

1 **High antibody titres induced by protein subunit**
2 **vaccines against Buruli ulcer using *Mycobacterium***
3 ***ulcerans* antigens Hsp18 and MUL_3720**

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6 Kirstie M. Mangas¹, Nicholas Tobias^{2,3}, Estelle Marion³, J r mie Babonneau³, Laurent
7 Marsollier³, Jessica L. Porter¹, Sacha J. Pidot¹, Chinn Yi Wong¹, David C. Jackson¹, Brendon Y.
8 Chua^{1,*} and Timothy P. Stinear^{1,*}.

9
10 ¹ Department of Microbiology and Immunology, Doherty Institute, University of Melbourne,
11 Melbourne, Victoria, Australia

12 ² Molekulare Biotechnologie, Fachbereich Biowissenschaften, Goethe-Universit t Frankfurt,
13 Frankfurt am Main, Germany

14 ³ LOEWE Centre for Translational Biodiversity in Genomics (TBG), Germany

15 ⁴ CRCINA, INSERM, Universit  de Nantes, Universit  d'Angers, Angers, France

16
17 Corresponding Authors:

18 Timothy Stinear, University of Melbourne, 792 Elizabeth St, Melbourne, Victoria, 3000,
19 Australia

20 Brendon Chua, University of Melbourne, 792 Elizabeth St, Melbourne, Victoria, 3000, Australia

21 Email addresses: tstinear@unimelb.edu.au, bychua@unimelb.edu.au

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23

24 **Abstract**

25 **Background.** *Mycobacterium ulcerans* is the causative agent of a debilitating skin and soft tissue
26 infection known as Buruli ulcer (BU). There is no vaccine against BU. The purpose of this study
27 was to investigate the vaccine potential of two previously described immunogenic *M.*
28 *ulcerans* proteins, MUL_3720 and Hsp18, using a mouse tail infection model of BU.

29 **Methods.** Recombinant versions of the two proteins were each electrostatically coupled with a
30 previously described lipopeptide adjuvant. Seven C57BL/6 and seven BALB/c mice were
31 vaccinated and boosted with each of the formulations. Vaccinated mice were then challenged
32 with *M. ulcerans* via subcutaneous tail inoculation. Vaccine performance was assessed by time-
33 to-ulceration compared to unvaccinated mice.

34 **Results.** The MUL_3720 and Hsp18 vaccines induced high titres of antigen-specific antibodies
35 that were predominately subtype IgG₁. However, all mice developed ulcers by day-40 post-*M.*
36 *ulcerans* challenge. No significant difference was observed in the time-to-onset of ulceration
37 between the experimental vaccine groups and unvaccinated animals.

38 **Conclusions.** These data align with previous vaccine experiments using Hsp18 and MUL_3720
39 that indicated these proteins may not be appropriate vaccine antigens. This work highlights the
40 need to explore alternative vaccine targets and different approaches to understand the role
41 antibodies might play in controlling BU.

42

43 **Introduction**

44 Buruli ulcer (BU) is a disease caused by *Mycobacterium ulcerans*. *M. ulcerans* infects
45 subcutaneous tissue and commonly presents as a skin nodule (in Africa) or papule (in Australia),
46 sometimes accompanied by redness; however, oedema is another common initial presentation.
47 As the disease progresses the skin around the infected area breaks down and an ulcer develops
48 [1, 2]. Ulcers typically present with deep undermined edges and have a necrotic core comprised
49 of slough of bacteria, dead skin and immune cells [3, 4]. Infections are rarely fatal but untreated
50 ulcers can destroy fat tissue, blood vessels, muscles and bone [5, 6].

51
52 The transmission of BU is likely caused by the introduction of *M. ulcerans* beneath the skin. This
53 could be achieved through the puncture of *M. ulcerans*-contaminated skin (with examples in the
54 literature of infections following human bite, bullet and land mine wounds, or vaccination) or by
55 the introduction of *M. ulcerans* contaminated objects into the subcutaneous tissue, such as
56 following insect bites [7-9]. BU endemic areas are focused in certain rural regions across west,
57 sub-Saharan and central Africa, including Nigeria, Ghana, Togo, Cameroon, Benin, Democratic
58 Republic of Congo and Côte d'Ivoire. The disease also occurs in Australia – primarily on the
59 Bellarine and Mornington Peninsulas near the major metropolitan centre of Melbourne [10-12].
60 The disease can affect all age groups and ethnicities [13]. In Australia, ulcers are predominately
61 reported on upper (27%) and lower limbs (70%) [14].

62
63 *M. ulcerans* is a slow-growing bacterium, with a doubling time of greater than 48 hours. As such,
64 symptoms of BU can take months to appear after primary infection. If diagnosed early, BU can
65 be treated effectively by combination antibiotic therapy [15]. Unfortunately, in many cases the
66 disease can initially be misdiagnosed as other more common skin infections [16, 17]. Delayed

67 diagnosis and treatment can lead to extensive lesions that leave victims with life-long
68 disfigurement and disability. Reparative surgery is often required for severe cases [18]. A
69 retrospective study in Australia showed that most diagnoses (87%) occurred once ulceration has
70 been reached [19] and in Ghana 66% cases were diagnosed with active lesions [20]. There is
71 currently no protective treatment for BU and no distinct mechanism of transmission.
72 Furthermore, treatment can be difficult to access for those in rural areas. Thus, there is a need to
73 develop an effective vaccine to protect those particularly in highly endemic areas.

74

75 The *M. bovis* 'BCG' vaccine has been shown to delay the onset of BU symptoms and decrease
76 bacterial load in both experimental animal BU infection models and in studies of human
77 populations [21-25]. Therefore, the BCG vaccine is the benchmark for assessing potential *M.*
78 *ulcerans* vaccines. Some studies have assessed the efficacy of putative BU vaccines although
79 none have reached clinical trials [21, 22, 26-37]. All these vaccines were tested in murine
80 challenge models and were not capable of preventing the eventual onset of disease.

81

82 One approach to vaccination is to use antigens specific for a specific pathogen (e.g. certain
83 proteins(s) that are recognized by the immune system and induce neutralizing antibodies [38,
84 39]. For rapid immune recognition these proteins would ideally be cell surface associated. Two
85 *M. ulcerans* proteins MUL_3720 and Hsp18 have been identified as potential candidates for
86 vaccine antigens. Hsp18 is a protein associated with biofilm formation and *M. ulcerans*-infected
87 individuals produce antibodies against Hsp18 [40, 41]. MUL_3720 is a highly expressed cell-
88 wall associated protein with a putative role in cell-wall biosynthesis [42, 43].

89

90 As protein antigens may be poorly immunogenic on their own, adjuvants are used to enhance
91 antigenic potency. A lipopeptide adjuvant known as R₄Pam₂Cys has been found to increase
92 antigen uptake, increase dendritic cell trafficking to lymph nodes and enhance antibody
93 production against antigens derived from pathogens including influenza and hepatitis C in
94 murine models [44-47].

