information services
gwasanaethau gwybodaeth

1 **BclA and toxin antigens augment each other to protect NMRI mice from lethal Bacillus**

2 **anthracis challenge**

3

4 *Susanne M. Köhler[a]*, Les W. Baillie[b] and Wolfgang Beyer[a]*

5

6 [a] Institute of Environmental and Animal Hygiene, University of Hohenheim, Garbenstr. 30, D-

7 70599 Stuttgart, Germany

8 [b] Cardiff School of Pharmacy and Pharmaceutical Science Cardiff University, King Edward VII

9 Avenue, Cardiff, CF10 3NB, UK

10 * Corresponding author. Tel.: +49 151 20022217; E-mail address: SuMe.koehler@gmail.com

11

14

15 **Abstract**

16 While proving highly effective in controlling Anthrax in farm animals all over the world currently

17 attenuated live anthrax vaccines employed in a veterinary context suffer from drawbacks such as

18 residual virulence, short term protection, variation in quality and, most importantly, lack of efficacy

19 if administered simultaneously with antibiotics. These limitations have stimulated the development

20 of non-living component vaccines which induce a broad spectrum immune response capable of

21 targeting both toxaemia (as in the case of PA based vaccines) and bacteraemia. To contribute to this

22 several new approaches were tested in outbred NMRI mice for antibody titres and protectiveness.

23 Plasmids encoding a recombinant toxin derived fusion peptide and a spore surface derived peptide

24 were tested as DNA-vaccines in comparison to their protein counterparts utilising two adjuvant

25 approaches and two DNA-vector backbones. The combination of two plasmids encoding

26 LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1, when delivered by GeneGun, protected 90% of

27 the animals against a lethal challenge with $25LD_{50}$ spores of the Ames strain of *Bacillus anthracis*.

28 Single applications of either antigen component showed significantly lower protection rates,

29 indicating the beneficial interaction between anti-spore and anti-toxin components for an acellular

30 vaccine formulation.

31

32 **1. Introduction**

33 Anthrax is caused by *Bacillus anthracis*, a Gram-positive, spore forming, rod-shaped bacterium [1].

34 Spores gain access via cutaneous, oral or inhalational routes where they germinate and develop into

35 vegetative bacilli which then replicate and produce toxins which eventually kill the host [2]. The

36 pathogen expresses two major plasmid encoded virulence factors, a gamma-linked poly-D-glutamic

37 acid capsule (pX02 [3]) and a tripartite toxin (pX01 [4]) comprised of Protective Antigen (PA)[1],

38 Lethal Factor (LF)[2] and Edema Factor (EF)[3] [5][6].

39 Current live attenuated veterinary anthrax vaccines are less than ideal. They can cause problems in

40 sensitive animals such as goats and llamas, protection is short term, variation in vaccine quality can

41 cause vaccine failure and most importantly the live nature of the vaccine prevents its efficacy if

42 delivered at the same time as antibiotics [7][8]. These limitations have stimulated the development

43 of non-living, component vaccines capable of inducing a broad spectrum immune response which

44 targets both toxaemia and bacteraemia.

45 The strong correlation between toxin neutralising activity (tna)[4] of PA-specific antibodies and

46 protection [9] has prompted efforts to develop vaccines based solely on domains which stimulate

47 antibodies with tna [10][11][12]. One such study which employed a fusion protein comprised of

48 domain 4 of PA (receptor binding site) and domain 1 of LF (PA binding site) protected mice against

---

1   PA – Protective Antigen
2   LF – Lethal Factor
3   EF – Edema Factor
4   tna – toxin neutralising activity

49 a subsequent lethal challenge with *B. anthracis* spores [13]. To further assess the immunogenic

50 value of this protein we administered it as a DNA-vaccine in two different vectors and compared its

51 activity to that seen against full length rPA83.

52 In addition to neutralising the action of toxins the spore can also be targeted to prevent the pathogen

53 from gaining a foothold in the infected individual [14][15]. Vaccination experiments with live

54 nonvirulent or formaldehyde-inactivated spores have shown that spore specific immune responses

55 can enhance the level of protection when given in combination with PA [16].

56

57 One such component is the *Bacillus* collagen like protein of *anthracis* (BclA)[5] which forms hair-

58 like structures projecting from the spore surface and represents a major spore immunogen [17][18].

59 The removal of the collagen-like region (CLR[6], domain 2) from BclA has no detrimental effect on

60 immunogenicity and results in a smaller peptide which is easier to incorporate into a

61 multicomponent vaccine [19][20]. In this study we determined the immunogenicity of a CLR-

62 deficient version of BclA called rBclAD1D3 when administered as a DNA-vaccine in two different

63 vectors.

64

65 For the DNA vaccine studies we employed two different plasmid backbones (pDNAVaccUltra and

66 NTC7382) which varied with regards to intracellular routing signals and immune stimulatory

67 elements [21]. To improve *in vivo* antigen presentation we utilised intracellular routing signals

68 which directed vaccine peptides to the MHC I and MHC II pathways. To target the MHC II

69 pathway [22] we employed tissue plasminogen activator (TPA)[7] which routes newly expressed

70 proteins to the secretion pathway [23] and lysosome-associated membrane protein (LAMP1)[8] which

71 directs proteins to the endosome [24][25]. To enhance MHC I presentation we employed ubiquitin

---

5   BclA – *Bacillus* collagen like protein of *anthracis*
6   CLR – collagen-like region
7   TPA – tissue plasminogen activator
8   LAMP1 – lysosome-associated membrane protein

72 which directs the associated protein to the proteasome [26][27].

