Prospects for the development of a subunit vaccine against *Mycobacterium ulcerans* disease (Buruli ulcer)

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Dekan

Dedicated to my parents and grandparents

verantwortung und opposition

verantwortlich ist
wer antworten muss

antworten muss:
auf fragen

fragen an den,
der verantwortlich ist

(auch wenn sie ihm nicht
behagen)

es braucht also welche
die fragen

(Mani Matter, Rumpelbuch)

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Summary

Buruli ulcer (BU) is a slow progressing disease of the skin and subcutaneous tissue caused by *Mycobacterium ulcerans*. It presents in different clinical forms ranging from small non-ulcerative nodules to large ulcers and sometimes multiple ulcerations. The highest prevalence of the focally occurring disease is found in rural areas of West African countries. Both the mode of transmission as well as the potential environmental reservoir of *M. ulcerans* remain unidentified to date. In Cameroon, the remote Mapé dam region has recently been identified as a new BU endemic area. To assess the age-adjusted prevalence and local geographic distribution of BU, a house-by-house survey in the Bankim health district was conducted. Results showed that children between the age of five to 15 and elderly people were over proportionally affected by BU. To confirm the clinical diagnosis of BU during and after the health survey in Bankim, fine needle aspirates and swabs from undermined ulcer edges were transported to the Swiss Tropical and Public Health Institute for laboratory confirmation by quantitative real time PCR. In parallel we developed a protocol for primary culture initiation of *M. ulcerans* from patient lesions after a long time span between sampling and processing in a BSL3 culture laboratory. The established two sets of Cameroonian patient isolates from the Mapé and the Nyong river valleys were used for a comparative genome sequencing study revealing the presence of two distinct phylogenetic clonal complexes in Cameroon. Despite the fact that BU can be treated with antibiotics, the socioeconomic impact of the disease on affected populations remains devastating. As long as it is not clearly known how the disease is contracted, interruption of transmission is not an option. A vaccine against *M. ulcerans* on the other hand could be used both as preventive measure and therapeutically. While sero-epidemiological studies imply the presence of protective immunity in some individuals, no vaccine is available to date. Within the framework of the EU funded collaborative project BuruliVac we investigated the potential for developing a protein subunit vaccine against *M. ulcerans* by delivering vaccine candidate antigens with three different systems: i. as recombinant proteins with an adjuvant, ii. as vesicular stomatitis virus replicon and iii. incorporated into a genetically modified mouse malaria parasite (*Plasmodium berghei*) in an infection - treatment - approach. All three formulations were assessed for their immunogenicity and their protective potential in a mouse model of BU. Although all three vaccination approaches elicited strong humoral immune responses, no full protection was observed for any of the formulations. However a slight partial protection was seen for a replicon - prime / recombinant protein boost regimen with a vesicular stomatitis virus replicon incorporating an expression cassette for the *M. ulcerans* protein MUL2232. Additionally, a transient delay of foot pad swelling was observed in mice receiving infection - treatment - vaccination with *P. berghei* expressing MUL4987. Despite the mainly extracellular

nature of *M. ulcerans* in infected tissue, antibody production against the protein vaccine candidates thus does not seem to be sufficient for protection. Considering marked differences between the mouse footpad model of BU and the disease in humans, we aimed at developing an animal model that better reflects local pathogenesis and host-pathogen interactions in the human BU lesions. Hence, we developed the pig as novel animal model for BU and showed that the observed histopathological changes in the infected pig skin closely represent those of human BU. Therefore the pig model has great potential for the validation of new therapeutic and prophylactic interventions.

Zusammenfassung

Das Buruli Ulkus (BU) ist eine langsam voranschreitende Erkrankung der Haut und des Unterhautgewebes, welche durch *Mycobacterium ulcerans* verursacht wird. Die Erkrankung zeigt sich in verschiedenen klinischen Formen, die von kleinen, geschlossenen Knoten bis zu grossen Ulzera und manchmal mehreren Läsionen reichen. Die grösste Prävalenz der lokal gehäuft auftretenden Erkrankung ist in ländlichen Gebieten westafrikanischer Länder zu finden. Sowohl die Art der Übertragung als auch mögliche Umweltreservoire von *M. ulcerans* konnten bisher nicht vollständig identifiziert werden. In Kamerun wurde kürzlich die abgelegene Region um den Mapé Staudamm als neues endemisches BU Gebiet beschrieben. Um die altersangepasste Prävalenz und die lokale geographische Verteilung des BU festzustellen, wurde eine Umfrage in sämtlichen Haushalten des Gesundheitsdistrikts Bankim durchgeführt. Die Resultate zeigten, dass Kinder im Alter von 5 bis 15 Jahren und ältere Personen überproportional vom BU betroffen sind. Zur Bestätigung der klinischen BU Diagnose während und nach der Umfrage in Bankim wurden Feinnadelaspirationen und Abstriche aus den unterhöhlten Ulzerrändern vorgenommen und im Schweizerischen Tropen- und Public Health Institut im Labor mit quantitativer real time PCR untersucht. Parallel dazu wurde ein Protokoll für das Ansetzen von *M. ulcerans* Primärkulturen aus Patientenproben etabliert, welches die lange Zeitspanne zwischen der Probenentnahme und der Verarbeitung in einem BSL3 Kulturlabor berücksichtigt. Vergleichende Genomsequenzstudien von so etablierten Isolat-Gruppen von kamerunischen Patienten aus dem Mapé und dem Nyong Flusstal zeigten, dass in Kamerun zwei verschiedene phylogenetisch klonale *M. ulcerans* Komplexe vorhanden sind. Obwohl das BU mit Antibiotika behandelt werden kann, sind die sozioökonomischen Auswirkungen der Krankheit auf die betroffenen Bevölkerungsgruppen verheerend. Solange unklar ist wie die Übertragung erfolgt, ist Ansteckungsprophylaxe keine Option. Hingegen könnte eine Impfung gegen *M. ulcerans* sowohl als vorbeugende Massnahme als auch therapeutisch eingesetzt werden. Seroepidemiologische Studien implizieren zwar, dass sich bei Einzelnen eine natürliche Immunität entwickelt, eine Impfung ist allerdings bisher nicht verfügbar. Im Rahmen des EU-finanzierten Konsortiums BuruliVac haben wir das Potenzial für die Entwicklung einer Protein-Untereinheits-Impfung gegen *M. ulcerans* untersucht. Hierfür haben wir Impfstoff Kandidaten Antigene mit drei verschiedenen Formulierungen verabreicht. i.) rekombinante Proteine mit einem Adiuvans, ii.) mittels Vesikulärem Stomatitis Virus (VSV) Replikons und schliesslich iii.) mittels genetisch veränderter Maus Malaria Parasiten (*Plasmodium berghei*) in einem Infektions - Behandlungs - Ansatz. Für alle drei Formulierungen wurden die Immunogenität und das Schutzpotenzial in einem BU Mausmodell beurteilt. Obwohl alle drei Impfansätze starke Antikörperantworten hervorriefen,

konnte mit keiner der drei Strategien ein vollständiger Schutz erreicht werden. Eine leichte, teilweise Schutzwirkung wurde für die Replikon prime / rekombinantes Protein boost Impfung mit MUL2232 verzeichnet. Zudem beobachteten wir für MUL4987 eine vorübergehende Verzögerung der Schwellung des infizierten Fusses bei Mäusen, welche mit einem Infektions - Behandlungs - Ansatz mit *P. berghei* geimpft wurden. Obwohl sich *M. ulcerans* im infizierten Gewebe hauptsächlich extrazellulär entwickelt, scheint die Antikörperproduktion gegen die Proteinimpfstoffkandidaten nicht für eine Schutzwirkung auszureichen. Angesichts der deutlichen Unterschiede zwischen dem Mausmodell und der Erkrankung im Menschen wollten wir ein Tiermodell entwickeln, welches die Pathogenese und die Wechselwirkung zwischen Wirt und Krankheitserreger in menschlichen Ulzera besser reflektiert. Daher haben wir das Schwein als neues experimentelles Tiermodell für das BU entwickelt. Wir konnten zeigen, dass die beobachteten histopathologischen Veränderungen der infizierten Schweinehaut denen des BU im Menschen sehr ähnlich sehen. Das von uns etablierte Schweinemodell hat grosses Potenzial für die Validierung von neuen therapeutischen und vorbeugenden Massnahmen.

Introduction

Buruli ulcer (BU), caused by infection with *Mycobacterium ulcerans,* is a slow progressing, ulcerative disease of the skin and subcutaneous tissue. After years of disregard, the World Health Organization (WHO) recognized BU in 1998 as an emerging health problem and launched the Global Buruli Ulcer Initiative (GBUI) with the goal of coordinating BU control and research efforts [1]. At the same time, a first BU conference was organized by the WHO, drawing attention to the disease and leading to the Yamoussoukro Declaration [2], which clearly stated the will of the research community to solve the problems caused by BU.

Despite all the efforts and advances in understanding BU, many major research questions are still unsolved, such as the existence of an environmental reservoir and transmission of the disease.

History and geographical distribution of Buruli ulcer

The discovery of BU presumably took place in Africa as early as 1897, when Sir Albert Cook described large ulcerations occurring on limbs, most probably caused by *M. ulcerans* [3]. In the 1920s Kleinschmidt described similar ulcerations in patients living in the former Northeast Zaire [4]. Only a little later, in 1937, the first cases were discovered in Australia, more precisely in Bairnsdale (Victoria) [5]. Australia is also the place where the first description of the etiological agent of these unusual skin infections eventually originated; MacCallum and colleagues described a total of six patients with ulcerative skin infections and isolated a *Mycobacterium* from one of these wounds, that could only be cultured at temperatures lower than those required for *M. tuberculosis* [6]. Two years later, the new microorganism was named *M. ulcerans* by Fenner [7]. BU, the name used nowadays for the disease caused by *M. ulcerans* infection, originates from reports of the Uganda Buruli group. *Clancey et al*. named the disease after a geographic area in Uganda, the Buruli county, where in the 1960s a large epidemic of BU occurred [8,9]. In the 1980s, cases were also reported from other Central and West African countries like Congo (now Democratic Republic of the Congo and The Republic of Congo), Nigeria, Cameroon and Ghana [10–13]. Since then, case numbers in West African countries have increased to the extent that BU is now more prevalent than leprosy in some regions of high burden countries [14].

Today the highest prevalence of BU is still found in West Africa, especially in Côte d'Ivoire, Cameroon, Ghana and Benin (Fig. 1). As seen in Figure 1, Australia and Japan are also reporting considerable numbers of BU patients [15,16], whereas in other countries of the over 30 that have reported BU cases until now, the disease occurs only sporadically [17].

Only rarely, and always as a result of travel, has *M. ulcerans* infection occurred in North America and Europe [18–20].

Most likely, the 2630 cases reported to the WHO in 2013 [21] are an underestimation of the true number of cases due to the remote location of most endemic areas and limited access of patients to the formal health system and the lack of a point-of-care diagnostic test. On the other hand, the proportion of BU cases among the patients reporting at the health facilities may be an overestimation [22].

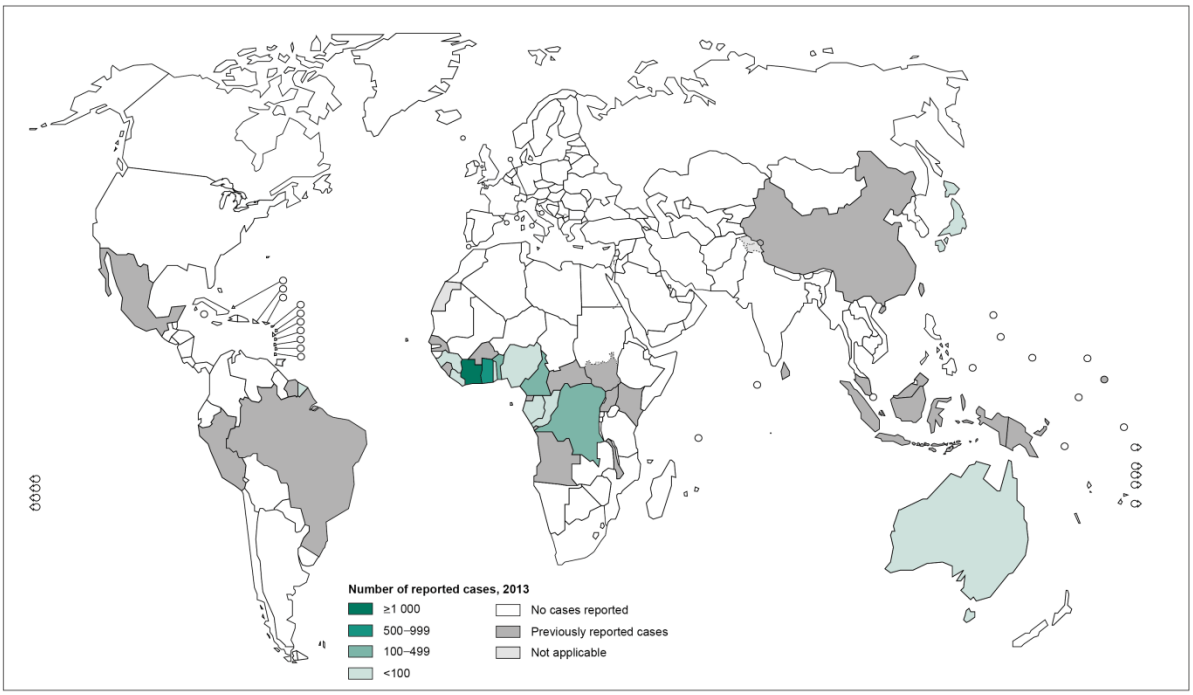

Figure 1: Worldwide distribution of Buruli Ulcer 2013 [23].

In Cameroon, BU was first described already in 1969. The reported cases at that time originated from a region in the Center Province, the Nyong River valley between the villages of Ayos and Akonolinga [12,24]. A second BU endemic region more in the north of Cameroon was reported by *Marion et al.* in 2011 [20]. In the Bankim health district, close to the Mapé river and dam, the first clinically suspected cases had already been reported in 2004 and numbers increased until 2009.

Evolution and genomic diversity of *M. ulcerans*

M. ulcerans is a member of the genus *Mycobacterium*, which comprises over 120 species. Many of these are important pathogens for animals and humans. *M. leprae* (the causative agent of leprosy) and *M. tuberculosis* (the causative agent of tuberculosis) are two examples for human pathogens, whereas *M. marinum* (fish pathogen) and *M. bovis* (causative agent of bovine tuberculosis) represent two (primarily) animal pathogens [25]. Early work based on multilocus sequence typing had already revealed a very close genetic relationship between *M. ulcerans* and *M. marinum* with an average nucleotide sequence identity of over 98 % in eight housekeeping and structural genes. It also indicated that all *M. ulcerans* strains had diverged from a common *M. marinum* progenitor once in evolution [26,27]. This finding was later confirmed when the first genome sequence of *M. ulcerans* became available in 2007 [28]. Furthermore, it became clear that a hallmark in the divergence of *M. ulcerans* and *M. marinum* was the acquisition of pMUM001, a virulence plasmid of 174 kb that is required for production of mycolactone, a toxin produced by *M. ulcerans* [29,30]. A second genetic difference clearly distinguishing *M. ulcerans* from its progenitor is the presence of two insertion sequence elements (IS2404 and IS2606). While they are completely absent in the genome of *M. marinum*, they expanded to high copy numbers in the genome of *M. ulcerans*, and represent the target of the current most sensitive diagnostic method for BU [31]. The expansion of IS2404 to over 200 copies and IS2606 to over 80 copies has mediated the loss of around 1 Mbp of DNA compared to *M. marinum.* Furthermore the *M. ulcerans* genome contains over 700 pseudogenes. Among the genes rendered non-functional or completely lost are genes important for pigment biosynthesis, anaerobiosis, intracellular growth [28] and potent T-cell antigens [32]. Some of the changes in the genome are reflected in the phenotype of *M. ulcerans*, for example the very slow growth rate compared to *M. marinum,* with estimated replication times of 72 hours compared to 4-6 hours, respectively [33].

All the data described above indicate that *M. ulcerans* has passed an evolutionary bottleneck and is adapting to a niche, most likely a more stable environment. Genomic information suggests that this new environment is dark, at least microaerophilic and extracellular. Additionally the slow growth rate, the thermosensitivity, the loss of important immunogens and the production of an immunosuppressive toxin would be of selective advantage for long term survival in a mammalian host [28,34,35].

The classical and the ancestral lineages of *M. ulcerans* causing clinical disease in humans [36], and the few other mycolactone-producing mycobacteria (MPM) that have been discovered so far like fish and frog pathogens, are genetically very closely related. Considering proper criteria for species differentiation, it is suggested to name all MPM *M. ulcerans* [35,37]. Analysis of insertion-deletion polymorphisms by Käser *et al.* has demonstrated the existence of two major lineages of *M. ulcerans* among clinical isolates: the

ancestral lineage, which is more closely related to *M. marinum* and comprises strains from South America, China and Japan, and the so called classical lineage with strains from Australia and Africa [36]. While cases caused by strains of the ancestral lineage are sporadic, the focal prevalence of cases caused by the classical lineage is much higher. Other conventional methods for genetic fine-typing were so far only able to discriminate between continental lineages and revealed the high clonality of circulating strains in different West African countries [38–41].

The highest resolution in deciphering the phylogenetic relationship between locally found *M. ulcerans* strains in West Africa can be achieved by single nucleotide polymorphism (SNP) typing, based on whole genome SNP analysis. With this method it was possible to demonstrate the highly clonal population structure of African *M. ulcerans* and the focal transmission of BU in the Densu river valley in Ghana [42,43]. It was hypothesized that only whole genome sequencing approaches in microepidemiological studies would provide sufficient resolution for tracing transmission pathways [33,42,43].

Results Chapter 3 of this thesis describes the findings of such a microepidemiological study in two BU endemic regions of Cameroon, where we analysed whole genome sequences of 91 *M. ulcerans* isolates.

Reservoir and Transmission

M. ulcerans is traditionally considered to be an environmental bacterium like its progenitor *M. marinum*. The Uganda Buruli group, which described a first big outbreak of BU in the 1960s, reported that there was no evidence of spread from person to person and that the disease “occurs characteristically but inexplicably near to large rivers” [44]. Until today this opinion is still preserved. Transmission from human to human is reported very rarely and the idea that introduction of *M. ulcerans* through minor wounds from an environmental reservoir is an important part of BU transmission remains [45,46]. However the definitive environmental reservoir and the exact mode of transmission are still not known [47].

To get further insight into the risk of contracting BU and the association of the disease to riverine areas, swamps and marshlands, a number of studies investigated risk factors in endemic areas of different West African countries. Results repeatedly confirmed that exposure to riverine areas by swimming and wading in swamps increased the risk for developing BU [48–50]. As pointed out by *Meritt et al*., almost all epidemiological studies have associated occurrence of BU with human-disturbed aquatic habitats due to deforestation, damming of streams and rivers or increased agricultural activities. In accordance with these findings, the habit of wearing protective clothing while farming was shown to reduce the risk for BU [47].

Since the introduction of polymerase chain reaction (PCR)-based methods for the detection of *M. ulcerans* DNA in the environment, various studies investigating the occurrence of the bacteria in the environment have been conducted. Specimens that have tested positive include soil, water filtrates, detritus, feces, fish, snails, frogs and insects [51–55]. Although the PCR method applied is very specific for diagnostic purposes, cross reaction with other mycobacteria in the environment cannot be completely excluded. Hence, the results of studies on environmental samples should be interpreted with caution, especially in the light of the very few positive samples in highly BU endemic areas, as well as the detection of positive samples in non-endemic regions [47,51]. Furthermore, positive PCR samples do not necessarily prove the presence of intact and live *M. ulcerans* in the environment. Due to its very slow growth rate and the presence of many other faster growing environmental organisms, cultivation of *M. ulcerans* from the environment remains very difficult [56]. Therefore the ultimate proof that *M. ulcerans* persists freely and alive in the environment is still missing.

With the detection of *M. ulcerans* DNA associated with aquatic insects, the idea of transmission by such vectors arose [57]. Especially water bugs were suspected to play a major role in transmission of the disease [58,59]. However, the significance of transmission by water bugs controversial, because these insects rarely bite humans. Furthermore, no significant difference was found in abundance of water bugs and/or positivity of water bugs for *M. ulcerans* DNA between BU endemic and non-endemic sites [60]. Mosquitoes have been proposed as vectors for the disease in Australia, where the disease is most prevalent in the temperate south-eastern state of Victoria [52]. Recently it was also reported that more than 20 % of ringtail and brushtail possums in endemic foci of Victoria had skin lesions caused by *M. ulcerans* [52,61]. While possums in Australia seem to represent a substantial animal reservoir, the search for a comparable reservoir in Africa has remained unsuccessful [62,63].

In conclusion, the exact mode of transmission of BU remains unknown. Possibly multiple modes of transmission occur, depending on the geographical and epidemiological setting [47]. As concluded by *Meritt et al*., studies on human behaviour patterns in highly endemic African villages would be of great benefit in order to better understand exposure to *M. ulcerans*, identify transmission patterns and ultimately develop preventive measures for the affected communities [47].

Pathogenesis and the role of mycolactone

BU affects all age groups, but lesions in children between the age of 5 and 15 are overrepresented [44,64]. In most studies an equal gender distribution has been found [65]. Lesions are typically observed on limbs of the patients, although they also occur less frequently on other body parts [44].

BU is primarily an infection of the skin with very restricted systemic dissemination [66]. After infection via an unknown mechanism, clinical signs typically start to emerge in the form of a movable, subcutaneous nodule after an incubation time that is estimated to be 2 - 3 months (Figure 2, A) [67]. Other non-ulcerative, early forms of BU are plaques and oedema (Figure 2, B and C) which tend to expand, leading to large ulcerations later in disease progression [68]. Additionally to this classification, the WHO has introduced a classification system that is mainly based on lesion size: Category I (single, small lesions < 5 cm in diameter), Category II (5 – 15 cm in diameter) and Category III (osteomyelitis and lesions with > 15 cm in diameter, disseminated forms, lesions in the head and neck region and in the face) [69]. Early forms of BU, especially nodules, can either heal spontaneously or progressive necrosis of the subcutaneous tissue eventually leads to collapse of the overlying dermis and epidermis, resulting in ulcers (Figure 2, D) [69]. Typical BU ulcerated lesions have undermined edges, slowly expand over time and are often painless, unless secondary infections occur [70]. The painless nature of the disease may be a consequence of nerve damage at the lesion site [71]. If left untreated, BU lesions can span entire body parts and can lead to complex and sometimes multiple lesions and to osteomyelitis [67]. It appears that co-infection with HIV would lead to more severe forms of BU but up to now, systematic studies on this topic are rare [72].

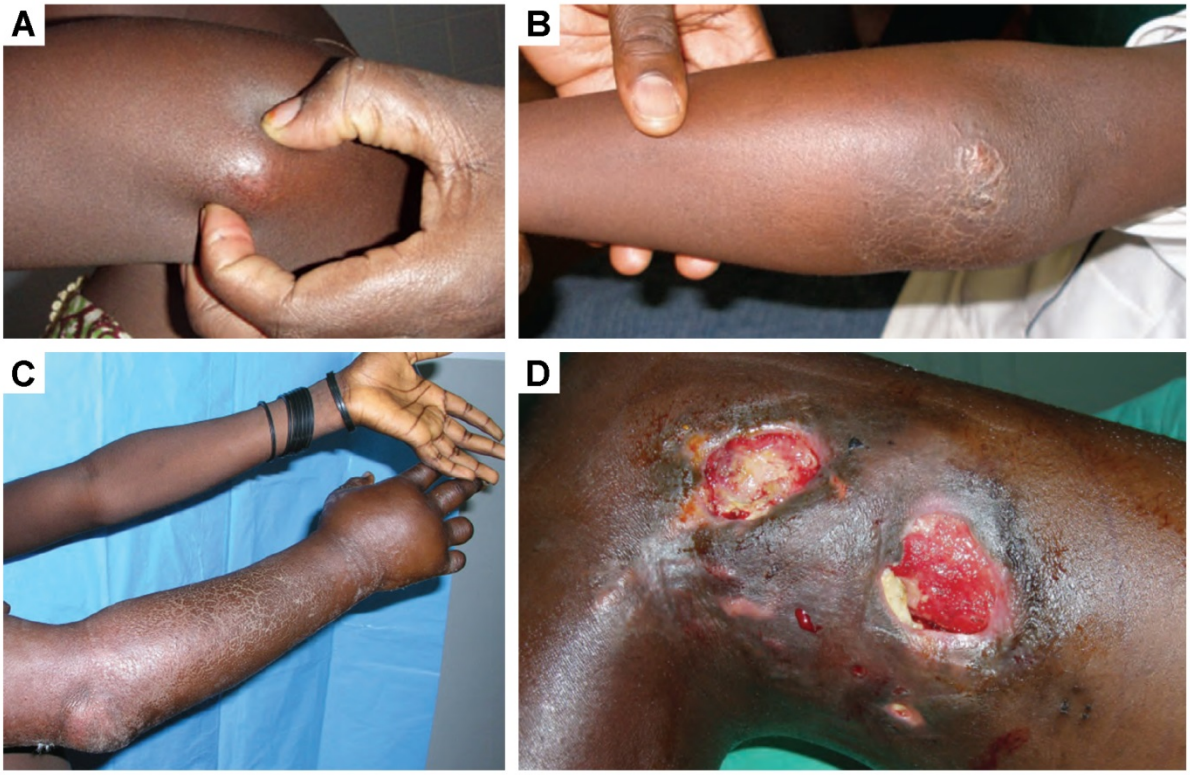

Figure 2: Typical clinical presentations of three non-ulcerative forms of BU, the nodule (A), plaque (B) and oedema (C). Representative of the typical open forms, a medium sized ulcer is shown (D) [69].

Skin tropism, slow progression of the wounds and necrosis of the skin are explained by the low optimal growth temperature of the pathogen, its slow growth rate and the production of the unique toxin, mycolactone, respectively [73].

The ability to produce this toxin depends entirely on the presence of the virulence plasmid pMUM01. This large plasmid encodes the polyketide synthases, which are needed to produce a family of macrolide exotoxins, generally referred to as mycolactones [73]. So far, six different variants of mycolactones have been described and named A to F. Different lineages of *M. ulcerans* are known to produce these different variants whereof mycolactone A/B produced by the African isolates is the most potent toxin of this unique family of molecules [74–76].

Mycolactone affects a broad range of mammalian cell types *in vitro* as well as *in vivo*. Added in small amounts to cell culture, it induces cell cycle arrest followed by apoptosis in fibroblast cell lines or reduces the phagocytic activity of cultured macrophages [73,77,78]. When injected into the skin of guinea pigs, the toxin causes ulcerations similar to the ones observed in human lesions [73,79]. Mycolactone injection results also in other species in the development of typical signs of BU on a histopathological level [80,81]. It is assumed that mycolactone is the main cause of the tissue destruction seen in BU lesions in humans,

however it is not entirely clear how it exerts its effects [82]. Local and systemic immunosuppressive effects of the toxin are also not entirely understood [83]. The diffusible nature of the lipid-toxin and technical restrictions in the quantification of mycolactone are limiting our current knowledge on tissue concentration and *in vivo* half-life of the toxin in human lesions.

Histopathologically, BU lesions are characterized by the presence of clusters of extracellular acid-fast bacilli (AFB), coagulative necrosis, the presence of fat cell ghosts, epidermal hyperplasia and very limited cellular infiltration [84,85]. Typically the distribution of AFB in ulcerative lesions is very inhomogeneous [86]. All histopathological hallmarks of the disease were basically described from tissue excised during surgical treatment of patients before the era of antibiotic treatment or from punch biopsies. Tissue samples from early BU lesions (nodules) are rarely available, resulting in very limited knowledge on the histopathology of early stages of the disease [83]. From studies in the mouse model it became clear that despite its late extracellular location, *M. ulcerans* may also have an initial, transient intracellular stage [87,88,80]. Lack of cellular infiltration is confined to the central, necrotic part of a lesion which is typically surrounded by different layers of inflammatory infiltrates [68].

It is currently thought that initial infection results in small accumulations of AFB (globi), which are needed to build up a sufficient mycolactone concentration to prevent elimination of the mycobacteria by infiltrating cells. As the amount of AFB, and with it the mycolactone cloud, increases, the infiltrating cells undergo apoptosis and a central necrotic area is developing [68,80,83].

Diagnosis and Treatment

Diagnosis of BU at health posts or hospitals in BU endemic areas is often based only on clinical grounds. Well trained staff can achieve an accuracy of up to 95 % but accuracy of clinical diagnosis is usually much lower [89]. Particularly in regions of low endemicity, where health workers are less experienced in diagnosing BU, laboratory confirmation is important to appropriately manage the disease [22,31].

Although urgently needed, no point of care diagnostic test for BU is currently available. Four methods for microbiological confirmation are recommended by the WHO:

- Direct smear examination for AFB
- *In vitro* culture
- Polymerase chain reaction (PCR) targeting the genomic multi-copy IS2404 sequence
- Histopathological examination of tissue

Of the four methods, only direct smear examination of wound exudates stained with Ziehl-Neelsen/Methylene blue is easily applicable at a local level [31]. However this method has a low sensitivity and does not differentiate between BU and cutaneous tuberculosis.

The main drawback of culturing *M. ulcerans* is the slow growth of the bacteria as well as the low sensitivity. On the other hand, cultures of *M. ulcerans* can be used for monitoring development of drug resistance and treatment success [31,90]. Additionally, culturing is required for establishing a strain collection for microepidemological studies. Histopathology requires a sophisticated laboratory and an invasive sampling technique but can be used for studying the local immune response, wound healing problems and treatment success [68,91,92]. Currently, the most sensitive and specific method for BU diagnosis is PCR for IS2404, especially by real-time (q) PCR methods and has therefore become the gold standard for BU diagnosis [31,93].

According to WHO recommendations, suspected BU cases should be laboratory confirmed by at least one of the above mentioned methods, preferably two. In 2013, it was proposed that national control programs should ensure that at least 70 % of all reported cases are laboratory confirmed by PCR. However there are many reasons for the discord between clinical and laboratory diagnosis, such as incorrect clinical diagnosis, inadequate sampling or laboratory errors [22,31].

For sampling of BU lesions, different methods exist, depending on the nature of the lesion (open or closed), the method of choice for laboratory confirmation and the place where specimens are collected. Closed lesions are typically sampled by fine-needle aspiration [31,94], whereas ulcers can be swabbed with a cotton wool swab. Both types of samples can be used for all laboratory confirmation methods except for histopathology for which punch biopsies or surgical excisions are needed [31].

Historically, surgical excision with wide margins into healthy tissue was the only treatment option for BU [69,72]. Based on data in mice and humans [67,95,96], in 2004 the WHO published provisional guidelines for treating BU with an antibiotic regimen consisting of oral rifampin (10 mg/kg) and intramuscular streptomycin (15 mg/kg) (R/S) for the duration of eight weeks [97]. A randomized trial by *Nienhuis et al*. and a number of observational studies confirmed the effectiveness of this treatment for different forms of BU and all lesion sizes [98–100]. It became clear that with this therapy, recurrence rates were reduced to 2 % as compared to 16 - 45 % for surgical treatment [67,69,100,101]. Therefore current WHO recommendations for treatment of BU are a combination of specific antibiotics for eight weeks, wound care, prevention of disability and surgery to remove necrotic tissue, covering of large skin defects and correcting of deformities [67]. Due to the need for daily injections with streptomycin, research efforts are directed towards replacing streptomycin with

clarithromycin, an oral drug with less toxicity. First studies testing this alternative drug regimen are promising [102–105].

Histopathological descriptions from humans as well as mice indicate that R/S therapy reduces or abolishes mycolactone production early after initiation of therapy [106]. Chronic leukocyte infiltration is observed and defined granuloma structures develop, allowing for specific immune responses against *M. ulcerans*. The majority of AFB after eight weeks of chemotherapy have beaded appearance, reflecting their destruction [68,107,108]. The massive immunostimulation observed during R/S therapy is considered to be a potential reason for the development of new ulcerations during or after antibiotic therapy, a phenomenon called “paradoxical reaction” [68]. The name is also used for the often observed clinical worsening of a BU lesion, for example an advanced progression of early plaques to ulceration, shortly after start of R/S [109–111].

An alternative therapy approach to antibiotic treatment is based on the sensitivity of *M. ulcerans* to temperatures above its optimal growth temperature of 32°C. Meyers and colleagues successfully treated eight patients in Zaire with heat already in the nineteen seventies [112], but complicated devices and electricity requirements led to the abandonment of this approach. In 2009 application of heat by phase change material filled bags proved an easy, cheap and promising alternative to antibiotic therapy [113]. A follow-up clinical trial of thermotherapy after proof of principle in 2009 confirmed these results in a larger and more diverse patient cohort (Manuscript submitted).

Immune response to *M. ulcerans* and prospects for a vaccine

As already described in the section on the pathogenesis of BU, the local immune response in the affected tissue is greatly influenced by the production of mycolactone. Over time a necrotic focus develops at the site of infection that contains abundant clumps of extracellular bacilli and cellular debris. At the border of the necrotic areas, neutrophils and macrophages with intracellular bacilli are found. This entire structure is typically surrounded by intact mixed infiltration as observed in human and mouse lesions [83,88].

The broad cross-reactivity with *M. tuberculosis* and *M. bovis* antigens complicates the analysis of *M. ulcerans*-specific immune responses and only limited data on humoral immune responses against *M. ulcerans* are available [68]. Studies using *M. ulcerans* culture filtrates found Immunoglobulin (Ig) G and IgM antibody responses in sera of BU patients, but also in sera of tuberculosis patients and Bacille Camette-Guérin (BCG)-immunized individuals [114,115]. The 18 kDa small heat shock protein of *M. ulcerans*, which has only a homologue in *M. leprae* but not in *M. tuberculosis* and *M. bovis*, was successfully used in serological analysis to distinguish between patients and individuals living in BU non-endemic areas

[116]. However, healthy household contacts in BU endemic regions showed reactivity as well, indicating that exposure to the pathogen is more frequent than the development of disease and that specific humoral responses develop in exposed individuals [116–118].

It is not entirely clear to what extent systemic cellular immune responses develop in BU patients. Skin testing with burulin, a lysate of *M. ulcerans*, frequently elicited delayed-type hypersensitivity responses in patients with healing or healed lesions but was rare in individuals with early BU lesions [114,119]. Such a reaction would be indicative of development of cell-mediated immunity but again, cross reaction with responses to BCG vaccination complicates the interpretation of these results. Further studies on systemic cellular responses against *M. ulcerans* were mainly conducted by *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) isolated from BU patients or whole blood assay approaches revealed large individual variations as well as variations between individual studies. However impairment of IFNγ secretion was a common denominator of the reports [120–125]. Most probably early T_H1 cellular responses that appear important for control of *M. ulcerans* infections are down regulated and the T_H1/T_H2 balance is shifted towards a T_H2 response, which does not seem beneficial for healing [120–122,125,126]. This scenario was illustrated in a case report from Australia, where development of a BU lesion coincided with the shift of a T_H1 cytokine response before disease onset to a T_H2 cytokine profile [127].

It is being debated to what extent systemic immunosuppression occurs and to what extent and how mycolactone influences systemic immune responses [68,83]. Results from mouse studies indicate that there may not be no systemic immunosuppression at all [128], but on the other hand systemic suppression of IFNγ responses was reported to resolve after surgical excision of BU lesions [123].

To date no specific vaccine against *M. ulcerans* is available [129]. The development of such a vaccine is complicated by the fact that *M. ulcerans* is not clearly an intracellular or a clearly extracellular bacterium and it is impossible to study early host-pathogen interactions due to the unknown mode of transmission of the bacilli [83]. Additionally, we do not know much about correlates of protection, except that a T_H1 type of cellular response might be protective [129,130]. Furthermore, the toxin mycolactone is not highly immunogenic and the relative importance of specific antibodies against the toxin as well as against cell surface structures of extracellular *M. ulcerans* remains unknown [68]. Nevertheless vaccine development against *M. ulcerans* seems possible. The fact that not all infected individuals develop disease and reports on self-healing of BU indicate, that protective immunity naturally develops in some people living in BU endemic areas and hence it might be possible to induce such immunity by vaccination [116,117,131,132].

Vaccination with BCG appears to have a transient and only partial protective effect against the development of BU [133,134]. Osteomyelitis, the most severe form of the disease, was reported to occur less frequently in BCG-vaccinated individuals [135]. It was speculated that the major component conferring protection in BCG vaccination, the antigen (Ag) 85 complex, could offer increased protection against *M. ulcerans* if the species-specific variants were used for vaccination. Accordingly, immunization with Ag85A in a DNA vaccination approach conferred partial protection against *M. ulcerans* in a mouse model of the disease [136,137]. The limited number of other protein subunit vaccination approaches tested so far as well as immunization with a mycolactone-negative strain of *M. ulcerans* could not confer full protection in mouse models neither [138–140].

Results Chapters 4 to 6 of this thesis describe the results of our studies employing different delivery systems for a potential protein subunit vaccine against *M. ulcerans*.

Animal models for Buruli ulcer

A range of animals (cats, dogs, alpacas) was reported over the years to be naturally infected with *M. ulcerans* [62,141–146]. Most of them were rare cases and not suitable as animal models in BU research. Actively tested as models for BU were the armadillo and the monkey but both are not used today [147,148]. The guinea pig model that was established 40 years ago is only rarely used as it does not offer substantial advantages over the mouse model described below [149,150].

The most widely used animal model for BU today has its origin in the nineteen fifties when it was developed by Frank Fenner, an Australian virologist. He discovered that sub-cutaneous (s.c.) inoculation of *M. ulcerans* into mouse foot pads led to the development of swelling and lesions 1-16 weeks after injection in a dose-dependent manner [151]. The model is basically used in the same way today, mainly for testing antimicrobial compounds but also in studies of vaccine efficacy [104,137,139,140,152,153]. Possible read-outs for the progression of infection in the infected mouse feet are measurements of foot pad swelling by caliper or a grading system for the severity of infection, the so called lesion index (Figure 3) [72,88]. At any given time point, bacterial load in the foot pads can be determined by lysis of the foot and subsequent enumeration of *M. ulcerans* by counting methods according to Shepard and McRae or classical colony forming unit (cfu) plating [139,154,155]. For the enumeration of bacteria in our mouse model of *M. ulcerans,* we adapted a qPCR technique developed by *Fyfe et al*. [156,157]. This is illustrated in Results Chapters 4 - 6 of this thesis.

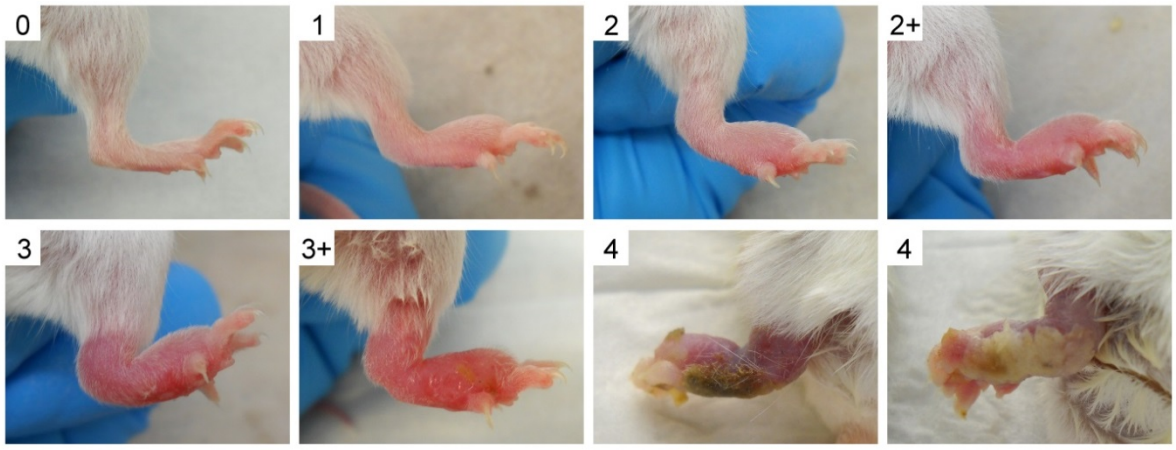

Figure 3: Mouse foot pad model of BU. S.c. injection of AFB into the healthy foot pad (0) leads to progressive swelling and inflammation. (1) Grade 1, slight swelling; (2 and 2+) Grade 2, swelling with inflammation; (3 and 3+) Grade 3, swelling with inflammation of the leg; (4) Grade 4, swelling with inflammation and possible ulceration, cage bedding sticking to the foot.

Additionally to the determination of the bacterial load, the local immune response in mouse foot pads can be characterized by histopathology [88]. As opposed to typically affected human tissue, mouse feet contain only little subcutaneous fat. An alternative model for BU that is closer to the human in terms of physiology and skin structure is the pig. Results Chapter 7 of this thesis describes the successful evaluation of the pig as an *M. ulcerans* infection model for studying early pathogenesis of BU and for the development of new therapeutic and prophylactic interventions.

References

1. WHO | The history of GBUI (n.d.). WHO. Available: http://www.who.int/buruli/gbui/en/. Accessed 30 September 2014.
2. WHO | The Yamoussoukro Declaration on Buruli ulcer (n.d.). WHO. Available: http://www.who.int/buruli/yamoussoukro_declaration/en/. Accessed 2 September 2014.
3. Cook A (1970) Mengo Hospital Notes, 1897. Makerere Medical School Library, Kampala, Uganda. BMJ 2: 378–379. doi:10.1136/bmj.2.5706.378-a.
4. Meyers WM, Connor DH, McCullough B, Bourland J, Moris R, et al. (1974) Distribution of *Mycobacterium ulcerans* infections in Zaire, including the report of new foci. Ann Société Belge Médecine Trop 54: 147–157.
5. Alsop DG (1972) The bairnsdale ulcer. Aust N Z J Surg 41: 317–319.
6. MacCALLUM P, Tolhurst JC (1948) A new mycobacterial infection in man. J Pathol Bacteriol 60: 93–122.
7. Fenner F (1950) The significance of the incubation period in infectious diseases. Med J Aust 2: 813–818.
8. Clancey J, Dodge R, Lunn HF (1962) Study of a mycobacterium causing skin ulceration in Uganda. Ann Société Belge Médecine Trop 42: 585–590.
9. Clancey JK (1964) MYCOBACTERIAL SKIN ULCERS IN UGANDA: DESCRIPTION OF A NEW *MYCOBACTERIUM* (MYCOBACTERIUM BURULI). J Pathol Bacteriol 88: 175–187.
10. Smith JH (1970) Epidemiologic observations on cases of Buruli ulcer seen in a hospital in the Lower Congo. Am J Trop Med Hyg 19: 657–663.
11. Oluwasanmi JO, Solankee TF, Olurin EO, Itayemi SO, Alabi GO, et al. (1976) *Mycobacterium ulcerans* (Buruli) skin ulceration in Nigeria. Am J Trop Med Hyg 25: 122–128.
12. Ravisse P (1977) [Skin ulcer caused by *Mycobacterium ulcerans* in Cameroon. I. Clinical, epidemiological and histological study]. Bull Société Pathol Exot Ses Fil 70: 109–124.
13. Bayley AC (1971) Buruli ulcer in Ghana. Br Med J 2: 401–402.
14. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, et al. (2004) *Mycobacterium ulcerans* disease (Buruli ulcer) in rural hospital, Southern Benin, 1997-2001. Emerg Infect Dis 10: 1391–1398. doi:10.3201/eid1008.030886.
15. Boyd SC, Athan E, Friedman ND, Hughes A, Walton A, et al. (2012) Epidemiology, clinical features and diagnosis of *Mycobacterium ulcerans* in an Australian population. Med J Aust 196. Available: https://www.mja.com.au/journal/2012/196/5/epidemiology-clinical-features-and-diagnosis-mycobacterium-ulcerans-australian. Accessed 2 September 2014.
16. Yotsu RR, Nakanaga K, Hoshino Y, Suzuki K, Ishii N (2012) Buruli ulcer and current situation in Japan: A new emerging cutaneous *Mycobacterium* infection. J Dermatol 39: 587–593. doi:10.1111/j.1346-8138.2012.01543.x.
17. Johnson PDR, Stinear T, Small PLC, Pluschke G, Merritt RW, et al. (2005) Buruli ulcer (*M. ulcerans* infection): new insights, new hope for disease control. PLoS Med 2: e108. doi:10.1371/journal.pmed.0020108.
18. McGann H, Stragier P, Portaels F, Gascoyne-Binzi D, Collyns T, et al. (2009) Buruli Ulcer in United Kingdom Tourist Returning from Latin America. Emerg Infect Dis 15: 1827–1829. doi:10.3201/eid1511.090460.
19. Lindo SD, Daniels F, Jr. (1974) Buruli ulcer in new york city. JAMA 228: 1138–1139. doi:10.1001/jama.1974.03230340040028.

20. Evans M r. w., Mawdsley J, Bull R, Lockwood D n. j., Thangaraj H, et al. (2003) Buruli ulcer in a visitor to London. Br J Dermatol 149: 907–909. doi:10.1046/j.1365-2133.2003.05562.x.
21. World Health Organization (n.d.) Number of new cases of Buruli ulcer reported (per year). Available: http://apps.who.int/neglected_diseases/ntddata/buruli/buruli.html.
22. Portaels F, Silva MT, Meyers WM (2009) Buruli ulcer. Clin Dermatol 27: 291–305. doi:10.1016/j.clindermatol.2008.09.021.
23. WHO Annual meeting on Buruli ulcer, 2014 (2014) Distribution of buruli ulcer, 2013.
24. Boisvert H (1977) [Skin ulcer caused by *Mycobacterium ulcerans* in Cameroon. II. Bacteriological study]. Bull Société Pathol Exot Ses Fil 70: 125–131.
25. Tortoli E (2006) The new *mycobacteria*: an update. FEMS Immunol Med Microbiol 48: 159–178. doi:10.1111/j.1574-695X.2006.00123.x.
26. Stinear TP, Jenkin GA, Johnson PD, Davies JK (2000) Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. J Bacteriol 182: 6322–6330.
27. Yip MJ, Porter JL, Fyfe JAM, Lavender CJ, Portaels F, et al. (2007) Evolution of *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria from a common *Mycobacterium marinum* progenitor. J Bacteriol 189: 2021–2029. doi:10.1128/JB.01442-06.
28. Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, et al. (2007) Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. Genome Res 17: 192–200. doi:10.1101/gr.5942807.
29. Stinear TP, Mve-Obiang A, Small PLC, Frigui W, Pryor MJ, et al. (2004) Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. Proc Natl Acad Sci U S A 101: 1345–1349. doi:10.1073/pnas.0305877101.
30. Stinear TP, Pryor MJ, Porter JL, Cole ST (2005) Functional analysis and annotation of the virulence plasmid pMUM001 from *Mycobacterium ulcerans*. Microbiol Read Engl 151: 683–692. doi:10.1099/mic.0.27674-0.
31. WHO | Laboratory diagnosis of buruli ulcer (n.d.). WHO. Available: http://www.who.int/buruli/laboratory_diagnosis/en/. Accessed 2 June 2014.
32. Huber CA, Ruf M-T, Pluschke G, Käser M (2008) Independent loss of immunogenic proteins in *Mycobacterium ulcerans* suggests immune evasion. Clin Vaccine Immunol CVI 15: 598–606. doi:10.1128/CVI.00472-07.
33. Röltgen K, Stinear TP, Pluschke G (2012) The genome, evolution and diversity of *Mycobacterium ulcerans*. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis 12: 522–529. doi:10.1016/j.meegid.2012.01.018.
34. Rondini S, Käser M, Stinear T, Tessier M, Mangold C, et al. (2007) Ongoing genome reduction in *Mycobacterium ulcerans*. Emerg Infect Dis 13: 1008–1015. doi:10.3201/eid1307.060205.
35. Doig KD, Holt KE, Fyfe JAM, Lavender CJ, Eddyani M, et al. (2012) On the origin of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. BMC Genomics 13: 258. doi:10.1186/1471-2164-13-258.
36. Käser M, Rondini S, Naegeli M, Stinear T, Portaels F, et al. (2007) Evolution of two distinct phylogenetic lineages of the emerging human pathogen *Mycobacterium ulcerans*. BMC Evol Biol 7: 177. doi:10.1186/1471-2148-7-177.
37. Pidot SJ, Asiedu K, Käser M, Fyfe JAM, Stinear TP (2010) *Mycobacterium ulcerans* and other mycolactone-producing *mycobacteria* should be considered a single species. PLoS Negl Trop Dis 4: e663. doi:10.1371/journal.pntd.0000663.

38. Portaels F, Fonteyene PA, de Beenhouwer H, de Rijk P, Guédénon A, et al. (1996) Variability in 3' end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to geographic origin of isolates. J Clin Microbiol 34: 962–965.
39. Ablordey A, Kotlowski R, Swings J, Portaels F (2005) PCR amplification with primers based on IS2404 and GC-rich repeated sequence reveals polymorphism in *Mycobacterium ulcerans*. J Clin Microbiol 43: 448–451. doi:10.1128/JCM.43.1.448-451.2005.
40. Ablordey A, Swings J, Hubans C, Chemlal K, Locht C, et al. (2005) Multilocus variable-number tandem repeat typing of *Mycobacterium ulcerans*. J Clin Microbiol 43: 1546–1551. doi:10.1128/JCM.43.4.1546-1551.2005.
41. Chemlal K, De Ridder K, Fonteyne PA, Meyers WM, Swings J, et al. (2001) The use of IS2404 restriction fragment length polymorphisms suggests the diversity of *Mycobacterium ulcerans* from different geographical areas. Am J Trop Med Hyg 64: 270–273.
42. Vandelannoote K, Jordaens K, Bomans P, Leirs H, Durnez L, et al. (2014) Insertion sequence element single nucleotide polymorphism typing provides insights into the population structure and evolution of *Mycobacterium ulcerans* across Africa. Appl Environ Microbiol 80: 1197–1209. doi:10.1128/AEM.02774-13.
43. Röltgen K, Qi W, Ruf M-T, Mensah-Quainoo E, Pidot SJ, et al. (2010) Single nucleotide polymorphism typing of *Mycobacterium ulcerans* reveals focal transmission of buruli ulcer in a highly endemic region of Ghana. PLoS Negl Trop Dis 4: e751. doi:10.1371/journal.pntd.0000751.
44. Epidemiology of *Mycobacterium ulcerans* infection (Buruli ulcer) at Kinyara, Uganda (1971). Trans R Soc Trop Med Hyg 65: 763–775.
45. Debacker M, Zinsou C, Aguiar J, Meyers WM, Portaels F (2003) First Case of *Mycobacterium ulcerans* Disease (Buruli Ulcer) Following a Human Bite. Clin Infect Dis 36: e67–e68. doi:10.1086/367660.
46. Meyers WM, Shelly WM, Connor DH, Meyers EK (1974) Human *Mycobacterium ulcerans* infections developing at sites of trauma to skin. Am J Trop Med Hyg 23: 919–923.
47. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, et al. (2010) Ecology and transmission of Buruli ulcer disease: a systematic review. PLoS Negl Trop Dis 4: e911. doi:10.1371/journal.pntd.0000911.
48. Raghunathan PL, Whitney EAS, Asamoa K, Stienstra Y, Taylor TH, et al. (2005) Risk factors for Buruli ulcer disease (*Mycobacterium ulcerans* Infection): results from a case-control study in Ghana. Clin Infect Dis Off Publ Infect Dis Soc Am 40: 1445–1453. doi:10.1086/429623.
49. Debacker M, Portaels F, Aguiar J, Steunou C, Zinsou C, et al. (2006) Risk factors for Buruli ulcer, Benin. Emerg Infect Dis 12: 1325–1331. doi:10.3201/eid1209.050598.
50. Pouillot R, Matias G, Wondje CM, Portaels F, Valin N, et al. (2007) Risk factors for buruli ulcer: a case control study in Cameroon. PLoS Negl Trop Dis 1: e101. doi:10.1371/journal.pntd.0000101.
51. Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, et al. (2008) Distribution of *Mycobacterium ulcerans* in buruli ulcer endemic and non-endemic aquatic sites in Ghana. PLoS Negl Trop Dis 2: e205. doi:10.1371/journal.pntd.0000205.
52. Johnson PDR, Azuolas J, Lavender CJ, Wishart E, Stinear TP, et al. (2007) *Mycobacterium ulcerans* in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. Emerg Infect Dis 13: 1653–1660. doi:10.3201/eid1311.061369.
53. Bratschi MW, Ruf M-T, Andreoli A, Minyem JC, Kerber S, et al. (2014) *Mycobacterium ulcerans* persistence at a village water source of Buruli ulcer patients. PLoS Negl Trop Dis 8: e2756. doi:10.1371/journal.pntd.0002756.

54. Marsollier L, Sévérin T, Aubry J, Merritt RW, Saint André J-P, et al. (2004) Aquatic snails, passive hosts of *Mycobacterium ulcerans*. Appl Environ Microbiol 70: 6296–6298. doi:10.1128/AEM.70.10.6296-6298.2004.
55. Willson SJ, Kaufman MG, Merritt RW, Williamson HR, Malakauskas DM, et al. (2013) Fish and amphibians as potential reservoirs of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer disease. Infect Ecol Epidemiol 3. doi:10.3402/iee.v3i0.19946.
56. Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, et al. (2008) First cultivation and characterization of *Mycobacterium ulcerans* from the environment. PLoS Negl Trop Dis 2: e178. doi:10.1371/journal.pntd.0000178.
57. Marsollier L, Robert R, Aubry J, Saint André J-P, Kouakou H, et al. (2002) Aquatic insects as a vector for *Mycobacterium ulcerans*. Appl Environ Microbiol 68: 4623–4628.
58. Marion E, Eyangoh S, Yeramian E, Doannio J, Landier J, et al. (2010) Seasonal and regional dynamics of *M. ulcerans* transmission in environmental context: deciphering the role of water bugs as hosts and vectors. PLoS Negl Trop Dis 4: e731. doi:10.1371/journal.pntd.0000731.
59. Doannio JMC, Konan KL, Dosso FN, Koné AB, Konan YL, et al. (2011) [*Micronecta* sp (*Corixidae*) and *Diplonychus* sp (*Belostomatidae*), two aquatic Hemiptera hosts and/or potential vectors of *Mycobacterium ulcerans* (pathogenic agent of Buruli ulcer) in Cote d'Ivoire]. Médecine Trop Rev Corps Santé Colon 71: 53–57.
60. Benbow ME, Williamson H, Kimbirauskas R, McIntosh MD, Kolar R, et al. (2008) Aquatic invertebrates as unlikely vectors of Buruli ulcer disease. Emerg Infect Dis 14: 1247–1254. doi:10.3201/eid1408.071503.
61. Fyfe JAM, Lavender CJ, Handasyde KA, Legione AR, O'Brien CR, et al. (2010) A major role for mammals in the ecology of *Mycobacterium ulcerans*. PLoS Negl Trop Dis 4: e791. doi:10.1371/journal.pntd.0000791.
62. O'Brien CR, Handasyde KA, Hibble J, Lavender CJ, Legione AR, et al. (2014) Clinical, microbiological and pathological findings of *Mycobacterium ulcerans* infection in three Australian Possum species. PLoS Negl Trop Dis 8: e2666. doi:10.1371/journal.pntd.0002666.
63. Durnez L, Suykerbuyk P, Nicolas V, Barrière P, Verheyen E, et al. (2010) Terrestrial small mammals as reservoirs of *Mycobacterium ulcerans* in benin. Appl Environ Microbiol 76: 4574–4577. doi:10.1128/AEM.00199-10.
64. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, et al. (2004) *Mycobacterium ulcerans* disease: role of age and gender in incidence and morbidity. Trop Med Int Health TM IH 9: 1297–1304. doi:10.1111/j.1365-3156.2004.01339.x.
65. Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, et al. (2013) Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. PLoS Negl Trop Dis 7: e2252. doi:10.1371/journal.pntd.0002252.
66. Junghanss T, Johnson RC, Pluschke G (2014) *Mycobacterium ulcerans* disease. Manson's tropical diseases. Saunders. pp. 519–531.
67. Walsh DS, Portaels F, Meyers WM (2011) Buruli ulcer: Advances in understanding *Mycobacterium ulcerans* infection. Dermatol Clin 29: 1–8. doi:10.1016/j.det.2010.09.006.
68. Schütte D, Pluschke G (2009) Immunosuppression and treatment-associated inflammatory response in patients with *Mycobacterium ulcerans* infection (Buruli ulcer). Expert Opin Biol Ther 9: 187–200. doi:10.1517/14712590802631854.
69. WHO | Treatment of *Mycobacterium ulcerans* disease (Buruli Ulcer) (n.d.). WHO. Available: http://www.who.int/buruli/treatment/en/. Accessed 11 September 2014.

70. Yeboah-Manu D, Kpeli GS, Ruf M-T, Asan-Ampah K, Quenin-Fosu K, et al. (2013) Secondary bacterial infections of Buruli ulcer lesions before and after chemotherapy with streptomycin and rifampicin. PLoS Negl Trop Dis 7: e2191. doi:10.1371/journal.pntd.0002191.
71. Goto M, Nakanaga K, Aung T, Hamada T, Yamada N, et al. (2006) Nerve damage in *Mycobacterium ulcerans*-infected mice: probable cause of painlessness in buruli ulcer. Am J Pathol 168: 805–811. doi:10.2353/ajpath.2006.050375.
72. Converse PJ, Nuermberger EL, Almeida DV, Grosset JH (2011) Treating *Mycobacterium ulcerans* disease (Buruli ulcer): from surgery to antibiotics, is the pill mightier than the knife? Future Microbiol 6: 1185–1198. doi:10.2217/fmb.11.101.
73. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, et al. (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283: 854–857.
74. Mve-Obiang A, Lee RE, Portaels F, Small PLC (2003) Heterogeneity of mycolactones produced by clinical isolates of *Mycobacterium ulcerans*: implications for virulence. Infect Immun 71: 774–783.
75. Scherr N, Gersbach P, Dangy J-P, Bomio C, Li J, et al. (2013) Structure-activity relationship studies on the macrolide exotoxin mycolactone of *Mycobacterium ulcerans*. PLoS Negl Trop Dis 7: e2143. doi:10.1371/journal.pntd.0002143.
76. Ortiz RH, Leon DA, Estevez HO, Martin A, Herrera JL, et al. (2009) Differences in virulence and immune response induced in a murine model by isolates of *Mycobacterium ulcerans* from different geographic areas. Clin Exp Immunol 157: 271–281. doi:10.1111/j.1365-2249.2009.03941.x.
77. Adusumilli S, Mve-Obiang A, Sparer T, Meyers W, Hayman J, et al. (2005) *Mycobacterium ulcerans* toxic macrolide, mycolactone modulates the host immune response and cellular location of *M. ulcerans* in vitro and in vivo. Cell Microbiol 7: 1295–1304. doi:10.1111/j.1462-5822.2005.00557.x.
78. Coutanceau E, Decalf J, Martino A, Babon A, Winter N, et al. (2007) Selective suppression of dendritic cell functions by *Mycobacterium ulcerans* toxin mycolactone. J Exp Med 204: 1395–1403. doi:10.1084/jem.20070234.
79. George KM, Pascopella L, Welty DM, Small PL (2000) A *Mycobacterium ulcerans* toxin, mycolactone, causes apoptosis in guinea pig ulcers and tissue culture cells. Infect Immun 68: 877–883.
80. Bolz M, Ruggli N, Ruf M-T, Ricklin ME, Zimmer G, et al. (2014) Experimental infection of the pig with *Mycobacterium ulcerans*: a novel model for studying the pathogenesis of Buruli ulcer disease. PLoS Negl Trop Dis 8: e2968. doi:10.1371/journal.pntd.0002968.
81. Hong H, Coutanceau E, Leclerc M, Caleechurn L, Leadlay PF, et al. (2008) Mycolactone diffuses from *Mycobacterium ulcerans*-infected tissues and targets mononuclear cells in peripheral blood and lymphoid organs. PLoS Negl Trop Dis 2: e325. doi:10.1371/journal.pntd.0000325.
82. Hall B, Simmonds R (2014) Pleiotropic molecular effects of the *Mycobacterium ulcerans* virulence factor mycolactone underlying the cell death and immunosuppression seen in Buruli ulcer. Biochem Soc Trans 42: 177–183. doi:10.1042/BST20130133.
83. Silva MT, Portaels F, Pedrosa J (2009) Pathogenetic mechanisms of the intracellular parasite *Mycobacterium ulcerans* leading to Buruli ulcer. Lancet Infect Dis 9: 699–710. doi:10.1016/S1473-3099(09)70234-8.
84. Hayman J (1993) Out of Africa: observations on the histopathology of *Mycobacterium ulcerans* infection. J Clin Pathol 46: 5–9.
85. Guarner J, Bartlett J, Whitney EAS, Raghunathan PL, Stienstra Y, et al. (2003) Histopathologic features of *Mycobacterium ulcerans* infection. Emerg Infect Dis 9: 651–656.

86. Rondini S, Horsfield C, Mensah-Quainoo E, Junghanss T, Lucas S, et al. (2006) Contiguous spread of *Mycobacterium ulcerans* in Buruli ulcer lesions analysed by histopathology and real-time PCR quantification of mycobacterial DNA. J Pathol 208: 119–128. doi:10.1002/path.1864.

87. Coutanceau E, Marsollier L, Brosch R, Perret E, Goossens P, et al. (2005) Modulation of the host immune response by a transient intracellular stage of *Mycobacterium ulcerans*: the contribution of endogenous mycolactone toxin. Cell Microbiol 7: 1187–1196. doi:10.1111/j.1462-5822.2005.00546.x.

88. Ruf M-T, Schütte D, Chauffour A, Jarlier V, Ji B, et al. (2012) Chemotherapy-associated changes of histopathological features of *Mycobacterium ulcerans* lesions in a Buruli ulcer mouse model. Antimicrob Agents Chemother 56: 687–696. doi:10.1128/AAC.05543-11.

89. Mensah-Quainoo E, Yeboah-Manu D, Asebi C, Patafuor F, Ofori-Adjei D, et al. (2008) Diagnosis of *Mycobacterium ulcerans* infection (Buruli ulcer) at a treatment centre in Ghana: a retrospective analysis of laboratory results of clinically diagnosed cases. Trop Med Int Health TM IH 13: 191–198. doi:10.1111/j.1365-3156.2007.01990.x.

90. Marsollier L, Honoré N, Legras P, Manceau AL, Kouakou H, et al. (2003) Isolation of three *Mycobacterium ulcerans* strains resistant to rifampin after experimental chemotherapy of mice. Antimicrob Agents Chemother 47: 1228–1232.

91. Andreoli A, Ruf M-T, Sopoh GE, Schmid P, Pluschke G (2014) Immunohistochemical monitoring of wound healing in antibiotic treated Buruli ulcer patients. PLoS Negl Trop Dis 8: e2809. doi:10.1371/journal.pntd.0002809.

92. Ruf M-T, Chauty A, Adeye A, Ardant M-F, Koussemou H, et al. (2011) Secondary Buruli ulcer skin lesions emerging several months after completion of chemotherapy: paradoxical reaction or evidence for immune protection? PLoS Negl Trop Dis 5: e1252. doi:10.1371/journal.pntd.0001252.

93. Beissner M, Herbinger K-H, Bretzel G (2010) Laboratory diagnosis of Buruli ulcer disease. Future Microbiol 5: 363–370. doi:10.2217/fmb.10.3.

94. Yeboah-Manu D, Danso E, Ampah K, Asante-Poku A, Nakobu Z, et al. (2011) Isolation of *Mycobacterium ulcerans* from swab and fine-needle-aspiration specimens. J Clin Microbiol 49: 1997–1999. doi:10.1128/JCM.02279-10.

95. Dega H, Bentoucha A, Robert J, Jarlier V, Grosset J (2002) Bactericidal activity of rifampin-amikacin against *Mycobacterium ulcerans* in mice. Antimicrob Agents Chemother 46: 3193–3196.

96. Bentoucha A, Robert J, Dega H, Lounis N, Jarlier V, et al. (2001) Activities of new macrolides and fluoroquinolones against *Mycobacterium ulcerans* infection in mice. Antimicrob Agents Chemother 45: 3109–3112. doi:10.1128/AAC.45.11.3109-3112.2001.

97. WHO | Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer) (n.d.). WHO. Available: zotero://attachment/245/. Accessed 19 June 2014.

98. Nienhuis WA, Stienstra Y, Thompson WA, Awuah PC, Abass KM, et al. (2010) Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial. Lancet 375: 664–672. doi:10.1016/S0140-6736(09)61962-0.

99. Chauty A, Ardant M-F, Adeye A, Euverte H, Guédénon A, et al. (2007) Promising clinical efficacy of streptomycin-rifampin combination for treatment of buruli ulcer (*Mycobacterium ulcerans* disease). Antimicrob Agents Chemother 51: 4029–4035. doi:10.1128/AAC.00175-07.

100. Sarfo FS, Phillips R, Asiedu K, Ampadu E, Bobi N, et al. (2010) Clinical efficacy of combination of rifampin and streptomycin for treatment of *Mycobacterium ulcerans* disease. Antimicrob Agents Chemother 54: 3678–3685. doi:10.1128/AAC.00299-10.

101. Kibadi K, Boelaert M, Fraga AG, Kayinua M, Longatto-Filho A, et al. (2010) Response to treatment in a prospective cohort of patients with large ulcerated lesions suspected to be Buruli Ulcer (*Mycobacterium ulcerans disease*). PLoS Negl Trop Dis 4: e736. doi:10.1371/journal.pntd.0000736.

102. Gordon CL, Buntine JA, Hayman JA, Lavender CJ, Fyfe JAM, et al. (2010) All-oral antibiotic treatment for Buruli ulcer: a report of four patients. PLoS Negl Trop Dis 4: e770. doi:10.1371/journal.pntd.0000770.

103. Chauty A, Ardant M-F, Marsollier L, Pluschke G, Landier J, et al. (2011) Oral treatment for *Mycobacterium ulcerans* infection: results from a pilot study in Benin. Clin Infect Dis Off Publ Infect Dis Soc Am 52: 94–96. doi:10.1093/cid/ciq072.

104. Almeida D, Converse PJ, Ahmad Z, Dooley KE, Nuermberger EL, et al. (2011) Activities of rifampin, Rifapentine and clarithromycin alone and in combination against *mycobacterium ulcerans* disease in mice. PLoS Negl Trop Dis 5: e933. doi:10.1371/journal.pntd.0000933.

105. Friedman ND, Athan E, Hughes AJ, Khajehnoori M, McDonald A, et al. (2013) *Mycobacterium ulcerans* disease: experience with primary oral medical therapy in an Australian cohort. PLoS Negl Trop Dis 7: e2315. doi:10.1371/journal.pntd.0002315.

106. Schütte D, Umboock A, Pluschke G (2009) Phagocytosis of *Mycobacterium ulcerans* in the course of rifampicin and streptomycin chemotherapy in Buruli ulcer lesions. Br J Dermatol 160: 273–283. doi:10.1111/j.1365-2133.2008.08879.x.

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

108. Ruf M-T, Sopoh GE, Brun LV, Dossou AD, Barogui YT, et al. (2011) Histopathological changes and clinical responses of Buruli ulcer plaque lesions during chemotherapy: a role for surgical removal of necrotic tissue? PLoS Negl Trop Dis 5: e1334. doi:10.1371/journal.pntd.0001334.

109. O'Brien DP, Robson ME, Callan PP, McDonald AH (2009) "Paradoxical" immune-mediated reactions to *Mycobacterium ulcerans* during antibiotic treatment: a result of treatment success, not failure. Med J Aust 191: 564–566.

110. Nienhuis WA, Stienstra Y, Abass KM, Tuah W, Thompson WA, et al. (2012) Paradoxical responses after start of antimicrobial treatment in *Mycobacterium ulcerans* infection. Clin Infect Dis Off Publ Infect Dis Soc Am 54: 519–526. doi:10.1093/cid/cir856.

111. O'Brien DP, Robson M, Friedman ND, Walton A, McDonald A, et al. (2013) Incidence, clinical spectrum, diagnostic features, treatment and predictors of paradoxical reactions during antibiotic treatment of *Mycobacterium ulcerans* infections. BMC Infect Dis 13: 416. doi:10.1186/1471-2334-13-416.

112. Meyers WM, Shelly WM, Connor DH (1974) Heat treatment of *Mycobacterium ulcerans* infections without surgical excision. Am J Trop Med Hyg 23: 924–929.

113. Junghanss T, Um Boock A, Vogel M, Schuette D, Weinlaeder H, et al. (2009) Phase change material for thermotherapy of Buruli ulcer: a prospective observational single centre proof-of-principle trial. PLoS Negl Trop Dis 3: e380. doi:10.1371/journal.pntd.0000380.

114. Dobos KM, Spotts EA, Marston BJ, Horsburgh CR, King CH (2000) Serologic response to culture filtrate antigens of *Mycobacterium ulcerans* during Buruli ulcer disease. Emerg Infect Dis 6: 158–164. doi:10.3201/eid0602.000208.

115. Okenu DMN, Ofielu LO, Easley KA, Guarner J, Spotts Whitney EA, et al. (2004) Immunoglobulin M antibody responses to *Mycobacterium ulcerans* allow discrimination between cases of active Buruli ulcer disease and matched family controls in areas where the disease is endemic. Clin Diagn Lab Immunol 11: 387–391.

116. Diaz D, Döbeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, et al. (2006) Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. Clin Vaccine Immunol CVI 13: 1314–1321. doi:10.1128/CVI.00254-06.