95

96 The aim of this study was to try to develop a preventative vaccine against Buruli ulcer,
97 comprising two highly expressed cell-wall associated proteins, MUL_3720 or Hsp18, bound to
98 an R₄Pam₂Cys-based lipopeptide adjuvant.

99

100 **Materials & Methods**

101 *Strains and culture conditions*

102 *Escherichia coli* Rosetta2 containing plasmid pET30b-Hsp18 (strain TPS681) or pDest17-
103 MUL_3720 (strain TPS682) was grown at 37°C in Luria-Bertani (LB) broth (Difco, Becton
104 Dickinson, MD, USA) supplemented with 100 µg/ml ampicillin (Sigma-Aldrich, USA) or 50
105 µg/ml kanamycin to express 6xHIS-tagged Hsp18 or MUL_3720 recombinant protein.
106 *Mycobacterium ulcerans* (strain Mu_1G897) was grown at 30°C in 7H9 broth or 7H10 agar
107 (Middlebrook, Becton Dickinson, MD, USA) supplemented with oleic acid, albumin, dextrose
108 and catalase growth supplement (OADC) (Middlebrook, Becton Dickinson, MD, USA), and
109 0.5% glycerol (v/v). *M. bovis* BCG (strain Sanofi Pasteur) used for vaccinations was grown at
110 37°C in 7H9 broth or 7H10 agar supplemented with OADC. Mycobacterial colony counts from
111 cultures or tissue specimens were performed using spot plating as previously described [48].

112

113 *Recombinant protein expression*

114 Overnight cultures of strains TPS681 and TPS682 were diluted to OD₆₀₀ = 0.05 in LB broth. Each
115 culture was incubated at 37°C with shaking at 200 rpm until OD₆₀₀ = 0.6-0.7, then 1 mM IPTG
116 (Isopropyl β-D-1-thiogalactopyranoside) was added to induce protein expression. The cells were
117 incubated for a further four hours to express the protein. To harvest the protein, cells were
118 resuspended in wash buffer (8 M urea, 150 mM sodium chloride, 10% glycerol) and sonicated at
119 amplitude 60 (QSonica Ultrasonic Liquid Processor S-4000, Misonix) until the solution turned
120 clear. The lysate was filtered with a 0.22 µm filter (Millipore) to remove cellular debris and the
121 protein was column-purified using anti-histidine resin (ClonTech). The resin was washed ten
122 times with 10x column volumes of wash buffer mixed with an increasing proportion of Tris
123 buffer (20 mM Tris-HCl, 150 mM sodium chloride, 10% glycerol) until the column was washed

124 with only tris buffer. The resin was washed a further two times with tris buffer containing 20
125 mM imidazole. Protein was eluted in tris buffer containing 200 mM imidazole and dialysed in
126 phosphate buffered saline (PBS) before concentration using a 3K MWCO PES concentration
127 column (Pierce).

128

129 *Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

130 Samples were denatured in an equal volume of 2 x sample loading buffer (40% (v/v) 0.5M Tris-
131 HCL pH 6.8, 10% glycerol, 1.7% (w/v) SDS, 10% 2- β -mercaptoethanol, 0.13% (w/v)
132 bromophenol blue in distilled water) at 100°C for 5 minutes. Ten microlitres of each sample and
133 SeeBlue® Plus2 pre-stained protein standard (Invitrogen) were loaded into a 0.5mm 12%
134 polyacrylamide gel under reducing conditions, as previously described [49]. The gel was run in
135 running buffer (0.3% (w/v) Tris, 1.44% (w/v) glycine and 0.1% (w/v) SDS in distilled water) for
136 1 hour at 150 volts (Mini-protean vertical electrophoresis cell, Bio-Rad). The gels were stained
137 in Coomassie stain (45% methanol, 10% acetic acid 0.25% (w/v) Coomassie brilliant blue in
138 distilled water) for 1 hour and destained in Coomassie destain (33% Methanol, 10% acetic acid,
139 60% distilled water) until the protein bands could be identified.

140

141 *Western Blotting*

142 Proteins were separated on a 12% polyacrylamide gel as per the method for SDS-PAGE. After
143 separation proteins were transferred to a nitrocellulose membrane in tris-glycine transfer buffer
144 (1.5 mM Tris, 12mM glycine, 15 % methanol (v/v) in distilled water) for 1 hour at 100 volts
145 (Mini Trans-Blot Cell, Bio-Rad). The nitrocellulose membrane was blocked in blocking buffer
146 (5% (w/v) skim milk powder and 0.1% Tween-20 in PBS) overnight at 4°C. The membrane was

147 incubated in blocking buffer containing anti-6xHIS-HRP antibody (Roche Applied Science) at
148 1:500 dilution. The membrane was washed in PBS containing 0.1% Tween-20 and then exposed
149 to developing solution (Western Lighting Chemiluminescence kit, Perkin Elmer) according to
150 manufacturer's guidelines. Chemiluminescence was detected using an MF ChemiBIS gel
151 imaging system (DNR Bio-Imaging Systems).

152

153 *Analysis of electrostatic interaction between protein antigen and lipopeptide formulations*

154 The association between each protein and R₄Pam₂Cys was measured by mixing 25 µg of protein
155 with increasing amounts of lipopeptide in 50 µl PBS in a 96-well plate (Nunc, Thermo
156 Scientific). The formation of protein-lipopeptide complexes through electrostatic interaction was
157 measured by an increase in light absorbance. Plates were read at dual wavelengths of 505 and
158 595 nm on plate reader (LabSystems Multiskan Multisoft microplate reader).

159

160 *Lipopeptide vaccine preparation*

161 Each vaccine dose contained 25 µg protein added to R₄Pam₂Cys at a ratio of 1:5 mole of protein
162 to lipopeptide. PBS was added to a final volume of 100 µl and the combination sonicated in a
163 water bath for 30 seconds. Control vaccine preparations were made containing 25 µg protein
164 alone or R₄Pam₂Cys lipopeptide alone and sonicated before administration.

165

166 *Ethics statement for animal experiments*

167 All animal experiments were performed in full compliance with national guidelines (articles
168 R214-87 to R214-90 from French "rural code") and European guidelines (directive 2010/63/EU
169 of the European Parliament and of the council of September 22, 2010 on the protection of

170 animals used for scientific purposes). All protocols were approved by the Ethics Committee of
171 region Pays de la Loire under protocol nos. CEEA 2009.14 and CEEA 2012.145. Animals were
172 maintained under specific pathogen-free conditions in the animal house facility of the Centre
173 Hospitalier Universitaire, Angers, France (agreement A 49 007 002). Six-week old female
174 C57BL/6 and BALB/c mice were obtained from Charles River Laboratories (Saint-Germain-
175 Nuelles, France) and housed at CHU Angers. Food and water were given *ad libitum*.