73

74 To enhance the immunogenicity of the expressed proteins we investigated the utility of two

75 molecular adjuvants. Mouse interferon-ß promoter stimulator 1 (mIPS-1)[9] incorporated into the

76 backbone of the antigen encoding plasmid significantly induces type I interferon and interferon-

77 stimulated genes in a TLR-independent matter [28][29][30]. Mouse class II MHC trans-activator

78 (CIITA)[10] up-regulates MHC expression [31][32] and was co-administered on a separate plasmid.

79 In comparison to the DNA vaccines, full length rPA and rBclA were tested as proteins alone and in

80 combination in the presence of a previously tested and approved lipopeptide adjuvant comprising

81 Pam3Cys-SKKKK, a TLR2/1 activator admixed with Pam_3Cys conjugated to the promiscuitive T-

82 helper-cell epitope of the sperm whale myoglobin SFISEAIIHVLHSRHPG [33][34].

83 The overall aim of this study was to determine the ability of BclA to confer additional

84 protectiveness when given together with a toxin-specific vaccine.

85

86

87 **2. Material and Methods**

88 **2.1. Antigen preparation**

89 E. coli BL21-CodonPlus-RIL cells (Stratagene, La Jolla, CA) harboring the plasmid pREP 4

90 (Qiagen, Venlo, Netherland) and pQE-30 (Qiagen) encoding either rPA83, rBclA or rLF were grown

91 and purified as described previously [35]. Proteins used for ELISA received no further treatment

92 while proteins used for vaccination were tested for endotoxin using the Limulus Amoebocyte Lysate

93 Endochrome-K test kit (Charles River, Wilmington, MA) as described by the manufacturer.

94 Endotoxin removal was performed via EndoTrap blue endotoxin removal system (Hyglos, Bernried,

95 Germany).

---

9   mIPS-1 – mouse interferon-ß promoter stimulator 1
10   CIITA – mouse class II MHC trans-activator

96

2.2. Preparation of DNA-vaccines

Providing of vector-backbones (pDNAVaccUltra and NTC7382) including signal sequences, cloning of respective gene sequences and purification of the constructs was undertaken by the Nature Technology Corporation (Lincoln, NE). The sequence for murine CIITA (Mn01492) was acquired from GeneCopoeia (Rockville, MD) and the sequence of mIPS-1 was used as given for pUNO1-mIPS1 (Genebank: NM_144888.2). Antigens used comprised of rPA83 (2208 bp) [35], LFD1PAD4 (1300 bp) [13] and BclAD1D3 (480 bp) [20]. Vaccines were prepared and applied as described previously [35].

2.3. Administration of vaccines and challenge

Trials were performed using 8-12 weeks old female outbred NMRI mice (Charles River). Immunisation groups comprised of 10 animals, while groups which only received an adjuvant (lipopeptide or CIITA) contained 5 individuals.

Mice were immunised 3 times at intervals of 2 weeks. Challenge was performed 3 weeks after the last immunisation and mice were monitored for survival for up to 4 weeks. Blood was taken prior to immunisation, before challenge and after the end of the challenge. All data presented is referring to sera taken before the challenge unless stated otherwise. Preparation, vaccination and challenge of the mice was performed under anesthesia with Isofluran (Actavis, Weiterstadt, Germany) using a ventilated Box.

Mice immunised with protein received a 200 µl dose s.c. in the neck containing 25 µg of each antigen and 50 µg of the lipopeptide adjuvant (EMC microcollections, Tuebingen Germany) diluted in sterile endotoxin free PBS (Sigma-Aldrich, St. Louis, MO). Mice immunised with DNA-vaccines were shaved 1-2 days before the immunisation. Each mouse received 2 cartridges containing a total of 3 µg of DNA (6 µg for the combination) per immunisation applied via GeneGun (tab 1). For the

121 challenge, a dose of 200 µl of a fully virulent Ames strain containing ~1000 spores ($25LD_{50}$) for all

122 DNA-vaccines and ~2000 spores ($50LD_{50}$) for all protein vaccines was administered s.c. in the

123 neck. The increased challenge dose for the protein vaccines was utilised due to the residual

124 protectiveness of the lipopeptide alone observed when challenged with lower spore doses (data not

125 shown), thus normalising its effect to allow for a better discrimination between different antigens

126 and applications. Mice that died during the challenge or were killed via CO_2 after the end of the

127 challenge were dissected to prepare liver and spleen which were then plated on blood agar to verify

128 the presence of the pathogen.

129

130 **2.4. Serology**

131 ELISA was performed as described previously [35]. Secondary antibodies comprised of horseradish

132 peroxidase conjugated polyclonal goat anti-mouse IgG (Sigma-Aldrich), IgG1 and IgG2a (Acris,

133 San Diego, CA). Endpoint titres were defined as the reciprocal of the highest serum dilution that

134 resulted in an absorbance greater than two standard deviations above the average of the negative

135 control serum sample (pooled naive sera of the according group) with a minimum OD_{414nm} value of

136 0.1.

137 Sera were analysed for neutralising antibody titres via in vitro toxin neutralising assay (TNA) as

138 published previously [35]. The neutralisation titre (NT) corresponds to the inverse serum dilution at

139 which the toxin neutralisation yielded 50% (NT_{50}). The NT_{50} was estimated using the SigmaPlot

140 regression wizard (4-parameter sigmoid regression curve). Detailed Titres are only described for

141 sera taken before the challenge.

