1   **Antimicrobial activity of Mycobacteriophage D29 Lysin B during *Mycobacterium ulcerans***

2   **infection**

3

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14   Short Title: Lysin treatment against experimental Buruli ulcer

15

16 **ABSTRACT**

17   Buruli Ulcer (BU) is a cutaneous disease caused by *Mycobacterium ulcerans*. The

18 pathogenesis of this disease is closely related to the secretion of the toxin mycolactone that

19 induces extensive destruction of the skin and soft tissues. Although the World Health Organization

20 recommends a combination of rifampicin and streptomycin for the treatment of BU, clinical

21 management of advanced stages often requires extensive surgical resection of the infected

22 tissue. Therefore, it is important to develop alternative strategies for the treatment of BU.

23   Endolysins (lysins) are phage encoded enzymes that degrade peptidoglycan of bacterial

24 cell walls. Over the past years, lysins have been emerging as alternative antimicrobial agents

25 against Gram-positive bacteria. Amongst Gram-positive bacteria, mycobacteria have an unusual

26 outer membrane that is covalently attached to the mycolylarabinogalactan-peptidoglycan

27 complex. To overcome this additional barrier to phage-mediated lysis, some mycobacteriophages

28 encode a lipolytic enzyme, Lysin B (Lys B).

29   In this study, we demonstrated for the first time that recombinant Lys B displays lytic

30 activity against *M. ulcerans* isolates. Moreover, using a mouse model of *M. ulcerans* footpad

31 infection, we show that subcutaneous treatment with Lys B leads to a reduction in bacterial

32 burdens, associated with IFN-γ and TNF production in the draining lymph node. These findings

33 highlight the potential use of lysins as a novel therapeutic approach against this neglected tropical

34 disease.

AUTHOR SUMMARY

Buruli Ulcer (BU) is a necrotizing skin disease caused by *Mycobacterium ulcerans*. Standard treatment for BU lesions consists of a combination of rifampicin and streptomycin for 8 weeks. However, clinical management of advanced stages of the disease often requires extensive surgical resection of the infected tissue. Therefore, it is important to develop alternative strategies for the treatment of BU. In that sense, we tested the efficacy of Lysin B (Lys B), a phage encoded lipolytic enzyme that degrades the mycolylarabinogalactan-peptidoglycan complex present in the mycobacterial cell wall. In this study, we show that Lys B not only displays lytic activity against *M. ulcerans* isolates *in vitro*, but also leads to a reduction in bacterial burdens in *M. ulcerans*-infected mouse footpads. These findings highlight the potential use of lysins as a novel therapeutic approach against this neglected tropical disease.

INTRODUCTION

Buruli ulcer (BU) is a necrotic cutaneous disease caused by *Mycobacterium ulcerans* and represents the third most prevalent mycobacterial infection worldwide, after tuberculosis and leprosy [1-3].

BU's pathogenesis is closely related to the secretion of the heat-stable polyketide toxin mycolactone that presents cytotoxic and immunosuppressive properties [4-8]. Early presentations of active BU include a painless pre-ulcerative nodule, papule, plaque or edematous lesion, which can evolve into typical ulcers or, in the most extreme cases, may result in extensive skin destruction, multifocal lesions or bone involvement [1,3,7,9,10].

The antibiotic regimen recommended by the World Health Organization (WHO) (daily administration of rifampicin (RIF) and streptomycin (STR) for 8 weeks) was proven to be curative for small pre-ulcerative lesions, but not for advanced forms, often requiring extensive surgical intervention [11-13]. Additionally, antibiotherapy is associated with adverse side effects, especially ototoxic and nephrotoxic effects [11,14].

Our group has previously demonstrated that bacteriophage therapy has potential as an innovative and effective therapy against *M. ulcerans* infection [15]. Indeed, our results in the murine model show that treatment with mycobacteriophage D29 effectively decreases the proliferation of *M. ulcerans* in the subcutaneous tissue resulting in marked macroscopic improvement of skin lesions [15].

Endolysins (lysins) are phage encoded enzymes produced during the late phase of the bacteriophage infection cycle, so as to degrade the cell wall peptidoglycan of the bacterial host, enabling the release of viral progeny [16-18]. Over the past decade, the development, characterization and exogenous application of recombinant and purified bacteriophage lytic enzymes has been successfully evaluated in several animal models of human diseases, such as sepsis, endocarditis, pharyngitis, pneumonia, meningitis and mucosal and skin infection [19-27]. Moreover, the use of a commercial endolysin for the treatment of *Staphylococcus aureus* skin infections has already been approved [28].