176

177

178 *Vaccination of animals*

179 The synthesis and purification of the branched cationic lipopeptide, R₄Pam₂Cys, was performed
180 as previously described [45, 50, 51]. Each vaccine dose contained 25 µg protein formulated in
181 PBS with R₄Pam₂Cys at a 1:5 molar ratio of protein to lipopeptide in a final volume of 100 µl.
182 The protein alone control formulation contained 25 µg protein per dose diluted in PBS. The
183 R₄Pam₂Cys alone formulations contained the same amount of lipopeptide used in each of the
184 protein + adjuvant formulations, calculated by the 1:5 molecular ratio (with the omission of the
185 protein from the solution). The R₄Pam₂Cys alone formulations were diluted to the correct
186 concentration in PBS. Live-attenuated *M. bovis* BCG strain ‘Sanofi Pasteur’ was grown to log
187 phase and stored at -80°C in 20% glycerol until use. Bacteria were washed with PBS and
188 resuspended in 200µl, before administration at 4.7 x 10⁵ bacteria per dose. All vaccines and
189 control formulations were sonicated for 5 minutes in a waterbath sonicator before being
190 administered.

191

192

193 For vaccination using R₄Pam₂Cys, animals were inoculated subcutaneously at the base of tail
194 (100µl per dose at 50 µl per flank) and boosted 21 days later with the same formulations. Mice
195 vaccinated with approximately 1 x 10³ CFU *M. bovis* BCG resuspended in PBS at the base of tail
196 (100 µl per dose at 50µl per flank).

197

198 *M. ulcerans* challenge

199 Mice were challenged on day 35 by subcutaneous injection on the tail with 1 x 10⁴ CFU *M.*
200 *ulcerans* (Mu_1G897) resuspended in 50 µl PBS. Mice were allowed to recover and monitored
201 for up to 40 days after infection and euthanised when tail ulceration was observed wherein sera
202 were obtained for immunological analysis.

203

204 *Serum antibody titre measurements*

205 Serum was prepared from blood obtained from mice at day 0, day 18, day 33 and day 63.
206 Antibody titres were measured using enzyme linked immunosorbent assay (ELISA) as per
207 methods described in [45]. Briefly, ELISA plates (Nunc, Thermo Scientific) were coated
208 overnight with 5 µg protein diluted in PBSN₃ and blocked with BSA₁₀PBS for 2 hours at room
209 temperature. Plates were washed with PBS containing 0.05% Tween-20 (PBST). Neat sera were
210 sequentially diluted in BSA₅PBST and incubated at room temperature for 6 hours. Bound
211 antibody was detected by adding horse radish peroxidase conjugated rabbit anti-mouse IgG
212 (Dako, Glostrup, Denmark) at a concentration of 1:400 in BSA₅PBST for 2 hours. Plates were
213 developed with developing solution (hydrogen peroxide, citric acid and ABTS) and incubated for
214 10-15 min with gentle agitation to observe a colour change. The reaction was stopped with 50

215 mM sodium fluoride. Plates were read at dual wavelengths of 505 and 595 nm on plate reader
216 (LabSystems Multiskan Multisoft microplate reader).

217

218 *Statistical analysis*

219 Graphpad Prism software (GraphPad Software v7, CA, USA) was used to perform statistical
220 analyses on the antibody titre. Antibody titres were analysed using two-way ANOVA with
221 Tukey's correction for multiple comparisons. The time to ulceration data were displayed as a
222 Kaplan-Meier plot and statistical significance was determined using a Log-Rank (Mantel-Cox)
223 test. For all tests $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ and $****p < 0.0001$ were considered
224 statistically significant.

225

226

227 **Results**

228 MUL_3720 and Hsp18 have previously been shown to be immunogenic and cell-wall associated
229 [40, 43]. The adjuvant Pam₂Cys has been shown to induce strong antibody responses to proteins
230 from infectious agents such as influenza and hepatitis C [52-54]. Therefore, this study measures
231 the ability of MUL_3720 and Hsp18 based vaccines, incorporating the adjuvant Pam₂Cys, to
232 generate protein-specific antibodies and to protect against BU.

233

234 **Recombinant MUL_3720 and Hsp18 both bound to R₄Pam₂Cys**

235 Recombinant MUL_3720 and Hsp18, expressed from inducible *E. coli* expression vectors, were
236 prepared for use as antigens in the vaccine formulations (Table S1). Purification of the
237 recombinant proteins was confirmed by SDS-PAGE and Western blot analyses of the eluate (Fig.
238 1). DLS analysis was then performed to identify whether recombinant MUL_3720 or Hsp18
239 would electrostatically bind to either the positively charged lipopeptide adjuvant R₄Pam₂Cys, or

240 its negatively charged counterpart, E₈Pam₂Cys. The optical density of solutions containing these
241 constituents at a wavelength of 450nm (OD₄₅₀) is related to the particle size of molecules in
242 solution, reflecting the strength of the ionic interaction between protein and lipopeptide [45].
243 MUL_3720 preferentially bound to R₄Pam₂Cys compared to E₈Pam₂Cys (Fig. 2A). This is
244 shown as a gradual increase in optical density following the addition of increasing amounts of
245 R₄Pam₂Cys to a constant amount of MUL_3720. At a 5-fold molar excess of protein to
246 lipopeptide the OD₄₅₀ plateaued, suggesting MUL_3720 bound most strongly to R₄Pam₂Cys at a
247 1:5 protein to lipopeptide ratio. Conversely, when E₈Pam₂Cys was added to MUL_3720 the
248 optical density remained static and did not increase with increasing lipopeptide concentrations,
249 indicating a lack of binding. Hsp18 also appeared to bind preferentially to R₄Pam₂Cys and also at
250 a 1:5 ratio of Hsp18 to R₄Pam₂Cys (Figure 2B). Therefore, two protein-adjuvant formulations
251 were prepared using MUL_3720 with R₄Pam₂Cys and Hsp18 with R₄Pam₂Cys, both at a 1:5
252 protein to lipopeptide molar ratio.

253

254 **Vaccination induced strong protein-specific antibody responses**

255 Prior to challenge with *M. ulcerans*, the ability of the vaccine candidates to generate murine
256 immune responses was assessed. ELISAs were utilized to measure the antibody (IgG) titres in
257 sera obtained from two strains of mice (BALB/c and C57BL/6) immunized with either
258 MUL_3720 + R₄Pam₂Cys or Hsp18 + R₄Pam₂Cys after the primary vaccination dose (day 18)
259 and a secondary dose (day 33).