142

143 **2.5. Statistics**

144 Estimated antibody titres of different groups were compared via Mann-Whitney U-test. Survival

145 rates were analysed through log rank test, taking full days survived into consideration. Correlations

146 between titres and titres and survival were estimated via spearman-rank correlation test. For all

147 statistical purposes a P-value of 0.05 and smaller was considered significant, resulting in a critical

148 value of 0.564 for r_s for quantities of 10.

149

150 **3. Results and discussion**

151 **Addition of rBclA to rPA83 increased the level of protection when applied together as proteins**

152 Groups of mice vaccinated with either rPA83, rBclA or a combination of both together with

153 lipopeptide adjuvant induced significant IgG antibody titres with a strong IgG1 emphasis against

154 their respective antigens (fig. 1 and 2). The measured antibody titres as well as the NT_{50}-titres

155 (fig. 3) for the groups receiving either rPA83 or rBclA alone were similar to or higher than those

156 seen in the group given both proteins suggesting no synergistic effects or shift in subclass

157 dominance.

158 Each antigen when given alone did not provide significant protection while the combination

159 achieved 70% protection (tab. 1; fig. 4A) indicating that rBclA and rPA83 augment each others

160 ability to protect. These findings are similar to those reported in a previous study where A/J mice

161 were challenged with spores of the Sterne strain of *B. anthracis* [14]. The authors of this study

162 concluded that BclA enhanced protection by inducing the opsonization of spores and stimulating the

163 production of antibodies which also inhibited germination. In addition PA-specific antibodies have

164 been shown to promote spore opsonization and the inhibition of germination in a similar manner to

165 BclA [36]. Thus the protection seen in animals given rPA83 and rBclA may be the result of a

166 synergy in the anti-spore effect rather than a feature of anti-toxin properties. This would explain

167 why we saw no correlation between survival and antibody titres ($r_s \leq \pm 0.53$). This applied also to

168 the NT_{50}-titres of animals immunised with rPA83 as they had generated high toxin neutralising

169 antibodies, a feature which has previously been shown to correlate with protection in other animal

170 models [9][37][38][39]. However to our knowledge such a correlation has not been recorded for

171 outbred mice challenged with Ames.

172 Comparison of the IgG subclass spectrum revealed a strong Th_2 bias, with IgG1 titres equating IgG

173 titres strongly ($r_s \geq +0.81$). Nonetheless significant, but highly scattered IgG2a antibody titres

174 against both antigens were raised in the majority of the animals.

175

176 **DNA-vectors encoding for toxin components were able to significantly protect outbred NMRI**

177 **mice from lethal challenge**

178 In a previous study we reported that the fusion protein LFD1PAD4 was able to fully protect A/J

179 mice against i.p. challenge with ~ 10^5 spores of the Sterne vaccine strain [13]. To determine if a

180 similar level of protection could be achieved when the antigen was presented in the context of a

181 DNA vaccine we introduced the gene encoding the fusion protein into two different DNA vectors

182 and compared the resulting immune responses to those seen using a DNA vaccine expressing full

183 length rPA83. The latter had been shown to significantly protect A/J mice from a lethal challenge

184 with the attenuated STI-1 vaccine strain [35] but not outbred mice from challenge with spores of the

185 fully virulent Ames strain [40].

186 The DNA-vaccines elicited significantly lower (1-2 log10) anti-PA-antibody and NT_{50}-titres

187 (fig. 1and 3) than the protein vaccines which contained rPA83 (tab 1). While the TPA-LFD1PAD4-

188 mIPS1 and TPA-rPA83-LAMP1 constructs stimulated similar anti-rPA83-IgG and -IgG1 titres they

189 were significantly higher than those seen using TPA-LFD1PAD4-LAMP1 which failed to elicit anti-

190 rPA83 titres in the majority of the animals and thus was not distinctive of the pre-immune sera.

191 Of the two DNA constructs which contained LF the TPA-LFD1PAD4-mIPS1 vector induced higher

192 anti-rLF-IgG and -IgG1 titres (fig. 5) and for both constructs the LF specific antibody response

193 before the challenge exceeded that seen against PA as has been noted previously [13]. A positive

194 correlation between anti-rPA83- and anti-rLF-IgG titres to NT_{50}-titres ($r_s \geq +0.66$) was seen for

195 TPA-LFD1PAD4-mIPS1 suggesting that the fusion protein stimulated the production of toxin

196   neutralising antibodies. Indeed significant NT_{50}-titres were only raised in individuals immunised

197   with TPA-LFD1PAD4-mIPS1. With regards to the IgG subclass balance all of the DNA constructs

198   failed to stimulate an IgG2a response for rPA83 and rLF indicating a strong Th_2-specific immune

199   response often seen with GeneGun applications [40][41][41]

200   When challenged with $25LD_{50}$ of fully virulent Ames strain spores a level of protection of between

201   30-40% was accomplished (tab. 1). Interestingly, only the surviving individuals immunised with

202   TPA-LFD1PAD4-LAMP showed a measurable anti-rPA83-IgG titre before the challenge whereas

203   the individuals that succumbed to infection did not, resulting in a strong correlation of anti-rPA83-

204   IgG titres with survival (r_s = +0.888). Furthermore, anti-rLF-IgG titres were induced in all animals

205   and showed a correlation to survival (r_s = +0.685) and anti-rPA83-IgG titres (r_s = +0.885). A notable

206   difference concerning the subclass bias of the two adjuvants tested (CIITA and mIPS-1) was not

207   present since their mode of action results in an up-regulation of the MHC-genes neither favouring

208   IgG1 nor IgG2a responses. However the variable immunogenicity of the different vectors might be

209   attributed to the different adjuvants utilised. In conclusion the toxin vectors examined in this study

210   elicited slightly lower PA-specific antibody titres than seen in an earlier study, but were equally

211   protective [40].