117. Röltgen K, Bratschi MW, Ross A, Aboagye SY, Ampah KA, et al. (2014) Late onset of the serological response against the 18 kDa small heat shock protein of *Mycobacterium ulcerans* in children. PLoS Negl Trop Dis 8: e2904. doi:10.1371/journal.pntd.0002904.

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

119. Stanford JL, Revill WD, Gunthorpe WJ, Grange JM (1975) The production and preliminary investigation of Burulin, a new skin test reagent for *Mycobacterium ulcerans* infection. J Hyg (Lond) 74: 7–16.

120. Gooding TM, Johnson PDR, Smith M, Kemp AS, Robins-Browne RM (2002) Cytokine profiles of patients infected with *Mycobacterium ulcerans* and unaffected household contacts. Infect Immun 70: 5562–5567.

121. Prévot G, Bourreau E, Pascalis H, Pradinaud R, Tanghe A, et al. (2004) Differential production of systemic and intralesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. Infect Immun 72: 958–965.

122. Westenbrink BD, Stienstra Y, Huitema MG, Thompson WA, Klutse EO, et al. (2005) Cytokine responses to stimulation of whole blood from patients with Buruli ulcer disease in Ghana. Clin Diagn Lab Immunol 12: 125–129. doi:10.1128/CDLI.12.1.125-129.2005.

123. Yeboah-Manu D, Peduzzi E, Mensah-Quainoo E, Asante-Poku A, Ofori-Adjei D, et al. (2006) Systemic suppression of interferon-gamma responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen *Mycobacterium ulcerans*. J Leukoc Biol 79: 1150–1156. doi:10.1189/jlb.1005581.

124. Phillips R, Horsfield C, Kuijper S, Sarfo SF, Obeng-Baah J, et al. (2006) Cytokine response to antigen stimulation of whole blood from patients with *Mycobacterium ulcerans* disease compared to that from patients with tuberculosis. Clin Vaccine Immunol CVI 13: 253–257. doi:10.1128/CVI.13.2.253-257.2006.

125. Schipper HS, Rutgers B, Huitema MG, Etuaful SN, Westenbrink BD, et al. (2007) Systemic and local interferon-gamma production following *Mycobacterium ulcerans* infection. Clin Exp Immunol 150: 451–459. doi:10.1111/j.1365-2249.2007.03506.x.

126. Gooding TM, Johnson PD, Campbell DE, Hayman JA, Hartland EL, et al. (2001) Immune response to infection with *Mycobacterium ulcerans*. Infect Immun 69: 1704–1707. doi:10.1128/IAI.69.3.1704-1707.2001.

127. Gooding TM, Kemp AS, Robins-Browne RM, Smith M, Johnson PDR (2003) Acquired T-helper 1 lymphocyte anergy following infection with *Mycobacterium ulcerans*. Clin Infect Dis Off Publ Infect Dis Soc Am 36: 1076–1077. doi:10.1086/368315.

128. Fraga AG, Cruz A, Martins TG, Torrado E, Saraiva M, et al. (2011) *Mycobacterium ulcerans* triggers T-cell immunity followed by local and regional but not systemic immunosuppression. Infect Immun 79: 421–430. doi:10.1128/IAI.00820-10.

129. Einarsdottir T, Huygen K (2011) Buruli ulcer. Hum Vaccin 7: 1198–1203. doi:10.4161/hv.7.11.17751.

130. Huygen K, Adjei O, Affolabi D, Bretzel G, Demangel C, et al. (2009) Buruli ulcer disease: prospects for a vaccine. Med Microbiol Immunol (Berl) 198: 69–77. doi:10.1007/s00430-009-0109-6.

131. Revill WDL, Morrow RH, Pike MC, Ateng J (1973) A CONTROLLED TRIAL OF THE TREATMENT OF *MYCOBACTERIUM ULCERANS* INFECTION WITH CLOFAZIMINE. The Lancet 302: 873–877. doi:10.1016/S0140-6736(73)92005-9.

132. Gordon CL, Buntine JA, Hayman JA, Lavender CJ, Fyfe JA, et al. (2011) Spontaneous Clearance of *Mycobacterium ulcerans* in a Case of Buruli Ulcer. PLoS Negl Trop Dis 5. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3201911/. Accessed 20 August 2014.

133. BCG vaccination against *mycobacterium ulcerans* infection (Buruli ulcer). First results of a trial in Uganda (1969). Lancet 1: 111–115.

134. Smith PG, Revill WD, Lukwago E, Rykushin YP (1976) The protective effect of BCG against *Mycobacterium ulcerans* disease: a controlled trial in an endemic area of Uganda. Trans R Soc Trop Med Hyg 70: 449–457.

135. Portaels F, Aguiar J, Debacker M, Guédénon A, Steunou C, et al. (2004) *Mycobacterium bovis* BCG vaccination as prophylaxis against *Mycobacterium ulcerans* osteomyelitis in Buruli ulcer disease. Infect Immun 72: 62–65.

136. Tanghe A, Content J, Van Vooren J-P, Portaels F, Huygen K (2001) Protective Efficacy of a DNA Vaccine Encoding Antigen 85A from *Mycobacterium bovis* BCG against Buruli Ulcer. Infect Immun 69: 5403–5411. doi:10.1128/IAI.69.9.5403-5411.2001.

137. Tanghe A, Dangy J-P, Pluschke G, Huygen K (2008) Improved protective efficacy of a species-specific DNA vaccine encoding mycolyl-transferase Ag85A from *Mycobacterium ulcerans* by homologous protein boosting. PLoS Negl Trop Dis 2: e199. doi:10.1371/journal.pntd.0000199.

138. Coutanceau E, Legras P, Marsollier L, Reysset G, Cole ST, et al. (2006) Immunogenicity of *Mycobacterium ulcerans* Hsp65 and protective efficacy of a *Mycobacterium leprae* Hsp65-based DNA vaccine against Buruli ulcer. Microbes Infect Inst Pasteur 8: 2075–2081. doi:10.1016/j.micinf.2006.03.009.

139. Roupie V, Pidot SJ, Einarsdottir T, Van Den Poel C, Jurion F, et al. (2014) Analysis of the vaccine potential of plasmid DNA encoding nine mycolactone polyketide synthase domains in *Mycobacterium ulcerans* infected mice. PLoS Negl Trop Dis 8: e2604. doi:10.1371/journal.pntd.0002604.

140. Fraga AG, Martins TG, Torrado E, Huygen K, Portaels F, et al. (2012) Cellular immunity confers transient protection in experimental Buruli ulcer following BCG or mycolactone-negative *Mycobacterium ulcerans* vaccination. PloS One 7: e33406. doi:10.1371/journal.pone.0033406.

141. Fyfe JAM, Lavender CJ, Handasyde KA, Legione AR, O'Brien CR, et al. (2010) A major role for mammals in the ecology of *Mycobacterium ulcerans*. PLoS Negl Trop Dis 4: e791. doi:10.1371/journal.pntd.0000791.

142. Mitchell PJ, McOrist S, Bilney R (1987) Epidemiology of *Mycobacterium ulcerans* infection in koalas (*Phascolarctos cinereus*) on Raymond Island, southeastern Australia. J Wildl Dis 23: 386–390.

143. Sakaguchi K, Iima H, Hirayama K, Okamoto M, Matsuda K, et al. (2011) *Mycobacterium ulcerans* infection in an Indian flap-shelled turtle (*Lissemys punctata punctata*). J Vet Med Sci Jpn Soc Vet Sci 73: 1217–1220.

144. O'Brien C, Kuseff G, McMillan E, McCowan C, Lavender C, et al. (2013) *Mycobacterium ulcerans* infection in two alpacas. Aust Vet J 91: 296–300. doi:10.1111/avj.12071.

145. Van Zyl A, Daniel J, Wayne J, McCowan C, Malik R, et al. (2010) *Mycobacterium ulcerans* infections in two horses in south-eastern Australia. Aust Vet J 88: 101–106. doi:10.1111/j.1751-0813.2009.00544.x.

146. Elsner L, Wayne J, O'Brien CR, McCowan C, Malik R, et al. (2008) Localised *Mycobacterium ulcerans* infection in a cat in Australia. J Feline Med Surg 10: 407–412. doi:10.1016/j.jfms.2008.03.003.

147. Walsh DS, Meyers WM, Krieg RE, Walsh GP (1999) Transmission of *Mycobacterium ulcerans* to the nine-banded armadillo. Am J Trop Med Hyg 61: 694–697.

148. Walsh DS, Dela Cruz EC, Abalos RM, Tan EV, Walsh GP, et al. (2007) Clinical and histologic features of skin lesions in a cynomolgus monkey experimentally infected with *Mycobacterium ulcerans* (Buruli ulcer) by intradermal inoculation. Am J Trop Med Hyg 76: 132–134.

149. Krieg RE, Hockmeyer WT, Connor DH (1974) Toxin of *Mycobacterium ulcerans*. Production and effects in guinea pig skin. Arch Dermatol 110: 783–788.

150. Williamson HR, Mosi L, Donnell R, Aqqad M, Merritt RW, et al. (2014) *Mycobacterium ulcerans* Fails to Infect through Skin Abrasions in a Guinea Pig Infection Model: Implications for Transmission. PLoS Negl Trop Dis 8. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3983084/. Accessed 3 September 2014.

151. Fenner F (1956) The pathogenic behavior of *Mycobacterium ulcerans* and *Mycobacterium balnei* in the mouse and the developing chick embryo. Am Rev Tuberc 73: 650–673.

152. Converse PJ, Xing Y, Kim KH, Tyagi S, Li S-Y, et al. (2014) Accelerated detection of mycolactone production and response to antibiotic treatment in a mouse model of *Mycobacterium ulcerans* disease. PLoS Negl Trop Dis 8: e2618. doi:10.1371/journal.pntd.0002618.

153. Converse PJ, Almeida DV, Nuermberger EL, Grosset JH (2011) BCG-mediated protection against *Mycobacterium ulcerans* infection in the mouse. PLoS Negl Trop Dis 5: e985. doi:10.1371/journal.pntd.0000985.

154. Shepard CC, McRae DH (1968) A method for counting acid-fast bacteria. Int J Lepr Mycobact Dis Off Organ Int Lepr Assoc 36: 78–82.

155. Martins TG, Trigo G, Fraga AG, Gama JB, Longatto-Filho A, et al. (2012) Corticosteroid-induced immunosuppression ultimately does not compromise the efficacy of antibiotherapy in murine *Mycobacterium ulcerans* infection. PLoS Negl Trop Dis 6: e1925. doi:10.1371/journal.pntd.0001925.

156. Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, et al. (2007) Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. Appl Environ Microbiol 73: 4733–4740. doi:10.1128/AEM.02971-06.

157. Lavender CJ, Fyfe JAM (2013) Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples. Methods Mol Biol Clifton NJ 943: 201–216. doi:10.1007/978-1-60327-353-4_13.

Objectives

Within the framework of the establishment of a new field site in the remote Mapé dam region of Cameroon, specific objectives of this thesis were to:

1. describe the epidemiology of Buruli ulcer in the Mapé Basin of Cameroon with a special focus on the laboratory confirmation of the clinically diagnosed patients by quantitative real time PCR and *M. ulcerans* cultivation
2. initiate the build-up of a *M. ulcerans* isolate collection from Cameroonian patients by devising an optimal procedure for transport and decontamination of clinical samples after a long time span between sampling and culture initiation
3. study the phylogeny of the cultivated Cameroonian *M. ulcerans* strains by whole genome sequencing with the aim of gaining insight into transmission by combining genomic and geographical data

A vaccine against *M. ulcerans* would be of great benefit if used for prophylaxis as well as therapeutically. As a partner in the EU funded collaborative project BuruliVac we aimed at evaluating the potential for developing a protein subunit-vaccine against *M. ulcerans* and at gaining insight into the nature of protective immune responses against *M. ulcerans* and correlates of protection.

Within the framework of the BuruliVac project specific objectives of this thesis were to:

4. identify and produce *M. ulcerans* protein vaccine candidates as recombinant proteins in *E. coli* and formulate the purified proteins with immunostimulatory molecules (adjuvants)
5. generate vesicular stomatitis virus (VSV) replicons encoding for *M. ulcerans* protein vaccine candidates, study their expression in VSV-infected cells and define an optimal immunization protocol in mice
6. deliver *M. ulcerans* protein vaccine candidates in genetically modified *Plasmodium berghei* parasites in an infection treatment vaccination approach
7. investigate the immunogenicity and the protective potential of the three different delivery systems in a mouse model of Buruli ulcer
8. develop the pig as animal model for Buruli ulcer for the profiling of advanced candidate vaccines and to study early forms of the disease

Results Chapter 1

Geographic Distribution, Age Pattern and Sites of Lesions in a Cohort of Buruli Ulcer Patients from the Mapé Basin of Cameroon

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Abstract

Buruli ulcer (BU), a neglected tropical disease of the skin, caused by *Mycobacterium ulceran*s, occurs most frequently in children in West Africa. Risk factors for BU include proximity to slow flowing water, poor wound care and not wearing protective clothing. Man-made alterations of the environment have been suggested to lead to increased BU incidence. *M. ulcerans* DNA has been detected in the environment, water bugs and recently also in mosquitoes. Despite these findings, the mode of transmission of BU remains poorly understood and both transmission by insects or direct inoculation from contaminated environment have been suggested. Here we investigated the BU epidemiology in the Mapé basin of Cameroon where the damming of the Mapé River since 1988 is believed to have increased the incidence of BU. Through a house-by-house survey in spring 2010, which also examined the local population for leprosy and yaws, and continued surveillance thereafter, we identified, till June 2012, altogether 88 RT-PCR positive cases of BU. We found that, the age adjusted cumulative incidence of BU was highest in young teenagers and in individuals above the age of 50 and that very young children (<5) were underrepresented among cases. BU lesions clustered around the ankles and at the back of the elbows. This pattern neither matches any of the published mosquito biting site patterns, nor the published distribution of small skin injuries in children, where lesions on the knees are much more frequent. The option of multiple modes of transmission should thus be considered. Analyzing the geographic distribution of cases in the Mapé Dam area revealed a closer association with the Mbam River than with the artificial lake.

Author Summary

Buruli ulcer (BU) is an infectious disease caused by *Mycobacterium ulcerans* that is affecting mostly children in endemic areas of West Africa. Proximity to slow flowing water is a risk factor, but the exact mode of transmission of BU remains unclear. Man-made environmental changes, such as sand mining, damming of rivers and irrigation have been implicated with increases in disease incidence. Here we report findings from a survey for BU and continued case detection thereafter in the Bankim Health District of Cameroon. In this area, the local population believed that the damming of the Mapé River has led to the emergence of BU. In 28 months we identified 88 laboratory confirmed cases of BU. Studying these cases, we found that the age adjusted cumulative incidence of BU in the elderly is similar to that in children and that the distribution pattern of BU lesions neither matches mosquito biting patterns nor the distribution of small skin injuries. Multiple modes of transmission should therefore be considered. Our data further showed that the patients appear to have closer contact to the local Mbam River than to the artificial Mapé dam reservoir.

Introduction

Buruli ulcer (BU), a neglected tropical disease (NTD) of the skin, is caused by *Mycobacterium ulcerans* [1] and if untreated, can lead to disability. Worldwide, local BU incidence rates are highest in West Africa and Australia, where the classical lineage of *M. ulcerans* is found [2–4] and the disease occurs at different foci in the endemic countries. Both sexes can be affected by the disease and although individuals of all ages can get BU, most of the patients are less than 15 years old [5]. In Cameroon, BU was first described in 1969 in the Nyong river valley where during a cross-sectional survey in 2001, a total of 436 clinically diagnosed cases of active or inactive BU were found [6]. Since then, the Bankim Health District (HD) has been identified as an additional BU endemic area in Cameroon [7]. In this area, where our research has been carried out, the local population suspects that the creation of an artificial lake, by damming of the Mapé River in 1988, has led to an increase in BU incidence. Risk factors for BU include proximity to slow flowing water, poor wound care and not wearing protective clothing [8]. However, the exact mode of transmission has not yet been elucidated [9,10]. Clinically, BU presents with symptoms ranging from nodules, plaques and oedemas to ulcers [11]. The cytotoxic and immunosuppressive toxin, mycolactone, uniquely produced by *M. ulcerans*, is believed to account for most of the pathology of BU [12]. The severity of cases is classified into three categories, with ‘1’ being patients with small (≤ 5cm dimeter) lesions, ‘2’ patients with medium size lesions (5–15 cm) and ‘3’ being patients with large (> 15cm) lesions, multiple lesions or lesions at critical sites [13]. Many BU cases identified in rural areas are still diagnosed based on clinical symptoms only, although the use of laboratory diagnosis is highly recommended by the World Health Organization (WHO). In 2004, the WHO introduced the use of the combination of streptomycin and rifampicin given daily for 8 weeks as treatment [14]. However, surgery and wound management remain critical aspects of BU care [15,16].

During our investigations of BU in the Bankim HD, we also examined the local population for two other NTDs of the skin, namely yaws and leprosy. Yaws is caused by *Treponema pallidum* (*T. pallidum*) subspecies *pertunu*, and is transmitted through skin and mucous membrane contact [17,18]. After an initial single lesion, the disease progresses to secondary multiple lesions and in about 10% of cases it causes permanent disability [18]. Leprosy is caused by *Mycobacterium leprae*, which is believed to be transmitted by the respiratory route and can cause major disabilities through nerve damage. Diagnosis of yaws and leprosy relies mainly on physical examinations [17,19] and treatment of both diseases is feasible with antibiotics [17,20].

The objectives of the present study were i) to conduct an exhaustive survey for BU, yaws and leprosy in the Bankim HD; ii) to continuously monitor the occurrence of BU in the Mapé Dam area; and iii) to examine the age distribution, geographic origin and distribution of lesions of the real-time polymerase chain reaction (RT-PCR) confirmed cases of BU to underpin future environmental and social science studies.

Materials and Methods

Ethical Statement

Approval for the survey and the subsequent continuous enrolment of cases was obtained from the Cameroon National Ethics Committee (N°041/CNE/DNM/09 and N°172/CNE/SE/2011) and the Ethics Committee of Basel (EKBB, reference no. 53/11). Participation in all aspects of the study was voluntary and all patients, independent of their study participation, were treated according to national treatment standards. All clinically confirmed cases who participated in the study provided written informed consent.

Study Area

The study was conducted in the Mapé Dam region of Cameroon (Figure 1) at two different geographical scales. The initial phase of the study was conducted in the Bankim HD which consists of seven Health Areas (HA): Atta; Songkolong, Somié, Nyamboya, Bandam, Bankim Urban and Bankim Rural. The health care infrastructure of the Bankim HD consists of one public district hospital, six primary and four private health centres (HC). All of these facilities employ two medical doctors and approximately 30 nurses. For the later part of the study, bordering regions in the 4 HD surrounding the Bankim HD (Nwa HD, Malantouen HD, Mayo Darle HD, Yoke HD) were also included in the study area. The main environmental features of the area are the Mapé Dam and the Mbam River.

Survey procedures

In early 2010 (March 22 to April 19), we conducted an exhaustive cross-sectional house-by-house survey for BU, leprosy and yaws in the 88 villages of the Bankim HD (Figure 1). Eleven teams of three trained field workers, namely one local nurse and two local community relays, were employed to interview all inhabitants. Field workers were trained for two days on the use of the questionnaire and the clinical signs of the three diseases investigated. At the household level, demographic information of all inhabitants was collected and posters with photographs of the clinical presentations of the diseases were shown. Households with suspected cases were re-visited by staff with extensive experience in the diagnosis of BU, leprosy and yaws. From clinically confirmed BU cases, samples were collected for laboratory confirmation as follows. Two or three dry swabs were collected from ulcerative lesions or a fine needle aspirate (FNA) was drawn from non-ulcerative lesions [21]. To facilitate handling of FNA samples, they were transferred onto a swab.

Prospective BU surveillance

Following the survey, we continued to monitor the occurrence of all new cases of BU in the Bankim HD by community and HC based case referral and regular supervision until the end

of June 2012. For this, a health worker, trained and experienced in the diagnosis of BU, regularly visited all HC in the Bankim HD and areas of the adjacent Malantouen HD. During these visits, suspected cases who independently came forward or who were referred to the HC by community or family members, were evaluated and if clinically confirmed, asked to come for treatment. Before treatment, swabs or an FNA were collected for laboratory confirmation as described above. In addition to demographic and clinical information, the houses where the patients lived for at least a year before disease onset were mapped using a GPS device. From the GPS device, coordinates were only recorded once the GPS receiver showed an accuracy of below 10m. Details of the location of the lesions on the patient's bodies were also collected and documented by photographs. Both clinically confirmed BU cases identified in the survey and during the continuous case detection were included in the cohort of patients investigated here.

Laboratory Confirmation of BU Cases

Samples were locally stored at 4ºC before transport to the laboratory where definite BU diagnosis was obtained by insertion sequence (*IS*) 2404 RT-PCR. Analysis was done according to the protocol developed by Fyfe *et al*. [22,23]. In brief, swabs were transferred into glass bottles containing glass beads with 2-5mL of PBS, and the bottle vortexed for 1.5 minutes. From 1mL of the solution, DNA was extracted and RT-PCR performed. DNA was amplified in a StepOnePlus Real-Time PCR System (Applied Biosystems) and data analyzed using the Applied Biosystems StepOne Software (2.2.2).

Analysis of lesion distribution

Using published age specific relative body surface areas (RBSA) [24] and the number of patients in each of the age groups, the weighted average RBSAs of a model person (all ages), a model child (<15), and a model adult (≥15) were computed. If required to perform a Fisher's exact test, RBSA were converted to counts which add up to the observed number of lesions. The shape file used to analyze lesion localizations is found in Dataset S1.

Statistical analysis

Continuous variables were summarized as means and standard deviation or medians and interquartile ranges and categorical ones as counts and percentages. The Fisher's exact or Chi-squared tests were used to compare categorical characteristics between groups and Student t-tests or Mann-Whitney U-test in the case of continuous variables. Multiple comparisons were adjusted for using a Bonferroni correction. The software, SAS (SAS Institute, Cary, USA; release 9.3), RStudio (RStudio, Boston, USA, version 0.95.262) and R (The R Foundation for Statistical Computing; version 2.15.1) were used to perform the statistical analysis. Geographic data and the localisation of lesions were analyzed with ArcGIS ArcMap (Economic and Social Research Institute, Redlands, USA; version 10.0).

Results

Survey for BU, leprosy and yaws in the Bankim HD

In the course of the survey, a total of 48 962 individuals in 9 344 households (Figure 1) were interviewed. The population of one village (approximately 550 people) refused to participate in the study. Assessing demographics and living conditions in the Bankim HD (Table 1), we found that the local population is very young with an average age of 19.3±17.0 (median = 14.0, interquartile range = 6.0 to 28.0), that 51.4% of the population are women and that overall, 61.2% of the population have attended school at some point in their lives. We further observed that Christianity is the most common (64.9%) religion and that, apart from the young members of society which are either students (32.2%) or children (23.5%), the most common professions in the district are farming (16.9%) and household work (17.4%). In terms of living conditions we found that there are on average 5.2 individuals living in each household and 26.8% of the households have a mosquito net. Further, our data showed that only 38.3% of the population have access to clean drinking water that comes at least from a fortified well and that the roofs and floors of the local houses are often very poorly constructed. Table 1 also shows that, the main local differences in the level of development in the HD exist between the six rural HA and the Bankim Urban HA (BA HA), which includes the town of Bankim (77.4% of the BA HA population) and nine small settlements around it. The higher level of development in the BA HA is reflected by the significantly higher percentage of people having gone to school at some point in their lives (p-value < 0.0001) or by the significantly better access to clean drinking water (p-value < 0.0001). Furthermore, in the BA HA the proportion of houses with better flooring (p-value < 0.0001) and walls (p-value = 0.0037) is also significantly higher compared to the other HA.

In the survey, we identified 32 cases of leprosy, 29 cases of yaws and 25 cases of BU based on clinical symptoms. With 32 cases of leprosy, the population-adjusted prevalence was at 6.5 cases per 10’000. The majority (70%) of the identified leprosy cases suffered from the multibacillary form of the disease and 22% of them were previously known but had abandoned their treatment and needed treatment re-initiation. Of the 29 yaws cases identified, 28% presented with the advanced symptom of hyperkeratosis and of the BU cases, 23% (6 cases) could be re-confirmed by RT-PCR.

Laboratory confirmation of BU

In the five months after the survey (April 2010 to August 2010), only two new RT-PCR reconfirmed BU cases were identified (Figure 2). Following this lag, between September 2010 and June 2012 (22 months) there was a steady flow of about 2.5 new RT-PCR confirmed BU cases per month from the Bankim HD. During this period, RT-PCR confirmed BU patients from the surrounding HDs (about 1.2 per month) also reported to BU treatment

facilities in the Bankim HD. Overall, our study identified 157 clinically confirmed cases of BU of which 88 (56%) could be confirmed by RT-PCR. Of the non-confirmed patients, 48 (31%) tested negative in RT-PCR and of 21 patients (13%) no samples were collected. Gender ratio, age distribution, average disease duration prior to consultation, and proportion of category 3 cases were comparable between the RT-PCR positive and negative patients. Only age differed significantly (p-value = 0.034) between the RT-PCR confirmed and the non-confirmed cases with the average age of the confirmed cases being 21.2 and that of the non-confirmed ones being 29.3. To ensure the reliability of our conclusions we focused the remaining analysis only on the 88 RT-PCR confirmed BU cases. Age distribution (p-value = 0.4754) and professions (p-value = 0.5161) did not differ significantly between the population of the Bankim HD and the confirmed BU patients. The gender distribution among patients was moderately different (p-value = 0.061) from that of the overall population with a larger proportion of males among the confirmed BU cases.

Spatial distribution of BU cases in the Mapé basin

To better describe BU epidemiology in the Mapé basin we set out to identify the exact geographic origin of all 88 laboratory confirmed cases in our cohort. Based on information from the patients or their close relatives we were able to determine the HD of origin for 86 (98%) of the cases (Figure S1). For the remaining 2 cases we could only determine that they did not live in the Bankim HD for the year before the onset of symptoms, but we could not conclusively determine which HD they were from. Studying the distribution of cases by HD, we found that the proportion of category 1 cases among the patients originating from inside the Bankim HD (24/62) was significantly higher (p-value = 0.039) compared to the cases from the surrounding HDs (5/26; Figure S1). For the 62 cases that originated from within the Bankim HD we were also able to determine their HA of origin. Using the population data as collected by the survey, we were then able to calculate the cumulative incidence rate of BU per HA in the Bankim HD during our study. As shown in Figure 3A, the cumulative incidence rate of BU in the Bankim HD is highest in the BR HA (5.08/1’000). The cumulative incidence rate in this HA is significantly higher compared to all other HA in the HD (p-value < 0.001). Interestingly the cumulative incidence rate is also significantly higher in the southern HA (BR, BA, BD, NY) compared to the northern HA (AT, SO, SG) of the Bankim HD (p-value < 0.001) (Figure 3A). Finally, for more detailed spatial analysis, the exact domiciles of 79 (89.8%) of the confirmed BU cases were mapped (Figure 3B). For 7 of the remaining cases (Bankim HD: 3 from the Bandam HA, 1 from the Somié HA; surrounding HD: 1 from the Malantouen HD, and 2 of unknown origin) we could not conclusively identify the exact house where they lived before the onset of BU. An additional two cases (1 each from the Nwa HD and Mayo Darle HD) are not considered in the analysis because they originated from outside of the Mapé basin. Based on the known exact origin of the cases that came from within the Bankim

HD (n=58) and who were therefore identified by the same case finding strategy, a Kernel function was used to compute the density of BU in the Bankim HD (Figure 3B). This BU density map shows that most of the cases occur in the southern part of the Bankim HD, particularly along the Mbam River and in the area between the Mapé Dam reservoir and that river. The exact origins of 80.8% (21/26) of the BU cases from outside of the Bankim HD, indicate that the local BU focus expands outside of the Bankim HD, in particular westwards into the Malantouen HD (Figure 3B).

Age and gender distribution of cases

The median age of the 88 RT-PCR confirmed cases was 12.5 (interquartile range= 8.0 to 30.0). The age of patients ranged from 0.5 to 73, 52 out of 88 (59.1%) were children (age <15) and 11 (12.5%) were older than 50. The gender ratio of all cases was 1.44 male / female. In children this ratio was 1.89, in the 15 to 50 year olds it was 0.79 and in the above 50 year olds, it was 1.75. The age dependent variation in the gender ratio was not statistically significant (p-value = 0.20).

With the ages of the 62 (70.5%) cases of BU which originated from within the Bankim HD and the population age distribution as collected in the survey (Figure 4A and Table 1), we computed the age adjusted cumulative incidence rate of BU in the Bankim HD for the period of the study. As shown in Figure 4B, we observed a low age adjusted cumulative incidence rate of BU in individuals aged below four years. The rate then peaked in children aged between four and < 14 years of age, with the 12 to <14 year olds particularly affected (34.4 cases per 10’000 inhabitants). Interestingly, the age adjusted cumulative incidence rate peaks again in the over 50 year olds (27.0 cases per 10’000 inhabitants; Figure 4B).

Localisation of BU lesions

In the laboratory confirmed BU patients studied here, 49/88 (55.7%) lesions occurred on the lower limbs, 27/88 (30.7%) on the upper limbs, 2/88 (2.3%) on the head and neck and 10/88 (11.4%) on the trunk. One of the trunk lesions occurred on the genitals. Two patients had multiple lesions and only the initial lesion was considered for analysis. The distribution of lesions differed significantly (p-value <0.001) from the relative body surface area (RBSA; Table S1). Interestingly, most of the lesions (52.3%) occurred in close proximity to joints with clusters around the ankles (19.2 %) and elbows (15.9 %; Figure 5A, 5B and Table S2). When analyzing the occurrence of lesions on the different body parts, we did not observe any statistically significant difference between lesions occurring on the right or left or front or back of the patients. However, when analyzing the occurrence of lesions on the joints, we did observe a statistically significant difference between the lesions on the front or back of the joints (p-value = 0.012), in particular there was a significant difference between the occurrence of lesion on the front or back of the elbow (p-value = 0.005). No such difference

was observed when analyzing the joint lesions on the right of left of the patients' bodies. Analyzing the distribution of the lesions by body part, we found a moderately significant difference between males and females (p-value = 0.076) with the percentage of lesions on the trunk being significantly higher (p-value = 0.033) in males (Table S1).

The distribution of lesions by body parts in children (Figure 5C and Table S1) was significantly different (p-value = 0.009) from the RBSA of children. Interestingly, only children (n=2) had lesions on the head and neck. Overall, the lesions appear more dispersed in children (Figure 5C and 5D). While, the difference in the general lesion distribution by body parts between adults and children (Table S1) was not statistically significant (p-value = 0.154), there was a significant difference (p-value = 0.011) between the distribution of lesions at joints in children and adults. In particular, most joint lesions in adults occurred at the ankle (36.1%), whereas most joint lesions in children occurred at the elbow (19.2%). Finally in adults, lesions occurred mainly on the front and back of the feet and the distribution also differed significantly (p-value = 0.004) from what is expected based on the RBSA of adults (Figure 5C).

Discussion

The 2011 Cameroon Demographic Health Survey (DHS) examined approximately 22'000 adults (>14 years old) and found that the population is very young with roughly 24% being 15-19 years old [25]. In the Bankim HD, we also observed a population that is strongly skewed towards young individuals and we found that living conditions and access to clean drinking water are very poor. Given the basic health infrastructure, these factors pose big challenges when addressing any health care related issues [26,27].

Although Cameroon has achieved nationwide leprosy elimination as defined by the WHO (< 1 case per 10'000 inhabitants) [28], our data showed that leprosy remains endemic in the Bankim HD. The substantial proportion of leprosy patients that had previously abandoned treatment further demonstrated that the oral treatment regimen requires better patient monitoring to achieve good compliance. In 2010 Cameroon reported 800 cases of yaws [29] and our survey confirmed that the Bankim HD is a yaws focus. Studies on the use of oral antibiotics have again raised hope for the eradication of yaws [30–32]. However, until eradication is possible, the focus of leprosy and yaws care should be early detection, complete cure and prevention of disabilities. To achieve this, front line medical staff needs to be trained on clinical diagnosis and efficient case management.

Characteristics of BU and the remote areas where it occurs have been suggested to necessitate active case searches for early case detection [33,34]. Indeed, house-by-house surveys have helped to elucidate BU epidemiology in Ghana and Ivory Coast [35,36]. In Cameroon a study around the Nyong River, identified 135 PCR confirmed cases of BU [6]. In the survey described here, the number of RT-PCR confirmed BU cases identified was smaller than expected. However, the lag of new cases during the first months after the survey indicated that the survey identified the cases present at that time. It cannot be excluded however, that a proportion of BU patients seeks to avoid contact with the formal health system. By continuous case detection, also accounting for the trust needed for cases to come forward [37], we identified 157 clinically diagnosed cases of BU (from April 2010 to June 2012). To increase validity of the findings [38,39], our analysis focused on the 88 (56.1%) RT-PCR confirmed cases among them. False negative RT-PCR results are unlikely since we analyzed multiple samples from each patient (data not shown). Although accurate BU clinical diagnosis is possible [40], misdiagnosis rates of up to 40% have been reported emphasizing the pressing need for a point-of-care rapid diagnostic test [5,38,39,41–43].

Based on the number of BU cases in each of the HA in the Bankim HD and their respective populations, the BR HA was determined to have the highest cumulative incidence rate of BU in the Bankim HD. Furthermore, by detailed mapping of cases and through the use of a

geographic information system (GIS), we identified hot-spots of BU transmission along the Mbam River. With only few cases living in the immediate proximity of only the Mapé Dam reservoir, our data does not support the suspected direct importance of this man-made lake. This does not exclude that environmental changes associated with the damming of the Mapé River may have had a more indirect effect on the spread of BU in the wider area. Whether the relatively large proportion of patients living in the town of Bankim (11/79 GPS mapped cases), contracted BU there, remains to be investigated. By also mapping cases from outside of the Bankim HD, we found that the local BU endemic area is larger then previously described [7]. Indeed it is possible that, because of the differences in case finding strategy inside and outside of the Bankim HD, our findings from outside the HD under represent the true degree of BU endemicity in the areas surrounding the Bankim HD. Further studies are therefore needed to investigate BU endemicity in the entire Mapé basin in more detail. Ongoing environmental and social science research at the identified hot-spots of disease is aiming to further elucidate the mode of transmission of BU.

BU affects individuals of all ages [15,34] but in the African endemic regions most patients are children [9,44]. However, when adjusting for the population age distribution, studies in Benin [45] and in Australia [46] showed that 75 to 79 year olds or the ≥74 olds, respectively, have the highest risk of contracting BU. Our data similarly showed that the age adjusted risk of BU is as high in the > 50 year olds as in children, a trend possibly associated with immunosenescence, the gradual deterioration of the immune system associated with natural age advancement [46,47]. It is interesting to note that cases among very young (< 5) children, which make up an even larger part of society than the 5 – 10 year olds, are relatively rare. This may indicate that compared to older children the very young children are less exposed to risk factors due to a smaller movement radius away from the house [10]. In the exposed individuals, host factors are likely to contribute to the degree of susceptibility [48]; seroepidemiological studies indicate that only a small proportion of exposed individuals develop clinical disease [49,50].

Detection of *M. ulcerans* DNA-positive mosquitoes in an Australian BU focus [46] as well as identification of the failure to wear protective clothing as a risk factor and of the use of mosquito repellent as a protective factor for BU [8], support the hypothesis that insects are involved in *M. ulcerans* transmission [10]. Most biting arthropods selectively feed at specifics sites based on visual, physical or chemical cues such as distance of the ground, breath and skin temperature of the bait [51–55]. The resulting feeding patterns are often focused either on the feet and ankles or the head of the human subject [52]. Interestingly for vector transmitted parasitic diseases with local manifestations such as cutaneous leishmaniasis and filariasis, it has been found that the lesion distribution correlates with the biting sites of the responsible vectors [56,57]. BU lesions occur mostly on the lower limbs [15,45,58–60] and in

adults, a focus on joints, specifically the elbows and ankles, has been reported [15,58]. Studies on the distribution of lesions also show that they are usually equally distributed between the left and right side of the body and compared to adults, children tend to have more lesions on the trunk [45,60]. Using GIS methodology we observed in this study that lesions cluster at specific locations on the limbs. We found that, particularly in adults, lesions occur mostly at locations where the skin is not commonly protected with clothing. As previously described, in females, which are more likely to cover their upper body with clothing, we found that there are less lesions on the trunk. In rural African villages children may often have their upper body exposed explaining the more dispersed distribution of their lesions.

Detection of *M. ulcerans* DNA in the environment [10] and identification of poor wound care and failure to wear protective clothing as risk factors for BU [8] have led investigators to speculate that transmission may alternatively occur by skin trauma and direct contact with *M. ulcerans* contaminated environment [10]. A study in Canadian children found that children 9 months and older have on average >3.5 recent skin injuries [61]. In 5 to 17 year olds injuries most often occur where the bones are close to the skin, i.e. at shins, knees, elbows and forearms. Injuries on the head were most common in children less than 5 years of age and lesions on genitals were rare in all ages [61]. While this study may have, due to differences in dress code and activities, limited relevance for Cameroonian children, it is remarkable that both BU lesions on the head in our cohort occurred in patients under the age of 5 [61]. While BU lesion distribution does not seem to correlate closely with the published distribution of insect bites, inoculation of skin injuries by a contaminated environmental source should lead, for example, to more lesions on the knees. Based on these data, the option of multiple modes of transmission should be considered.

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Author Contributions

Conceived and designed the experiments: MWB MB LG ENT DN AUB GP. Performed the experiments: MWB MB JCM LG FGW SK ENT MTR FM. Analyzed the data: MWB LG GP. Wrote the paper: MWB MB LG GP.

Tables

Table 1. Sociodemographic characteristics of the Bankim Health District population.

	Health Areas							
Characteristic	**AT**	**SG**	**SO**	**NY**	**BD**	**BA**	**BR**	**Total**
Individuals								
Inhabitants surveyed (n)	7 621	11 346	7 308	5 602	5 421	8 514	3 150	48 962
Age in years (mean ± SD)	19.9±17.0	19.8±17.2	20.2±18.2	18.2±16.3	17.4±16.5	19.3±16.1	19.1±16.5	19.3±17.0
Gender (% female)	49.8	50.9	52.9	53.1	51.7	51.1	51.1	51.4
Education* (%)	62.2	64.9	55.9	53.8	49.8	72.0	61.5	61.2
Religion (%)								
Christian	72.9	65.2	61.2	59.6	52.2	70.6	69.6	64.9
Muslim	26.7	34.6	38.8	40.4	47.8	28.7	30.2	34.8
Profession (%)								
Farming	25.6	13.3	17.0	12.5	13.7	12.7	32.9	16.9
Fishing	0.1	2.4	0.1	2.2	1.1	0.7	2.2	1.2
Household work	13.4	21.3	21.9	18.9	21.6	13.3	4.1	17.4
Student	32.9	33.6	26.8	30.6	24.0	40.5	33.2	32.2
Child	21.3	22.4	27.5	26.1	32.6	16.2	23.0	23.5
Households								
Number of households (n)	1788	2100	1332	1053	942	1532	597	9344
Individuals per household (mean ± SD)	4.3±2.9	5.4±2.9	5.5±3.3	5.3±3.0	5.8±3.7	5.6±3.4	5.3±2.9	5.3±3.2
Mosquito net** (%)	16.1	22.3	28.6	36.9	31.6	21.8	57.6	26.8
Clean drinking water*** (%)	27.0	34.5	47.9	17.2	25.0	76.7	23.2	38.3
Concrete or mood walls (%)	99.1	97.3	98.6	99.2	91.3	98.8	98.5	97.8
Tile or metal sheet roof (%)	40.6	40.8	32.0	39.4	31.4	78.1	42.8	44.7
Cemented or tailed floor (%)	8.0	10.9	7.8	9.7	6.8	41.6	11.6	14.3

*Attended school anytime during their life.
**Lived in household with a mosquito net.
***Had access to drinking water from a tap or a concrete fortified well.
SD: standard deviation.
AT = Atta.
SG = Songkolong.
SO = Somié.
NY = Nyamboya.
BD = Bandam.
BA = Bankim Urban.
BR = Bankim Rural.
doi:10.1371/journal.pntd.0002252.t001

Figures

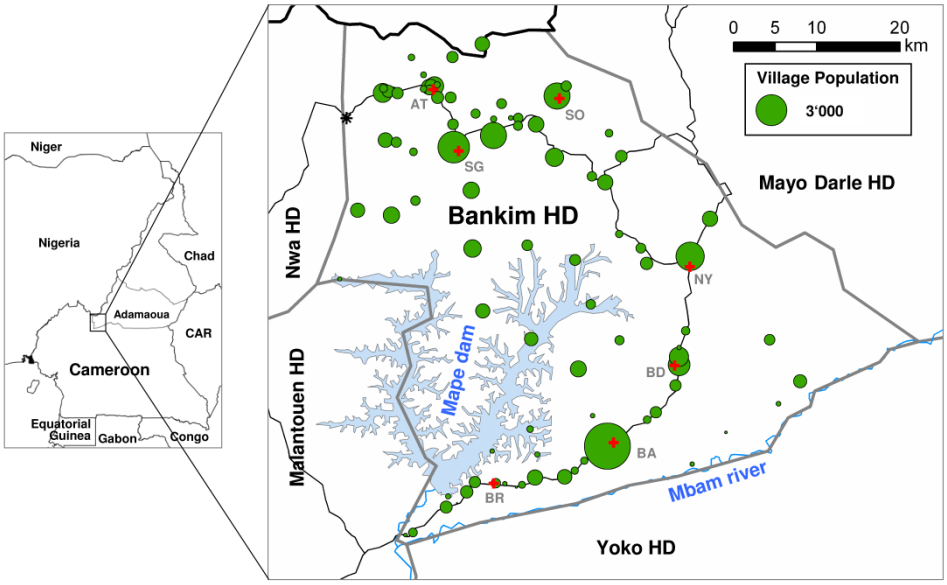

Figure 1: Environmental Features and Population of the Bankim HD.

The Bankim HD is located in the South-Western corner of the Adamaoua Region of Cameroon and encompasses most of the Mapé basin. The main environmental features of the area are the Mapé Dam reservoir and the Mbam River. The Bankim HD consists of 7 HA (red crosses depict the location of the main HC in each of the HA; BR: Bankim Rural; BA: Bankim Urban; BD: Bandam; NY: Nyamboya; SO: Somié; SG: Songkolong; AT: Atta) and is surrounded by four other HD. In early 2010 we conducted an exhaustive house-by-house survey and examined a total of 48'962 individuals in all HA of the district; population sizes of villages based on this survey are indicated by green circles. The village of Koumtchoum (black star; estimated population of 550) as a whole refused to participate in the survey and the village of Djaouro Tchi Arouna (470 inhabitants, located in the Somié HA between the HC of Somié and the town at the Nigerian border to the North-West of it) could not be mapped.

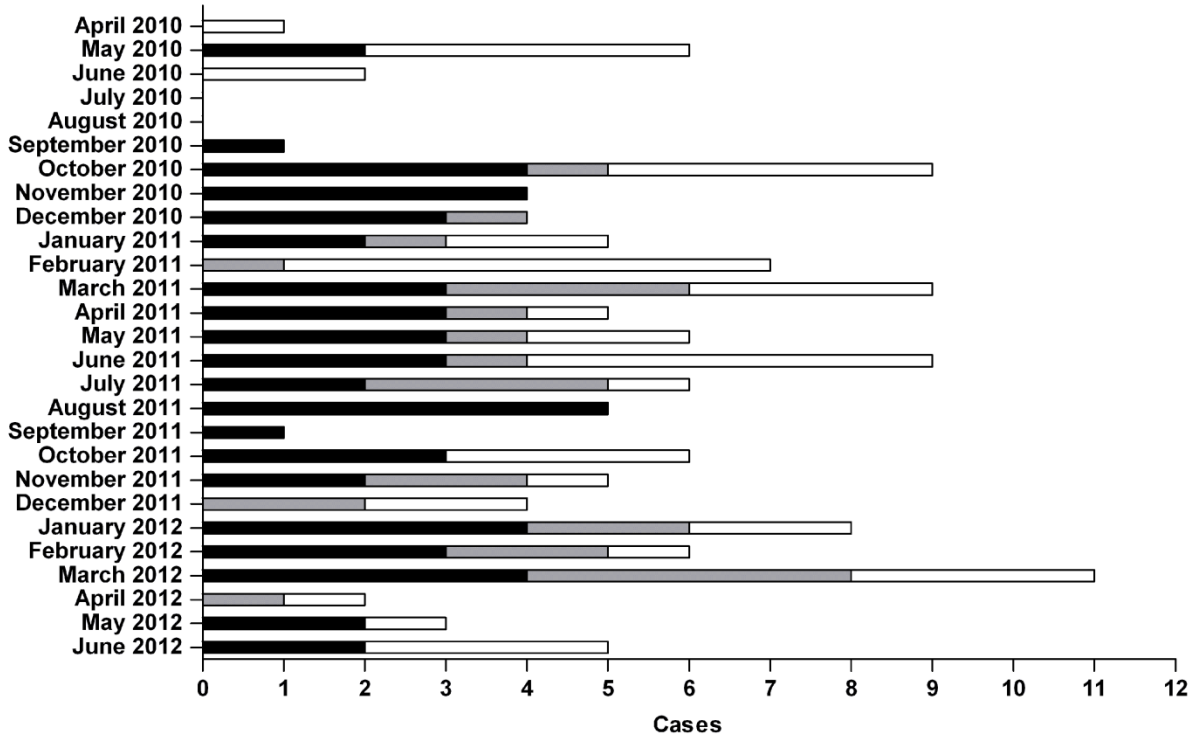

Figure 2: Identification of BU Cases in the Mapé Basin following the Survey.

In the 27 months following the exhaustive survey all clinically diagnosed BU cases in the Bankim area were included in a cohort study. Cases are separated into the RT-PCR confirmed cases which occurred inside (black) and outside (grey) of the Bankim HD. RT-PCR negative and non-laboratory examined cases occurring anywhere in the Mapé basin during the same time period are also shown (white).

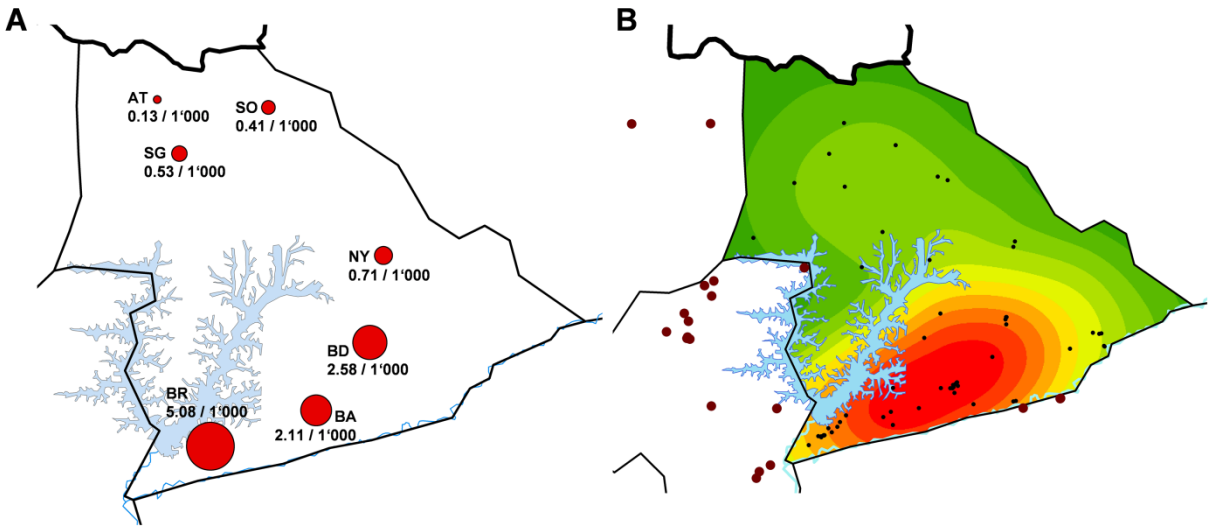

Figure 3: Geographic Distribution of BU in the Mapé Basin.

Based on the HA of origin of the 62 BU cases who originated within the Bankim HD and the population data collected in the survey, we computed the per HA cumulative incidence rate of BU in the Bankim HD (A). For detailed analysis, the places of residence of 58 (black points) of the 62 cases from the Bankim HD were mapped using a GPS device (B). For the remaining 4 RT-PCR confirmed cases which occurred inside the Bankim HD (3 from the Bandam HA, 1 from the Somié HA) we could not identify their home. Using a Kernel function the density of BU in the Bankim HD was computed based on the mapped cases (red: highest BU density). Panel B further shows the places of residence of 21 of the 26 RT-PCR confirmed cases of BU who originated from outside of the Bankim HD (brown points). For three of the remaining cases (1 from the Malantouen HD, and 2 of unknown origin) we could not identify their exact origin and two additional cases (1 each from the Nwa HD and Mayo Darle HD) are not shown because they originated from outside of the region shown on the map.

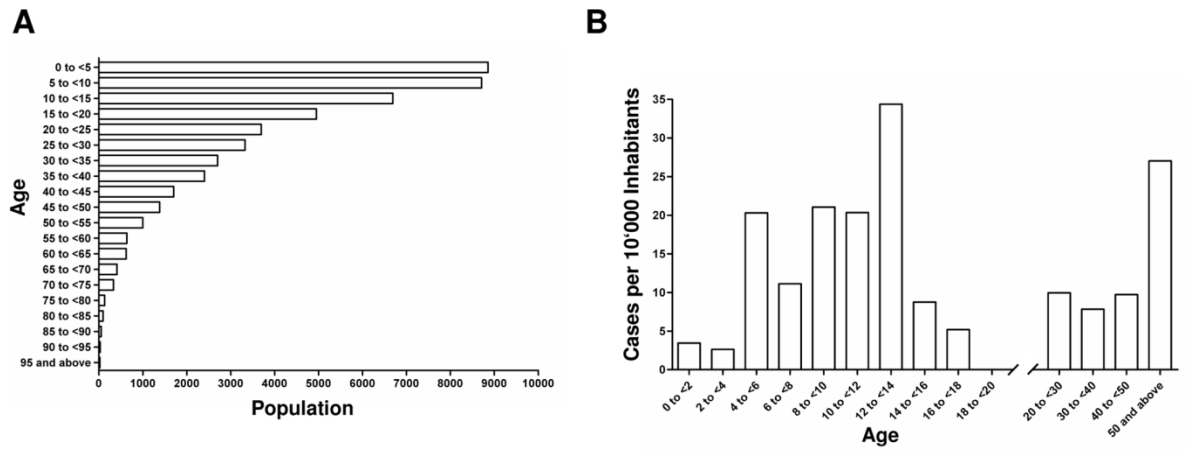

Figure 4: Population Age Distribution and Age Adjusted Cumulative BU Incidence Rate in the Bankim HD.

In the course of the exhaustive survey, data on the population age structure of the Bankim HD were collected (A). Using this age distribution and the ages of the RT-PCR confirmed BU cases which occurred inside of the Bankim HD (n=62), the age adjusted cumulative BU incidence rate (cases per 1'000 inhabitants) for the duration of the study could be computed (B).

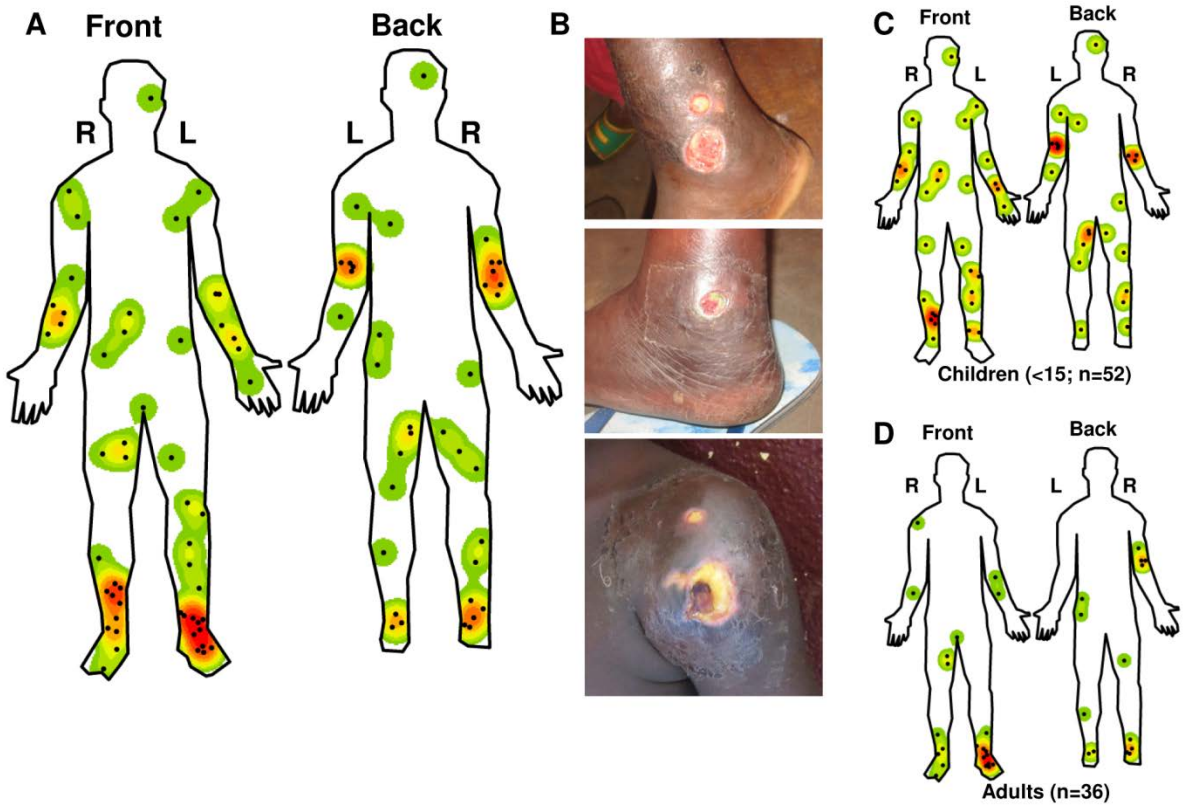

Figure 5: Lesion Localization.

The localization of the lesions of all the RT-PCR confirmed BU patients (88) were mapped in detail and Kernel function was used to create a heat map of the lesion distribution (A). The localization of the lesions on the front and back and left (L) and right (R) of the patient's bodies are shown. Studying the distribution of lesions, it was noted that they often occur at joints (B, example of two lesions on the ankle and one on the shoulder). Distribution of lesions in children (C, n=52) and adults (D, n=36) were also analyzed separately.

Supplementary Table 1: Lesion Distribution by Body Parts.

	All			Children (< 15 years old)			Adults		
Lesion location*	**All (n=88)**	**Male (n=52)**	**Female (n=36)**	**All (n=52)**	**Male (n=34)**	**Female (n=18)**	**All (n=36)**	**Male (n=18)**	**Female (n=18)**
Head or neck	2 (2.3)	2 (3.8)	0 (0)	2 (3.8)	2 (5.9)	0 (0)	0 (0)	0 (0)	0 (0)
Upper limbs	27 (30.7)	16 (30.8)	11 (30.6)	19 (36.5)	14 (41.2)	5 (27.8)	8 (22.2)	2 (11.1)	6 (33.3)
Trunk	10 (11.4)	9 (17.3)	1 (2.8)	7 (13.5)	7 (20.6)	0 (0)	3 (8.3)	2 (11.1)	1 (5.6)
Lower limbs	49 (55.7)	25 (48.1)	24 (66.7)	24 (46.2)	11 (32.4)	13 (72.2)	25 (69.4)	14 (77.8)	11 (61.1)

* number of patients with lesion at the given location and percentage in parenthesis

Supplementary Table 2: Lesion on Joints.

	All			Children (< 15 years old)			Adults		
Lesion Location *	**All (n=88)**	**Male (n=52)**	**Female (n=36)**	**All (n=52)**	**Male (n=34)**	**Female (n=18)**	**All (n=36)**	**Male (n=18)**	**Female (n=18)**
Ankle	17 (19.3)	10 (19.2)	7 (19.4)	4 (7.7)	1 (2.9)	3 (16.7)	13 (36.1)	9 (50.0)	4 (22.2)
Elbow	14 (15.9)	9 (17.3)	5 (13.9)	10 (19.2)	7 (20.6)	3 (16.7)	4 (11.1)	2 (11.1)	2 (11.1)
Hip	3 (3.4)	3 (5.8)	0 (0)	2 (3.8)	2 (5.9)	0 (0)	1 (2.8)	1 (5.6)	0 (0)
Knee	3 (3.4)	3 (5.8)	0 (0)	3 (5.8)	3 (8.8)	0 (0)	0 (0)	0 (0)	0 (0)
Shoulder	2 (2.3)	1 (1.9)	1 (2.8)	1 (1.9)	1 (2.9)	0 (0)	1 (2.8)	0 (0)	1 (5.6)
Toe	1 (1.1)	0 (0)	1 (2.8)	0 (0)	0 (0)	0 (0)	1 (2.8)	0 (0)	1 (5.6)
Wrist	2 (2.3)	2 (3.8)	0 (0)	2 (3.8)	2 (5.9)	0 (0)	0 (0)	0 (0)	0 (0)
Not Joint	46 (52.3)	24 (46.2)	22 (61.1)	30 (57.7)	18 (52.9)	12 (66.7)	16 (44.4)	6 (33.3)	10 (55.6)

* number of patients with lesion at the given location and percentage in parenthesis

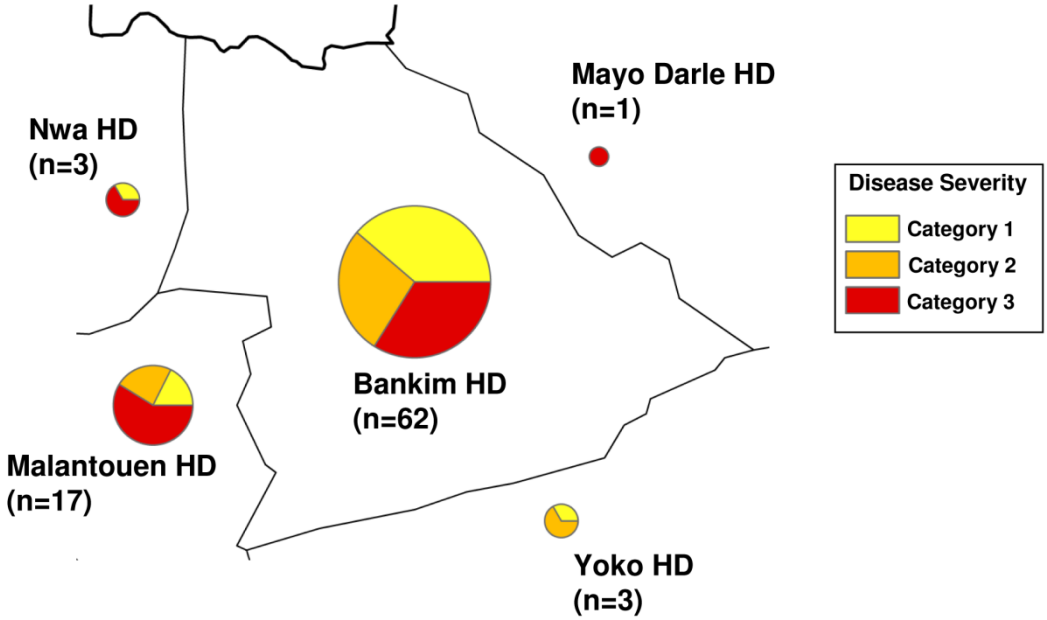

Figure S1: Disease Category by Health District.

Most of the RT-PCR confirmed cases that were identified in the Bankim area originated from within the Bankim HD (n=62). However, patients from all of the surrounding HD also came to Bankim for BU treatment (Malantouen: 17; Nwa: 3; Yoko: 3; Mayo Drale: 1). The number of cases that occurred in each of the HD are classified by disease severity (red: category 3, orange: category 2, yellow: category 1). Two RT-PCR confirmed cases (both category 3, both from outside of the Bankim HD) could not be displayed because the location where the patient first showed symptoms of BU could not be conclusively determined.

References

1. Maccallum P, Tolhurst JC, Buckle G, Sissons HA (1948) A new mycobacterial infection in man. The Journal of Pathology and Bacteriology 60: 93–122. doi:10.1002/path.1700600111.
2. Rondini S, Käser M, Stinear T, Tessier M, Mangold C, et al. (2007) Ongoing genome reduction in *Mycobacterium ulcerans*. Emerging Infect Dis 13: 1008–1015.
3. Käser M, Rondini S, Naegeli M, Stinear T, Portaels F, et al. (2007) Evolution of two distinct phylogenetic lineages of the emerging human pathogen *Mycobacterium ulcerans*. BMC Evol Biol 7: 177. doi:10.1186/1471-2148-7-177.
4. Doig KD, Holt KE, Fyfe JA, Lavender CJ, Eddyani M, et al. (2012) On the origin of *Mycobacterium ulcerans*, the causative agent of buruli ulcer. BMC genomics 13: 258. doi:10.1186/1471-2164-13-258.
5. Portaels F, Silva MT, Meyers WM (2009) Buruli ulcer. Clin Dermatol 27: 291–305. doi:10.1016/j.clindermatol.2008.09.021.
6. Noeske J, Kuaban C, Rondini S, Sorlin P, Ciaffi L, et al. (2004) Buruli ulcer disease in Cameroon rediscovered. Am J Trop Med Hyg 70: 520–526.
7. Marion E, Landier J, Boisier P, Marsollier L, Fontanet A, et al. (2011) Geographic expansion of Buruli ulcer disease, Cameroon. Emerging Infect Dis 17: 551–553. doi:10.3201/eid1703.091859.
8. Jacobsen KH, Padgett JJ (2010) Risk factors for *Mycobacterium ulcerans* infection. International Journal of Infectious Diseases 14: e677–e681. doi:10.1016/j.ijid.2009.11.013.
9. Walsh DS, Portaels F, Meyers WM (2011) Buruli Ulcer: Advances in Understanding *Mycobacterium ulcerans* Infection. Dermatologic Clinics 29: 1–8. doi:10.1016/j.det.2010.09.006.
10. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, et al. (2010) Ecology and transmission of Buruli ulcer disease: a systematic review. PLoS Negl Trop Dis 4: e911. doi:10.1371/journal.pntd.0000911.
11. World Health Organization (2000) Buruli ulcer *Mycobacterium ulcerans* infection. Available: http://whqlibdoc.who.int/hq/2000/who_cds_cpe_gbui_2000.1.pdf. Accessed 03 May 2012
12. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, et al. (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283: 854–857.
13. World Health Organization (2012) Buruli ulcer (*Mycobacterium ulcerans* infection) Fact sheet N°199. Available: http://www.who.int/mediacentre/factsheets/fs199/en/. Accessed 18 December 2012
14. World Health Organization (2004) Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer). Available: http://www.who.int/buruli/information/antibiotics/en/index.html. Accessed 03 May 2013
15. Adu E, Ampadu E, Acheampong D (2011) Surgical Management of Buruli Ulcer Disease: A Four-Year Experience from Four Endemic Districts in Ghana. Ghana Med J 45: 4–9.
16. Ruf M-T, Sopoh GE, Brun LV, Dossou AD, Barogui YT, et al. (2011) Histopathological changes and clinical responses of Buruli ulcer plaque lesions during chemotherapy: a role for surgical removal of necrotic tissue? PLoS Negl Trop Dis 5: e1334. doi:10.1371/journal.pntd.0001334.

17. Walker SL, Hay RJ (2000) Yaws-a review of the last 50 years. Int J Dermatol 39: 258–260.

18. Antal GM, Lukehart SA, Meheus AZ (2002) The endemic treponematoses. Microbes Infect 4: 83–94.

19. Suzuki K, Akama T, Kawashima A, Yoshihara A, Yotsu RR, et al. (2012) Current status of leprosy: epidemiology, basic science and clinical perspectives. J Dermatol 39: 121–129. doi:10.1111/j.1346-8138.2011.01370.x.

20. Jacobson RR, Krahenbuhl JL (1999) Leprosy. Lancet 353: 655–660. doi:10.1016/S0140-6736(98)06322-3.

21. Eddyani M, Fraga AG, Schmitt F, Uwizeye C, Fissette K, et al. (2009) Fine-needle aspiration, an efficient sampling technique for bacteriological diagnosis of nonulcerative Buruli ulcer. J Clin Microbiol 47: 1700–1704. doi:10.1128/JCM.00197-09.

22. Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, et al. (2007) Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. Appl Environ Microbiol 73: 4733–4740. doi:10.1128/AEM.02971-06.

23. Lavender CJ, Fyfe JAM (2013) Direct Detection of *Mycobacterium ulcerans* in Clinical Specimens and Environmental Samples. Methods Mol Biol 943: 201–216. doi:10.1007/978-1-60327-353-4_13.

24. US EPA National Center for Environmental Assessment WD, Moya J (n.d.) Exposure Factors Handbook 2011 Edition (Final). Available:http://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=236252#Download. Accessed 1 November 2012.

25. National Institute of Statistics Republic of Cameroon (2012) Demographic and Health survey and Multiple Indicators Cluster Survey DHS-MICS 2011: Preliminary Report. Available: http://www.statistics-cameroon.org/downloads/EDS-MICS11/DHSMICS_2011_preliminary_report.pdf. Accessed 10 December 2012

26. Bartram J, Cairncross S (2010) Hygiene, sanitation, and water: forgotten foundations of health. PLoS Med 7: e1000367. doi:10.1371/journal.pmed.1000367.

27. Cairncross S, Blumenthal U, Kolsky P, Moraes L, Tayeh A (1996) The public and domestic domains in the transmission of disease. Trop Med Int Health 1: 27–34.

28. The International Federation of Anti-Leprosy Associations (2012) Cameroon The History of Leprosy in this Country. Available: http://www.ilep.org.uk/ilep-co-ordination/leprosy-around-the-world/africa/cameroon/. Accessed 11 December 2012

29. World Health Organization (2012) Weekly Epidemiological Record 18 May 2012, vol. 87, 20 (pp 189–200). Available: http://www.who.int/wer/2012/wer8720/en/index.html. Accessed 11 December 2012.

30. World Health Organization (2010) First WHO Report on Neglected Tropical Diseases. Available: http://www.who.int/neglected_diseases/2010report/en/index.html. Accessed 5 December 2012

31. Maurice J (2012) WHO plans new yaws eradication campaign. The Lancet 379: 1377–1378. doi:10.1016/S0140-6736(12)60581-9.

32. Mitjà O, Hays R, Ipai A, Penias M, Paru R, et al. (2012) Single-dose azithromycin versus benzathine benzylpenicillin for treatment of yaws in children in Papua New Guinea: an open-label, non-inferiority, randomised trial. The Lancet 379: 342–347. doi:10.1016/S0140-6736(11)61624-3.

33. Kanga JM, Kacou ED (2001) [Epidemiologicl aspects of Buruli ulcer in Côte d'Ivoire: results of a national survey]. Bull Soc Pathol Exot 94: 46–51.

34. Amofah G, Bonsu F, Tetteh C, Okrah J, Asamoa K, et al. (2002) Buruli ulcer in Ghana: results of a national case search. Emerging Infect Dis 8: 167–170. doi:10.3201/eid0802.010119.

35. Marston BJ, Diallo MO, Horsburgh CR, Diomande I, Saki MZ, et al. (1995) Emergence of Buruli ulcer disease in the Daloa region of Cote d'Ivoire. Am J Trop Med Hyg 52: 219–224.

36. Amofah GK, Sagoe-Moses C, Adjei-Acquah C, Frimpong EH (1993) Epidemiology of Buruli ulcer in Amansie West district, Ghana. Trans R Soc Trop Med Hyg 87: 644–645.

37. Porten K, Sailor K, Comte E, Njikap A, Sobry A, et al. (2009) Prevalence of Buruli ulcer in Akonolinga health district, Cameroon: results of a cross sectional survey. PLoS Negl Trop Dis 3: e466. doi:10.1371/journal.pntd.0000466.

38. Phanzu DM, Bafende EA, Dunda BK, Imposo DB, Kibadi AK, et al. (2006) *Mycobacterium ulcerans* disease (Buruli ulcer) in a rural hospital in Bas-Congo, Democratic Republic of Congo, 2002-2004. Am J Trop Med Hyg 75: 311–314.

39. Saka B, Landoh DE, Kobara B, Djadou KE, Yaya I, et al. (2012) [Profile of Buruli ulcer treated at the National Reference Centre of Togo: a study of 119 cases.]. Bull Soc Pathol Exot. doi:10.1007/s13149-012-0241-1.

40. Mensah-Quainoo E, Yeboah-Manu D, Asebi C, Patafuor F, Ofori-Adjei D, et al. (2008) Diagnosis of *Mycobacterium ulcerans* infection (Buruli ulcer) at a treatment centre in Ghana: a retrospective analysis of laboratory results of clinically diagnosed cases. Trop Med Int Health 13: 191–198. doi:10.1111/j.1365-3156.2007.01990.x.

41. Bratschi MW, Njih Tabah E, Bolz M, Stucki D, Borrell S, et al. (2012) A case of cutaneous tuberculosis in a Buruli ulcer-endemic area. PLoS Negl Trop Dis 6: e1751. doi:10.1371/journal.pntd.0001751.

42. Bretzel G, Huber KL, Kobara B, Beissner M, Piten E, et al. (2011) Laboratory confirmation of Buruli ulcer disease in Togo, 2007-2010. PLoS Negl Trop Dis 5: e1228. doi:10.1371/journal.pntd.0001228.