260

261 Vaccination with MUL_3720 recombinant protein alone or MUL_3720 + R₄Pam₂Cys were
262 capable of inducing MUL_3720-specific antibody titres in both BALB/c and C57BL/6 strains of

263 mice (Fig. 3A, B). Primary vaccination with MUL_3720 protein alone induced MUL_3720-
264 specific antibody responses that significantly increased ($p < 0.0001$) following a vaccine boost (p
265 = 0.0234). Additionally, MUL_3720 + R₄Pam₂Cys generated MUL_3720 specific antibody
266 responses after primary vaccination ($p < 0.0001$ in BALB/c and C57BL/6), which were increased
267 after the secondary boost ($p < 0.0001$ in BALB/c and not statistically significant in C57BL/6).
268 The titres after the boost in particular were greater than MUL_3720 alone vaccination ($p =$
269 0.0031 in BALB/c and $p = 0.006$ in C57BL/6). Mice that were not vaccinated with recombinant
270 MUL_3720 (R₄Pam₂Cys alone and BCG) did not have an increase in MUL_3720-specific
271 antibodies compared to naïve mice.

272

273 Vaccination with Hsp18 recombinant protein alone or Hsp18 + R₄Pam₂Cys induced Hsp18-
274 specific antibody titres in both strains of mice (Fig. 3C, D). Vaccine boost with Hsp18
275 recombinant protein alone induced significantly higher Hsp18-specific antibody responses in
276 BALB/c mice compared to a single vaccination with Hsp18 protein ($p < 0.0001$). Boosting with
277 protein alone in C57BL/6 did not significantly increase antibody titres. Hsp18 + R₄Pam₂Cys
278 induced Hsp18-specific antibody responses in both mouse strains after primary vaccination ($p <$
279 0.0001 in BALB/c and $p = 0.0165$ in C57BL/6) and the Hsp18-specific antibody titre
280 significantly increased after booster vaccination ($p < 0.0001$ in BALB/c and $p = 0.0016$ in
281 C57BL/6). In all strains, the antibody titres induced by Hsp18 + R₄Pam₂Cys were significantly
282 higher than vaccination with Hsp18 protein alone ($p = 0.0004$ in BALB/c and $p < 0.0001$ in
283 C57BL/6) (Fig. 3C, D) with negligible levels of antibodies seen in mice vaccinated with only
284 R₄Pam₂Cys, or BCG.

285

286 **Measurement of IgG antibody subtypes following MUL_3720 + R₄Pam₂Cys and Hsp18 +**
287 **R₄Pam₂Cys vaccination**

288 Quantifying levels of IgG antibody shows that the predominant isotypes produced by
289 MUL_3720 were IgG₁ and IgG_{2b} (Fig. 3E) with no significant difference between these isotype
290 titres. Vaccination with MUL_3720 + R₄Pam₂Cys produces significantly more IgG₁ and IgG_{2b}
291 antibodies ($p = 0.0076$). The antibody titres for both isotypes were highest prior to infection with
292 *M. ulcerans* (day 33) and decreased after infection by day 63. This vaccine was capable of
293 inducing IgG_{2a} antibodies, which was detected also on day 33, however in smaller amounts than
294 IgG₁ and IgG_{2b} ($p = 0.0399$ for MUL_3720 + R₄Pam₂Cys) (Fig. 3E).

295

296 Similar to vaccination with MUL_3720, Hsp18 was also capable of inducing strong IgG
297 antibody titres. The predominant isotype was IgG₁ which Hsp18 + R₄Pam₂Cys elicited more than
298 any other isotype (Fig. 3F) including IgG_{2a} and IgG_{2b}. Again, these titres was highest at day 33
299 and decreased significantly after infection on day 63. This trend was also observed after
300 vaccination with Hsp18 alone ($p = 0.0018$ vs IgG_{2a} and $p = 0.0076$ vs IgG_{2b}, respectively at day
301 33).

302

303 **MUL_3720 + R₄Pam₂Cys and Hsp18 + R₄Pam₂Cys do not protect against the onset of BU**

304 As both vaccines were capable of inducing protein-specific antibody responses, they were tested
305 in a murine challenge model to measure their protective efficacy. Efficacy was measured by time
306 delay to the onset of ulceration in a mouse tail infection model. There are a progression of
307 clinical symptoms for Buruli ulcer in this model (Fig. 4). Once ulceration has been reached the

308 disease would likely continue until the tail became necrotic. Therefore, the experimental
309 endpoint was deemed to be the point of ulceration.

310

311 After the scheduled vaccinations, mice were challenged via subcutaneous tail inoculation with 1
312 $\times 10^4$ CFU of *M. ulcerans* and observed for up to 40 days. In BALB/c and C57BL/6 mice there
313 was no significant difference between the time to ulceration between control mice (mice not
314 vaccinated with recombinant protein, such as R₄Pam₂Cys alone and BCG) and mice vaccinated
315 with either MUL_3720 + R₄Pam₂Cys or Hsp18 + R₄Pam₂Cys (Figure 5A and 5B). There was
316 also no significant difference in the time to ulceration between mice that were vaccinated with
317 MUL_3720 + R₄Pam₂Cys or Hsp18 + R₄Pam₂Cys and BCG, the benchmark for mycobacterial
318 vaccine efficacy. Signs of infection in all BALB/c and C57BL/6 mice were visible by day 33
319 (Table 1 and Table 2) and all mice reached ulceration by day 63, 30 days post-*M. ulcerans*
320 challenge (Fig. 5A, B).

321

322 **Antibody titres do not correlate with protection against *M. ulcerans***

323 High antibody titres were observed in all mice vaccinated with either recombinant MUL_3720 or
324 Hsp18, particularly in the secondary response after booster vaccination (Fig. 3A-D) prior to *M.*
325 *ulcerans* challenge. However, mice vaccinated with protein alone or protein plus lipopeptide
326 adjuvant all succumbed to infection by day 75. The sera from mice at the day 63 was used to
327 quantify antibody titres during infection. At day 63 all mice still had detectable protein-specific
328 antibodies against the recombinant protein with which they were vaccinated (Figure 3A-3D). In
329 BALB/c mice (Fig. 3A, C) the antibody titres at day 63 were lower than after the secondary
330 response prior to challenge ($p < 0.0001$ for both Hsp18 + R₄Pam₂Cys and MUL_3720 +

331 R₄Pam₂Cys) but remained significantly higher than at day 0 ($p < 0.0001$ for both Hsp18 +
332 R₄Pam₂Cys and MUL_3720 + R₄Pam₂Cys). In C57BL/6 mice (Fig. 3B and 3D), antibody titres
333 against MUL_3720 or Hsp18 from mice vaccinated with either protein alone or protein plus
334 lipopeptide adjuvant were also significantly decreased at day 63 compared to the secondary
335 response at day 35 ($p < 0.0001$ and $p = 0.0406$ for MUL_3720 + R₄Pam₂Cys and Hsp18 +
336 R₄Pam₂Cys, respectively). Similar to BALB/c mice, the day 63 respective protein-specific
337 antibodies for MUL_3720 + R₄Pam₂Cys and Hsp18 + R₄Pam₂Cys were significantly higher than
338 at day 0 ($p < 0.0001$ and $p = 0.0004$ for MUL_3720 + R₄Pam₂Cys and Hsp18 + R₄Pam₂Cys,
339 respectively).