212

213   **DNA-vectors encoding for BclAD1D3 possibly stimulated sterile immunity and protected 50%**

214   **of the animals**

215   BclAD1D3 was cloned into two different DNA vaccine vectors, one in which the gene was flanked

216   by TPA and LAMP1 and a second in which BclAD1D3 was directed to the proteasome by the

217   incorporation of an N-terminal ubiquitin signal sequence. Both constructs elicited robust anti-

218   rBclA-IgG, -IgG1 and -IgG2a antibody responses (fig. 2) comparable to those seen for full length

219   rBclA given as a protein. We saw no significant difference in the magnitude of the immune response

220   for either DNA vaccine suggesting that the various routing signals had no detectable influence. The

221 only discernable difference between both concerned the IgG2a titres, which were detectable in all

222 individuals immunised with TPA-BclAD1D3-LAMP1 but not when BclAD1D3-Ubiquitin was

223 applied. It is possible that by using a GeneGun to deliver each DNA vaccine any beneficial effect

224 provided by targeting routing was masked [41][42]. Our previous study with full length BclA

225 applied via GeneGun in a vector containing a different secretion signal, depicted a similar antibody

226 spectrum with a distinctive IgG1 bias [40].

227 Both of the vaccines tested in our study protected 50% of the animals when challenged with fully

228 virulent spores (fig. 1B). Analysis of the blood of animals which have survived challenge gave the

229 indication of a possible sterile infection as inferred from lack of PA antibodies [43]. To our

230 knowledge the level of protection achieved in this study following immunisation with BclAD1D3

231 exceeds that previously reported for BclA in protein or DNA vaccine form [14][40][44][20].

232

233 **A combined DNA vaccine comprising TPA-LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1**

234 **stimulated significant protection against a lethal spore challenge**

235 To determine if we could further increase the level of protective immunity we co-immunised mice

236 with TPA-LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1 and subjected them to a lethal spore

237 challenge. The TPA-BclAD1D3-LAMP vector was selected for these studies as it had previously

238 been reported that DNA-vectors containing ubiquitin reacted adversely in the presence of other

239 DNA vector signal sequences [45]. Following a lethal spore challenge ($25LD_{50}$) 90% of the animals

240 survived and there was an increase in time to death for the single animal that succumbed to

241 infection (tab 1).

242 This increased protection was mirrored by an increase in the magnitude of the anti-rPA83-IgG and -

243 IgG1 titres (fig. 1) in that they significantly exceeded those of the single vectors although they were

244 still significantly lower than those seen in animals who had received protein vaccines containing

245 rPA83. The enhanced antibody titres against rPA83 were not associated with a higher NT_{50}-titre

246   suggesting that the immune response was directed against other epitopes, possibly targets that

247   promote opsonization or inhibit germination. While the boost in anti-rPA83 titres as a consequence

248   of co-administration of spore antigens has been seen previously, it was in animals who had been

249   immunised with a protein formulation [44]. Our corresponding protein combination did not mimic

250   this trend.

251   The anti-rLF-IgG titres observed following co-administration were on the whole similar to those

252   seen after immunisation with TPA-LFD1PAD4-mIPS-1 alone and showed less scattering. The NT_{50}-

253   titre correlated strongly with anti-rLF-IgG (r_s = +0.85) and -IgG1 titres (r_s = +0.918) and weakly

254   with anti-rPA83 IgG and -IgG1 titres ($r_s \geq$ +0.61). This was also true when TPA-LFD1PAD4-mIPS1

255   was given alone although the correlations were much weaker, thus the tna was positively influenced

256   for both parts of the antigen alike when combined with BclA under the presence of both adjuvants

257   while the overall titres generated were diversified.

258   Surprisingly the anti-rBclA titre of the DNA-vaccine combination and to a lesser extend that of the

259   protein combination, was significantly lower than that seen for either single vaccine application

260   (fig. 2). While an antagonistic effect of BclA on PA titre has been observed in other studies and has

261   been ascribed in part to "antigenic competition" [14][46][44] a negative effect of PA on BclA titres,

262   as seen here, has not been reported. Apart from the possibility of DNA-vector interferences [45] and

263   the possible influence of the presence of the adjuvant mIPS-1 in addition to CIITA we have no

264   explanation for this phenomenon.

265   However, the overall individual titres measured for the DNA-combination showed less scattering

266   within the group compared to the titres seen in the groups given a single vector. Thus BclA and PA

267   given together might have a balancing effect leading to a more stable immune response which

268   might contribute to enhanced survival.

269   In conclusion a DNA vaccine comprising a combination of plasmids encoding spore and toxin

270   targets conferred significantly greater protection than that seen for the individual DNA plasmids

11

271 (P ≤ 0.042), the single protein components (P ≤ 0.00008) and the vectors tested in our previous

272 study [40]. Furthermore it was found to be as effective as a comparable protein combination

273 (P = 0.251). To our knowledge this is the first example of a vaccination strategy against Anthrax in

274 which the protective immunity achieved using a DNA based approach exceeded that seen with

275 protein equivalents [40].

276

284

285

References

[1] Turnbull PCB. Introduction: Anthrax History, Disease and Ecology. In: Koehler TM, editors. Anthrax. Current topics in microbiology and immunology, Berlin Heidelberg: Springer-Verlag; 2002, p. 1-19.

