberculosis, conferred protection against BU only for a few months, despite the high homology of the amino acid sequence with the *M. ulcerans* orthologue [167, 168]. Therefore, better targets for immunization are still to be found and, to this end, a comparative genomics screening study identified several essential components of *M. ulcerans* that could provide important hints to direct future vaccination strategies [169].

So, why did not any of these immunization attempts succeed? Even if a transient protective response against *M. ulcerans* is generated, bacteria are consistently able to overcome it at some point. This likely implies that, unless elimination of the replication of the bacilli is achieved at the very early stages post-infection, accumulation of mycolactone will eventually begin to significantly damage the surrounding tissue including immune cells, thus defending the pathogen and downplaying any protective immune mechanism. Indeed, the administration of either antibiotics or bacteriophages in *M. ulcerans* infected mice corroborate this interpretation, since the killing of *M. ulcerans* is accompanied by an increase in the cellular immune response associated with cytokines such as IFN-γ [85, 170, 171].

In this sense, neutralization of mycolactone itself seems to be an appealing target both for passive or active immunization strategies, as it would open the necessary opportunity for the immune response against *M. ulcerans* to develop and maintain effectively. This is not a novel concept, but has been for long hampered by the lipid-like nature of mycolactone, which makes it poorly immunogenic [168, 172]. However, recently, anti-mycolactone IgG antibodies were generated in mice through the administration of a protein conjugate of a truncated non-cytotoxic form of the toxin [173]. Although these antibodies were able to bind to mycolactone and to

neutralize its effect in in vitro assays with L929 fibroblast cell lines, it remains to be clarified if the antibodies are protective in vivo and if immunization with the truncated form of mycolactone induces a long-lasting protective humoral response [173]. Nevertheless, these are promising steps for future strategies targeting both active immunization efforts of endemic populations and passive immunization of BU patients.

7 Novel Models for Investigation and Future Perspectives

Research on BU includes a wide range of animal models, ranging from mammals and reptiles to birds, in an attempt to find one mimicking human disease best and allowing a detailed study of the pertaining cellular and molecular events [3, 174–177]. Nonetheless, most of the evidence on BU pathophysiology and the host immune response has originated from experimentally infected mice, which, in spite of mimicking most of the clinical features of human BU, such as erythema, edema and ulcers, tend to progress very rapidly to necrosis [50]. In an attempt to directly tackle this issue, in vitro sub-culturing of a *M. ulcerans* Ghanaian isolate (NM20/02) resulted in an attenuated strain that allowed a more prolonged evaluation of the cellular infiltrates [32]. Additionally, the extended kinetics facilitated the confirmation of other studies disclosing murine strain-specificities in the response against *M. ulcerans* infection, with overall evidence indicating that BALB/c mice are able to delay bacterial growth and respond more effectively to BCG immunization than their C57BL/6 counterparts [21, 32, 157]. This critical point to be considered when extrapolating data to the human pathophysiology, can eventually be overcome by the addition of information from other animal models with more similarities with the human skin, such as pigs and guinea pigs [174, 175, 178, 179].

Intriguingly, whereas many of the above models have been very useful to learn about the disease pathogenesis, very little progress was made concerning the mechanisms behind natural resistance to the disease. In fact, for long it has been known that natural resistance to *M. ulcerans* infection occurs in some animal species and some studies also indicated the potential for spontaneous healing of BU lesions in humans [3, 86, 176, 180–183]. On the other hand, it is estimated that even with proper antibiotic and surgical treatment, more than 20% of patients develop permanent sequelae [111]. What causes lie between these two extremes? Two recent studiesaimed to shed some light on this question, by exploring the ability of the Hartley guinea pigs and the FVB/N mice to spontaneously heal BU [21, 184]. Strikingly, whereas the guinea pigs were able to achieve sterilizing immunity, FVB/N mice appear to halt the production of mycolactone by *M. ulcerans* without being able to completely eliminate the pathogen [21, 184]. Considering for instance, that changes in the sugar content of the growth medium of *M. ulcerans* can affect its production of mycolactone and antigens, this raises the hypothesis whether FVB/N mice deal with the infection by manipulating the local environment [185]. It is intriguing to observe that both models control the infection regardless of the initial inoculum,

which most likely suggests that there are key host factors that can effectively hamper the progression of the infection and that have never been so far considered as preventive or therapeutic approaches [21, 184]. Hence, it is highly likely that answers to this conundrum lie within the genetic makeup of BU patients that spontaneously recover from the disease, but with the current widespread use of antibiotics, the task of identifying such cases is extremely difficult. Nevertheless, the existence of animal models of resistance already constitutes an exciting opportunity that should be seized to unravel what factors could be exploited in order to overturn a progressive disease into a state of full resolution.

8 Conclusions

For a long time, research on *M. ulcerans* infection has been highly focused on experimental models. While these led to many important advances, integration of recent progress in epidemiological, clinical, cellular and molecular research constitutes an opportunity to further our understanding of BU pathogenesis. In this context, several lines of evidence point towards the importance of CMI in the local interaction with invading *M. ulcerans* bacilli, but the scope of this contact is still widely unknown. Likewise, bearing in mind that mycolactone can act beyond the site of infection, the consequences of its influence on the systemic production of cytokines or metabolic networks of the immune cells are yet to be understood. As such, it would be important to determine how immune recognition of *M. ulcerans* occurs at the molecular level, what host factors influence cytokine production upon this recognition and what the effect of this interaction is on CMI effector mechanisms. As for the humoral immunity, it would also be paramount to understand what its importance is and to what extent it prevents or delays the progression of the disease. It is only if answers for these fundamental questions are found, that the way to tailor more effective vaccination and prophylactic measures will become clear.

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