Amongst Gram-positive bacteria, mycobacteria have an unusual outer membrane composed by mycolic acids esterified with arabinogalactan (AG) which is linked to peptidoglycan, forming the mycolylarabinogalactan-peptidoglycan (mAGP) complex, a potential barrier to phage-

4

76   mediated lysis [29]. Mycobacteriophage genome sequences show that, in addition to lysins that

77   degrade the peptidoglycan layer of bacterial cell walls [30-32], some mycobacteriophages also

78   encode a second lysin that targets the mAGP complex, known as Lysin B (Lys B) [33-35]. As

79   described by Payne *et al.* [33], mycobacteriophage D29 encodes Lys B, a mycolylarabinogalactan

80   esterase that cleaves the ester linkage joining the mycolic acid-rich outer membrane to AG,

81   releasing free mycolic acids [36]. Although few studies have shown that Lys B can also act

82   externally, suggesting its promising antimicrobial effect, there is no proven efficacy *in vivo* [37].

83   In the present study, following the *in vitro* evaluation of the lytic activity of recombinant

84   mycobacteriophage D29 Lys B against *M. ulcerans* isolates, the therapeutic effect of Lys B during

85   *M. ulcerans* infection was evaluated in the mouse footpad model of infection. The progression of

86   macroscopic/microscopic pathology and bacterial loads, as well as the cytokine profiles, were

87   evaluated in the footpad and the draining lymph node (DLN).

88 **MATERIALS AND METHODS**

89 **Expression and purification of Lys B**

90   The mycobacteriophage D29 Lys B gene (GenBank: accession number AF022214) was

91 amplified by PCR from of mycobacteriophage D29 DNA using the primer: 5´-

92 CCCTGGAACATATGAGCAAGCCC-3´ (nt 6606 to 7370). The amplification product was cloned

93 into the expression vector pET28a (Novagem) fused to an N-terminal 6xHis tag. The resulting

94 plasmid pET28a–Lys B was used to overexpress Lys B using *E. coli* BL21 (DE3) (Novagem) as

95 a host for expression. Expression cultures were grown to an optical density (OD) between 0.4

96 and 0.6, at 600 nm, in Luria-Bertani broth containing kanamycin (50 µg/mL). Protein expression

97 was induced with 1mM isopropyl-D-thiogalactopyranoside (IPTG) with shaking for 4 h at 37°C.

98 Bacterial cells were harvested by centrifugation (10000x g, 5 min, 4°C), resuspended in

99 phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8), sonicated on ice for

100 5x10 s pulses separated by 10 s rests and then centrifuged (10000x g, 5 min, 4°C). The

101 supernatant was applied to a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen) and

102 the protein was eluted under native conditions with 500mM imidazole in phosphate buffer

103 according to the manufacturer's instructions. The purity of the protein was analyzed by 12%

104 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie

105 blue staining. Protein-containing fractions were combined and filter sterilized (0.22 µm pore size)

106 for use in *in vitro* and *in vivo* studies. Protein concentration was determined using NanoDrop ND-

107 1000 (NanoDrop Technologies). The lipolytic activity of the purified Lys B was confirmed by

108 spotted in Middlebrook 7H9 plates containing substrates as follows: 1% (v/v) Tween 80 or 1%

109 (v/v) Tween 20 with 1 mM $CaCl_2$ and plates were incubated at 37°C for at least 24 h. Enzymatic

110 activity was indicated by the formation of a white precipitate spot on Tween plates [34,38].

111

112 ***In vitro* Lys B activity against *M. ulcerans***

113   In order to evaluate the antimicrobial activity of purified Lys B against *M. ulcerans* strains,

114 a plate lysis assay was performed [39]. Representative isolates of *M. ulcerans* from endemic BU

115 areas were selected based on their genetic and phenotypic characteristics, including the type of

116 mycolactone produced and their virulence for mice (see Table 1) [8,40-44]. The strains were

117 obtained from the collection of the Institute of Tropical Medicine (ITM), Antwerp, Belgium.