[2] Dixon TC, Meselson M, Guillemin J, Hanna PC. Anthrax. N Engl J Med 1999; 341:815–26.

[3] Uchida I, Sekizaki T, Hashimoto K, Terakado N. Association of the Encapsulation of Bacillus anthracis with a 60 Megadalton Plasmid. J Gen Microbiol 1985; 131:363-7.

[4] Mikesell P, Ivins BE, Ristroph JD, Dreier TM. Evidence for Plasmid-Mediated Toxin Production in Bacillus anthracis. Infect Immun 1983; 39:371-6.

[5] Smith H, Keppie J, Stanley JL. The chemical basis of the virulence of Bacillus anthracis. V: The specific toxin produced by B. anthracis in vivo. Br J Exp Pathol 1955; 36:460-72.

[6] Leppla SH. Anthrax Toxin. In: Aktories K, Just I, editors. Bacterial protein toxins, Berlin Heidelberg: Springer-Verlag; 2000, p. 445-72.

[7] Turnbull PCB. Anthrax vaccines: past, present and future. Vaccine 1991; 9:533-9.

[8] Stepanov AV, Marinin LI, Pomerantsev AP, Staritsin NA. Development of novel vaccines against anthrax in man. J Bacteriol 2006; 44:155-160.

[9] Little S, Ivins B, Fellows P, Pitt M, Norris S, Andrews G. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. Vaccine 2004; 22:422-30.

[10] Price BM, Liner AL, Park S, Leppla SH, Mateczun A, Galloway DR. Protection against Anthrax Lethal Toxin Challenge by Genetic Immunization with a Plasmid Encoding the Lethal Factor Protein. Infect Immun 2001; 69:4509-15.

[11] Flick-Smith HC, Walker NJ, Gibson P, Bullifent H, Hayward S, Miller J et al. A Recombinant Carboxy-Terminal Domain of the Protective Antigen of Bacillus anthracis Protects Mice against Anthrax Infection. Infect Immun 2002; 70:1653-6.

[12] Stokes MGM, Titball RW, Neeson B, Galen JE, Walker NJ, Stagg AJ et al. Oral Administration of a Salmonella enterica-Based Vaccine Expressing Bacillus anthracis Protective Antigen Confers Protection against Aerosolized B. anthracis. Infect Immun 2007; 75:1827-34.

[13] Baillie LW, Huwar TB, Moore S, Mellado-Sanchez G, Rodriguez L, Neeson BN et al. An anthrax subunit vaccine candidate based on protective regions of Bacillus anthracis protective antigen and lethal factor. Vaccine 2010; 28:6740-8.

[14] Brahmbhatt TN, Darnell SC, Carvalho HM, Sanz P, Kang TJ, Bull RL, Rasmussen SB et al. Recombinant exosporium protein BclA of bacillus anthracis is effective as a booster for mice primed with suboptimal amounts of protective antigen. Infect Immun 2007; 75:5240-7.

[15] Enkhtuya J, Kawamoto K, Kobayashi Y, Uchida I, Neeraj R, Makino S. Significant passive protective effect against anthrax by antibody to Bacillus anthracis inactivated spores that lack two virulence plasmids. Microbiology 2006; 152:3103-10.

[16] Brossier F, Levy M, Mock M. Anthrax Spores Make an Essential Contribution to Vaccine Efficacy. Infect Immun 2002; 70:661-4.

[17] Sylvestre P, Couture-Tosi E, Mock M. A collagen-like surface glycoprotein is a structural component of the Bacillus anthracis exosporium. Mol Microbiol 2002; 45:169-78.

[18] Steichen C, Chen P, Kearney JF, Turnbough Jr C. Identification of the immunodominant protein and other proteins of the Bacillus anthracis exosporium. J Bacteriol 2003; 185:1903-10.

[19] Boydston JA, Chen P, Steichen CT, Turnbough CL. Orientation within the exosporium and structural stability of the collagen-like glycoprotein BclA of Bacillus anthracis. J Bacteriol 2005; 187:5310-7.

[20] Liu C-Q, Nuttall SD, Tran H, Wilkins M, Streltsov VA, Alderton MR. Construction, Crystal Structure and Application of a Recombinant Protein That Lacks the Collagen-Like Region of BclA

From Bacillus anthracis Spores. Biotechnol Bioeng 2007; 99:774-82.

[21] Williams JA, Luke J, Johnson L, Hodgson C. pDNAVACCultra vector family: high throughput intracellular targeting DNA vaccine plasmids. Vaccine 2006; 24:4671-6.

[22] Nuchtern JG, Biddison WE, Klausner RD. Class II MHC molecules can use the endogenous pathway of antigen presentation. Nature 1990; 343:74-6.

[23] Li Z, Howard A, Kelley C, Delogu G, Collins F, Morris S. Immunogenicity of DNA vaccines expressing tuberculosis proteins fused to tissue plasminogen activator signal sequences. Infect Immun 1999; 67:4780-6.

[24] Drake JR, Lewis TA, Condon KB, Mitchell RN, Webster P. Involvement of MIIC-like late endosomes in B cell receptor mediated antigen processing in murine B cells. J Immunol 1999; 162:1150-5.

[25] Chen JW, Murphy TL, Willingham MC, Pastan I, August JT. Identification of two lysosomal membrane glycoproteins. J Cell Biol 1985; 101:85-95.

[26] Johnson E, Bartel B, Seufert W, Varshavsky A. Ubiquitin as a degradation signal. EMBO J 1992; 11:497-505.