43. Yeboah-Manu D, Asante-Poku A, Asan-Ampah K, Ampadu EDE, Pluschke G (2011) Combining PCR with microscopy to reduce costs of laboratory diagnosis of buruli ulcer. Am J Trop Med Hyg 85: 900–904. doi:10.4269/ajtmh.2011.11-0362.

44. Raghunathan PL, Whitney EAS, Asamoa K, Stienstra Y, Taylor TH, et al. (2005) Risk factors for Buruli ulcer disease (*Mycobacterium ulcerans* Infection): results from a case-control study in Ghana. Clin Infect Dis 40: 1445–1453. doi:10.1086/429623.

45. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, et al. (2004) *Mycobacterium ulcerans* disease: role of age and gender in incidence and morbidity. Trop Med Int Health 9: 1297–1304. doi:10.1111/j.1365-3156.2004.01339.x.

46. Johnson PDR, Azuolas J, Lavender CJ, Wishart E, Stinear TP, et al. (2007) *Mycobacterium ulcerans* in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. Emerging Infect Dis 13: 1653–1660.

47. Agarwal S, Busse PJ (2010) Innate and adaptive immunosenescence. Ann Allergy Asthma Immunol 104: 183–190; quiz 190–192, 210. doi:10.1016/j.anai.2009.11.009.

48. Stienstra Y, Van Der Graaf WTA, Te Meerman GJ, The TH, De Leij LF, et al. (2001) Susceptibility to development of *Mycobacterium ulcerans* disease: review of possible risk factors. Tropical Medicine & International Health 6: 554–562. doi:10.1046/j.1365-3156.2001.00746.x.

49. Diaz D, Döbeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, et al. (2006) Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. Clin Vaccine Immunol 13: 1314–1321. doi:10.1128/CVI.00254-06.

50. Yeboah-Manu D, Röltgen K, Opare W, Asan-Ampah K, Quenin-Fosu K, et al. (2012) Sero-epidemiology as a tool to screen populations for exposure to *Mycobacterium ulcerans*. PLoS Negl Trop Dis 6: e1460. doi:10.1371/journal.pntd.0001460.

51. Knols BG, Takken W, De Jong R (1994) Influence of human breath on selection of biting sites by Anopheles albimanus. J Am Mosq Control Assoc 10: 423–426.

52. De Jong R, Knols BG (1996) Selection of biting sites by mosquitoes. Ciba Found Symp 200: 89–100; discussion 101–108.

53. Dekker T, Takken W, Knols BGJ, Bouman E, Van de Laak S, et al. (1998) Selection of biting sites on a human host by Anopheles gambiae s.s., An. arabiensis and An. quadriannulatus. Entomologia Experimentalis et Applicata 87: 295–300. doi:10.1046/j.1570-7458.1998.00334.x.

54. De Jong R, Knols BG (1995) Selection of biting sites on man by two malaria mosquito species. Experientia 51: 80–84.

55. DUKE BO, BEESLEY WN (1958) The vertical distribution of Simulium damnosum bites on the human body. Ann Trop Med Parasitol 52: 274–281.

56. Hashiguchi Y, De Coronel VV, Gomez Landires EA (n.d.) An epidemiological study of Leishmaniasis in a plantation «cooperativa 23 de Febrero» newly established in Ecuador. Kiseichugaku Zasshi 33: 393–401.

57. KERSHAW WE, DUKE BO, BUDDEN FH (1954) Distribution of microfilariae of O. volvulus in the skin; its relation to the skin changes and to eye lesions and blindness. Br Med J 2: 724–729.

58. Boyd SC, Athan E, Friedman ND, Hughes A, Walton A, et al. (2012) Epidemiology, clinical features and diagnosis of *Mycobacterium ulcerans* in an Australian population. Med J Aust 196: 341–344.

59. Walsh DS, Portaels F, Meyers WM (2010) Recent advances in leprosy and Buruli ulcer (*Mycobacterium ulcerans* infection). Curr Opin Infect Dis 23: 445–455. doi:10.1097/QCO.0b013e32833c2209.

60. Hospers IC, Wiersma IC, Dijkstra PU, Stienstra Y, Etuaful S, et al. (2005) Distribution of Buruli ulcer lesions over body surface area in a large case series in Ghana: uncovering clues for mode of transmission. Trans R Soc Trop Med Hyg 99: 196–201. doi:10.1016/j.trstmh.2004.05.004.

61. Labbé J, Caouette G (2001) Recent skin injuries in normal children. Pediatrics 108: 271–276.

Results Chapter 2

Primary cultivation: factors affecting contamination and *Mycobacterium ulcerans* growth after long turnover time of clinical specimens

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Abstract

Background: While cultivation of pathogens represents a foundational diagnostic approach in the study of infectious diseases, its value for the confirmation of clinical diagnosis of Buruli ulcer is limited by the fact that colonies of *Mycobacterium ulcerans* appear only after about eight weeks of incubation at 30ºC. However, for molecular epidemiological and drug sensitivity studies, primary isolation of *M. ulcerans* remains an essential tool. Since for most of the remote Buruli ulcer endemic regions of Africa cultivation laboratories are not easily accessible, samples from ulcers often have to be stored for extended periods of time prior to processing. The objective of the current study therefore was to determine which transport medium, decontamination method or other factors decrease the contamination rate and increase the chance of primary isolation of *M. ulcerans* bacilli after long turnover time.

Methods: Swab and FNA samples for the primary cultivation were collected from clinically confirmed Buruli ulcer patients in the Mapé Basin of Cameroon. The samples were either stored in the semi-solid transport media 7H9 or Amies or dry for extended period of time prior to processing. In the laboratory, four decontamination methods and two inoculation media were evaluated and statistical methods applied to identify factors that decrease culture contamination and factors that increase the probability of *M. ulcerans* recovery.

Results: The analysis showed: i) that the use of moist transport media significantly increased the recovery rate of *M. ulcerans* compared to samples kept dry; ii) that the choice of the decontamination method had no significant effect on the chance of *M. ulcerans* isolation; and iii) that Löwenstein-Jensen supplemented with antibiotics as inoculation medium yielded the best results. We further found that, ten extra days between sampling and inoculation lead to a relative decrease in the isolation rate of *M. ulcerans* by nearly 20 %. Finally, collection and processing of multiple samples per patient was found to significantly increase the *M. ulcerans* isolation rate.

Conclusions: Based on our analysis we suggest a procedure suitable for the primary isolation of *M. ulcerans* strains from patients following long delay between sample collection and processing to a *M. ulcerans* strain collection for research purposes.

Background

Buruli ulcer (BU), a neglected tropical disease of the skin caused by *Mycobacterium ulcerans*, has been reported from over 30 countries worldwide with most cases occurring in West Africa. Clinically BU presents with both non-ulcerative lesions, such as nodules, plaques and edema, as well as ulcers. The major burden of the disease falls on children between 5 and 15 years of age [1, 2]. Despite intensive research efforts, both the reservoir and the mode of transmission of *M. ulcerans* have remained unclear [1]. Currently available methods for laboratory diagnosis of BU are microscopy, histopathology, PCR for the *M. ulcerans* specific insertion sequence 2404 (IS2404) or primary culturing. Based on the high sensitivity and specificity, the IS2404 PCR test is considered the gold standard in BU diagnosis [2]. Historically, BU was treated by wide excision of lesions and tissue samples could easily be obtained for laboratory diagnosis. Since the introduction of rifampicin and streptomycin combination therapy in 2004 [3], samples for laboratory testing are fine needle aspirates (FNA) taken from closed lesions and swab specimens taken from the undermined edges of ulcers [4].

Although primary culturing of *M. ulcerans* can provide a definitive BU diagnosis, the value of this method for primary diagnosis is strongly hampered by the fact that colonies take two to three months to appear and even under optimal conditions the sensitivity of the method is limited [5, 6]. For clinical diagnosis, culturing therefore only represents an auxiliary to other methods for the laboratory confirmation of BU. However, for studies on treatment efficacy, molecular epidemiology, and drug sensitivity, primary isolation of *M. ulcerans* remains crucial [7, 8].

To prevent overgrowth with other faster growing microorganisms, primary culturing requires decontamination of clinical samples prior to culture inoculation [6]. The commonly used decontamination methods use NaOH and HCl (Petroff and reverse Petroff method) or oxalic acid (OA). Although necessary to prevent overgrowth, all of these methods have been shown to have a detrimental effect on the viability of *M. ulcerans* [6, 9, 10]. As for the culturing media on which *M. ulcerans* is isolated, PANTA, a mixture of the antibiotics polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin, can be used to prevent the growth of a range of microorganisms but does not have an inhibitory effect on the growth of *M. ulcerans* [10].

Since for cultivation of *M. ulcerans* a sophisticated laboratory infrastructure is required, clinical specimens from BU patients often cannot be processed in a timely manner. It has been shown that from tissue specimens transported in semisolid transport medium, positive cultures can be obtained even if samples were kept at ambient temperature for more than

two months [11]. For FNA samples transported in liquid transport medium containing PANTA and processed within 2 weeks, Eddyani *et al*. further found a sensitivity of *in vitro* culture of 17.6 %, which was not significantly lower than the culture positivity rate of 25.0 % obtained by culturing tissue specimens from the same patients [12]. Yeboah-Manu *et al*. observed culture sensitivities of 41.7 % for FNA samples from non-ulcerative lesions and of 43.8 % for swab samples if they were transported on ice in transport media containing PANTA and processed within 24 hours [13]. The same study also yielded similar results when comparing the Petroff and the OA decontamination methods [13]. Further in a subset of samples in the same study, 33.3 % of swabs transported dry and processed between seven days and one month after collection were culture positive [13].

For the current study we set out to determine the best procedure for the cultivation of *M. ulcerans* from swab and FNA samples stored over extended periods of time prior to processing. Specifically, the objectives of the current study were to determine how transport medium, decontamination method, inoculated media, transport time or other factors decrease the contamination rate and increase the chance of primary isolation of *M. ulcerans*. The procedure devised here is suitable for the primary isolation of *M. ulcerans* strains from patients following long delay between sample collection and processing to establish a strain collection for research purposes.

Materials and Methods

Patient recruitment and ethical statement

For the current study samples were collected from BU patients attending health facilities in the Mapé Basin of Cameroon [14] between August 2010 and July 2012. Locally patients were diagnosed and treatment initiated based on clinical symptoms and if available Ziehl-Neelsen staining. Samples for laboratory confirmation were collected and processed as described below. Ethical clearance for the study was obtained from the Cameroon National Ethics Committee (N°041/CNE/DNM/09 and N°172/CNE/SE/2011) and the Ethics Committee of Basel (EKBB, reference no. 53/11). Participation was voluntary and all patients who participated in the study or their legal guardian provided written informed consent.

Sample collection, storage and transport

Prior to the start of medical treatment FNA were collected from patients with non-ulcerative lesions and swabs from patients with ulcers. The number of swabs depended on the number of lesions, lesion size and clinical judgment. FNA were collected with sterile needles and swabs using individually packed sterile cotton swabs. To minimize the handling of needles and avoid any dilution of the samples in transport medium, FNA were transferred onto a cotton swab immediately after collection. These swabs were then processed the same as the swabs collected from ulcers and, in the remainder of the manuscript we refer to all samples as swabs. Swabs were stored dry, in 7H9 medium containing PANTA (7H9) or in the antibiotics free Amies medium (VWR International); the latter two being semi-solid transport media. Sterile 7H9 (Difco Middlebrook, Becton Dickinson and Company) transport medium was prepared to contain 0.5 % Agar-Agar (Merck), 0.2 % glycerol (Sigma), 2 % PANTA (Becton Dickinson and Company) and 10 % OADC enrichment (Becton Dickinson and Company) [11]. Briefly after autoclaving the dissolved 7H9 powder and the agar, the glycerol, PANTA and OADC were added and 5ml portions of the still warm medium was filled into empty cotton swab tubes (Copan). The transport medium was stored at 4ºC until use. Following sample collection, swabs were inserted into the tube containing the transport medium and locally stored at 4ºC whenever possible. Due to the remoteness of the BU endemic areas in which clinical samples for this study were collected, timely transport to adequately equipped laboratories with sufficient capacity was difficult. Therefore at 4-6 month intervals, samples were transported to the laboratory at the Swiss Tropical and Public Health Institute at ambient temperature. Once in the laboratory, swabs were stored at 4ºC until processing.

PCR, decontamination and primary inoculation

For DNA extraction and culturing, swabs were transferred to 14 mL McCartney glass bottles (Marienfeld, Germany) that were filled approximately to one third with 3 mm diameter glass beads (Marienfeld, Germany) and 2-5 mL of sterile phosphate buffered saline (PBS). The bottles were vortexed for 1.5 minutes and DNA was extracted from 1 mL of the solution as described by Lavender and Fyfe [15]. Extracts were analyzed in duplicate by IS2404 real-time PCR (qPCR) as previously described [15].

Decontamination of 1 mL of the remaining extract in PBS was performed with 1 mL of 1 M NaOH for 10 minutes (NaOH_10min), 1 mL of 1 M NaOH for 20 minutes (NaOH_20min), 1 mL of 5 % OA for 30 minutes (OA_30min) or 1 mL of 5 % OA for 1 hour (OA_1h) at room temperature with occasional vortexing. Some but not all extracts were decontaminated with multiple decontamination methods in parallel (Additional Table 1). Decontaminated extracts were diluted with 20 mL of sterile PBS. The decontaminated samples were centrifuged for 30 minutes at 3000g, the supernatant decanted and the pellet re-suspended in 0.15 to 0.25 mL of sterile PBS. Of the re-suspended pellet, 0.1 mL was transferred to Löwenstein-Jensen (LJ) medium slants with glycerol (Becton Dickinson and Company) or the same LJ medium slants supplemented with 2 % PANTA (LJ_PANTA). As with the decontamination methods above, some but not all re-suspended pellets were inoculated on both media in parallel.

Culture processing and *M. ulcerans* identification

All inoculated cultures were incubated at 30ºC until *M. ulcerans* growth could be observed. Slants were regularly examined and discarded if contamination, i.e. overgrowth with other faster growing microorganisms, was detected. All inoculations with no growth were kept for a minimum of 25 weeks before discarding. Suspected *M. ulcerans* growth was confirmed by colony PCR using primers MU154 (5'-ggcagttacttcactgcaca-3') and MU155 (5'-cggtgatcaagcgttcacga-3') and amplification for 32 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minute at 72°C. PCR products were resolved in a 1.5 % agarose gel.

Statistical analysis

To identify factors that affect the rate of contamination and recovery of *M. ulcerans* primary cultures, three sets of univariate and multivariate statistical analyses, one with all inoculations and two with subsets, were performed. The factors investigated included: transport medium, decontamination method, inoculation media, swab qPCR Ct value, patient qPCR result, the time from sampling to inoculation and the number of weeks before diagnosis as reported by the patient. The first analysis identified differences between inoculations that did not contaminate *versus* those that did result in contamination (non-contamination vs. contamination). This analysis aimed at identifying factors that affect contamination of cultures of samples taken from lesions clinically suspected of BU,

independent of the presence of *M. ulcerans* on the swab. Specifically, the data set used for this analysis included both qPCR positive and negative swabs and inoculations with any of the three possible outcomes: *M. ulcerans* growth, contamination with another microorganisms or no growth. Swabs with a negative qPCR result in this data set were assigned a Ct value of 76.80 (reciprocal of the midpoint between zero and the minimum of 1/Ct). These qPCR negative swabs were included in the analysis to investigate general factors that affect contamination of wound exudates and if the presence of *M. ulcerans* affects the rate of contamination. In the second analysis, a subset of inoculations was analyzed to identify factors that affect *M. ulcerans* growth (*M. ulcerans* growth vs. no growth). All inoculations with no realistic probability of resulting in *M. ulcerans* growth, i.e. contaminated inoculations and inoculations from swabs that were qPCR negative were excluded from this analysis. Finally in the third analysis, we studied factors that affected the recovery of *M. ulcerans* if some inoculations resulted in contamination (*M. ulcerans* growth vs. contamination or no growth). This subset included all inoculations originating from qPCR positive swabs independent of their outcome, i.e. also including swabs for which growth may have been undetectable because of overgrowth by other microorganisms. In this third analysis, the outcomes “no growth” and “contamination” were grouped together as the undesired outcome. Workflows of the swabs included in the three data sets are shown in Figure 1 and the numbers of samples decontaminated with two decontamination methods in parallel are listed in Additional Table 1.

For the identification of factors that contribute to the outcomes studied, generalized linear mixed models with patient identification and identification of each individual swab as random effects were used. Factors included in the one-to-one analysis were: transport medium, decontamination method, inoculation medium, swab qPCR Ct value, per patient qPCR result, time from sampling to inoculation and the duration of the disease before diagnosis. Factors that individually had a p-value of the association of less than 0.2 were included in the multivariate analyses. The software SAS (SAS Institute, Cary, USA; release 9.3), RStudio (RStudio, Boston, USA, version 0.95.262) and R (The R Foundation for Statistical Computing; version 2.15.1) were used to perform the analyses.

Results

Factors affecting the rate of contamination

In total 443 swabs, collected from 73 qPCR confirmed and 22 non-confirmed patients were included in the analysis for factors affecting the rate of contamination. Of all these swabs, 302 were qPCR positive with an average Ct value of 28.09 (Figure 1A). Of the 1125 culture inoculations from these swabs, 7.8 % (88/1125) resulted in *M. ulcerans* growth. The 88 *M. ulcerans* culture positive inoculations originated from samples collected from 31 patients, which corresponds to a per patient positivity of 32.6 % (Table 1). Further 52.5 % (591/1125) of the inoculations yielded no growth and 39.6 % (446/1125) resulted in contamination. The median time to detection of contamination was 5.0 (IQR = 4.0 to 11.0) days (Table 1). One-to-one analysis was used to identify factors that should be included in the multivariate analysis (Additional Table 2). When studying the combined effect of factors on the rate of contamination, we found that the transport medium had an overall significant (p-value: 0.008) effect on the probability of contamination. Specifically, swabs transported dry had a 57.7 times lower probability of contamination compared to swabs transported in Amies medium (Table 2). We found no significant difference in terms of the rate of contamination between swabs transported in 7H9 or Amies medium. As also shown in Table 2, there was no overall significant difference (p-value: 0.266) in the rate of contamination between the four decontamination methods evaluated here. The inoculated culture medium on the other hand significantly (p-value: <0.001) influenced the rate of contamination, with cultures on LJ having a 3.8 (1/0.264) times higher probability of contamination than cultures inoculated onto LJ_PANTA. The multivariate analysis further showed that there was a significant (p-value: 0.007) interaction between transport medium and the number of days from sampling to inoculation (Table 2). Finally, both an increase in the Ct value of the swab (p-value: 0.236) and the storage time from sampling to inoculation (p-value: 0.606) did not have a significant effect on the rate of contamination of the cultures (Table 2). Overall, this analysis to identify conditions that were best suited to prevent contamination of *M. ulcerans* primary cultures suggested that swabs should be stored dry, any of the evaluated decontamination methods can be used, cultures should be inoculated onto LJ_PANTA and neither the Ct value of the IS2404 qPCR nor the time from sampling to inoculation had a significant effect on the rate of contamination.

Factors affecting the rate of *M. ulcerans* recovery in primary culturing

To identify factors that affect the recovery of *M. ulcerans*, 440 inoculations from 220 qPCR positive swabs originating from 66 qPCR positive patients were analyzed. Only the subset of swabs with a realistic probability of resulting in *M. ulcerans* growth, as defined in Material and

Methods above, were used for this analysis. The average Ct value of the swabs was 27.9. Of all the inoculations included in the analysis, 88 inoculations of exudates collected from 31 patients resulted in *M. ulcerans* growth and the rest yielded no growth. This corresponds to a per inoculation positivity rate of 20.0 % (88/440) and a per patient positivity rate of 47.0% (31/66). Because it was not possible to determine if the contaminated inoculations could have resulted in *M. ulcerans* growth, they were not included in this analysis (Materials and Methods; Table 1). The median time to detectable *M. ulcerans* growth was 67.0 (IQR = 55.0 to 105.2) days (Table 1). One-to-one analyses were again used to identify factors to be included in the multivariate analysis (Additional Table 3). In this multivariate analysis, we found that the transport medium had an overall significant effect (p-value: <0.001) on the probability of *M. ulcerans* growth (Table 3). Specifically, swabs transported dry had a 97.7 % reduced chance (p-value < 0.001) of *M. ulcerans* growth compared to samples transported in Amies medium (Table 3). Between swabs transported in Amies or 7H9 medium no significant difference was found, although transport in Amies medium had a tendency to increase the chance of *M. ulcerans* recovery (odds ratio for transport in 7H9: 0.304; Table 3). Further in the multivariate analysis there was no significant difference in the chance of *M. ulcerans* recovery between the decontamination methods evaluated (p-value: 0.519) and inoculation on either LJ or LJ_PANTA (p-value: 0.216). However, with an increase of the Ct value by one unit, the probability of *M. ulcerans* growth was reduced by 12.1 % (p-value: 0.044; Table 3, 1 - OR) and with every 10 extra days of storage between sampling and inoculation, the probability of *M. ulcerans* growth decreased significantly (p-value: 0.001) by 45.9 %. Our analysis further detected a moderately significant (p-value: 0.074) interaction between the transport time and the transport medium, with Amies medium having the best chance of *M. ulcerans* growth for an increase in transport time by 10 days.

Overall, the analysis to evaluate factors that affect the recovery of *M. ulcerans* revealed that: i) storage in either Amies or 7H9 medium was significantly better than keeping swabs dry; ii) none of the decontamination methods tested had a significantly better effect on the growth of *M. ulcerans* and iii) the *M. ulcerans* recovery was not affected by inoculation of samples onto media containing PANTA. On the other hand, the analysis showed that both a one unit increase in the Ct value of the IS2404 qPCR and a 10 day increase in the turnover time of the samples had a significantly negative effect on the rate of *M. ulcerans* recovery.

M. ulcerans recovery versus no growth or contamination

For the identification of factors that affect *M. ulcerans* recovery in a scenario where some cultures are contaminated, 700 inoculated cultures from 302 qPCR positive swabs (average Ct value of 28.09) taken from 72 qPCR positive patients were analyzed. As shown in Table 1, 88 inoculations collected from 31 patients of the total 700 inoculations resulted in *M. ulcerans* growth. This corresponded to a per patient positivity rate of 43.1 %. Further, 37.1 %

(260/700) of the inoculations were contaminated and 50.3 % (352/700) did not result in any growth. The observed culture positivity rate corresponded to a per patient culture positivity of 43.1 %. Analysis of these inoculations in a multivariate analysis showed, that transport medium had a significant effect (p-value: 0.019) on the recovery of *M. ulcerans* (Table 4). Specifically, swabs transported in Amies medium showed the best recovery rate of *M. ulcerans*, although not significantly better than 7H9 medium (95% CI of OR: 0.269 - 1.415). As also seen in the analysis for non-contamination (Table 2) and *M. ulcerans* growth (Table 3), the decontamination methods evaluated here did not significantly differ in their effect on the chance of *M. ulcerans* recovery (p-value: 0.295; Table 4). On the other hand the use of the inoculated media had a significant (p-value: 0.003) impact on the chance of *M. ulcerans* growth, with the use of LJ as compared to LJ_PANTA reducing the probability of *M. ulcerans* growth by 65.5 %. As further shown in Table 4, a one unit increase in the Ct value away from the mean Ct value of the inoculated swabs decreased the chance of *M. ulcerans* recovery by 10.8 % (p-value: 0.011). Furthermore, consistent with the analysis of factors affecting *M. ulcerans* growth (Table 3), the time span from sampling to inoculation significantly (p-value: 0.006) reduced the chance of *M. ulcerans* isolation, with a decrease of 19.1 % for every 10 extra days of storage compared to the mean storage time of 80.2 days (Table 4, Figure 2). As shown in Figure 2, the predicted probability of *M. ulcerans* recovery decreased from 61.2 % at zero days of storage to 26.8 % within 70 days of storage. Specifically for swabs with a qPCR Ct value of 27.8, that were transported in Amies medium, decontaminated using NaOH for 10 minutes and inoculated onto LJ with PANTA, a probability of *M. ulcerans* growth of 61.2 % can be expected if samples were stored for zero days. If the samples are stored for 50 or 100 days, the predicted probability decreases to 35.3 % and 15.9 % respectively.

Overall our analysis of the sample set consisting of only qPCR positive swabs but including all possible outcomes of the inoculations, showed that either of the moist transport media increased the chance of *M. ulcerans* recovery compared to samples kept dry. None of the decontamination methods yielded superior results and *M. ulcerans* culturing was favored on PANTA supplemented LJ medium. Further the analysis showed that both a one unit increase in the Ct value of the IS2404 qPCR and a 10 day increase in the turnover time of the samples negatively impacted the chance of *M. ulcerans* isolation.

Discussion

In the present study we evaluated the effect of various factors on the rate of *M. ulcerans* primary isolation from clinical specimens to develop a method for *M. ulcerans* recovery from samples collected from very remote BU endemic areas. For this, we examined three sets of inoculations, i) to identify factors that reduce the rate of contamination of the primary cultures, ii) to determine which factors increase the rate of *M. ulcerans* recovery in a scenario where none of the cultures are contaminated and iii) to evaluate the effect of factors in the realistic setting where some *M. ulcerans* growth will be missed due to the contamination of cultures.

In a study on IS2404 PCR positive tissue biopsies which were stored for up to 26 weeks in semi-solid transport medium, Eddyani *et al*. were able to achieve a culture positivity rate of 45.2 %. This showed that the establishment of an *M. ulcerans* strain collection from remote BU endemic areas from tissue samples is possible [11]. Other studies using either tissue biopsies or swab and FNA samples and shorter transport times have also reported culture positivity rates of about 50 % with the rest of the cases, although clinically diagnosed and PCR confirmed, remaining culture negative [6, 13, 16, 17]. In a study comparable to the present analysis, Eddyani *et al*. cultured *M. ulcerans* from FNA that were stored for two weeks before processing, a per sample positivity rate of 17.6 % was achieved [12]. In spite of long storage intervals prior to processing, we have achieved here a per PCR–reconfirmed-sample positivity rate of 23.5 %, corresponding to a PCR-reconfirmed-patient *M. ulcerans* culture positivity rate from swab and FNA samples of 43.1 %. Given the similarity in these *M. ulcerans* recovery rates in several studies employing different clinical specimens and approaches, the reason for lack of growth of *M. ulcerans* from certain patients should be further investigated [18].

To increase *M. ulcerans* recovery rates, Yeboah-Manu *et al.* have suggested collecting and processing multiple swab or FNA samples per patient. To increase the chance of culture positivity, we have ensured that lesion exudates were collected from all around ulcers. Further, we collected and processed several (median = 4; IQR = 2 to 6) swabs from most patients and this repeated sampling did indeed affect the rate of recovery of *M. ulcerans* per patient. Only considering qPCR positive swabs, we observed a significant difference (p-value 0.003) in the number of swabs collected from culture positive patients (median: 5; IQR: 3 to 6 swabs) versus the number of swabs collected from culture negative patients (median: 2; IQR: 1 to 5 swabs). Based on these results, we recommend the collection of up to five swab samples per patient prior to treatment start to increase the probability of recovering the infecting *M. ulcerans* strain.

In both our analyses for *M. ulcerans* growth and for contamination of cultures, there was no significant difference between swabs transported in 7H9 or Amies medium. However, based on the lower costs of Amies transport medium compared to the 7H9 medium (approximately 0.7 USD per Amies swab and 2.8 USD per 7H9 swab with PANTA and OADC), we recommend the use of Amies medium for the transport of swabs collected from BU patients.

In our analysis, the number of days for which samples were stored did not significantly affect the rate of contamination of the inoculated cultures but longer storage did significantly reduce the rate of *M. ulcerans* recovery (Tables 3 and 4, Figure 2). This is contrary to a previous report of culturing from tissue biopsies which found that that storage time did not affect the rate of *M. ulcerans* recovery [11]. *M. ulcerans* may thus survive better if transported in the context of a tissue biopsy than as swab sample. As can be expected, the Ct value of the IS2404 qPCR had a significant effect on the rate of *M. ulcerans* recovery (Tables 3 and 4). On the other hand, neither the qualitative nor the quantitative qPCR result of a swab nor the BU status, i.e. the overall patient qPCR result, had a significant effect on the rate of contamination of the primary cultures in the one-by-one or the multivariate analysis (Table 2 and Additional Table 2). In line with a recent report on secondary infections of BU lesions [19], this finding does not support the assumption that BU lesions are less prone to contamination with secondary microorganisms than other wounds [20].

In our analysis there was no significant difference between the four decontamination methods evaluated, although in the univariate analysis for *M. ulcerans* growth, NaOH for 10 minutes appeared to outperform the other decontamination options with borderline significance (p-value: 0.076, Additonal Table 3). Since 10 min NaOH is also the quickest decontamination method, we therefore suggest this one to be used.

Our analysis of *M. ulcerans* growth vs. contamination or no growth (Figure 1C), showed that the use of LJ_PANTA compared to LJ had a significant positive effect (p-value: 0.003) on the rate of recovery of *M. ulcerans*. This is similar to what has been found by Yeboah-Manu *et al*. where *M. ulcerans* recovery was also significantly improved (p-value < 0.001) on LJ_PANTA compared to LJ alone [13].

By evaluating a small set of swabs, Yeboah-Manu *et al.* have previously been able to show that culturing from dry cotton swabs is feasible [13]. In our study we have confirmed this finding. However the chance of *M. ulcerans* recovery from swabs stored dry is reduced by 75.2 % compared to swabs transported in Amies (Table 4) and in our analysis dry swabs only achieved a per patient culture positivity rate of 13.1 %.

Conclusions

In conclusion, our results show that primary culturing of *M. ulcerans* from cotton swabs after long turnover time is possible. Based on our findings we suggest that this type of samples should be transported in Amies medium, that they should be decontaminated in 0.5 M NaOH for 10 minutes and that cultures should be inoculated onto LJ medium supplemented with 2 % PANTA. Furthermore, multiple samples (approximately 5) should be collected from each patient and only the PCR positive swabs should be inoculated for culturing.

Overall, the here identified method can help to establish *M. ulcerans* strain collections from very remote BU endemic areas. An increased number of available *M. ulcerans* strains from all endemic areas will be a valuable resource for studies to increase our understanding of pathology, transmission and many other aspects of BU.

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Key Words

Buruli ulcer, *Mycobacterium ulcerans*, primary cultivation, long turnover time

Author Contributions

MWB and JCM performed the sampling of BU patients in the Mapé Basin endemic area. MWB, MB, SK and MTR prepared transport media and performed the laboratory work. MWB and LG performed the statistical analysis and modeling. MWB, MB and GP wrote the manuscript. MWB, MB and GP conceived the study and MWB, AUB, DY and GP coordinated and supervised the study.

All authors read and approved the final manuscript.

Tables

Table 1 Outcomes of *M. ulcerans* primary culturing

Attribute		Non-contamination vs. contamination[#]	*M. ulcerans* growth vs. no growth[##]	*M. ulcerans* growth vs. contamination or no growth[###]
M. ulcerans growth per swab (%)		71/443 (16.03)	71/220 (32.27)	71/302 (23.50)
M. ulcerans growth per decontamination procedure (%)	Total	80/680 (11.76)	80/315 (25.40)	80/466 (17.17)
	NaOH_10min	21/106 (19.81)	21/59 (35.59)	21/99 (21.21)
	NaOH_20min	19/214 (8.88)	19/113 (16.81)	19/139 (13.67)
	OA_1h	17/157 (10.83)	17/73 (23.29)	17/118 (14.41)
	OA_30min	23/203 (11.33)	23/70 (32.86)	23/110 (20.91)
M. ulcerans growth per inoculation (%)		88/1125 (7.82)	88/440 (20.00)	88/700 (12.57)
M. ulcerans growth per patient (%)		31/95 (32.63)	31/66 (46.97)	31/72 (43.06)
Contamination per swab (%)		284/443 (64.11)	-	188/302 (62.25)
Contamination per decontamination procedure (%)	Total	346/680 (50.88)	-	223/466 (47.85)
	NaOH_10min	54/106 (50.94)	-	48/99 (48.48)
	NaOH_20min	97/214 (45.33)	-	58/139 (41.73)
	OA_1h	74/157 (47.13)	-	54/118 (45.76)
	OA_30min	121/203 (59.61)	-	63/110 (57.27)
Contamination per inoculation (%)		446/1125 (39.64)	-	260/700 (37.14)
No growth (%)		591/1125 (52.53)	352/440 (80.00)	352/700 (50.29)
Days to primary outcome*		5.0 (4.0; 11.0)	67.0 (55.0; 105.2)	67.0 (55.0; 105.2)

[#]Analysis of 1125 inoculations from 95 patients with contamination as the primary outcome.
[##]Analysis of 440 inoculations from 66 patients with *M. ulcerans* growth as the primary outcome.
[###]Analysis of 700 inoculations from 72 patients with *M. ulcerans* growth as the primary outcome.
*Median with IQR in parentheses.

Table 2 Association[§] between non-contamination and all factors of interest

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect p-value
Transport medium[§§]	7H9	1.579	0.632 - 3.944	0.008
	dry	57.675	23.704 - 140.334	
	Amies (ref. level)	1.000		
Decontamination medium	OA_1h	0.715	0.362 - 1.412	0.266
	OA_30min	0.540	0.260 - 1.122	
	NaOH_20min	0.879	0.383 - 2.016	
	NaOH_10min (ref. level)	1.000		
Inoculation media[§§]	LJ	0.264	0.162 - 0.429	<0.001
	LJ_PANTA (ref. level)	1.000		
Swab qPCR Ct value	for an increase in 1 unit Ct	0.992	0.978 - 1.006	0.236
Time from sampling to inoculation	for an increase in 10 days			0.606
Interaction of transport medium and days from sampling to inoculation	when 7H9[§§§]	0.982	0.824 - 1.171	0.007
	when dry[§§§]	1.167	1.032 - 1.321	
	when Amies[§§§] (ref. level)	0.799	0.643 - 0.993	

[§]Adjusted for random effects of the patient and swab.
[§§]An interaction (p-value: <0.001) between transport medium and inoculation media was observed.
[§§§]For an increase in 10 days from the mean number of days from sampling to inoculation.

Table 3 Association[§] between *M. ulcerans* growth and all factors of interest with a relevant effect

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect p-value
Transport medium	7H9	0.304	0.046 - 2.026	<0.001
	dry	0.023	0.004 - 0.143	
	Amies (ref. level)	1.000		
Decontamination medium	OA_1h	0.414	0.102 - 1.683	0.519
	OA_30min	0.697	0.127 - 3.837	
	NaOH_20min	1.242	0.204 - 7.546	
	NaOH_10min (ref. level)	1.000		
Inoculation media	LJ	0.478	0.148 - 1.545	0.216
	LJ_PANTA (ref. level)	1.000		
Swab qPCR Ct value	for an increase in 1 unit Ct	0.879	0.775 - 0.996	0.044
Time from sampling to inoculation	for an increase in 10 days			0.001
Interaction of transport medium and days from sampling to inoculation	when 7H9[§§]	0.531	0.320 - 0.881	0.074
	when dry[§§]	0.880	0.704 - 1.099	
	when Amies[§§] (ref. level)	0.541	0.347 - 0.844	

[§]Adjusted for random effects of the patient and swab.
[§§]For an increase in 10 days from mean number of days from sampling to inoculation.

Table 4 Model describing the association[§] between *M. ulcerans* growth versus no growth or contamination and all factors of interest with a relevant effect

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect p-value
Transport medium	7H9	0.617	0.269 - 1.415	0.019
	dry	0.248	0.094 - 0.655	
	Amies (ref. level)	1.000		
Decontamination medium	OA_1h	0.440	0.182 - 1.062	0.295
	OA_30min	0.529	0.203 - 1.375	
	NaOH_20min	0.739	0.236 - 2.313	
	NaOH_10min (ref. level)	1.000		
Inoculation media	LJ	0.345	0.169 - 0.703	0.003
	LJ_PANTA (ref. level)	1.000		
Swab qPCR Ct value	for an increase in 1 unit Ct	0.892	0.818 - 0.974	0.011
Time from sampling to inoculation	for an increase in 10 days	0.809	0.695 - 0.941	0.006

[§]Adjusted for random effects of the patient and swab.

Figures

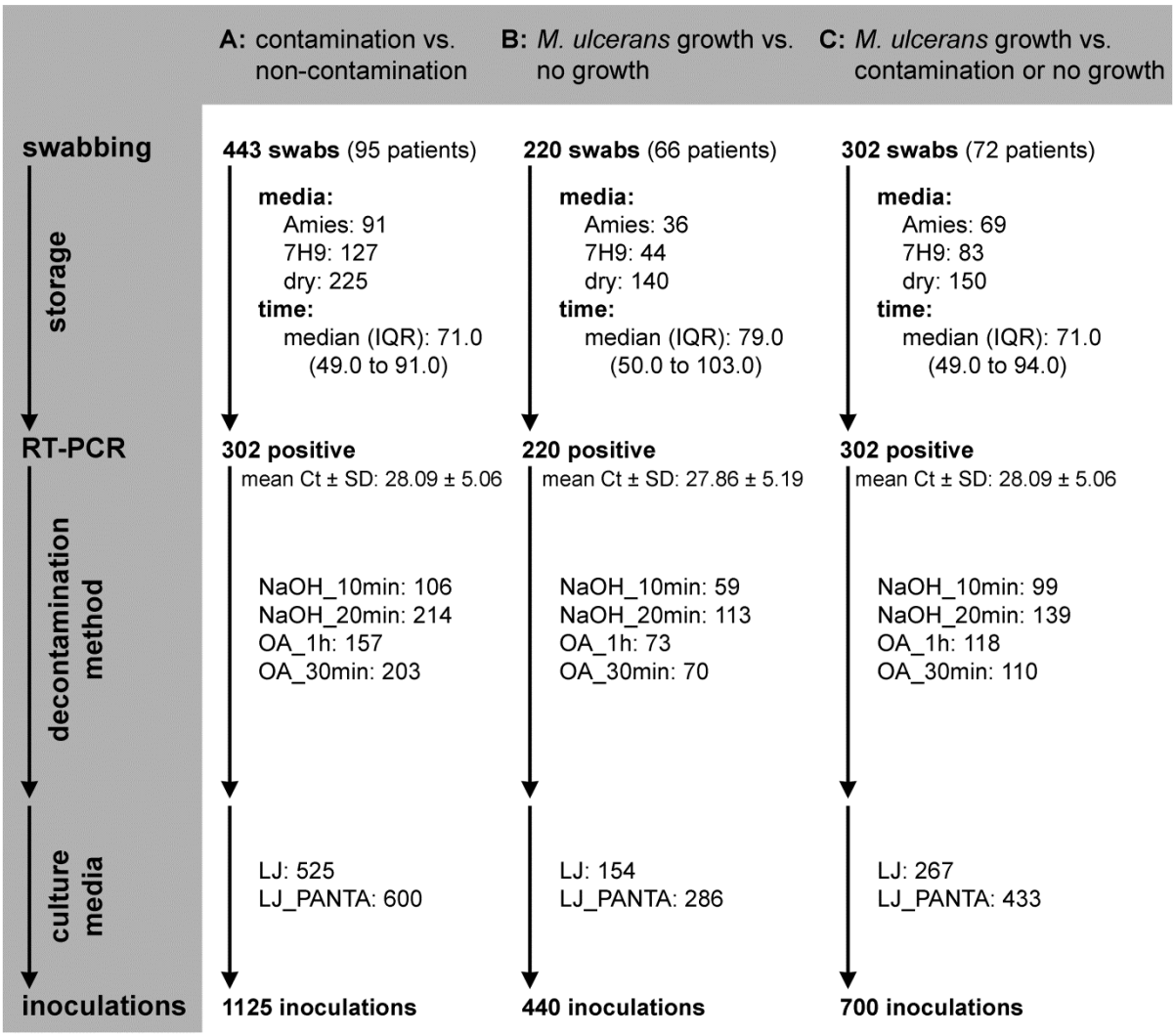

Figure 1: Workflow for swabs included in the statistical analysis.

The complete set of *M. ulcerans* primary culturing inoculations was used to identify factors that affect the rate of contamination (non-contamination vs. contamination; A). A subset of the inoculations was used to identify factors that influence the growth of *M. ulcerans* in the absence of any contamination (*M. ulcerans* growth vs. no growth; B) and a second subset was used to identify factors that affect *M. ulcerans* growth if some of the inoculations resulted in contamination (*M. ulcerans* growth vs. contamination or no growth; C). The number of swabs collected as well as the transport media used and the storage time are shown. Further, the number of qPCR positive swabs with their average Ct value are given and the numbers of decontaminations as well as inoculations performed are shown. Finally the number of total inoculations in each of the data sets is indicated.

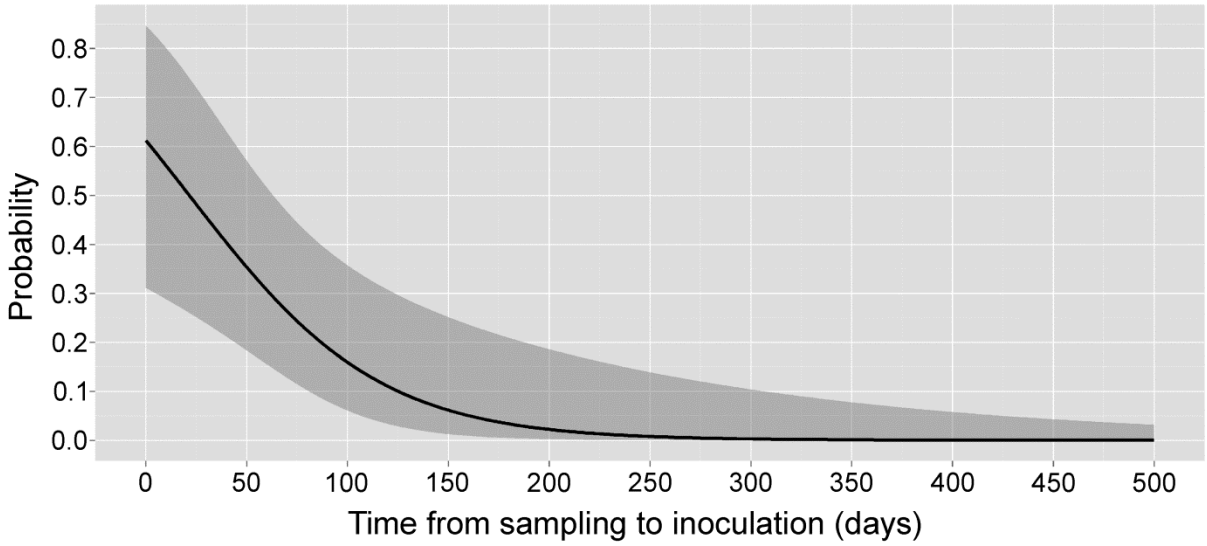

Figure 2: Predicted probabilities for *M. ulcerans* growth.

Based on the *M. ulcerans* growth vs. no growth or contamination model the probability of *M. ulcerans* growth was predicted as a function of transport time for samples transported in Amies medium, decontaminated with NaOH for 10 minutes, inoculated onto LJ medium supplemented with PANTA and if the Ct value of the qPCR was 27.8. Mean predicted probability of the *M. ulcerans* growth rate and 95% confidence intervals are shown.

Supplementary Material

Additional Table 1: Number of samples decontaminated with multiple decontamination methods in parallel

Decontamination method*	Non-contamination vs. contamination	*M. ulcerans* growth vs. no growth	*M. ulcerans* growth vs. contamination or no growth
NaOH_10 and OA_30min	19	3	13
NaOH_10 and OA_1h	87	44	86
NaOH_20 and OA_30min	61	25	33
OA_1h and OA_30min	70	23	32

* Number of clinical samples decontaminated with the combinations of decontamination methods indicated for the three set of samples analysed.

Additional Table 2: Association[§] between non-contamination of *M. ulcerans* cultures and each of the factors of interest

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect (p-value)
Transport Medium	7H9	0.840	0.462 - 1.527	<0.001
	dry	24.314	13.268 - 44.555	
	Amies (ref. level)	1.000		
Decontamination medium	OA_1h	0.853	0.465 - 1.567	0.021
	OA_30min	0.749	0.396 - 1.414	
	NaOH_20min	1.777	0.860 - 3.673	
	NaOH_10min (ref. level)	1.000		
Inoculation media	LJ	0.441	0.307 - 0.634	<0.001
	LJ_PANTA (ref. level)	1.000		
Swab qPCR Ct value	for an increase in 1 unit Ct	0.990	0.978 - 1.002	0.119
Patient qPCR result	negative	0.799	0.379 - 1.682	0.554
	positive (ref. level)	1.000		
Time from sampling to inoculation	for an increase in 10 day	1.402	1.282 - 1.534	<0.001
Weeks before diagnosis*	for an increase by 1 week	0.992	0.976 - 1.007	0.285

[§] Adjusted for random effects of the patient and swab identification.

* Information as reported by the patient.

Additional Table 3: Association[§] between *M. ulcerans* growth and each of the factors of interest.

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect p-value
Transport medium	7H9	0.343	0.099 - 1.192	<0.001
	dry	0.004	0.001 - 0.015	
	Amies (ref. level)	1.000		
Decontamination medium	OA_1h	0.373	0.135 - 1.032	0.076
	OA_30min	0.396	0.136 - 1.157	
	NaOH_20min	0.229	0.071 - 0.734	
	NaOH_10min (ref. level)	1.000		
Inoculation media	LJ	0.510	0.239 - 1.089	0.082
	LJ_PANTA (ref. level)	1.000		
Swab qPCR Ct value	for an increase in 1 unit Ct	0.947	0.868 - 1.033	0.216
Time from sampling to inoculation	for an increase in 10 day	0.621	0.522 - 0.738	<0.001
Number of days of treatment before sampling	for an increase in 1 day	1.033	0.965 - 1.106	0.343
Weeks before diagnosis*	1 week	1.002	0.976 - 1.029	0.865

[§] Adjusted for random effects of the patient and swab identification.

* Information as reported by the patient.

References

1. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, Benbow ME, Boakye DA: **Ecology and transmission of Buruli ulcer disease: a systematic review**. *PLoS Negl Trop Dis* 2010, **4**:e911.

2. Walsh DS, Portaels F, Meyers WM: **Buruli ulcer: Advances in understanding *Mycobacterium ulcerans* infection**. *Dermatol Clin* 2011, **29**:1–8.

3. **WHO | Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer)** [http://www.who.int/buruli/information/antibiotics/en/index.html]

4. **Buruli ulcer : diagnosis of *Mycobacterium ulcerans* disease : a manual for health care providers / edited by: Françoise Portaels, Paul Johnson, Wayne M. Meyers** [http://apps.who.int/iris/handle/10665/67000]

5. Yeboah-Manu D, Bodmer T, Mensah-Quainoo E, Owusu S, Ofori-Adjei D, Pluschke G: **Evaluation of decontamination methods and growth media for primary isolation of *Mycobacterium ulcerans* from surgical specimens**. *J Clin Microbiol* 2004, **42**:5875–5876.

6. Portaels F, Agular J, Fissette K, Fonteyne PA, De Beenhouwer H, de Rijk P, Guédénon A, Lemans R, Steunou C, Zinsou C, Dumonceau JM, Meyers WM: **Direct detection and identification of *Mycobacterium ulcerans* in clinical specimens by PCR and oligonucleotide-specific capture plate hybridization**. *J Clin Microbiol* 1997, **35**:1097–1100.

7. Röltgen K, Qi W, Ruf M-T, Mensah-Quainoo E, Pidot SJ, Seemann T, Stinear TP, Käser M, Yeboah-Manu D, Pluschke G: **Single nucleotide polymorphism typing of *Mycobacterium ulcerans* reveals focal transmission of buruli ulcer in a highly endemic region of Ghana**. *PLoS Negl Trop Dis* 2010, **4**:e751.

8. Beissner M, Awua-Boateng N-Y, Thompson W, Nienhuis WA, Klutse E, Agbenorku P, Nitschke J, Herbinger K-H, Siegmund V, Fleischmann E, Adjei O, Fleischer B, van der Werf TS, Loscher T, 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* 2010, **83**:1059–1065.

9. Petroff SA: **A NEW AND RAPID METHOD FOR THE ISOLATION AND CULTIVATION OF TUBERCLE BACILLI DIRECTLY FROM THE SPUTUM AND FECES**. *J Exp Med* 1915, **21**:38–42.

10. Palomino JC, Portaels F: **Effects of decontamination methods and culture conditions on viability of *Mycobacterium ulcerans* in the BACTEC system**. *J Clin Microbiol* 1998, **36**:402–408.

11. Eddyani M, Debacker M, Martin A, Aguiar J, Johnson CR, Uwizeye C, Fissette K, Portaels F: **Primary culture of *Mycobacterium ulcerans* from human tissue specimens after storage in semisolid transport medium**. *J Clin Microbiol* 2008, **46**:69–72.

12. Eddyani M, Fraga AG, Schmitt F, Uwizeye C, Fissette K, Johnson C, Aguiar J, Sopoh G, Barogui Y, Meyers WM, Pedrosa J, Portaels F: **Fine-needle aspiration, an efficient sampling technique for bacteriological diagnosis of nonulcerative Buruli ulcer**. *J Clin Microbiol* 2009, **47**:1700–1704.

13. Yeboah-Manu D, Danso E, Ampah K, Asante-Poku A, Nakobu Z, Pluschke G: **Isolation of *Mycobacterium ulcerans* from swab and fine-needle-aspiration specimens**. *J Clin Microbiol* 2011, **49**:1997–1999.

14. Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, Kerber S, Njih Tabah E, Ruf M-T, Mou F, Noumen D, Um Boock A, Pluschke G: **Geographic distribution, age pattern and sites of lesions in a cohort of buruli ulcer patients from the mapé basin of cameroon**. *PLoS Negl Trop Dis* 2013, **7**:e2252.

15. Lavender CJ, Fyfe JAM: **Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples**. *Methods Mol Biol Clifton NJ* 2013, **943**:201–216.

16. Beissner M, Herbinger K-H, Bretzel G: **Laboratory diagnosis of Buruli ulcer disease**. *Future Microbiol* 2010, **5**:363–370.

17. Mensah-Quainoo E, Yeboah-Manu D, Asebi C, Patafuor F, Ofori-Adjei D, Junghanss T, Pluschke G: **Diagnosis of *Mycobacterium ulcerans* infection (Buruli ulcer) at a treatment centre in Ghana: a retrospective analysis of laboratory results of clinically diagnosed cases**. *Trop Med Int Health TM IH* 2008, **13**:191–198.

18. Williamson H, Phillips R, Sarfo S, Wansbrough-Jones M, Small P: **Genetic diversity of PCR-positive, culture-negative and culture-positive *Mycobacterium ulcerans* isolated from buruli ulcer patients in Ghana**. *PloS One* 2014, **9**:e88007.

19. Yeboah-Manu D, Kpeli GS, Ruf M-T, Asan-Ampah K, Quenin-Fosu K, Owusu-Mireku E, Paintsil A, Lamptey I, Anku B, Kwakye-Maclean C, Newman M, Pluschke G: **Secondary bacterial infections of buruli ulcer lesions before and after chemotherapy with streptomycin and rifampicin**. *PLoS Negl Trop Dis* 2013, **7**:e2191.

20. Demangel C, Stinear TP, Cole ST: **Buruli ulcer: reductive evolution enhances pathogenicity of *Mycobacterium ulcerans***. *Nat Rev Microbiol* 2009, **7**:50–60.

Results Chapter 3

Locally Confined Clonal Complexes of *Mycobacterium ulcerans* in Two Buruli Ulcer Endemic Regions of Cameroon

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Abstract

Background: Mycobacterium ulcerans is the causative agent of the necrotizing skin disease Buruli ulcer (BU), which has been reported from over 30 countries worldwide. The majority of notified patients come from West African countries, such as Côte d'Ivoire, Ghana, Benin and Cameroon. All clinical isolates of M. ulcerans from these countries are closely related and their genomes differ only in a limited number of single nucleotide polymorphisms (SNPs).

Methodology/Principal Findings: We performed a molecular epidemiological study with clinical isolates from patients from two distinct BU endemic regions of Cameroon, the Nyong and the Mapé river basins. Whole genome sequencing of the M. ulcerans strains from these two BU endemic areas revealed the presence of two phylogenetically distinct clonal complexes. The strains from the Nyong river basin were genetically more diverse and less closely related to the M. ulcerans strain circulating in Ghana and Benin than the strains causing BU in the Mapé river basin.

Conclusions: Our comparative genomic analysis revealed that M. ulcerans clones diversify locally by the accumulation of SNPs. Case isolates coming from more recently emerging BU endemic areas, such as the Mapé river basin, may be less diverse than populations from longer standing disease foci, such as the Nyong river basin. Exchange of strains between distinct endemic areas seems to be rare and local clonal complexes can be easily distinguished by whole genome sequencing.

Author Summary

Buruli ulcer (BU) is a progressively necrotizing disease of the skin, caused by infection with *Mycobacterium ulcerans*. BU occurs very focally with highest incidence in West Africa. The mode of transmission and the nature and role of potential environmental reservoirs are currently not entirely understood. In this study we sequenced whole genomes of sets of *M. ulcerans* case isolates from two BU endemic regions in Cameroon. We identified two distinct phylogenetic lineages, which directly correlated with the two endemic regions. Furthermore, we showed that the genetic diversity of *M. ulcerans* is higher in the older endemic region of Cameroon (Nyong river basin) compared to the more recently emerged infection focus in the same country (Mapé river basin). Together, our results demonstrate that *M. ulcerans* is developing local clonal complexes by the accumulation of single nucleotide polymorphisms (SNPs) and that these complexes often remain confined to individual endemic foci. The gene encoding for rpoB, which is known to harbour drug resistance mutations against rifampicin in *M. tuberculosis*, was not affected by SNPs in any of the analysed *M. ulcerans* strains.

Introduction

Buruli ulcer (BU), the third most common mycobacterial disease affecting humans after tuberculosis and leprosy, is caused by *Mycobacterium ulcerans* [1]. The disease is characterized by progressive necrosis of the skin and subcutaneous tissue, leading to sometimes extensive ulcerations. Even though standard antibiotic treatment for eight weeks with rifampicin and streptomycin, as it is currently recommended by the World Health Organization (WHO), is highly effective in killing the bacterium, quite a number of patients still require surgery for wound debridement and/or skin grafting and can remain with scarring and disabilities [2]. BU has been reported from over 30 countries worldwide but has its highest incidence in West Africa, where it occurs very focally in rural areas, which are associated with wetlands, marshes and riverine zones [3].

The distinct pathology of *M. ulcerans* infections is mainly attributed to mycolactone, a macrolide exotoxin produced by the mycobacteria [4]. Mycolactone is highly toxic to mammalian cells and is also believed to have immunomodulatory functions [5]. The polyketide synthases required to produce this potent toxin are encoded as three large genes on a giant virulence plasmid, pMUM001, whose acquisition represents a crucial step in the divergence of *M. ulcerans* from its progenitor *M. marinum* [6,7]. Further hallmarks of *M. ulcerans* evolution include the proliferation of two distinct insertion sequence (IS) elements (IS2404 and IS2606), the accumulation of a large number of pseudogenes and considerable genome downsizing. These findings indicate that *M. ulcerans* has recently passed through an evolutionary bottleneck and is adapting to a new and more stable environment [7]. This new niche is suspected to be aerobic, osmotically stable, dark and possibly extracellular. Production of the immunosuppressive toxin mycolactone and the loss of a set of highly immunogenic proteins [8] may represent an adaptation to an environment that is screened by an immune system.

Possums, an Australian marsupial species, seem to be especially susceptible to the disease and may function as an animal reservoir in BU endemic foci of Victoria, Australia. However, attempts to identify an animal reservoir in Africa have not been successful to date [9,10]. Therefore it is assumed that there may be other environmental reservoirs of *M. ulcerans* in association with stagnant water bodies. Contact with such wetlands is a known risk factor for contracting BU, and patients may become infected through microtrauma of the skin or inoculation by an unknown insect vector [3]. *M. ulcerans* DNA has been detected by IS2404 specific PCR in environmental samples, but the cultivation of the slow growing mycobacteria from such samples is exceptionally difficult and has only succeeded once so far [11,12]. Therefore it remains unclear what relevance the presence of DNA in the environment has

and how *M. ulcerans* is transmitted. For a long time the highly clonal population structure of *M. ulcerans* represented a major obstacle for molecular epidemiological studies. Conventional typing methods such as restriction fragment length polymorphism, multilocus sequence typing and variable number tandem repeat analysis provide insufficient resolution [13]. To date the best typing resolution was attained with single nucleotide polymorphism (SNP) typing assays [14]. With this method *Röltgen et al*. were able to demonstrate focal transmission in the Densu river basin of Ghana [14]. Furthermore, with an extended set of SNPs sufficient phylogenetic signal could be obtained to reconstruct recent evolutionary events in *M. ulcerans* on a continental scale [15]. A drawback of this method is, however, that it requires prior knowledge of the relevant SNPs.

With its decreasing costs, whole genome sequencing (WGS) is now replacing SNP typing for *M. ulcerans*. Here we report on a genomic epidemiological study aimed at inferring evolutionary patterns of *M. ulcerans* in two BU endemic regions of Cameroon (the Mapé and the Nyong river basins) by using WGS combined with fine-scale geographic information on the origin of the patients from which the *M. ulcerans* strains were isolated.

Materials and Methods

Ethical statement and patient recruitment

Samples for this study were collected from patients recruited between August 2010 and July 2012 in the Mapé river basin of Cameroon [16] and at the district hospital in Ayos in southern Cameroon [17]. Ethical clearance for the collection and processing of samples was obtained from the Cameroon National Ethics Committee (N°041/CNE/DNM/09, N°006/CNE/SE/2010, and N°172/CNE/SE/2011), the Ethics Committee of the Heidelberg University Hospital, Germany (N°ISRCTN72102977) and the Ethics Committee of Basel (EKBB, reference n. 53/11). Participation was voluntary and all patients who participated in the study or their legal guardian provided written informed consent.

M. ulcerans cultivation

Prior to the start of medical treatment, cotton swabs were collected from each patient for diagnosis of *M. ulcerans* disease by quantitative polymerase chain reaction (qPCR) targeting the *M. ulcerans* specific IS2404 [18] and for cultivation of the bacteria. Wound exudates in phosphate buffered saline (PBS), that were produced from cotton swabs for DNA extraction as described by Lavender and Fyfe [19], were decontaminated as described by *Bratschi et al*. [20] and cultures initiated on Löwenstein-Jensen (LJ) medium slants (with glycerol; Becton Dickinson and Company) and/or LJ medium slants supplemented with 2 % PANTA. Inoculated cultures were incubated at 30°C until growth could be observed. Detected growth was confirmed to be *M. ulcerans* by colony PCR using primers MU154 (5'-ggcagttacttcactgcaca-3') and MU155 (5'-cggtgatcaagcgttcacga-3') and amplification for 32 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minute at 72°C. PCR products were resolved in a 1.5 % agarose gel. Confirmed *M. ulcerans* cultures were expanded on 7H10 agar plates (Becton Dickinson and Company) until enough bacteria could be harvested for DNA extraction as described below.

DNA extraction

M. ulcerans DNA for WGS was extracted as described by *Käser et al*. [21]. Briefly, *M. ulcerans* bacteria were transferred from 7H10 agar plates into a 1.5ml screw-cap tube and suspended in lysis buffer (15 % sucrose, 50 mM Tris (pH8.5) and 50 mM EDTA). After Incubation with lysozyme for 1h at 37°C, sodium dodecyl sulfate (SDS) and proteinase K (PK) were added and the bacteria lysed with a tissue homogenizer (Precellys24, Bertin Technologies) in tough micro-organism lysis tubes containing beads (Bertin Technologies). DNA from lysate supernatant was extracted by the Phenol-chlorophorm / Ethyl alcohol (EtOH) method [21]. Amount and purity of the extracted mycobacterial DNA was assessed

with a Qubit 2.0 Fluorometer according to the manufacturer's protocol (Qubit dsDNA HS Assay Kit, Invitrogen).

Whole genome sequencing

All processing and sequencing of genomic DNA was performed by the core sequencing teams at the Wellcome Trust Sanger Institute. All samples were sequenced as multiplexed libraries using Illumina HiSeq 2000 analyzers on 75-bp paired-end runs as described by *Harris et al*. [22].

Read alignment and SNP detection

Variation, in the form of SNPs, was detected using a mapping approach. The paired-end Illumina reads were mapped against the *M. ulcerans* reference genome of the strain Agy99 (accession number CP000325) and against the *M. ulcerans* Agy99 pMUM001 plasmid, with an insert size ranging from 50 to 400 bp using SMALT version 0.7.4 (http://www.sanger.ac.uk/resources/software/smalt/) with a word length of 13 and skip size of 1. The maximum insert size was 1000, the minimum insert size was 50, resulting, on average, in a 25x depth coverage for more than 92.1 % of the reference genome. The default mapping parameters recommended for reads were employed, except for the minimum score required for mapping, which was increased to 30 to make the mapping more conservative. Candidate SNPs were identified using SAMtools [23] mpileup as previously described [24]. Base calls for all isolates were filtered to remove those at sites with a SNP quality score below 30, where the called based was in less than 75 % of mapped reads on each strand, or where fewer than two reads mapped to each strand. SNPs called in repetitive regions of the *M. ulcerans* reference genome (737,280 bp) were excluded from the analysis and only the SNPs mapped in the core genome (4,894,326 bp) were used to construct the phylogenetic tree. Repetitive regions were defined as exact repetitive sequences of ≥20 bp in length, identified using rep repeat-match [25]. If 10 % of the genomes under study had an ambiguous base in a called SNP, these positions were removed from the analysis. The information on the potentially problematic regions is provided in S1 Table.

Phylogenetic analyses

A maximum-likelihood phylogenetic tree was generated from the whole genome sequences based on the SNPs called by SMALT. Published *M. ulcerans* genomes from strains isolated in Ghana (NM14_01, NM49_02, NM54_02, NM43_02, Agy99), Benin (Mu_06-3845, Mu_06-3846, Mu_07-1082) and the *M. marinum* genome (Mu_06-3844) of an isolate from Belgium were included in the analysis [13]. The *M. marinum* genome was used to root the tree. In total 91 strains were included in this study (S2 Table). Separate maximum-likelihood trees for the plasmid and the chromosome were reconstructed based on one *M. marinum* genome and 53 *M. ulcerans* genomes. The *M. ulcerans* genomes include 45 Cameroonian isolates

with each patient being represented by one strain. Maximum-likelihood phylogenetic trees were constructed using RAxML v7.0.4 und a general time-reversible (GTR) substitution model with γ correction for among-site rate variation. Support for nodes on the trees was assessed using 100 bootstrap replicates.

SNP pairwise distance

To compute the pairwise distance based on the genome wide SNP count between the isolates we used MEGA6 [26]. The average pairwise SNP distances per genome within the lineages were plotted using software package R (http://www.r-project.org/) and statistical significance was assessed by applying the Wilcoxon rank sum test.

Nucleotide diversity

Genetic diversity was assessed by calculating the average pair-wise nucleotide differences per site (Pi) for both main lineages of *M. ulcerans* in Cameroon, using the program VariScan [27]. We calculated the nucleotide diversity for 1.5 kb non-overlapping windows.

Median joining networks

We produced two median joining networks using Network 4.6.1.2 [28], based on 107 variable nucleotide positions from 34 isolates of the Mapé river basin and 117 variable nucleotide positions from 11 isolates of the Nyong river basin. For the analysis of the association between geographic and genetic distances of the sampled populations we performed a Mantel test using the function “mantel” asking for the Pearson’s product –moment correlation with 999 permutations in the R package “vegan” [29]. We did this for two sets of matrices, a smaller subset of 11 samples corresponding to the Nyong isolates, and the entire set of 33 samples corresponding to the Mapé isolates.

Results

M. ulcerans phylogeny in Cameroon

We sequenced the genomes of 82 *M. ulcerans* strains isolated from 45 IS2404 qPCR confirmed Cameroonian BU patients. Patients were identified between 2010 and 2012 and came from two geographically separated BU endemic regions of Cameroon, the Mapé and the Nyong river basins. Prior to treatment, ulcerative lesions were sampled with a cotton swab for laboratory confirmation of the clinical diagnosis, primary isolation of the disease causing organism and WGS of the isolated *M. ulcerans* strains.

The mapping of the obtained Illumina sequencing reads against the reference strain resulted in an average coverage of 380 reads per position per genome. We reconstructed the phylogenetic relationship among 45 Cameroonian (one strain per patient), five Ghanaian and three Beninese *M. ulcerans* isolates based on 26,740 variable nucleotide positions, rooting the tree using a published *M. marinum* genome (Fig. 1). The phylogenetic tree showed a very strong geographical structure. The chromosome tree of the Cameroonian *M. ulcerans* isolates showed two distinct lineages, the first one containing all the Nyong river basin isolates (Nyong lineage) and the second one all the isolates from the Mapé region (Mapé lineage). The Mapé river basin isolates were more closely related to a set of published genomes [13] of Ghanaian and Beninese isolates that we included in the analysis, than to the Nyong lineage. The two Cameroonian clonal complexes differed in altogether 828 SNPs shared by all members of the respective lineages (Fig. 1).

The plasmid phylogeny reflected the topology of the *M. ulcerans* chromosome phylogenetic tree (Fig. 1), supporting the hypothesis of a unique acquisition of the plasmid during the emergence of *M. ulcerans* [30] followed by parallel evolution between the chromosome and the plasmid.

Genetic diversity among the Cameroonian isolates

We analysed the genetic diversity within the two Cameroonian geographical lineages separately (Fig. 2). The genetic diversity observed for the Nyong river basin isolates (median pairwise SNP difference = 26.2 SNPs) was significantly higher (*p*-value < 0.0001) than for the Mapé basin isolates (median pairwise SNP difference = 7.6 SNPs). Furthermore, an analysis of the pairwise geographic distance of seven isolates from the Eastern Nyong river basin (approximately 1090 km^2) and of four isolates from Western Nyong (approximately 625km^2) still yielded values that were higher (median 24 and 36 pairwise SNP difference) than for the Mapé isolates (Fig. 2). The higher genetic diversity thus does not seem to be related to the broader geographical distribution for the Nyong river basin isolates

(approximately 8600 km^2) compared to the Mapé river basin isolates (approximately 6400 km^2) (Fig. 3, A2 and B2). These results were also reflected in the phylogenetic tree, where branch lengths were longer for the Nyong river basin strains than for the Mapé river basin isolates (Fig. 1). The gene encoding for rpoB, which is known to harbour drug resistance mutations against rifampicin in *M. tuberculosis* [31], was not affected by SNPs in any of the *M. ulcerans* strains analysed here.

When analysing the nucleotide diversity distribution along the chromosome by calculating the average nucleotide pairwise diversity per site (Pi) for both lineages, 99.9 % of the genome was found to be highly conserved (S1 Fig.). However, the average nucleotide diversity per site for the Nyong river basin lineage was 3.2 times higher than for the Mapé river basin lineage ($4.10e^{-6}$ *versus* $1.3e^{-6}$). The regions of the genome with higher nucleotide diversity ($0.375e^{-4}$ and $1.25e^{-4}$, respectively) seemed randomly distributed across the chromosome for both lineages (S2 Fig.) and the gene content of these regions varied between the two Cameroonian lineages, comprising affected genes of diverse functionalities (S3 Table).

Phylogeographic analysis of the Cameroonian *M. ulcerans* isolates

In order to analyse the distribution of genetic variants within the endemic areas, we reconstructed median joining networks for the sequenced strains and mapped the places of residence of the patients from which the strains originated (Fig. 3).

The network of the Mapé river basin isolates had a star structure with two isolates (BP130 and BP140) at the centre. All the other isolates were connected to this centre and separated by three to nine SNPs. While the SNP distance between the two central strains was zero, the geographical distance between the corresponding residence places of the two corresponding patients was 19.5 km. A total of four clusters were distinguished in the network: blue formed by two isolates, green and yellow formed by three isolates each and the red cluster as a complex structure formed by nine strains. The strains belonging to the red cluster shared two SNPs, the green ones also two SNPs, the yellow ones four and the blue ones shared three SNPs. All the grey strains were not forming clusters and differed by 1 to 11 SNPs from the central strains. In the network of the Nyong river basin isolates we observed only three clusters formed by three (green), seven (red) and one strain (blue). The isolates from the red cluster shared six SNPs, while the isolates from the green one shared only two SNPs. Overall, for both BU endemic areas in Cameroon we did not find a clear correlation between the genetic networks and the geographic distribution of the houses where the patients lived in the year prior to the onset of BU disease (Fig. 3). Statistical analysis with the Mantel test for the smaller subset of Nyong samples resulted in a positive and marginally significant correlation between the geographic and genetic distances ($r = 0.2785$, p-value = 0.054),

whereas the test performed for the Mapé set of isolates resulted in a small non-significant negative correlation (r = -0.04774, *p*-value = 0.676).

Rate of acquisition of SNPs

In the course of this genomic epidemiological study we obtained from three patients isolates from two or three different time points during the course of their disease (Table 1). When comparing the SNP diversity between the isolates from the same patient, only one SNP difference was observed between two sequential isolates (Table 1). The affected gene (MUL_1383) encoded for a hypothetical protein and the detected mutation was synonymous. No SNP difference was observed between two strains isolated from two distant ulcers of one patient (Table 1).

