BclA and toxin antigens augment each other to protect NMRI mice from lethal Bacillus anthracis challenge

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Abstract

While proving highly effective in controlling Anthrax in farm animals all over the world currently attenuated live anthrax vaccines employed in a veterinary context suffer from drawbacks such as residual virulence, short term protection, variation in quality and, most importantly, lack of efficacy if administered simultaneously with antibiotics. These limitations have stimulated the development of non-living component vaccines which induce a broad spectrum immune response capable of targeting both toxaemia (as in the case of PA based vaccines) and bacteraemia. To contribute to this several new approaches were tested in outbred NMRI mice for antibody titres and protectiveness. Plasmids encoding a recombinant toxin derived fusion peptide and a spore surface derived peptide were tested as DNA-vaccines in comparison to their protein counterparts utilising two adjuvant approaches and two DNA-vector backbones. The combination of two plasmids encoding

LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1, when delivered by GeneGun, protected 90% of the animals against a lethal challenge with $25LD_{50}$ spores of the Ames strain of *Bacillus anthracis*. Single applications of either antigen component showed significantly lower protection rates, indicating the beneficial interaction between anti-spore and anti-toxin components for an acellular vaccine formulation.

1. Introduction

Anthrax is caused by *Bacillus anthracis*, a Gram-positive, spore forming, rod-shaped bacterium [1]. Spores gain access via cutaneous, oral or inhalational routes where they germinate and develop into vegetative bacilli which then replicate and produce toxins which eventually kill the host [2]. The pathogen expresses two major plasmid encoded virulence factors, a gamma-linked poly-D-glutamic acid capsule (pX02 [3]) and a tripartite toxin (pX01 [4]) comprised of Protective Antigen (PA)[1], Lethal Factor (LF)[2] and Edema Factor (EF)[3] [5][6].

Current live attenuated veterinary anthrax vaccines are less than ideal. They can cause problems in sensitive animals such as goats and llamas, protection is short term, variation in vaccine quality can cause vaccine failure and most importantly the live nature of the vaccine prevents its efficacy if delivered at the same time as antibiotics [7][8]. These limitations have stimulated the development of non-living, component vaccines capable of inducing a broad spectrum immune response which targets both toxaemia and bacteraemia.

The strong correlation between toxin neutralising activity (tna)[4] of PA-specific antibodies and protection [9] has prompted efforts to develop vaccines based solely on domains which stimulate antibodies with tna [10][11][12]. One such study which employed a fusion protein comprised of 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

a subsequent lethal challenge with *B. anthracis* spores [13]. To further assess the immunogenic value of this protein we administered it as a DNA-vaccine in two different vectors and compared its activity to that seen against full length rPA83.

In addition to neutralising the action of toxins the spore can also be targeted to prevent the pathogen from gaining a foothold in the infected individual [14][15]. Vaccination experiments with live nonvirulent or formaldehyde-inactivated spores have shown that spore specific immune responses can enhance the level of protection when given in combination with PA [16].

One such component is the *Bacillus* collagen like protein of *anthracis* (BclA)[5] which forms hair-like structures projecting from the spore surface and represents a major spore immunogen [17][18]. The removal of the collagen-like region (CLR[6], domain 2) from BclA has no detrimental effect on immunogenicity and results in a smaller peptide which is easier to incorporate into a multicomponent vaccine [19][20]. In this study we determined the immunogenicity of a CLR-deficient version of BclA called rBclAD1D3 when administered as a DNA-vaccine in two different vectors.

For the DNA vaccine studies we employed two different plasmid backbones (pDNAVaccUltra and NTC7382) which varied with regards to intracellular routing signals and immune stimulatory elements [21]. To improve *in vivo* antigen presentation we utilised intracellular routing signals which directed vaccine peptides to the MHC I and MHC II pathways. To target the MHC II pathway [22] we employed tissue plasminogen activator (TPA)[7] which routes newly expressed proteins to the secretion pathway [23] and lysosome-associated membrane protein (LAMP1)[8] which 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

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

To enhance the immunogenicity of the expressed proteins we investigated the utility of two molecular adjuvants. Mouse interferon-ß promoter stimulator 1 (mIPS-1)[9] incorporated into the backbone of the antigen encoding plasmid significantly induces type I interferon and interferon-stimulated genes in a TLR-independent matter [28][29][30]. Mouse class II MHC trans-activator (CIITA)[10] up-regulates MHC expression [31][32] and was co-administered on a separate plasmid.
In comparison to the DNA vaccines, full length rPA and rBclA were tested as proteins alone and in combination in the presence of a previously tested and approved lipopeptide adjuvant comprising Pam3Cys-SKKKK, a TLR2/1 activator admixed with Pam_3Cys conjugated to the promiscuitive T-helper-cell epitope of the sperm whale myoglobin SFISEAIIHVLHSRHPG [33][34].
The overall aim of this study was to determine the ability of BclA to confer additional protectiveness when given together with a toxin-specific vaccine.