[27] Delogu G, Howard A, Collins FM, Morris SL. DNA Vaccination against Tuberculosis: Expression of a Ubiquitin-Conjugated Tuberculosis Protein Enhances Antimycobacterial Immunity. Infect Immun 2000; 68:3097-102.

[28] Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H. IPS-1, an adaptor triggering RIG-I- and MDA5-mediated type 1 interferon induction. Nat Immunol 2005; 6:981-8.

[29] Johnson CL, Gale M. Jr. CARD games between virus and host get a new player. Trends Immunol 2006; 27:1-4.

[30] Yonehama M, Fujita T. RNA recognition and signal transduction by RIG-1-like receptors. Immunology 2009; 227:54-65.

[31] Kim D, Hoory T, Monie A, Ting JP-Y, Hung C-F, Wu T-C. Enhancement of DNA vaccine potency through co-administration of CIITA DNA with DNA vaccines via gene gun. J Immunol 2008; 180:7019-27.

[32] Ballachanda ND, Singer SS. CIITA and its dual roles in MHC gene transcription. Front Immun 2013; 4:1-6.

[33] Ghielmetti M, Reschner A, Zwicker M, Padovan E. Synthetic bacterial lipopeptide analogs: structural requirements for adjuvanticity. Immunbiol 2005; 210:211-5.

[34] Wiesmüller K-H, Fleckenstein B, Jung G. Peptide Vaccines and Peptide Libraries. Biol Chem 2001; 382:571-579.

[35] Hahn UK, Alex M, Czerny CP, Böhm R, Beyer W. Protection of mice against challenge with Bacillus anthracis STI spores after DNA vaccination. Int J Med Microbiol 2004; 294:35-44.

[36] Cote CK, Rossi CA, Kang AS, Morrow PR, Lee JS, Welkos SL. The detection of protective antigen (PA) associated with spores of Bacillus anthracis and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb Pathogenesis 2005; 38:209-25.

[37] Pitt MLM, Little SF, Ivins BE, Fellows P, Barth J, Hewetson J et al. In vitro correlate of immunity in a rabbit model of inhalational anthrax. Vaccine 2001; 19:4768-73.

[38] Ionin B, Hopkins RJ, Pleune B, Sivko GS, Reid FM, Clement KH et al. Evaluation of Immunogenicity and Efficacy of Anthrax Vaccine Adsorbed for Postexposure Prophylaxis. Clin Vacc Immunol 2013; 20:1016-26.

[39] Abboud N, Casadevall A. Immunogenicity of Bacillus anthracis Protective Antigen Domains and Efficacy of Elicited Antibody Responses Depend on Host Genetic Background. Clin Vaccine Immunol ; 15:1115-23.

[40] Hahn UK, Boehm R, Beyer W. DNA vaccination against anthrax in mice—combination of anti-spore and anti-toxin components. Vaccine 2006; 24:4569-71.

[41] Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J Immunol 1997;

158:2278-84.

[42] Weiss R, Scheiblhofer S, Freund J, Ferreira F, Livey I, Thalhamer J. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that overrules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. Vaccine 2002; 20:3148-54.

[43] Hermanson G, Whitlow V, Parker S, Tonsky K, Rusalov D, Ferrari et al. A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores. PNAS 2004; 101:13601-6.

[44] Cote CK, Kaatz L, Reinhardt J, Bozue J, Tobery SA, Bassett AD et al. Characterization of a multi-component anthrax vaccine designed to target the initial stages of infection as well as toxaemia. J Med Microbiol 2012; 61:1380-92.

[45] Midha S, Bhatnagar R. Anthrax protective antigen administered by DNA vaccination to distinct subcellular locations potentiates humoral and cellular immune responses. Eur J Immunol 2009; 39:159-177.

[46] Cybulski RJ Jr, Sanz P, McDaniel D, Darnell S, Bull RL, O'Brien AD. Recombinant Bacillus anthracis spore proteins enhance protection of mice primed with suboptimal amounts of protective antigen. Vaccine 2008; 26:4927-39.

Table 1

Groups	Antibody titres [d]				Vaccine efficacy (%)	survivors / total	Mean time to death [g]
	PA IgG	LF IgG	BclA IgG	TNA			
Protein vaccination							
Lipopeptide adjuvant	<200	n.d.	<200	<100	0% [e]	(0/5) [e]	4.2 ± 3.3
Lipopeptide + rBclA	<200	n.d.	257066 ± 149351	<100	10% [e]	(1/10) [e]	3.4 ± 0.7
Lipopeptide + rPA83	561803 ± 113102	n.d.	<200	14564 ± 4543	10% [e]	(1/10) [e]	3.7 ± 1.4
Lipopeptide + rBclA + rPA83	415030 ± 199480	n.d.	123442 ± 42637	15581 ± 9683	70% [e]	(7/10) [e]	5.2 ± 3.2
DNA vaccination							
CIITA [a]	<200	<200	<200	<100	0% [f]	(0/5) [f]	3.1 ± 0.7
TPA-LFD1PAD4-mIPS1 [a]	22488 ± 28511	80682 ± 93200	<200	691 ± 772	30% [f]	(3/10) [f]	4.1 ± 1.2
CIITA + TPA-LFD1PAD4-LAMP1 [b]	2135 ± 4065	14443 ± 9708	<200	<100	40% [f]	(4/10) [f]	4.2 ± 0.7
CIITA + TPA-PA83-LAMP1 [b]	31645 ± 26067	<200	<200	239 ± 524	30% [f]	(3/10) [f]	4.0 ± 1.0
CIITA + TPA-BclAD1D3-LAMP1 [b]	<200	<200	166381 ± 106094	<100	50% [f]	(5/10) [f]	4.2 ± 1.6
CIITA + BclAD1D3-Ubiquitin [b]	<200	<200	221171 ± 150564	<100	50% [f]	(5/10) [f]	4.5 ± 1.7
CIITA + TPA-LFD1PAD4-mIPS1 + TPA-BclAD1D3-LAMP1 [c]	60015 ± 32158	57728 ± 37181	34528 ± 15256	463 ± 682	90% [f]	(9/10) [f]	9.0 ± 0.0