Discussion

Due to the limitations of conventional typing methods for the differentiation of strains belonging to the highly monomorphic African *M. ulcerans* population, use of WGS was suggested to reach sufficient analytical depth for molecular epidemiology studies [32]. Here our comparative genomic analysis of strains from two geographically separated BU endemic areas of Cameroon, the Mapé and the Nyong river basins, identified two phylogenetically distinct lineages of *M. ulcerans*. These data support previous findings that the spread of local clonal lineages between endemic areas only rarely occurs [13,14]. In a previous IS element - SNP based typing study most strains from the central region of Cameroon had the same SNP types as strains from neighbouring Gabon. The IS element - SNP type found in a strain from the Mapé river basin was also present across entire Central and West-Africa leading to the hypothesis that this lineage represents the founder of the other observed IS element - SNP types [15]. Our WGS analysis showed that the strains from the Mapé river basin are in fact more closely related to the *M. ulcerans* strain circulating in Ghana and Benin than the strains belonging to the Nyong river basin lineage. Additional WGS data with strains from all BU endemic African countries are required to shed more light on the spread and evolution of *M. ulcerans* in Africa and the origin of the locally observed two distinct Cameroonian lineages.

Analysis of the pairwise SNPs distance and nucleotide diversity distribution revealed a lower genetic diversity among the Mapé river basin strains than among the Nyong river basin strains. Epidemiological data suggest that *M. ulcerans* has expanded in the Mapé river area more recently than in the Nyong river basin. Descriptions of BU cases in the Nyong river basin exist since 1969 [17]. In contrast, clinically suspected cases of BU in the Mapé river area have been reported first only in 2004 [33]. While the disease may have preexisted there, epidemiological data strongly indicate that BU incidence has recently increased in the Mapé river basin [16,33]. Recent expansion of a clone may thus explain the more limited genetic diversity of the *M. ulcerans* lineage present in the Mapé river basin. It was speculated, that this expansion was associated with environmental changes caused by the damming of the Mapé river in 1989 [16,33]. Although it has been shown that cases associate more with the Mbam river as opposed to the Mapé dam directly [11,16], damming may have had an indirect effect on groundwater level and stagnant water bodies in the area.

We have compared the genome sequences of strains isolated at different time points over the course of the BU infection of three patients. In only one case, we detected a single SNP in one of the isolates compared to the strain isolated earlier from the same patient. It is not possible to conclude whether this observed single polymorphism is related to a re-infection

by a variant strain or to a point mutation that occurred either in the patient or during the *in vitro* cultivation. However, these data support the expectation of a low mutation rate in *M. ulcerans.*

Our analysis shows that WGS is an important tool for studying the local diversity and population structure of *M. ulcerans* in endemic areas and for resolving the evolutionary history of the pathogen. A combination of phylogenetic analysis with geographical information on the patient's home at the time of disease onset did not reveal a clear distribution pattern of the genetic variants. This may in part be related to the limited resolution of the comparative genomic analysis performed here. Resolution of the WGS typing could be further increased by inclusion of repetitive regions of the genome and the virulence plasmid that we so far excluded from the analysis, such as the IS2404 and PE/PPE regions. On the other hand, our sero-epidemiological analyses have provided evidence that exposure to *M. ulcerans* does not primarily occur at the homes of patients [34], but may rather be associated with more peripheral environmental water contact sites. Furthermore, for patients from the Mapé river basin it has been found that many of them move over long distances (in some cases >15 km) from their homes towards the Mbam river for their farming activities [11,16]. For genomic epidemiology studies it may therefore be necessary to establish detailed individual movement and environmental water contact patterns to follow the spatial-temporal spread of genetic variants.

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Author Contributions

Conceived and designed the experiments: MB MWB SK JCM AUB GP ALC. Performed the experiments: MB MWB SK MV PFB TJ ALC. Analyzed the data: MB MWB DB SRH GP ALC. Contributed reagents/materials/analysis tools: MV PFB TJ SRH JP. Wrote the paper: MB MWB DB SRH JP GP ALC.

Tables

Table 1. Genomic diversity among strains isolated from consecutive swab samples from the same BU lesion and among isolates originating from different lesions of the same patient.

Patient ID	Target lesion	Sampling time (days of/after treatment) or sampling location	Swab ID	Big pellet ID	Number of SNPs compared to the first sampling time point or the other sampling location respectively
BU02_038	1	Day 89	MB2_0057	BP39	
		Day 232	MB2_0192	BP29	0
		Day 337	MB2_0373	BP58	0
BU02_51	1	Day 0	MB2_061	BP53	
		Day 128	MB2_0305	BP25	0
BU02_061	1	Day 0	MB2_0292	BP71	
		Day 25	MB2_0477	BP59	1
BU2011_49	1	shoulder	MB2_0385	BP48	
	2	ankle	MB2_0473	BP67	0

Figures

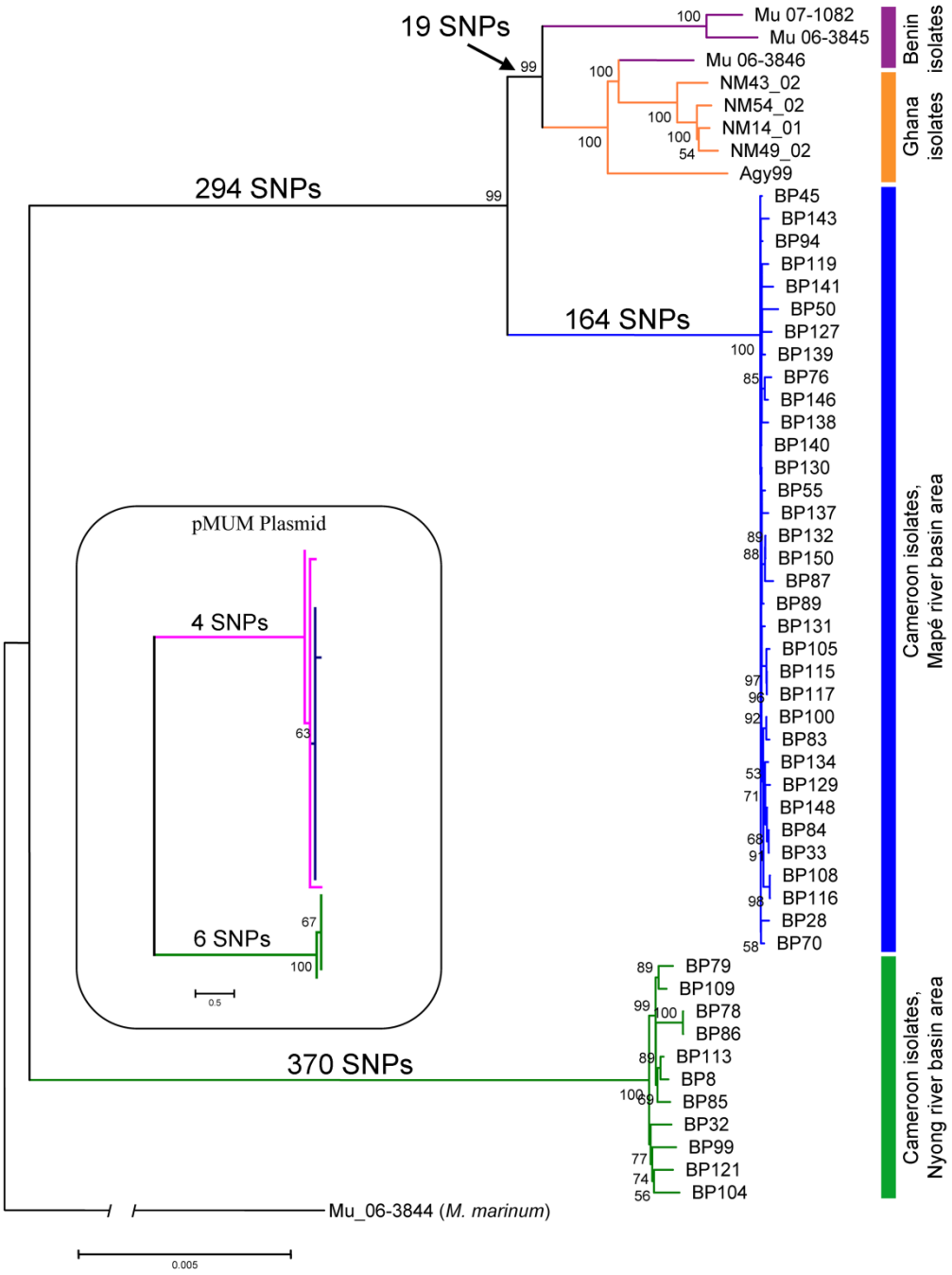

Figure 1: Phylogenetic reconstruction of Africa *M. ulcerans* strains.

Maximum-likelihood phylogenetic tree based on 26,740 variable nucleotide positions across 53 *M. ulcerans* strains by RAxML. The tree was rooted using *M. marinum* (Mu_06-3844) as outgroup. The geographical origin of the strains is indicated to the right of the tree and branches are coloured according to the region of isolation of the strains. Bootstrap values

higher than 50 % are shown along the branches. The insert shows the pMUM plasmid SNP tree based on 21 SNPs with the topology matching the corresponding chromosome-based tree.

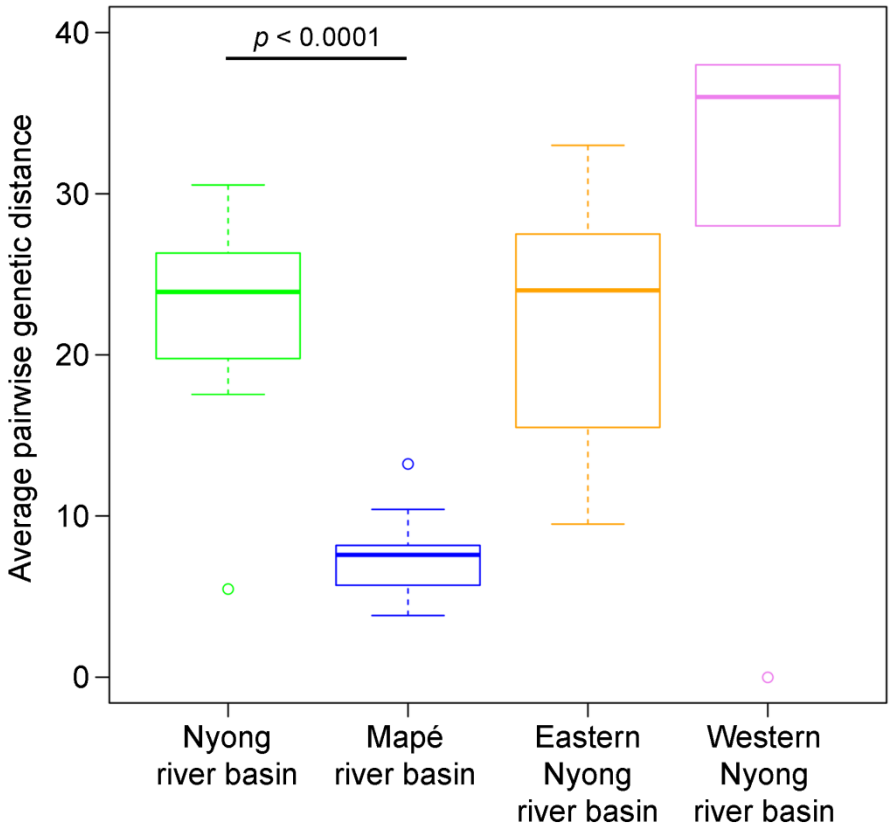

Figure 2: SNP pairwise distance between isolates from the same lineage.

The variation in the average SNP pairwise distance per genome for each lineage (Mapé and Nyong river basin strains) is shown in a box plot, with circles representing outlier sequence pairs. Two sub-areas of the Nyong river basin: the Eastern Nyong river basin and Western Nyong river basin, which are each smaller than the Mapé river basin area, are additionally shown. Statistical significance was assessed with the Wilcoxon rank sum test.

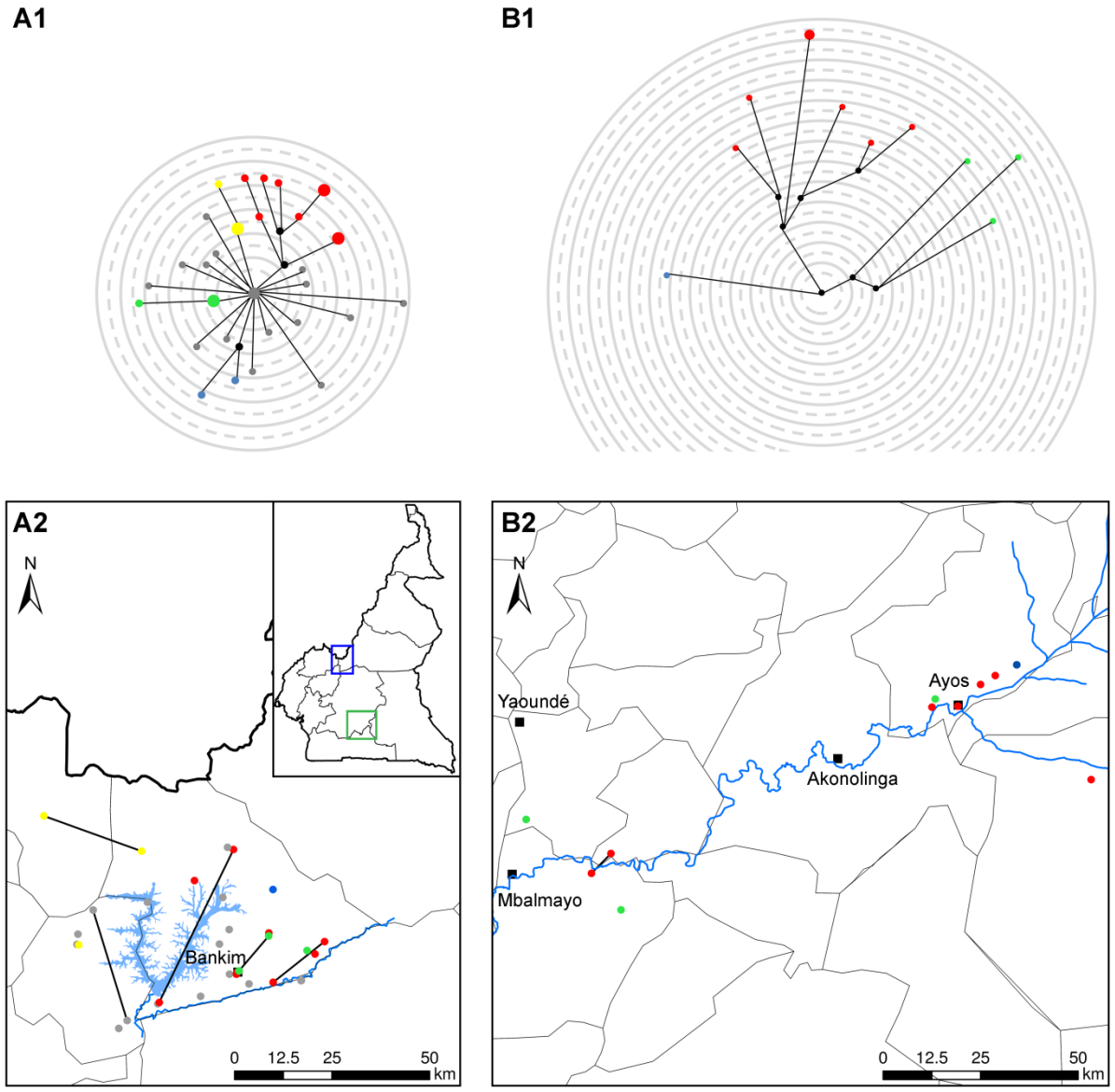

Figure 3: Phylogeographic analysis of *M. ulcerans* in the Mapé and Nyong river basins of Cameroon.

Detailed information about the places of residence of the patients for the year before the onset of BU disease was collected from all patients from whom *M. ulcerans* was isolated. Micro-evolutionary diversity of *M. ulcerans* at the two BU endemic sites is shown in panel A1 (Mapé river basin) and panel B1 (Nyong river basin). Median joining networks using 117 variable single nucleotide positions among whole genome sequences of *M. ulcerans* isolates from the Mapé river basin and 107 positions in isolates from the Nyong river basin area are depicted. Branch lengths correspond to the number of SNP differences, circle sizes correspond to the number of isolates and circle colours correspond to the sub-clusters defined. Black circles indicate median vectors (mv) and are hypothetical genotypes. Panels A2 (Mapé river basin) and B2 (Nyong river basin) show the geographic localization of the

patient’s residences with the locations colour coded according to the lineages shown in panel A1 and B1. When isolates of two patients were exactly the same, the respective homes of the patients are connected by a line. Major towns in the areas are indicated by black squares.

Supplementary Material

Supplementary Table 1: Potentially problematic regions for SNP calling identified by repeat-match.

Please use the link provided with the actual publication.

Supplementary Table 2: Mapping statistics and metadata for the bacterial isolates used in the study.

Isolate	Accession Number	Pellet Type	Year	Country	District	Match_to_ref	Yield Matching_kb
BP33	ERR350089	bulk	2010	Cameroon	Bankim	95.4	358,752
BP134	ERR350149	bulk	2012	Cameroon	Bankim	94.7	386,185
BP132	ERR350148	bulk	2012	Cameroon	Nwa	95.1	375,389
BP117	ERR350137	bulk	2012	Cameroon	Bankim	95	347,643
BP129	ERR350145	bulk	2012	Cameroon	Bankim	94.6	452,945
BP140	ERR350153	bulk	2012	Cameroon	Bankim	94.1	451,221
BP130	ERR350146	bulk	2012	Cameroon	Malantouen	94.6	451,126
BP139	ERR350151	bulk	2012	Cameroon	Bankim	94.8	369,387
BP144	ERR350156	bulk	2012	Cameroon	Bankim	93.8	358,116
BP143	ERR350154	bulk	2012	Cameroon	Malantouen	95.5	433,066
BP146	ERR350158	bulk	2012	Cameroon	Foumban	95.5	369,167
BP141	ERR350155	bulk	2012	Cameroon	Bankim	94.2	355,503
BP150	ERR350160	bulk	2012	Cameroon	Bankim	95.5	355,612
BP148	ERR350159	bulk	2012	Cameroon	NA	95.1	361,366
BP46	ERR350095	bulk	2011	Cameroon	Malantouen	95.1	399,657
BP52	ERR350098	bulk	2011	Cameroon	Malantouen	94.9	399,164
BP89	ERR350124	single	2011	Cameroon	Malantouen	94.5	366,566
BP72	ERR350112	bulk	2011	Cameroon	Bankim	93.3	427,248
BP83	ERR350119	single	2011	Cameroon	Bankim	95.4	438,866
BP100	ERR350127	bulk	2011	Cameroon	Bankim	93.7	425,243
BP31	ERR350087	bulk	2011	Cameroon	Malantouen	94.9	403,037
BP40	ERR350093	bulk	2011	Cameroon	Malantouen	95.1	344,030
BP66	ERR350106	bulk	2011	Cameroon	Malantouen	95.1	422,133
BP81	ERR350117	single	2011	Cameroon	Malantouen	95.1	363,826
BP87	ERR350123	single	2011	Cameroon	Malantouen	94.5	354,147
BP20	ERR350079	bulk	2011	Cameroon	Yoko	95.1	359,093
BP50	ERR350097	single	2011	Cameroon	Yoko	93.3	406,405
BP28	ERR350085	bulk	2011	Cameroon	Bankim	95.6	340,807
BP21	ERR350080	bulk	2011	Cameroon	Malantouen	94.9	354,131

BP62	ERR350104	bulk	2011	Cameroon	Malantouen	95.1	191,466
BP65	ERR350105	bulk	2011	Cameroon	Malantouen	95.2	376,201
BP70	ERR350110	bulk	2011	Cameroon	Malantouen	94.7	439,510
BP45	ERR350094	bulk	2011	Cameroon	Bankim	94.8	355,521
BP105	ERR350129	single	2011	Cameroon	Bankim	95.8	415,398
BP68	ERR350108	bulk	2011	Cameroon	Bankim	94.6	330,494
BP127	ERR350144	single	2011	Cameroon	Bankim	94.9	417,149
BP24	ERR350082	bulk	2011	Cameroon	Bankim	94.9	357,606
BP69	ERR350109	bulk	2011	Cameroon	Bankim	94.7	417,625
BP108	ERR350130	single	2011	Cameroon	Bankim	95.5	400,877
BP48	ERR350096	bulk	2011	Cameroon	Bankim	94.2	415,040
BP67	ERR350107	bulk	2011	Cameroon	Bankim	94.9	366,190
BP114	ERR350134	bulk	2011	Cameroon	Bankim	93.4	401,856
BP116	ERR350136	bulk	2011	Cameroon	Bankim	94.7	375,683
BP131	ERR350147	bulk	2011	Cameroon	Malantouen	94.4	386,429
BP115	ERR350135	bulk	2011	Cameroon	Bankim	94.4	388,573
BP120	ERR350139	bulk	2011	Cameroon	Bankim	94.9	331,099
BP119	ERR350138	bulk	2011	Cameroon	Yoko	95.3	399,766
BP137	ERR350150	NA	2011	Cameroon	Malantouen	94.9	416,258
BP38	ERR350091	bulk	2010	Cameroon	Bankim	94.9	357,979
BP76	ERR350114	single	2010	Cameroon	Bankim	94.6	441,493
BP75	ERR350113	bulk	2010	Cameroon	Bankim	94.7	427,529
BP84	ERR350120	single	2010	Cameroon	Bankim	95	405,420
BP126	ERR350143	single	2010	Cameroon	Bankim	95.3	84,645
BP55	ERR350100	bulk	2010	Cameroon	Bankim	95.2	361,665
BP94	ERR350125	bulk	2010	Cameroon	Bankim	95.8	357,855
BP22	ERR350081	bulk	NA	Cameroon	Mbalmayo	95.7	382,036
BP86	ERR350122	single	NA	Cameroon	Mbalmayo	96.1	431,925
BP27	ERR350084	bulk	NA	Cameroon	Mfou	95.6	296,495
BP29	ERR350086	bulk	NA	Cameroon	Mfou	96	335,712
BP34	ERR350090	bulk	NA	Cameroon	Mfou	95.6	452,999
BP39	ERR350092	bulk	NA	Cameroon	Mfou	95.4	375,911
BP58	ERR350101	bulk	NA	Cameroon	Mfou	95.5	359,868
BP6	ERR350076	bulk	NA	Cameroon	Mfou	95.7	353,664
BP78	ERR350115	single	NA	Cameroon	Mfou	95.8	380,280
BP32	ERR350088	bulk	NA	Cameroon	Ayos	95.4	380,516
BP25	ERR350083	bulk	NA	Cameroon	Ayos	95.9	326,183
BP53	ERR350099	bulk	NA	Cameroon	Ayos	95.3	380,115
BP79	ERR350116	single	NA	Cameroon	Ayos	95.6	380,813
BP82	ERR350118	single	NA	Cameroon	Ayos	94.5	386,405
BP8	ERR350077	bulk	NA	Cameroon	Ayos	95.6	393,378

BP112	ERR350133	bulk	NA	Cameroon	Ayos	95.4	361,027
BP113	ERR350157	bulk	NA	Cameroon	Ayos	95.5	382,611
BP104	ERR350128	single	NA	Cameroon	Ayos	95.6	426,105
BP110	ERR350132	single	NA	Cameroon	Ayos	95.8	397,694
BP59	ERR350102	bulk	NA	Cameroon	Ayos	95.4	384,369
BP71	ERR350111	bulk	NA	Cameroon	Ayos	95.7	416,769
BP121	ERR350140	bulk	NA	Cameroon	Mbalmayo	95.6	335,505
BP5	ERR350075	bulk	NA	Cameroon	Abong Mbang	95.6	363,269
BP85	ERR350121	single	NA	Cameroon	Abong Mbang	96.3	388,938
BP109	ERR350131	single	NA	Cameroon	Ayos	95.2	375,262
BP60	ERR350103	bulk	NA	Cameroon	Ayos	94.7	396,155
BP99	ERR350126	bulk	NA	Cameroon	Mbalmayo	94.8	467,194
BP138	ERR350152	bulk	2011	Cameroon	Bankim	95.2	363,763
NM 14_01	SRR073310	NA	2001	Ghana	Ga District	99.8	159,537
NM 43_02	SRR073314	NA	2002	Ghana	Ga District	99.8	156,814
NM 54_02	SRR073313	NA	2002	Ghana	Ga District	99.8	157,658
NM 49_02	SRR073312	NA	2002	Ghana	Ga District	99.8	149,352
Mu_06-3845	ITM001441	NA	2000	Benin	Houedja	91.6	80,435
Mu_06-3846	ITM971116	NA	1997	Benin	Lalo	91.0	69,676
Mu_07-1082	ITM030216	NA	2003	Benin	Adjohoun	90.8	69,823
Mu_06-3844	ITM063844	NA	2006	Belgium		89.3	210,427

Supplementary Table 3: Areas across the genomes with high Pi.

Area	Pi	Region	Genes	product	SNPs
Mapé	2.674E-04	38001..40900	MUL_0040	transcriptional regulatory protein (Whib-like), WhiB5	3S
Mapé	1.72E-04	4907201..4910100	MUL_4425	monooxygenase, wich function probably is involved in cellular metabolism	1S
Nyong basin	2.91E-04	159201..162100	MUL_0156	conserved hypothetical protein	1S
Nyong basin	2.91E-04	652901..655800	MUL_0630	conserved hypothetical membrane protein	1STOP
			MUL_0631	conserved hypothetical membrane protein	1N
			MUL_0632	3-oxoacyl-[acyl-carrier-protein] synthase III FabH	1N
Nyong basin	3.39E-04	4821301..4824200	MUL_4346	multi-functional enzyme with acyl-CoA-reductase activity AcrA1_1	1N, 3S
Nyong basin	4.36E-04	293401..296300	MUL_0288	conserved hypothetical protein	3N

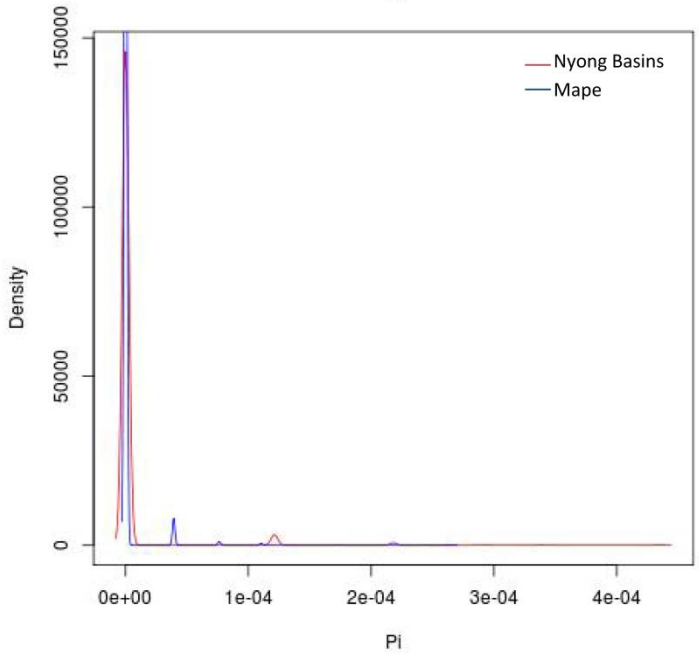

Figure S1: Distribution of chromosomal nucleotide diversity statistics.

Pi calculated on non-overlapping 1,500 pair sliding windows. Plots were drawn using the R density function.

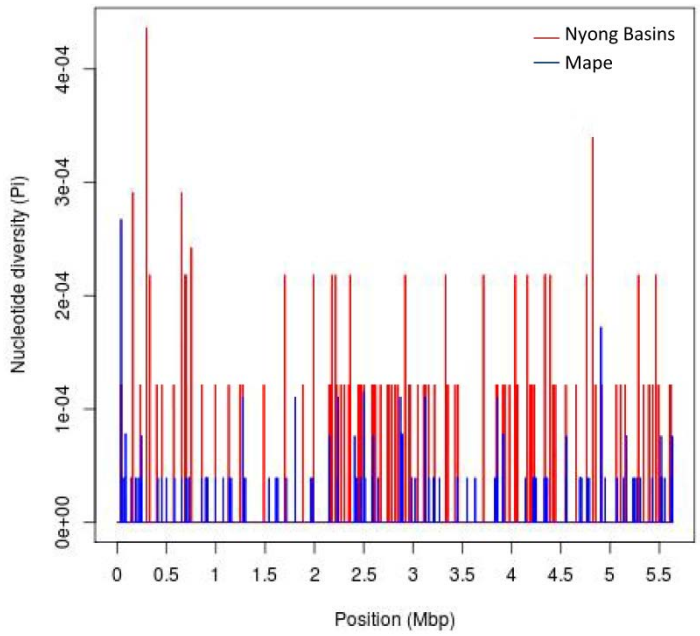

Figure S2: Nucleotide diversity (Pi) values along the length of the chromosome.

Chromosomes are represented linearly, using the coordinate system of the respective reference genomes with 0 on the far left (and far right).

References

1. Johnson PDR, Stinear T, Small PLC, Pluschke G, Merritt RW, et al. (2005) Buruli ulcer (*M. ulcerans* infection): new insights, new hope for disease control. PLoS Med 2: e108. doi:10.1371/journal.pmed.0020108.
2. WHO | Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer) (n.d.). WHO. Available: http://www.who.int/buruli/information/antibiotics/en/index16.html. Accessed 4 February 2014.
3. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, et al. (2010) Ecology and Transmission of Buruli Ulcer Disease: A Systematic Review. PLoS Negl Trop Dis 4: e911. doi:10.1371/journal.pntd.0000911.
4. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, et al. (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283: 854–857.
5. Hall B, Simmonds R (2014) Pleiotropic molecular effects of the *Mycobacterium ulcerans* virulence factor mycolactone underlying the cell death and immunosuppression seen in Buruli ulcer. Biochem Soc Trans 42: 177–183. doi:10.1042/BST20130133.
6. Stinear TP, Jenkin GA, Johnson PD, Davies JK (2000) Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. J Bacteriol 182: 6322–6330.
7. Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, et al. (2007) Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. Genome Res 17: 192–200. doi:10.1101/gr.5942807.
8. Hayman J (1993) Out of Africa: observations on the histopathology of *Mycobacterium* infection. J Clin Pathol 46: 5–9.
9. Fyfe JAM, Lavender CJ, Handasyde KA, Legione AR, O'Brien CR, et al. (2010) A major role for mammals in the ecology of *Mycobacterium ulcerans*. PLoS Negl Trop Dis 4: e791. doi:10.1371/journal.pntd.0000791.
10. Durnez L, Suykerbuyk P, Nicolas V, Barrière P, Verheyen E, et al. (2010) Terrestrial small mammals as reservoirs of *Mycobacterium ulcerans* in benin. Appl Environ Microbiol 76: 4574–4577. doi:10.1128/AEM.00199-10.
11. Bratschi MW, Ruf M-T, Andreoli A, Minyem JC, Kerber S, et al. (2014) *Mycobacterium ulcerans* persistence at a village water source of Buruli ulcer patients. PLoS Negl Trop Dis 8: e2756. doi:10.1371/journal.pntd.0002756.
12. Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, et al. (2008) First cultivation and characterization of *Mycobacterium ulcerans* from the environment. PLoS Negl Trop Dis 2: e178. doi:10.1371/journal.pntd.0000178.
13. Röltgen K, Stinear TP, Pluschke G (2012) The genome, evolution and diversity of *Mycobacterium ulcerans*. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis 12: 522–529. doi:10.1016/j.meegid.2012.01.018.
14. Doig KD, Holt KE, Fyfe JAM, Lavender CJ, Eddyani M, et al. (2012) On the origin of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. BMC Genomics 13: 258. doi:10.1186/1471-2164-13-258.
15. Röltgen K, Qi W, Ruf M-T, Mensah-Quainoo E, Pidot SJ, et al. (2010) Single nucleotide polymorphism typing of *Mycobacterium ulcerans* reveals focal transmission of buruli ulcer in a highly endemic region of Ghana. PLoS Negl Trop Dis 4: e751. doi:10.1371/journal.pntd.0000751.

16. Vandelannoote K, Jordaens K, Bomans P, Leirs H, Durnez L, et al. (2014) Insertion Sequence Element Single Nucleotide Polymorphism Typing Provides Insights into the Population Structure and Evolution of *Mycobacterium ulcerans* across Africa. Appl Environ Microbiol 80: 1197–1209. doi:10.1128/AEM.02774-13.

17. Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, et al. (2013) Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. PLoS Negl Trop Dis 7: e2252. doi:10.1371/journal.pntd.0002252.

18. Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, et al. (2007) Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. Appl Environ Microbiol 73: 4733–4740. doi:10.1128/AEM.02971-06.

19. Lavender CJ, Fyfe JAM (2013) Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples. Methods Mol Biol Clifton NJ 943: 201–216. doi:10.1007/978-1-60327-353-4_13.

20. Käser M, Ruf M-T, Hauser J, Marsollier L, Pluschke G (2009) Optimized method for preparation of DNA from pathogenic and environmental *mycobacteria*. Appl Environ Microbiol 75: 414–418. doi:10.1128/AEM.01358-08.

21. Harris SR, Clarke IN, Seth-Smith HMB, Solomon AW, Cutcliffe LT, et al. (2012) Whole-genome analysis of diverse Chlamydia trachomatis strains identifies phylogenetic relationships masked by current clinical typing. Nat Genet 44: 413–419, S1. doi:10.1038/ng.2214.

22. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, et al. (2009) The Sequence Alignment/Map format and SAMtools. Bioinforma Oxf Engl 25: 2078–2079. doi:10.1093/bioinformatics/btp352.

23. Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, et al. (2011) Rapid pneumococcal evolution in response to clinical interventions. Science 331: 430–434. doi:10.1126/science.1198545.

24. Holt KE, Parkhill J, Mazzoni CJ, Roumagnac P, Weill F-X, et al. (2008) High-throughput sequencing provides insights into genome variation and evolution in *Salmonella Typhi*. Nat Genet 40: 987–993. doi:10.1038/ng.195.

25. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725–2729. doi:10.1093/molbev/mst197.

26. Vilella AJ, Blanco-Garcia A, Hutter S, Rozas J (2005) VariScan: Analysis of evolutionary patterns from large-scale DNA sequence polymorphism data. Bioinforma Oxf Engl 21: 2791–2793. doi:10.1093/bioinformatics/bti403.

27. Noeske J, Kuaban C, Rondini S, Sorlin P, Ciaffi L, et al. (2004) Buruli Ulcer Disease in Cameroon Rediscovered. Am J Trop Med Hyg 70: 520–526.

28. Marion E, Landier J, Boisier P, Marsollier L, Fontanet A, et al. (2011) Geographic Expansion of Buruli Ulcer Disease, Cameroon. Emerg Infect Dis 17: 551–553. doi:10.3201/eid1703091859.

Results Chapter 4

Vaccination with the surface proteins MUL_2232 and MUL_3720 of *Mycobacterium ulcerans* induces antibodies but fails to provide protection against Buruli ulcer

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Abstract

Background: Buruli ulcer, caused by infection with *Mycobacterium ulcerans,* is a chronic ulcerative neglected tropical disease of the skin and subcutaneous tissue that is most prevalent in West African countries. *M. ulcerans* produces a cytotoxic macrolide exotoxin called mycolactone, which causes extensive necrosis of infected subcutaneous tissue and the development of characteristic ulcerative lesions with undermined edges. While cellular immune responses are expected to play a key role against early intracellular stages of *M. ulcerans* in macrophages, antibody mediated protection might be of major relevance against advanced stages, where bacilli are predominantly found as extracellular clusters.

Methodology/Principal Findings: To assess whether vaccine induced antibodies against surface antigens of *M. ulcerans* can protect against Buruli ulcer we formulated two surface vaccine candidate antigens, MUL_2232 and MUL_3720, as recombinant proteins with the synthetic Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion. The candidate vaccines elicited strong antibody responses without a strong bias towards a T_H1 type cellular response, as indicated by the IgG2a to IgG1 ratio. Despite the cross-reactivity of the induced antibodies with the native antigens, no significant protection was observed against progression of an experimental *M. ulcerans* infection in a mouse footpad challenge model.

Conclusions: Even though vaccine-induced antibodies have the potential to opsonise the extracellular bacilli they do not have a protective effect since infiltrating phagocytes might be killed by mycolactone before reaching the bacteria, as indicated by lack of viable infiltrates in the necrotic infection foci.

Author Summary

Buruli ulcer is a slow progressing ulcerative disease of the skin and subcutaneous tissue that is most prevalent in West African rural communities. *Mycobacterium ulcerans*, the causative agent of the disease, produces a toxin called mycolactone, which is held responsible for the extensive tissue damage seen in advanced Buruli ulcer lesions. To date, no effective vaccine against the disease exists and it is unclear to what extent antibodies against cell surface antigens of *M. ulcerans* play a role in protection. To assess whether vaccine induced antibodies against cell surface proteins can protect against Buruli ulcer, we formulated two surface vaccine candidate antigens, MUL_2232 and MUL_3720, as adjuvanted recombinant proteins and investigated their protective potential in a mouse model of *M. ulcerans* infection. Despite the induction of strong antibody responses against the surface molecules and cross-reactivity of the induced antibodies with the antigens in their native context, we did not observe protection against the disease. While the vaccine-induced antibodies could opsonize the extracellular bacilli, infiltrating phagocytes might be killed early by mycolactone.

Introduction

Buruli ulcer (BU) is a neglected tropical disease of the skin and subcutaneous tissue reported from over 30 countries worldwide. BU is most prevalent in West African countries like Cote d'Ivoire, Cameroon, Benin and Ghana [1,2]. *Mycobacterium ulcerans*, the causative agent of BU, produces a macrolide exotoxin called mycolactone, which is responsible for extensive necrosis of infected subcutaneous tissue leading to the development of large ulcerative lesions, if not treated at an early stage [3]. While extensive surgical removal of the diseased tissue has been the only treatment approach for a long time, since 2004 the WHO recommends eight weeks of combination chemotherapy with rifampicin and streptomycin [4]. This change in treatment strategy has substantially decreased both the amount of surgery required for treatment of extensive skin lesions as well as recurrence rates [5–7]. Nevertheless, BU has remained a huge socioeconomic burden in endemic regions of Africa. Affected populations are typically living in rural regions with limited access to health care services and limited financial resources, frequently resulting in delayed health care seeking and presentations with large ulcerative lesions, which take long time to heal [8,9].

Sero-epidemiological studies have shown that active BU only develops in some of the people exposed to *M. ulcerans* [10,11]. Together with reports on spontaneous healing of BU lesions [12,13] and the fact that the risk for young adults to develop BU is much smaller than for children [14], this observation suggests that development of protective immunity against BU is possible [15]. However, it is not clear which immune effector functions are important for protection. Cellular immunity is expected to play a key role in the early intracellular growth phase of *M. ulcerans* in macrophages [16–18]. However, induction of T_H1 responses by vaccination with Bacillus Calmette-Guérin (BCG) or a mycolactone negative *M. ulcerans* strain conferred only transient protection in an experimental mouse infection model [19]. Likewise, BCG vaccination seems to lead to cross-reactive immunity to severe forms of BU in clinical trials [20,21], but the BCG mediated induction of cellular response was not able to protect completely from *M. ulcerans* disease in either mice or humans [19–21]. In advanced BU lesions, in which clusters of extracellular bacilli dominate, antibodies against surface proteins of *M. ulcerans* may be of major importance for conferring protection [18,22,23]. In order to study this hypothesis, two *M. ulcerans* surface antigens, MUL_2232 and MUL_3720, were chosen in this study as vaccine candidate antigens. MUL_2232, the 18 kDa small heat shock protein of *M. ulcerans*, is an immunodominant cell wall associated protein with a homologue found in *M. leprae,* but not in *M. bovis* or *M. tuberculosis* [10]. MUL_3720 is a 22 kDa molecule with a predicted N-terminal lectin domain and a C-terminal peptidoglycan-binding domain with a putative role in cell attachment and cell-cell interaction [24] that is highly expressed on the surface of the bacilli [25].

Within the framework of a collaborative project funded by the European Commission (BuruliVac) our goal was to assess whether vaccine induced antibody responses against surface proteins of *M. ulcerans* are protective against BU. Here we present immunogenicity studies of MUL_2232 and MUL_3720 formulated as adjuvanted recombinant proteins with Alum, Sigma adjuvant (a squalene oil-in-water emulsion containing Monophosphoryl Lipid A (MPL) and synthetic trehalose dicorynomycolate) or EM048 (glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) adjuvant system [26,27]). Further, we assessed the potential of the induced immune responses to confer protection against experimental infection in a murine *M. ulcerans* infection model.

Materials and Methods

Ethical statement

All animal experiments performed were approved by the animal welfare committee of the Canton of Basel (authorization number 2375) and the Canton of Vaud (authorization number 2261) and were conducted in compliance with the Swiss animal protection law. Infection experiments with *M. ulcerans* were conducted under Biosafety-level-3 conditions at the École polytechnique fédérale de Lausanne (EPFL).

Expression and purification of recombinant *M. ulcerans* proteins

The potential protein vaccine candidate antigens MUL_2232 (GenBank accession number 4550596) and MUL_3720 (GenBank accession number 4553013) of *M. ulcerans* Agy99 were ordered as codon optimized genes for expression in human cells (GenScript) and received in pUC57 plasmids. Expression of the antigens as recombinant proteins in *E. coli* was achieved with the pET28a expression system (Novagen, modified to contain an ampicillin selection cassette). Briefly, restriction sites required for further cloning were attached by the use of specifically designed primers for amplification of the codon optimized sequences by polymerase chain reaction (PCR). Primer sequences for MUL_22232 amplification were 5'-TTCCTTCATATGCTGATGAGAACCGACCCTTTTAGA-3' and 5'-TTCCTTGCGGCCGCTCAAGCCTCAATCACTTCGGGA. Primer sequences for MUL_3720 amplification were 5'-TTCCTTCATATGAGCGATACTCTGACTGAAGGACAG-3' and 5'-TTCCTTGCGGCCGCGCTCAAGGAATAGTCAGGACCTCT-3'. PCR products were cut by the restriction enzymes NdeI and NotI (New England Biolabs) and subsequently ligated into pET28 to attach an N-terminal 6xHis-tag. After propagation of the generated plasmids in Top10 *E. coli* (Invitrogen), control restriction and sequencing of the plasmids ensured selection of appropriate clones for expression of the proteins. Protein expression was induced in *E. coli* BL21(DE3) strains (Invitrogen) by addition of 1 mM isopropyl thiogalacoside (Calbiochem) for 4 h at 37°C in lysogeny broth (LB) medium supplemented with Ampicillin. After screening for high level recombinant protein expression by analysis of small induced cultures, larger amounts of recombinant proteins were produced by selected expression clones.

Protein lysates were produced by dilution of the bacterial pellet in PBS, the addition of lysozyme and sonication. After removal of cellular debris by centrifugation, the 6xHis-tagged recombinant proteins were purified by nickel-nitrilotriacetic acetic (Ni-NTA) metal-affinity chromatography. Proteins were eluted with increasing concentrations of imidazole and the integrity and purity of proteins was assessed by SDS-page separation and Coomassie Blue staining (S1 Fig). Final concentrations of the produced recombinant proteins rMUL2232 and

rMUL3720 were determined by BCA assay (Pierce) according to the manufacturer's instructions.

Adjuvant formulations

Oil-in-Water formulated TLR-4 agonist (GLA-SE, EM048) was produced by the Infectious Disease Research Institute (IDRI) [26,27]. EM048 was mixed with recombinant protein in PBS to a final concentration of 50 µg/ml EM048 and 200 µg/ml recombinant protein. Sigma adjuvant system (Sigma) was reconstituted according to the manufacturer's instructions and combined with recombinant protein to a final concentration of 200 µg/ml recombinant protein. Imject Alum (Thermo Scientific) was mixed with recombinant protein according to the manufacturer's instructions to a final volume ratio of Imject Alum to immunogen of 1:1 and a final concentration of 200 µg/ml recombinant protein.

Immunization of mice

Immunogenicity of the described vaccine formulations was studied in 8 week old female BALB/c mice (Janvier). Groups of five mice were immunized three times by the subcutaneous (s.c.) route in the scruff of the neck with 100 µl of the adjuvanted proteins in three week intervals. Prior to the first immunization as well as before every new immunization mice were bled by the tail vein and serum gained by centrifugation of the blood in SST Microtainer tubes (Becton, Dickinson and Company). An additional blood collection was performed three weeks and six months after the last immunization.

Enzyme-linked immunosorbent assays (ELISA)

Serum immunoglobulin G (IgG) antibody titers were determined by ELISA on recombinant protein with all incubation steps performed at room temperature (RT). 10 µg/ml of rMUL2232 or rMUL3720, respectively, were coated on ELISA plates (Maxisorp; Nunc) by incubation overnight. After blocking with 5% skim milk/PBS for 1 hour, plates were incubated with dilution series of sera from immunized mice in 0.5% skim milk/PBS for two hours, washed and incubated with alkaline phosphatase-conjugated goat anti-mouse monoclonal antibody (mAb; Sigma) as secondary antibodies for 1 hour. Plates were washed prior to development with *p*-nitrophenyl phosphate (Sigma) as substrate. The optical density (OD) of the reaction product was measured at 405 nm with a microplate absorbance reader (Sunrise Absorbance Reader; Tecan). The threshold for endpoint titer determination was defined as the double of the mean measurements plus the mean standard deviation of a dilution series done without primary antibody and a dilution series done with pre-bleed serum. Individual serum dilution series were approximated with sigmoidal dose-response curves and the reciprocal dilution of the intersection between the curve and the threshold was defined as individual endpoint titer (GraphPad Prism software, Graph-Pad Software Inc.).

For the determination of IgG subclasses, ELISA was performed as described above with the use of subclass specific alkaline phosphate-conjugated secondary antibodies (Southern Biotech).

Western blot analysis

According to the manufacturer's instructions, 10 µg of *M. ulcerans* whole cell lysate was loaded on a prefabricated 4-12% gradient gel (NuPAGE Novex 4-12% Bis-Tris Gel; Invitrogen) with MES running buffer under reducing conditions. A dry-blotting system (iBlot; Invitrogen) was used to electrophoretically transfer the separated proteins to nitrocellulose membranes, which were subsequently blocked in 5% skim milk/PBS over night at 4°C. Membranes were cut into strips and individually incubated with appropriate dilutions of serum of immunized mice in 1% skim milk/0.05% Tween20 / PBS for 1 hour at RT. After several washing steps in 1% skim milk/0.05% Tween20/PBS, a HRP-conjugated goat anti-mouse IgG γ-chain mAb (Southern Biotech) was used as secondary antibody and incubated for 1 hour at RT. Excess antibody and skim milk residuals were washed away with PBS and blots were then developed using ECL Western blotting detection reagents (ECL Western blotting Substrate; Pierce).

For the determination of the Western blot endpoint titers, individual dilution series of sera were processed as described above. Development of the entire set of strips by the ECL system was done on one single film. Development time was chosen as the shortest time needed for detecting a specific signal in at least one strip for every serum dilution, e.g. lowest dilution, and for every dilution series of individual sera at least one strip with no specific signal, e.g. highest dilution. Western blot endpoint titers were defined as the reciprocal value of the last dilution that yielded a specific band in Western blotting on the film.

Immunofluorescence assays on paraffin embedded *M. ulcerans*

Immunofluorescence assays with sera of immunized mice on paraffin embedded *M. ulcerans* bacteria were performed as previously described [25]. Briefly, African *M. ulcerans* isolates were embedded into paraffin, cut into 3 µm thin sections and mounted on Superforst Plus glass slides (Thermo Scientific). Sections were then deparaffinised, rehydrated and pre-treated with 1mM EDTA buffer pH=8 for epitope retrieval as described for tissue sections in immunohistochemistry [28]. Unspecific binding was prevented by incubation of the bacteria in 1.5% goat serum in PBS for 1 hour at RT. Appropriately diluted mouse mAbs specific for MUL_2232 and MUL_3720 were used as primary antibodies. Detection of the specific binding of primary antibodies was done with an Alexa488 labelled secondary goat anti-mouse total IgG (H+L) antibodies (Life Technologies). Image acquisition was performed on a confocal laser microscope (Carl Zeiss, Axiovert 200M).

Active immune protection experiments in mice

Active immune protection experiments were conducted with groups of eight female 8 weeks old BALB/c mice. Mice were immunized twice s.c. in the scruff of the neck with 100 µl of the adjuvanted proteins in three week intervals. Three weeks after the second immunization and prior to infection with *M. ulcerans,* mice were bled by the tail vein and successful immunization was verified by testing the sera for specific antibodies in ELISA and Western blotting as described above. All *M. ulcerans* infection experiments were conducted under BSL-3 conditions.

The *M. ulcerans* strain S1013 used for the experimental infection of mice was isolated in 2010 from the ulcerative lesion of a Cameroonian BU patient [29]. Bacteria were cultivated in BacT/ALERT medium (Biomerieux) for six weeks, recovered by centrifugation and diluted in sterile PBS to 125 mg/ml wet weight. Mice were infected with 1.5×10^6 (high dose) or 1.5×10^5 (low dose) bacteria in PBS into the left hind foot pad three weeks after the last immunization.

The course of the infection was followed by weekly measurements of the foot pad thickness with a caliper. At days 63 (high dose) and 87 (low dose) after experimental infection, mice were euthanised, blood samples harvested through cardiac puncture and foot pads aseptically removed for enumeration of *M. ulcerans* bacteria or histopathology. Mouse foot pads designated for enumeration of *M. ulcerans* bacteria were dipped in 70% ethanol, dried under the laminar flow, cut into four pieces with a scalpel and transferred to reinforced hard tissue grinding tubes (MK28-R, Precellys) containing 750 µl of BacT/ALERT medium. Tissue homogenization was performed with a Precellys 24-Dual tissue homogenizer (3 x 20 s at 5000 rpm with 30 s break), the lysate was transferred to a clean tube and the lysis tube still containing tissue residuals refilled with additional 750 µl of BacT/ALERT medium. The remains were homogenized a second time as described above and the individual two lysates were pooled [30].

DNA from 100 µl of a 1:50 dilution of the foot pad lysate in PBS was extracted as described by Lavender and Fyfe [31]. Extracted DNA was then analysed for insertion sequence (IS) 2404 by quantitative (q) PCR as previously described [31]. For graphic representation of the results, cycle threshold (Ct) values were converted into genome copy numbers per foot pad by applying a standard curve established for IS2404 by *Fyfe et al*. [32].

Histopathology

Mouse foot pads designated for histopathological analysis were removed above the ankle and immediately transferred to 10% neutral-buffered formalin solution (approx. 4% formaldehyde, Sigma) for fixation during 24 hours at room temperature. Subsequently, the foot pads were decalcified in 0.6 M EDTA and 0.25 M citric acid for 12 days at 37°C and

transferred to 70% ethanol for storage and transport. Foot pad samples were dehydrated and embedded into paraffin. 5 µm thin sections were cut, deparaffinised, rehydrated, and stained with Haematoxylin/Eosin (HE, Sigma, J.T. Baker) or Ziehl-Neelsen/Methylene blue (ZN, Sigma) according to WHO standard protocols [33]. Stained sections were mounted with Eukitt mounting medium (Fluka). Pictures were taken with a Leica DM2500B microscope or with an Aperio scanner.

Results

GLA-SE adjuvanted recombinant *M. ulcerans* protein formulations elicited specific antibody responses

The two *M. ulcerans* vaccine candidate antigens MUL_2232 and MUL_3720 were expressed as 6xHis-tagged recombinant proteins in *E. coli* and purified via a Ni-NTA column (S1 Fig). Mice were immunized three times with either 20 µg of MUL_2232 or MUL_3720 formulated with the human-compatible GLA-SE adjuvant EM048. Alum and Sigma adjuvant were used as control adjuvants. ELISA with mouse sera on the respective recombinant proteins showed that all formulations elicited robust antigen specific serum IgG responses (Fig 1) dominated by IgG1 and only a minor proportion of IgG3 (Fig 2, A1 and B1).

Western blotting analyses of sera against *M. ulcerans* lysates showed specific bands of the expected molecular weight of MUL2232 and MUL3720 (Fig 3A, S2 Fig, A). Sera of immunized mice also recognized *M. ulcerans* bacteria in an indirect immunofluorescence assay (IFA) performed on paraffin embedded *M. ulcerans* bacteria (Fig 3B, S2 Fig, B). For both target antigens the previously demonstrated surface localization [25] was confirmed. Six months after the last immunization, antibody responses in all groups of immunized mice had dropped significantly. However, specific antibody titres were still higher than in the pre-immune sera and sufficient to elicit signals in Western blotting analyses (Fig 2, A3 and B3).

Assessment of protectivity of the vaccine induced antibody responses in an experimental *M. ulcerans* infection mouse model

Given that antigens formulated with the human-compatible EM048 elicited higher total IgG responses after two injections and with similar IgG1, but higher IgG2a, IgG2b, IgG3 antibody levels compared to formulation with Alum, we determined the protective potential of these vaccine formulations in an experimental *M. ulcerans* infection mouse model. Because the increase of total IgG titers after a third immunization was not significant (Fig 1), groups of eight mice were immunized twice with rMUL3720/EM048. Three weeks after the second immunization mice were infected into the left hind foot pad with an inoculum of 1.5×10^6 (high dose) or 1.5×10^5 (low dose) of *M. ulcerans* bacilli. The course of the infection was followed by weekly measurements of the foot pad thickness with a caliper. Mice in all groups infected with the high dose of bacteria showed first signs of inflammation and foot pad swelling seven weeks after infection (Fig 4, A). Swelling gradually increased over time, until mice had to be euthanised at day 63 and the bacterial load was determined by qPCR (Fig 4, B). Compared to the amount of *M. ulcerans* DNA that was contained in the inoculum, a roughly 250 times increase had occurred both in immunized and control animals during the 63 days of infection (Fig 4, B).

Histopathological analysis of representative foot pads revealed the presence of oedema (Fig 5, B1, B4) and slight infiltration at the site of infection (Fig 5, B6) as well as at the heel of the foot pad (Fig 5, B2). Acid fast bacilli (AFB) were found at all sites where infiltration occurred (Fig 5, B3, B5, B7).

In mice infected with the low dose inoculum, foot pad swelling started seven to eight weeks after infection (Fig 4, A) and the increase in *M. ulcerans* DNA content was about 9500 fold in 87 days. Also with the lower challenge dose no difference in bacterial load was observed between immunized and control immunized animals (Fig 4, B). Large clumps of AFB were found in all infected foot pads irrespective of the immunization status of the mice (Fig 5, C1 - C2, D1. AFB occurred in clumps (Fig 5, C2), organized within filamentous structures principally located in oedematous tissue (Fig 5, C3, D3) and in close contact with infiltrating cells (Fig 5, D2). Similarly, rMUL2232/EM048 did not induce any protective effect (S3 Fig).

M. ulcerans infection failed to boost immunization-induced antibody responses

Antibody titers in immunized mice before infection (S4 Fig) and after 63 days of infection were directly compared to elucidate whether the immunization-induced antibody responses were increased by exposure to the target antigens in the native context of the infecting bacilli. No booster effect was observed after infection with the high dose inoculum of *M. ulcerans* (Fig 6, A1 and A2). On the contrary, both ELISA and Western blotting analyses demonstrated a significant drop in specific antibody titres after infection (Fig 6, B). Furthermore, neither non-immunized nor control-immunized animals raised a specific antibody response against rMUL2232 (Fig 6, A1) or rMUL3720 (Fig 6, A2) and Western blotting analyses of these control sera revealed a profound lack of any *M. ulcerans* specific antibody responses in the course of infection (Fig 6, B).

Discussion

Currently, there is no highly effective vaccine against the mycobacterial diseases tuberculosis, leprosy and BU available. BCG was originally developed as a vaccine against tuberculosis, but, dependent on the study site, a protective efficacy ranging from 20% to 90% was also observed against leprosy [34,35]. Similarly, BCG was found to offer a short-lasting protection of 47% against BU in a controlled clinical trial in Uganda [20], reconfirming results of a previous smaller trial [36]. However, case-control studies have failed to provide evidence of a lasting protective effect of routine BCG vaccination against BU [37–39]. Yet in *M. ulcerans* mouse infection models any other vaccine candidate has so far outperformed the effectiveness of BCG [18].

As a partner in the collaborative research project BuruliVac, we seeked to assess the potential to develop a protein based subunit vaccine against BU. Here we report results obtained with two cell surface exposed *M. ulcerans* proteins formulated with the adjuvant EM048. In spite of the development of robust humoral immune responses, none of the vaccination formulations tested conferred protection in the experimental *M. ulcerans* mouse foot pad infection model.

Choosing potential protective antigens for the inclusion into a protein subunit vaccine against BU was difficult, since the nature of protective immune responses against the disease is unclear. Given the mainly extracellular location of *M. ulcerans* in advanced lesions, the choice of surface exposed proteins seemed most attractive. Additionally, *M. ulcerans* specific proteins were of preference, because it was already observed that proteins from the closely related pathogens *M. bovis* and *M. leprae* were not very effective in conferring cross-protectivity, despite high sequence homology [40,41]. We have chosen MUL_2232 for its known surface localization, its strong immunogenicity and its missing homolog in *M. tuberculosis* [10]. The second candidate, MUL_3720, was identified in a screen for potential diagnostic antigens conducted in our laboratory [24]. Homologs of MUL_3720 are absent in other mycobacterial pathogens prevalent in BU endemic areas. Furthermore, it is highly expressed and most importantly it is located on the surface of *M. ulcerans* [25].

In light of current literature available on protective immunity to BU we have chosen to formulate the selected antigens with the human compatible adjuvant GLA-SE. GLA is a synthetic Toll-like receptor 4 agonist, that has been demonstrated to confer potent adjuvant activity for various antigens [26,42–44] when formulated with a squalene based oil-in-water stable emulsion (SE). While SE is an adjuvant on its own, the addition of GLA biases the induced cell mediated immunity (CMI) toward a T_H1 type immune response, an observation made for several antigens tested so far [42,45]. However, formulations of the recombinant

proteins rMUL2232 and rMUL3720 with EM048, the specific GLA-SE adjuvant used in this study, did not lead to such a clear shift of CMI towards T_H1. Yet compared to Alum, which is still the adjuvant most commonly used in human vaccines, EM048 induced significantly higher antibody titres with both recombinant proteins investigated. All mice immunized with adjuvanted recombinant protein vaccine-candidates and later challenged with *M. ulcerans* had mounted strong specific antibody responses, which were cross-reactive with the antigens in the native context on the bacterial cell surface. Nevertheless, these mice were not protected from disease. Opsonisation of the bacteria by the immunization-induced antibodies may not lead to protection, since the clusters of the extracellular *M. ulcerans* found in advanced BU lesions are imbedded in necrotic tissue. Infiltrating macrophages are therefore not able to reach the bacteria, since infiltrating cells seem to be killed by mycolactone before reaching the infection foci. On the other hand, it is not completely ruled out that antibodies against other target structures may be protective. Neutralizing antibodies against the poorly immunogenic macrolide toxin mycolactone for example could potentially confer protection against disease. The fact that the response of immunized mice was not boosted upon progressive infection with *M. ulcerans* could hint to another obstacle for vaccine development against BU. The apparent general lack of antibody responses in non-immunized challenged control mice that was observed by us and others [23] is surprising, and requires further investigation. A number of studies have reported systemic T-cell anergy in patients with BU, but development of antibody responses against *M. ulcerans* was detected in a majority of patients [10,46–48].

In the framework of this study we have developed methods to evaluate the protective capacity of candidate vaccines in the *M. ulcerans* mouse footpad infection model. The fact that we did not see protection in immunized animals could have many explanations; including insufficient bias towards a T_H1 type of CMI. Most likely immunization with only one antigen is generally not sufficient for protection against disease. Considering that vaccination with a mycolactone deficient mutant strain of *M. ulcerans* did not lead to full protection in the mouse model [19], the development of a multivalent subunit vaccine may be the right strategy to pursue.

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Author Contributions

Conceived and designed the experiments: MB GP. Performed the experiments: MB, SK, AV. Analyzed the data: MB GP. Contributed reagents/materials/analysis tools: AMD, WO, MS, MSD. Wrote the paper: MB AB GP.

Figures

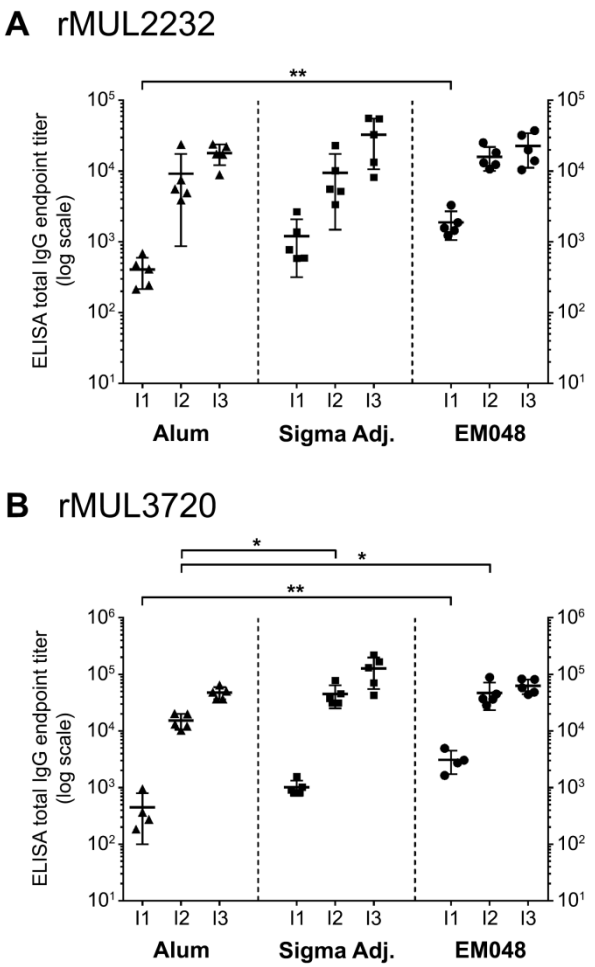


Figure 1. Immunogenicity of recombinant protein/EM048 formulation in comparison to commercially available adjuvants for mice.

Groups of five BALB/c mice were immunized three times in three week intervals with 20 µg of rMUL2232 (A) or rMUL3720 (B) formulated with Alum, Sigma Adjuvant or EM048. Serum three weeks after every immunization (I1, I2 and I3) was analysed in ELISA on the respective recombinant protein. Depicted are individual endpoint IgG titers as determined in one single ELISA, the mean (line) ± standard deviation. Overall statistical significance was calculated by Kruskal-Wallis test per immunization time point (A: P_{I1} = 0.0029, P_{I2} and P_{I3} = ns.; B: P_{I1} = 0.0009, P_{I2} = 0.0029, P_{I3} = ns.). Individual statistical differences between groups were assessed by the Dunn procedure and are depicted if detected (* $p \leq 0.05$; ** $p \leq 0.01$).

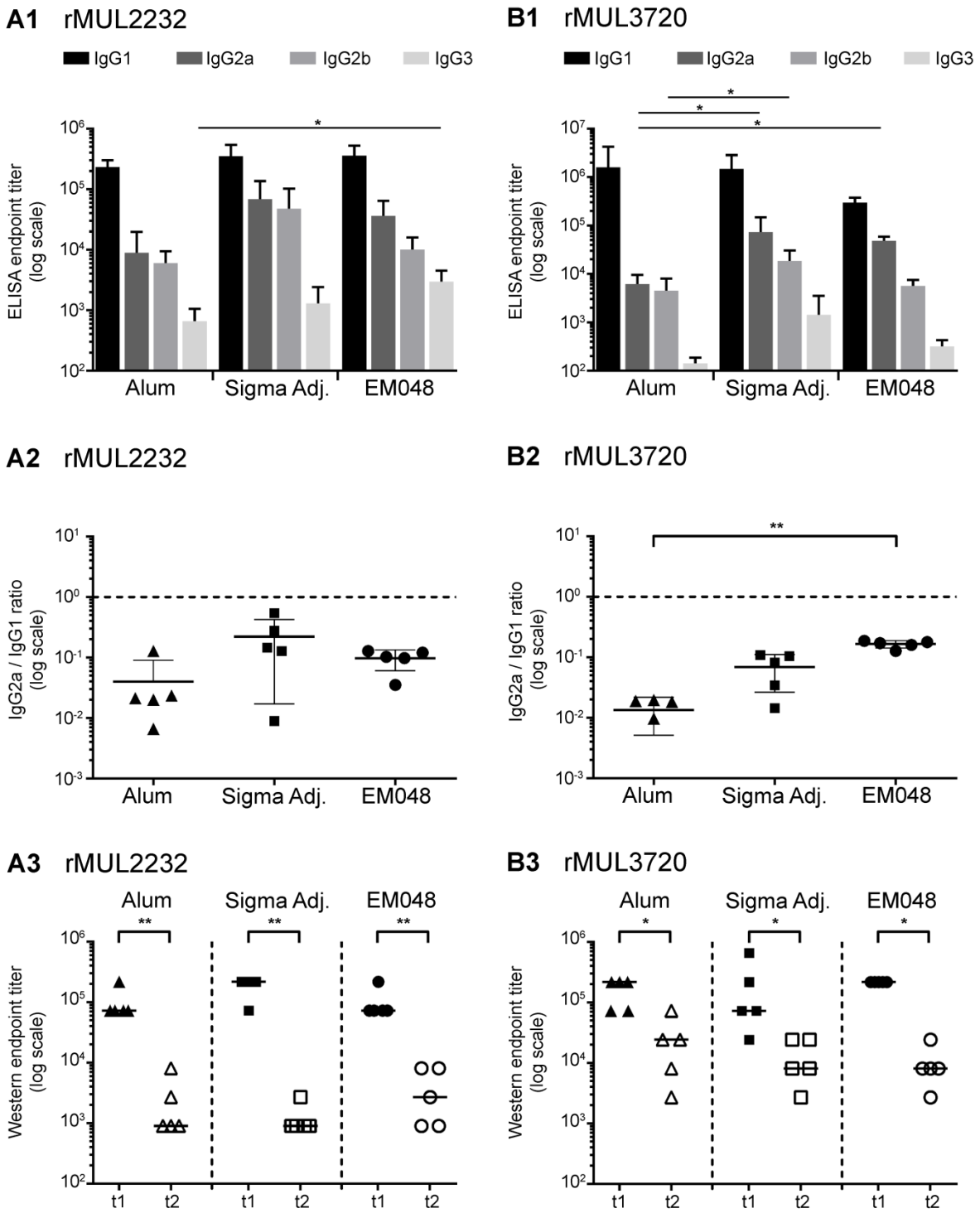


Figure 2. Assessment of Immunoglobulin G subclasses and the longevity of antibody responses induced by immunization.

Immunoglobulin G (IgG) subclasses were determined in serum collected three weeks after the third immunization with rMUL2232 (A1) and the indicated adjuvant or rMUL3720 (B1) and the indicated adjuvant. ELISA on recombinant protein was performed with secondary antibodies specific for four IgG subclasses: IgG1 (black), IgG2a (dark grey), IgG2b (light

grey), IgG3 (very light grey). Depicted are the mean individual endpoint IgG titers determined in one single ELISA (bar), and the standard deviation (error bar). Overall statistical significance was calculated by Kruskal-Wallis test (A1: P_{IgG1} = ns., P_{IgG2a} = 0.0396, P_{IgG2b} = ns., P_{IgG3} = 0.0375; B1: P_{IgG1} = ns., P_{IgG2a} = 0.0029, P_{IgG2b} = 0.0431, P_{IgG3} = ns.). Individual statistical differences between groups were assessed by the Dunn procedure and are depicted if detected (* $p \leq 0.05$).

The ratio of IgG2a to IgG1 was determined for individual animals accordingly (A2 and B2). Depicted are the individual values, the mean IgG2a to IgG1 ratio (bar) and the standard deviation (error bar). Overall statistical significance was calculated by Kruskal-Wallis test (A2: P = ns.; B2: P = 0.0001). Individual statistical differences between groups were assessed by the Dunn procedure and are depicted if detected (** $p \leq 0.01$).

Total IgG titers in individual immunized mice three weeks (t1) and six months (t2) after the third immunization were compared by Western blotting on *M. ulcerans* lysate. Depicted are individual Western blot endpoint titers and the median per group (line) for rMUL2232 immunized animals (A3) and rMUL3720 immunized animals (B3). Statistical significance was assessed by Mann-Whitney test and is depicted if detected (* $p \leq 0.05$; ** $p \leq 0.01$).