340

341 **Challenge with *M. ulcerans* did not induce protein-specific antibody levels comparable to**
342 **vaccination with MUL_3720 or Hsp18.**

343 MUL_3720 and Hsp18 recombinant proteins are immunogenic and capable of inducing protein-
344 specific antibody responses after vaccination. However, only minor detectable antibody
345 responses against either recombinant MUL_3720 or Hsp18 at day 63 (Fig. 3A-D) were found in
346 mice vaccinated with R₄Pam₂Cys alone or BCG then challenged with *M. ulcerans*. These
347 responses are much lower than the protein-specific antibody responses generated from
348 MUL_3720 or Hsp18 vaccinated mice, particularly in C57/BL6 mice ($p < 0.0001$) (Fig. 6).
349 Animals from both mouse strains that were vaccinated with R₄Pam₂Cys alone or BCG showed
350 no increase in protein-specific antibody responses against either recombinant MUL_3720 and
351 Hsp18 on day 63 post-*M. ulcerans* challenge (Fig. 3A-D), even though these two proteins are
352 both expressed in *M. ulcerans*.

353

354 Discussion

355 This study aimed to develop a vaccine against *M. ulcerans* utilizing two previously described
356 cell-wall associated proteins, Hsp18 and MUL_3720 [40-43]. Both the MUL_3720 and Hsp18-
357 based vaccines were capable of inducing high antibody titres, but these responses were not
358 associated with protection (Fig. 5). This may indicate that these proteins, while strongly
359 immunogenic, play no major role in pathogenesis, so targeting them with potentially neutralizing
360 antibodies induced by the vaccine has no impact on disease. Alternatively, antibodies raised by
361 these vaccines may not have had the functional potential to control infection. In addition to
362 antigen binding, antibodies engage via their Fc domains with Fc γ receptors (Fc γ R) present on
363 innate immune cells (NK cells, monocytes, macrophages and neutrophils) to rapidly recruit the
364 anti-microbial activity of the innate immune system. Antibodies with these functions can
365 promote control of a pathogen through the activation of multiple effector cell functions,
366 including Ab dependent cellular cytotoxicity, cellular phagocytosis and/or cytokine and enzyme
367 secretion [55-57]. Recent research has shown that mice lacking antibodies have increased
368 susceptibility to *M. tuberculosis* infection [58] and non-human primates treated to deplete B cells
369 also exhibit increased bacterial burden [59]. Despite the findings of this research, it is likely that
370 B cells and antibody responses still play a role in controlling *M. ulcerans* infection in this model,
371 albeit with different specificities. Future research could use human BU patient cohorts and
372 mouse infection models to attempt to characterize the targets, functional and structural aspects of
373 antibody responses that differentiate subjects able to control BU from susceptible subjects. It
374 might then be possible to use B cell probe technologies to isolate Ag-specific memory B cells
375 from individuals that control *M. ulcerans* infection and then clone the immunoglobulin gene
376 sequences identified [60]. Antigen-specific monoclonal Abs (mAbs) could then be generated and

377 characterized for their *in vitro* anti-microbial activity and used in *in vivo* mouse passive transfer
378 studies to determine potential use as mAb therapeutics against BU.

379

380 Another explanation for the ineffectiveness of antibodies in this study may be due to the
381 localized immune suppression induced by the *M. ulcerans* toxin mycolactone at the site of
382 infection. Mycolactone diffuses into tissue surrounding the bacteria [61-63]. Mycolactone is a
383 cytotoxin that modulates the function of several immune cells [63, 64]. The toxin inhibits the
384 Sec61 translocon, affecting T cell activation, impairing T cell responsiveness and distorting
385 cytokine production [62, 63]. The mycolactone-induced depletion of T cell homing to peripheral
386 lymph nodes affects subsequent B-cell activation and migration from the lymphatics [65]. The
387 antibodies induced by the vaccine in this study may be functional but unable to access bacteria
388 within the infection or it may be that multiple effector cell functions have been modulated by
389 mycolactone exposure through interference with receptor expression on key innate immune cells,
390 rendering these cells poorly responsive to antibodies. Suppression of protein-specific antibody
391 production in the presence of mycolactone has been observed [66]. Mycolactone administered to
392 a different location to the antigen caused no reduction to systemic antigen-specific IgG titres
393 [66], similar to the observations from our study.

394

395 The greatest antibody responses were of the IgG1 subclass. Typical antibody responses against
396 proteins occur via B cell isotype switching from IgM (non-specific antibody isotype) to IgG.
397 There are 4 subclasses of IgG (IgG1, IgG2, IgG3 and IgG4) and isotype switching to
398 predominantly IgG1 suggests refinement of immune responses to respond specifically to either
399 MUL_3720 or Hsp18, as IgG1 is capable of binding to protein antigens [67]. IgG1 can also bind

400 all forms of Fc γ R which is required to elicit and mediate effector immune functions as described
401 above [68]. The presence of IgG2 suggest further isotype switching from IgG1 to IgG2_{a/b} as the
402 immune response develops. IgG2 is less effective at inducing phagocytosis and fixing
403 complement and is more commonly associated with polysaccharide antigens. Though tests on the
404 recombinant proteins had undetectable levels of lipopolysaccharide (LPS), there could be trace
405 amounts from the *E. coli* expression vector boosting IgG2 responses. Studies analysing
406 antibodies generated during leprosy and TB infection show a switch from IgG1 to IgG2
407 antibodies for leprosy and a persistence of IgG1 and IgG3 antibodies for TB [69]. As isotype
408 switching of antibodies requires help by T helper cells, future work could therefore also
409 incorporate studies on the effect of vaccination and subsequent *M. ulcerans*-infection on T cells
410 as well as antibody responses.

411
412 In this study, all mice succumbed to infection in a relatively short period (40 days) compared to
413 previous mouse tail infection models [70] and human BU, where the incubation period is
414 estimated at 4.8 months before the onset of ulceration [71]. All BALB/c and C57BL/6 mice
415 succumbed to infection by 40 days after MU infection, even mice that were vaccinated by *M.*
416 *bovis* BCG. *M. bovis* BCG has been previously shown to delay the onset of disease on average
417 by at least 6 weeks [21, 22, 31]. In this study however, there was no significant difference
418 between mice vaccinated with either MUL_3720 or Hsp18 protein alone or with both proteins
419 plus R₄Pam₂Cys. This suggests that *M. bovis* BCG is ineffective at protecting mice in this model
420 of *M. ulcerans* vaccination. This failure to observe any protective impact of *M. bovis* BCG might
421 be a reflection of the challenge strain of *M. ulcerans* used (strain Mu_1G897) and/or the high
422 challenge dose used (10⁴ bacteria). High concentrations (>10⁴ bacteria) have not been reported in

423 environmental sources of *M. ulcerans* [9, 72-75], consistent with the hypothesis that a relatively
424 small bacterial inoculum is required to establish BU [9]. At the time this study was conducted the
425 minimum infectious dose (ID₅₀) for BU had not been determined, however the ID₅₀ has since
426 been identified as approximately 3 CFU [48]. Future studies should therefore use a murine model
427 that is more representative of a natural *M. ulcerans* infection, reflected both in the mode of *M.*
428 *ulcerans* entry into the subcutaneous tissue and in the dose of bacteria used for challenge.