[a] immunisation with uniform bullets, resulting in a dose of 3 µg per vector per immunisation

[b] immunisation with mixed bullets (1:1) reducing the effectively applied amount of each vaccine vector to 1.5 µg per vaccination
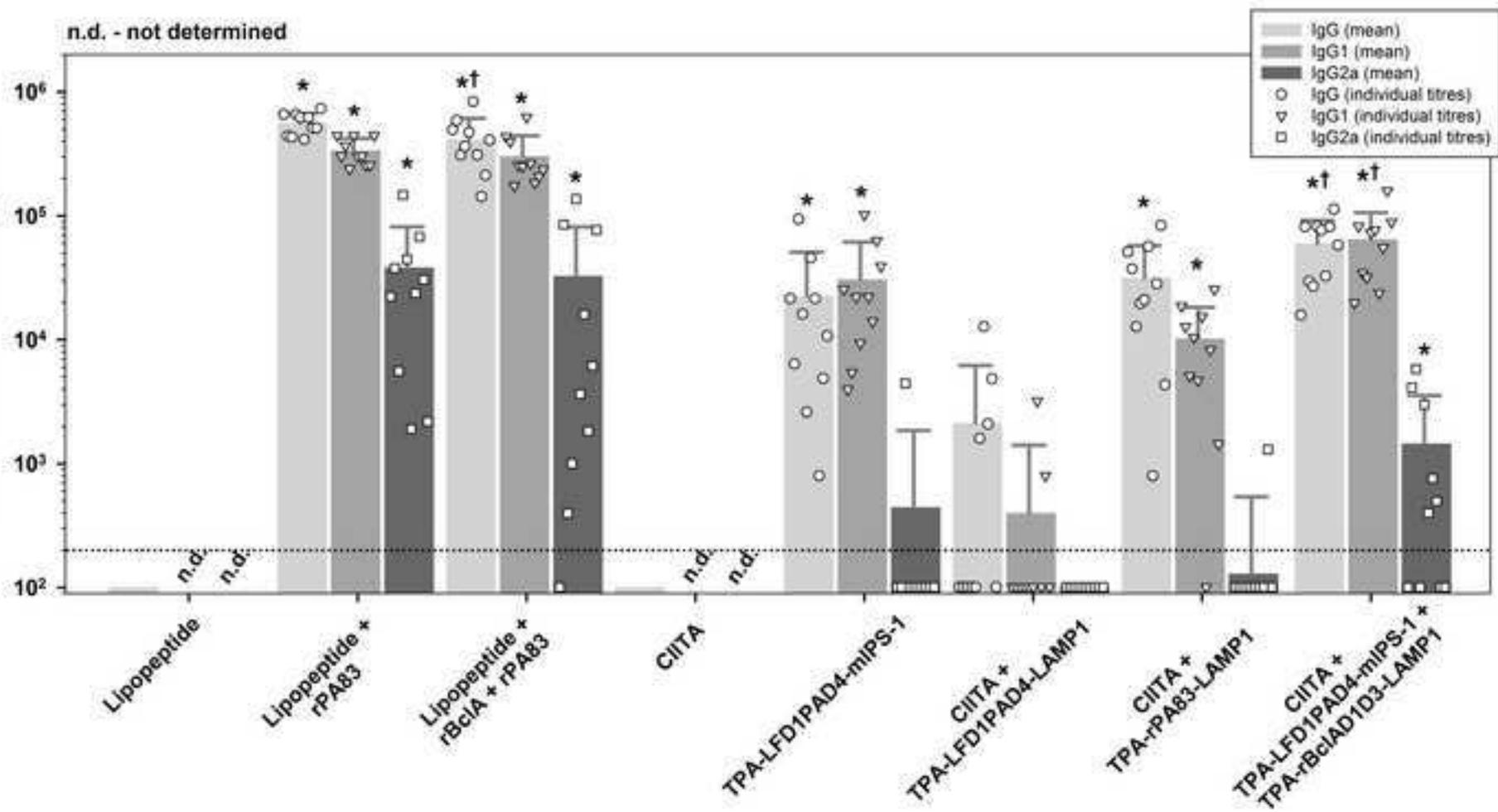
[c] immunisation with mixed bullets (1:2:1) reducing the effectively applied amount of each vaccine vector to 1.5, 3 and 1.5 µg respectively per vaccination