6

118   Clinical isolates were grown on Middlebrook 7H9 broth medium at 32ºC to an OD_{600} of

119   1.0 and clumps were dispersed by passing the bacterial suspension several times through a 25-

120   gauge needle. The bacterial suspension (10^5 CFU/mL) was plated on Middlebrook 7H9 agar

121   medium. A stock solution of purified Lys B was serially diluted in phosphate buffer (final

122   concentration 10-0.1 µg) and spotted onto bacterial lawns that air dried for 30 min. Phosphate

123   buffer was spotted as a negative control. Plates were incubated at 32ºC for approximately 6-8

124   weeks. Antimicrobial activity of Lys B was indicated by a clear lysis zone within the lawn where

125   *M. ulcerans* growth was prevented.

126

127   *In vivo* **bioavailability and enzymatic activity of Lys B**

128   The bioavailability and lipolytic enzymatic activity of Lys B were evaluated *in vivo*. Mice sera

129   were collected by retro-orbital bleeding. Footpad and DLN suspensions were centrifuged for 10 min

130   at 5000 rpm, and supernatant was considered the total protein extract. Protein concentration was

131   determined using a NanoDrop ND-1000. The enzymatic activity of Lys B in samples was assessed

132   by a lipase assay, as described above. For the bioavailability evaluation of the protein in samples, a

133   western blot was performed. Briefly, samples were loaded and resolved by a 12% SDS-PAGE and

134   transferred to 0.2 µm Nitrocellulose membranes (Bio-Rad) with the semi-dry Trans-Blot Turbo system

135   (Bio-Rad). Membranes were blocked and subjected to immunoblotting with anti-mouse 6xHis

136   antibody peroxidase conjugate (Clontech Lab. Inc., Takara). The 6xHis-tagged Lys B was detected

137   with SuperSignal (Thermo Scientific #34095) in a Universal Hood II (Bio-Rad).

138

139   **Animals**

140   Eight-week-old female BALB/c mice were obtained from Charles River (Barcelona, Spain)

141   and were housed under biosafety level 3 conditions with food and water *ad libitum*.

142

143   **Footpad mouse model of *M. ulcerans* infection**

144   *M. ulcerans* 98-912 is a mycolactone D producing strain, isolated in China from an

145   ulcerative case and is highly virulent for mice, as previously described [8,42,43,45]. The isolate

146   was grown on Middlebrook 7H9 agar medium at 32ºC for approximately 6-8 weeks. For the

147   preparation of inoculum, *M. ulcerans* was recovered, diluted in phosphate-buffered saline (PBS)

7

148   and vortexed using glass beads. Mice were infected in the left hind footpad with 0.03 ml of *M.*

149   *ulcerans* suspension containing 5.5 $\log_{10}$ CFU.

150

151   **Treatment of *M. ulcerans*-infected mice with Lys B**

152   The treatment was initiated when footpads of mice were swollen to 2.7 mm and was

153   performed by two subcutaneous (s.c.) injections in the infected footpad with 50 µg of Lys B in

154   PBS at 10- and 13-days post-infection. Control-infected mice were injected with PBS without

155   protein. Two groups of uninfected animals were also injected with Lys B or vehicle PBS buffer

156   alone, as controls.

157

158   **Assessment of footpad swelling**

159   After infection, as an index of lesion development, footpad swelling of mice was monitored

160   throughout the experiment, using a caliper to measure the diameter of the frontal area of the

161   footpad. For ethical reasons, mice were sacrificed after the emergence of ulceration and no further

162   parameters were evaluated.

163

164   **Bacterial growth**

165   *M. ulcerans* growth was evaluated in footpad tissues. Briefly, footpad tissue specimens

166   were minced, resuspended in PBS (Sigma) and vortexed with glass beads to obtain homogenized

167   suspensions. Serial dilutions of the footpad were plated on Middlebrook 7H9 agar medium. *M.*

168   *ulcerans* numbers were counted after 6 to 8 weeks of incubation at 32ºC and expressed as colony

169   forming units (CFU).

170

171   **Detection of cytokines**

172   The levels of the cytokines tumor necrosis factor (TNF) and gamma interferon (IFN-γ) in

173   the supernatant of homogenized suspensions from DLN of mice were quantified by using a

174   Quantikine Murine ELISA kit (eBioscience Inc), according to the manufacturer's instructions.

175

176   **Detection of antibodies**

177   The levels of antibodies against Lys B in the sera of mice were quantified by ELISA. Mice

178   sera were collected by retro-orbital bleeding 3 days after the second s.c. Lys B injection. Briefly,

179   polystyrene microtitre plates (Nunc) were coated with Lys B (10 µg/ml) and incubated overnight

180   at 4ºC. The wells were blocked with 0.05% (v/v) Tween 20 in PBS for 1h at room temperature.