2. Material and Methods

2.1. Antigen preparation

E. coli BL21-CodonPlus-RIL cells (Stratagene, La Jolla, CA) harboring the plasmid pREP 4 (Qiagen, Venlo, Netherland) and pQE-30 (Qiagen) encoding either rPA83, rBclA or rLF were grown and purified as described previously [35]. Proteins used for ELISA received no further treatment while proteins used for vaccination were tested for endotoxin using the Limulus Amoebocyte Lysate Endochrome-K test kit (Charles River, Wilmington, MA) as described by the manufacturer. Endotoxin removal was performed via EndoTrap blue endotoxin removal system (Hyglos, Bernried, Germany).

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

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

challenge, a dose of 200 µl of a fully virulent Ames strain containing ~1000 spores ($25LD_{50}$) for all DNA-vaccines and ~2000 spores ($50LD_{50}$) for all protein vaccines was administered s.c. in the neck. The increased challenge dose for the protein vaccines was utilised due to the residual protectiveness of the lipopeptide alone observed when challenged with lower spore doses (data not shown), thus normalising its effect to allow for a better discrimination between different antigens and applications. Mice that died during the challenge or were killed via CO_2 after the end of the challenge were dissected to prepare liver and spleen which were then plated on blood agar to verify the presence of the pathogen.

2.4. Serology

ELISA was performed as described previously [35]. Secondary antibodies comprised of horseradish peroxidase conjugated polyclonal goat anti-mouse IgG (Sigma-Aldrich), IgG1 and IgG2a (Acris, San Diego, CA). Endpoint titres were defined as the reciprocal of the highest serum dilution that resulted in an absorbance greater than two standard deviations above the average of the negative control serum sample (pooled naive sera of the according group) with a minimum OD_{414nm} value of 0.1.

Sera were analysed for neutralising antibody titres via in vitro toxin neutralising assay (TNA) as published previously [35]. The neutralisation titre (NT) corresponds to the inverse serum dilution at which the toxin neutralisation yielded 50% (NT_{50}). The NT_{50} was estimated using the SigmaPlot regression wizard (4-parameter sigmoid regression curve). Detailed Titres are only described for sera taken before the challenge.

2.5. Statistics

Estimated antibody titres of different groups were compared via Mann-Whitney U-test. Survival rates were analysed through log rank test, taking full days survived into consideration. Correlations

between titres and titres and survival were estimated via spearman-rank correlation test. For all statistical purposes a P-value of 0.05 and smaller was considered significant, resulting in a critical value of 0.564 for r_s for quantities of 10.

3. Results and discussion

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

Groups of mice vaccinated with either rPA83, rBclA or a combination of both together with lipopeptide adjuvant induced significant IgG antibody titres with a strong IgG1 emphasis against their respective antigens (fig. 1 and 2). The measured antibody titres as well as the NT_{50}-titres (fig. 3) for the groups receiving either rPA83 or rBclA alone were similar to or higher than those seen in the group given both proteins suggesting no synergistic effects or shift in subclass dominance.

Each antigen when given alone did not provide significant protection while the combination achieved 70% protection (tab. 1; fig. 4A) indicating that rBclA and rPA83 augment each others ability to protect. These findings are similar to those reported in a previous study where A/J mice were challenged with spores of the Sterne strain of *B. anthracis* [14]. The authors of this study concluded that BclA enhanced protection by inducing the opsonization of spores and stimulating the production of antibodies which also inhibited germination. In addition PA-specific antibodies have been shown to promote spore opsonization and the inhibition of germination in a similar manner to BclA [36]. Thus the protection seen in animals given rPA83 and rBclA may be the result of a synergy in the anti-spore effect rather than a feature of anti-toxin properties. This would explain why we saw no correlation between survival and antibody titres ($r_s \leq \pm 0.53$). This applied also to the NT_{50}-titres of animals immunised with rPA83 as they had generated high toxin neutralising antibodies, a feature which has previously been shown to correlate with protection in other animal models [9][37][38][39]. However to our knowledge such a correlation has not been recorded for

outbred mice challenged with Ames.

Comparison of the IgG subclass spectrum revealed a strong Th_2 bias, with IgG1 titres equating IgG titres strongly ($r_s \geq +0.81$). Nonetheless significant, but highly scattered IgG2a antibody titres against both antigens were raised in the majority of the animals.