429

430 **Conclusions**

431 Vaccination with either MUL_3720 or Hsp18 proteins induced high antibody titres. These
432 responses were augmented when either protein was linked with the lipopeptide adjuvant
433 R₄Pam₂Cys. However, robust antibody responses did not correlate with protection against
434 challenge with *M. ulcerans*. Future work could test different *M. ulcerans* antigens in vaccine
435 formulations against Buruli ulcer. As mycolactone is a key virulence factor, neutralising this
436 toxin early in infection by targeting the PKS enzymes required for its biosynthesis could be a
437 focus for future vaccination developments. Using a low *M. ulcerans* inoculum as a more realistic
438 vaccine challenge dose is also warranted.

439

440 **Acknowledgements**

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442 of recombinant Hsp18.

443

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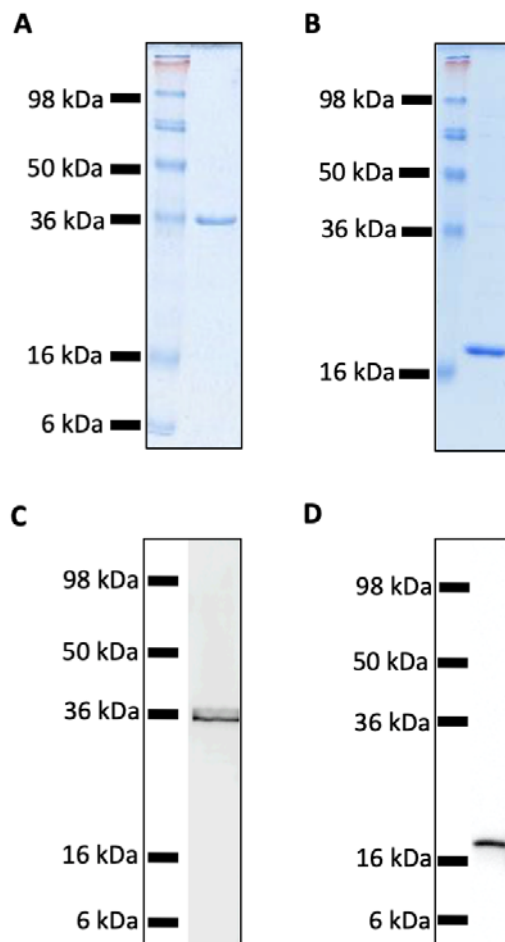
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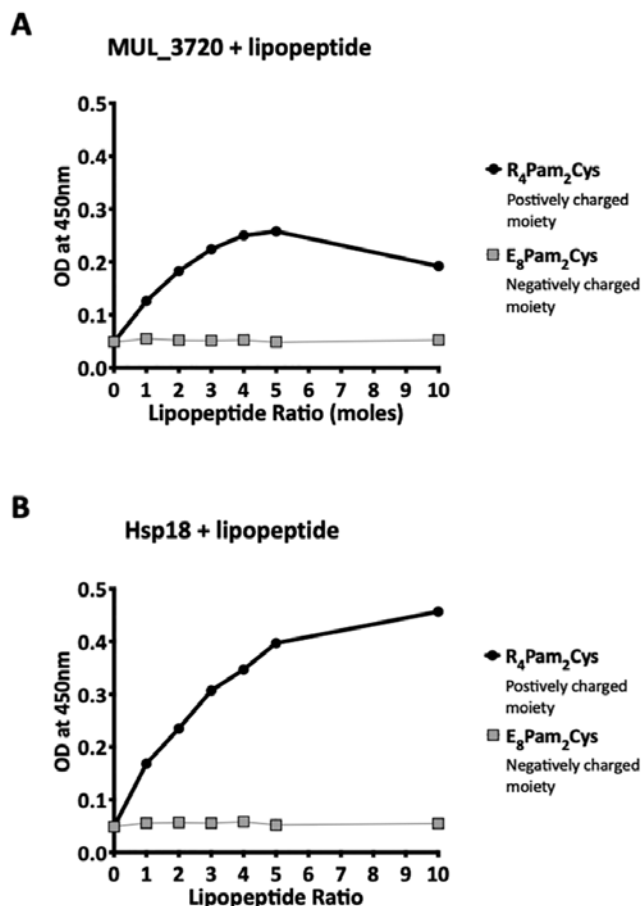
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631 Figures