181   Serial dilutions of samples were plated and incubated for 2 h at room temperature. After washing,

182   bound antibodies were detected with anti-mouse IgG antibody peroxidase conjugate (Sigma) for

183   1 h at room temperature. The antibodies against Lys B were detected with TMB substrate solution

184   (eBioscience) at 450 nm. ELISA antibody titres were expressed as the reciprocal of the highest

185   dilution giving an absorbance above that of the control (no Lys B injection).

186

187   **Ethics Statement**

188   This study was approved by the Portuguese national authority for animal experimentation

189   *Direção Geral de Alimentação e Veterinária* (DGAV 8421 from 2018). Animals were kept and

190   handled in accordance with the Directive 2010/63/EU of the European Parliament and of the

191   Council, on the protection of animals used for scientific purposes (transposed to Portuguese law

192   – *Decreto-Lei* 2013/113, 7th of august).

193

194   **Statistical analysis**

195   Differences between the means of experimental groups were analyzed with the two-tailed

196   Student t test. Differences with a P value of ≤ 0.05 were considered significant.

197

198   **RESULTS**

199   **Lys B shows *in vitro* antimicrobial activity against *M. ulcerans* isolates**

200   Mycobacteriophage D29 Lys B was expressed and purified as a His$_6$-tagged protein and

201   its lipolytic activity was tested using Middlebrook 7H9 agar plates containing Tween 80 (Figure

202   1A) and Tween 20 (Figure 1B) as substrates [34]. Tween 80 and Tween 20 are esters of oleic

203   (C18) and lauric (C12) acids, respectively, and can be cleaved by lipolytic enzymes to produce

204   fatty acids and alcohol. The presence of Ca^{2+} in the medium causes the formation of an insoluble

205   fatty acid salt that presents itself as a white precipitate [34,39]. As observed in Figure 1A-B, Lys

206   B produced a spot of white precipitate, confirming the enzymatic lipolytic activity of purified

207   recombinant mycobacteriophage D29 Lys B. No activity was observed with the phosphate buffer

208   used as a negative control (data not shown).

209   Purified Lys B was tested for its antimicrobial activity against several *M. ulcerans* isolates

210   (Table 1) in a plate lysis assay. Our results show that Lys B has antimicrobial activity against *M.*

211   *ulcerans*. Indeed, all *M. ulcerans* isolates tested were susceptible to the action of 10 to 0.1 μg of

212   Lys B protein, causing a clear spot zone indicating cell lysis in *M. ulcerans* lawns. Moreover, only

213   two of the tested *M. ulcerans* isolates, 97-1116 and 94-1331, were not susceptible to the lowest

214   protein concentration used, 0.1 μg.

215

216   **Lys B is detected and has enzymatic activity *in vivo***

217   To determine the bioavailability and presence of enzymatically active Lys B *in vivo* after

218   s.c injection in the footpad, the protein was measured in footpads, DLN, and sera. In a western

219   blot analysis of footpad supernatant, Lys B was consistently detected, and the levels remained

220   stable during the first 4h after administration (Figure 2A). At 6 h after injection, Lys B was still

221   detected, although at lower levels (Figure 2A). In order to analyze if Lys B maintained its lipolytic

222   enzymatic activity *in vivo,* samples were spotted on Tween plates [34,39]. As shown in Figure 2B,

223   lipolytic activity was detected in footpad suspensions until 8h after injection. Regarding sera

224   samples, although Lys B was not detected by western blot analysis (data not shown), lipolytic

225   activity was observed (Figure 2B). No lipolytic activity or presence of Lys B was detected in the

226   DLN (data not shown).

227

10

Treatment with Lys B decreases the *M. ulcerans* bacterial load in the footpad

To investigate the efficacy of Lys B treatment for the control of *M. ulcerans in vivo*, we used the footpad mouse model of infection [15,42-44]. Mice were s.c. infected in footpads with 5.5 $\log_{10}$ CFU of *M. ulcerans* strain 98-912. After 10 days, when footpad swelling reached 2.7 mm (Figure 3A), and at 13 days post-infection, mice were treated subcutaneously in the footpad with 50 μg of Lys B in PBS buffer. As shown in Figure 3B, *M. ulcerans* proliferated in infected footpads of non-treated mice over the course of experimental infection (P<0.01), while a significant 1$\log_{10}$ reduction in CFU counts (P<0.01) was observed in Lys B treated-mice at day 16 post-infection. The administration of vehicle PBS buffer or Lys B alone in uninfected footpads did not induce significant swelling of the footpad (data not shown). Moreover, our results show (Table 2) detectable levels of IgG antibodies to Lys B in sera of both Lys B-treated infected and non-infected mice at day 16 post-infection. Collectively, these results show that the administration of Lys B to *M. ulcerans* infected tissue has a protective effect, significantly reducing local bacterial burdens despite the presence of Lys B-specific antibodies in circulation.