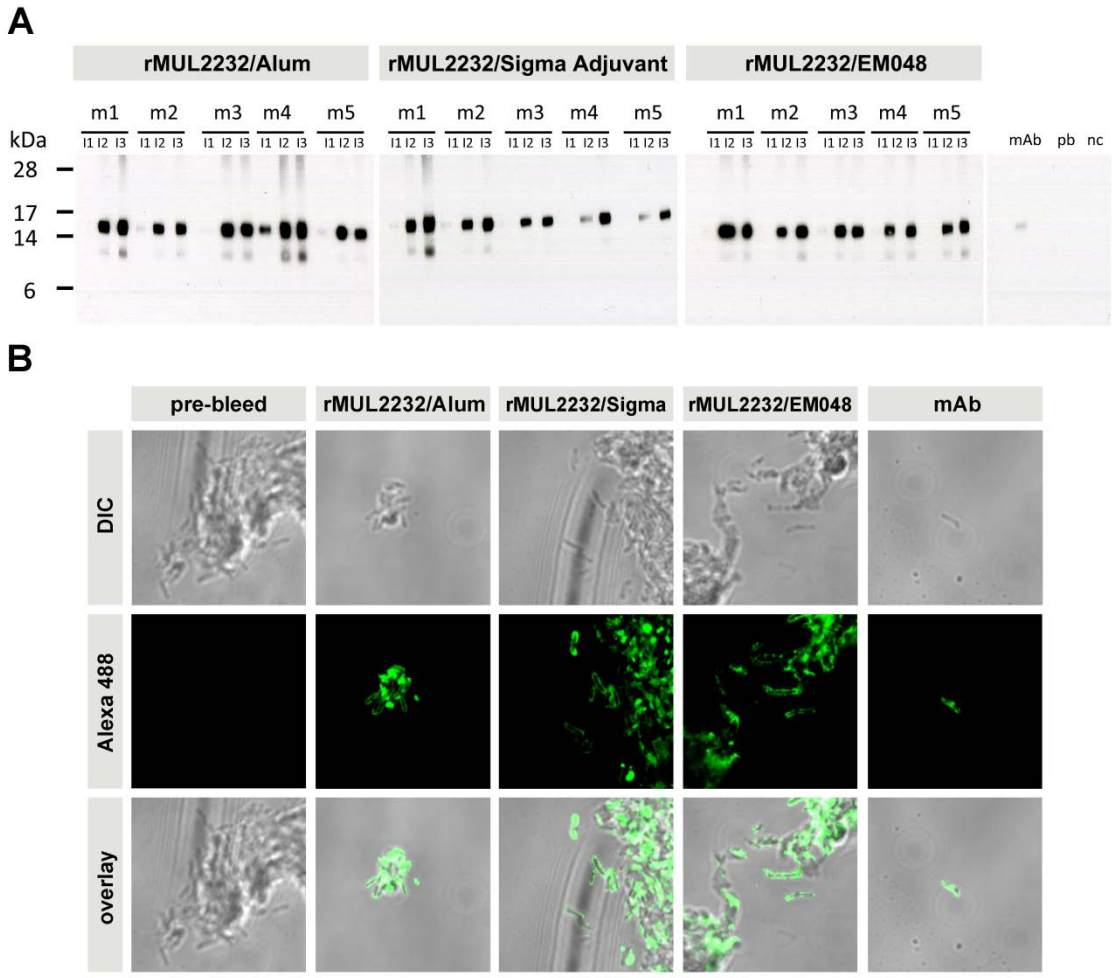


Figure 3. Cross reactivity of the immune sera with *M. ulcerans*.

(A) Groups of five BALB/c mice (m1 – m5) were immunized three times in three week intervals with 20 µg of rMUL2232 formulated with Alum, Sigma Adjuvant or EM048. Serum three weeks after every immunization (I1, I2 and I3) was analysed by Western blotting on *M. ulcerans* lysate. Monoclonal anti-MUL_2232 antibody (mAb) served as positive control, pre-bleed (pb) serum or no primary antibody (nc) as negative controls.

(B) Sera from three weeks after the third immunization with rMUL2232 and indicated adjuvant were used for indirect immunofluorescence staining on paraffin embedded *M. ulcerans* bacteria with an Alexa488 labelled secondary antibody. Pre-bleed serum did not stain the bacteria. Sera of immunized mice (a mix of sera from five individual mice per group) did reveal surface staining similar to the staining achieved with anti-MUL_2232 monoclonal antibody (mAb).

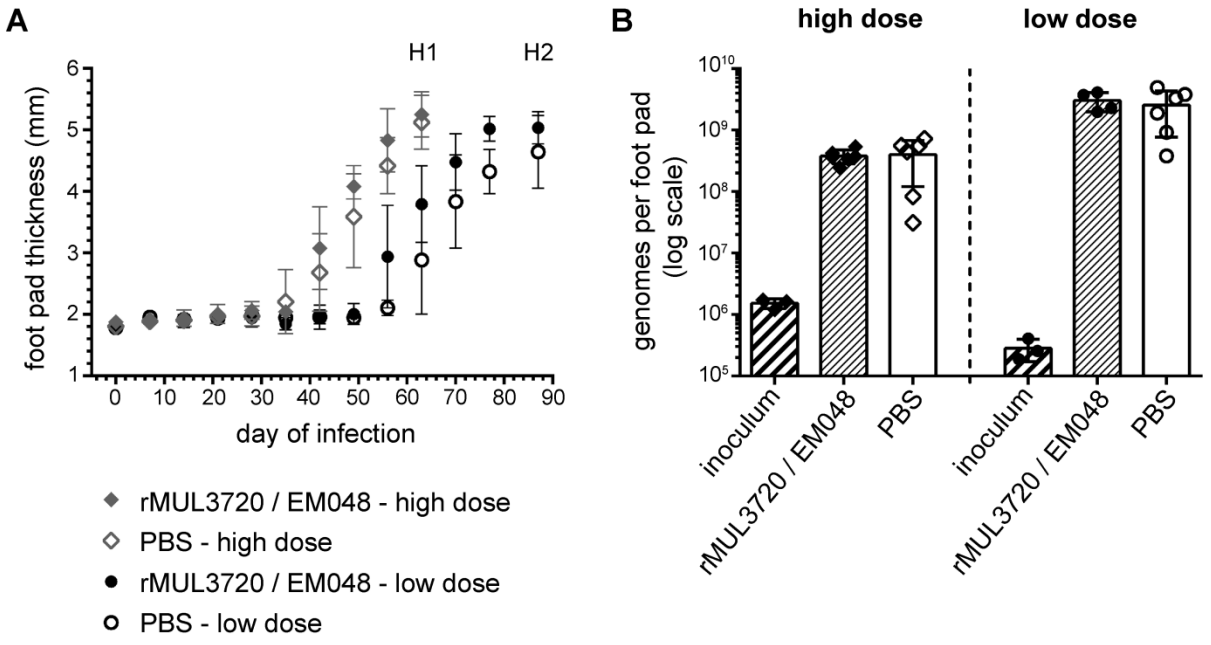


Figure 4. Evaluation of the protective potential of immunization with rMUL3720/EM048 formulation in a *M. ulcerans* infection mouse model.

Groups of eight BALB/c mice were immunized twice with 20 µg of rMUL3720/EM048 or PBS alone as infection control. Three weeks after the last immunization mice were challenged with a high dose or a low dose of *M. ulcerans* (inoculum) into the left hind foot pad. Infection was followed by measuring foot pad thickness with a caliper (A) until mice were euthanized at day 63 after infection (high dose, H1) or at day 87 after infection (low dose, H2). Depicted is the mean foot pad thickness (diamond/circle) ± standard deviation of the differently immunized groups. (B) Bacterial load in infected foot pads was determined by qPCR for six mice per group. Depicted are individual measurements as genome copies per foot pad, the mean (line) ± standard deviation.

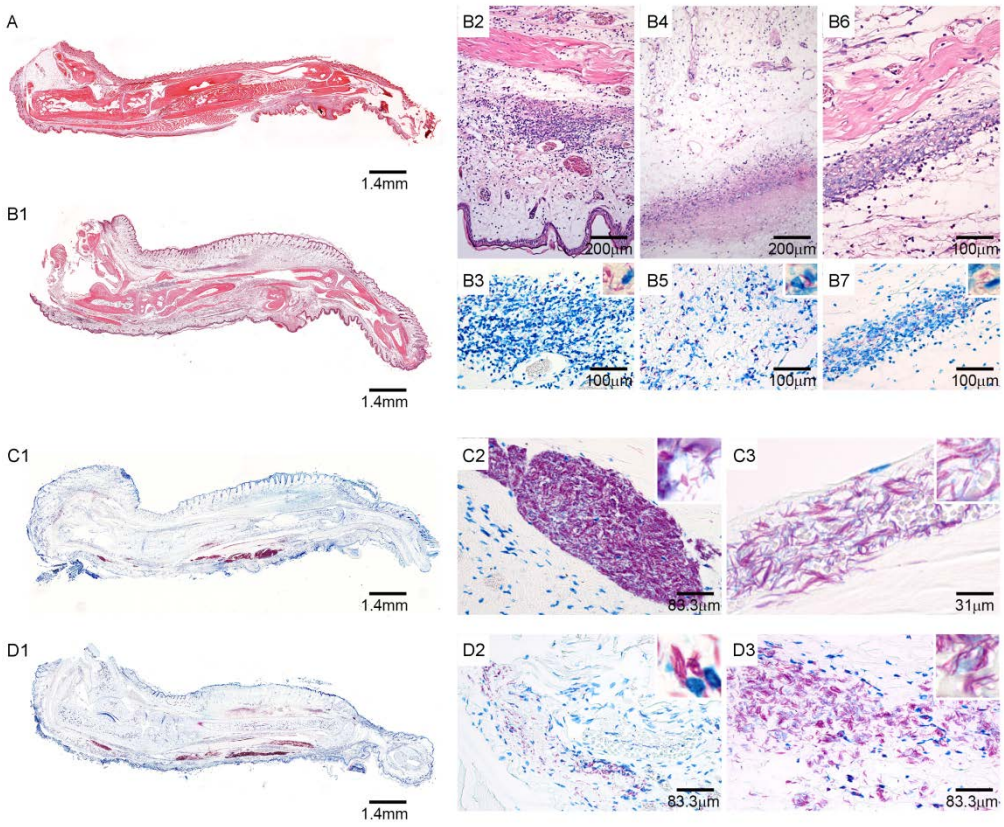


Figure 5. Histopathological evaluation of mouse footpads following *M. ulcerans* infection.

Histological sections of foot pads from *M. ulcerans*-infected mice were stained with Haematoxylin/Eosin (A, B1 - B7) or Ziehl-Neelsen/Methylene blue (ZN) (C1 - C4, D1 - D4). Histological sections of one lymph node were stained with ZN (E1 – E3).

Mice challenged with a high dose of *M. ulcerans* developed typical signs of infection in the mouse foot pad model until day 63 after infection. Compared to a control foot pad (A) the infected foot pad of a representative immunized mouse (B1) showed necrosis and infiltration interspersed with AFB at the ankle (B2, B3) as well as at the base of the foot (B6, B7). Oedema was marked on top of the foot pad (B4) where bacteria were also found (B5). Immunized mice (C1) as well as control mice (D1) challenged with a lower dose of *M. ulcerans* developed strong infection foci until day 87 after infection. AFB appeared as big, dense clumps (C2) or in close association with infiltrating cells (D2) towards the heel of the infected foot pads as well as in the middle of the foot (C1, D1). AFB were also present in oedematous tissue in the upper half of the foot (D3) and appeared in filamentous organization (C3).

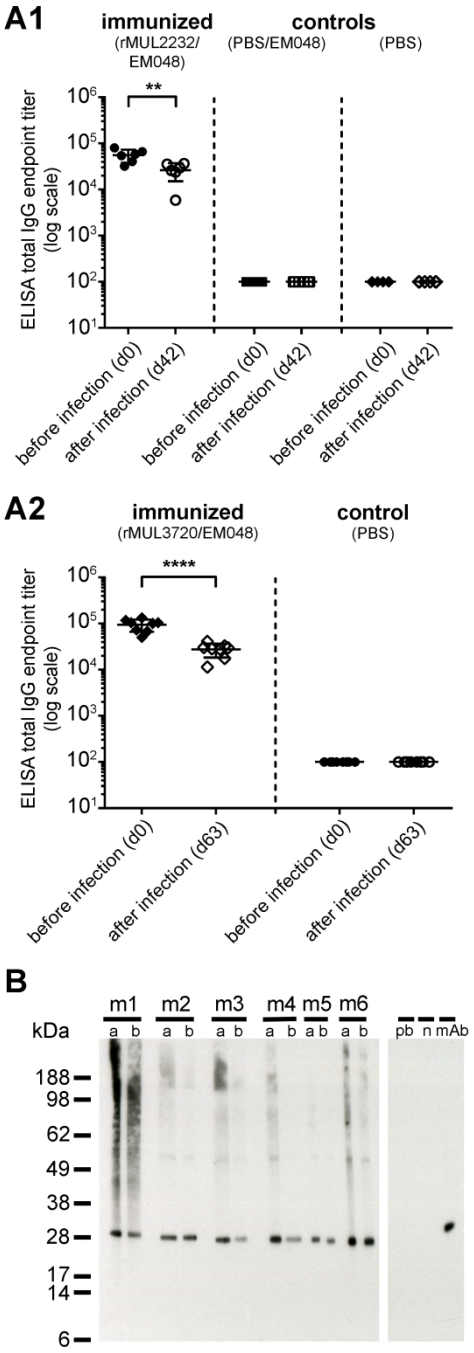


Figure 6. Evaluation of the booster effect of *M. ulcerans* infection on the pre-existing antibody response in rMUL3720/EM048 immunized and subsequently infected mice.

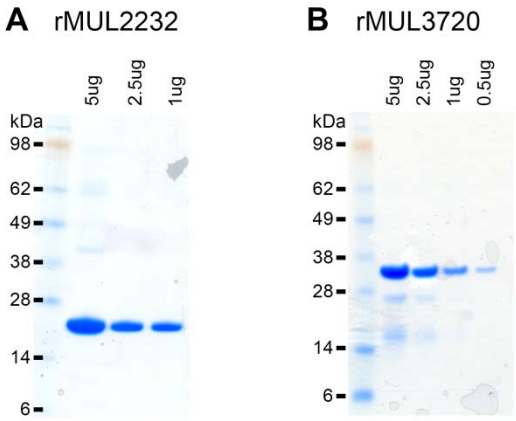
(A1) Serum of six BALB/c mice immunized with rMUL2232/EM048 prior to infection (before infection) with *M. ulcerans* was compared to serum of the same animals after 42 days of infection (after infection) in ELISA on rMUL2232. Control immunized animals had received PBS/EM048 or only PBS prior to infection. Depicted are individual endpoint IgG titers as

determined in one single ELISA, the group mean (line) ± standard deviation. Statistical significance was calculated by the Student's t- test (** $p \leq 0.01$).

(A2) Serum of eight BALB/c mice immunized with rMUL3720/EM048 prior to infection with *M. ulcerans* was compared to serum of the same animals 63 days after infection with *M. ulcerans* bacteria in ELISA on rMUL3720. Control immunized animals (control) had only received PBS prior to infection. Depicted are individual endpoint IgG titers as determined in one single ELISA, the mean (line) ± standard deviation. Statistical significance was calculated by the Student's t- test (**** $p \leq 0.0001$).

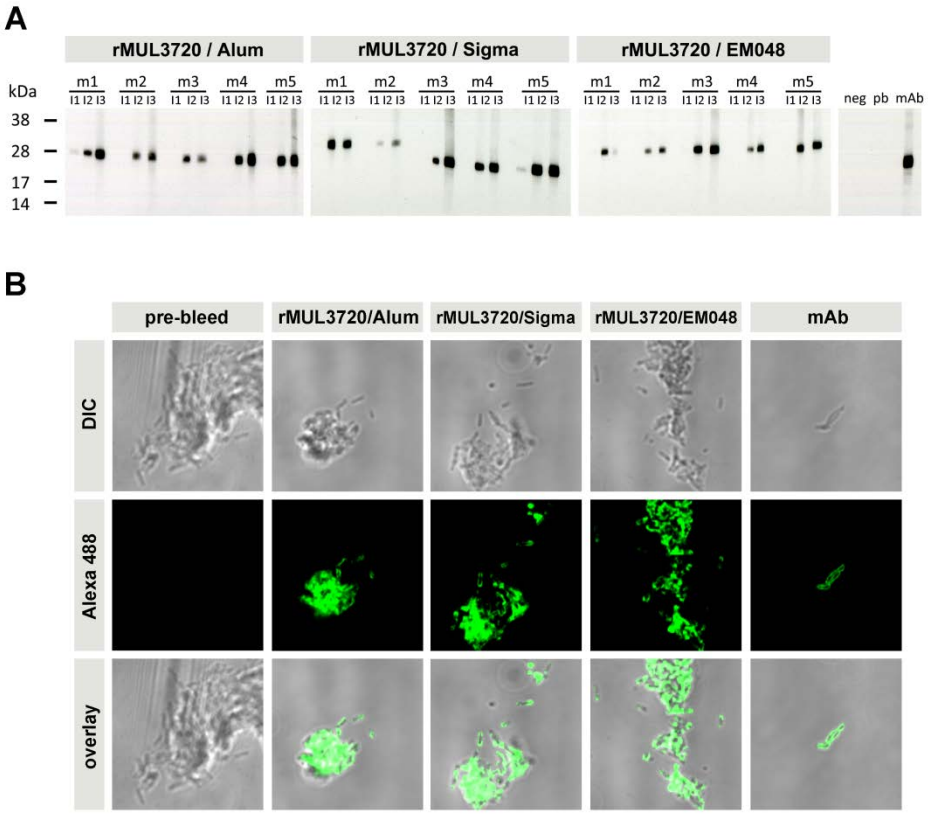
(B) Serum of six BALB/c mice (m1 – m6) immunized with rMUL3720/EM048 prior to infection with *M. ulcerans* (a) was compared to serum of the same animals after 42 days of infection (b) by Western blotting on *M. ulcerans* whole cell lysate. Monoclonal anti-MUL_3720 antibody (mAb) served as positive control, pre-bleed (pb) serum or no primary antibody (nc) as negative controls.

Supplementary Material



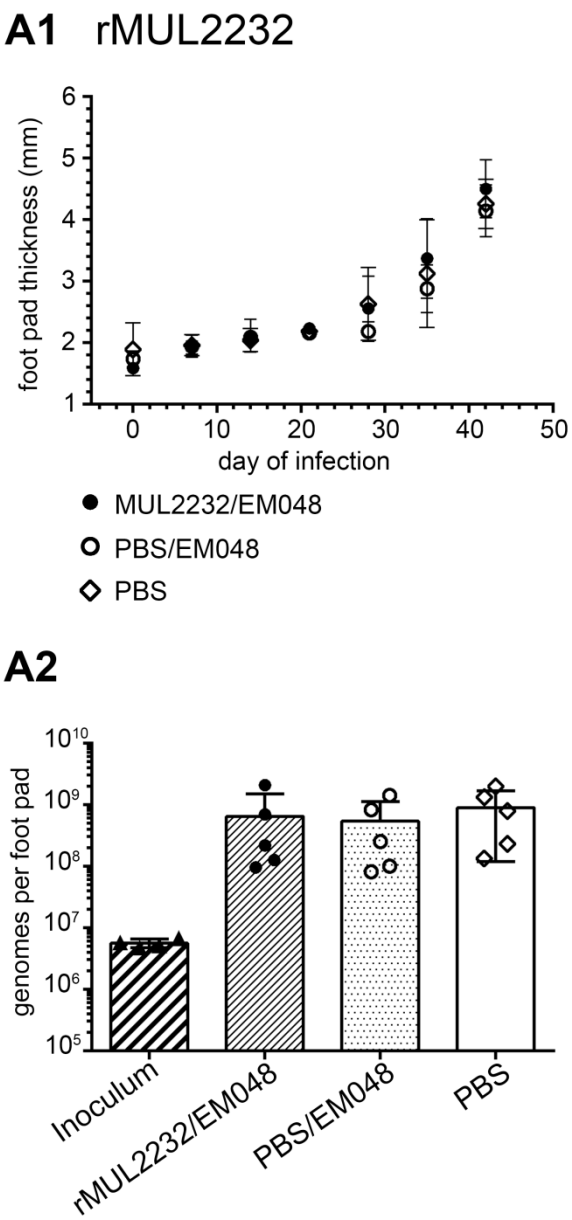
S1 Figure. Two *M. ulcerans* candidate vaccine antigens expressed as recombinant proteins in *E. coli*.

Indicated amounts of rMUL2232 (A) or rMUL3720 (B) were resolved on SDS-page and stained with Coomassie blue.



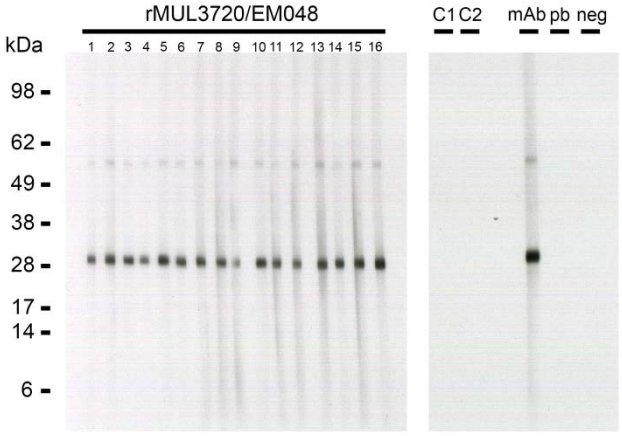
S2 Figure. Cross reactivity of immune sera with *M. ulcerans*.

(A) Groups of five BALB/c mice (m1 – m5) were immunized three times in three week intervals with 20 µg of rMUL3720 formulated with Alum, Sigma Adjuvant or EM048. Serum after every immunization (I1, I2 and I3) was analysed by Western blotting on *M. ulcerans* lysate. Monoclonal anti-MUL_3720 antibody (mAb) served as positive control, pre-bleed (pb) serum or no primary antibody (nc) as negative controls. (B) Sera from three weeks after the third immunization with rMUL3720 and indicated adjuvant were used for indirect immunofluorescence staining on paraffin embedded *M. ulcerans* bacteria with an Alexa488 labelled secondary antibody. Pre-bleed serum did not stain the bacteria. Sera of immunized mice (a mix of sera from five individual mice per group) did reveal surface staining similar to the staining achieved with anti-MUL_3720 monoclonal antibody (mAb).



S3 Figure. Evaluation of the protective potential of immunization with rMUL2232/EM048 formulation in a *M. ulcerans* infection mouse model.

Groups of six BALB/c mice were immunized twice with 20 µg of rMUL2232/EM048, PBS/EM048 or PBS alone as infection control. Three weeks after the last immunization mice were challenged with *M. ulcerans* (inoculum) into the left hind foot pad. Infection was followed by measuring foot pad thickness with a caliper (A1) until mice were euthanized at day 42 after infection. Depicted is the mean foot pad thickness (diamond/dot) ± standard deviation of the differently immunized groups. (A2) Bacterial load in infected foot pads was determined by qPCR for five mice per group. Depicted are individual measurements as genome copies per foot pad, the mean (line) ± standard deviation.



S4 Figure. Reactivity of immune sera on *M. ulcerans* lysate.

Groups of eight BALB/c mice were immunized twice with 20 µg of rMUL3720/EM048 or PBS only as infection control. Serum prior to infection with *M. ulcerans* was analysed by Western blotting on *M.ulcerans* lysate. Monoclonal anti-MUL_3720 antibody (mAb) served as positive control, pre-bleed (pb) serum or no primary antibody (neg) as negative controls. C1 and C2 each represent a mix of sera of eight mice immunized with PBS only.

References

1. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, Benbow ME, et al. Ecology and transmission of Buruli ulcer disease: a systematic review. PLoS Negl Trop Dis. 2010;4: e911. doi:10.1371/journal.pntd.0000911
2. Junghanss T, Johnson RC, Pluschke G. *Mycobacterium ulcerans* disease. Manson's tropical diseases. 23rd ed. Saunders; 2014. pp. 519–531.
3. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, et al. Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. Science. 1999;283: 854–857.
4. WHO | Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer). In: WHO [Internet]. [cited 4 Feb 2014]. Available: http://www.who.int/buruli/information/antibiotics/en/index16.html
5. Nienhuis WA, Stienstra Y, Thompson WA, Awuah PC, Abass KM, Tuah W, et al. Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial. Lancet. 2010;375: 664–672. doi:10.1016/S0140-6736(09)61962-0
6. Sarfo FS, Phillips R, Asiedu K, Ampadu E, Bobi N, Adentwe E, et al. Clinical efficacy of combination of rifampin and streptomycin for treatment of *Mycobacterium ulcerans* disease. Antimicrob Agents Chemother. 2010;54: 3678–3685. doi:10.1128/AAC.00299-10
7. Chauty A, Ardant M-F, Adeye A, Euverte H, Guédénon A, Johnson C, et al. Promising clinical efficacy of streptomycin-rifampin combination for treatment of buruli ulcer (*Mycobacterium ulcerans* disease). Antimicrob Agents Chemother. 2007;51: 4029–4035. doi:10.1128/AAC.00175-07
8. WHO | Laboratory diagnosis of buruli ulcer. In: WHO [Internet]. [cited 2 Jun 2014]. Available: http://www.who.int/buruli/laboratory_diagnosis/en/
9. WHO | Treatment of *Mycobacterium ulcerans* disease (Buruli Ulcer). In: WHO [Internet]. [cited 11 Sep 2014]. Available: http://www.who.int/buruli/treatment/en/
10. Diaz D, Döbeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, Soder N, et al. Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. Clin Vaccine Immunol CVI. 2006;13: 1314–1321. doi:10.1128/CVI.00254-06
11. Röltgen K, Bratschi MW, Ross A, Aboagye SY, Ampah KA, Bolz M, et al. Late onset of the serological response against the 18 kDa small heat shock protein of *Mycobacterium ulcerans* in children. PLoS Negl Trop Dis. 2014;8: e2904. doi:10.1371/journal.pntd.0002904
12. Revill WDL, Morrow RH, Pike MC, Ateng J. A CONTROLLED TRIAL OF THE TREATMENT OF *MYCOBACTERIUM ULCERANS* INFECTION WITH CLOFAZIMINE. The Lancet. 1973;302: 873–877. doi:10.1016/S0140-6736(73)92005-9
13. Gordon CL, Buntine JA, Hayman JA, Lavender CJ, Fyfe JA, Hosking P, et al. Spontaneous Clearance of *Mycobacterium ulcerans* in a Case of Buruli Ulcer. PLoS Negl Trop Dis. 2011;5. doi:10.1371/journal.pntd.0001290
14. Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, Kerber S, et al. Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. PLoS Negl Trop Dis. 2013;7: e2252. doi:10.1371/journal.pntd.0002252

15. Huygen K, Adjei O, Affolabi D, Bretzel G, Demangel C, Fleischer B, et al. Buruli ulcer disease: prospects for a vaccine. Med Microbiol Immunol (Berl). 2009;198: 69–77. doi:10.1007/s00430-009-0109-6

16. Torrado E, Fraga AG, Castro AG, Stragier P, Meyers WM, Portaels F, et al. Evidence for an intramacrophage growth phase of *Mycobacterium ulcerans*. Infect Immun. 2007;75: 977–987. doi:10.1128/IAI.00889-06

17. Coutanceau E, Marsollier L, Brosch R, Perret E, Goossens P, Tanguy M, et al. Modulation of the host immune response by a transient intracellular stage of *Mycobacterium ulcerans*: the contribution of endogenous mycolactone toxin. Cell Microbiol. 2005;7: 1187–1196. doi:10.1111/j.1462-5822.2005.00546.x

18. Einarsdottir T, Huygen K. Buruli ulcer. Hum Vaccin. 2011;7: 1198–1203. doi:10.4161/hv.7.11.17751

19. Fraga AG, Martins TG, Torrado E, Huygen K, Portaels F, Silva MT, et al. Cellular immunity confers transient protection in experimental Buruli ulcer following BCG or mycolactone-negative *Mycobacterium ulcerans* vaccination. PloS One. 2012;7: e33406. doi:10.1371/journal.pone.0033406

20. Smith PG, Revill WD, Lukwago E, Rykushin YP. The protective effect of BCG against *Mycobacterium ulcerans* disease: a controlled trial in an endemic area of Uganda. Trans R Soc Trop Med Hyg. 1976;70: 449–457.

21. Portaels F, Aguiar J, Debacker M, Guédénon A, Steunou C, Zinsou C, et al. *Mycobacterium bovis* BCG vaccination as prophylaxis against *Mycobacterium ulcerans* osteomyelitis in Buruli ulcer disease. Infect Immun. 2004;72: 62–65.

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

23. Watanabe M, Nakamura H, Nabekura R, Shinoda N, Suzuki E, Saito H. Protective effect of a dewaxed whole-cell vaccine against *Mycobacterium ulcerans* infection in mice. Vaccine. 2015;33: 2232–2239. doi:10.1016/j.vaccine.2015.03.046

24. Dreyer A, Röltgen K, Dangy JP, Ruf MT, Scherr N, Bolz M, et al. Identification of the *Mycobacterium ulcerans* Protein MUL_3720 as a Promising Target for the Development of a Diagnostic Test for Buruli Ulcer. PLoS Negl Trop Dis. 2015;9: e0003477. doi:10.1371/journal.pntd.0003477

25. Vettiger A, Scherr N, Ruf M-T, Röltgen K, Pluschke G. Localization of Mycobacterial Antigens by Immunofluorescence Staining of Agarose Embedded Cells. J Mycobact Dis. 2014;4: 150.

26. Coler RN, Bertholet S, Moutaftsi M, Guderian JA, Windish HP, Baldwin SL, et al. Development and characterization of synthetic glucopyranosyl lipid adjuvant system as a vaccine adjuvant. PloS One. 2011;6: e16333. doi:10.1371/journal.pone.0016333

27. Treanor JJ, Essink B, Hull S, Reed S, Izikson R, Patriarca P, et al. Evaluation of safety and immunogenicity of recombinant influenza hemagglutinin (H5/Indonesia/05/2005) formulated with and without a stable oil-in-water emulsion containing glucopyranosyl-lipid A (SE+GLA) adjuvant. Vaccine. 2013;31: 5760–5765. doi:10.1016/j.vaccine.2013.08.064

28. Portaels F, Johnson P, Meyers WM, Initiative WHOGBU. Buruli ulcer : diagnosis of *Mycobacterium ulcerans* disease : a manual for health care providers / edited by: Françoise Portaels, Paul Johnson, Wayne M. Meyers [Internet]. 2001 [cited 6 Feb 2014]. Available: http://apps.who.int/iris/handle/10665/67000

29. Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, Kerber S, et al. Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. PLoS Negl Trop Dis. 2013;7: e2252. doi:10.1371/journal.pntd.0002252

30. Bolz M, Kerber S, Zimmer G, Pluschke G. Use of Recombinant Virus Replicon Particles for Vaccination against *Mycobacterium ulcerans* Disease. PLoS Negl Trop Dis. 2015;9: e0004011. doi:10.1371/journal.pntd.0004011

31. Lavender CJ, Fyfe JAM. Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples. Methods Mol Biol Clifton NJ. 2013;943: 201–216. doi:10.1007/978-1-60327-353-4_13

32. Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, Azuolas J, et al. Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. Appl Environ Microbiol. 2007;73: 4733–4740. doi:10.1128/AEM.02971-06

33. Portaels F, Organization WH. Laboratory diagnosis of buruli ulcer: a manual for health care providers / edited by Françoise Portaels. Diagnostic de l'ulcère de Buruli au laboratoire : manuel destiné au personnel de santé / edité par Françoise Portaels. 2014; Available: http://apps.who.int//iris/handle/10665/111738

34. Setia MS, Steinmaus C, Ho CS, Rutherford GW. The role of BCG in prevention of leprosy: a meta-analysis. Lancet Infect Dis. 2006;6: 162–170. doi:10.1016/S1473-3099(06)70412-1

35. Merle CSC, Cunha SS, Rodrigues LC. BCG vaccination and leprosy protection: review of current evidence and status of BCG in leprosy control. Expert Rev Vaccines. 2010;9: 209–222. doi:10.1586/erv.09.161

36. BCG vaccination against *mycobacterium ulcerans* infection (Buruli ulcer). First results of a trial in Uganda. Lancet. 1969;1: 111–115.

37. Phillips RO, Phanzu DM, Beissner M, Badziklou K, Luzolo EK, Sarfo FS, et al. Effectiveness of routine BCG vaccination on buruli ulcer disease: a case-control study in the Democratic Republic of Congo, Ghana and Togo. PLoS Negl Trop Dis. 2015;9: e3457. doi:10.1371/journal.pntd.0003457

38. Debacker M, Portaels F, Aguiar J, Steunou C, Zinsou C, Meyers W, et al. Risk factors for Buruli ulcer, Benin. Emerg Infect Dis. 2006;12: 1325–1331. doi:10.3201/eid1209.050598

39. Raghunathan PL, Whitney EAS, Asamoa K, Stienstra Y, Taylor TH, Amofah GK, et al. Risk factors for Buruli ulcer disease (*Mycobacterium ulcerans* Infection): results from a case-control study in Ghana. Clin Infect Dis Off Publ Infect Dis Soc Am. 2005;40: 1445–1453. doi:10.1086/429623

40. Tanghe A, Content J, Van Vooren JP, Portaels F, Huygen K. Protective efficacy of a DNA vaccine encoding antigen 85A from *Mycobacterium bovis* BCG against Buruli ulcer. Infect Immun. 2001;69: 5403–5411.

41. Coutanceau E, Legras P, Marsollier L, Reysset G, Cole ST, Demangel C. Immunogenicity of *Mycobacterium ulcerans* Hsp65 and protective efficacy of a *Mycobacterium leprae* Hsp65-based DNA vaccine against Buruli ulcer. Microbes Infect Inst Pasteur. 2006;8: 2075–2081. doi:10.1016/j.micinf.2006.03.009

42. Fox CB, Moutaftsi M, Vergara J, Desbien AL, Nana GI, Vedvick TS, et al. TLR4 ligand formulation causes distinct effects on antigen-specific cell-mediated and humoral immune responses. Vaccine. 2013;31: 5848–5855. doi:10.1016/j.vaccine.2013.09.069

43. Baldwin SL, Shaverdian N, Goto Y, Duthie MS, Raman VS, Evers T, et al. Enhanced humoral and Type 1 cellular immune responses with Fluzone adjuvanted with a synthetic TLR4 agonist formulated in an emulsion. Vaccine. 2009;27: 5956–5963. doi:10.1016/j.vaccine.2009.07.081

44. Duthie MS, Coler RN, Laurance JD, Sampaio LH, Oliveira RM, Sousa ALM, et al. Protection against *Mycobacterium leprae* Infection by the ID83/GLA-SE and ID93/GLA-SE Vaccines Developed for Tuberculosis. Infect Immun. 2014;82: 3979–3985. doi:10.1128/IAI.02145-14

45. Orr MT, Fox CB, Baldwin SL, Sivananthan SJ, Lucas E, Lin S, et al. Adjuvant formulation structure and composition are critical for the development of an effective vaccine against tuberculosis. J Control Release Off J Control Release Soc. 2013;172: 190–200. doi:10.1016/j.jconrel.2013.07.030

46. Gooding TM, Johnson PD, Campbell DE, Hayman JA, Hartland EL, Kemp AS, et al. Immune response to infection with *Mycobacterium ulcerans*. Infect Immun. 2001;69: 1704–1707. doi:10.1128/IAI.69.3.1704-1707.2001

47. Gooding TM, Kemp AS, Robins-Browne RM, Smith M, Johnson PDR. Acquired T-helper 1 lymphocyte anergy following infection with Mycobacterium ulcerans. Clin Infect Dis Off Publ Infect Dis Soc Am. 2003;36: 1076–1077. doi:10.1086/368315

48. Dobos KM, Spotts EA, Marston BJ, Horsburgh CR, King CH. Serologic response to culture filtrate antigens of *Mycobacterium ulcerans* during Buruli ulcer disease. Emerg Infect Dis. 2000;6: 158–164.

Results Chapter 5

Use of Recombinant Virus Replicon Particles for Vaccination against *Mycobacterium ulcerans* Disease

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Abstract

Buruli ulcer, caused by infection with *Mycobacterium ulcerans*, is a necrotizing disease of the skin and subcutaneous tissue, which is most prevalent in rural regions of West African countries. The majority of clinical presentations seen in patients are ulcers on limbs that can be treated by eight weeks of antibiotic therapy. Nevertheless, scarring and permanent disabilities occur frequently and Buruli ulcer still causes high morbidity. A vaccine against the disease is so far not available but would be of great benefit if used for prophylaxis as well as therapy. In the present study, vesicular stomatitis virus-based RNA replicon particles encoding the *M. ulcerans* proteins MUL2232 and MUL3720 were generated and the expression of the recombinant antigens characterized *in vitro*. Immunisation of mice with the recombinant replicon particles elicited antibodies that reacted with the endogenous antigens of *M. ulcerans* cells. A prime-boost immunization regimen with MUL2232-recombinant replicon particles and recombinant MUL2232 protein induced a strong immune response but only slightly reduced bacterial multiplication in a mouse model of *M. ulcerans* infection. We conclude that a monovalent vaccine based on the MUL2232 antigen will probably not sufficiently control *M. ulcerans* infection in humans.

Author Summary

Infection with *Mycobacterium ulcerans* can lead to a slow progressing, ulcerative disease of the skin and underlying soft tissue called Buruli ulcer. The disease is most prevalent in rural African communities with limited access to health care facilities. The most efficient means to prevent the disease, a vaccine against Buruli ulcer is not available to date. In the present study we investigated the immunogenicity and protective potential of a single cycle virus system expressing the two *M. ulcerans* antigens MUL2232 and MUL3720. Immunization of mice with those vesicular stomatitis virus replicon particles led to the induction of humoral as well as cellular immune responses in the immunized animals. Subsequent challenge experiments in a mouse model of *M. ulcerans* infection demonstrated only a limited reduction of bacterial burden in mice immunized with a prime-boost approach with MUL2232. Most probably, a vaccine formulation with only one antigen will not be able to provide protection against Buruli ulcer in humans.

Introduction

Mycobacterium ulcerans causes Buruli ulcer (BU), a disease of the skin and underlying subcutaneous tissue, which is reported from over 30 countries worldwide [1]. BU is most prevalent in West African countries and mainly affects children less than 15 years of age, living in remote, rural areas [2]. The natural reservoir of *M. ulcerans* has not been identified so far and also the mode of transmission of this pathogen remains unclear [3].

M. ulcerans produces a macrolide exotoxin called mycolactone, which induces apoptosis in mammalian cells and leads to the typical clinical presentation of ulcerative BU skin lesions after the overlying epidermis has collapsed [4]. Non-ulcerative forms of the disease are nodules or papules, oedema and plaques [5]. BU was traditionally treated by wide surgical excision of the affected skin and skin grafting if necessary. Since 2004, treatment of patients for eight weeks with the antibiotics rifampicin and streptomycin is recommended as standard therapy by the World Health Organization (WHO) [6]. Even though the use of antibiotics has reduced recurrence rates to less than 2% [7–9], patients are often left with scars and lifelong disabilities [10]. A vaccine against BU would therefore be of high value to prevent and treat the disease [11].

As opposed to *M. tuberculosis* and *M. leprae*, which are both intracellular pathogens for which T helper 1 (T_H1) cellular immune responses are essential for infection control [12], *M. ulcerans* is found in extracellular clumps in the necrotic subcutaneous skin tissue of advanced lesions. However, there is evidence for an initial intracellular stage in macrophages during the early phase of infection [13,14]. Correlates of protection have not been identified, and in particular it is not known, whether antibodies specific for surface antigens of *M. ulcerans* have protective activity [15].

Vaccination with Bacille Calmette-Guérin (BCG) seems to confer a transient protection from BU and a shorter duration of ulcers [16–18]. In a mouse model of *M. ulcerans* infection, vaccination with either BCG or a mycolactone-negative *M. ulcerans* mutant strain delayed the onset of foot pad swelling [19,20]. A limited protective efficacy has also been achieved with monovalent DNA-based protein subunit vaccine formulations targeting either the mycolyl-transferase antigen (Ag) 85A from *M. bovis* or *M. ulcerans* [21,22] or mycolactone polyketide synthase domains that are encoded on the giant *M. ulcerans* plasmid pMUM001 [23].

Vesicular stomatitis virus (VSV) is a member of the virus family *Rhabdoviridae* and has a non-segmented single-stranded RNA genome of negative polarity. VSV is transmitted by insects and causes a vesicular-like disease in livestock and Flu-like symptoms in humans. The VSV seroprevalence is very low in the human population, indicating that human

infections are rare. The non-persisting replication of VSV in the cytosol of the host cell, low virulence and low pre-existing immunity in humans, and the option of simple genetic manipulation [24] has recommended VSV as viral vector system for vaccination [25,26]. Several VSV-based vaccines for protection against viral and bacterial pathogens have been generated in recent years and have been evaluated in a number of animal models [27–30]. More recently, single-cycle VSV vectors have been developed, which lack the genetic information for the VSV glycoprotein G and are complemented with this protein *in trans*. Although these VSVΔG vectors are restricted to a single round of infection, they were shown to be as immunogenic as propagation-competent VSV vectors [31].

In the present study we used this safe vaccine vector platform to characterize the immunogenicity of two *M. ulcerans* protein antigens. In view of the mainly extracellular nature of *M. ulcerans*, we chose antigens known to be highly expressed on the surface of the bacteria. The 18 kDa small heat shock protein (MUL2232) was identified amongst the most immunodominant antigens expressed by *M. ulcerans* when serum responses from people living in endemic areas were analysed [32]. A homologue of MUL2232 was found in the genome of *M. leprae* but neither in *M. bovis* nor in *M. tuberculosis*. Similar to the *M. leprae* homologue, MUL2232 is associated with the cell wall fraction of *M. ulcerans* [32,33]. The second antigen chosen, MUL3720, is a 21 kDa protein with a putative lectin and a peptidoglycoan-binding domain that does not have homologues in *M. leprae* or *M. tuberculosis* [34,35]. It is highly expressed by *M. ulcerans* and may play a role in cell attachment and cell-cell interactions [35], making it a candidate antigen for vaccine development. We generated recombinant single-cycle VSV vectors encoding the selected antigens individually and analysed their expression in mammalian cell lines. We further analysed the immunogenicity of the generated VSV vectors and evaluated two short immunization protocols. Finally, we investigated the protective potential of such immunization schemes in an experimental mouse model of *M. ulcerans* infection.

Materials and Methods

Ethics statement

All animal experiments were conducted in compliance with the Swiss animal protection law and approved by the animal welfare committee of the Canton of Basel (authorization number 2375) and the Canton of Vaud (authorization number 2657).

Cells

Baby hamster kidney 21 (BHK-21) cells were obtained from the German Cell Culture Collection (DSZM, Braunschweig). Cells were grown in Earle's minimal essential medium (MEM, Life Technologies) supplemented with 5% foetal bovine serum (FBS, Biowest). BHK-G43, a transgenic BHK-21 cell line expressing the VSV G protein in an inducible manner, was maintained as described previously [36]. Murine L929 fibroblasts (ATCC, The Global Bioresource Center) were cultivated in RPMI medium (Gibco) supplemented with 10% foetal calf serum (FCS, Sigma), 2 mM glutamine (Gibco) and 0.05 mM β-mercaptoethanol (Gibco).

Generation of recombinant vesicular stomatitis virus replicon particles expressing *M. ulcerans* codon optimized antigens

The potential protein vaccine candidate antigens MUL_2232 (GenBank accession number 4550596) and MUL_3720 (GenBank accession number 4553013) of *M. ulcerans* Agy99 were ordered as codon optimized genes for expression in humans (GenScript) and received in pUC57 plasmids. For generation of recombinant VSV replicon particles (VRPs), codon optimized target antigens were amplified by PCR and inserted into the pVSV*ΔG plasmid using single MluI and BstEII restriction sites upstream and downstream of the fourth transcription unit, replacing the VSV G gene [37]. Sequence integrity of the resulting plasmids was confirmed by Sanger sequencing. Recombinant VRPs were generated as described previously [38]. In brief, BHK-G43 cells were infected with recombinant MVA-T7 virus expressing T7 RNA polymerase [39]. Subsequently, the infected cells were transfected with plasmids driving T7 RNA polymerase-mediated expression of the VSV proteins N, P, and L, and with pVSV*ΔG(MUL2232) or pVSV*ΔG(MUL3720) driving T7 RNA polymerase-mediated transcription of VSV antigenomic (negative-sense) RNA. Expression of the VSV G protein was induced by adding mifepristone (Sigma) to the cell culture medium. At 24 hours post transfection cells were detached with trypsin and seeded along with an equal number of fresh BHK-G43 cells into T-75 flasks. Cells were then incubated at 37°C for another 24 hours in the presence of mifepristone. Cell culture supernatant was clarified by low-speed centrifugation and by passage through a 0.2 µm pore filter. The VRPs in the clarified cell culture supernatant were further propagated on mifepristone-induced BHK-G43 cells and

stored at -70°C. VRPs were titrated on BHK-21 cells taking advantage of the eGFP reporter protein.

Western blot analysis

Detection of vaccine antigens in VRP-infected cells

Equal numbers of confluent L929 fibroblasts were infected with VRPs at a multiplicity of infection (MOI) of 10 for 6 hours at 37°C. Cells were washed with ice-cold phosphate buffered saline (PBS; Sigma) and harvested for lysis. Protein lysates of both the soluble and insoluble fraction of the cells were produced with a cell fractionation kit (Abcam) according to the manufacturer's instructions. Equal amounts of protein lysates as well as *M. ulcerans* lysate in an appropriate dilution were resolved on prefabricated 4-12% gradient gels (NuPAGE Novex 4-12% Bis-Tris Gel; Invitrogen) with MES running buffer (Invitrogen) according to the manufacturer's directions. A dry-blotting system (iBlot; Invitrogen) was used to transfer proteins to nitrocellulose membranes. Membranes were blocked and specific proteins detected with in house anti-MUL2232 or anti-MUL3720 mouse mAbs followed by HRP-conjugated goat anti-mouse IgG γ-chain mAb (Southern Biotech, 1030-05). Blots were developed using ECL Western blotting detection reagents (ECL Western blotting Substrate; Pierce).

Cross-reactivity of the elicited antibodies in mouse sera with the native antigen in *M. ulcerans* lysate

10 µg of *M. ulcerans* whole cell lysate was resolved on a one well prefabricated 4-12% gradient gel (NuPAGE Novex 4-12% Bis-Tris Gel; Invitrogen) with MES running buffer according to the manufacturer's instructions. The blocked membrane was subsequently cut into thin strips that were individually incubated with appropriate dilutions of serum of immunized mice for 2 hours. Secondary antibody and development were performed as described above.

Immunofluorescence analyses

For indirect immunofluorescence analysis, BHK-21 cells were grown on 12 mm diameter cover slips (2×10^5 cells/well) and infected with VRPs (10^6 infectious units/well) for 6 hours at 37°C. Cells were fixed with 3% paraformaldehyde (PFA) and washed with PBS containing 0.1 M of glycine. The cells were permeabilized with 0.25% (v/v) Triton X-100 and subsequently incubated for 1 hour with anti-MUL2232 or anti-MUL3720 mAbs in appropriate dilutions in 1% bovine serum albumin (Sigma). For detection of antigen-bound primary antibodies, cells were incubated with an Alexa Flour 546 labelled anti-mouse IgG secondary antibody (1/500; Molecular Probes, A-11018). The cells were washed with distilled water,

embedded in Mowiol 4-88 (Sigma) mounting medium, and analyzed with a Leica TCS SL confocal microscope and LCS software (Leica Microsystems AG, Glattbrugg, Switzerland).

ELISA on recombinant protein

ELISA plates (Maxisorp; Nunc) were coated with 10ug/ml purified recombinant MUL2232 (rMUL2232) or MUL3720 (rMUL3720) protein produced in *Escherichia coli* [40]. After blocking, plates were incubated with serially diluted sera from immunized mice. Alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma) was used as secondary antibody and *p*-nitrophenyl phosphate (Sigma) served as substrate. The optical density (OD) of the reaction product was measured at 405 nm with a microplate reader (Sunrise Absorbance Reader; Tecan). The threshold for endpoint titer determination was defined as the double of the mean measurements plus the mean standard deviation of a dilution series done without primary antibody and a dilution series done with pre-bleed serum. Individual serum dilution series were approximated with sigmoidal dose-response curves and the reciprocal dilution of the intersection between the curve and the threshold was defined as individual endpoint titer.

Mouse immunization studies

All animal studies were conducted in 8 weeks old female BALB/c mice (Janvier). VRPs (10^7 fluorescence-forming units) were either applied subcutanously (s.c.) in the neck (for evaluation of the vaccination protocol) or intramuscularly (i.m.) into the right caudal tight muscle using a volume of 100 µl or 30 µl, respectively. When several immunizations were conducted, injections were performed in three week intervals. For prime-boost vaccination regimen, 30 µg of non-adjuvanted recombinant protein were applied s.c. into the neck. Blood was collected from the tail vein prior to every immunization as well as 5/6 and 14 days after the protein boost. Serum was prepared by centrifugation of the blood in SST Microtainer tubes (Becton, Dickinson and Company).

Analysis of cellular immune responses

Immunized mice were euthanized 3 weeks after the second immunization. Heart blood was collected and spleens were aseptically removed and homogenized by passing through a 70 µm cell strainer (BD Falcon). Cells were then pelleted and red blood cells lysed by incubating the pellet in red blood cell lysing buffer (Sigma) for 1 minute. Remaining cells were washed several times with stemline T-cell expansion medium (Sigma) supplemented with 4mM L-Glutamine (Gibco), 1% Pen-Strep (Gibco), 2.5 ug/ml Amphotericin B (Sigma) and 0.05 mM β-mercaptoethanol (Gibco) and finally adjusted to 4×10^6 white blood cells/ml. 7.2×10^5 cells per well were incubated in round-bottom microwell plates (BD Falcon) in a humidified CO_2 incubator and stimulated with Concanavalin A (2 µg/ml, Sigma) as positive control or recombinant proteins at 5 µg/ml. Supernatants were harvested after 24h for Interleukin 2 (IL-2) and 96h for Interleukin 10 (IL-10) and Interferon gamma (IFNγ) assays and stored frozen

at -20°C until analysis for the selected cytokines. Amount of cytokines in supernatants was determined with Quantikine ELISA kits for IL-2, IL-10 and IFNγ (R&D Systems).

Challenge infection experiments

The *M. ulcerans* strain (S1013) used for the experimental infection of mice was isolated in 2010 from the ulcerative lesion of a Cameroonian BU patient [2]. Bacteria were cultivated for 6 weeks in Bac/T medium (Biomerieux), recovered by centrifugation, and suspended in sterile PBS to 125 mg/ml wet weight corresponding to 2.8 x 10^5 CFU/ml as determined by plating serial dilutions on 7H9 agar plates (Difco). Three weeks after the last immunization, mice were infected with 30 µl of *M. ulcerans* suspension (1/100 of the stock solution in PBS) into the left hind foot pad. Development of the infection was followed by weekly measurements of the foot pad thickness with a caliper. At day 60 after experimental infection, mice were sacrificed and foot pads aseptically removed for enumeration of *M. ulcerans* bacteria or histopathology. Draining inguinal lymph nodes of designated animals were removed and fixed in formalin as well. All *M. ulcerans* infection experiments were conducted under BSL3 conditions.

Colony forming unit (CFU) plating and enumeration of bacterial load by real-time PCR (qPCR)

Mouse feet designated for enumeration of *M. ulcerans* bacteria were immediately removed above the ankle after euthanasia, shortly dipped into 70% ethanol, then dried under the laminar flow, cut in 4 pieces with a scalpel and transferred to 750 µl of Bac/T medium in reinforced hard tissue grinding tubes (MK28-R, Precellys). Tissue homogenization was performed with a Precellys 24-Dual tissue homogenizer (3 x 20 s at 5000 rpm with 30 s break), the lysate was transferred to a new tube and the lysis tube still containing tissue remains was refilled with 750 µl of Bac/T medium. The remains were homogenized a second time and the two lysates pooled.

CFU plating

250 µl of the foot pad lysate was decontaminated with 0.5 M NaOH as described previously [41]. The pellet of decontaminated lysate was dissolved in 500 µl of Bac/T medium and appropriate dilution series plated on 7H9 agar plates. Following 150 days of incubation at 30°C colonies were counted and the concentration of bacteria expressed as CFU.

DNA extraction and qPCR

DNA from 100 µl of a 1 to 50 dilution of the foot pad lysate in PBS was extracted as described by Lavender and Fyfe [42]. Extracted DNA was analysed for IS2404 by qPCR as previously described [42]. Ct values were converted into genome copy numbers per foot pad by applying the standard curve established for IS2040 by *Fyfe et al*. [43].

Histopathology

Mouse feet designated for histopathological analysis were removed above the ankle and immediately transferred to 10% neutral-buffered Formalin solution (approx. 4% formaldehyde, Sigma) for fixation during 24 hours at room temperature. Subsequently, the feet were decalcified in 0.6 M EDTA and 0.25 M citric acid for 12 days at 37°C and transferred to 70% ethanol for storage and transport. The samples were dehydrated and embedded into paraffin. 5 µm thin sections were cut, deparaffinised, rehydrated, and stained with Haematoxylin/Eosin (HE, Sigma, J.T. Baker) or Ziehl-Neelsen/Methylene blue (ZN, Sigma) according to WHO standard protocols [44]. Stained sections were mounted with Eukitt mounting medium (Fluka). Pictures were taken with a Leica DM2500B microscope or with an Aperio scanner.

Statistical analysis and image processing

Differences of bacterial load in infected foot pads were statistically analysed by the Mann-Whitney test using Graph Pad Prism (Version 6.03). Results of cytokine production were subjected to $\log_{10}$ transformation and subsequently analysed with SAS software (SAS Institute, Cary, USA, release 9.3) using linear mixed models adjusted for random effects. Image processing and picture panel assembly was performed with Photoshop software (Adobe Photoshop CS6 Extended, version 13.0.1).

Results

Expression of the *M. ulcerans* vaccine candidate antigens MUL2232 and MUL3720 by cells infected with recombinant virus replicon particles

Candidate vaccines were generated by replacing the VSV glycoprotein G gene with the *M. ulcerans* genes MUL2232 or MUL3720 (Fig. 1, A). In order to ease virus detection and titration, the coding sequence for the enhanced green fluorescent protein (eGFP) was inserted as an additional transcription cassette downstream of the *M. ulcerans* genes. The recombinant viruses VSV*ΔG(MUL2232) and VSV*ΔG(MUL3720) as well as the control virus VSV*ΔG, which only contained the eGFP gene in place of the VSV surface glycoprotein G (Fig. 1, A), were produced and propagated in the helper cell line BHK-G43 providing the VSV G protein *in trans.* As expected from other studies with similar constructs, the trans-complemented particles were able to infect a variety of different mammalian cell lines but were unable to release progeny viruses [27,45]. We thus refer to them as to virus replicon particles (VRPs).

In order to study the expression of MUL2232 and MUL3720 in infected cells, BHK-21 cells were infected with the three different VRPs generated and analysed by using MUL2232 and MUL3720-specific mouse monoclonal antibodies (mAbs) in immunofluorescence microscopy. Both antigens accumulated in the cytosol of the infected BHK-21 cells (Fig. 1, B1 and B2). While in the immunofluorescence analysis MUL2232 appeared to be expressed at lower levels than MUL3720, staining intensities in Western blotting analyses were comparable for both proteins (Fig.1, C). MUL2232 was mainly detected in the soluble fraction of infected L929 fibroblasts and to a smaller part in the insoluble fraction (Fig. 1, C1) while MUL3720 was found in the soluble fraction only (Fig. 1, C2). Both proteins co-migrated with the corresponding proteins in *M. ulcerans* lysate according to the predicted molecular mass (Fig. 1, C).

Immunization of mice with virus replicon particles induces *M. ulcerans* cross-reactive antibody responses

To characterize the immune responses elicited by the recombinant VRPs, we compared different immunization regimens in BALB/c mice. Humoral immune responses were assessed by ELISA on immobilized recombinant protein. Sub-cutaneous (s.c.) administration of 10^7 VRPs did not lead to measureable antibody responses against the target antigens (Fig. 2, A and B). However, when VSV*ΔG(MUL2232) was given i.m., Immunoglobulin (Ig) G antibodies were elicited in all immunized animals. In contrast, only some mice produced antibodies following i.m. immunization with VSV*ΔG(MUL3720) (Fig. 2, A and B). Therefore induction of antibodies solely by i.m. immunization with VSV*ΔG(MUL3720) was no longer

pursued in subsequent experiments. Independently of the route of administration, both VRPs primed the immune system, as demonstrated by the fast humoral immune response to an adjuvant-free booster immunization (s.c.) with 30 µg of the corresponding recombinant protein (Fig. 2, A and B). Six days after the booster injection, antibody titers were generally higher in mice primed i.m. than in mice primed s.c. (Fig. 2, A and B). Therefore, only the i.m. route was employed for VRP administration in subsequent experiments. In addition, a dose of 10^7 VRPs per immunization was generally used.

In a next step, we assessed the potential of a shorter prime-boost immunization strategy by immunizing the animals only once i.m. with VSV*ΔG(MUL2232) or VSV*ΔG(MUL3720) and three weeks later with 30 µg of rMUL2232 or rMUL3720 via the subcutaneous route in the absence of adjuvant. Five days after the rMUL2232 boost, the ELISA IgG titers were only marginally higher than those observed prior to the boost, but increased further in the subsequent two weeks (Fig. 3, A). On the other hand, administration of rMUL3720 led to high antibody titers already eight days after the boost (Fig. 3, C). Importantly, the elicited antibodies were not only cross-reactive with the recombinant proteins produced in *E. coli* but also with the target proteins expressed by *M. ulcerans,* as demonstrated by Western blotting on *M. ulcerans* lysate (Fig. 3, B and D).

To evaluate whether this immunization regimen would also elicit antigen-specific cellular immune responses, antigen-specific cytokine secretion of spleen cells was studied *in vitro* after primary immunization of mice with VSV*ΔG(MUL2232) and a second immunization with either rMUL2232 or VSV*ΔG(MUL2232). Three weeks after the second immunization, spleen cells were stimulated with either rMUL2232 or the unrelated rMUL3720 as control. Stimulation with Concanavalin A (ConA) served as positive control for confirming the viability of the cultured spleen cells. The stimulated cell culture supernatants were analysed by ELISA for the production of the T_H1 cytokine Interferon gamma (IFNγ) (Fig. 4, A), the pleiotropic [46] cytokine Interleukin 2 (IL-2) (Fig. 4, B) and the T_H2 cytokine Interleukin 10 (IL-10) [47] (Fig. 4, C). Overall, no marked difference in terms of cytokine production was observed between the two immunization schedules. In both cases all three cytokines were produced in significantly higher amounts when cultured spleen cells were stimulated with the corresponding rMUL2232 antigen. In contrast, stimulation with the unrelated antigen rMUL3720 or mock stimulation had no significant effect (Fig. 4). The most pronounced response was found for IL-10 (Fig. 4, C), indicating a slight polarization towards a T_H2 type response. The same experimental setup with VSV*ΔG(MUL3720) was not successful due to technical problems.

Vaccination has no marked inhibitory effect on bacterial proliferation in an experimental BU mouse model

In a last step, we explored the protective efficacy of the VRP-based immunization in an experimental *M. ulcerans* infection model. Groups of six BALB/c mice were immunized according to the VSV*ΔG(MUL2232)/rMUL2232 prime-boost regimen or two times i.m. with VSV*ΔG(MUL2232). Respective control groups were immunized either with the control VRP VSV*ΔG and an rMUL2232 boost (prime-boost control) or twice with VSV*ΔG (Fig. 5). Three weeks after the last immunization, the left hind foot pad of the mice was infected with 8.4 x 10^3 *M. ulcerans* bacilli. The slowly progressing infection was followed by weekly measurements of the thickness of the infected foot pads with a caliper (Fig. 5, A). To determine the bacterial multiplication, mice were euthanized at day 60 after infection and foot pads either processed for histopathological analysis or lysed for enumeration of *M. ulcerans* by standard CFU plating. Quantification was additionally performed by an adapted qPCR method, suitable for the detection of bacterial proliferation in mouse foot pads over the course of the infection (Fig. 5, C). No differences in the kinetics of footpad swelling were observed between immunized mice and the respective control animals (Fig. 5, A). However, 60 days post infection the number of CFU per foot pad was slightly but significantly lower in animals immunized with the VRP prime-protein boost regimen as compared to the prime-boost control immunized animals (Fig. 5, B). Quantification of *M. ulcerans* DNA in the footpads by insertion sequence (IS) 2404 specific qPCR yielded similar results, i.e. a slight but significant reduction in bacterial multiplication caused by the VRP prime-protein boost immunization, but not by two subsequent immunization with VRPs (Fig. 5, B and D). The same experiment with MUL3720 VRPs did not result in any difference between immunized and control groups (S2 Figure).

A histopathological analysis of representative foot pads confirmed the findings of a slight reduction of bacterial burden in the VSV prime-protein boost immunized animals as compared to the prime-boost control animals. The non-infected right foot pads served as control and appeared completely normal (Fig. 6, A1) with intact muscle tissue (Fig. 6, A2) and no apparent oedematous changes (Fig. 6, A3). In comparison, the infected left foot pads of control mice showed typical histopathological signs of BU with strong oedema (Fig. 6, B1 and B4), necrotic sole of foot (Fig. 6, B2), and inflammatory infiltration and extensive haemorrhages all over the foot pad (Fig. 6, B3). Ziehl-Neelsen/Methylene blue (ZN) staining revealed large clumps of acid fast bacilli (AFB) (Fig. 6, C1), not only located where they were initially injected, but also more towards the heel of the foot (Fig. 6, C2) and in the oedematous tissue in the upper part of the foot pad (Fig. 6, C5). AFB were associated with remains of infiltrating immune cells (Fig. 6, C3) and were also found as fibrous structures in completely necrotic tissue (Fig. 6, C4). In animals that received a VRP prime-protein boost

immunization, a trend towards a reduction in the number of AFB clusters was observed (Fig. 6, D1). Furthermore, AFB appeared to be more often in close contact with infiltrating cells or were found intracellular (Fig. 6, D3).

Discussion

Despite substantial control efforts and improvements in treatment and diagnosis, the socioeconomic impact of BU on affected communities remains high. Long treatment regimens with daily i.m. injections in rural settings of West Africa, late reporting to health facilities, scarring and resulting permanent disabilities create high morbidity that could be prevented by a vaccine against *M. ulcerans*. Although no such vaccine is available at the moment, reports on self-healing in patients with early stages of the disease [48,49] as well as sero-epidemiological studies in BU-endemic countries [32,50] indicate that the human organism is in principle capable of inducing a protective immune responses against BU. Furthermore, protein subunit vaccination approaches have demonstrated partial protection in a BU mouse infection model [21,22,51].

In the present study, we used a viral replicon particle system for delivering *M. ulcerans* protein antigens to the immune system. Immunization with a high number of VRPs induced *M. ulcerans* specific antibody responses with i.m. application being superior to s.c. application. Our VRP prime-recombinant protein boost regimen not only induced a humoral but also a cellular immune response. This is in line with previous work showing that VRPs are able to trigger humoral as well as cellular immune responses [52].

The bacterial proteins MUL2232 and MUL3720 were chosen as vaccine antigens because previous work suggested that these antigens are not only highly immunogenic but also associated with the outer surface of *M. ulcerans* [32,34,35]. Therefore, the strong humoral immune response observed following immunization of mice was expected to allow antibody-mediated opsonization of the bacteria with consequent enhanced phagocytosis, complement activation, or antibody-dependent cellular cytotoxicity. While we did not observe vaccination-induced reductions in foot pad swelling in our murine *M. ulcerans* infection model, assessment of the bacterial load by qPCR as well as CFU plating revealed a slight, but significant reduction in bacterial multiplication in VRP prime-protein boost immunized mice. While measurement of foot pad swelling over time is a parameter that can be followed without euthanizing mice [53,54], our results illustrate that foot pad swelling not necessarily reflects the extent of bacterial proliferation.

Despite a slight inhibitory effect on bacterial proliferation, immunization with only one target antigen in a VRP prime-protein boost regimen was not sufficient to confer full protection against the experimental infection. Several factors, like the choice or number of antigens included into the VRPs, could have let to this negative result. Since it is not known, which immune effector functions are relevant for protection against *M. ulcerans* disease [15], it is not clear whether lack of a strong T_H1-polarisation is of major relevance for the failure to

achieve a strong protective efficacy with the immunization regimens tested. One of the advantages of the replicon system lies in the ability of VSV to tolerate incorporation of long stretches of foreign DNA into its genome [25,26]. As a modular system, it offers the possibility to design a multivalent subunit vaccine by combining several *M. ulcerans* proteins in one replicon. Furthermore the versatility of the system allows to engineer the location of the expressed protein in the infected cell, or to target specific immune cells by including genes for co-stimulatory molecules or receptors to be expressed on the surface of the infected cells [25]. Here we have demonstrated that RNA replicon particles are a very good delivery system for mycobacterial antigens, which is in particular encouraging future development of VRP-based multivalent subunit vaccines.

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Author Contributions

Conceived and designed the experiments: MB GZ GP. Performed the experiments: MB SK GZ. Analyzed the data: MB GZ GP. Wrote the paper: MB GZ GP.

Figures

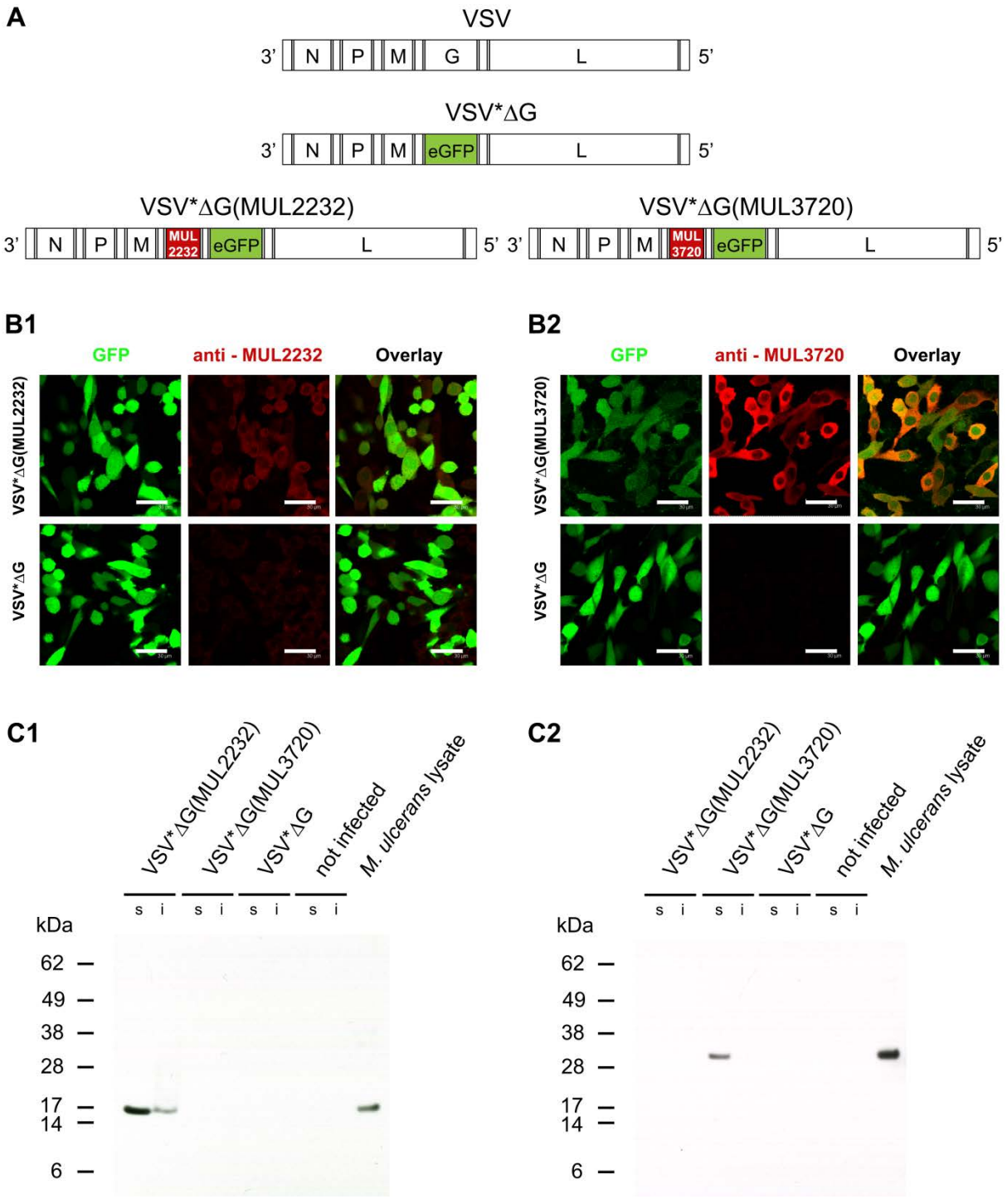


Figure 1: Expression of *M. ulcerans* proteins by VRP-infected cells.

(A) Genome maps of recombinant VSV: The genome of VSV encodes for the nucleoprotein N, phosphoprotein P, matrix protein M, glycoprotein G, and the large RNA polymerase L. In VSV*ΔG, the glycoprotein G coding region was replaced by the coding sequence for eGFP.

In VSV*ΔG(MUL22332) and VSV*ΔG(MUL3720), the VSV G gene was replaced by the *M. ulcerans* genes MUL2232 and MUL3720, respectively. The reporter protein eGFP is encoded by an additional transcription cassette located downstream of the *M. ulcerans* genes. (B) BHK-21 cells were infected with the indicated replicons and indirect immunofluorescence analysis performed with mAbs specific for MUL2232 (B1) or MUL3720 (B2) (red fluorescence). The infection of cells with VRPs is indicated by eGFP expression (green fluorescence). Scale bar equals 30 µm. (C) Equal numbers of L929 fibroblasts were infected with the indicated VRPs at a MOI of 10. At 6 hours post infection, the cells were harvested and protein lysates of the soluble (s) and insoluble (i) fraction analysed by Western blotting for expression of *M. ulcerans* antigens using mAbs specific for MUL2232 (C1) and MUL3720 (C2), respectively.

