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1 # HLA class II polymorphism influences the immune response to
2 # protective antigen and susceptibility to *Bacillus anthracis*
3
4 **Short title**: HLA class II and anthrax PA
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21
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23 HLA-binding; bacterial immunity
24
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32 # Abstract

33 The causative agent of anthrax, *Bacillus anthracis*, evades the host immune response and
34 establishes infection through the production of binary exotoxins composed of Protective
35 Antigen (PA) and one of two subunits, lethal factor (LF) or edema factor (EF). The
36 majority of vaccination strategies have focused upon the antibody response to the PA
37 subunit. We have used a panel of humanised HLA class II transgenic mouse strains to
38 define HLA-DR-restricted and HLA-DQ-restricted CD4+ T cell responses to the
39 immunodominant epitopes of PA. This was correlated with the binding affinities of
40 epitopes to HLA class II molecules, as well as the responses of two human cohorts:
41 individuals vaccinated with the Anthrax Vaccine Precipitated (AVP) vaccine (which
42 contains PA and trace amounts of LF), and patients recovering from cutaneous anthrax
43 infections. The infected and vaccinated cohorts expressing different HLA types were
44 found to make CD4+ T cell responses to multiple and diverse epitopes of PA. The effects
45 of HLA polymorphism were explored using transgenic mouse lines, which demonstrated
46 differential susceptibility, indicating that HLA-DR1 and HLA-DQ8 alleles conferred
47 protective immunity relative to HLA-DR15, HLA-DR4 and HLA-DQ6. The HLA
48 transgenics enabled a reductionist approach, allowing us to better define CD4+ T cell
49 epitopes. Appreciating the effects of HLA polymorphism on the variability of responses
50 to natural infection and vaccination will be vital in planning protective strategies against
51 anthrax.

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53
Author Summary

54 The bacterium responsible for causing the disease anthrax, Bacillus anthracis, produces a
55 binary toxin composed of Protective Antigen (PA) and either Lethal Factor (LF) or
56 Edema Factor (EF). Previous vaccination strategies have focused upon the antibody
57 response to the PA subunit. However, within the field of bacterial immunity, there is a
58 growing appreciation of the importance of the adaptive immune response, specifically led
59 by CD4+ T cells. We identified long-term CD4+ T cell responses to PA epitopes
60 following cutaneous human anthrax infection and vaccination, indicating that this toxin
61 component is a principle B. anthracis antigen. To characterise the impact of
62 polymorphism in HLA class II alleles at DR and DQ loci, we used transgenic mice to
63 map the immunodominant epitopes from PA. This was correlated with survival in the
64 transgenic lines following live anthrax challenge. We were able to demonstrate the
65 differential impact of HLA class II alleles upon the CD4+ T cell immunodominant
66 epitopes which shaped the immune hierarchy and therefore susceptibility to anthrax
67 infection.

68

69
Introduction

70 Anthrax is an acute zoonotic disease that primarily affects grazing mammals, although
71 the causative agent, *Bacillus anthracis*, also infects humans and is found in many parts of
72 the developing world, where the majority of natural human infection occurs [1].
73 Infections in humans, which may be fatal, depending upon the route of infection, are
74 usually confined to agricultural workers, those who eat infected carcasses and those who
75 handle the skins and coats of infected animals [2]. Over past decades, the need to protect