DNA-vectors encoding for toxin components were able to significantly protect outbred NMRI mice from lethal challenge

In a previous study we reported that the fusion protein LFD1PAD4 was able to fully protect A/J mice against i.p. challenge with ~ 10^5 spores of the Sterne vaccine strain [13]. To determine if a similar level of protection could be achieved when the antigen was presented in the context of a DNA vaccine we introduced the gene encoding the fusion protein into two different DNA vectors and compared the resulting immune responses to those seen using a DNA vaccine expressing full length rPA83. The latter had been shown to significantly protect A/J mice from a lethal challenge with the attenuated STI-1 vaccine strain [35] but not outbred mice from challenge with spores of the fully virulent Ames strain [40].

The DNA-vaccines elicited significantly lower (1-2 log10) anti-PA-antibody and NT_{50}-titres (fig. 1and 3) than the protein vaccines which contained rPA83 (tab 1). While the TPA-LFD1PAD4-mIPS1 and TPA-rPA83-LAMP1 constructs stimulated similar anti-rPA83-IgG and -IgG1 titres they were significantly higher than those seen using TPA-LFD1PAD4-LAMP1 which failed to elicit anti-rPA83 titres in the majority of the animals and thus was not distinctive of the pre-immune sera.

Of the two DNA constructs which contained LF the TPA-LFD1PAD4-mIPS1 vector induced higher anti-rLF-IgG and -IgG1 titres (fig. 5) and for both constructs the LF specific antibody response before the challenge exceeded that seen against PA as has been noted previously [13]. A positive correlation between anti-rPA83- and anti-rLF-IgG titres to NT_{50}-titres ($r_s \geq +0.66$) was seen for 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

only discernable difference between both concerned the IgG2a titres, which were detectable in all individuals immunised with TPA-BclAD1D3-LAMP1 but not when BclAD1D3-Ubiquitin was applied. It is possible that by using a GeneGun to deliver each DNA vaccine any beneficial effect provided by targeting routing was masked [41][42]. Our previous study with full length BclA applied via GeneGun in a vector containing a different secretion signal, depicted a similar antibody spectrum with a distinctive IgG1 bias [40].

Both of the vaccines tested in our study protected 50% of the animals when challenged with fully virulent spores (fig. 1B). Analysis of the blood of animals which have survived challenge gave the indication of a possible sterile infection as inferred from lack of PA antibodies [43]. To our knowledge the level of protection achieved in this study following immunisation with BclAD1D3 exceeds that previously reported for BclA in protein or DNA vaccine form [14][40][44][20].

A combined DNA vaccine comprising TPA-LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1 stimulated significant protection against a lethal spore challenge

To determine if we could further increase the level of protective immunity we co-immunised mice with TPA-LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1 and subjected them to a lethal spore challenge. The TPA-BclAD1D3-LAMP vector was selected for these studies as it had previously been reported that DNA-vectors containing ubiquitin reacted adversely in the presence of other DNA vector signal sequences [45]. Following a lethal spore challenge ($25LD_{50}$) 90% of the animals survived and there was an increase in time to death for the single animal that succumbed to infection (tab 1).

This increased protection was mirrored by an increase in the magnitude of the anti-rPA83-IgG and -IgG1 titres (fig. 1) in that they significantly exceeded those of the single vectors although they were still significantly lower than those seen in animals who had received protein vaccines containing rPA83. The enhanced antibody titres against rPA83 were not associated with a higher NT_{50}-titre

suggesting that the immune response was directed against other epitopes, possibly targets that promote opsonization or inhibit germination. While the boost in anti-rPA83 titres as a consequence of co-administration of spore antigens has been seen previously, it was in animals who had been immunised with a protein formulation [44]. Our corresponding protein combination did not mimic this trend.

The anti-rLF-IgG titres observed following co-administration were on the whole similar to those seen after immunisation with TPA-LFD1PAD4-mIPS-1 alone and showed less scattering. The NT_{50}-titre correlated strongly with anti-rLF-IgG (r_s = +0.85) and -IgG1 titres (r_s = +0.918) and weakly with anti-rPA83 IgG and -IgG1 titres ($r_s \geq +0.61$). This was also true when TPA-LFD1PAD4-mIPS1 was given alone although the correlations were much weaker, thus the tna was positively influenced for both parts of the antigen alike when combined with BclA under the presence of both adjuvants while the overall titres generated were diversified.

Surprisingly the anti-rBclA titre of the DNA-vaccine combination and to a lesser extend that of the protein combination, was significantly lower than that seen for either single vaccine application (fig. 2). While an antagonistic effect of BclA on PA titre has been observed in other studies and has been ascribed in part to “antigenic competition” [14][46][44] a negative effect of PA on BclA titres, as seen here, has not been reported. Apart from the possibility of DNA-vector interferences [45] and the possible influence of the presence of the adjuvant mIPS-1 in addition to CIITA we have no explanation for this phenomenon.