Treatment with Lys B induces increased levels of IFN-γ and TNF in the DLN

Previous studies from our laboratory showed that *M. ulcerans* disseminates to the DLN, where the differentiation/expansion of mycobacteria-specific specific T cells occurs, contributing for the control of *M. ulcerans* proliferation through the production of IFN-γ [43,45] and TNF [8]. To determine whether Lys B treatment impacts host immune response, we carried out a comparative analysis of cytokine kinetics in the DLN.

Treatment with Lys B resulted in a significant increase in the levels of IFN-γ in the DLN (P<0.01) at day 16 post-infection (six days after the beginning of the treatment), as compared with non-treated mice (Figure 4A). The protein levels of the pro-inflammatory cytokine TNF were low in the DLN of non-treated mice (Figure 4B). In contrast, in Lys B-treated mice, significant levels of TNF (P<0.01) were detectable at day 16 post-infection (day 6 post-treatment) (Figure 4B).

DISCUSSION

256

257   The RIF/STR regimen recommended by the WHO for the treatment of BU is often not

258   effective in advanced ulcerative stages of the disease, for which the adjunction of surgical

259   resection of infected skin followed by skin grafting is required [12,13]. Regarding the development

260   of new and innovative anti-microbial strategies, we have previously shown in the mouse footpad

261   model of *M. ulcerans* infection that a single s.c. injection of the lytic mycobacteriophage D29 can

262   effectively decrease the proliferation of *M. ulcerans* resulting in marked macroscopic improvement

263   of skin lesions [15]. Importantly, these results uncovered a second line of investigation. The

264   potential of purified recombinant bacteriophage lytic enzymes (lysins) has been regarded as an

265   alternative method to control bacterial pathogens, including *S. aureus* [23,24,39,46,47],

266   *Streptococcus pneumoniae* [19,20,22,25,26], group B streptococci [24], *Bacillus anthracis* [48].

267   Recently, a new engineered endolysin (Artilysin Art-175) has shown activity against gram-

268   negative pathogens, such as *Pseudomonas aeruginosa* [49]. In addition, endolysins have

269   advantageous characteristics that avoid most of the common resistance mechanisms against

270   antibiotics [48]. Although the ability of exogenously applied lysins as antibacterial agents is

271   typically limited to bacteria without an outer membrane or surface lipids and waxes (i.e. Gram-

272   positive bacteria), the mycobacteriophage Lys B has the potential to reduce cell viability due to

273   the mechanical weakening and increased permeability of the cell wall. Lys B is a

274   mycolylarabinogalactan esterase with activity in the mAGP, hydrolyzing the ester linkage that

275   joins the mycolic acid-rich outer membrane to arabinogalactan, releasing free mycolic acids [33].

276   Due to the importance of the mAGP-complex for the stability of the mycobacterial cell envelope

277   [50], we were prompted to test the therapeutic potential of Lys B in the context of *M. ulcerans*

278   infection.

279   Herein, we have demonstrated the potential of mycobacteriophage D29 Lys B therapy

280   against *M. ulcerans* infection in the mouse footpad model. Indeed, we show that treatment with

281   Lys B can effectively decrease the proliferation of the high virulent mycolactone-producing *M.*

282   *ulcerans* strain 98-912. Importantly, our *in vitro* results show a high susceptibility of several *M.*

283   *ulcerans* isolates to Lys B, indicating that its activity *in vivo* is not limited to *M. ulcerans* 98-912.

284   The therapeutic efficacy of Lys B treatment will depend on the presence of biologically active Lys

285   B *in vivo*. As previously described, a rapid decrease in lysin levels after administration can result

286   in the decrease of the amount of active lysin [22,51]. Indeed, in a pneumococcal meningitis mouse

287   model, the bacterial load in the cerebrospinal fluid increased as the concentration of Cpl-1 lysin

288   decreased over time [22]. Based on these observations, and in order to improve the Lys B

289   bioavailability in mice footpads, we performed two administrations and shown detectable levels

290   of Lys B protein with enzymatic activity in mouse footpads after s.c. injection.