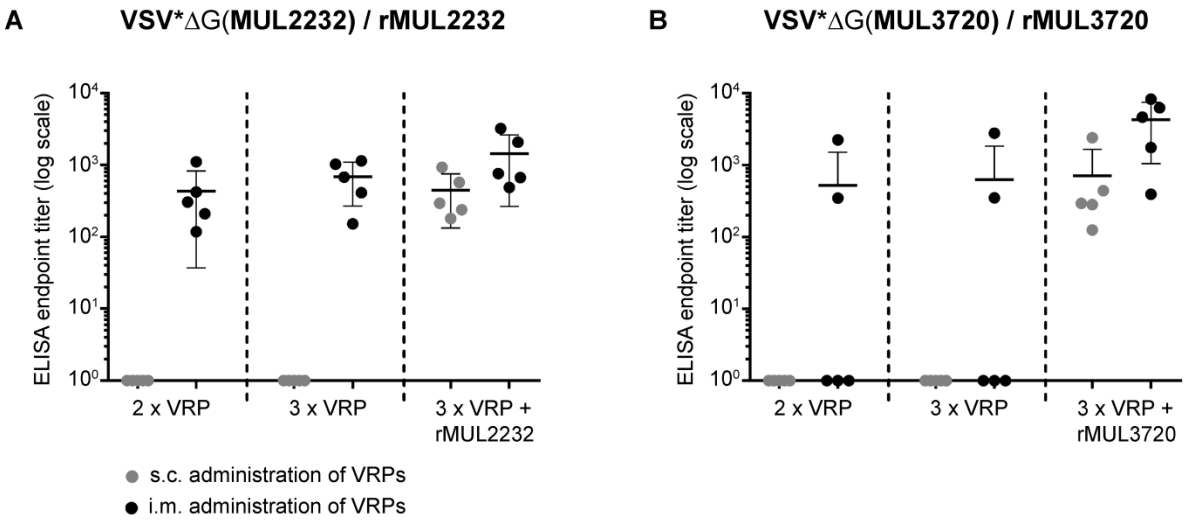


Figure 2: Comparison of immunization routes.

Groups of five female BALB/c mice were sequentially immunized with VSV*ΔG(MUL2232) (A) or VSV*Δ(MUL3720) (B) via the sub-cutaneous (grey dots) or intra-muscular (black dots) route. Prior to every new immunization, blood was taken and serum analysed by ELISA on the corresponding recombinant proteins (rMUL2232 or rMUL3720). After the third immunization, all mice were boosted (s.c) with the VRP correspondent, non-adjuvanted recombinant protein. Endpoint total IgG titers were determined for individual animals in one single ELISA. Mean values (line) ± standard deviations are shown.

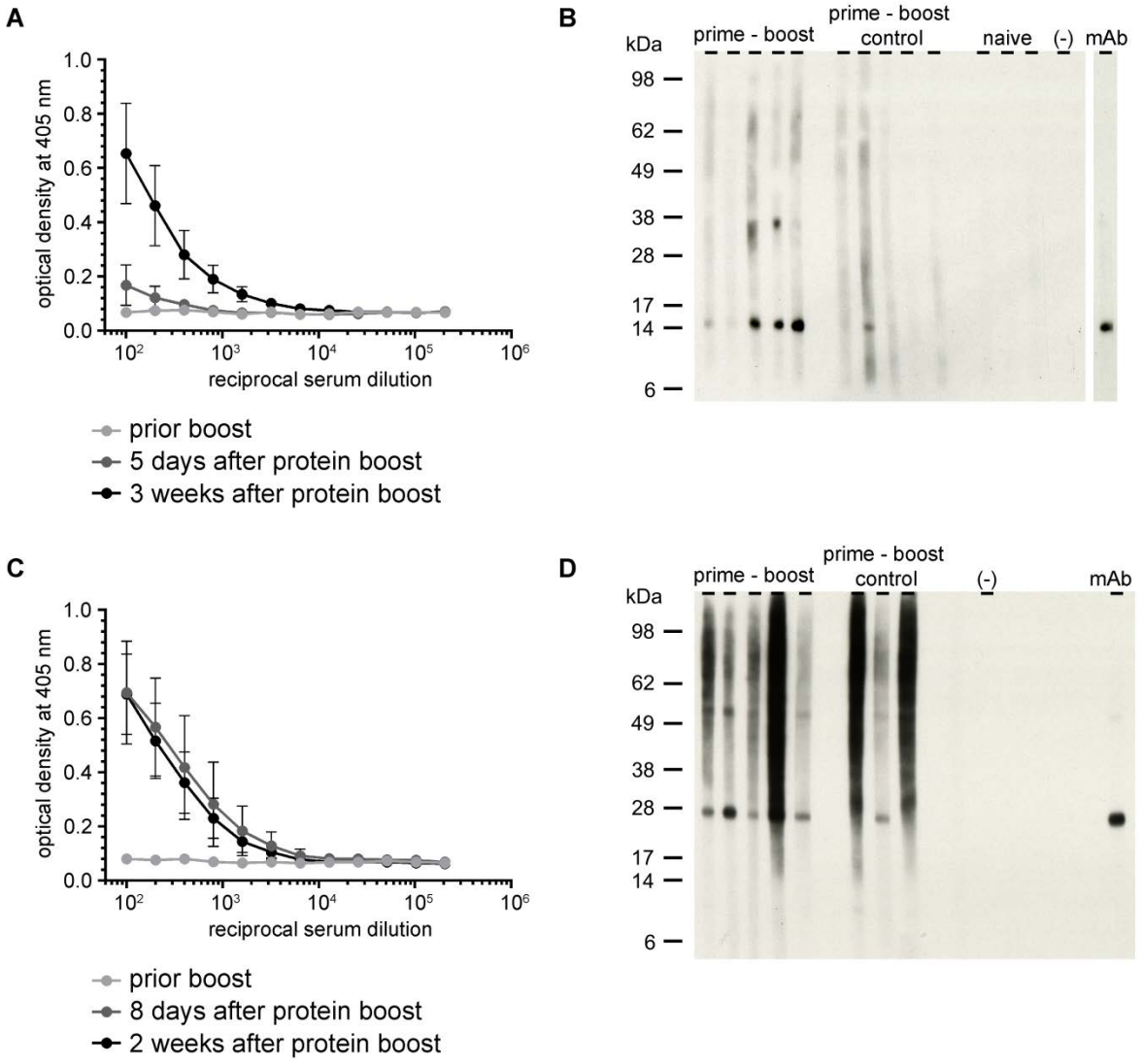


Figure 3: Induction of antigen-specific antibodies by a short prime-boost immunization protocol.

Female BALB/c mice (n = 5) were immunized once with VSV*ΔG(MUL2232) or VSV*ΔG(MUL3720) and boosted with rMUL2232 or rMUL3720 three weeks later. Control animals (n = 5 and n = 3) were immunized with empty replicon prior to recombinant protein boost three weeks later. (A) Analysis of serially diluted immune sera by ELISA on immobilized rMUL2232. Mean values (dot) ± standard deviations are shown. (B) Western blotting analysis on *M. ulcerans* lysate using the indicated immune sera taken three weeks after protein boost. (C) Analysis of serially diluted immune sera by ELISA on immobilized rMUL3720. Mean values (dot) ± standard deviation are shown. (D) Western blotting analysis on *M. ulcerans* lysate using the indicated immune sera taken two weeks after protein boost.

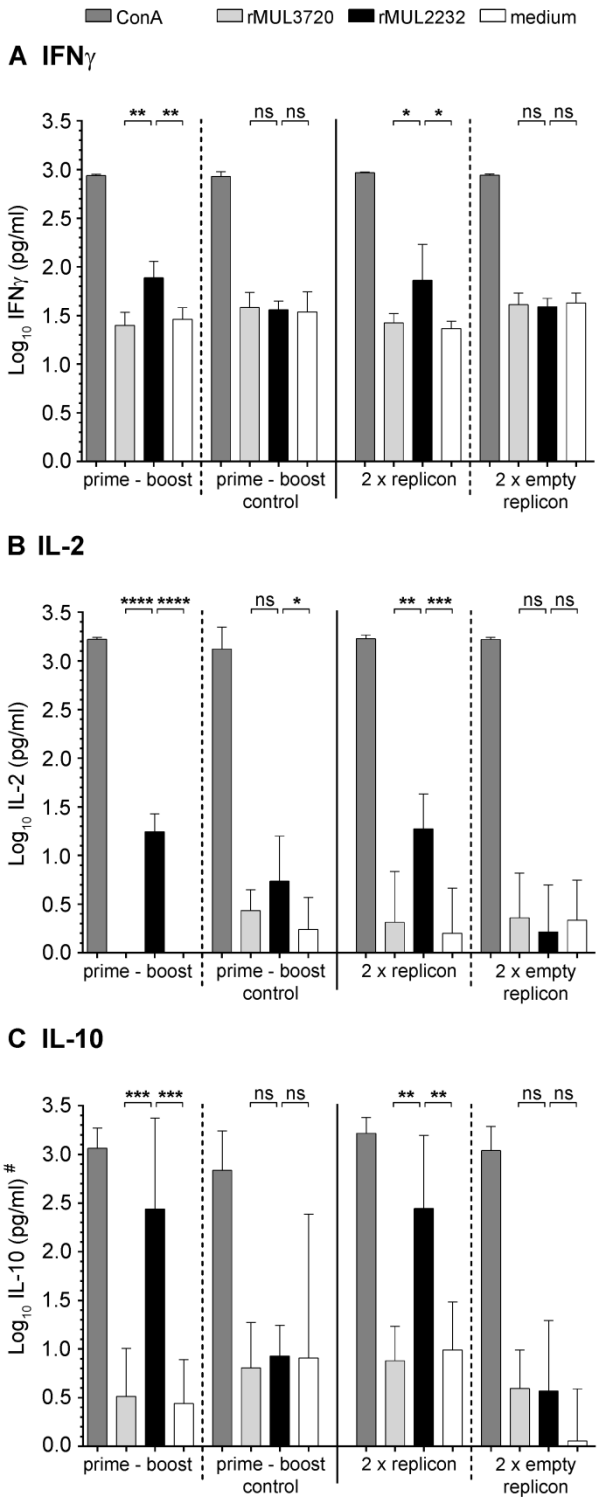


Figure 4: Analysis of cellular immune responses.

Female BALB/c mice (n = 5) were immunized once with VSV*ΔG(MUL2232) (i.m.) and boosted with rMUL2232 (s.c., prime-boost) or were immunized with VSV*ΔG (i.m.) and boosted with rMUL2232 (s.c., prime-boost control). Alternatively, the animals were immunized twice with either VSV*ΔG(MUL2232) (2 x VRP) or the control vector VSV*ΔG (2 x

empty VRP). Three weeks after the final immunization, mice were euthanized. Spleen cells were taken into culture and stimulated with either Concanavalin A (ConA), rMUL2232, rMUL3720 or left unstimulated (medium). Levels of secreted cytokines were determined by commercially available ELISA kits at 24 hours (IL-2, B) and 96 hours (IFNγ, A; IL-10, C) after stimulation. Mean values (bar) ± standard deviations for 5 mice tested are shown and expressed as $\log_{10}$ (pg/ml). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$ (p – value adjusted for random effects in a linear mixed model). Ns equals non-significance. # Due to high non-specific rMUL3720 stimulation presumably caused by contaminants in the recombinant protein preparation, values were normalized to those of a non-immunized group of mice and z-values are depicted for all stimulations except ConA.

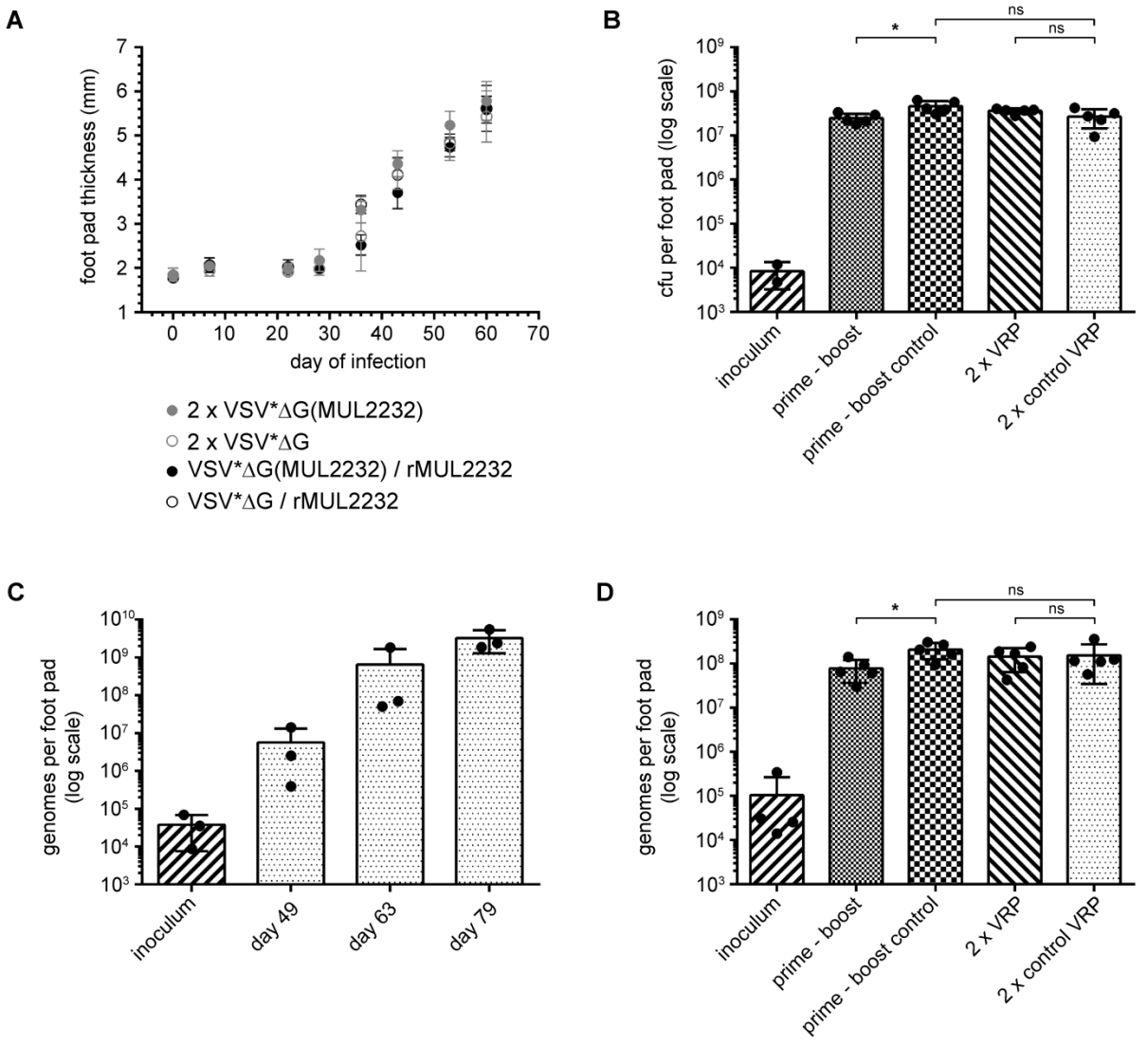


Figure 5: Infection of immunized mice with *M. ulcerans*.

Groups of six immunized, female BALB/c mice were infected by s.c. injection of *M. ulcerans* suspension (30 µl) into the left hind foot pad. (A) Development of the infection was followed by weekly measurements of the foot pad thickness with a caliper. The mean foot pad thickness (dot) ± standard deviation is shown for each animal group. (B) At day 60 after infection, mice were sacrificed and the number of *M. ulcerans* bacilli in foot pads determined by classical CFU plating. (C) Quantification of *M. ulcerans* in foot pad lysates by IS2404 specific qPCR. Genome equivalents are shown. (D) Determination of *M. ulcerans* genome equivalents in immunized and infected mice. * $p \leq 0.05$ (Mann-Whitney test).

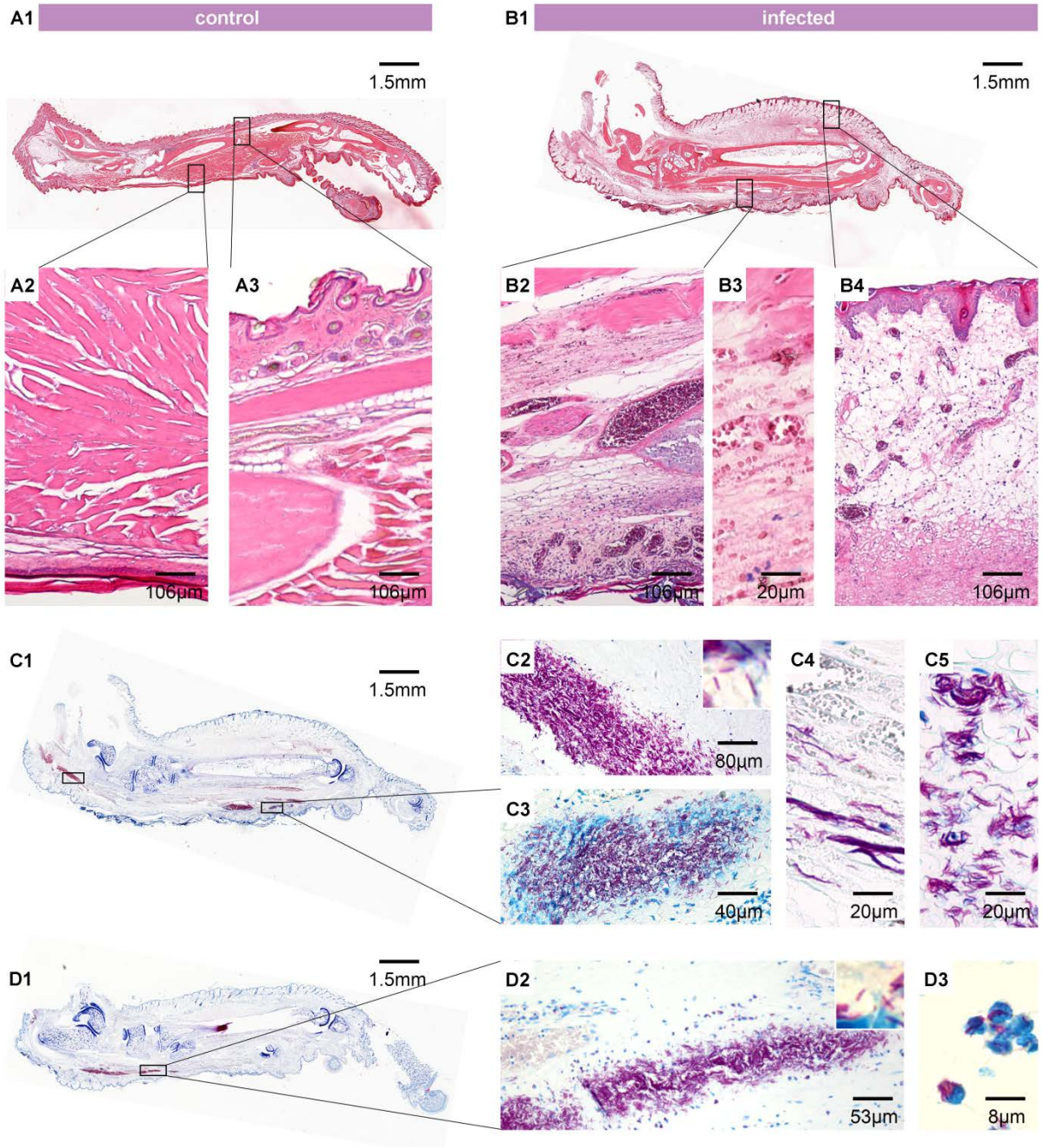
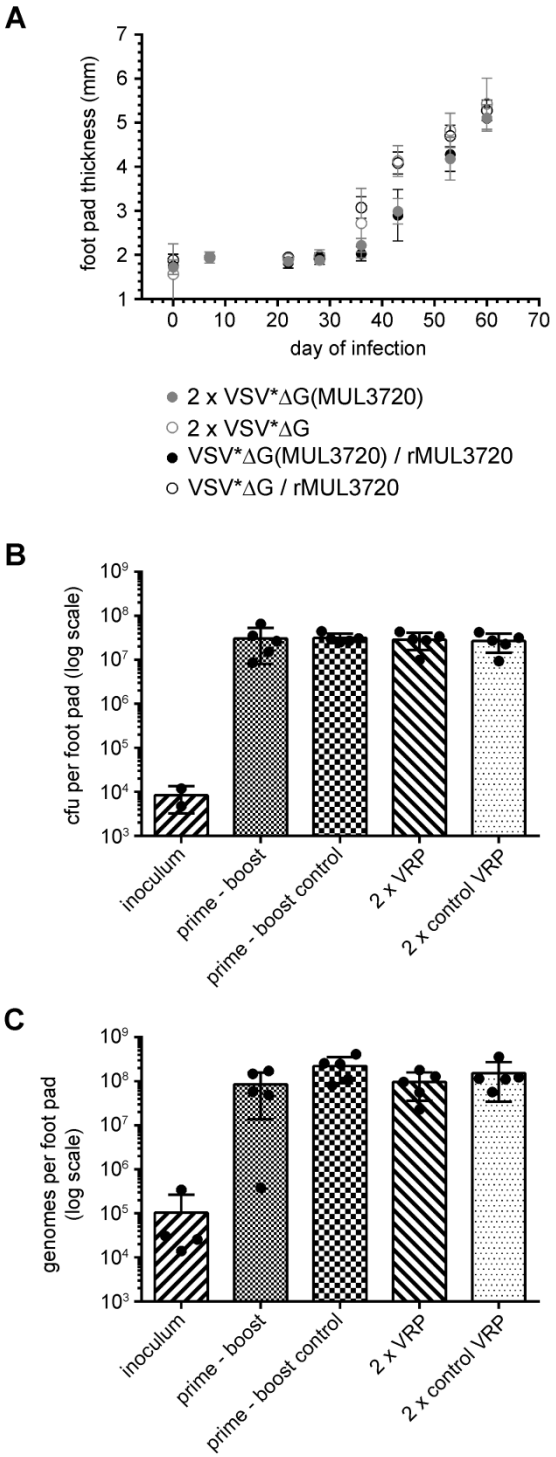


Figure 6: Histopathological analysis of mouse foot pads following infection with *M. ulcerans*.

Histological sections of foot pads from *M. ulcerans*-infected mice were stained with Haematoxylin/Eosin (A1-A3, B1-B4) or Ziehl-Neelsen/Methylene blue (ZN) (C1-C5, D1-D3). The right, non-infected foot pad showed no swelling (A1), intact muscle tissue (A2) and absence of oedema in the upper part of the foot (A3). As opposed to this, the left foot of a mouse immunized with VSV*ΔG/rMUL2232 (prime-boost control) was strongly swollen 60 days post infection (B1). Cellular infiltration and beginning necrosis of the tissue was explicitly strong at the base of the foot pad, where bacteria were injected (B2) and muscle

fibres started to become destroyed (B3). The upper part of the foot is highly oedematous (B4). Staining of the same foot pad for AFB revealed large amounts of bacteria in close contact to infiltrating immune cells at the base of the foot (C3). Appearance of AFB in the destroyed muscle tissue (C4). Big clumps of bacteria were also found towards the ankle of the foot (C2) and in the oedematous tissue in the upper half of the foot pad (C5). The foot pad of a mouse immunized with VSV*ΔG(MUL2232)/rMUL2232 (prime-boost) contained large amounts of AFB (D1) which were less spread in the whole foot pad. Intracellular AFB were frequently observed (D3).

Supplementary Material



S1 Figure: Infection of immunized mice with *M. ulcerans* (MUL3720 immunizations).

Groups of six immunized, female BALB/c mice were infected into the left hind foot pad with 30 µl of *M. ulcerans* suspension (s.c.). (A) Development of the infection was followed by

weekly measures of the foot pad thickness with a caliper. Depicted is the mean foot pad thickness (dot) ± standard deviation of the individual differently immunized groups. (B) At day 60 after infection, mice were sacrificed and the number of *M. ulcerans* bacilli in foot pads determined by classical CFU plating. (C) Determination of *M. ulcerans* genome equivalents in immunized and infected mice.

References

1. Van der Werf TS, Van der Graaf WT, Tappero JW, Asiedu K. *Mycobacterium ulcerans* infection. The Lancet. 1999;354: 1013–1018. doi:10.1016/S0140-6736(99)01156-3
2. Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, Kerber S, et al. Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. PLoS Negl Trop Dis. 2013;7: e2252. doi:10.1371/journal.pntd.0002252
3. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, Benbow ME, et al. Ecology and transmission of Buruli ulcer disease: a systematic review. PLoS Negl Trop Dis. 2010;4: e911. doi:10.1371/journal.pntd.0000911
4. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, et al. Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science. 1999;283: 854–857.
5. WHO | Laboratory diagnosis of buruli ulcer. In: WHO [Internet]. [cited 2 Jun 2014]. Available: http://www.who.int/buruli/laboratory_diagnosis/en/
6. WHO | Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer). In: WHO [Internet]. [cited 19 Jun 2014]. Available: zotero://attachment/245/
7. Chauty A, Ardant M-F, Adeye A, Euverte H, Guédénon A, Johnson C, et al. Promising clinical efficacy of streptomycin-rifampin combination for treatment of buruli ulcer (*Mycobacterium ulcerans* disease). Antimicrob Agents Chemother. 2007;51: 4029–4035. doi:10.1128/AAC.00175-07
8. Nienhuis WA, Stienstra Y, Thompson WA, Awuah PC, Abass KM, Tuah W, et al. Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial. Lancet. 2010;375: 664–672. doi:10.1016/S0140-6736(09)61962-0
9. Sarfo FS, Phillips R, Asiedu K, Ampadu E, Bobi N, Adentwe E, et al. Clinical efficacy of combination of rifampin and streptomycin for treatment of *Mycobacterium ulcerans* disease. Antimicrob Agents Chemother. 2010;54: 3678–3685. doi:10.1128/AAC.00299-10
10. Agbenorku P, Donwi IK, Kuadzi P, Saunderson P. Buruli ulcer: treatment challenges at three centres in ghana. J Trop Med. 2012;2012: 371915. doi:10.1155/2012/371915
11. Einarsdottir T, Huygen K. Buruli ulcer. Hum Vaccin. 2011;7: 1198–1203. doi:10.4161/hv.7.11.17751
12. Prezzemolo T, Guggino G, La Manna MP, Di Liberto D, Dieli F, Caccamo N. Functional Signatures of Human CD4 and CD8 T Cell Responses to *Mycobacterium tuberculosis*. Front Immunol. 2014;5: 180. doi:10.3389/fimmu.2014.00180
13. Coutanceau E, Marsollier L, Brosch R, Perret E, Goossens P, Tanguy M, et al. Modulation of the host immune response by a transient intracellular stage of *Mycobacterium ulcerans*: the contribution of endogenous mycolactone toxin. Cell Microbiol. 2005;7: 1187–1196. doi:10.1111/j.1462-5822.2005.00546.x
14. Torrado E, Fraga AG, Castro AG, Stragier P, Meyers WM, Portaels F, et al. Evidence for an intramacrophage growth phase of *Mycobacterium ulcerans*. Infect Immun. 2007;75: 977–987. doi:10.1128/IAI.00889-06
15. Schütte D, Pluschke G. Immunosuppression and treatment-associated inflammatory response in patients with *Mycobacterium ulcerans* infection (Buruli ulcer). Expert Opin Biol Ther. 2009;9: 187–200. doi:10.1517/14712590802631854

16. Nackers F, Dramaix M, Johnson RC, Zinsou C, Robert A, DE Biurrun Bakedano E, et al. BCG vaccine effectiveness against Buruli ulcer: a case-control study in Benin. Am J Trop Med Hyg. 2006;75: 768–774.
17. Smith PG, Revill WD, Lukwago E, Rykushin YP. The protective effect of BCG against *Mycobacterium ulcerans* disease: a controlled trial in an endemic area of Uganda. Trans R Soc Trop Med Hyg. 1976;70: 449–457.
18. BCG vaccination against *mycobacterium ulcerans* infection (Buruli ulcer). First results of a trial in Uganda. Lancet. 1969;1: 111–115.
19. Converse PJ, Almeida DV, Nuermberger EL, Grosset JH. BCG-mediated protection against *Mycobacterium ulcerans* infection in the mouse. PLoS Negl Trop Dis. 2011;5: e985. doi:10.1371/journal.pntd.0000985
20. Fraga AG, Martins TG, Torrado E, Huygen K, Portaels F, Silva MT, et al. Cellular immunity confers transient protection in experimental Buruli ulcer following BCG or mycolactone-negative *Mycobacterium ulcerans* vaccination. PloS One. 2012;7: e33406. doi:10.1371/journal.pone.0033406
21. Tanghe A, Content J, Van Vooren J-P, Portaels F, Huygen K. Protective Efficacy of a DNA Vaccine Encoding Antigen 85A from *Mycobacterium bovis* BCG against Buruli Ulcer. Infect Immun. 2001;69: 5403–5411. doi:10.1128/IAI.69.9.5403-5411.2001
22. Tanghe A, Dangy J-P, Pluschke G, Huygen K. Improved protective efficacy of a species-specific DNA vaccine encoding mycolyl-transferase Ag85A from *Mycobacterium ulcerans* by homologous protein boosting. PLoS Negl Trop Dis. 2008;2: e199. doi:10.1371/journal.pntd.0000199
23. Roupie V, Pidot SJ, Einarsdottir T, Van Den Poel C, Jurion F, Stinear TP, et al. Analysis of the vaccine potential of plasmid DNA encoding nine mycolactone polyketide synthase domains in *Mycobacterium ulcerans* infected mice. PLoS Negl Trop Dis. 2014;8: e2604. doi:10.1371/journal.pntd.0002604
24. Schnell MJ, Buonocore L, Whitt MA, Rose JK. The minimal conserved transcription stop-start signal promotes stable expression of a foreign gene in vesicular stomatitis virus. J Virol. 1996;70: 2318–2323.
25. Zimmer G. RNA replicons - a new approach for influenza virus immunoprophylaxis. Viruses. 2010;2: 413–434. doi:10.3390/v2020413
26. Lichty BD, Power AT, Stojdl DF, Bell JC. Vesicular stomatitis virus: re-inventing the bullet. Trends Mol Med. 2004;10: 210–216. doi:10.1016/j.molmed.2004.03.003
27. Halbherr SJ, Brostoff T, Tippenhauer M, Locher S, Berger Rentsch M, Zimmer G. Vaccination with recombinant RNA replicon particles protects chickens from H5N1 highly pathogenic avian influenza virus. PloS One. 2013;8: e66059. doi:10.1371/journal.pone.0066059
28. Cobleigh MA, Buonocore L, Uprichard SL, Rose JK, Robek MD. A vesicular stomatitis virus-based hepatitis B virus vaccine vector provides protection against challenge in a single dose. J Virol. 2010;84: 7513–7522. doi:10.1128/JVI.00200-10
29. Klas SD, Lavine CL, Whitt MA, Miller MA. IL-12-assisted immunization against *Listeria monocytogenes* using replication-restricted VSV-based vectors. Vaccine. 2006;24: 1451–1461. doi:10.1016/j.vaccine.2005.05.046
30. Rose NF, Marx PA, Luckay A, Nixon DF, Moretto WJ, Donahoe SM, et al. An Effective AIDS Vaccine Based on Live Attenuated Vesicular Stomatitis Virus Recombinants. Cell. 2001;106: 539–549. doi:10.1016/S0092-8674(01)00482-2
31. Publicover J, Ramsburg E, Rose JK. A single-cycle vaccine vector based on vesicular stomatitis virus can induce immune responses comparable to those generated by a replication-competent vector. J Virol. 2005;79: 13231–13238. doi:10.1128/JVI.79.21.13231-13238.2005

32. Diaz D, Döbeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, Soder N, et al. Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. Clin Vaccine Immunol CVI. 2006;13: 1314–1321. doi:10.1128/CVI.00254-06

33. Marques MA, Chitale S, Brennan PJ, Pessolani MC. Mapping and identification of the major cell wall-associated components of *Mycobacterium leprae*. Infect Immun. 1998;66: 2625–2631.

34. Vettiger A, Scherr N, Ruf M-T, Röltgen K, Pluschke G. Localization of Mycobacterial Antigens by Immunofluorescence Staining of Agarose Embedded Cells. J Mycobact Dis. 2014;4: 150.

35. Dreyer A, Röltgen K, Dangy JP, Ruf MT, Scherr N, Bolz M, et al. Identification of the *Mycobacterium ulcerans* Protein MUL_3720 as a Promising Target for the Development of a Diagnostic Test for Buruli Ulcer. PLoS Negl Trop Dis. 2015;9: e0003477. doi:10.1371/journal.pntd.0003477

36. Hanika A, Larisch B, Steinmann E, Schwegmann-Wessels C, Herrler G, Zimmer G. Use of influenza C virus glycoprotein HEF for generation of vesicular stomatitis virus pseudotypes. J Gen Virol. 2005;86: 1455–1465. doi:10.1099/vir.0.80788-0

37. Kalhoro NH, Veits J, Rautenschlein S, Zimmer G. A recombinant vesicular stomatitis virus replicon vaccine protects chickens from highly pathogenic avian influenza virus (H7N1). Vaccine. 2009;27: 1174–1183. doi:10.1016/j.vaccine.2008.12.019

38. Berger Rentsch M, Zimmer G. A vesicular stomatitis virus replicon-based bioassay for the rapid and sensitive determination of multi-species type I interferon. PloS One. 2011;6: e25858. doi:10.1371/journal.pone.0025858

39. Sutter G, Ohlmann M, Erfle V. Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. FEBS Lett. 1995;371: 9–12.

40. Baneyx F. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol. 1999;10: 411–421.

41. Bratschi MW, Bolz M, Grize L, Kerber S, Minyem JC, Um Boock A, et al. Primary cultivation: factors affecting contamination and *Mycobacterium ulcerans* growth after long turnover time of clinical specimens. BMC Infect Dis. 2014;14: 636. doi:10.1186/s12879-014-0636-7

42. Lavender CJ, Fyfe JAM. Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples. Methods Mol Biol Clifton NJ. 2013;943: 201–216. doi:10.1007/978-1-60327-353-4_13

43. Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, Azuolas J, et al. Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. Appl Environ Microbiol. 2007;73: 4733–4740. doi:10.1128/AEM.02971-06

44. Portaels F, Organization WH. Laboratory diagnosis of buruli ulcer: a manual for health care providers / edited by Françoise Portaels. Diagnostic de l'ulcère de Buruli au laboratoire : manuel destiné au personnel de santé / edité par Françoise Portaels. 2014; Available: http://apps.who.int//iris/handle/10665/111738

45. Zimmer G, Locher S, Berger Rentsch M, Halbherr SJ. Pseudotyping of vesicular stomatitis virus with the envelope glycoproteins of highly pathogenic avian influenza viruses. J Gen Virol. 2014;95: 1634–1639. doi:10.1099/vir.0.065201-0

46. Liao W, Lin J-X, Leonard WJ. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013;38: 13–25. doi:10.1016/j.immuni.2013.01.004

47. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. Nat Rev Immunol. 2014;14: 24–35. doi:10.1038/nri3567

48. Revill WDL, Morrow RH, Pike MC, Ateng J. A CONTROLLED TRIAL OF THE TREATMENT OF *MYCOBACTERIUM ULCERANS* INFECTION WITH CLOFAZIMINE. The Lancet. 1973;302: 873–877. doi:10.1016/S0140-6736(73)92005-9

49. Gordon CL, Buntine JA, Hayman JA, Lavender CJ, Fyfe JA, Hosking P, et al. Spontaneous Clearance of *Mycobacterium ulcerans* in a Case of Buruli Ulcer. PLoS Negl Trop Dis. 2011;5. doi:10.1371/journal.pntd.0001290

50. Röltgen K, Bratschi MW, Ross A, Aboagye SY, Ampah KA, Bolz M, et al. Late onset of the serological response against the 18 kDa small heat shock protein of *Mycobacterium ulcerans* in children. PLoS Negl Trop Dis. 2014;8: e2904. doi:10.1371/journal.pntd.0002904

51. Coutanceau E, Legras P, Marsollier L, Reysset G, Cole ST, Demangel C. Immunogenicity of *Mycobacterium ulcerans* Hsp65 and protective efficacy of a *Mycobacterium leprae* Hsp65-based DNA vaccine against Buruli ulcer. Microbes Infect Inst Pasteur. 2006;8: 2075–2081. doi:10.1016/j.micinf.2006.03.009

52. Johnson JE, McNeil LK, Megati S, Witko SE, Roopchand VS, Obregon JH, et al. Non-propagating, recombinant vesicular stomatitis virus vectors encoding respiratory syncytial virus proteins generate potent humoral and cellular immunity against RSV and are protective in mice. Immunol Lett. 2013;150: 134–144. doi:10.1016/j.imlet.2012.12.005

53. Martins TG, Trigo G, Fraga AG, Gama JB, Longatto-Filho A, Saraiva M, et al. Corticosteroid-induced immunosuppression ultimately does not compromise the efficacy of antibiotherapy in murine *Mycobacterium ulcerans* infection. PLoS Negl Trop Dis. 2012;6: e1925. doi:10.1371/journal.pntd.0001925

54. Trigo G, Martins TG, Fraga AG, Longatto-Filho A, Castro AG, Azeredo J, et al. Phage therapy is effective against infection by *Mycobacterium ulcerans* in a murine footpad model. PLoS Negl Trop Dis. 2013;7: e2183. doi:10.1371/journal.pntd.0002183

Results Chapter 6

Use of *Plasmodium berghei* blood stage parasites expressing mycobacterial vaccine candidate antigens for immunization under chloroquine cover

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Abstract

Malaria infection with viable *Plasmodium falciparum* parasites by bites of infected mosquitoes under simultaneous drug cover with chloroqine, so called infection-treatment-vaccination, has been shown to be an effective vaccination strategy in malaria-naïve human volunteers. Similarly, infection-treatment-vaccination with infected red blood cells in mouse models led to sterile immunity. Thinking further along this line, *Plasmodium* parasites could possibly be used as delivery systems for antigens originating from other disease causing agents and cross-protect against several diseases. As part of the evaluation of the potential for developing a protein subunit vaccine against Buruli ulcer, an ulcerative disease of the skin caused by *Mycobacterium ulcerans,* we tested an infection treatment vaccination approach with genetically modified *Plasmodium berghei* mouse malaria parasites. We confirmed the expression of the chosen protein vaccine candidates of *M. ulcerans*, MUL3720 and MUL4987 (Ag85A), in the generated *P. berghei* lines and demonstrated the induction of potent specific antibody responses by infection of mice under chloroquine cover. However, the elicited immune responses were not sufficient to produce solid protection in an experimental mouse footpad infection model of Buruli ulcer.

Introduction

Already in 1975 it has been shown that immunization with attenuated whole *Plasmodium falciparum* sporozoites applied by mosquito bites can elicit sterilizing immune protection against experimental malaria infection in human volunteers [1]. These and other findings led to increased efforts to develop whole parasite-based vaccination as alternative to a subunit vaccine against malaria. Recently it has been shown that protection against controlled human malaria infection can be achieved by repeated intravenous injection of attenuated, cryopreserved *P. falciparum* sporozoites [2]. More generally speaking, whole parasite vaccination approaches can be separated into two key concepts: infection with irradiated or attenuated sporozoites or infection with viable parasites under drug cover. Infection with viable malaria parasites under drug cover, also called infection – treatment - vaccination (ITV), has been shown to be effective in malaria-naive volunteers immunized by bites of infected mosquitoes while simultaneously preventing disease with chloroquine prophylaxis [3]. In mouse malaria models sterile immunity has also been achieved by infection with infected red blood cells (iRBC) under curative drug cover [4,5]. With the goal to address whether the subcellular location of an antigen influences the nature and magnitude of antigen-specific T-cell responses, Lin *et al*. recently reported the introduction of the model antigen Ovalbumin (OVA) in *P. berghei* parasites [6]. Whether OVA was expressed on the parasitophorous vacuole membrane (PVM) or in the parasite cytosol influenced its expression levels as well as the induction and magnitude of parasite-specific T-cell responses [6]. For the study presented here we chose to assess whether mycobacterial antigens could be expressed instead of OVA and whether such modified *P. berghei* parasites could be used for ITV for a mycobacterial disease.

Mycobacterium ulcerans, the donor of the heterologous antigens for this study, causes a slowly progressing, necrotising disease of the skin and subcutaneous tissue in humans [7]. The so called Buruli ulcer (BU) is typically found very focally in rural communities in West African countries, but has been reported from more than 30 countries worldwide [8]. Until today both the natural reservoir/s as well as the mode(s) of transmission are not clearly identified [8,9]. With 8 weeks of daily doses of rifampicin and streptomycin, treatment for BU is very long and could potentially be shortened by the use of a vaccine [10]. Furthermore a vaccine could prevent the disease and its associated socioeconomic impact on the affected population completely. Unfortunately no such vaccine is available at the moment [11]. Little is known about correlates of protection in BU patients, although sero-epidemiological studies and reports on spontaneous healing indicate the existence of a protective immune response [12–16]. Cellular mediated immunity (CMI) of a T-helper 1 (T_H1) type was suggested to be essential against first intracellular stages of *M. ulcerans* [11]. However, later in the

development of BU, the majority of bacilli are found extracellular in the affected subcutaneous tissue [17,18]. Against those stages, the presence of natural- or vaccine-induced antibodies against surface proteins could be of major importance [19]. In the study presented here we focused on the use of *Plasmodium* parasites in ITV as delivery system for the two heterologous protein antigens MUL3720 and MUL4987 of *M. ulcerans*. We studied the humoral immune response induced by ITV and evaluated the protective potential of the vaccine formulations in a mouse challenge model. If feasible, the resulting vaccine would be one step of many to go towards multi-disease vaccination against malaria and other infectious diseases.

Materials and Methods

Ethical Statement

All animal experiments performed were approved by the animal welfare committee of the Canton of Basel (authorization number 1731) and the Canton of Vaud (authorization number 2657) and were conducted in compliance with the Swiss animal protection law. All infection experiments with *M. ulcerans* were conducted under Biosafety-level-3 (BSL-3) conditions at the École polytechnique federal de Lausanne (EPFL).

Generation of transgenic *P. berghei* parasites

P. berghei expressing codon optimized MUL_3720 and MUL_4987 as fusion to the PVM protein EXP1/HEP17 (exported protein 1, hepatocyte erythrocyte protein 17 kD) were generated as previously described [6].

P. berghei immunization of mice by infection and subsequent cure with chloroquine

Mice were infected by the intravenous (i.v.) route with whole blood of a donor mouse previously infected with genetically modified *Plasmodium berghei* by the intra-peritoneal (i.p.) route. Donor mouse blood was diluted with sterile phosphate buffered saline (PBS) to contain 1 % of infected erythrocytes in order to infect every mouse of the experimental group with 2 x 10^7 iRBC, ensuing high in group consistency of the infection rate [20]. Treatment of the mice was started 48 hours after i.v. infection by administering chloroquine (Sigma) in the drinking water (0.288 mg/ml) during fourteen days. To make the water more palatable for the animals, glucose at a concentration of 15 mg/ml was additionally added to the drinking water. Three weeks after infection with *P. berghei,* blood collection by the tail vein was performed and serum gained by centrifugation of the blood in SST Microtainer tubes (Becton, Dickinson and Company).

Lysates of *P. berghei* and *M. ulcerans*

For production of *P. berghei* protein lysate, blood of highly infected mice was collected by cardiac puncture after euthanasia. iRBC were harvested by centrifugation and washed several times with PBS. The pellet of iRBC was dissolved in five times its volume in RIPA buffer containing protease inhibitors (cOmplete Mini, EDTA-free, Roche), incubated 15 minutes on ice and lysate cleared by centrifugation. Total protein content was determined by BCA assay (Pierce) according to the manufacturer's instructions. For protein lysates of *M. ulcerans,* 7.5 ml fully grown culture in BacT/ALERT medium (Biomerieux) was pelleted by centrifugation, resuspended in 1 ml PBS and incubated at 85°C for 1h. Bacteria were pelleted again, washed in PBS and finally resuspended in 375 µl 1% SDS with protease

inhibitors and transferred to lysis tubes (Tough micro-organism lysing tubes, Precellys). Final lysis of the bacteria was performed with a Precellys 24-Dual tissue homogenizer (2 x 50 s at 5000 rpm with 5 s break) and lysate cleared from bacterial debris by centrifugation. Total protein content was determined by BCA assay (Pierce).

Western blotting

All Western blotting analysis was conducted according to the manufacturer's instructions on prefabricated 4-12% gradient gels (NuPAGE Novex 4-12% Bis-Tris Gel; Invitrogen) with MES running buffer. A dry-blotting system (iBlot; Invitrogen) was used to transfer proteins to nitrocellulose membranes by electrophoresis. Membranes were blocked in 5% skim milk/PBS over night at 4°C. For Western blotting on *P. berghei* lysates, individual lysates were separated on different lines of the gel and the blocked membrane incubated with appropriately diluted anti-MUL3720 monoclonal antibody (mAb) or anti-MUL4987 mAb in 1% skim milk/0.05 % Tween20/PBS for 1 hour at room temperature (RT). After several washing steps in 1% skim milk/0.05% Tween20/PBS, HRP-conjugated goat anti-mouse IgG γ-chain mAb (Southern Biotech) was used as secondary antibody and incubated for 1 hour at RT. Excess antibody and skim milk residuals were washed away with PBS and blots were then developed using ECL Western blotting detection reagents (ECL Western blotting Substrate; Pierce). For Western blotting on *M. ulcerans* lysate, 10 µg of total protein was separated on a one well gel, the blocked membrane cut into thin strips and individually incubated with appropriate dilutions of serum of immunized mice in 1% skim milk/0.05% Tween20/PBS for 1 hour at RT. Washing and development were then conducted as described above.

Expression of recombinant proteins and enzyme linked immunosorbent assays (ELISA)

Expression of the vaccine candidate antigens MUL3720 and MUL4987 (Ag85A) was achieved with the pET28a expression system (Novagen, modified to contain an ampicillin selection cassette) in *E. coli* BL21(DE3) strains (Invitrogen) by addition of 1mM isopropyl thiogalacoside (Calbiochem) for 4h at 37°C. Purification of the 6xHis-tagged proteins from the bacterial pellet in PBS (recombinant MUL3720, rMUL3720) or 8 M Urea (recombinant MUL4987, rMUL4987) was done by nickel-nitrilotriacetic acetic (Ni-NTA) metal affinity chromatography [21]. Proteins were eluted with increasing concentrations of imidazole and decrease of pH respectively, and integrity and purity of proteins assessed by SDS-page separation and Coomassie Blue staining (Add Ref. Adj. paper). Final amount of the so produced recombinant proteins (rMUL3720 and rMUL4987) was determined by BCA assay (Pierce) according to the manufacturer's instructions.

Sera of immunized mice were analysed for specific antibodies against the vaccine candidate antigens by ELISA on recombinant proteins. All incubation steps were performed at RT. 10

µg/ml of rMUL3720 or rMUL4987 were coated on ELISA plates (Maxisorp; Nunc) by incubation overnight. Plates were first blocked in 5% skim milk/PBS for 1 hour and incubated with dilution series of sera from immunized mice in 0.5% skim milk/PBS for two hours. After washing the plate with dH_2O/0.1% Tween20, alkaline phosphatase-conjugated goat anti-mouse monoclonal antibody (mAb; Sigma) was incubated for 1 hour. Plates were washed again and subsequently developed with *p*-nitrophenyl phosphate (Sigma) as substrate. The optical density (OD) of the reaction product was measured at 405 nm with a microplate absorbance reader (Sunrise Absorbance Reader; Tecan). The threshold for endpoint titer determination was defined as the double of the mean measurements plus the mean standard deviation of a dilution series done without primary antibody and a dilution series done with serum taken prior to all immunizations (pre-bleed). Individual serum dilution series were approximated with sigmoidal dose-response curves (Graph Pad prism) and the reciprocal dilution of the intersection between the curve and the threshold was defined as individual endpoint titer.

Immunofluorescence assays

Infected parasites cultures were stained for immunofluorescence analysis (IFA) as previously described [22]. Briefly, whole blood of infected mice was harvested and iRBC fixed with 4% formaldehyde/0.01% glutaraldehyde and permeabilized with 0.1% Triton X-100. Staining was performed with anti-MUL3720 mAb as primary antibody and Alexa 568 labelled donkey anti-mouse IgG (Invitrogen) as secondary antibody. Pictures were acquired with a Zeiss LSM 700 confocal microscope (Carl Zeiss GmbH, Jena, Germany). Immunofluorescence assays with sera of immunized mice on paraffin embedded *M. ulcerans* bacteria were performed as previously described [23]. Briefly, African *M. ulcerans* isolates were embedded into paraffin, cut into 3 µm thin sections and mounted on Superforst Plus glass slides (Thermo Scientific). Sections were deparaffinised, rehydrated and pre-treated with 1mM EDTA buffer pH=8 for epitope retrieval as described for tissue sections in immunohistochemistry [24]. Unspecific binding was prevented by incubation of the bacteria in 1.5% goat serum in PBS for 1 hour at RT. Appropriately diluted mouse mAbs specific for MUL3720 and MUL4987 were used as primary antibodies. Detection of the specific binding of primary antibodies was achieved with an Alexa488 labelled secondary goat anti-mouse total IgG (H+L) antibody (Life Technologies). Image acquisition was performed on a applied precision widefield Delta Vision microscope (Olympus IX71).

M. ulcerans infection

Groups of 15 female, seven weeks old BALB/c mice (Janvier) were immunized by *P. berghei* infection and subsequent chloroquine cure via drinking water as described above. Four weeks after ITV mice were challenged with 2.4×10^2 colony forming unit (CFU) *M. ulcerans*

(S1013, [25,26]) into the left hind foot pad. For infection bacteria were cultivated in BacT/ALERT medium (Biomerieux) for six weeks, recovered by centrifugation and diluted in sterile PBS to 125 mg/ml wet weight. 30 µl of a 1:100 dilution of this stock solution was used for infection. Inoculum dose was determined by CFU plating on 7H9 agar plates.

The course of infection was followed by weekly measurements of the foot pad thickness with a caliper. At day 39 after infection (time point 1, T1) five mice per experimental group were euthanized and all foot pads analysed for bacterial load by qPCR. At day 49 after infection (time point 2, T2) a total of seven mice per experimental group were euthanized. Four foot pads were analysed for bacterial load and three processed for histopathological analysis. The remaining three mice were euthanized at day 63 (time point 3, T3) and all analysed for bacterial load by qPCR. When mice were euthanized, blood was collected by cardiac puncture and foot pads aseptically removed. Foot pads designated for histopathological analysis were immediately transferred to 10% neutral-buffered Formalin solution (approx. 4% formaldehyde) for fixation during 24 hours at RT. Foot pads designated for enumeration of *M. ulcerans* bacteria were dipped in 70% ethanol, dried under the laminar flow and cut into four pieces with a scalpel. After transfer to reinforced hard tissue grinding tubes (MK28-R, Precellys) foot pads were homogenized in 750 µl of 7H9 medium (Becton, Dickinson and Company). Tissue homogenization was performed with a Precellys 24-Dual tissue homogenizer (3 x 20 s at 5000 rpm with 30 s break), the lysate was transferred to a new tube and the lysis tube still containing tissue remains refilled with additional 750 µl of 7H9 medium. The remains were homogenized a second time and the correspondent two lysates pooled.

Quantification of bacterial load by qPCR and CFU plating

For enumeration of bacteria in the foot pad lysate, DNA from 100 µl of a 1:40 dilution of the foot pad lysate in PBS was extracted as described by Lavender and Fyfe [27]. Extracted DNA was then analysed for insertion sequence (IS) 2404 qPCR as previously described [27]. For graphic representation of the results, cycle threshold (Ct) values were converted into genome copy numbers per foot pad by applying the standard curve established for IS2404 by *Fyfe et al*. [28]. Extractions were done two times from independent foot pad lysate dilutions, qPCR performed in one run and the average of the two Ct values taken for conversion into genome copy numbers per foot pad. For enumeration of bacteria by CFU plating, 250 µl of foot pad lysate was decontaminated with 0.5 M NaOH as described previously [29]. The pellet of decontaminated lysate was dissolved in 500 µl of 7H9 medium and appropriate dilution series plated on 7H9 agar plates. Following 250 days of incubation at 30°C colonies were counted and the concentration of bacteria expressed as CFU.

Histopathology

Formalin fixed foot pads were decalcified in 0.6 M EDTA and 0.25 M citric acid for 12 days at 37°C and transferred to 70% ethanol for storage and transport. The samples were dehydrated and embedded into paraffin. 5 µm thin sections were cut, deparaffinised, rehydrated, and stained with Haematoxylin/Eosin (HE, Sigma, J.T. Baker) or Ziehl-Neelsen/Methylene blue (ZN, Sigma) according to WHO standard protocols [30]. Immunohistochemical staining was performed as previously described [31]. Stained sections were mounted with Eukitt mounting medium (Fluka). Pictures were taken with a Leica DM2500B microscope or with an Aperio scanner.

Results

Genetically modified *P. berghei* parasites express *M. ulcerans* vaccine candidate antigens

Coding sequences for the two protein vaccine candidate antigens MUL3720 and MUL4987 of *M. ulcerans* were introduced into the chromosome of *P. berghei* parasites. Expressed as fusion protein to HEP17 this strategy resulted in parasite lines cl2205 (MUL_3720::Hep17) and cl2224 (MUL_4987::Hep17), for which the *M. ulcerans* antigens were expected to be located on the PVM surrounding the parasites in the iRBC. Additionally, two parasite lines in which the candidate antigens are C-terminally tagged with the fluorescent protein mCherry and are expressed under the control of the strong *hsp*70 promoter were generated. The resulting parasite lines cl2222 (MUL_3720::$mCherry_{hsp70}$) and cl2225 (MUL4987::$mCherry_{hsp70}$) were expected to express the proteins in the parasite cytosol. Western blotting analyses on lysates of the genetically modified parasites confirmed the expression of MUL3720 in cl2205 (Fig. 1A, left panel) and of MUL4987 in cl2224 (Fig. 1A, right panel). Both proteins were detected only in the lysate of the correspondently modified *P. berghei* clone (Fig. 1A). Due to their nature as fusion proteins with HEP17, both proteins had an increased size compared to their counterparts expressed by *M. ulcerans* or the respective recombinant proteins (Fig. 1A). Expression of MUL3720 in the parasite line cl2222 was also confirmed by Western blot analyses (Fig. 1B) but no signal was obtained for MUL4987 expression in cl2225.

Although for both target proteins mouse monoclonal antibodies (mAb) were available to visualize the expression of the target protein by the *P. berghei* parasites in fixed RBC from infected mice by IFA, only staining with anti-MUL3720 mAb was successful (Fig. 1C). RBC infected with cl2205 showed a clear staining of the PVM as a dotted ring around the parasite (Fig. 1C, top panel row). Cl2222 showed the expression of MUL3720 in the parasite vacuole (Fig. 1C, middle panel row), whereas the genetically non-modified *P. berghei* strain ANKA showed no staining.

Immunization with genetically modified *P. berghei* under chloroquine coverage leads to specific antibody responses

In a pilot study, female NMRI mice were immunized twice by ITV with 2 x 10^7 *P. berghei* iRBC. Both times curative administration of chloroquine via the drinking water was initiated when parasitemia had reached around 1 %. Six weeks after the first and ten days after the second ITV sera of immunized mice was analysed for specific antibody responses by ELISA on the respective recombinant protein. One round of ITV resulted in specific antibody responses against antigens expressed on the PVM (Fig. 2A, cl2205; 2B, cl2224) but no

responses were observed when antigens were expressed in the parasite cytosol (Fig. 2A, cl2222; 2B, cl2225). Antibody responses were not significantly boosted by a second round of ITV (Fig. 2A and B). Sera of animals immunized twice by ITV with the control parasite strain ANKA slightly reacted in ELISA on rMUL3720 but not on rMUL4987.

Based on these results we only applied one ITV in subsequent *M. ulcerans* challenge experiments in BALB/c mice. Animals of this mouse strain immunized with cl2205 also mounted a strong antibody response against rMUL3720 as seen in ELISA with serum from three weeks after ITV (Fig. 3, A1). In comparison, antibody responses against rMUL4987 in cl2224 immunized mice were weaker and more heterogeneous (Fig. 3, B1 and B2). Control mice infected with genetically unmodified parasites (ANKA) as well as non-infected animals did not mount a specific antibody response against the recombinant antigens. Western blotting analysis with the individual sera on lysate of *M. ulcerans* and IFA on paraffin embedded bacteria revealed cross-reactivity of the elicited antibodies with MUL3720 and MUL4987 expressed by the mycobacteria (Fig. 3, A2 and B2, C1 and C2). Moreover, the analysis confirmed the more variable humoral immune response against MUL4987. None of the control mice developed antibodies cross-reactive with the two *M. ulcerans* target proteins (Fig. 3, A2 and B2).

In comparison to two other delivery systems we had previously used for the same *M. ulcerans* protein antigens [26] (Add Ref. Adjuvant) ITV with genetically modified *P. berghei* induced medium antibody titers (Fig. 4). For both candidate antigens two subsequent immunizations with recombinant proteins adjuvanted with GLA-SE (Add Ref. Adjuvant) induced the strongest responses, while formulation as virus replicon particles [26] induced only marginal titers (Fig. 4). In terms of immunoglobulin (Ig) G subclasses both parasite lines, cl2205 and cl2224, induced predominantly IgG1 and IgG2a and the subclass IgG spectrum did not change with a second ITV (Supplementary Fig. 1).

Infection of *P. berghei* immunized animals with *M. ulcerans*

To assess the protective potential of ITV with the genetically modified *P. berghei* lines against *M. ulcerans* infection, we challenged immunized mice in an experimental infection model with 3.5×10^4 *M. ulcerans* bacilli into the hind left foot pad. The development of the infection was followed by weekly measurements of the footpad thickness with a caliper (Fig. 5A). 39 (T1) and 49 (T2) days after infection randomly selected animals were sacrificed to determine the bacterial load in the infected foot pads. Footpad swelling started around day 28 after infection and gradually progressed over the following five weeks until all remaining mice were euthanized at day 63 (T3). Transiently smaller foot pad swelling was observed in cl2224 mice compared to the infection control group. This difference was significant at days 35 (Fig. 5B, $p = 0.0074$) and 44 ($p = 0.0199$), but not at day 39 and at later time points.

Accordingly, no difference in amount of oedema was detected in the histopathological analysis of representative foot pads done at T2 (Supplementary Fig. 2). All animals showed cellular infiltration that mainly consisted of neutrophils in close association with acid fast bacilli (AFB). In contrast, footpad swelling was significantly increased in mice immunized with cl2205 (at day 35, 39 and 49; p = 0.0178, 0.0347 and 0.0006, respectively) or ANKA (at day 35 and 49; p = 0.0320, and 0.0016, respectively) (Fig. 5B) compared to infection control animals. When we determined the bacterial load in the infected foot pads after 39 days (T1), 49 days (T2) and 63 days (T3) by IS2404 qPCR based quantification of *M. ulcerans* DNA, differences between groups were perceivable but not statistically significant (Fig. 6), which was confirmed by the results from CFU plating (Supplementary Fig. 3).

Comparing total IgG titers in sera collected after ITV with total IgG titers in sera after ITV and additional *M. ulcerans* infection, no booster effect of the infection on animals immunized with cl2205 was observed (Fig. 7A). In contrast, a slight significant boost by the exposure to the antigen in the native context of an infection was detected in animals that had received cl2224 by ITV (Fig. 7B). Interestingly, infection alone did not induce antibodies against rMUL3720 nor rMUL4987 (Fig. 7).

Discussion

Although vaccination by ITV with malaria parasites expressing heterologous antigens is still far from application, this approach could serve in the future as multi-disease vaccination. Recently it has been demonstrated that the model antigen ovalbumin (OVA) expressed in *P. berghei* parasites induced OVA specific T-cell responses in an ITV approach with infection by injection of iRBCs [6]. Along these lines we have demonstrated that *P. berghei* can serve as delivery system for heterologous mycobacterial antigens.

The subcellular location of the model antigen OVA in the malaria parasites had a strong influence on the nature and magnitude of the antigen-specific T-cell responses [6]. Expression on the PVM by fusion of OVA to HEP17 led to significantly stronger splenic and intracerebral OVA-specific $CD8^+$ and $CD4^+$ T-cell responses than cytosolic OVA expression [6]. Similarly, *M. ulcerans* proteins fused to Hep17 led to more stable expression of the candidate antigens and induced significant specific antibody titers in ITV immunized mice as compared to cytosolic expression. Protein-dependent effects were additionally observed in the presented study, in which both fusion protein expression, as well as the induced humoral immune response were stronger in MUL3720::Hep17 expressing parasites than in the MUL4987::Hep17 parasites. As expected, the MUL3720::Hep17 fusion protein was associated with the PVM; due to lack of a suitable antibody the localisation of the MUL4987::Hep17 fusion protein with the PVM could not be reconfirmed.

ITV induced strong antibody responses that were cross-reactive with the native protein antigens on the surface of *M. ulcerans* cells. Classically, a mouse foot pad model of *M. ulcerans* infection is used to test new drug treatments for Buruli ulcer or study the protective potential of new vaccines [32]. In this challenge model mice immunized with unmodified and MUL3720-expressing parasites showed a tendency of accelerated swelling and inflammation compared to non-immunized mice. This may be related to an unspecific increased immunological alert elicited by the malaria infection. Taking into account that immunization by ITV enhanced *M. ulcerans* infection related footpad swelling, the observed delay in swelling in mice immunized with MUL4987 expressing parasites may reflect development of partial specific immune protection. In line with this observation, also priming with a MUL4987 (Ag85A) DNA vaccine and boosting with recombinant Ag85A protein has been shown before to delay footpad swelling [33,34]. To what extent antibody responses play a role in protection against the mainly extracellular *M. ulcerans* bacilli is not clear [12]. From the data presented here, we conclude that antibody responses against MUL3720 and MUL4987 are not of major importance in conferring protection.

Acknowledgments

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Figures

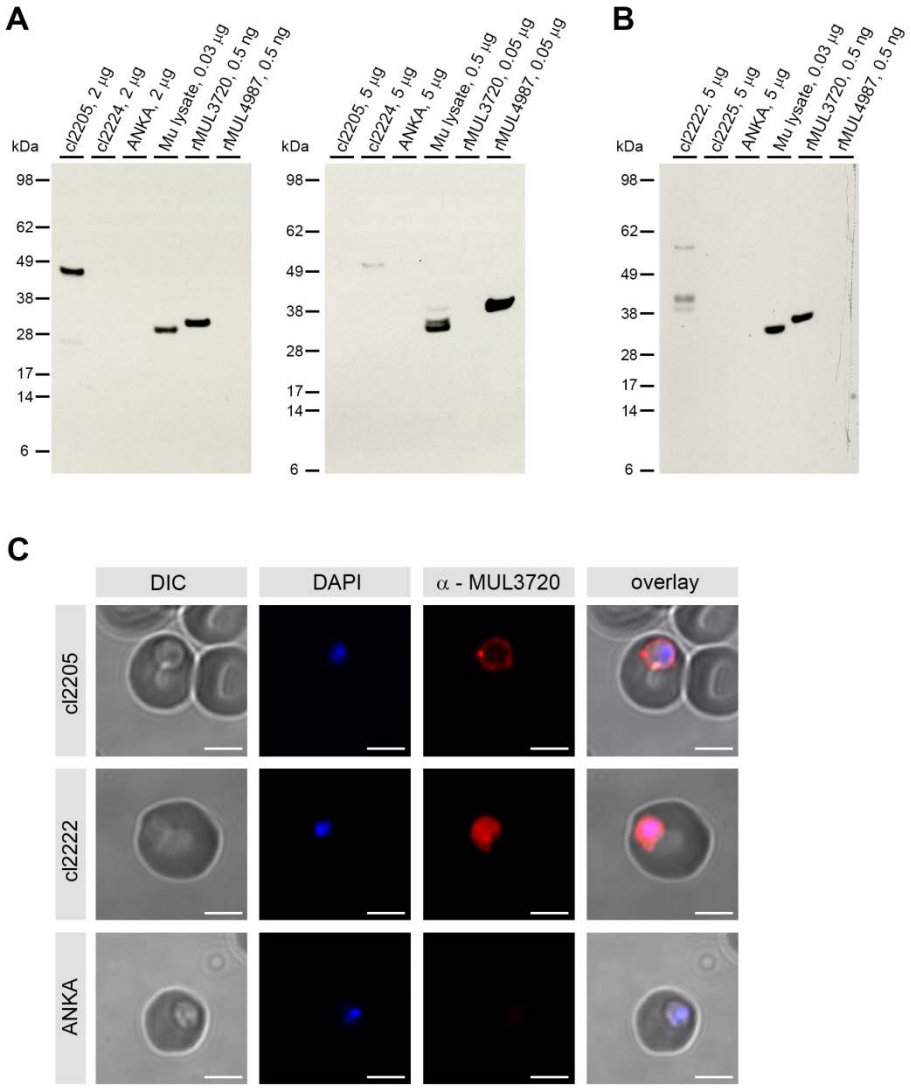


Figure 1: Expression of the vaccine candidate antigens MUL_3720 and MUL_4987 by genetically modified *P. berghei* parasites.

(A) Western blot analysis on lysates of different *P. berghei* lines with mAb's against MUL3720 (left panel) and MUL4987 (right panel). Indicated amounts of lysates of *P. berghei* parasites, *M. ulcerans* lysate (Mu lysate) and recombinant proteins were loaded. Both proteins were expressed by the specific parasite line at the size expected, which due to their fusion to *P. berghei* HEP17 was bigger than the proteins in *M. ulcerans* lysate or the recombinant forms of the proteins. **(B)** Western blot analysis on lysates of different *P. berghei* lines probed with anti-MUL372 mAb. Indicated amounts of lysates of *P. berghei* parasites, *M. ulcerans* lysate (Mu lysate) and recombinant proteins were loaded. **(C)** Indirect immunofluorescence staining of red blood cells infected with genetically modified *P. berghei* parasites. Formalin/Glutaraldehyde fixed iRBC were probed with anti-MUL3720 mAb (red)

and nuclei stained with DAPI (blue). Infection with the parasite line cl2205 led to expression of MUL3720 on the PVM. Control infection with ANKA parasites did not lead to expression of MUL3720. Scale bar represents 3 µm.

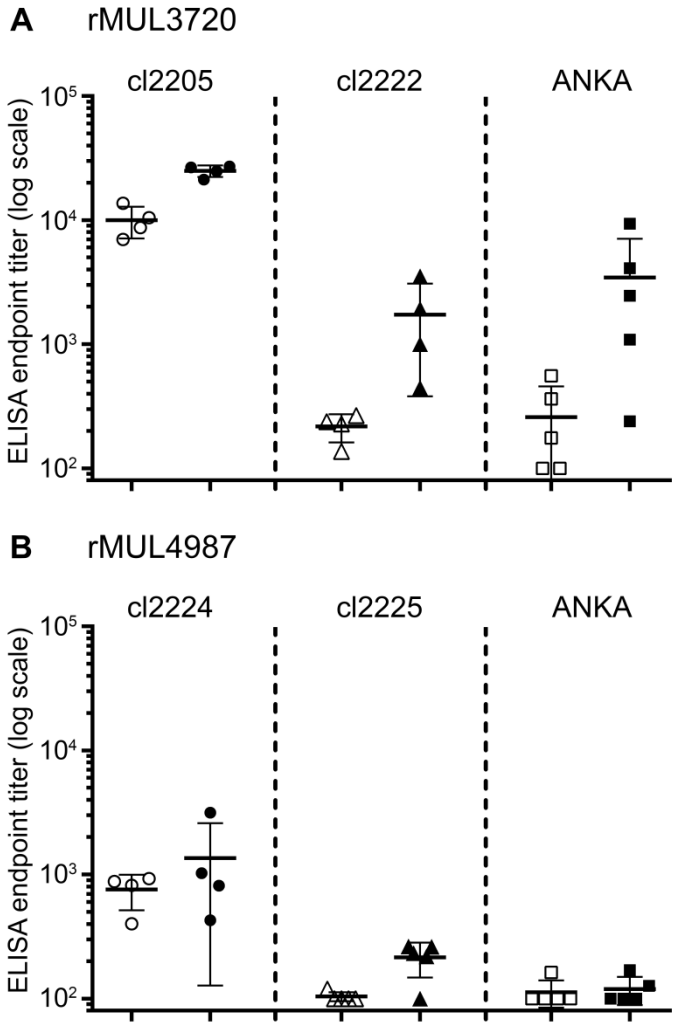


Figure 2: Humoral immune response to two sequential ITVs with different *P. berghei* constructs.

Female NMRI mice (n = 4 - 5) were immunized by ITV with the indicated *P. berghei* lines. Six weeks after first ITV serum was collected (open symbols) and the procedure was repeated (closed symbols). Sera after each ITV were analysed by ELISA on the *P. berghei* line corresponding recombinant protein (A: rMUL3720; B: rMUL4987). Endpoint total IgG titers were determined for individual animals in one single ELISA. Mean values (line) ± standard deviations are shown. Differences in total IgG endpoint titers after first and second ITV were not statistically significant (Wilcoxon Rank-Sum test).