However, the overall individual titres measured for the DNA-combination showed less scattering within the group compared to the titres seen in the groups given a single vector. Thus BclA and PA given together might have a balancing effect leading to a more stable immune response which might contribute to enhanced survival.

In conclusion a DNA vaccine comprising a combination of plasmids encoding spore and toxin targets conferred significantly greater protection than that seen for the individual DNA plasmids

($P \leq 0.042$), the single protein components ($P \leq 0.00008$) and the vectors tested in our previous study [40]. Furthermore it was found to be as effective as a comparable protein combination ($P = 0.251$). To our knowledge this is the first example of a vaccination strategy against Anthrax in which the protective immunity achieved using a DNA based approach exceeded that seen with protein equivalents [40].

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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

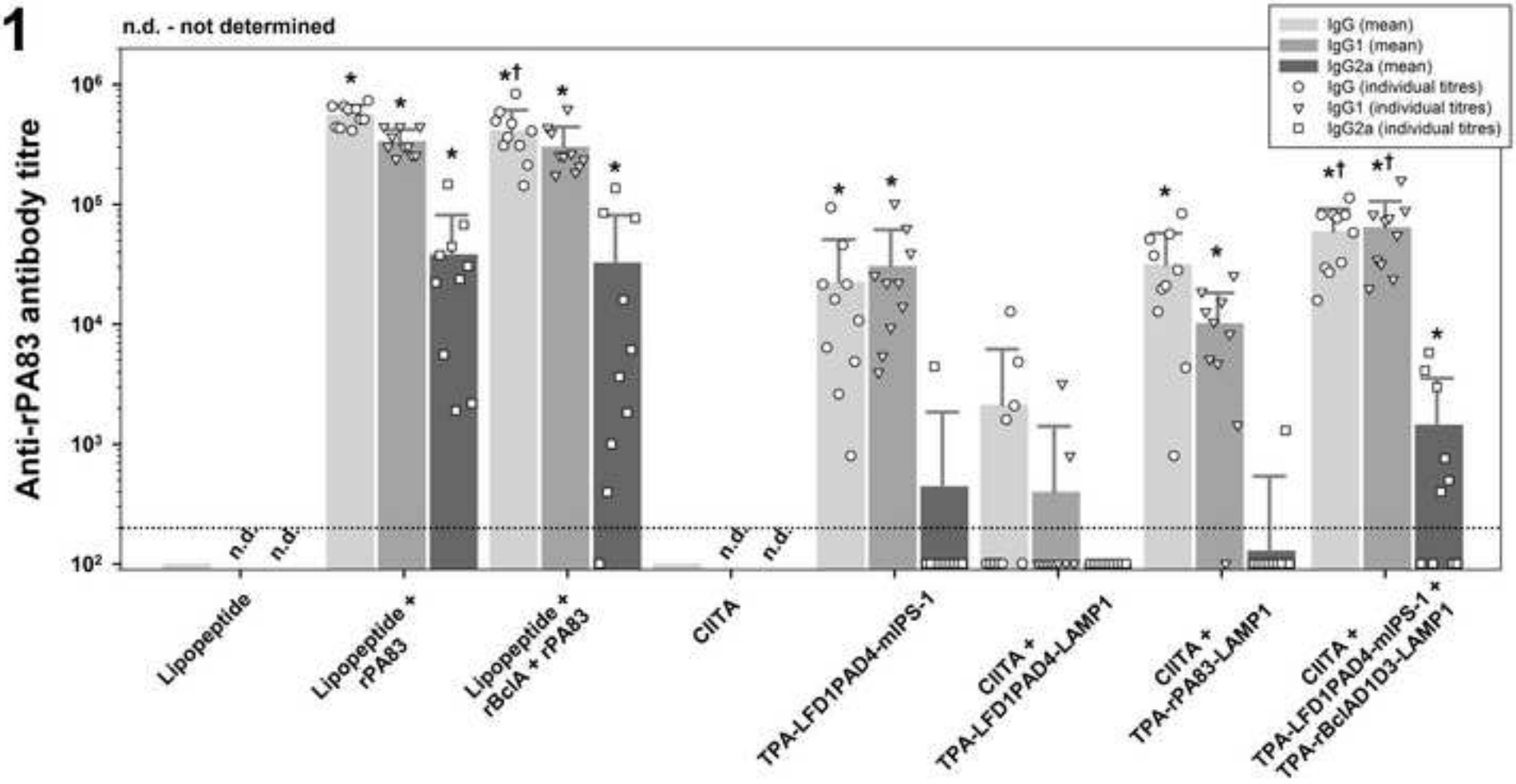
[d] group mean IgG or NT_{50} titres ± standard deviation before the challenge