291   It is known that the differentiation/proliferation of IFN-γ producing mycobacteria-specific

292   lymphocytes occurs in mouse DLN early after experimental *M. ulcerans* infection [43]. However,

293   this transient protective host response is not sufficient to inhibit the proliferation of virulent *M.*

294   *ulcerans* in mice, as increasing concentrations of mycolactone at the infection site impair the

295   effector activity of macrophages and induce cell and tissue destruction [8,42,45] In our study, Lys

296   B administration effectively decreased the bacterial load in the infected tissue, allowing the

297   development of a protective host immune response. Indeed, we observed that Lys B treatment in

298   infected mice results in a significant increase in IFN-γ levels in the DLN.

299   Regarding antibody production, it has been demonstrated in several studies using

300   different lysins and pathogens (*S. pneumoniae, S. aureus*, *Streptococcus pyogens* and *B.*

301   *anthracis*) [52] that antibodies against lysins are non-neutralizing and therefore lysins can be used

302   repeatedly to treat bacterial infections without adverse effects or loss of efficacy. In fact, when

303   naïve and lysin-immunized mice were challenged with *S. pneumonia* and afterwards treated with

304   Cpl-1 lysin, no differences were observed between the groups of mice regarding reduction of

305   bacterial numbers [51]. Indeed, in the present study the presence of IgG antibodies against Lys

306   B did not affect the control of viable bacteria in treated footpads, suggesting that produced

307   antibodies did not inhibit Lys B activity.

308   For therapeutic use, an antimicrobial agent should not affect mammalian cells and only

309   targeting the pathogen. Lysins target structures unique and highly conserved to bacterial cells

310   and as such should not present a potential toxic threat to humans and animals [53]. This has been

311   supported by several reports using lysins in preclinical studies in mouse models [26,51,54] and,

312   in agreement, in our study, since Lys B injection was not associated with detectable side effects

313   until the end of the experimental period.

314   To our knowledge, this is the first study on mycobacteriophage Lys B activity against *M.*

315   *ulcerans* infection. It should be pointed out that mice were treated at an advanced stage of *M.*

13

316   *ulcerans* infection, which is relevant for human infection since BU patients often seek medical

317   treatment in advanced stages of the disease.

318   Although the development of a therapeutic regimen using Lys B will involve the

319   determination of the proper doses and dosing schedules, our *in vivo* results are proof of concept

320   of their potential against BU disease.

321

322   **Acknowledgments**

326

327   **TABLES AND FIGURES**

328   **Table 1.** Antimicrobial activity of Lys B again*st M. ulcerans* isolates*.*

329

M. ulcerans strain	Origin	Geographical origin	Type of Mycolactone	Min. Lysin B (µg) *
98-912	Ulcer	China	D	> 0.1
97-1116	Plaque	Benin	A/B	> 0.5
1615	Ulcer	Malaysia	A/B	> 0.1
94-1331	nd	Papua New Guinea	A/B	> 0.5
94-1327	Ulcer	Australia	C	> 0.1
5114	Ulcer	Mexico	-	> 0.1
00-1441	Aquatic insect	Benin	A/B	> 0.1
94-1324	Ulcer	Australia	C	> 0.1

330   * Minimum amount of Lys B causing a lysis zone in *M. ulcerans* lawns. The results are

331   representative of three independent assays.

332

333

334   **Table 2.** Antibodies titers against Lys B

Challenge [a]	Antibody titres [b, c]
M. ulcerans	ND
M. ulcerans + Lys B	1166 ± 196
Lys B	1041 ± 165
PBS	ND

335

336   [a] Mice were injected subcutaneously in the left footpad with: *M. ulcerans* 98-912, Lys B or

337   phosphate buffer. After the emergence of macroscopic lesion (10 days post-infection; footpad

338   swelling of 2.7mm) infected mice were subjected to treatment with two doses of subcutaneous

339   injection of Lys B (10 and 13 days post-infection).

340   [b] Results are from one representative experiment of two independent experiments; values

341   represent the mean ± SD (n=6)

342   [c] ELISA IgG antibody titres are expressed as the reciprocal of the highest dilution giving an

343   absorbance above that of the control (no Lys B injection).