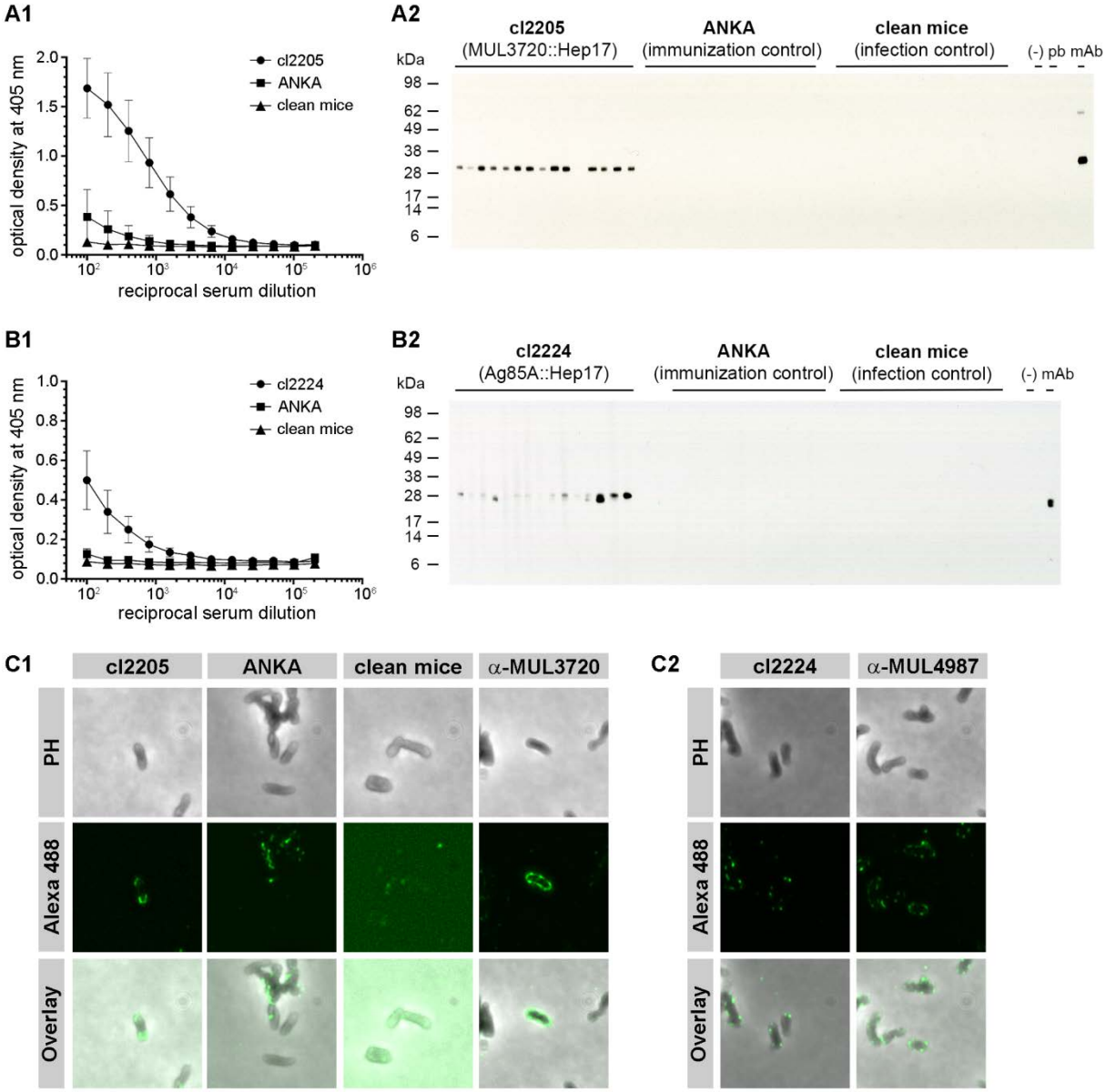


Figure 3: Humoral immune response to the chloroquine covered infection with modified *P. berghei* parasites in mice.

Sera of groups of 15 *P. berghei* infected and subsequently chloroquine treated, female BALB/c mice were analysed by ELISA on rMUL3720 (A1) or rMUL4987 (A2). Serial dilutions of sera are shown as mean (dot) ± standard deviation. While strong antibody response upon infection with cl2205 was detected in immunized mice (A1) the response upon infection with cl2224 was less pronounced (A2). Cross reactivity of the infection-induced antibodies with the native antigens was demonstrated by Western blotting on *M. ulcerans* lysate (B1 and B2). Anti-MUL3720 mAb (B1) or anti-MUL4987 mAb (B2) served as positive controls, pre-bleed (pb) serum or no primary antibody (nc) as negative controls. All but one mouse in the cl2205 infected group showed strong specific response against MUL3720 (B1). Antibody

response to MUL4987 was specific to the native antigen in Western blotting, but again not very pronounced (B2). (C1 and C2) Cross reactivity of the infection-induced antibodies in serum of representative animals with surface structures on paraffin embedded *M. ulcerans* bacteria demonstrated in immunofluorescence analysis.

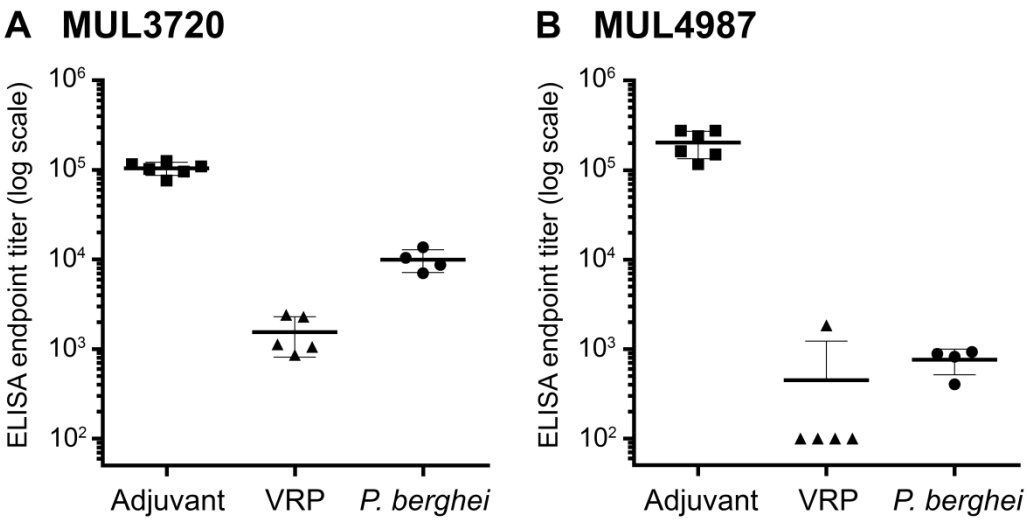


Figure 4: Comparison of different delivery systems for MUL3720 and MUL4987.
Groups of female mice were immunized with standard regimens of different delivery systems for the two protein vaccine candidate antigens MUL3720 (A) and MUL4987 (B). Sera were analysed two to three weeks after the last immunization. Adjuvant formulation: 20 µg of recombinant protein were formulated with GLA-SE/EM048 (Add Ref Adjuvant) and mice were immunized twice s.c. in the scruff of the neck in a three week interval. Virus replicon particle (VRP): Mice were immunized once intra-muscularly with 10^7 VSV*ΔG(MUL3720) (A) or VSV*ΔG(tPA-MUL4987) (B) and boosted three weeks later with 30 µg of the respective non-adjuvanted recombinant protein [26]. *P. berghei*: one round of ITV with cl2205 (A) or cl2224 (B) was done. Endpoint total IgG titers were determined for individual animals in one single ELISA per delivery system on recombinant protein. Mean values (line) ± standard deviations are shown.

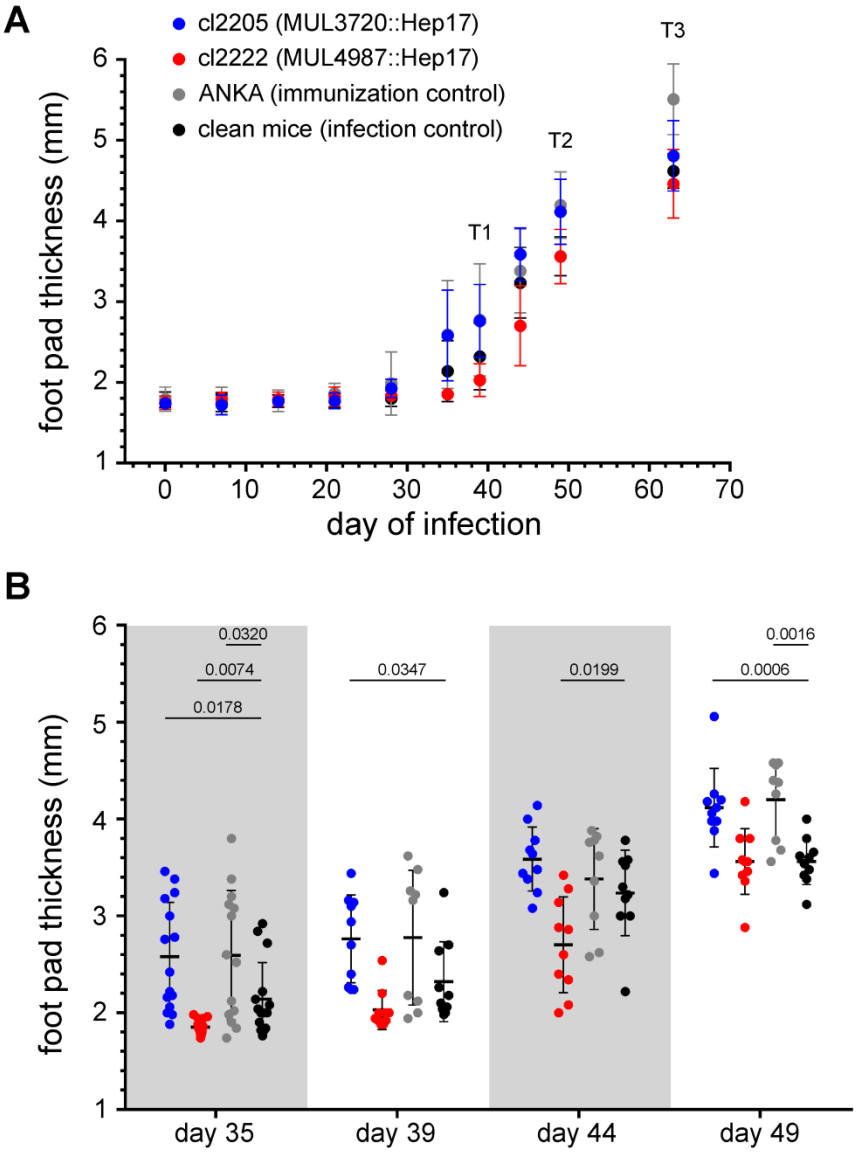


Figure 5: Course of infection with *M. ulcerans* in *P. berghei* immunized mice.

(A) Groups of 15 *P. berghei* immunized, female BALB/c mice were challenged with *M. ulcerans* injected s.c. into the left hind foot pad. Infection was followed by measuring foot pad thickness with a caliper until mice were euthanized at day 39 (T1, remaining n = 10), day 49 (T2, remaining n = 3 (2)) and day 63 (T3). Depicted is the mean foot pad thickness (dot) ± standard deviation of the individual differently immunized groups. Foot pad swelling started after 4 weeks of infection and progressively increased until mice were euthanized at day 63 after infection. Differences between immunized groups were highest between day 35 and day 49 of infection and are depicted in more detail in **(B)**.

(B) For infection between day 35 and day 49 foot pad thickness of individual mice per group is depicted (dots). Additionally, the mean value (line) ± standard deviation of each group is

given. Statistical significance was calculated by the Student's t- test and is indicated with the according *p*-value when detected. Mice immunized with cl2205 (blue) had a tendency to show stronger swelling than non-immunized mice (black) that was always statistically significant except at day 44. Mice immunized with cl2222 (red) showed a slight tendency to have less swelling than non-immunized mice.

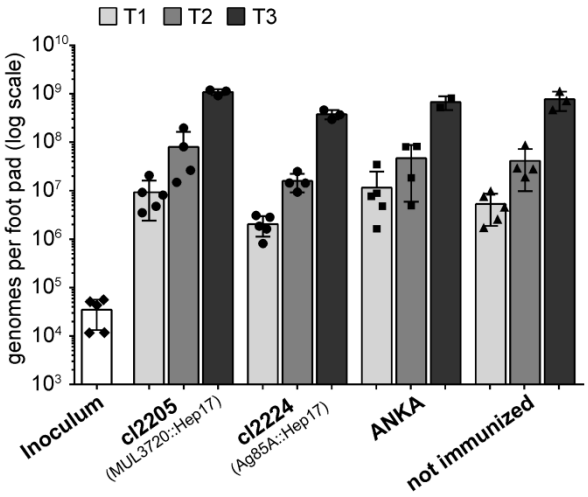


Figure 6: Bacterial load in foot pads of *P. berghei* immunized and *M. ulcerans* infected mice.

After infection with 3.5 x 10^4 *M. ulcerans* (Inoculum) bacterial load in infected foot pads was determined by qPCR at three time points during infection: 39 days (T1, n = 5), 49 days (T2, n = 4) and 63 days (T3, n = 2 – 3) after infection. Depicted are individual measurements as genome copies per foot pad, the mean (line) ± standard deviation. A slight tendency of reduction in bacterial load in cl2224 immunized animals is visible at all time points but not statistically significant (Kruskal-Wallis test).

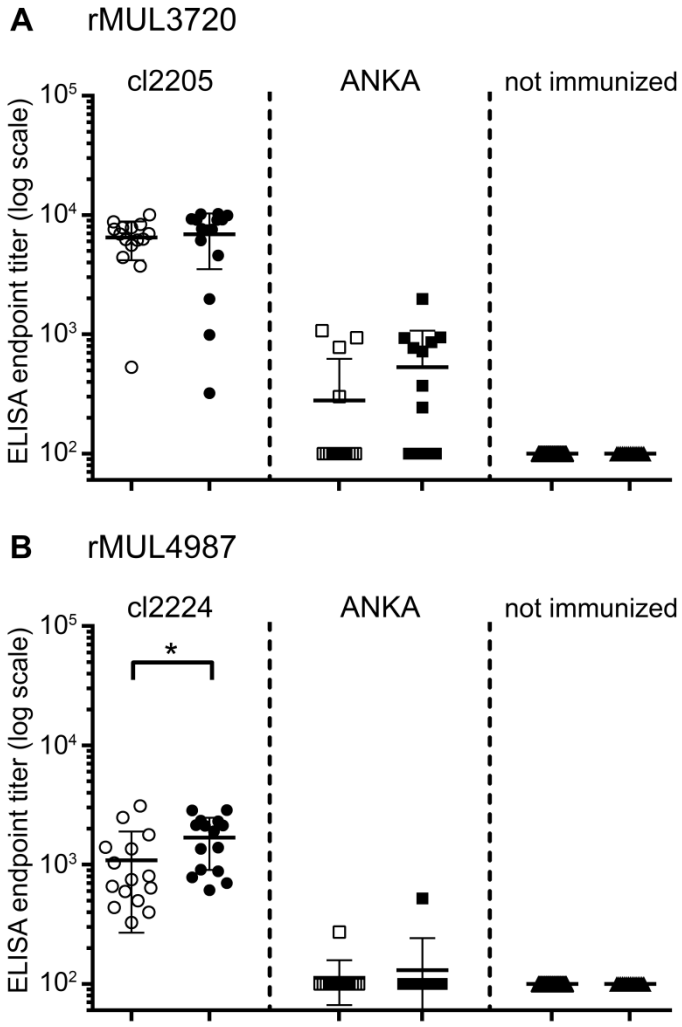
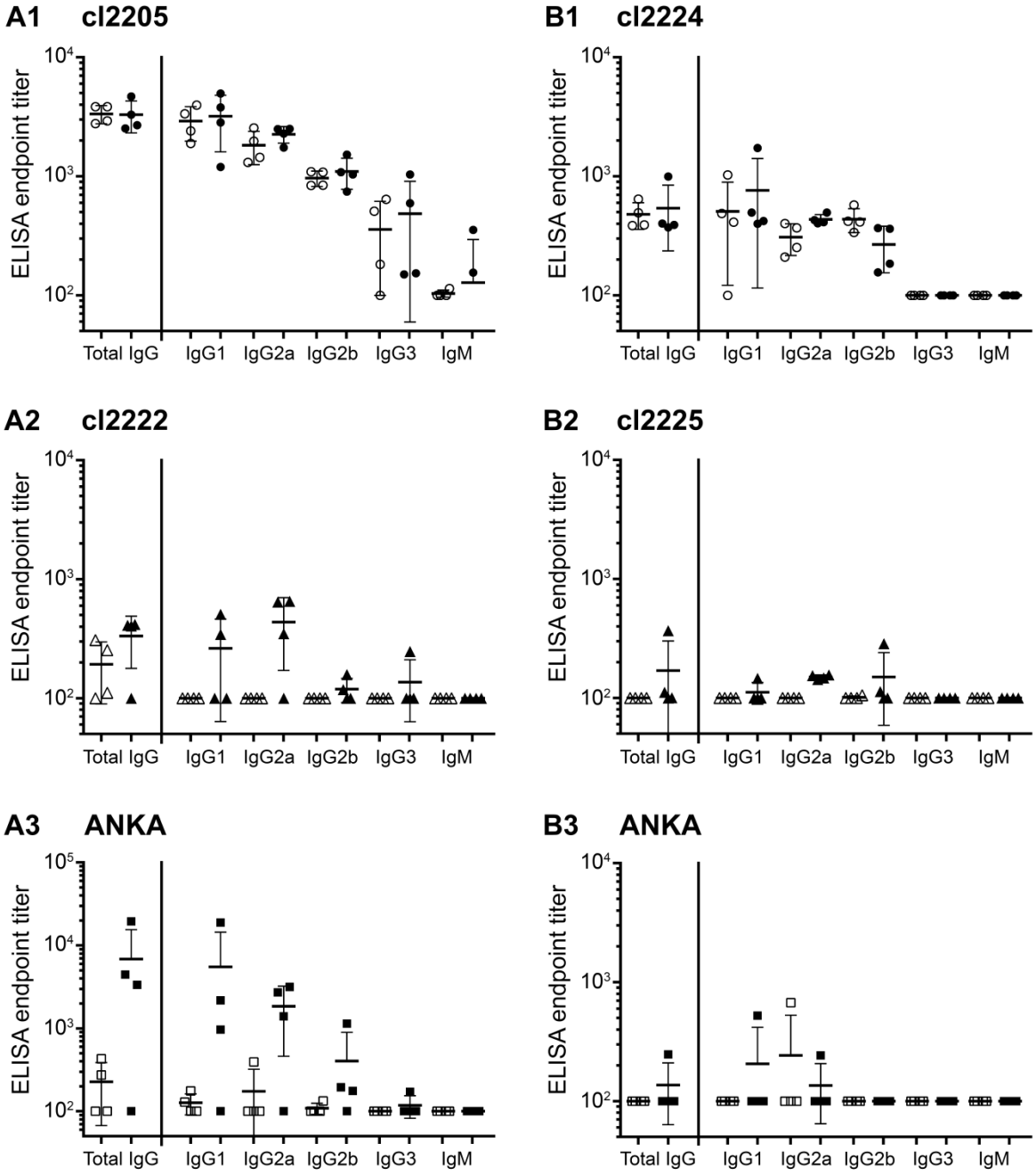


Figure 7: Vaccination-induced humoral immune responses are not boostered by infection with *M. ulcerans*.

Groups of 15 *P. berghei* immunized, female BALB/c mice were challenged with *M. ulcerans* injected s.c. into the left hind foot pad. Sera after ITV before *M. ulcerans* infection were compared to sera of the same animals after at least 39 days of *M. ulcerans* infection by ELISA on the *P. berghei* line corresponding recombinant protein (A: rMUL3720; B: rMUL4987). Endpoint total IgG titers were determined for individual animals in one single ELISA. Mean values (line) ± standard deviations are shown. Statistical differences were assessed by paired t-test and are shown when detected. * $p \leq 0.05$.

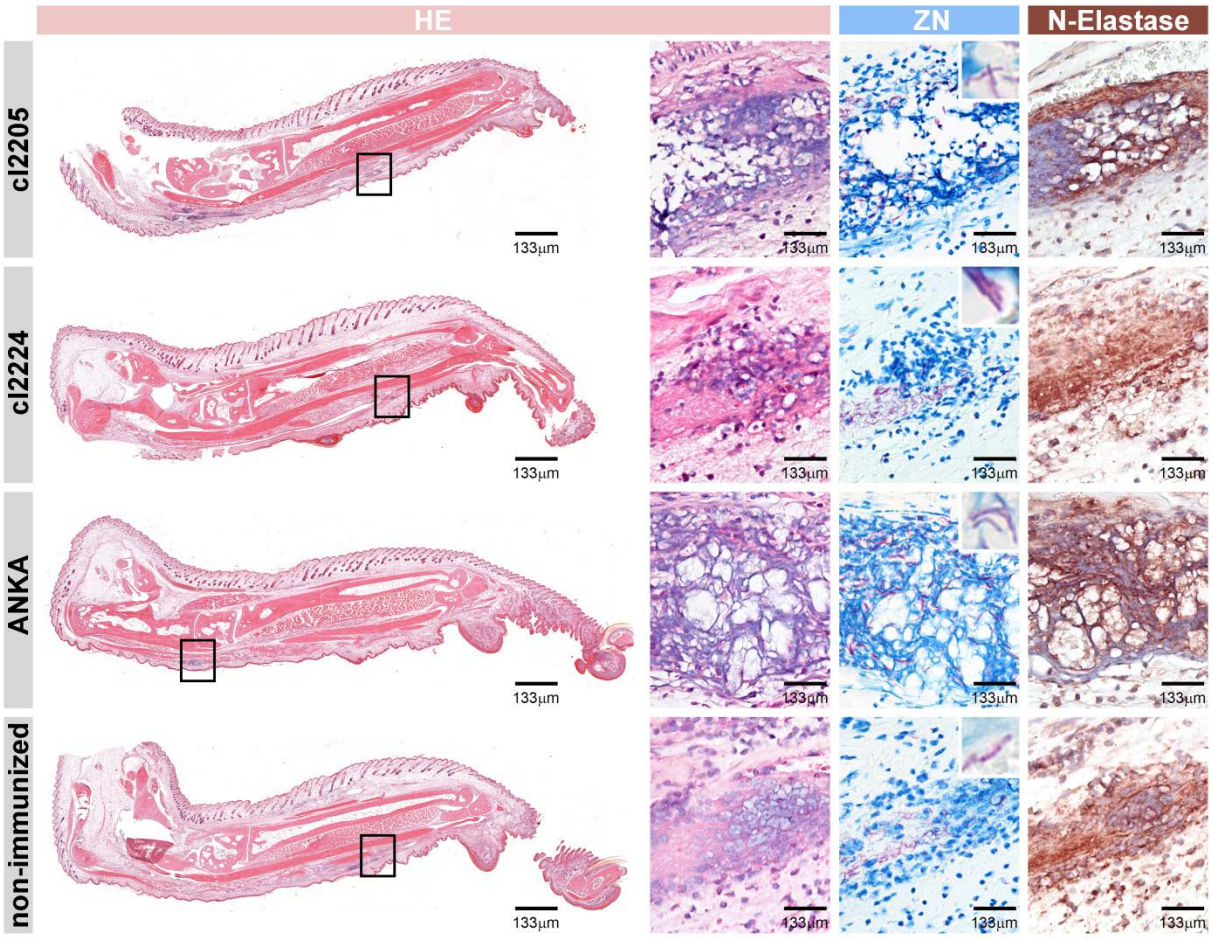
Supplementary Material



Supplementary Figure 1: Ig subclass distribution in sera of mice after two sequential ITVs.

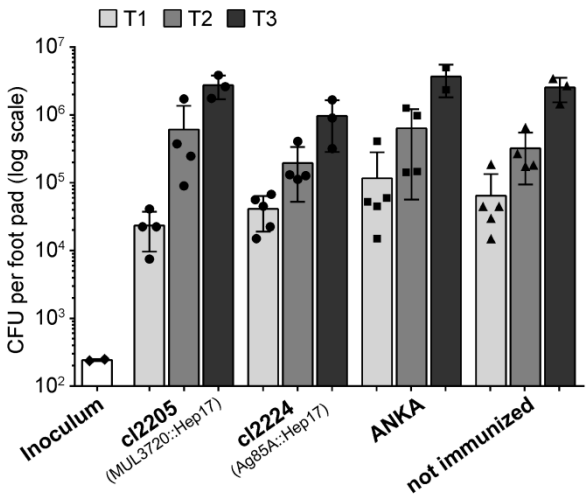
Female NMRI mice (n = 4 - 5) were immunized by ITV with the indicated *P. berghei* lines. Six weeks after first ITV serum was collected (open symbols) and the procedure was repeated (closed symbols). Sera after each ITV were analysed by ELISA on the *P. berghei* line corresponding recombinant protein (A1-A3: rMUL3720; B1-B3: rMUL4987). Ig subclass titers

were determined for individual animals in one single ELISA by development with isotype specific secondary antibodies. Mean values (line) ± standard deviations are shown.



Supplementary Figure 2: Histopathological analysis at T1.

All scans of representative infected foot pads of each experimental group showed slight oedema in the upper part of the foot pad and slight cellular infiltration and necrosis at the base of the foot pad (HE). ZN staining revealed AFB in close contact to infiltrating cells and cellular debris, which were mainly neutrophils (N-Elastase).



Supplementary Figure 3: Bacterial load in foot pads of ITV immunized and *M. ulcerans* infected mice.

After infection with *M. ulcerans* (Inoculum) bacterial load in infected foot pads was determined by CFU plating at three time points during infection: 39 days (T1, n = 5), 49 days (T2, n = 4) and 63 days (T3, n = 2 – 3) after inoculation. Depicted are individual measurements as CFU per foot pad, the mean (line) ± standard deviation.

References

[1] Clyde DF. Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of the University of Maryland studies, 1971-75. Bull World Health Organ 1990;68 Suppl:9–12.

[2] Seder RA, Chang L-J, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, et al. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 2013;341:1359–65. doi:10.1126/science.1241800.

[3] Roestenberg M, Teirlinck AC, McCall MBB, Teelen K, Makamdop KN, Wiersma J, et al. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. Lancet 2011;377:1770–6. doi:10.1016/S0140-6736(11)60360-7.

[4] Belnoue E, Voza T, Costa FTM, Grüner AC, Mauduit M, Rosa DS, et al. Vaccination with live *Plasmodium yoelii* blood stage parasites under chloroquine cover induces cross-stage immunity against malaria liver stage. J Immunol Baltim Md 1950 2008;181:8552–8.

[5] Doll KL, Butler NS, Harty JT. CD8 T cell independent immunity after single dose infection-treatment-vaccination (ITV) against *Plasmodium yoelii*. Vaccine 2014;32:483–91. doi:10.1016/j.vaccine.2013.11.058.

[6] Lin J-W, Shaw TN, Annoura T, Fougère A, Bouchier P, Chevalley-Maurel S, et al. The sub-cellular location of OVA in Plasmodium blood stages influences the magnitude of T-cell responses. Infect Immun 2014. doi:10.1128/IAI.01940-14.

[7] Junghanss T, Johnson RC, Pluschke G. *Mycobacterium ulcerans* disease. Mansons Trop. Dis. 23rd ed., Saunders; 2014, p. 519–31.

[8] Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, Benbow ME, et al. Ecology and transmission of Buruli ulcer disease: a systematic review. PLoS Negl Trop Dis 2010;4:e911. doi:10.1371/journal.pntd.0000911.

[9] Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, Kerber S, et al. Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. PLoS Negl Trop Dis 2013;7:e2252. doi:10.1371/journal.pntd.0002252.

[10] WHO | Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer). WHO n.d. http://www.who.int/buruli/information/antibiotics/en/index16.html (accessed February 4, 2014).

[11] Einarsdottir T, Huygen K. Buruli ulcer. Hum Vaccin 2011;7:1198–203. doi:10.4161/hv.7.11.17751.

[12] Schütte D, Pluschke G. Immunosuppression and treatment-associated inflammatory response in patients with *Mycobacterium ulcerans* infection (Buruli ulcer). Expert Opin Biol Ther 2009;9:187–200. doi:10.1517/14712590802631854.

[13] Diaz D, Döbeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, Soder N, et al. Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to Mycobacterium ulcerans. Clin Vaccine Immunol CVI 2006;13:1314–21. doi:10.1128/CVI.00254-06.

[14] Röltgen K, Bratschi MW, Ross A, Aboagye SY, Ampah KA, Bolz M, et al. Late onset of the serological response against the 18 kDa small heat shock protein of *Mycobacterium ulcerans* in children. PLoS Negl Trop Dis 2014;8:e2904. doi:10.1371/journal.pntd.0002904.

[15] Revill WDL, Morrow RH, Pike MC, Ateng J. A CONTROLLED TRIAL OF THE TREATMENT OF *MYCOBACTERIUM ULCERANS* INFECTION WITH CLOFAZIMINE. The Lancet 1973;302:873–7. doi:10.1016/S0140-6736(73)92005-9.

[16] Gordon CL, Buntine JA, Hayman JA, Lavender CJ, Fyfe JA, Hosking P, et al. Spontaneous Clearance of *Mycobacterium ulcerans* in a Case of Buruli Ulcer. PLoS Negl Trop Dis 2011;5. doi:10.1371/journal.pntd.0001290.

[17] Hayman J. Out of Africa: observations on the histopathology of *Mycobacterium ulcerans* infection. J Clin Pathol 1993;46:5–9.

[18] Ruf M-T, Sopoh GE, Brun LV, Dossou AD, Barogui YT, Johnson RC, et al. Histopathological changes and clinical responses of Buruli ulcer plaque lesions during chemotherapy: a role for surgical removal of necrotic tissue? PLoS Negl Trop Dis 2011;5:e1334. doi:10.1371/journal.pntd.0001334.

[19] Schütte D, Pluschke G. Immunosuppression and treatment-associated inflammatory response in patients with *Mycobacterium ulcerans* infection (Buruli ulcer). Expert Opin Biol Ther 2009;9:187–200. doi:10.1517/14712590802631854.

[20] Vennerstrom JL, Arbe-Barnes S, Brun R, Charman SA, Chiu FCK, Chollet J, et al. Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 2004;430:900–4. doi:10.1038/nature02779.

[21] Crowe J, Döbeli H, Gentz R, Hochuli E, Stüber D, Henco K. 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. Methods Mol Biol Clifton NJ 1994;31:371–87. doi:10.1385/0-89603-258-2:371.

[22] Oberli A, Slater LM, Cutts E, Brand F, Mundwiler-Pachlatko E, Rusch S, et al. A *Plasmodium falciparum* PHIST protein binds the virulence factor PfEMP1 and comigrates to knobs on the host cell surface. FASEB J 2014:fj.14–256057. doi:10.1096/fj.14-256057.

[23] Vettiger A, Scherr N, Ruf M-T, Röltgen K, Pluschke G. Localization of Mycobacterial Antigens by Immunofluorescence Staining of Agarose Embedded Cells. J Mycobact Dis 2014;4:150.

[24] Portaels F, Johnson P, Meyers WM, Initiative WHOGBU. Buruli ulcer : diagnosis of *Mycobacterium ulcerans* disease : a manual for health care providers / edited by: Françoise Portaels, Paul Johnson, Wayne M. Meyers 2001. http://apps.who.int/iris/handle/10665/67000 (accessed February 6, 2014).

[25] Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, Kerber S, et al. Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. PLoS Negl Trop Dis 2013;7:e2252. doi:10.1371/journal.pntd.0002252.

[26] Bolz M, Kerber S, Zimmer G, Pluschke G. Use of Recombinant Virus Replicon Particles for Vaccination against *Mycobacterium ulcerans* Disease. PLoS Negl Trop Dis 2015;9:e0004011. doi:10.1371/journal.pntd.0004011.

[27] Lavender CJ, Fyfe JAM. Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples. Methods Mol Biol Clifton NJ 2013;943:201–16. doi:10.1007/978-1-60327-353-4_13.

[28] Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, Azuolas J, et al. Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. Appl Environ Microbiol 2007;73:4733–40. doi:10.1128/AEM.02971-06.

[29] Bratschi MW, Bolz M, Grize L, Kerber S, Minyem JC, Um Boock A, et al. Primary cultivation: factors affecting contamination and *Mycobacterium ulcerans* growth after long turnover time of clinical specimens. BMC Infect Dis 2014;14:636. doi:10.1186/s12879-014-0636-7.

[30] Portaels F, Organization WH. Laboratory diagnosis of buruli ulcer: a manual for health care providers / edited by Françoise Portaels. Diagnostic de l'ulcère de Buruli au laboratoire : manuel destiné au personnel de santé / edité par Françoise Portaels 2014.

[31] Ruf M-T, Schütte D, Chauffour A, Jarlier V, Ji B, Pluschke G. Chemotherapy-associated changes of histopathological features of *Mycobacterium ulcerans* lesions in a Buruli ulcer mouse model. Antimicrob Agents Chemother 2012;56:687–96. doi:10.1128/AAC.05543-11.

[32] Converse PJ, Nuermberger EL, Almeida DV, Grosset JH. Treating *Mycobacterium ulcerans* disease (Buruli ulcer): from surgery to antibiotics, is the pill mightier than the knife? Future Microbiol 2011;6:1185–98. doi:10.2217/fmb.11.101.

[33] Tanghe A, Content J, Van Vooren J-P, Portaels F, Huygen K. Protective Efficacy of a DNA Vaccine Encoding Antigen 85A from *Mycobacterium bovis* BCG against Buruli Ulcer. Infect Immun 2001;69:5403–11. doi:10.1128/IAI.69.9.5403-5411.2001.

[34] Tanghe A, Dangy J-P, Pluschke G, Huygen K. Improved protective efficacy of a species-specific DNA vaccine encoding mycolyl-transferase Ag85A from *Mycobacterium ulcerans* by homologous protein boosting. PLoS Negl Trop Dis 2008;2:e199. doi:10.1371/journal.pntd.0000199.

Results Chapter 7

Experimental Infection of the Pig with *Mycobacterium ulcerans*: A novel Model for Studying the Pathogenesis of Buruli Ulcer Disease

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Abstract

Background Buruli ulcer (BU) is a slowly progressing, necrotising disease of the skin caused by infection with *Mycobacterium ulcerans*. Non-ulcerative manifestations are nodules, plaques and oedema, which may progress to ulceration of large parts of the skin. Histopathologically, BU is characterized by coagulative necrosis, fat cell ghosts, epidermal hyperplasia, clusters of extracellular acid fast bacilli (AFB) in the subcutaneous tissue and lack of major inflammatory infiltration. The mode of transmission of BU is not clear and there is only limited information on the early pathogenesis of the disease available.

Methodology/Principal findings For evaluating the potential of the pig as experimental infection model for BU, we infected pigs subcutaneously with different doses of *M. ulcerans*. The infected skin sites were excised 2.5 or 6.5 weeks after infection and processed for histopathological analysis. With doses of 2×10^7 and 2×10^6 colony forming units (CFU) we observed the development of nodular lesions that subsequently progressed to ulcerative or plaque-like lesions. At lower inoculation doses signs of infection found after 2.5 weeks had spontaneously resolved at 6.5 weeks. The observed macroscopic and histopathological changes closely resembled those found in *M. ulcerans* disease in humans.

Conclusion/Significance Our results demonstrate that the pig can be infected with *M. ulcerans.* Productive infection leads to the development of lesions that closely resemble human BU lesions. The pig infection model therefore has great potential for studying the early pathogenesis of BU and for the development of new therapeutic and prophylactic interventions.

Author Summary

Buruli ulcer caused by *Mycobacterium ulcerans* infection is a necrotizing disease of the skin and the underlying subcutaneous tissue. Since the skin of pigs (*Sus scrofa*) has striking structural and physiological similarities with human skin, we investigated whether it is possible to develop an experimental *M. ulcerans* infection model by subcutaneous injection of the mycobacteria into pig skin. Injection of 2×10^6 or 2×10^7 colony forming units of *M. ulcerans* led to the development of lesions that were both macroscopically and microscopically very similar to human Buruli ulcer lesions. In particular for the characterization of the pathogenesis of Buruli ulcer and of immune defence mechanisms against *M. ulcerans*, the pig model appears to be superior to the mouse foot pad model commonly used for the evaluation of the efficacy of chemotherapeutic regimens.

Introduction

Buruli ulcer (BU), caused by infection with *Mycobacterium ulcerans*, is a human disease of the skin primarily affecting subcutaneous fat tissue and leading to ulceration of the overlying dermal and epidermal layers [1,2]. The disease is reported from countries worldwide but has its highest prevalence in West Africa [3]. Natural reservoirs of *M. ulcerans* as well as the mode(s) of transmission are not clearly identified [3,4]. While for a long time wide surgical excision was the only treatment option for BU, since 2004 the World Health Organization (WHO) recommends antibiotic therapy with rifampicin and streptomycin for 8 weeks [5]. This change in standard treatment has reduced recurrence rates to less than 2% [6–9].

M. ulcerans produces the polyketide exotoxin mycolactone that is responsible for the necrotizing nature of BU [10]. Three distinct non-ulcerative stages of the disease are described: subcutaneous, painless and movable nodules or papules, oedema and plaques. All three stages may progress to ulceration once the destruction of the subcutis results in collapse of the overlying epidermis and dermis [11].

Ulcerative BU lesions have been histopathologically well described through the analysis of excised tissue from surgically treated patients. Coagulative necrosis, fat cell ghosts and epidermal hyperplasia together with the presence of extracellular clusters of acid fast bacilli (AFB) in the absence of major inflammatory infiltrates in central parts of the lesions are considered hallmarks of the disease and can also be used for histopathological diagnosis [12,13]. However, early, pre-ulcerative stages have been described less frequently, because in particular in the African BU endemic regions patients are rarely reporting at treatment centres during early stages of the disease. Furthermore, with the replacement of surgical treatment by chemotherapy, tissue samples are not easily available any longer. Therefore, a suitable experimental animal infection model is required to contribute to the understanding of early host-pathogen interactions and pathogenesis in BU.

A range of animal species have been reported of being naturally infected with *M. ulcerans* and of developing ulcerative lesions. These include koalas, possums, cats, dogs and horses [14–21]. Except for possums which appear to be unusually susceptible to the disease, these animal infections seem to occur only sporadically [22]. Experimental *M. ulcerans* infections have been performed with amphibians, armadillos, rats, mice, guinea pigs and monkeys, with a mouse foot pad model being most widely used for studying the efficacy of prophylactic and therapeutic interventions [23–29]. Here we propose the pig (*Sus scrofa*) as experimental *M. ulcerans* infection model, since pigs are closely related to humans in terms of many aspects of anatomy and physiology [30,31]. The pig is widely used as a model in dermatological studies because pig skin, in contrast to rodent skin, has striking similarities to human skin

[32]. Not only the thickness of the epidermis and the dermis are comparable to human skin [33], but also the presence of a subcutaneous fat cell layer is favouring the pig model over the mouse foot pad model commonly used for analysing BU pathogenesis. Furthermore, the porcine immune system reflects the human immune system in many aspects better than the murine immune system does [34,35]. For all these reasons we explored here the potential of the pig to serve as model for human *M. ulcerans* infection.

Materials and Methods

Ethical Statement

All animal experiments described here were approved by the Animal Welfare Committee of the Canton of Berne under licence number BE50/11, and conducted in compliance with the Swiss animal protection law and with other national and international guidelines.

Bacteria

The *M. ulcerans* strain used in this study was isolated in 2010 from a swab taken from the undermined edges of the ulcerative lesion of a Cameroonian BU patient [4]. Five passages of the strain after isolation were done in Bac/T medium (Biomerieux) at 30°C. For preparation of the inoculum, bacteria were cultivated in Bac/T medium for 6 weeks, recovered by centrifugation and diluted in sterile phosphate-buffered saline (PBS) to 375 mg/ml wet weight corresponding to 2 x 10^8 CFU/ml as determined by plating serial dilutions on 7H9 agar plates. From this stock solution suspension serial dilutions in PBS were prepared for infection.

Infection and inoculation with synthetic mycolactone A/B

Specific pathogen-free 2-month-old pigs (Large White) from the in-house breeding unit of the Institute of Virology and Immunology (IVI) were kept under BSL3 conditions one week prior and during the time of experimental infection. Animals were checked once daily for macroscopic signs of infection, had *ad libitum* access to water and were fed daily with complete pelleted food.

Pigs were infected on both flanks at four to six infection sites with 100 µl of *M. ulcerans* suspension, containing 2 x 10^7, 2 x 10^6, 2 x 10^5, 2 x 10^4 or 2 x 10^3 CFU. Injection areas were wiped with 70% ethanol and bacterial suspensions injected subcutaneously with a 26G needle. Individual infection sites were encircled with a black marker and the labelling renewed at least once a week. Animals were euthanized at 2.5 weeks or 6.5 weeks post-infection and tissue samples taken as described below.

In addition, the effect of mycolactone was studied directly by injecting 5 µg or 0.5 µg of synthetic mycolactone A/B [36] and analysing tissue specimens taken 2.5 weeks later.

Euthanasia and necropsy

Pigs were euthanized by intravenous injection of pentobarbital (150 mg/kg bodyweight) and subsequent exsanguination. Skin tissue at infection sites was extensively excised with a scalpel and scissors, including all layers of the skin down to the fascia, and samples were

immediately transferred to 10% neutral-buffered Formalin solution (approx. 4% formaldehyde).

Histopathological analysis

After fixation samples were transferred to 70% ethanol for storage and transport, dehydrated and embedded into paraffin. 5 µm thin sections were cut, deparaffinised, rehydrated and directly stained with Haematoxylin/Eosin (HE) or Ziehl-Neelsen/Methylene blue (ZN) according to WHO standard protocols [11]. Stained sections were mounted with Eukitt mounting medium (Fluka). Pictures were taken with a Leica DM2500B microscope or with an Aperio scanner.

Results

Macroscopic appearance of *Mycobacterium ulcerans* infected pig skin

In order to assess early effects of the subcutaneous experimental infection of pigs with doses of 2 x 10^3 to 2 x 10^7 *M. ulcerans* CFU, injection sites were closely monitored for macroscopic changes of the skin. At 2.5 weeks after injection of the bacteria, first changes in colouration and thickness of the skin became apparent at the sites inoculated with the highest inoculation doses, 2 x 10^7 and 2 x 10^6 CFU (Fig. 1, B1). Like nodular BU lesions in humans, these early lesions were elevated, movable, firm and palpable. When these skin areas were excised 2.5 weeks and 6.5 weeks after infection and vertically cut in half after fixation in formalin, roundish yellow structures reflecting coagulative necrosis in the dermis became macroscopically apparent (Fig. 1, B2). A belt with reddish colour, reflecting infiltrating cells and bleeding into the skin, was observed around the necrotic core. While these structures were larger at sites inoculated with a dose of 2 x 10^7 CFU than at sites inoculated with 2 x 10^6 CFU, the general architecture observed with both inoculation doses was similar. At sites inoculated with <2 x 10^5 CFU, no macroscopically visible alterations of the skin were found 2.5 weeks after infection (Fig. 1, A1 and A2).

At 6.5 weeks after experimental infection, sites injected with the highest inoculation dose had either enlarged to an indurated plaque (Fig.1 E1) or ulcerated (Fig. 1, D1). At sites injected with 2 x 10^6 CFU, nodular lesions were observed that were flatter and less palpable compared to those detected 2.5 weeks after infection (Fig. 1, C1). These lesions were macroscopically clearly visible in cross sections through the tissue (Fig. 1, C2). Nodular and ulcerative lesions exhibited greyish/reddish colour changes in the dermis and subcutis (Fig. 1, C2 and D2). The plaque lesion developing after injection with 2 x 10^7 CFU appeared as long cord-like structure with a centre made of yellowish necrotic slough, surrounded by several layers differing in colouration (Fig. 1, E2).

Histopathological features of the pig skin 2.5 weeks after experimental infection

Microcopically, infiltrating immune cells were found 2.5 weeks after infection at all sites inoculated with ≥2 x 10^4 CFU (Fig. 2, A1, B1, C1 and D1). As expected from the macroscopically observed signs, the most pronounced histopathological alterations were associated with the two highest inoculation doses (2 x 10^7 and 2 x 10^6 CFU). The non-ulcerative lesions that developed between the dermis and the underlying muscle tissue displaced the fat layer (Fig. 2, A1 and B1) and caused the macroscopically visible elevation of the skin (Fig. 1, B1). Microscopically, a necrotic core surrounded by large numbers of infiltrating cells and interspersed with fat cell ghosts was observed (Fig. 2, A2 and B2).

At sites infected with 2 x 10^5 CFU, no necrotic core structures but some fat cell ghosts and accumulations of infiltrating cells were found (Fig. 2, C1 and C2). The infection with 2 x 10^4 CFU caused a small accumulation of infiltrating cells (Fig. 2, D1 and D2) and no signs of infection and/or inflammation were observed at sites inoculated with the lowest dose (2 x 10^3 CFU).

Histopathological features of the pig skin 6.5 weeks after experimental infection

At 6.5 weeks after experimental infection, histopathological changes were only found at sites that had been injected with 2 x 10^7 or 2 x 10^6 CFU. In contrast, the skin appeared macro- and microscopically healthy following infection with lower doses of *M. ulcerans*, exhibiting intact epidermis and fat cells, undistorted collagen fibre networks and no marked inflammatory infiltration (Fig. 3, D1 and D2).

Where the infection focus had started to ulcerate, strong infiltration towards the destroyed epidermis was observed (Fig. 3, A1 and A2). No AFB were found in this region, indicative for loss of the necrotic core with the major burden of AFB through the ulceration (Fig. 3, A2, Fig. 4, B1 and B2). Small clusters of AFB were found at deeper sites in the tissue, lateral to the ulceration site (Fig. 4, B3 - B5). Infiltration and destruction of collagen fibres extended into the lower part of the dermis and the upper part of the subcutis, reaching far beyond the area where the epidermis was destroyed (Fig. 3, A1), indicating the formation of undermined edges (Fig. 3, A2, dotted line).

The overall architecture of the plaque lesion that had developed resembled the nodular stages seen 2.5 weeks after infection, i.e. a necrotic centre was surrounded by layers of infiltrating cells (Fig. 3, B1). While large clumps of extracellular AFB were found in the necrotic core after injection of 2 x 10^7 CFU (Fig. 3, B2), AFB were less abundant and bacterial clumps smaller when 2 x 10^6 CFU were used for infection (Fig. 3, C2). Fig. 4A depicts the complex architecture of a plaque lesion (2 x 10^7 CFU dose) with several distinct belts of infiltrating cells surrounding a central necrotic core which contained huge clusters of AFB but was completely devoid of infiltration (Fig. 4A, Ring 1, A1 and A2). In the surrounding ring 2, AFB were scarce and had mostly a beaded appearance. In addition to these single AFB, small globi-like clusters of AFB were found, along with Methylene blue stained remains of infiltrating cells (Fig. 4A, Ring 2, A3 and A4). Ring 3 contained mostly small infiltrating cells that appeared intact, and some acid-fast bacterial debris (Fig.4A, Ring 3, A5 and A6). The outermost layer that could be distinguished did not contain AFB and was mainly built by macrophages and lymphocytes (Fig. 4A, Ring 4, A7). Hence, the number and integrity of AFB decreased from the centre to the periphery of the lesion, whereas the integrity of the

cellular infiltration showed an opposite trend, most likely reflecting levels of the cytotoxic macrolide mycolactone decreasing from centre to periphery.

Histopathological resemblance of pig and human BU lesions

All key features of BU pathology in humans were also found in the experimentally infected pig skin. Already 2.5 weeks after infection, coagulative necrosis (Fig. 5, A1), fat cell ghosts (Fig. 5, A2) and extracellular clusters of AFB (Fig. 5, A3 and A4) were detected. Slight epidermal hyperplasia was already observed at 2.5 weeks and became more pronounced 6.5 weeks after infection (Fig. 5, A5 - A7). At this time, typical histopathological hallmarks of more advanced human BU lesions also emerged in the infected pig skin, namely formation of granulomas (Fig. 5, A8) and presence of giant cells (Fig. 5, A9). Not only experimental infection with *M. ulcerans* led to these typical alterations in the skin, but also the injection of synthetic mycolactone A/B (Fig. 5B).

Development of satellite infection foci

Besides the general histopathological changes, another similarity to findings in human BU [37] was observed: the formation of satellite infection foci adjacent to the primary lesion. A striking example for this is depicted in Fig. 4B where two satellite foci with small clusters of AFB in a necrotic core were found peripheral to the ulcerated main infection focus (Fig. 4B, Region 2). Likewise in the plaque lesion depicted in Fig. 4A, clusters of AFB were found near the main infection focus (Fig. 4A, Ring 5, A8).

Discussion

Detailed studies on the early pathogenesis of BU in an animal model closely mimicking human BU would be very important for a better understanding of host-pathogen interactions and the relative importance of different effector functions of the innate and adaptive immune system against *M. ulcerans.* Here we explored the potential of the pig to serve as model for human *M. ulcerans* infection. After having infected pigs subcutaneously with high doses (2 x 10^6 or 2 x 10^7 CFU) of *M. ulcerans* bacteria, we observed the development of different forms of BU lesions (nodules, plaques and ulcers). Macroscopic and histopathological changes closely mirrored human BU. Challenge with lower doses (2 x 10^3 to 2 x 10^5 CFU) resulted in limited tissue destruction and/or infiltration 2.5 weeks after infection, which resolved spontaneously until week 6.5. Likewise, the dose of bacteria transmitted may be of critical importance for the outcome of a natural *M. ulcerans* infection in humans. Sero-epidemiological analyses in human populations living in BU endemic areas have indicated that exposure to *M. ulcerans* often leads to self-resolving, non-symptomatic infections, as indicated by development of *M. ulcerans* specific antibody responses [38,39]. While macrophages and other immune cells might be able to eliminate smaller numbers of scattered *M. ulcerans* cells, microcolonies of a critical size may develop a protective cloud of mycolactone around them. If the local concentration of the macrolide cytotoxin exceeds a certain level, infiltrating cells may be killed before they can reach the bacteria. This leads to the characteristic picture of clusters of extracellular AFB located primarily in the necrotic core of advanced lesions, which is devoid of living infiltrating immune cells, but contains debris of early inflammatory infiltrates [40,41].

In our study, we observed round elevations of the skin already 2.5 weeks after infection. These alterations were firm, movable and clearly palpable and hence displayed the characteristic features of human BU nodules [11]. Microscopic investigation of the infected skin sites revealed that most histopathological hallmarks of BU had already developed during the first 2.5 weeks of infection if 2 x 10^6 or 2 x 10^7 CFU of *M. ulcerans* was used. The experimentally induced nodules exhibited a necrotic core containing extracellular AFB surrounded by infiltrating cells and fat cell ghosts. Subcutaneous injection of the bacteria led to the formation of an infection focus in the lower dermis and subcutis, where it is also typically found in human BU [13].

In ulcerative human BU lesions AFB are typically focally distributed and not evenly dispersed in the affected tissue [37,42]. Ulceration leads to the shedding of necrotic tissue containing masses of AFB. Therefore the bacterial burden is usually higher in non-ulcerative lesions than in ulcers, where the majority of the remaining AFB reside in the undermined edges of

the ulcers. Our histopathological analyses showed, that like in human BU disease [37], satellite lesions may develop near the primary lesion. These may emerge from globi-like accumulations of AFB originating from bacteria that were internalized and transported to distant sites by phagocytic cells. Globi-like accumulations are also found in human BU [43] and in experimentally infected mice [44–46]. Again, these microcolonies may have to reach a critical size to be able to develop a protective cloud of mycolactone around them. The emergence of only small numbers of newly established microcolonies may explain why borders of advanced ulcers often appear to be very heterogeneous with respect to disease activity with some regions displaying progressive tissue destruction and others showing spontaneous healing tendencies.

At 6.5 weeks after infection with 2 x 10^6 or 2 x 10^7 CFU of *M. ulcerans,* lesions that were still closed comprised a necrotic centre containing clumps of AFB surrounded by well stratified belts of infiltrating cells. Similarly, lesions consisting of a necrotic core surrounded by an inner belt of CD14 positive monocytes/macrophages and a more external belt of CD3 positive T-cells have been described in human BU [47]. The integrity and number of bacteria was decreasing to the outer rim of the lesion. In contrast, the density and integrity of the cellular infiltrates decreased towards the necrotic core.

In the pig model first macroscopic signs of infection (nodules) developed relatively fast after injection of a high number of bacteria. For human BU disease in Uganda and Southern Australia incubation periods of 4-13 weeks and 5-38 weeks have been estimated, respectively [48]. However, incubation periods as short as 2-3 weeks have also been described [49]. Despite extensive analyses we did not find bacteria in the tissue with low inoculation doses at the 6.5 week time point. Therefore we assume that also at later time points lesions would not develop with these low infection doses. It is possible that pigs are more resistant to *M. ulcerans* infection than humans. Consequently, the size of the inoculum to achieve productive experimental infection may be higher for pigs than for the natural infection of humans. This high experimental inoculation dose may have led to fast progression of the disease.

In conclusion, our findings indicate that the pig is a very good animal model to study many aspects of M. ulcerans infection. Pig skin represents a much closer model for human skin than murine foot pads, ears or tails with respect to physiology, structure and abundance of fat tissue [50]. In addition, the immune system of the pig resembles the human system more closely than that of the mouse [34]. In particular the development of new therapeutic and prophylactic interventions might benefit from the porcine M. ulcerans infection model.

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Author Contributions

Conceived and designed the experiments: MB MER GZ GP. Performed the experiments: MB NR MTR GZ. Analyzed the data: MB MTR GP. Contributed to the writing of the manuscript: MB MTR GZ GP.

Figures

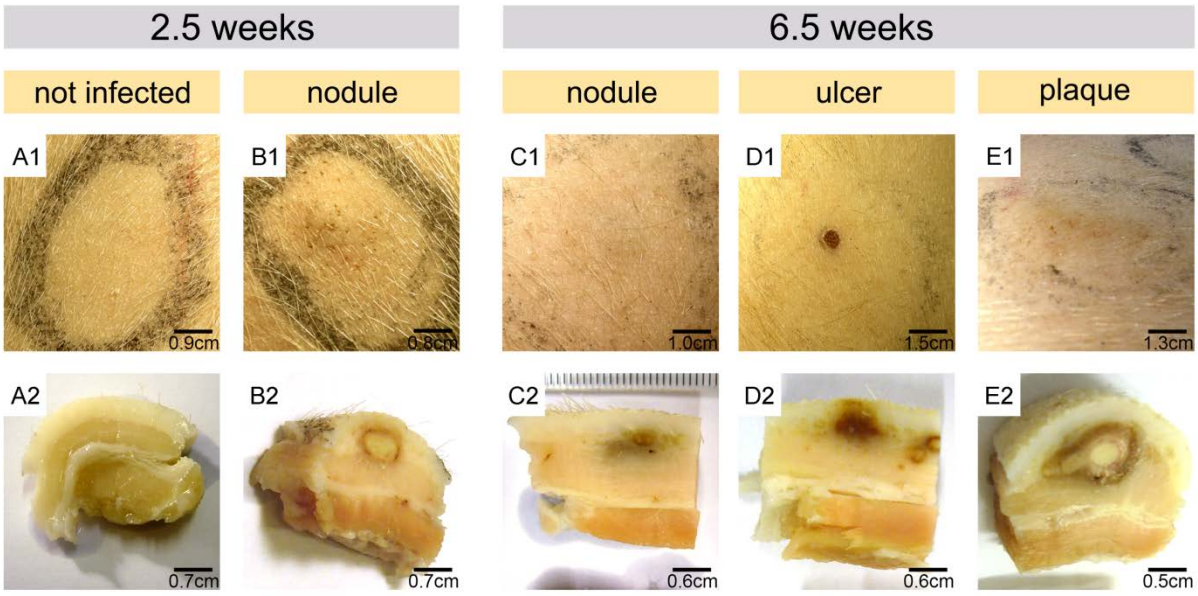


Figure 1: Macroscopic appearance of pig skin infected with *M. ulcerans.*

Development of representative lesions 2.5 weeks or 6.5 weeks after subcutaneous infection with 2 x 10^7 (B1, D1 and E1) or 2 x 10^6 (C1) CFU is depicted and compared to a site left uninfected (A1). Excised tissue specimens were fixed and vertically cut in half to visualize macroscopically visible alteration in tissue structure (A2 - E2). For high inoculation doses (≥2 x 10^6 CFU) the formation of nodules (B1) with necrotic centres was observed already 2.5 weeks after infection (B2). These yellow centres indicative for coagulative necrosis were surrounded by a reddish ring (B2). At 6.5 weeks after infection, these nodules had progressed into a small ulcer or a plaque (D1, E1) associated with marked macroscopically visible alterations in tissue structure (D2, E2). At sites injected with 2 x 10^6 CFU nodules with greyish discoloration of the dermis had developed 6.5 weeks after injection of *M. ulcerans* (C1, C2).

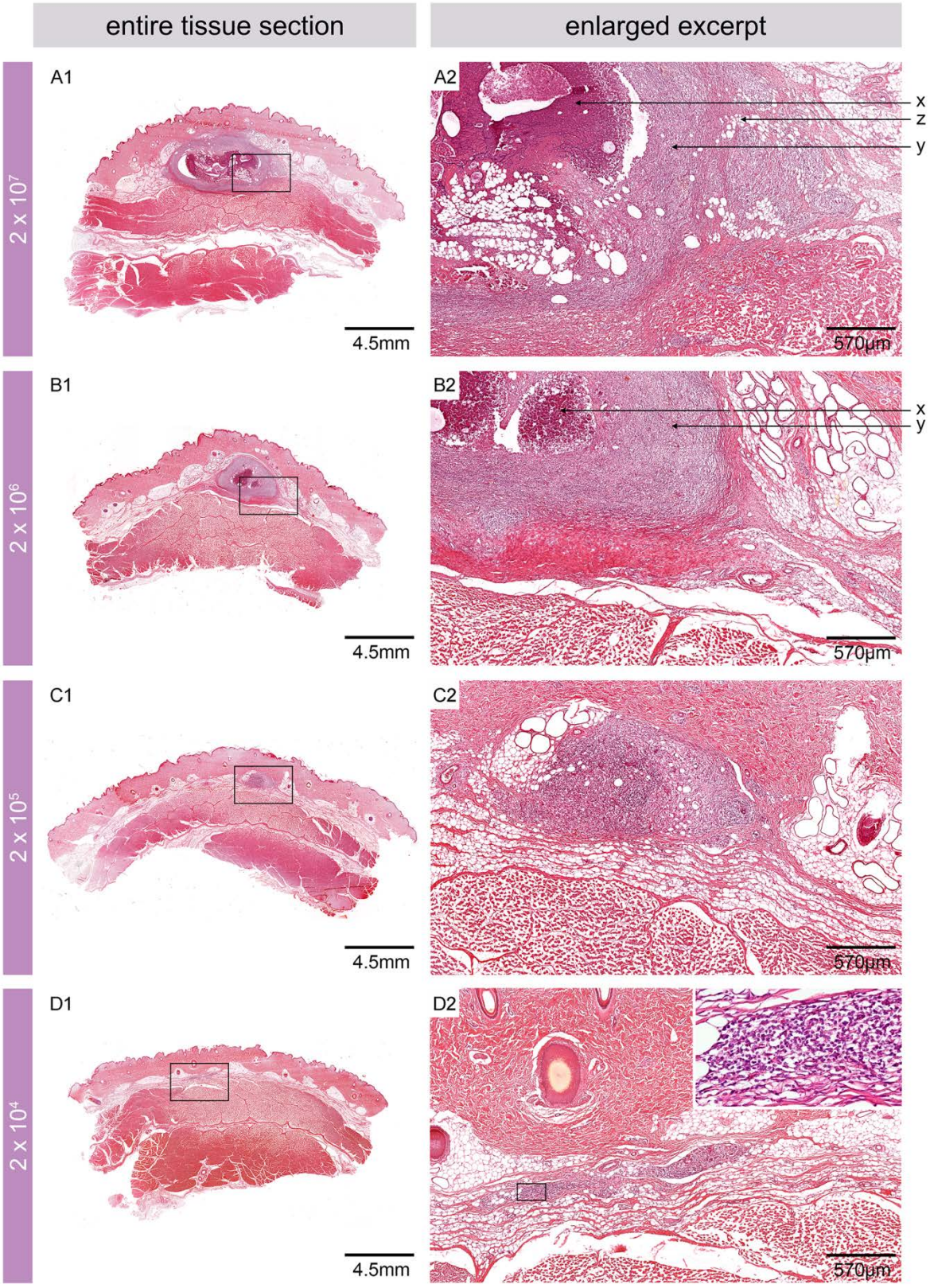

entire tissue section
enlarged excerpt
2 x 10⁷
A1
A2
x
z
y
4.5mm
570µm
2 x 10⁶
B1
B2
x
y
4.5mm
570µm
2 x 10⁵
C1
C2
4.5mm
570µm
2 x 10⁴
D1
D2
4.5mm
570µm

Figure 2: Microscopic appearance of pig skin 2.5 weeks after experimental infection.

Histologic sections stained with HE. Infiltrating cells were found in the fat layer between dermis and muscle tissue at sites infected with ≥2 x 10^4 CFU (A1, B1, C1 and D1). While the two highest inoculation doses led to the development of lesions with a necrotic core surrounded by strong infiltration (A2, B2), infiltration but no necrotic core was observed when doses of 2 x 10^4 and 2 x 10^3 CFU were used (C2, D2 insert). Fat cell ghosts were found at sites infected with the three highest inoculation doses (A2, B2 and C2). x: necrosis, y: infiltration, z: fat cell ghosts.

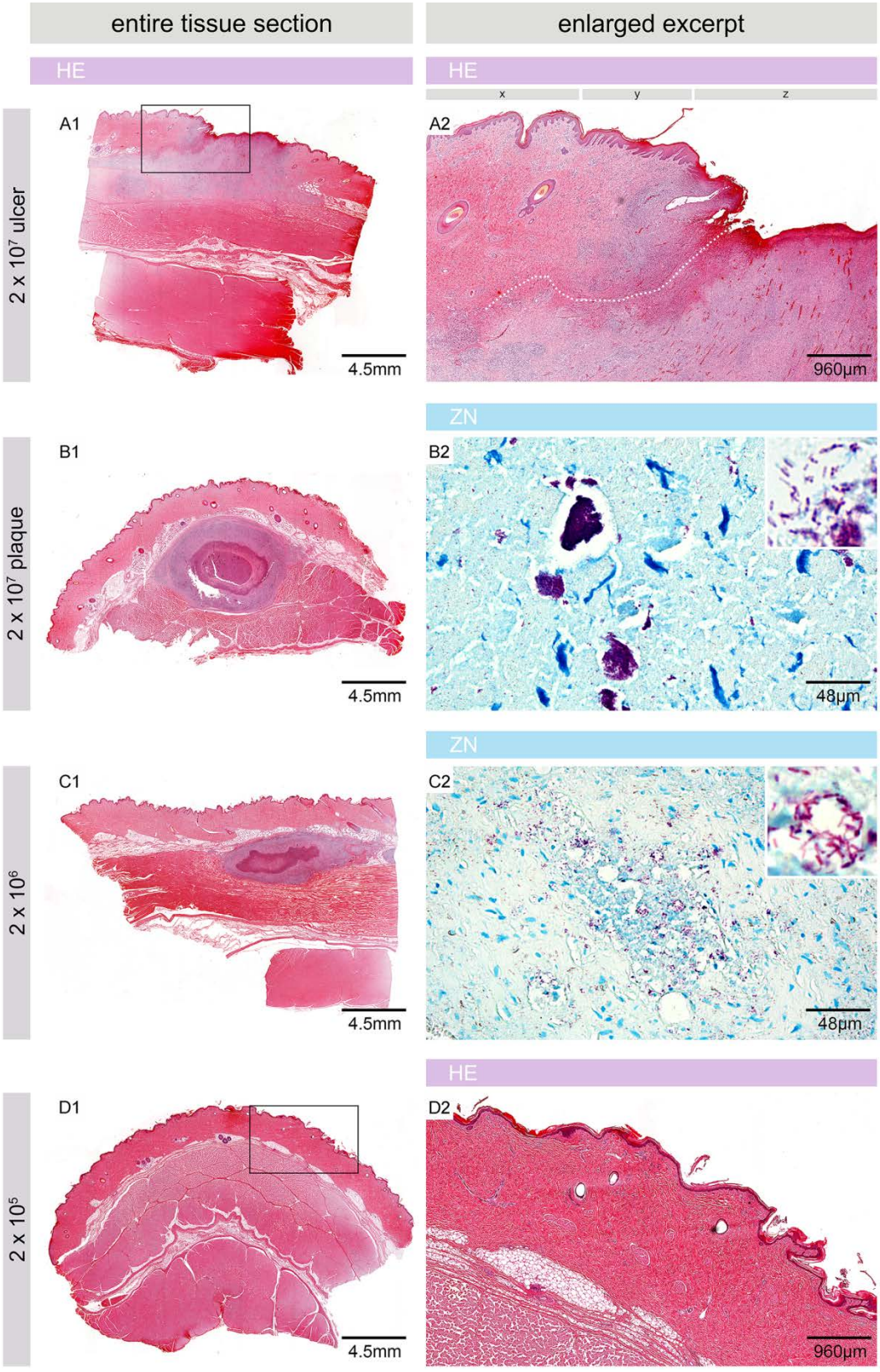
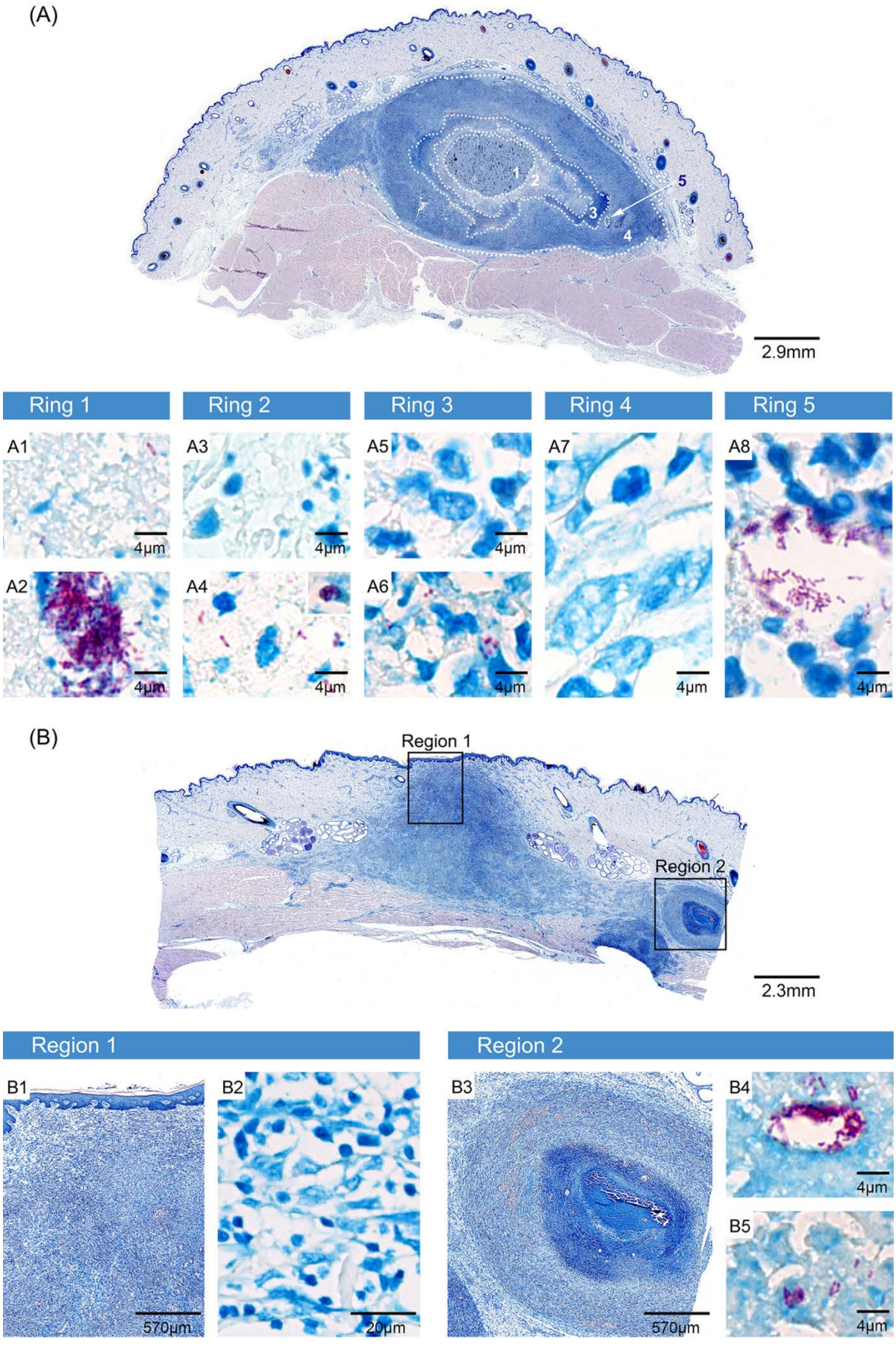

entire tissue section
enlarged excerpt
HE
HE
x
y
z
2 x 10⁷ ulcer
A1
A2
4.5mm
960µm
ZN
2 x 10⁷ plaque
B1
B2
4.5mm
48µm
ZN
2 x 10⁶
C1
C2
4.5mm
48µm
HE
2 x 10⁵
D1
D2
4.5mm
960µm

Figure 3: Microscopic appearance of pig skin 6.5 weeks after experimental infection.

Histologic sections stained with Haematoxylin/Eosin (HE) (A1, A2, B1, C1, D1 and D2) or Ziehl-Neelsen/Methylene blue (ZN) (B2, C2). At sites injected with 2 x 10^7 CFU nodules had either developed into a small ulcer with destroyed epidermis (z), strong infiltration and indications for the development of undermined edges (A1, A2, dotted line) or into a plaque with a necrotic core surrounded by infiltrating cells (B1). x: intact epidermis, y: epidermal hyperplasia, z: destroyed/missing epidermis.

The site infected with 2 x 10^6 CFU showed a similar architecture as the plaque but flatter, less organized and with a smaller overall circumference (C1). Both lesions comprised AFB in their necrotic cores, either in big clumps (B2) or in smaller numbers and smaller aggregations (C2). No signs of infection, inflammation and pathology were observed at sites inoculated with 2 x 10^5 CFU (D1, D2) or less (not shown).