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76 individuals from occupational exposure has combined with fears regarding the use of
77 anthrax as a bioweapon, to drive the development of vaccines based on the toxins
78 produced by the bacteria [1]. Such concerns have resurfaced recently in relation to
79 potential anthrax weaponisation [3]. Furthermore, there have been recent cases in
80 Northern Europe of anthrax infections in intravenous drug users as a consequence of
81 contaminated drug supplies [4]. There are also growing concerns regarding the effect of
82 climate change in the Arctic upon the release of potentially viable anthrax spores from
83 melting permafrost [5].
84
85 The three toxins of *B. anthracis*, Protective Antigen (PA), Lethal Factor (LF) and Edema
86 Factor (EF) combine in a binary fashion, so that coupling PA with LF or EF produces
87 Lethal Toxin (LT) or Edema Toxin (ET), respectively [6]. The two predominantly used
88 vaccines, the United States-licensed Anthrax Vaccine Adsorbed (AVA; trade name
89 BioThrax) and the United Kingdom-licensed vaccine, Anthrax Vaccine Precipitated
90 (AVP), are culture filtrate vaccines containing PA and variable amounts of LF and EF [7].
91 Both vaccines are administered intramuscularly: AVA is given as three initial doses at 0, 1
92 and 6 months, while AVP is administered as a primary series of four vaccinations at 0, 3, 6
93 and 32 weeks [6]; a booster vaccination at 12 months, after the primary series for each
94 vaccine, is then required. The requirement for an intensive vaccination regimen, as well as
95 concerns about adverse reaction rates as high as 11% for the UK vaccine [8], and up to 60%
96 for the US vaccine [9], have prompted interest in streamlined vaccination schedules or the
97 development of effective, safe, subunit vaccines [10, 11].
98

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99 Second-generation anthrax vaccines under development are based on the administration of
100 the immunogenic anthrax toxins, specifically recombinant protective antigen (rPA). Human
101 clinical trials have indicated that these rPA vaccines may be capable of eliciting robust
102 cellular and humoral immune responses, whilst avoiding the adverse reactions associated
103 with older filtrate-based vaccines [12-14].
104
105 PA-specific monoclonal antibodies generated from AVA-vaccinated humans were found to
106 neutralise LT in vitro, and passive transfer of these antibodies provided protection in mouse
107 models of LT challenge [15, 16]. Although it is possible to show passive transfer of immunity
108 with toxin-neutralising antibodies [17], Crowe *et al.* found that over half of AVA-vaccinated
109 individuals demonstrated no detectable toxin-neutralising effect; despite the presence of anti-
110 PA antibodies in the majority of vaccinated individuals [18]. Studies in rhesus macaques
111 have demonstrated that AVA administration is capable of providing protection from
112 subsequent spore challenge, with a Th1/Th2 profile predictive of survival, even in the
113 presence of very low levels of circulating anti-PA antibody [19].
114
115 Protection afforded by a response to PA in both rodent and non-human primate models has
116 been suggested to be T-cell mediated [20, 21]. Plasmid vaccination in mice induces high
117 antibody titres as well as PA-specific Th1 immunity and induction of a high level of IFNγ
118 secretion [22]. Doolan and colleagues reported that individuals exposed to anthrax spores in
119 the US mail service incident experienced dose-dependent priming of T cell immunity, and, to
120 a lesser extent, of B cell immunity against PA [23]; low-level anthrax exposure led to PA T
121 cell responses in the absence of detectable antibodies. While Glomski *et al* found that, in
122 contrast to humoral immunity, IFNγ production by CD4+ T cells protected mice against
123 capsulated B. anthracis infection [24].

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124

125 Work from our lab has shown that individuals naturally exposed to anthrax spores
126 demonstrate IFNγ secreting antigen-specific $CD4^+$ T cell immunity to PA and LF, which for
127 PA, showed correlation between the magnitude of response and the duration of the infection
128 [25, 26]. We also found that a survivor of injectional anthrax developed strong, potentially
129 protective, T cell immunity to several commonly immunodominant epitopes of PA and LF,
130 previously described in Turkish patients [27]. This evidence suggests that cellular immunity
131 has a critical role to play in vaccine mediated clearance of *B. anthracis*.

132

133 Whether the future of anthrax vaccinology lies with third-generation, subunit vaccines or
134 with improved protocols for priming with existing vaccines, the need has never been greater
135 to fully comprehend the nature of effective immunity to *B. anthracis,* and the impact of
136 immunogenetic diversity. Here we describe a combined approach to characterising $CD4^+$ T
137 cell immunity to the PA toxin. This encompasses comprehensive analysis of T cell epitopes
138 through investigation of HLA class II binding, mapping of responses in a panel of HLA class
139 II transgenic mice, live challenge studies in HLA transgenic mice and studies of infected or
140 vaccinated human donors. Our results show PA to be highly CD4+ T cell epitope-rich, with
141 variable immunodominance which is dependent on HLA class II genotype. As discussed
142 below, this has implications for wide-scale roll-out and assessment of PA-based vaccines.