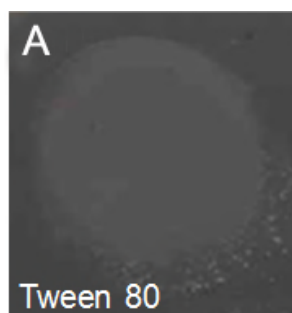344   ND, not detected

345

346 **Figure 1**

347

348

Figure 1. Lipolytic activity of Lys B. The lipolytic activity of the purified Lys B was confirmed by spotted in Middlebrook 7H9 plates containing substrates of **(A)** 1% (v/v) Tween 80 or **(B)** 1% (v/v) Tween 20 with 1 mM $CaCl_2$. Plates were incubated at 37ºC for at least 24 h. Enzymatic activity is indicated by the formation of a white precipitate spot on Tween plates
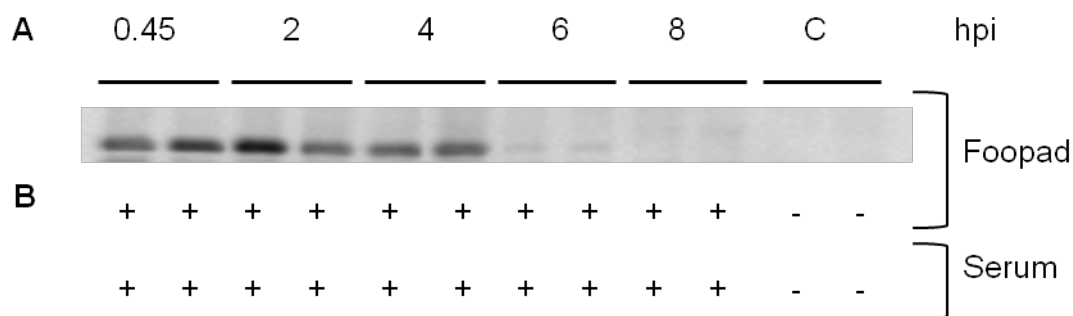
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354   **Figure 2**

355

356

357   **Figure 2. Assessment of bioavailability and enzymatic activity of Lys B in the footpad and**

358   **serum of mice.** Mice were injected subcutaneously in the left footpad with Lys B. At different time

359   points, the presence of Lys B was assessed by Western blot **(A)** and Lys B enzymatic activity

360   was determined by a lipase assay **(B)**, in footpad supernatant and serum; hpi, hours post-

361   injection; C, control mice; + lipase activity; - no activity. Results are from one representative

362   experiment of two independent experiments.
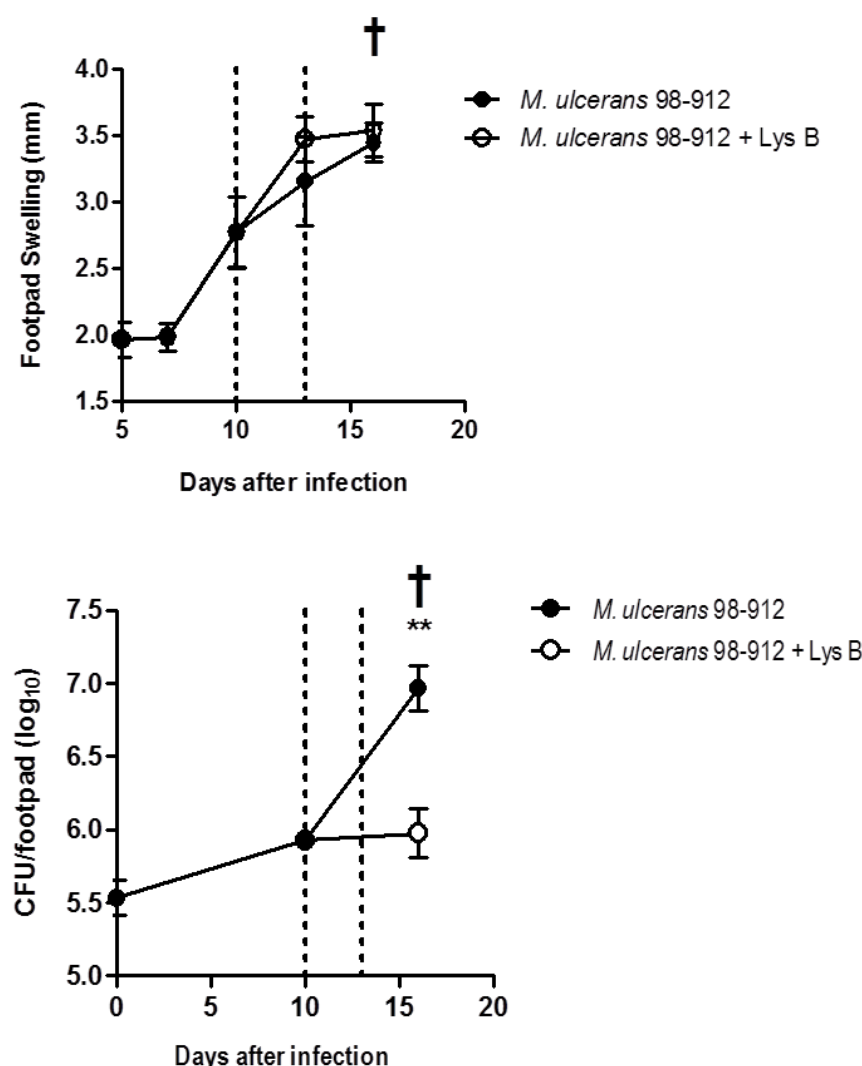
363