(A)
1
2
3
4
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2.9mm
Ring 1
Ring 2
Ring 3
Ring 4
Ring 5
A1
A2
A3
A4
A5
A6
A7
A8
4µm
(B)
Region 1
Region 2
2.3mm
Region 1
Region 2
B1
B2
B3
B4
B5
570µm
20µm
570µm
4µm
4µm

Figure 4: Containment of large amounts of AFB in the necrotic core and development of satellite microcolonies.

Histologic sections stained with ZN. A plaque (A) and a small ulcer (B) are shown that developed 6.5 weeks after infection with 2 x 10^7 CFU. The ulcerated lesion was strongly infiltrated at the site of ulceration, where no AFB were found (Region 1, B1, B2). Lateral and between dermis and muscle tissue infiltrating cells enclosed small necrotic areas (Region 2, B3), where AFB were found as satellite microcolonies (B4, B5). The plaque consisted of distinct layers of infiltrating cells encasing a necrotic core containing large clumps of bacteria (Ring 1, A1, A2). A second and third ring with decreasing bacterial load and integrity and increasing integrity of infiltrating cells were layered around this core (Ring 2, A3, A47 and Ring 3, A5, A6). A belt of intact cells was surrounding these three inner layers. It did not contain any AFB (Ring 4) except for a microcolony peripheral to the main bacterial burden (Ring 5, A5).

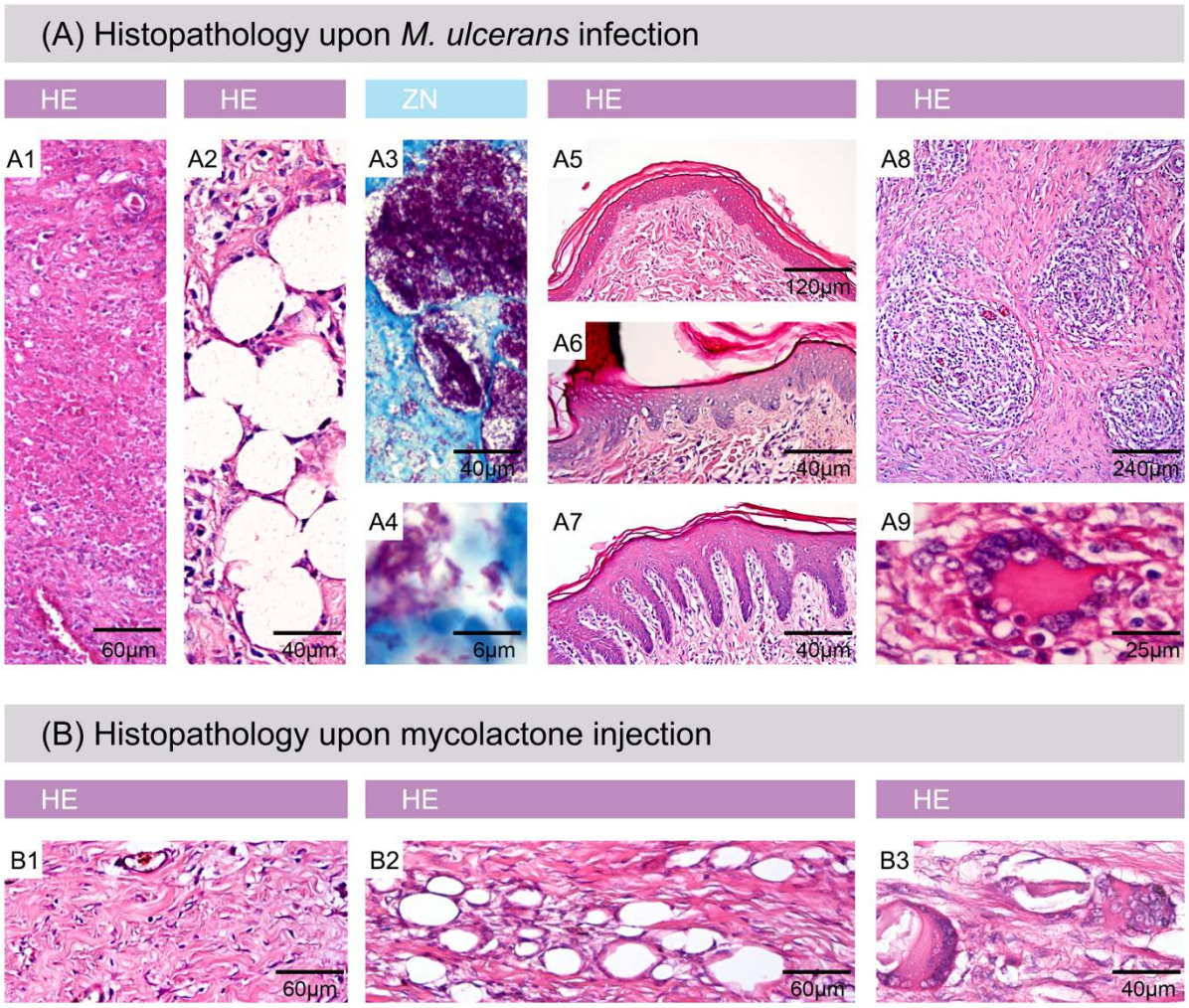

Figure 5: Histophathological hallmarks of Buruli ulcer in experimentally infected pig skin.

Histologic sections stained with Haematoxylin/Eosin (HE) (A1, A2, A5, A6, A7, A8, A9, B1, B2 and B3) or Ziehl-Neelsen/Methylene blue (ZN) (A3, A4).

A: All typical histopathological features of BU in humans were found in infected pig skin. A1: necrosis, A2: fat cell ghosts, A3 and A4: extracellular clusters of AFB, A5: healthy epidermis, A6: moderate epidermal hyperplasia, A7: strong epidermal hyperplasia, A8: granuloma formation, A9: giant cells.

B: Histopathological changes induced by mycolactone injection. B1: necrosis, B2: fat cell ghosts, B3: giant cells.

References

1. Buruli ulcer: *Mycobacterium ulcerans* infection (2000). Geneva: World Health Organization, Global Buruli Ulcer Initiative. 118 p.
2. Hayman J (1985) Clinical Features of *Mycobacterium Ulcerans* Infection. Australas J Dermatol 26: 67–73. doi:10.1111/j.1440-0960.1985.tb01819.x.
3. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, et al. (2010) Ecology and Transmission of Buruli Ulcer Disease: A Systematic Review. PLoS Negl Trop Dis 4: e911. doi:10.1371/journal.pntd.0000911.
4. Bratschi MW, Bolz M, Minyem JC, Grize L, Wantong FG, et al. (2013) Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. PLoS Negl Trop Dis 7: e2252. doi:10.1371/journal.pntd.0002252.
5. WHO | Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer) (n.d.). WHO. Available: http://www.who.int/buruli/information/antibiotics/en/index16.html. Accessed 4 February 2014.
6. Nienhuis WA, Stienstra Y, Thompson WA, Awuah PC, Abass KM, et al. (2010) Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial. The Lancet 375: 664–672. doi:10.1016/S0140-6736(09)61962-0.
7. Sarfo FS, Phillips R, Asiedu K, Ampadu E, Bobi N, et al. (2010) Clinical efficacy of combination of rifampin and streptomycin for treatment of *Mycobacterium ulcerans* disease. Antimicrob Agents Chemother 54: 3678–3685. doi:10.1128/AAC.00299-10.
8. Chauty A, Ardant M-F, Adeye A, Euverte H, Guedenon A, et al. (2007) Promising Clinical Efficacy of Streptomycin-Rifampin Combination for Treatment of Buruli Ulcer (*Mycobacterium ulcerans* Disease). Antimicrob Agents Chemother 51: 4029–4035. doi:10.1128/AAC.00175-07.
9. Kibadi K, Boelaert M, Fraga AG, Kayinua M, Longatto-Filho A, et al. (2010) Response to treatment in a prospective cohort of patients with large ulcerated lesions suspected to be Buruli Ulcer (*Mycobacterium ulcerans* disease). PLoS Negl Trop Dis 4: e736. doi:10.1371/journal.pntd.0000736.
10. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, et al. (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283: 854–857.
11. Portaels F, Johnson P, Meyers WM, Initiative WHOGBU (2001) Buruli ulcer : diagnosis of *Mycobacterium ulcerans* disease : a manual for health care providers / edited by: Françoise Portaels, Paul Johnson, Wayne M. Meyers. Available: http://apps.who.int/iris/handle/10665/67000. Accessed 6 February 2014.
12. Guarner J, Bartlett J, Whitney EAS, Raghunathan PL, Stienstra Y, et al. (2003) Histopathologic features of *Mycobacterium ulcerans* infection. Emerg Infect Dis 9: 651–656.
13. Hayman J (1993) Out of Africa: observations on the histopathology of *Mycobacterium ulcerans* infection. J Clin Pathol 46: 5–9.
14. Mitchell PJ, McOrist S, Bilney R (1987) Epidemiology of *Mycobacterium ulcerans* infection in koalas (*Phascolarctos cinereus*) on Raymond Island, southeastern Australia. J Wildl Dis 23: 386–390.
15. Mitchell PJ, Jerrett IV, Slee KJ (1984) Skin ulcers caused by *Mycobacterium ulcerans* in koalas near Bairnsdale, Australia. Pathology (Phila) 16: 256–260.

16. Trott KA, Stacy BA, Lifland BD, Diggs HE, Harland RM, et al. (2004) Characterization of a *Mycobacterium ulcerans*-like infection in a colony of African tropical clawed frogs (*Xenopus tropicalis*). Comp Med 54: 309–317.
17. O'Brien CR, Handasyde KA, Hibble J, Lavender CJ, Legione AR, et al. (2014) Clinical, Microbiological and Pathological Findings of *Mycobacterium ulcerans* Infection in Three Australian Possum Species. PLoS Negl Trop Dis 8: e2666. doi:10.1371/journal.pntd.0002666.
18. Elsner L, Wayne J, O'Brien CR, McCowan C, Malik R, et al. (2008) Localised *Mycobacterium ulcerans* infection in a cat in Australia. J Feline Med Surg 10: 407–412. doi:10.1016/j.jfms.2008.03.003.
19. Portaels F, Chemlal K, Elsen P, Johnson PD, Hayman JA, et al. (2001) *Mycobacterium ulcerans* in wild animals. Rev Sci Tech Int Off Epizoot 20: 252–264.
20. Sakaguchi K, Iima H, Hirayama K, Okamoto M, Matsuda K, et al. (2011) *Mycobacterium ulcerans* infection in an Indian flap-shelled turtle (*Lissemys punctata punctata*). J Vet Med Sci Jpn Soc Vet Sci 73: 1217–1220.
21. Van Zyl A, Daniel J, Wayne J, McCowan C, Malik R, et al. (2010) *Mycobacterium ulcerans* infections in two horses in south-eastern Australia. Aust Vet J 88: 101–106. doi:10.1111/j.1751-0813.2009.00544.x.
22. Fyfe JAM, Lavender CJ, Handasyde KA, Legione AR, O'Brien CR, et al. (2010) A major role for mammals in the ecology of *Mycobacterium ulcerans*. PLoS Negl Trop Dis 4: e791. doi:10.1371/journal.pntd.0000791.
23. Marcus LC, Stottmeier KD, Morrow RH (1976) Experimental alimentary infection of anole lizards (*Anolis carolinensis*) with *Mycobacterium ulcerans*. Am J Trop Med Hyg 25: 630–632.
24. Walsh DS, Meyers WM, Krieg RE, Walsh GP (1999) Transmission of *Mycobacterium ulcerans* to the nine-banded armadillo. Am J Trop Med Hyg 61: 694–697.
25. Singh NB, Srivastava A, Verma VK, Kumar A, Gupta SK (1984) *Mastomys natalensis*: a new animal model for *Mycobacterium ulcerans* research. Indian J Exp Biol 22: 393–394.
26. Addo P, Owusu E, Adu-Addai B, Quartey M, Abbas M, et al. (2005) Findings from a buruli ulcer mouse model study. Ghana Med J 39: 86–93.
27. Oliveira MS, Fraga AG, Torrado E, Castro AG, Pereira JP, et al. (2005) Infection with *Mycobacterium ulcerans* induces persistent inflammatory responses in mice. Infect Immun 73: 6299–6310. doi:10.1128/IAI.73.10.6299-6310.2005.
28. Read JK, Heggie CM, Meyers WM, Connor DH (1974) Cytotoxic Activity of *Mycobacterium ulcerans*. Infect Immun 9: 1114–1122.
29. Walsh DS, Dela Cruz EC, Abalos RM, Tan EV, Walsh GP, et al. (2007) Clinical and histologic features of skin lesions in a cynomolgus monkey experimentally infected with *Mycobacterium ulcerans* (Buruli ulcer) by intradermal inoculation. Am J Trop Med Hyg 76: 132–134.
30. Swindle MM, Makin A, Herron AJ, Clubb FJ Jr, Frazier KS (2012) Swine as models in biomedical research and toxicology testing. Vet Pathol 49: 344–356. doi:10.1177/0300985811402846.
31. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V (2012) The pig: a model for human infectious diseases. Trends Microbiol 20: 50–57. doi:10.1016/j.tim.2011.11.002.
32. Sullivan TP, Eaglstein WH, Davis SC, Mertz P (2001) The pig as a model for human wound healing. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc 9: 66–76.
33. Liu Y, Chen J, Shang H, Liu C, Wang Y, et al. (2010) Light microscopic, electron microscopic, and immunohistochemical comparison of Bama minipig (*Sus scrofa domestica*) and human skin. Comp Med 60: 142–148.

34. Dawson H (2011) A Comparative Assessment of the Pig, Mouse and Human Genomes: Structural and Functional Analysis of Genes Involved in Immunity and Inflammation. The Minipig in Biomedical Research. CRC Press. pp. 323–342. Available: http://www.crcnetbase.com/doi/abs/10.1201/b11356-28. Accessed 4 February 2014.

35. Fairbairn L, Kapetanovic R, Sester DP, Hume DA (2011) The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. J Leukoc Biol 89: 855–871. doi:10.1189/jlb.1110607.

36. Gersbach P, Jantsch A, Feyen F, Scherr N, Dangy J-P, et al. (2011) A ring-closing metathesis (RCM)-based approach to mycolactones A/B. Chem Weinh Bergstr Ger 17: 13017–13031. doi:10.1002/chem.201101799.

37. Rondini S, Horsfield C, Mensah-Quainoo E, Junghanss T, Lucas S, et al. (2006) Contiguous spread of *Mycobacterium ulcerans* in Buruli ulcer lesions analysed by histopathology and real-time PCR quantification of mycobacterial DNA. J Pathol 208: 119–128. doi:10.1002/path.1864.

38. Yeboah-Manu D, Röltgen K, Opare W, Asan-Ampah K, Quenin-Fosu K, et al. (2012) Sero-epidemiology as a tool to screen populations for exposure to *Mycobacterium ulcerans*. PLoS Negl Trop Dis 6: e1460. doi:10.1371/journal.pntd.0001460.

39. Diaz D, Döbeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, et al. (2006) Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. Clin Vaccine Immunol CVI 13: 1314–1321. doi:10.1128/CVI.00254-06.

40. Ruf M-T, Sopoh GE, Brun LV, Dossou AD, Barogui YT, et al. (2011) Histopathological changes and clinical responses of Buruli ulcer plaque lesions during chemotherapy: a role for surgical removal of necrotic tissue? PLoS Negl Trop Dis 5: e1334. doi:10.1371/journal.pntd.0001334.

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

42. Peduzzi E, Groeper C, Schütte D, Zajac P, Rondini S, et al. (2007) Local activation of the innate immune system in Buruli ulcer lesions. J Invest Dermatol 127: 638–645. doi:10.1038/sj.jid.5700593.

43. Schütte D, Umboock A, Pluschke G (2009) Phagocytosis of *Mycobacterium ulcerans* in the course of rifampicin and streptomycin chemotherapy in Buruli ulcer lesions. Br J Dermatol 160: 273–283. doi:10.1111/j.1365-2133.2008.08879.x.

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

45. Ruf M-T, Schütte D, Chauffour A, Jarlier V, Ji B, et al. (2012) Chemotherapy-associated changes of histopathological features of *Mycobacterium ulcerans* lesions in a Buruli ulcer mouse model. Antimicrob Agents Chemother 56: 687–696. doi:10.1128/AAC.05543-11.

46. Coutanceau E, Marsollier L, Brosch R, Perret E, Goossens P, et al. (2005) Modulation of the host immune response by a transient intracellular stage of *Mycobacterium ulcerans*: the contribution of endogenous mycolactone toxin. Cell Microbiol 7: 1187–1196. doi:10.1111/j.1462-5822.2005.00546.x.

47. Ruf M-T, Chauty A, Adeye A, Ardant M-F, Koussemou H, et al. (2011) Secondary Buruli ulcer skin lesions emerging several months after completion of chemotherapy: paradoxical reaction or evidence for immune protection? PLoS Negl Trop Dis 5: e1252. doi:10.1371/journal.pntd.0001252.

48. Trubiano JA, Lavender CJ, Fyfe JAM, Bittmann S, Johnson PDR (2013) The Incubation Period of Buruli Ulcer (*Mycobacterium ulcerans* Infection). PLoS Negl Trop Dis 7.

Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3789762/. Accessed 21 March 2014.

49. Reid IS (1967) *Mycobacterium ulcerans* infection: a report of 13 cases at the Port Moresby General Hospital, Papua. Med J Aust 1: 427–431.

50. Addo P, Owusu E, Adu-Addai B, Quartey M, Abbas M, et al. (2005) Findings from a Buruli Ulcer Mouse Model Study. Ghana Med J 39: 86–93.

Discussion

WHO currently lists 17 diseases as neglected tropical diseases (NTDs), whereof BU is one of four that are caused by bacterial pathogens. All NTDs have in common that they affect the world's poorest people and impair their physical and/or cognitive development and thus impede their ability to earn a living [1,2]. Undoubtedly, the acknowledgement of BU as a major public health problem by the WHO has substantially increased the research interest for the disease, which is reflected in the increased amount of scientific publications on *M. ulcerans* since the "Yamoussoukro Declaration" in 1998 [3,4]. On the other hand, a closer look at what we know today about BU and its causative agent *M. ulcerans* reveals that a substantial part of the knowledge originates from the early times of BU research (1960s and 1970s) and that our knowledge is far from being complete. Major questions, like the main environmental reservoir of *M. ulcerans,* remain unanswered and the exact mode/s of transmission of the disease has/have not yet been identified. Consequently, the incubation time of BU can only be estimated to be 2 - 3 months [5] and the mechanisms leading to the large variety of observed clinical pictures remain incomprehensible. Besides mere ignorance concerning certain BU-relevant questions, other aspects imperatively need optimization, such as early case detection and simple, highly sensitive and specific diagnostic tools for BU. Furthermore extended treatment options, especially treatment beyond antibiotics, remain major issues in BU research.

Within the framework of this thesis, we have tackled some of the unknown aspects of BU by establishing a new research site in the remote Mapé basin of Cameroon, where the disease has recently emerged [6]. We have studied the epidemiology in this region in detail (Results Chapter 1) and laid the foundations for subsequent biomedical studies (Results Chapter 2) as well as investigations into identifying transmission patterns by combining whole genome sequencing information with geographical data (Results Chapter 3). Furthermore, we have elucidated the potential for developing a protein subunit vaccine against *M. ulcerans* (Results Chapters 4 - 6) and established the pig as suitable animal model for studying early BU pathology and validating new interventions (Results Chapter 7).

Buruli ulcer endemic areas: What has been learned from the Mapé dam region of Cameroon?

Already in 1969, one BU endemic region was reported in Cameroon - the Nyong river basin located in the south-central region of the country [7,8]. At that time, a total of 47 cases were described from an area between the two villages of Ayos and Akonolinga. A cross-sectional survey in the same region in 2001 identified a total of 436 cases on clinical grounds, of which 135 were laboratory confirmed [9]. Reports on BU cases in a second region of Cameroon,

the Bankim Health District (HD, Mapé dam region), only emerged recently when *Marion et al*. described the discovery of 195 clinically diagnosed cases over a 2.5 year period from 2007 onwards [6]. Damming of the Mapé river and the resulting formation of a lake in 1988, was suspected to be the cause for the increased incidence of BU in this part of Cameroon. In collaboration with the foundation Fairmed, the Bankim HD was established as a new field site. As a starting point, a house-by-house survey was conducted in spring 2010, collecting epidemiological data of over 48'000 individuals and examining the local population for BU, leprosy and yaws (Results Chapter 1). Despite the notion that BU was highly endemic in the area, only 25 cases of BU were identified on clinical grounds and only 23 % of them could be reconfirmed by IS2404 qPCR. Although this survey strategy can be successful for detecting cases in remote communities, which are otherwise not discovered by the health care system, we would recommend another approach for future studies. Only selected communities should be chosen based on epidemiological data for maximising the cost-benefit ratio.

On the other hand, the exhaustive survey performed for initiating a new field site in a new BU endemic area did yield much more information than "just" newly discovered BU cases. The **awareness** for BU and the acceptance of the local control program in the population was certainly increased. This is reflected in the total number of new BU cases that were identified by passive case finding during the 27 months following the survey. A steady flow of patients from the Bankim HD as well as from surrounding HDs reported to the district hospital, were clinically diagnosed, laboratory confirmed and received treatment (Results Chapter 1).

The survey also demonstrates the **urgent need for improved tools for sensitive and specific BU diagnosis in the field** (a point-of-care diagnostic test). Although the quality of clinical diagnosis in Bankim has improved since the survey (increasing from 23 % laboratory confirmation to 56 % two years after the survey), the number of false positive clinical diagnoses is still very high. Prevention of unnecessary antibiotic treatment in those wrongly diagnosed patients is fundamental for preventing the development of resistant mycobacteria, especially because tuberculosis is highly endemic in most BU-endemic regions and the risk for misdiagnosis of cutaneous tuberculosis as BU is substantial [10]. Additionally, rifampicin and streptomycin both have side effects, especially in children, and treating BU-unrelated skin conditions with eight weeks of these drugs is immoderate at best. According to the WHO, laboratory confirmation of clinical BU diagnosis is essential for the following reasons: (1) to confirm the precise prevalence and incidence of BU in a given area, (2) to confirm new foci, especially where health care workers lack experience with BU, (3) to help manage the disease by surgical and/or antimycobacterial treatment and (4) to confirm and differentiate relapse and reinfection after treatment [11]. Of the available four methods for laboratory confirmation, only smear examination for AFB after Ziehl-Neelsen staining is feasible in a remote area like the Bankim HD. With positivity rates between 40 - 78 %, this method has a

low sensitivity and additionally only limited specificity [11]. For example *M. tuberculosis* from a cutaneous tuberculosis case also appears positive in the smear examination. The current gold standard of diagnosis, which is the highly sensitive and specific IS2404 qPCR, requires sophisticated laboratory equipment and trained personnel. Nevertheless the WHO recently recommended that by the end of 2014, at least 70 % of reported cases should be laboratory reconfirmed by a positive PCR result. It remains to be seen whether this goal is achievable, considering the costs of the methods, the logistic challenges in sample transport and the limited capacities of the laboratories in the respective countries. Special attention should also be given to quality control because the high sensitivity of the method requires strict procedures for avoiding cross-contamination and thus false positive results.

For the Bankim HD, swab samples were transported for laboratory confirmation to the Swiss TPH in Basel. Although a preliminary set up of qPCR in Bankim was feasible, frequent power failures and the lack of appropriate premises and trained personnel hinder the sustainable establishment of this diagnostic method at a peripheral level. Additionally the build-up of very good structures solely for BU diagnosis, without improvement of the standard of treatment at the local health facilities should be avoided. Besides laboratory confirmation of clinically diagnosed cases, **cultivation of the circulating *M. ulcerans* strains** was performed at the Swiss TPH (Results Chapter 2). Due to the slow growth rate, cultivation of *M. ulcerans* does not have significant diagnostic value. Furthermore culturing has a low sensitivity, especially when culture initiation is done long after sampling. With a turnover time of 4 - 6 months, we achieved a per patient positivity rate of 43.1 %, which is comparable to rates reported elsewhere [12,13]. But even though primary cultivation of *M. ulcerans* isolates is not crucial for immediate laboratory confirmation of the diagnosis, it remains a basis for studies on molecular typing, phylogenetic analyses and monitoring of drug resistance of the locally circulating strains (Results Chapter 3). The primary isolates obtained from the Mapé basin region build the core of a *M. ulcerans* strain collection stored at the Swiss TPH. The use of these strains, for example for identification of transmission patterns (Results Chapter 3) or the development of appropriate animal models for microbiological investigations (Results Chapter 4 - 7), should ideally lead to a long-term benefit for the BU-affected communities.

Another very important outcome of the survey in Bankim was the **detailed knowledge gained about the new field site** and the proper identification of all the villages and the origin of the patients (by GPS). Contrary to local beliefs, the reported BU cases were not primarily associated with the lake but rather with the Mbam river composing the Southern border of the Bankim HD (Results Chapter 1). Subsequent studies investigating possible environmental reservoirs in this area confirmed this first finding and identified village water sources as being repeatedly positive for *M. ulcerans* DNA [14]. The combination of the findings from whole genome sequencing of the above-mentioned primary isolates from

patients in the Mapé area with the information on the residential origin of the corresponding patients was expected to give further insight into BU transmission patterns in the region (Results Chapter 3). Unfortunately no clear correlation was seen and transmission of BU remains enigmatic.

Lastly, the survey revealed the **importance of socioeconomic factors** leading to late care seeking of BU-affected patients. Antimicrobial treatment of BU is in principle free of cost for the patients. Unfortunately streptomycin requires daily injections for which the patients have to come to the health post either every day or they have to stay for the entire treatment period when travelling distances are too long. As we have also seen in the initial survey, the proportion of affected children between the age of 5 and 15 is very high (Results Chapter 1). Very often when children have to stay for a prolonged time at a health facility, a caretaker has to accompany them, therefore abandoning the rest of the family and the daily work. During their stay, both the patient and the caretaker have to provide their own meals, which adds another financial burden besides the travelling costs. To further illustrate the problem, a study in central Cameroon has described that the cost burden of BU accounts for up to 25 % of a household's yearly earnings [15]. One of the obvious solutions to minimize the stay at treatment facilities would be a completely oral antibiotic treatment. Currently studies are under way to test the efficacy of fully or partially replacing streptomycin with clarithromycin, which would abolish the need for injections, and initial results appear promising [16–19]. On the other hand, proper wound care, which has a huge impact on good healing progression of BU, and improved diagnosis are problems that will have to be solved independently of an all-oral treatment strategy.

Ultimately, prevention of BU by a vaccine would eliminate a major part of the above-described problems; hence the development of such a vaccine remains one of the highly desirable achievements in BU research.

Is a vaccine against Buruli ulcer a utopia?

Together with sanitation, vaccination has been the most important contribution to public health during the last 100 years and probably the most effective measure in the prevention of infectious diseases. Since the development of the first vaccines by Jenner and Pasteur, progress in basic immunology and the development of novel technologies has resulted in many more vaccines being successfully used worldwide today [20]. Unfortunately tackling microbes that do not grow *in vitro* (*M. leprae*), that have an intracellular phase (*M. tuberculosis*) or are of high antigenic variability (*Plasmodium* species) has been unsuccessful thus far [21].

The evidence that protective immunity against *M. ulcerans* can develop in humans is incomplete but nevertheless there are indications that development of a vaccine is feasible.

The first piece of evidence comes from serological studies investigating antibody responses in BU affected and non-affected persons [22–26]. A study by *Dobos et al*. investigated humoral immune responses in BU patients to *M. ulcerans* culture filtrate antigens in order to identify novel diagnostic antigens. Specific IgG responses were frequently found in BU patients but interestingly also in some non-exposed US tuberculosis patients as well as in healthy controls from BU endemic areas [22]. While a part of these responses might be attributed to antigenic cross-reactivity of *M. tuberculosis* and *M. ulcerans*, this factor did not play a role in a study using the 18 kDa small heat shock protein (shsp, MUL2232) as a serological marker [23]. MUL2232 does not have a homolog in *M. bovis* and *M. tuberculosis*. Antibodies against the protein were frequently found in sera of BU patients and healthy household contacts but only rarely in controls from regions where the disease is not endemic. The authors of the study therefore concluded, that only a small portion of *M. ulcerans*-infected individuals actually develop clinical disease [23]. A second piece of evidence originates from a study done in the 1970s on the treatment of *M. ulcerans* infection with clofazimine, in which the authors report healing without surgery in the placebo group in 6 out of 21 patients [27]. Other scientific reports on self-healing of BU are very rare but anecdotal descriptions of early BU lesions just disappearing after a certain time are more frequent [28]. Both of the above-described phenomena may have several explanations, one of which would be the development of a protective immune response. Thus the immune system seems to be, under certain circumstances, able to cope with a *M. ulcerans* infection. When Yeboah-Manu and colleagues used the 18 kDa shsp as a serological marker for the identification of new BU endemic areas in Ghana, one of their conclusions was that unknown host factors as well as unknown pathogen factors might determine how frequently exposure to *M. ulcerans* leads to clinical disease [24]. Only one host-factor study for BU has been done so far [29] but more of such investigations might deliver important insights into the reasons for why only some people develop the disease. Moreover, such studies might explain the observed bimodality in age distribution (Results Chapter 1).

The third indication for the existence of a protective immune response against BU derives from histopathological investigations on secondary lesions emerging after chemotherapy. Analysis of tissue specimens from secondary lesions of two patients revealed typical BU histopathology of a cured lesion with massive leukocyte infiltrates and large B-cell clusters, indicating that treatment may lead to a priming of the response that is later sufficient to resolve secondary lesions [30]. Lastly, Bacille Calmette-Guérin (BCG) vaccination was found to confer limited protection against BU [31–33], indicating that vaccine-mediated protection against BU is possible, but the antigenic cross-reactivity between *M. ulcerans* and *M. bovis* might not be sufficient to achieve complete protection by BCG vaccination.

One frequently asked question is, where would such a vaccine be implemented once it is developed? Implementation would have to consider the very focal distribution of BU on a country level (as seen in Results Chapter 1). The goal for BU vaccination would certainly not be a mass vaccination program on a countrywide scale, but would rather be confined to the affected communities. Because a majority of the affected patients are children between the age of five and 15 (Results Chapter 1), immunization would especially need to protect young children. The focal distribution of BU would facilitate the introduction of a vaccine as a preventive measure, because fewer doses would have to be produced and distributed, thus considerably decreasing the logistic effort. Another important application could be therapeutic vaccination. BU patients could receive the vaccine as soon as they report to the health centre, because stimulation of the right immune mechanisms by vaccination might help the immune system to eliminate the infection.

What are possible vaccination approaches against *M. ulcerans*?

Toxin neutralization by antibodies

Mycolactone, the key virulence factor of *M. ulcerans,* is a primary target for vaccine development. Neutralizing antibodies could potentially prevent the pathology, although it is not entirely clear how good the chances are that bacterial proliferation without the presence of toxin activity could be inhibited by the immune system [34]. Unfortunately, mycolactone is a lipid molecule that is poorly immunogenic. Attempts to generate antisera against the molecule were not successful for a long time. Only recently, the generation of a set of monoclonal antibodies against mycolactone succeeded in our laboratory at the Swiss TPH (unpublished results) and the molecule used for immunization has just entered active immune protection experiments in mice. Another way of tackling mycolactone production was attempted by immunization of mice against polyketide synthase domains on the pMUM01 plasmid [35]. Despite good immunogenicity of the DNA vaccine prime - recombinant protein boost regimen, no significant protection was seen in the immunized mice when delay to foot pad swelling was assessed. The induced antibodies would have to reach the inside of the mycobacteria to possibly interfere with toxin production, a rationale which is difficult to comprehend.

Live attenuated mycobacteria

One of the most used vaccines is BCG, a live attenuated *M. bovis* strain used for vaccination against *M. tuberculosis*. BCG is known to induce significant cross-reactive immune responses against other mycobacteria thus BCG-mediated protection against BU was suspected [36]. Indeed, two very large clinical studies in Uganda reported timely limited protection in a proportion of people as well as a shortening of the duration of ulcers in patients [31,32]. Furthermore, BCG vaccinated children and adults seemed to develop

osteomyelitis less frequently than non-vaccinated individuals [33]. In line with these observations, BCG vaccination appears to delay the onset of foot pad swelling in a mouse model of BU [35,37]. Both in humans as well as in the animal model, the BCG-induced immune response is apparently insufficiently cross-reactive or not of the right nature to confer sustained protection against *M. ulcerans* infection. In order to induce more specific immune responses, live attenuated strains of *M. ulcerans* were generated by random transposon mutagenesis [38]. Immunization of mice with those mycolactone deficient *M. ulcerans* strains and BCG vaccination resulted in comparable protection from BU in the mouse model [39]. Besides the lack of increased protective potential compared to BCG, mycolactone deficient *M. ulcerans* vaccine strains would require at least one additional targeted mutation to address safety concerns.

Subunit vaccination

Subunit vaccines tend to have a substantially better safety profile than live attenuated vaccines, although this often comes at the cost of strongly reduced immunogenicity that has to be compensated for with an immunogenic delivery system [40]. Protein subunit vaccination was investigated for BU with two different protein vaccine candidates. Firstly, heat shock protein (Hsp) 65 of *M. leprae* was tested in a DNA vaccination approach but was only marginally able to reduce the *M. ulcerans* burden in infected mouse tails after six weeks of infection [41]. Secondly, Ag85A was successful in delaying foot pad swelling in mice immunized with a DNA prime encoding either the *M. tuberculosis* version of the protein or the homolog of *M. ulcerans* (MUL4987) and a recombinant protein boost [42]. Protection conferred with MUL4987 by this approach was comparable to BCG immunization and significantly better than the DNA vaccine encoding the *M. tuberculosis* Ag85A [42].

Encouraged by these data we further evaluated the potential to develop a protein subunit vaccine by formulating three protein vaccine candidate antigens with different delivery systems and by assessing their protective potential in a mouse foot pad model of BU (Results Chapter 4 - 6).

What is the right antigen for protein subunit vaccination against *M. ulcerans* disease?

The lack of correlates of protection made it difficult to choose vaccine candidates for the development of a protein subunit vaccine against BU. Because the whole genome sequence of *M. ulcerans* is available [43], the strategy of reverse vaccinology could have been applied [21]. This approach was successfully used for the first time to identify novel protein vaccine candidates against serogroup B *Neisseria meningitidis* (MenB) [44]. In a first step, the complete MenB genome was screened for open reading frames coding for putative surface-exposed or secreted proteins. In a second step, 350 of the approximately 600 predicted

genes were expressed in *E. coli* and mice were immunized with the purified proteins to obtain antisera. Those were tested for bactericidal antibodies in an *in vitro* assay, with the background knowledge that such antibodies correlate with protection in humans. Of the 28 identified antigens, five were finally combined to a multicomponent vaccine as the first vaccine against MenB [45]. An attempt to directly translate those single steps to the development of a vaccine against *M. ulcerans* would encounter several problems. A major obstacle is that it is not clear whether immune protection can be mediated by humoral and/or cellular immune responses. If binding of antibodies to the mycobacterial cells were suspected to be protective, screening of the *M. ulcerans* genome for surface-exposed and secreted proteins would be possible, but the expression of all hits in *E. coli* and the subsequent immunization of mice would be a challenging task for a research group working on BU with limited resources. The next major obstacle in the whole approach would be encountered when antisera would have to be tested in an *in vitro* format to reduce the number of candidates and to separate those that potentially confer protection *in vivo*. No correlates of protection for BU are known today (see next section), and the extremely slow growth rate of *M. ulcerans* would make opsonophagocytosis assays very complicated. The best approach may thus be the expression of all *M. ulcerans* proteins and their subsequent testing in the mouse foot pad model. Taking into account that the progression of the infection is slow and experiments would have to be performed under BSL-3 conditions, this approach would be extremely costly. Alternatively to a pure reverse vaccinology approach, antigens could be chosen based on experience from vaccine development against closely related pathogens. *M. ulcerans* homologs of lead vaccine candidates against tuberculosis could, for example, be considered. Unfortunately genome analysis has revealed that *M. ulcerans* has lost a substantial amount of genes during its evolution from *M. marinum* [46]. Among those are many of the known immunodominant antigens like the *M. marinum* homolog of the 6 kDa early secretory antigenic target protein (ESAT-6) as well as the 10 kDa culture filtrate protein (CFP-10) from *M. tuberculosis* [47].

How did we face those problems and what delivery strategies did we try?

Encountering all these difficulties, we identified two new *M. ulcerans* protein vaccine candidates based on the main criterion of surface exposure for antibody-mediated protection. The 18 kDa shsp (MUL2232) was identified among the immunodominant antigens by generating a panel of monoclonal antibodies after vaccination with whole cells. It has a homolog in *M. leprae* but not in *M. bovis* and *M. tuberculosis* [23]. The second candidate, MUL3720, has no homologs in other prevalent pathogenic mycobacteria. It is a highly interesting candidate because it is cell surface associated and highly expressed in *M. ulcerans*. Furthermore, its structure suggests an involvement in host-pathogen interaction.

Together with these two new candidates, we also included the previously tested Ag85A (MUL4987) into our panel of protein vaccine candidates.

With the most straight-forward approach of expressing the protein vaccine candidates as recombinant proteins in *E. coli* and administering them with an adjuvant, we did not see any protection in the mouse model (Results Chapter 4). The adjuvant (TRL-4 agonist) was superior compared to Alum in induction of humoral responses, but as opposed to previous reports with other antigens, we did not see a clear polarization of the cellular response towards a T_H1 type [48,49]. Already from the results of this study, we concluded that antibody responses against the protein vaccine candidates are not sufficient for protection against BU. Further confirmation of this conclusion came from the results of two studies employing systems to deliver the vaccine candidate antigens that are known to induce both humoral as well as strong cellular responses (Results Chapters 5 and 6). For the VSV replicon approach, we observed for MUL2232 a slight but significant reduction in bacterial load of mice immunized with a replicon prime - recombinant protein boost regimen (Results Chapter 5). Enhanced induction of cellular immunity could supposedly be the reason for this difference from the recombinant protein-adjuvant formulation. The most complicated immunization strategy in terms of applicability, infection-treatment-vaccination with genetically modified *P. berghei* parasites, did induce a slight transient delay in foot pad swelling with parasites expressing MUL4987 in the mouse model of BU (Results Chapter 6).

Overall, the results from subunit vaccination were somewhat disappointing, when comparing the effect of protein subunit vaccination with the different delivery systems to the effect of vaccination with live attenuated *M. ulcerans* performed in other laboratories [39]. The comparison of our findings with the published data on vaccination with live attenuated *M. ulcerans* indicated that the presence of several immunogens is required to achieve substantial protection. On the other hand, protection mediated by live attenuated vaccination is also not complete [39]. This result may be explained by the concept of deceptive imprinting [50]. Already described for other pathogens, such as the human deficiency virus (HIV), it describes the dominance of minor, non-protective antigens over antigens that would be protective if properly seen and processed by the immune system. We would therefore deduce that live attenuated vaccination is not necessarily the best way forward, and that a combination of antigens in a multi-component subunit vaccination is more promising. From the delivery systems we have evaluated, the VSV replicon holds especially great potential for easily combining several antigens to a multivalent subunit vaccine (Results Chapter 5). Based on our first results, we would most likely combine MUL2232 and MUL4987 in one replicon or alternatively co-administer two replicons simultaneously. Further, more candidate antigens should be tested.

What is the nature of a protective immune response against *M. ulcerans*?

As a partner in the EU-funded collaborative project BuruliVac, we not only aimed at evaluating the potential for developing a protein subunit-vaccine against *M. ulcerans,* but also at gaining insight into the nature of protective immune responses against *M. ulcerans* and correlates of protection. The initial idea was to test a defined set of protein vaccine candidates with different delivery systems in a mouse model of BU. For successful formulations, an in-depth analysis of the vaccination-induced immune response was planned. Because we did not observe substantial protection for any of the formulations, this last objective remained unrealized. As already pointed out earlier, we concluded that a profound antibody response against the chosen protein vaccine candidates is not sufficient for protection. Inferred from early histopathological findings, *M. ulcerans* was considered for a long time to be a purely extracellular pathogen [51,52]. Therefore, it was rational to consider that antibodies could be the major effector molecules in fighting the disease. Opsonisation of the mycobacteria could enhance phagocytosis, increase intra-macrophage killing or improve antigen presentation and the induction of protective T-cell responses [53]. In contrast to *M. ulcerans*, the closely related *M. tuberculosis* and *M. leprae* are both mainly intracellular pathogens for which T-cell responses with a T_H1 polarization are regarded as crucial for protection [54]. Yet, more recently, *in vitro* and experimental animal infection model studies indicated that an initial intracellular proliferation phase of *M. ulcerans* exists [55,56] and may have relevance for disease progression and the nature of protective immune responses [52]. Healing of BU in human patients is accompanied by granuloma formation [51,57], as well as the change of the Burulin skin test from negative to positive [22]. Analyses of the cytokine profiles have demonstrated the induction of a T_H2 response in patients, which is switched to a predominantly T_H1 profile during healing [58]. However some of the results in this field of research on BU are contradictory and conclusions should be drawn with caution [26,59–61].

Mycolactone has been reported to impair the responsiveness of T-cells to stimulation [62] and to inhibit tumor necrosis factors (TNF) and IL-10 secretion of human monocytes [63]. Furthermore the toxin apparently supresses dendritic cell functions [64]. Studies also imply that mycolactone diffuses into peripheral blood and to lymphoid organs and induces systemic immunosuppression [65,66]. Most of the results concerning effector functions of mycolactone are currently under debate. Strikingly, only a minority of mice developed antibodies against *M. ulcerans* proteins in all the different experiments we have performed so far (Results Chapter 4).

While it is not clear to what extent the toxin plays a role in inhibition of the systemic immune system, its local effects are devastating. In the end, the induction of anti-mycolactone antibodies by vaccination might therefore be the key for the development of a protective immune response against BU.

How to improve the animal models for BU?

Natural as well as experimental infection with *M. ulcerans* is possible in a range of animal species [67–75]. The most frequently used model for BU is the mouse foot pad infection in BALB/c or C57BL/6 mice [4]. There is some notion that the two different inbred mouse strains could have differential susceptibility to the disease, but formal investigations are missing. The *M. ulcerans* strain used for infection of the mice is incredibly important, because strains of different geographical origin induce different immune responses and pathology in mice [76]. Most likely this is in part due to the different type of mycolactone that is produced by these strains. Many studies on BU in mice employ a Malaysian *M. ulcerans* strain, which produces mycolactone A/B (strain 1615) and was isolated in 1964 [77]. To work with a strain that is as close as possible to the strains circulating in the countries with highest BU prevalence (for which a vaccine would be of greatest benefit) we used a low passage Cameroonian *M. ulcerans* strain that was isolated in 2010 (S1013) (Results Chapters 2, 4 - 6). Dosing of the inoculum remains a major problem. Although several research groups claim that counting of the AFB according to Shepard and McRae [78] is feasible, this approach was never successful in our hands. *M. ulcerans* cells have a strong tendency to clump in aqueous solutions, thus making dilutions for infection inaccurate. By applying as few steps in handling as possible and adjusting the infection dose to the wet weight of the bacteria, we achieved a quite reliable dosing scheme for mouse infections (Results Chapter 4 - 6). Read-outs for the progression of the infection remain difficult. An immediate approach is the measurement of foot pad swelling, although oedema does not always correlate with bacterial load (Results Chapter 6). Classical colony forming unit (CFU) plating remains an important tool for enumeration of *M. ulcerans* from infected foot pads. Unfortunately the slow growth rate of *M. ulcerans* makes this approach very time consuming, prone to contamination and again, clump formation is a problem. As an alternative, we have adapted an already published qPCR method [79,80] for use on lysed mouse foot pads (Results Chapter 4 - 6). It is not possible to distinguish dead from live bacteria with this method, but a comparison to the infection dose as well as between differently treated mice is possible and considerably faster than CFU plating. Because every time point for determination of the AFB load requires the entire foot pad, it is necessary to euthanize parts of the experimental groups if assessment during the course of the experiment is desired. The use of luminescent *M. ulcerans* would be an alternative to avoid this problem [81,82]. To successfully apply this method, one would have to ensure that the genetically altered luminescent *M. ulcerans* are as virulent as wild-type bacteria.

One of the major drawbacks of the mouse model of BU is the lack of similarity between mouse foot pads and human skin. Mouse feet are a small entire limb with almost no fat tissue but contain bone, whereas human skin is thicker, with more subcutaneous tissue that

contains fat in substantial amounts. To compare pathology and especially histopathology with human BU, the mouse is not ideal. We therefore developed an alternative model for studying certain aspects of BU - the pig (Results Chapter 7). Pig skin and human skin are almost not distinguishable microscopically [83]. Furthermore, the pig immune system resembles the human one more than the mouse immune system does [84,85]. As shown in Results Chapter 7, the pig is an ideal alternative model to study early infection phases of BU. Drawbacks of the pig model are the costs as well as the size of the animal, which requires special facilities and trained personnel. On the other hand, the size of the animals allows for replicates on the same individual, therefore decreasing the number of pigs used per experiment. It is possible to use the pig as a model for testing advanced vaccine candidates as well as therapeutic interventions. Both animal models have the major drawback that in Switzerland, the experiments have to be performed under BLS3 conditions. This fact complicates housing and handling of the animals, sample processing and increases the costs tremendously.

Conclusions

During and after a survey in 2010 in the BU-endemic Mapé basin of Cameroon, clinically diagnosed patients were laboratory confirmed at the Swiss TPH in Basel. By establishing an optimal procedure for culture initiation from patient swab samples, even after long time spans between sampling and processing, we facilitated the build-up of a *M. ulcerans* strain database at the Swiss TPH. The isolated strains can be used in the future for molecular typing, phylogenetic analyses and monitoring of drug resistance of the locally circulating strains. A first such study combined whole genome sequencing analysis with detailed information on the patient locations and thus revealed the presence of two phylogenetic clonal complexes of *M. ulcerans* in Cameroon. We did not gain deeper insight into transmission patterns of the disease, which is most probably due to the limited genomic diversity observed between the *M. ulcerans* strains.

As long as it is not clearly known how BU is contracted, interruption of transmission is not an option. A vaccine against *M. ulcerans* would therefore be the most effective measure for prevention of BU and could also be used therapeutically.

- Within the framework of this thesis, we profiled two new protein vaccine candidates of *M. ulcerans*, MUL2232 and MUL3720, selected based on their surface location. Additionally, one more antigen previously associated with partial protection (MUL4987) was included.
- We successfully delivered the vaccine candidate antigens with three different delivery systems: i) as recombinant proteins together with an adjuvant, ii) as VSV replicons and iii) with genetically modified *Plasmodium berghei* parasites in an infection - treatment - vaccination approach. All three delivery systems induced potent antibody responses and were evaluated for their protective potential in a BU mouse model.
- No full protection from BU was observed, but slight partial protection was achieved by immunization with a replicon - prime / recombinant protein boost regimen with a VSV replicon encoding MUL2232. Furthermore, a transient delay of foot pad swelling was observed in mice receiving infection – treatment – vaccination with *P. berghei* expressing MUL4987. This indicates that antibody responses against the chosen protein vaccine candidates are not sufficient to confer full protection. The combination of several antigens to a multivalent vaccine would be an option for potentially improving the protective effect. Due to its ability to accept large amounts of foreign stretches of RNA, the VSV replicon system offers great potential for developing such multivalent vaccines. Furthermore, a combination of different vaccination approaches

could also prove successful. In the end, the inclusion of a component inducing anti-mycolactone antibodies might be crucial to design a protective vaccine.

The model that is currently most used, the mouse foot pad model, has the advantage that large numbers of mice can easily be housed and handled. The pig model, which we have established within the framework of this thesis, has not only higher costs but also needs special housing, which is not easily available. On the other hand, the pathology of BU is reflected substantially better in the pig model. For studying host-pathogen interaction in early BU lesions as well as for evaluation of advanced vaccination and therapeutic approaches, the pig therefore represents an ideal BU animal model.

Overall, our contributions as well as the results from other partners within the EU-funded collaborative BuruliVac project demonstrated that a vaccine against BU is not easily accomplished. Future investments into vaccine development against BU should concentrate on multivalent vaccine approaches as well as early combination of different vaccination strategies. Vaccines for therapeutic use against BU could also play a major role in fighting *M. ulcerans* caused suffering.

References

1. WHO | The 17 neglected tropical diseases (n.d.). WHO. Available: http://www.who.int/neglected_diseases/diseases/en/. Accessed 27 September 2014.
2. CDC - Global Health - Neglected Tropical Diseases (n.d.). Available: http://www.cdc.gov/globalhealth/ntd/. Accessed 27 September 2014.
3. WHO | The Yamoussoukro Declaration on Buruli ulcer (n.d.). WHO. Available: http://www.who.int/buruli/yamoussoukro_declaration/en/. Accessed 2 September 2014.
4. Converse PJ, Nuermberger EL, Almeida DV, Grosset JH (2011) Treating *Mycobacterium ulcerans* disease (Buruli ulcer): from surgery to antibiotics, is the pill mightier than the knife? Future Microbiol 6: 1185–1198. doi:10.2217/fmb.11.101.
5. Trubiano JA, Lavender CJ, Fyfe JAM, Bittmann S, Johnson PDR (2013) The incubation period of Buruli ulcer (*Mycobacterium ulcerans* infection). PLoS Negl Trop Dis 7: e2463. doi:10.1371/journal.pntd.0002463.
6. Marion E, Landier J, Boisier P, Marsollier L, Fontanet A, et al. (2011) Geographic expansion of Buruli ulcer disease, Cameroon. Emerg Infect Dis 17: 551–553. doi:10.3201/eid1703.091859.
7. Ravisse P (1977) [Skin ulcer caused by *Mycobacterium ulcerans* in Cameroon. I. Clinical, epidemiological and histological study]. Bull Société Pathol Exot Ses Fil 70: 109–124.
8. Boisvert H (1977) [Skin ulcer caused by *Mycobacterium ulcerans* in Cameroon. II. Bacteriological study]. Bull Société Pathol Exot Ses Fil 70: 125–131.
9. Noeske J, Kuaban C, Rondini S, Sorlin P, Ciaffi L, et al. (2004) Buruli ulcer disease in Cameroon rediscovered. Am J Trop Med Hyg 70: 520–526.
10. Bratschi MW, Njih Tabah E, Bolz M, Stucki D, Borrell S, et al. (2012) A case of cutaneous tuberculosis in a Buruli ulcer-endemic area. PLoS Negl Trop Dis 6: e1751. doi:10.1371/journal.pntd.0001751.
11. WHO | Laboratory diagnosis of buruli ulcer (n.d.). WHO. Available: http://www.who.int/buruli/laboratory_diagnosis/en/. Accessed 2 June 2014.
12. Mensah-Quainoo E, Yeboah-Manu D, Asebi C, Patafuor F, Ofori-Adjei D, et al. (2008) Diagnosis of *Mycobacterium ulcerans* infection (Buruli ulcer) at a treatment centre in Ghana: a retrospective analysis of laboratory results of clinically diagnosed cases. Trop Med Int Health TM IH 13: 191–198. doi:10.1111/j.1365-3156.2007.01990.x.
13. Yeboah-Manu D, Danso E, Ampah K, Asante-Poku A, Nakobu Z, et al. (2011) Isolation of *Mycobacterium ulcerans* from swab and fine-needle-aspiration specimens. J Clin Microbiol 49: 1997–1999. doi:10.1128/JCM.02279-10.
14. Bratschi MW, Ruf M-T, Andreoli A, Minyem JC, Kerber S, et al. (2014) *Mycobacterium ulcerans* persistence at a village water source of Buruli ulcer patients. PLoS Negl Trop Dis 8: e2756. doi:10.1371/journal.pntd.0002756.
15. Grietens KP, Boock AU, Peeters H, Hausmann-Muela S, Toomer E, et al. (2008) "It is me who endures but my family that suffers": social isolation as a consequence of the household cost burden of Buruli ulcer free of charge hospital treatment. PLoS Negl Trop Dis 2: e321. doi:10.1371/journal.pntd.0000321.
16. Phillips RO, Sarfo FS, Abass MK, Abotsi J, Wilson T, et al. (2014) Clinical and bacteriological efficacy of rifampin-streptomycin combination for two weeks followed by rifampin and clarithromycin for six weeks for treatment of *Mycobacterium ulcerans* disease. Antimicrob Agents Chemother 58: 1161–1166. doi:10.1128/AAC.02165-13.
17. Chauty A, Ardant M-F, Marsollier L, Pluschke G, Landier J, et al. (2011) Oral treatment for *Mycobacterium ulcerans* infection: results from a pilot study in Benin. Clin Infect Dis Off Publ Infect Dis Soc Am 52: 94–96. doi:10.1093/cid/ciq072.

18. Gordon CL, Buntine JA, Hayman JA, Lavender CJ, Fyfe JAM, et al. (2010) All-oral antibiotic treatment for buruli ulcer: a report of four patients. PLoS Negl Trop Dis 4: e770. doi:10.1371/journal.pntd.0000770.
19. O'Brien DP, McDonald A, Callan P, Robson M, Friedman ND, et al. (2012) Successful outcomes with oral fluoroquinolones combined with rifampicin in the treatment of *Mycobacterium ulcerans*: an observational cohort study. PLoS Negl Trop Dis 6: e1473. doi:10.1371/journal.pntd.0001473.
20. Murphy KM (2012) Janeway's Immunobiology, 8th Edition. 8 edition. Garland Science. 888 p.
21. Finco O, Rappuoli R (2014) Designing vaccines for the twenty-first century society. Front Immunol 5: 12. doi:10.3389/fimmu.2014.00012.
22. Dobos KM, Spotts EA, Marston BJ, Horsburgh CR, King CH (2000) Serologic response to culture filtrate antigens of *Mycobacterium ulcerans* during Buruli ulcer disease. Emerg Infect Dis 6: 158–164. doi:10.3201/eid0602.000208.
23. Diaz D, Döbeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, et al. (2006) Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. Clin Vaccine Immunol CVI 13: 1314–1321. doi:10.1128/CVI.00254-06.
24. Yeboah-Manu D, Röltgen K, Opare W, Asan-Ampah K, Quenin-Fosu K, et al. (2012) Sero-epidemiology as a tool to screen populations for exposure to *Mycobacterium ulcerans*. PLoS Negl Trop Dis 6: e1460. doi:10.1371/journal.pntd.0001460.
25. Röltgen K, Bratschi MW, Ross A, Aboagye SY, Ampah KA, et al. (2014) Late onset of the serological response against the 18 kDa small heat shock protein of *Mycobacterium ulcerans* in children. PLoS Negl Trop Dis 8: e2904. doi:10.1371/journal.pntd.0002904.
26. Gooding TM, Johnson PDR, Smith M, Kemp AS, Robins-Browne RM (2002) Cytokine profiles of patients infected with *Mycobacterium ulcerans* and unaffected household contacts. Infect Immun 70: 5562–5567.
27. Revill WDL, Morrow RH, Pike MC, Ateng J (1973) A CONTROLLED TRIAL OF THE TREATMENT OF *MYCOBACTERIUM ULCERANS* INFECTION WITH CLOFAZIMINE. The Lancet 302: 873–877. doi:10.1016/S0140-6736(73)92005-9.
28. Huygen K, Adjei O, Affolabi D, Bretzel G, Demangel C, et al. (2009) Buruli ulcer disease: prospects for a vaccine. Med Microbiol Immunol (Berl) 198: 69–77. doi:10.1007/s00430-009-0109-6.
29. Stienstra Y, van der Werf TS, Oosterom E, Nolte IM, van der Graaf WTA, et al. (2006) Susceptibility to Buruli ulcer is associated with the SLC11A1 (NRAMP1) D543N polymorphism. Genes Immun 7: 185–189. doi:10.1038/sj.gene.6364281.
30. Ruf M-T, Chauty A, Adeye A, Ardant M-F, Koussemou H, et al. (2011) Secondary Buruli ulcer skin lesions emerging several months after completion of chemotherapy: paradoxical reaction or evidence for immune protection? PLoS Negl Trop Dis 5: e1252. doi:10.1371/journal.pntd.0001252.
31. BCG vaccination against *mycobacterium ulcerans* infection (Buruli ulcer). First results of a trial in Uganda (1969). Lancet 1: 111–115.
32. Smith PG, Revill WD, Lukwago E, Rykushin YP (1976) The protective effect of BCG against *Mycobacterium ulcerans* disease: a controlled trial in an endemic area of Uganda. Trans R Soc Trop Med Hyg 70: 449–457.
33. Portaels F, Aguiar J, Debacker M, Guédénon A, Steunou C, et al. (2004) *Mycobacterium bovis* BCG vaccination as prophylaxis against *Mycobacterium ulcerans* osteomyelitis in Buruli ulcer disease. Infect Immun 72: 62–65.
34. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, et al. (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283: 854–857.

35. Roupie V, Pidot SJ, Einarsdottir T, Van Den Poel C, Jurion F, et al. (2014) Analysis of the vaccine potential of plasmid DNA encoding nine mycolactone polyketide synthase domains in *Mycobacterium ulcerans* infected mice. PLoS Negl Trop Dis 8: e2604. doi:10.1371/journal.pntd.0002604.

36. Einarsdottir T, Huygen K (2011) Buruli ulcer. Hum Vaccin 7: 1198–1203. doi:10.4161/hv.7.11.17751.

37. Tanghe A, Adnet P-Y, Gartner T, Huygen K (2007) A booster vaccination with *Mycobacterium bovis* BCG does not increase the protective effect of the vaccine against experimental *Mycobacterium ulcerans* infection in mice. Infect Immun 75: 2642–2644. doi:10.1128/IAI.01622-06.

38. Stinear TP, Mve-Obiang A, Small PLC, Frigui W, Pryor MJ, et al. (2004) Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. Proc Natl Acad Sci U S A 101: 1345–1349. doi:10.1073/pnas.0305877101.

39. Fraga AG, Martins TG, Torrado E, Huygen K, Portaels F, et al. (2012) Cellular immunity confers transient protection in experimental Buruli ulcer following BCG or mycolactone-negative *Mycobacterium ulcerans* vaccination. PloS One 7: e33406. doi:10.1371/journal.pone.0033406.

40. Rappuoli R, Bagnoli F, editors (2011) Vaccine Design: Innovative Approaches and Novel Strategies. 1 edition. Norfolk, UK: Caister Academic Press. 394 p.

41. Coutanceau E, Legras P, Marsollier L, Reysset G, Cole ST, et al. (2006) Immunogenicity of *Mycobacterium ulcerans* Hsp65 and protective efficacy of a *Mycobacterium leprae* Hsp65-based DNA vaccine against Buruli ulcer. Microbes Infect Inst Pasteur 8: 2075–2081. doi:10.1016/j.micinf.2006.03.009.

42. Tanghe A, Dangy J-P, Pluschke G, Huygen K (2008) Improved protective efficacy of a species-specific DNA vaccine encoding mycolyl-transferase Ag85A from *Mycobacterium ulcerans* by homologous protein boosting. PLoS Negl Trop Dis 2: e199. doi:10.1371/journal.pntd.0000199.

43. Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, et al. (2007) Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. Genome Res 17: 192–200. doi:10.1101/gr.5942807.

44. Masignani V, Rappuoli R, Pizza M (2002) Reverse vaccinology: a genome-based approach for vaccine development. Expert Opin Biol Ther 2: 895–905. doi:10.1517/14712598.2.8.895.

45. Serruto D, Serino L, Masignani V, Pizza M (2009) Genome-based approaches to develop vaccines against bacterial pathogens. Vaccine 27: 3245–3250. doi:10.1016/j.vaccine.2009.01.072.

46. Doig KD, Holt KE, Fyfe JAM, Lavender CJ, Eddyani M, et al. (2012) On the origin of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. BMC Genomics 13: 258. doi:10.1186/1471-2164-13-258.

47. Huber CA, Ruf M-T, Pluschke G, Käser M (2008) Independent loss of immunogenic proteins in *Mycobacterium ulcerans* suggests immune evasion. Clin Vaccine Immunol CVI 15: 598–606. doi:10.1128/CVI.00472-07.

48. Orr MT, Fox CB, Baldwin SL, Sivananthan SJ, Lucas E, et al. (2013) Adjuvant formulation structure and composition are critical for the development of an effective vaccine against tuberculosis. J Control Release Off J Control Release Soc 172: 190–200. doi:10.1016/j.jconrel.2013.07.030.

49. Baldwin SL, Shaverdian N, Goto Y, Duthie MS, Raman VS, et al. (2009) Enhanced humoral and Type 1 cellular immune responses with Fluzone adjuvanted with a synthetic TLR4 agonist formulated in an emulsion. Vaccine 27: 5956–5963. doi:10.1016/j.vaccine.2009.07.081.

50. Tobin GJ, Trujillo JD, Bushnell RV, Lin G, Chaudhuri AR, et al. (2008) Deceptive imprinting and immune refocusing in vaccine design. Vaccine 26: 6189–6199. doi:10.1016/j.vaccine.2008.09.080.

51. Hayman J (1993) Out of Africa: observations on the histopathology of *Mycobacterium ulcerans* infection. J Clin Pathol 46: 5–9.

52. Silva MT, Portaels F, Pedrosa J (2009) Pathogenetic mechanisms of the intracellular parasite *Mycobacterium ulcerans* leading to Buruli ulcer. Lancet Infect Dis 9: 699–710. doi:10.1016/S1473-3099(09)70234-8.

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

54. Dorhoi A, Reece ST, Kaufmann SHE (2011) For better or for worse: the immune response against *Mycobacterium tuberculosis* balances pathology and protection. Immunol Rev 240: 235–251. doi:10.1111/j.1600-065X.2010.00994.x.

55. Torrado E, Fraga AG, Castro AG, Stragier P, Meyers WM, et al. (2007) Evidence for an intramacrophage growth phase of *Mycobacterium ulcerans*. Infect Immun 75: 977–987. doi:10.1128/IAI.00889-06.

56. Bolz M, Ruggli N, Ruf M-T, Ricklin ME, Zimmer G, et al. (2014) Experimental infection of the pig with *Mycobacterium ulcerans*: a novel model for studying the pathogenesis of Buruli ulcer disease. PLoS Negl Trop Dis 8: e2968. doi:10.1371/journal.pntd.0002968.

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

58. Yeboah-Manu D, Peduzzi E, Mensah-Quainoo E, Asante-Poku A, Ofori-Adjei D, et al. (2006) Systemic suppression of interferon-gamma responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen *Mycobacterium ulcerans*. J Leukoc Biol 79: 1150–1156. doi:10.1189/jlb.1005581.

59. Gooding TM, Johnson PD, Campbell DE, Hayman JA, Hartland EL, et al. (2001) Immune response to infection with *Mycobacterium ulcerans*. Infect Immun 69: 1704–1707. doi:10.1128/IAI.69.3.1704-1707.2001.

60. Prévot G, Bourreau E, Pascalis H, Pradinaud R, Tanghe A, et al. (2004) Differential production of systemic and intralesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. Infect Immun 72: 958–965.

61. Westenbrink BD, Stienstra Y, Huitema MG, Thompson WA, Klutse EO, et al. (2005) Cytokine responses to stimulation of whole blood from patients with Buruli ulcer disease in Ghana. Clin Diagn Lab Immunol 12: 125–129. doi:10.1128/CDLI.12.1.125-129.2005.

62. Boulkroun S, Guenin-Macé L, Thoulouze M-I, Monot M, Merckx A, et al. (2010) Mycolactone suppresses T cell responsiveness by altering both early signaling and posttranslational events. J Immunol Baltim Md 1950 184: 1436–1444. doi:10.4049/jimmunol.0902854.

63. Pahlevan AA, Wright DJ, Andrews C, George KM, Small PL, et al. (1999) The inhibitory action of *Mycobacterium ulcerans* soluble factor on monocyte/T cell cytokine production and NF-kappa B function. J Immunol Baltim Md 1950 163: 3928–3935.

64. Coutanceau E, Decalf J, Martino A, Babon A, Winter N, et al. (2007) Selective suppression of dendritic cell functions by *Mycobacterium ulcerans* toxin mycolactone. J Exp Med 204: 1395–1403. doi:10.1084/jem.20070234.

65. Hong H, Coutanceau E, Leclerc M, Caleechurn L, Leadlay PF, et al. (2008) Mycolactone diffuses from *Mycobacterium ulcerans*-infected tissues and targets mononuclear cells in peripheral blood and lymphoid organs. PLoS Negl Trop Dis 2: e325. doi:10.1371/journal.pntd.0000325.

66. Sarfo FS, Le Chevalier F, Aka N, Phillips RO, Amoako Y, et al. (2011) Mycolactone diffuses into the peripheral blood of Buruli ulcer patients--implications for diagnosis and disease monitoring. PLoS Negl Trop Dis 5: e1237. doi:10.1371/journal.pntd.0001237.

67. Fyfe JAM, Lavender CJ, Handasyde KA, Legione AR, O'Brien CR, et al. (2010) A major role for mammals in the ecology of *Mycobacterium ulcerans*. PLoS Negl Trop Dis 4: e791. doi:10.1371/journal.pntd.0000791.

68. Walsh DS, Dela Cruz EC, Abalos RM, Tan EV, Walsh GP, et al. (2007) Clinical and histologic features of skin lesions in a cynomolgus monkey experimentally infected with *mycobacterium ulcerans* (Buruli ulcer) by intradermal inoculation. Am J Trop Med Hyg 76: 132–134.

69. Mitchell PJ, McOrist S, Bilney R (1987) Epidemiology of *Mycobacterium ulcerans* infection in koalas (*Phascolarctos cinereus*) on Raymond Island, southeastern Australia. J Wildl Dis 23: 386–390.

70. Marcus LC, Stottmeier KD, Morrow RH (1976) Experimental alimentary infection of anole lizards (*Anolis carolinensis*) with *Mycobacterium ulcerans*. Am J Trop Med Hyg 25: 630–632.

71. Elsner L, Wayne J, O'Brien CR, McCowan C, Malik R, et al. (2008) Localised *Mycobacterium ulcerans* infection in a cat in Australia. J Feline Med Surg 10: 407–412. doi:10.1016/j.jfms.2008.03.003.

72. Singh NB, Srivastava A, Verma VK, Kumar A, Gupta SK (1984) *Mastomys natalensis*: a new animal model for *Mycobacterium ulcerans* research. Indian J Exp Biol 22: 393–394.

73. Sakaguchi K, Iima H, Hirayama K, Okamoto M, Matsuda K, et al. (2011) *Mycobacterium ulcerans* infection in an Indian flap-shelled turtle (Lissemys punctata punctata). J Vet Med Sci Jpn Soc Vet Sci 73: 1217–1220.

74. O'Brien C, Kuseff G, McMillan E, McCowan C, Lavender C, et al. (2013) *Mycobacterium ulcerans* infection in two alpacas. Aust Vet J 91: 296–300. doi:10.1111/avj.12071.

75. Van Zyl A, Daniel J, Wayne J, McCowan C, Malik R, et al. (2010) *Mycobacterium ulcerans* infections in two horses in south-eastern Australia. Aust Vet J 88: 101–106. doi:10.1111/j.1751-0813.2009.00544.x.

76. Ortiz RH, Leon DA, Estevez HO, Martin A, Herrera JL, et al. (2009) Differences in virulence and immune response induced in a murine model by isolates of *Mycobacterium ulcerans* from different geographic areas. Clin Exp Immunol 157: 271–281. doi:10.1111/j.1365-2249.2009.03941.x.

77. Torrado E, Adusumilli S, Fraga AG, Small PLC, Castro AG, et al. (2007) Mycolactone-mediated inhibition of tumor necrosis factor production by macrophages infected with *Mycobacterium ulcerans* has implications for the control of infection. Infect Immun 75: 3979–3988. doi:10.1128/IAI.00290-07.

78. Shepard CC, McRae DH (1968) A method for counting acid-fast bacteria. Int J Lepr Mycobact Dis Off Organ Int Lepr Assoc 36: 78–82.

79. Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, et al. (2007) Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. Appl Environ Microbiol 73: 4733–4740. doi:10.1128/AEM.02971-06.

80. Lavender CJ, Fyfe JAM (2013) Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples. Methods Mol Biol Clifton NJ 943: 201–216. doi:10.1007/978-1-60327-353-4_13.

81. Zhang T, Bishai WR, Grosset JH, Nuermberger EL (2010) Rapid assessment of antibacterial activity against *Mycobacterium ulcerans* by using recombinant luminescent strains. Antimicrob Agents Chemother 54: 2806–2813. doi:10.1128/AAC.00400-10.

82. Zhang T, Li S-Y, Converse PJ, Grosset JH, Nuermberger EL (2013) Rapid, serial, non-invasive assessment of drug efficacy in mice with autoluminescent *Mycobacterium ulcerans* infection. PLoS Negl Trop Dis 7: e2598. doi:10.1371/journal.pntd.0002598.

83. Liu Y, Chen J, Shang H, Liu C, Wang Y, et al. (2010) Light microscopic, electron microscopic, and immunohistochemical comparison of Bama minipig (*Sus scrofa domestica*) and human skin. Comp Med 60: 142–148.

84. Dawson H (2011) A Comparative Assessment of the Pig, Mouse and Human Genomes: Structural and Functional Analysis of Genes Involved in Immunity and Inflammation. The Minipig in Biomedical Research. CRC Press. pp. 323–342. Available: http://www.crcnetbase.com/doi/abs/10.1201/b11356-28. Accessed 4 February 2014.

85. Fairbairn L, Kapetanovic R, Sester DP, Hume DA (2011) The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. J Leukoc Biol 89: 855–871. doi:10.1189/jlb.1110607.