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144 # Results

145 ## *CD4+ T cell responses to B. anthracis PA epitopes in anthrax-recovered*
146 ## *patients and vaccinees*

147 We have previously described T cell memory responses to anthrax antigens in a cohort
148 of individuals who suffered clinical disease after natural, occupational exposure [25, 26,
149 28]. These were agricultural workers from the Kayseri region of Turkey who had been in
150 contact with infected livestock and been hospitalised with confirmed cutaneous anthrax
151 infections. PBMCs were collected for immune analysis at 0.4 to 7.5 years after recovery
152 under antibiotic therapy. In earlier studies, we described the fact that responses to
153 recombinant PA and LF antigens were higher in naturally exposed individuals than in
154 vaccinees receiving a full course of the UK AVP anthrax vaccine. Furthermore, immune
155 responses in naturally infected donors were characterised by a broad cytokine profile,
156 encompassing IL-2, IL-5, IL-9 and IL-13 [29]. In the present study we sought to analyse
157 in greater detail the epitope specificity of vaccinated and infected individuals to PA. PA
158 epitopes were screened by looking for ELISpot responses to a panel of 73 overlapping
159 peptides of 20mers overlapping by 10 amino acid residues and analysed in pools of six. A
160 total of 26 peptides were identified as epitopes in at least one AVP vaccinee (Fig 1), of
161 which only 7 epitopes were an immune target for more than one vaccinee. Of note is the
162 finding that only 4 vaccinees (AVP vaccinees donors 1-4) out of 10 responded to any
163 epitopes, and of these the majority of the responses were elicited in donor 3, who
164 responded to a total of 21 epitopes (Table S1). Although this study was not powered to
165 make assumptions regarding the involvement of HLA alleles in the presentation of

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166 anthrax peptides, it is interesting that HLA-DR11 and DR13 were over-represented in the
167 population of donors responding to the peptides contained within the vaccine. In contrast,
168 the majority of infected individuals (7 out of 9 donors) responded to at least one PA
169 epitope, and there did not appear to be any particular bias towards specific HLA alleles in
170 the responses (Fig S2), with 69 of the 73 peptides analysed in this cohort found to carry
171 infection-specific epitopes. Peptides such as PA 168-187 and PA 651-670 contained
172 epitopes that were recognised with a high frequency response by multiple individuals (PA
173 168-187 mean = 264.2 spots/million, ±123.2 SEM, and PA 651-670 mean = 273.4
174 spots/million, ±123.6 SEM) and encompassing diverse HLA class II alleles. However, it
175 is notable that although adjacent peptides (PA 161-187 and PA 641-660 respectively)
176 were identified as epitopes for one of the vaccinated individuals, neither of the infection-
177 specific epitopes, recognised in the context of multiple HLA alleles, were a focus of the
178 response in any vaccinees.
179

180 In both infected and vaccinated cohorts, the epitopes came from sequences within all four
181 domains of PA (Fig 1), indicating that, unlike LF, the majority of PA epitopes are not
182 clustered within a single domain of the protein [25]. This comparison also highlights the
183 fact that individuals who had been hyper-immunised on the standard UK schedule with
184 seven to 14 doses of the AVP vaccine over 3.5 to 10 years, responded to fewer epitopes
185 than infected individuals, with no epitopes identified that were present in the context of
186 vaccination alone. This supported the suggestion, which we originally made in regard to
187 LF; that live infection unveils cryptic anthrax epitopes not commonly recognised after
188 administration of the protein antigen.