364   **Figure 3**

365

366   **Figure 3. Lesion progression and *M. ulcerans* proliferation in footpads of infected mice.**

367   Mice were infected subcutaneously in the left footpad with 5.5 $\log_{10}$ CFU of *M. ulcerans* strain 98-

368   912. After the emergence of macroscopic lesion (footpad swelling of 2.7mm) mice were subjected

369   to treatment with two doses of subcutaneous injection of Lys B (10 and 13 days post-infection)

370   (dashed lines). **(A)** Lesion progression was assessed by measurement of footpad swelling (n=15)

371   and **(B)** bacterial proliferation was assessed by colony forming units in footpads (n=6). †, mice

372   were sacrificed for ethical reasons after the emergence of ulceration. Results are from one

373   representative experiment of two independent experiments. Data points represent the mean ±

374   SD. Significant differences between treated and non-treated mice were performed using

375   Student's t test (**, p≤0.01).

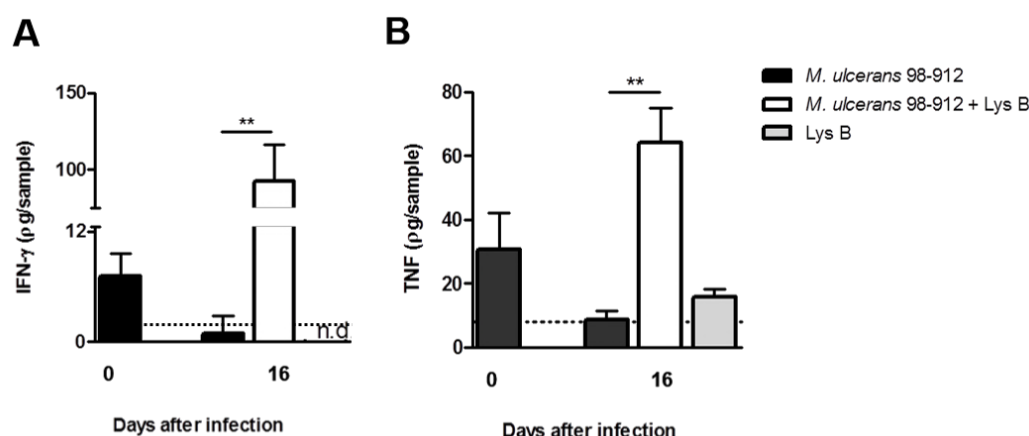376

19

377 **Figure 4.**

378

379 **Figure 4. Cytokine profile in DLN of Lys B-treated *M. ulcerans* infected mice.** Mice were

380 infected subcutaneously in the left footpad with 5.5 $\log_{10}$ CFU of *M. ulcerans* strain 98-912. After

381 the emergence of macroscopic lesion (footpad swelling of 2.7mm) mice were subjected to

382 treatment with two doses of subcutaneous injection of Lys B (10 and 13 days post-infection). **(A)**

383 Levels of IFN-γ and **(B)** TNF in DLN were quantified by ELISA. Results are from one

384 representative experiment of two independent experiments. Bars represent the mean ± SD (n=6).

385 n.d., not detected. Dashed lines represent the detection limit. Significant differences between

386 treated and non-treated mice were performed using Student's t test (**, p≤0.01).

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