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

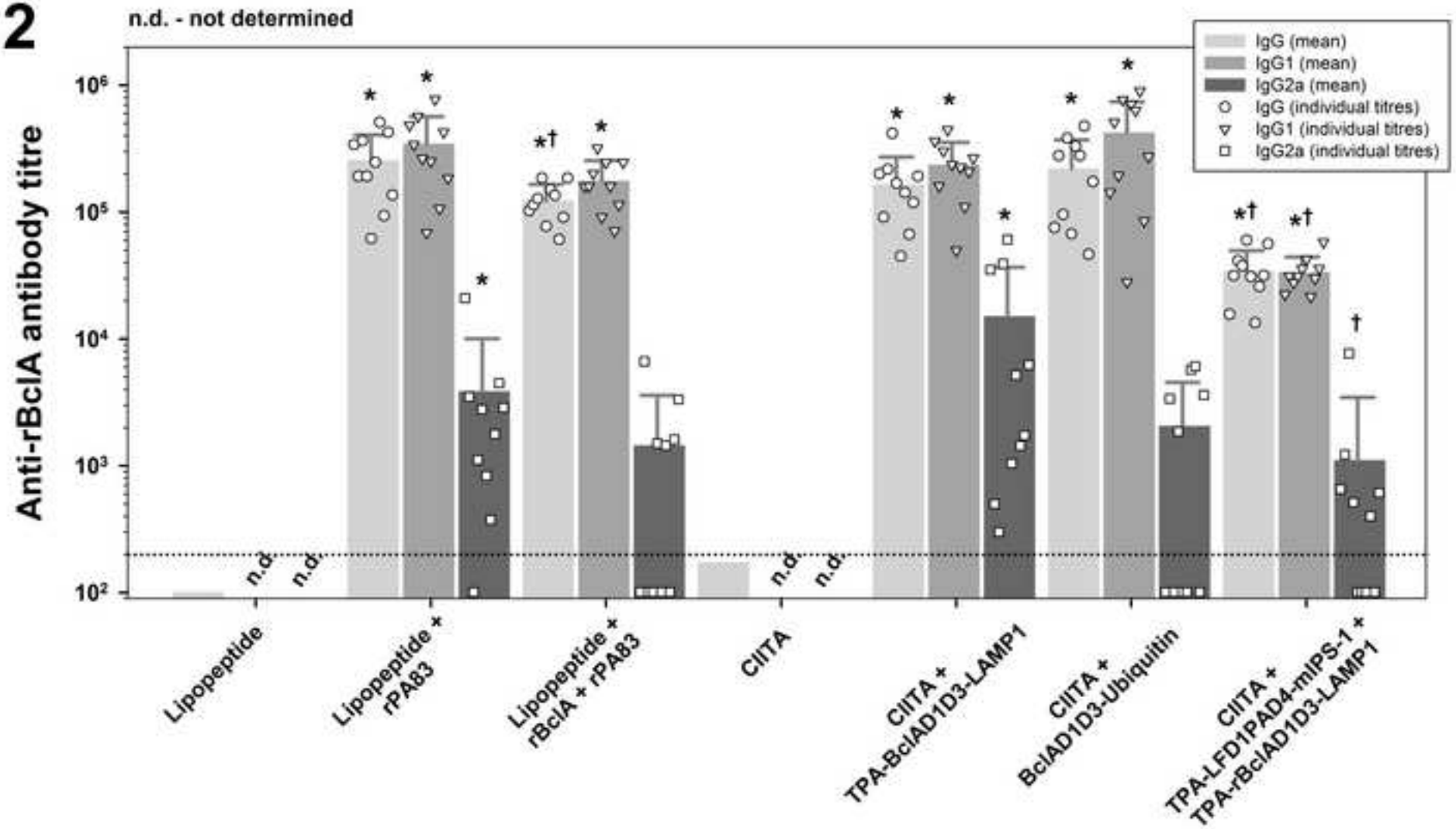
[f] DNA groups were challenged with $25LD_{50}$