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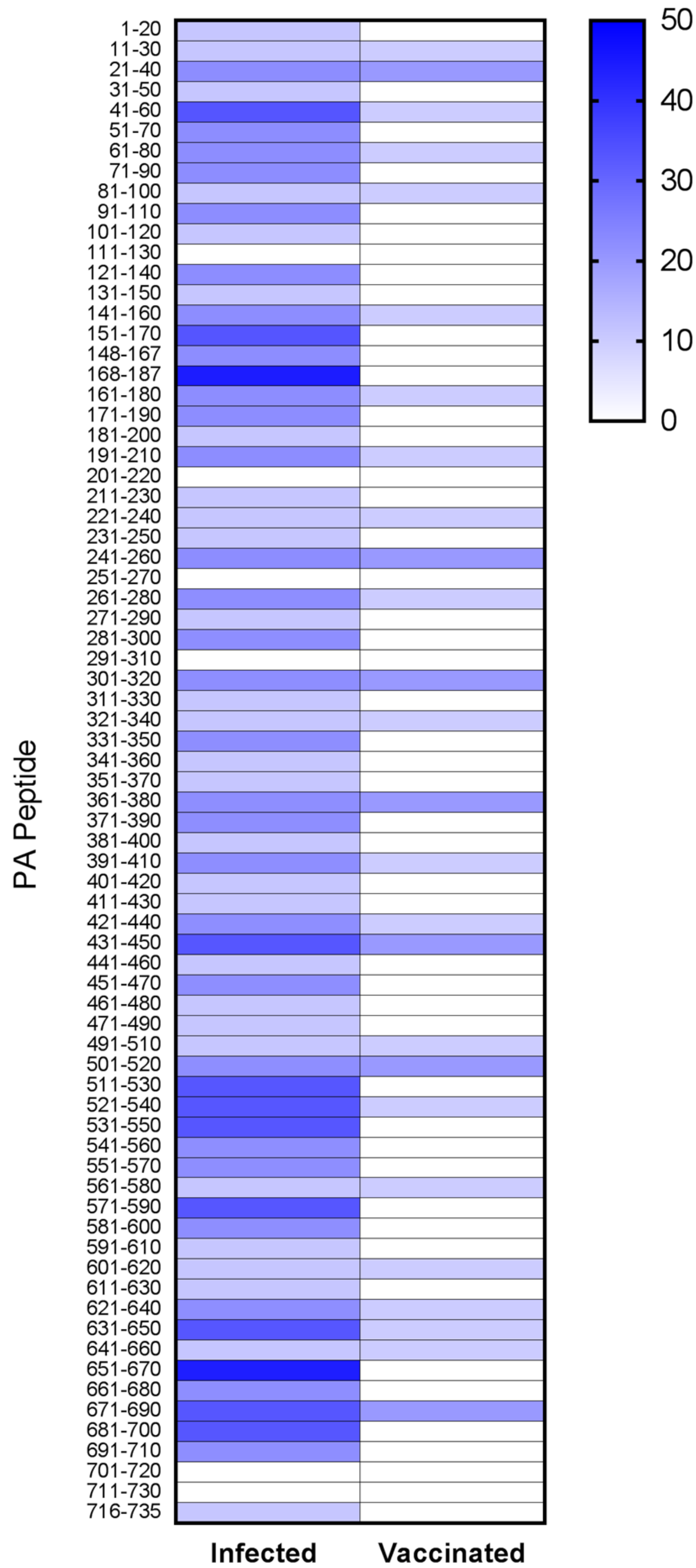

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190 **Figure 1.**

191

192 ***Differential susceptibility to B. anthracis challenge in HLA transgenic***

193 ***mice***

194 In order to more precisely define the contribution of different HLA class II alleles to
195 anthrax and PA immunity, we turned to HLA class II transgenic mice as a defined,
196 reductionist model allowing analysis of individual alleles in isolation.