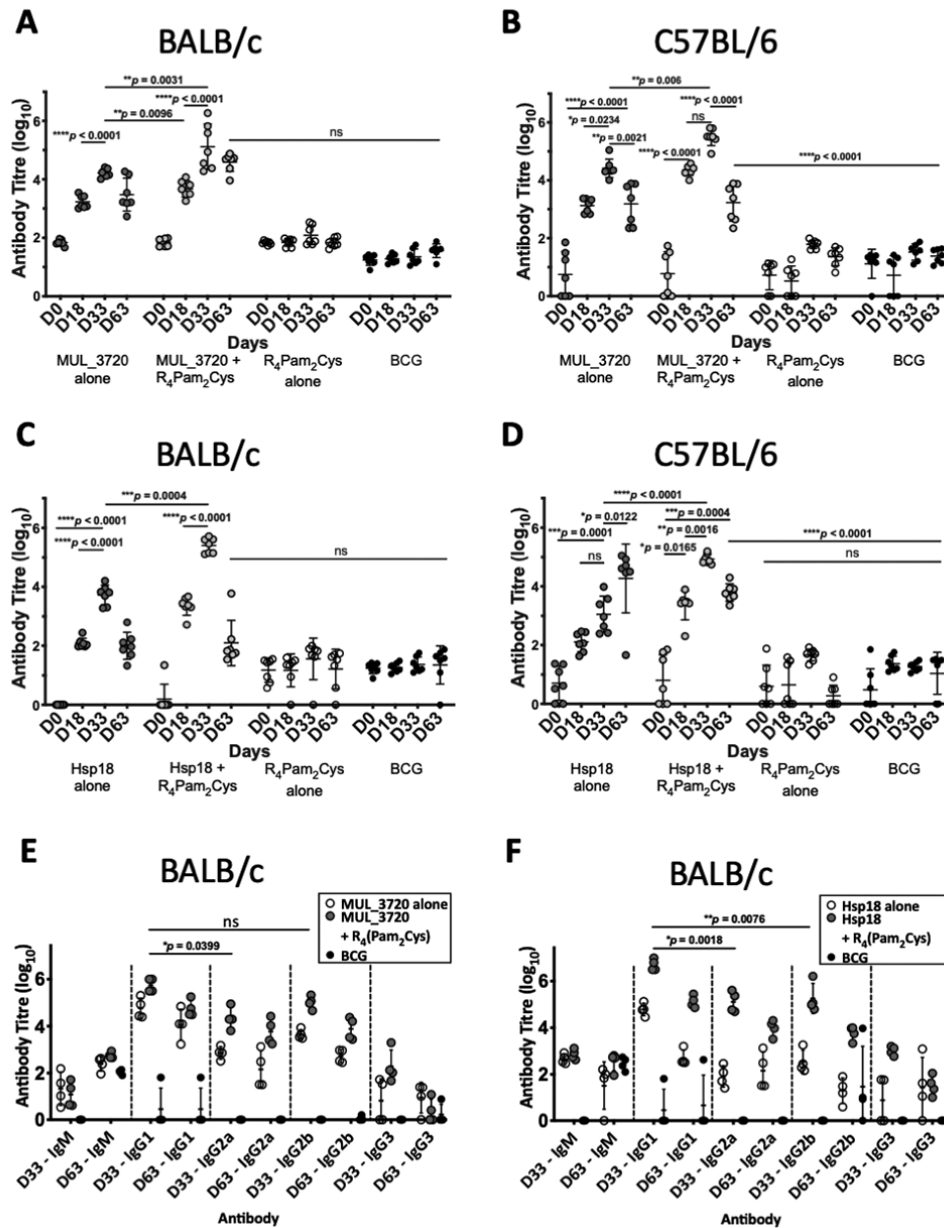
632
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634 **Figure 1. SDS-PAGE and Western Blot Analysis of purified recombinant MUL_3720 and**
635 **Hsp18 proteins.** (A) SDS-PAGE of MUL_3720 protein elution (containing 10 μ g protein)
636 shows a band ~36 kDa. (B) SDS-PAGE of Hsp18 protein elution (containing 10 μ g protein)
637 shows a band ~18 kDa. (C) Protein in the final MUL_3720 elute was analysed by Western Blot
638 using an anti-6xHIS-tag antibody to detect the presence of a single band corresponding to the
639 band as the SDS- PAGE analysis. (D) Protein in the final Hsp18 elute was analysed by Western
640 Blot using an anti-6xHIS-tag antibody to detect the presence of a single band corresponding to
641 the 18 kDa band as the SDS-PAGE analysis.



642

643 **Figure 2. Recombinant MUL_3720 and Hsp18 protein formulation linked with R₄Pam₂Cys.**

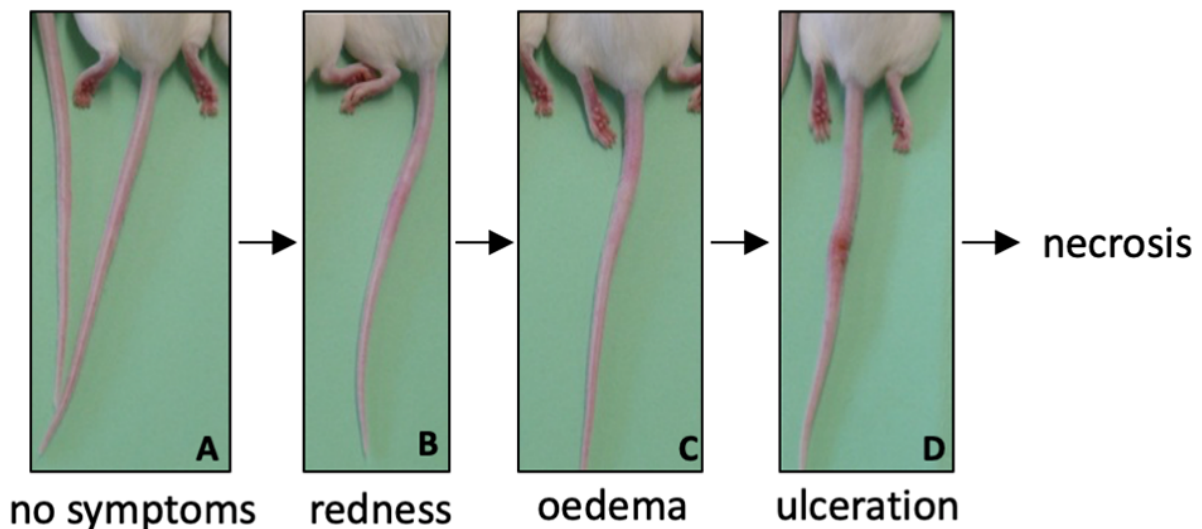
644 To analyse the formation of antigen-lipopeptide complexes, a constant amount of antigen (**A**)
645 MUL_3720 (25µg) and (**B**) Hsp18 (25µg) was mixed with lipopeptide at different
646 protein:lipopeptide molar ratios in 50 µl of PBS. These graphs depict the absorbance values of
647 these solutions at an optical density of 450nm (OD₄₅₀). In these assays either R₄Pam₂Cys or
648 E₈Pam₂Cys lipopeptides were added to the proteins at increasing amounts. The addition of
649 R₄Pam₂Cys is depicted with black circles and the addition of E₈Pam₂Cys is depicted with grey
650 squares. An increase in absorbance in correlation to an increase in lipopeptide was indicative of
651 protein binding to lipopeptide.



652 **Figure 3. Antibody titres from BALB/c and C57BL/6 mice immunized with recombinant**
 653 **MUL_3720 or Hsp18 linked to R₄Pam₂Cys lipopeptide adjuvant. MUL_3720-specific**
 654 **antibody titres from (A) BALB/c and (B) C57BL/6 mice. Mice were vaccinated with protein**
 655 **alone (MUL_3720) (grey circles), recombinant protein + R₄Pam₂Cys (blue circles), R₄Pam₂Cys**
 656 **alone (clear circles) and *M. bovis* BCG (black circles). A separate ELISA was performed to**
 657 **measure Hsp18-specific antibody titres in (C) BALB/c and (D) C57BL/6 mice. Mice were**

658 vaccinated with protein alone (Hsp18) (grey circles), recombinant protein + R₄Pam₂Cys (blue
659 circles), R₄Pam₂Cys alone (clear circles) and *M. bovis* BCG (black circles). IgG isotypes (IgG₁,
660 IgG_{2a}, IgG_{2b} and IgG₃) were quantified from BALB/c mice immunized with (E) MUL_3720 +
661 R₄Pam₂Cys and (F) Hsp18 + R₄Pam₂Cys. Mice were vaccinated with protein antigen alone
662 (either MUL_3720 or Hsp18) (clear circles), protein + R₄Pam₂Cys (grey circles) and BCG (black
663 circles). Results are shown as zero if below detectable limits. The null hypothesis (no difference
664 in mean antibody responses between treatment groups) was rejected at **p* < 0.05, ***p* < 0.01,
665 ****p* < 0.001 or *****p* < 0.0001. The error bars represent standard deviation (n=7).
666
667