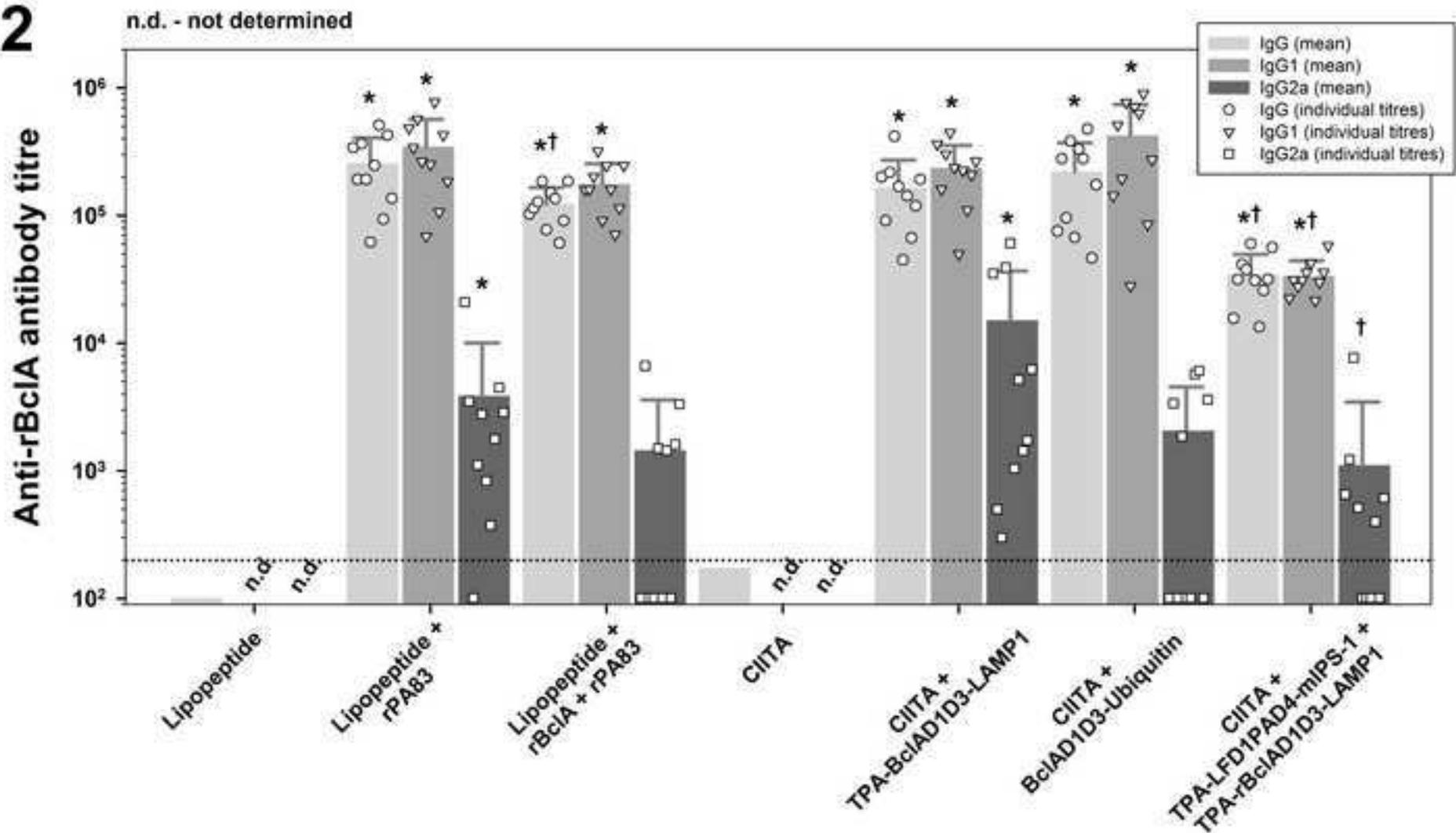[d] group mean IgG or NT_{50} titres ± standard deviation before the challenge

[e] protein groups were challenged with $50LD_{50}$

[f] DNA groups were challenged with $25LD_{50}$

[g] time to death in days post challenge ± standard deviation

Figure 1.

n.d. - not determined

2

Anti-rBclA antibody titre

Legend:
- IgG (mean)
- IgG1 (mean)
- IgG2a (mean)
- IgG (individual titres)
- IgG1 (individual titres)
- IgG2a (individual titres)

X-axis categories:
- Lipopeptide
- Lipopeptide + rPA83
- Lipopeptide + rBclA + rPA83
- CIITA
- CIITA + TPA-BclAD1D3-LAMP1
- CIITA + BclAD1D3-Ubiquitin
- CIITA + TPA-LFD1PAD4-mIPS-1 + TPA-rBclAD1D3-LAMP1

3

4A

Survivors [%]

Days post infection

- ▽ — Lipopeptide
- ● — Lipopeptide + rBclA
- □ — Lipopeptide + rPA83
- △ — Lipopeptide + rBclA + rPA83

4B

Survivors [%]

Days post infection

- ▽ — CIITA
- □ — TPA-LFD1PAD4-mtPS-1
- ● — TPA-LFD1PAD4-LAMP1 + CIITA
- ▽ — TPA-PA83-LAMP1 + CIITA
- ■ — TPA-BclA-LAMP1 + CIITA
- ○ — BclA-Ubiquitin + CIITA
- △ — TPA-LFD1PAD4-mtPS-1 + TPA-BclA-LAMP1 + CIITA

5

Legend:
- IgG (mean)
- IgG1 (mean)
- IgG2a (mean)
- ○ IgG (individual titres)
- ▽ IgG1 (individual titres)
- □ IgG2a (individual titres)

n.d. - not determined

Y-axis: Anti-rLF antibody titre (10^2, 10^3, 10^4, 10^5)

X-axis categories:
- CIITA
- TPA-LFD1PAD4-mIPS-1
- CIITA + TPA-LFD1PAD4-LAMP1
- CIITA + TPA-LFD1PAD4-mIPS-1 + TPA-rBclAD1D3-LAMP1