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

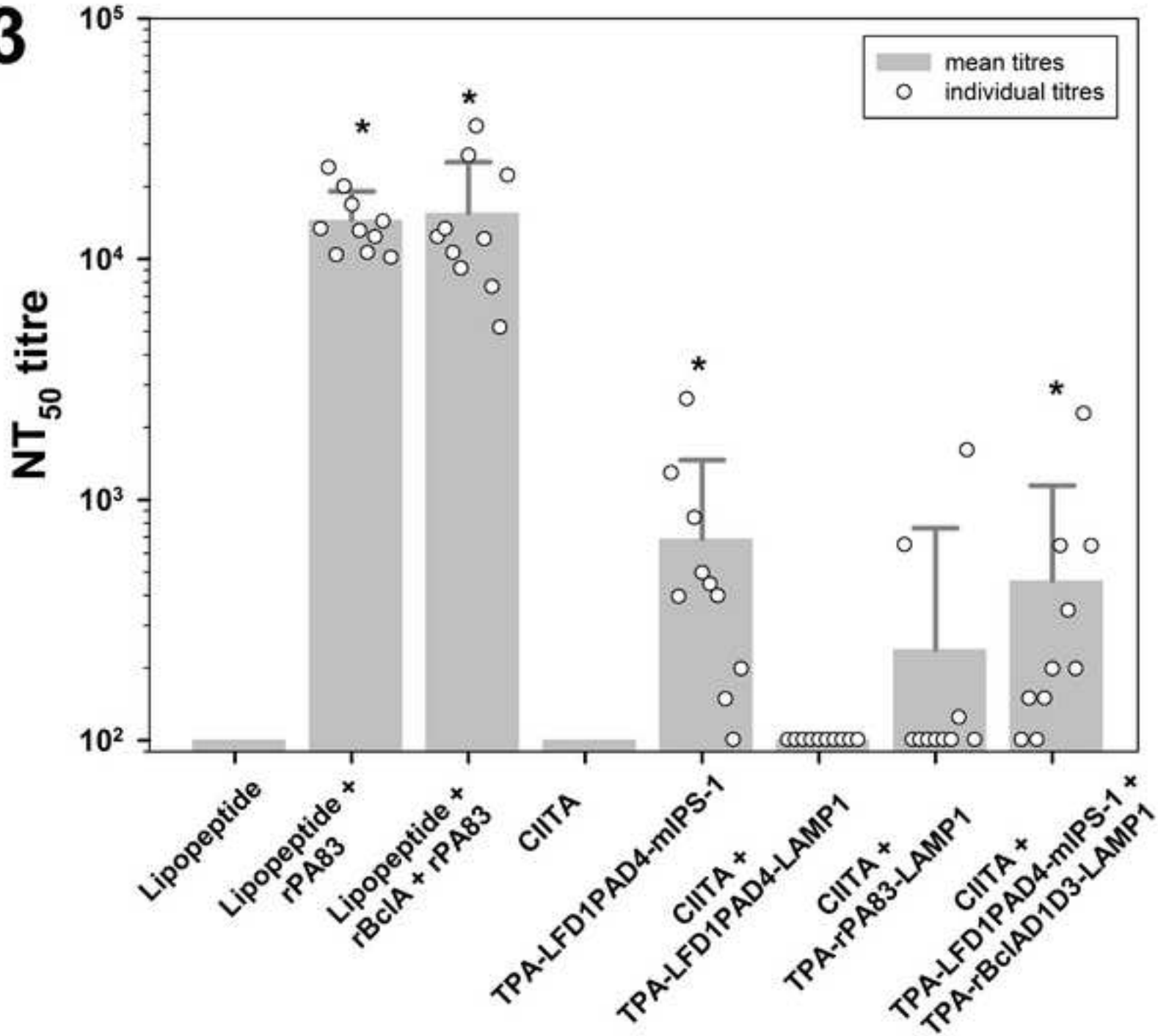
1

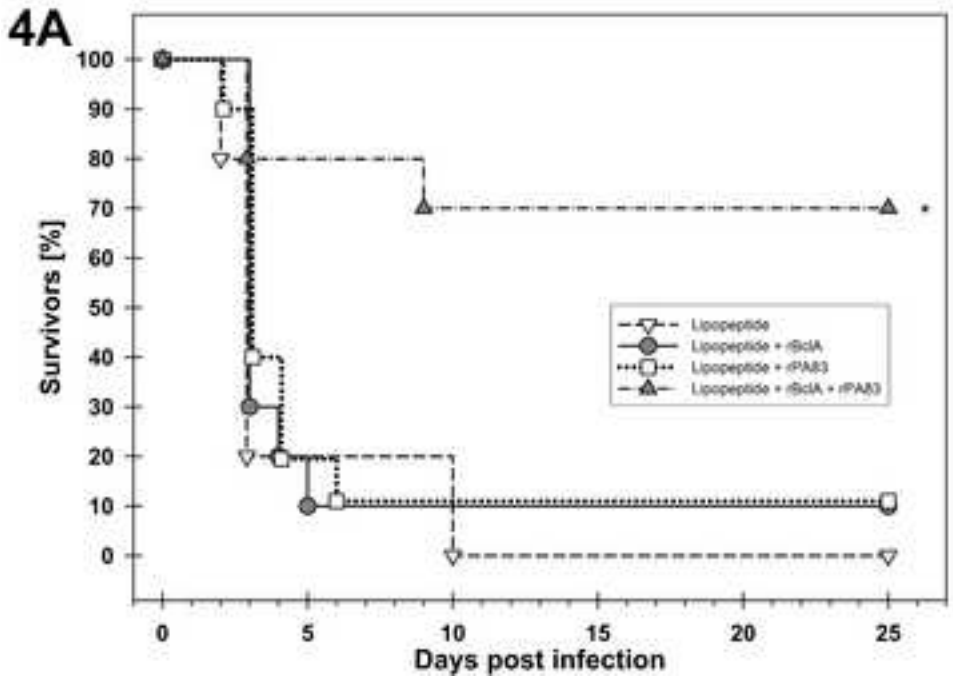

n.d. - not determined
IgG (mean)
IgG1 (mean)
IgG2a (mean)
IgG (individual titres)
IgG1 (individual titres)
IgG2a (individual titres)
Anti-rPA83 antibody titre
10⁶
10⁵
10⁴
10³
10²
n.d.
n.d.
Lipopeptide
Lipopeptide + rPA83
Lipopeptide + rBclA + rPA83
CIITA
TPA-LFD1PAD4-mIPS-1
CIITA + TPA-LFD1PAD4-LAMP1