Stages of *M. ulcerans* tail infection

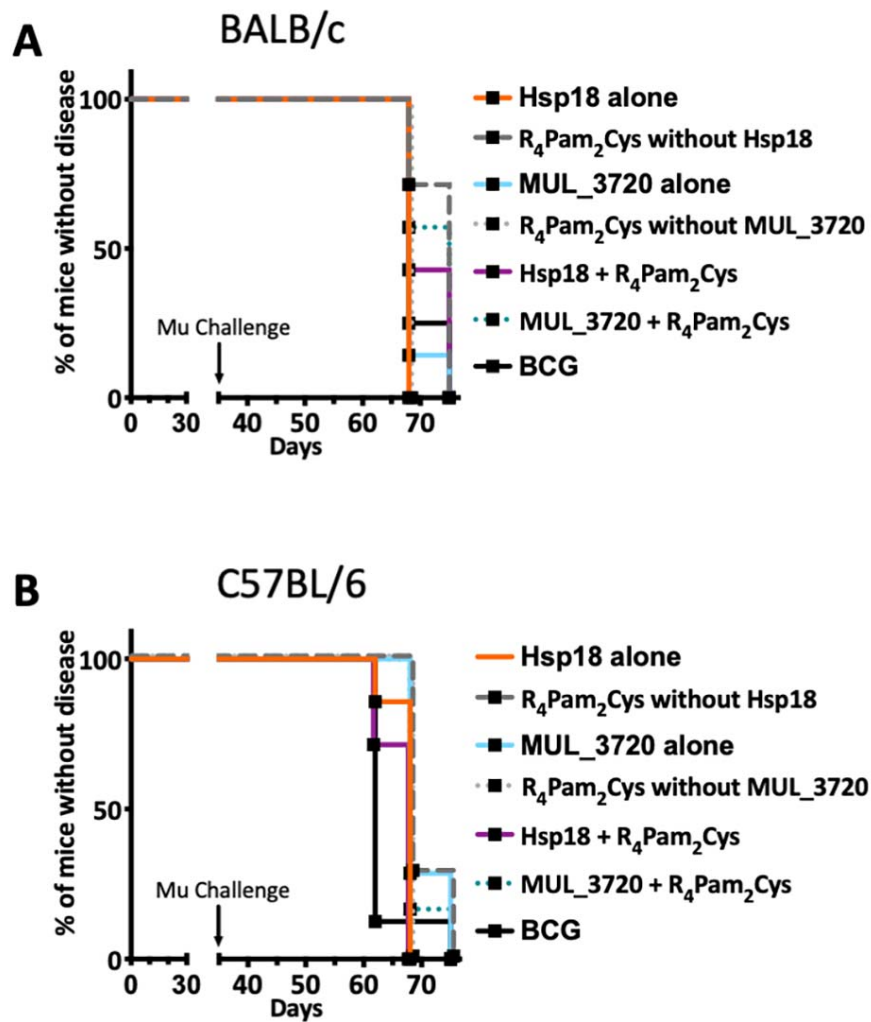


668 **Figure 4. Progression of BU in the murine tail infection model over time.** (A) Healthy mouse
669 tail. (B) Appearance of a small sign of redness at the site of tail infection. (C) Oedema
670 surrounding the initial site of redness. (D) Tail lesion at the point of ulceration. This is typically

671 identified by excessive oedema and redness at the site of imminent ulceration. Mice were culled
672 before ulcerative lesions appeared.

673

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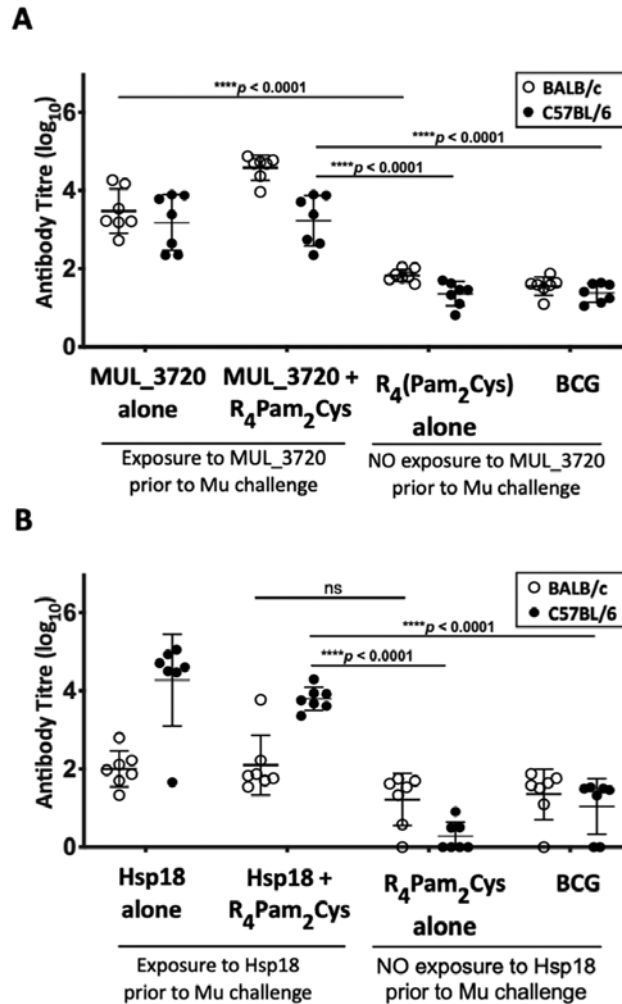
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676 **Figure 5. Vaccine performance using murine tail infection model of BU.**

677 Survival analysis showing the time taken (days) for each mouse to reach ulceration for different

678 vaccination groups post *M. ulcerans* challenge. (A) BALB/c mice (n=7) and (B) C57BL/6 mice

679 (n=7). The null hypothesis (no difference in mean antibody responses between treatment groups)
680 was rejected if $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ or $****p < 0.0001$.
681



682

683 **Figure 6. Antibody responses to recombinant MUL_3720 and Hsp18 in unvaccinated mice.**

684 (A) MUL_3720-specific antibody titres and (B) Hsp18-specific antibody titres from BALB/c
685 mice (clear circles) and C57BL/6 mice (black circles) at day 63 (post-MU exposure). The null
686 hypothesis (no difference in mean antibody responses between treatment groups) was rejected if

687 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$. The error bars are standard deviation
688 (n=7).