CIITA + TPA-rPA83-LAMP1
CIITA + TPA-LFD1PAD4-mIPS-1 + TPA-rBclAD1D3-LAMP1

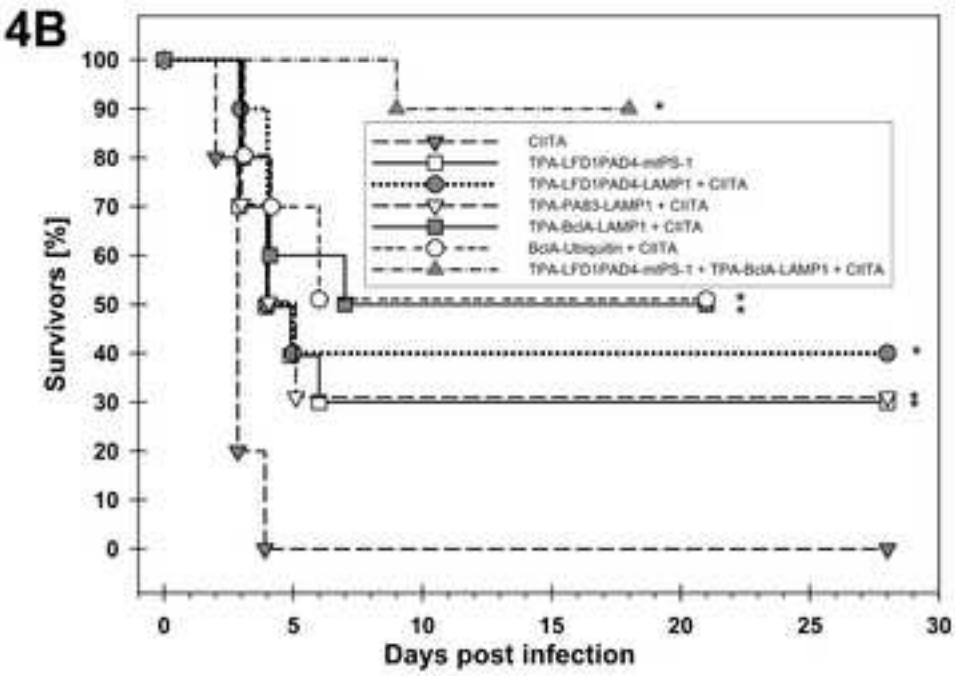
2


n.d. - not determined
Anti-rBclA antibody titre
IgG (mean)
IgG1 (mean)
IgG2a (mean)
IgG (individual titres)
IgG1 (individual titres)
IgG2a (individual titres)
Lipopeptide
Lipopeptide + rPA83
Lipopeptide + rBclA + rPA83
CIITA
CIITA + TPA-BclAD1D3-LAMP1
CIITA + BclAD1D3-Ubiquitin
CIITA + TPA-LFD1PAD4-mIPS-1 + TPA-rBclAD1D3-LAMP1

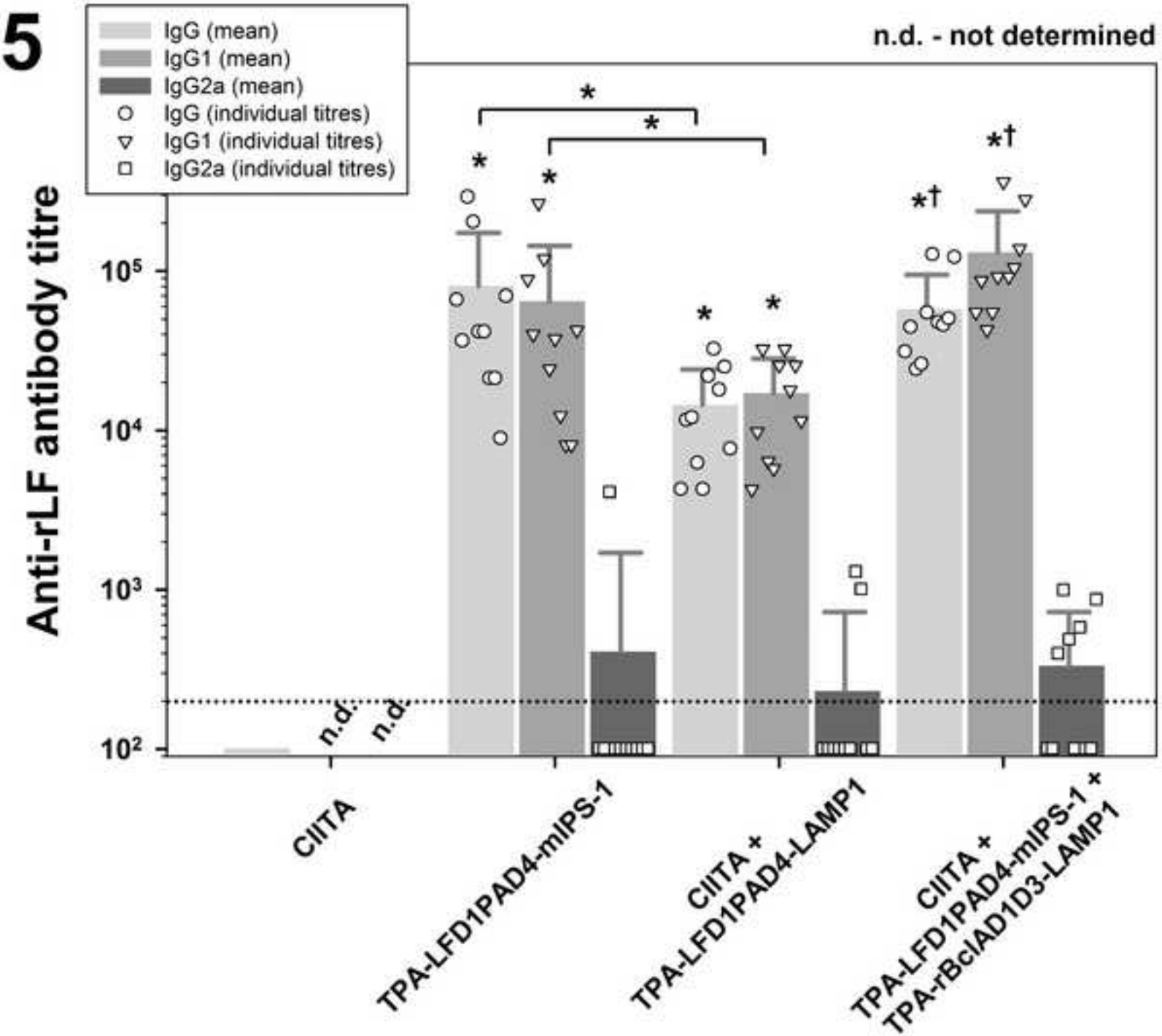
3


10^5
10^4
10^3
10^2
NT_{50} titre
mean titres
individual titres
*
Lipopeptide
Lipopeptide + rPA83
Lipopeptide + rBclA + rPA83
CIITA
TPA-LFD1PAD4-mIPS-1
CIITA + TPA-LFD1PAD4-LAMP1
CIITA + TPA-rPA83-LAMP1
CIITA + TPA-LFD1PAD4-mIPS-1 + TPA-rBclAD1D3-LAMP1


4A
Survivors [%]
Days post infection
Lipopeptide
Lipopeptide + rBclA
Lipopeptide + rPA83
Lipopeptide + rBclA + rPA83


4B
Survivors [%]
Days post infection
CitTA
TPA-LFD1/PAD4-mIPS-1
TPA-LFD1/PAD4-LAMP1 + CitTA
TPA-PA83-LAMP1 + CitTA
TPA-BclA-LAMP1 + CitTA
BclA-Ubiquitin + CitTA
TPA-LFD1/PAD4-mIPS-1 + TPA-BclA-LAMP1 + CitTA

5


IgG (mean)
IgG1 (mean)
IgG2a (mean)
IgG (individual titres)
IgG1 (individual titres)
IgG2a (individual titres)
n.d. - not determined
Anti-rLF antibody titre
10⁵
10⁴
10³
10²
n.d.
n.d.
*
*
*
*
*
*
*†
*†
CIITA
TPA-LFD1PAD4-mIPS-1
CIITA + TPA-LFD1PAD4-LAMP1
CIITA + TPA-LFD1PAD4-mIPS-1 + TPA-rBclAD1D3-LAMP1