197

198 We initially compared susceptibility of mice expressing either HLA-DR1 or HL-DQ8 to
199 challenge with $1x10^6$ CFU (10^3 median lethal doses, MLD) *B. anthracis* STI strain. HLA-
200 DR1 mice were resistant to *B. anthracis* STI challenge (MLD > 10^6 CFU), while HLA-
201 DQ8 mice were also relatively resistant, with 80% survival. The more susceptible HLA
202 class II transgenic mice demonstrated differential susceptibility to challenge at 10^5 CFU
203 (10^2 MLD *B. anthracis* STI) with the following survival rates: DQ6 mice (100%), DR4
204 (80%), and DR15 (55%). By comparison, the parent strain for the HLA class II
205 transgenics, C57BL6, showed 40% survival against a 10^5 CFU contemporaneous
206 challenge with the STI vaccine strain of *B. anthracis*.

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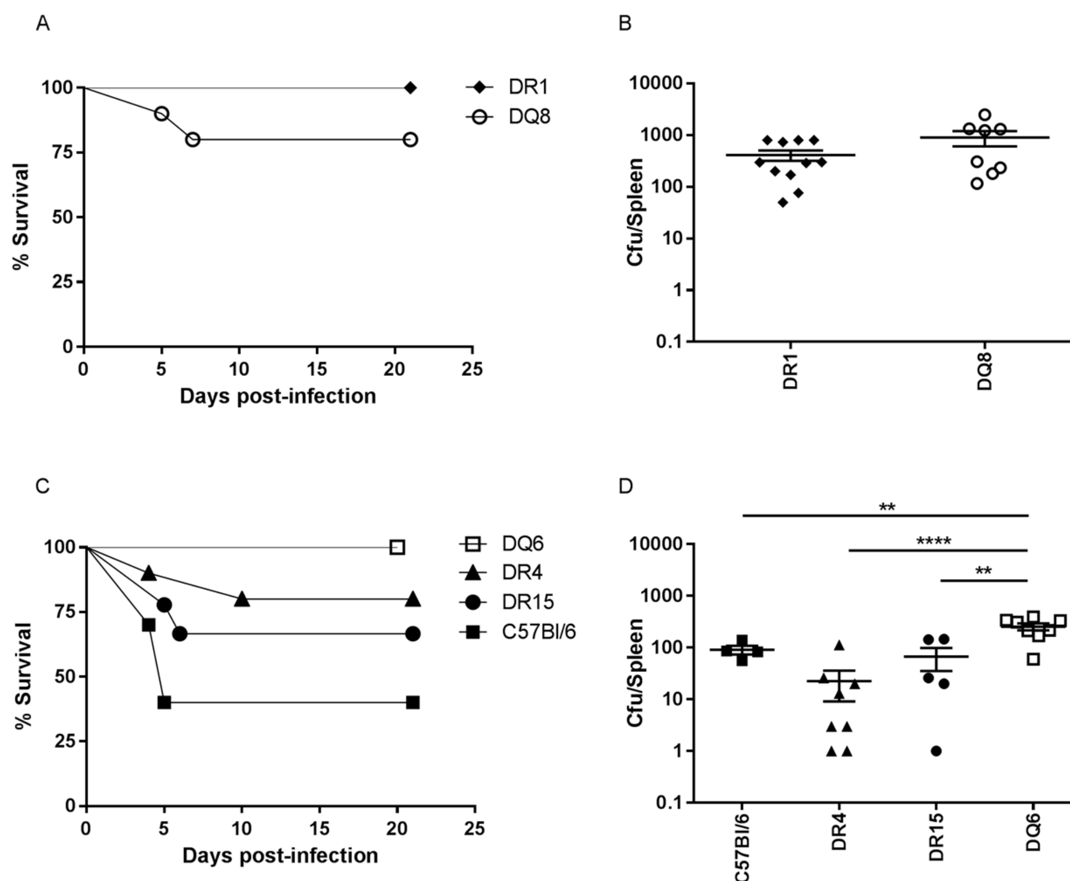

208

209 **Figure 2.**

210

211 The bacterial loads recovered from the spleens of individual surviving mice of each strain
212 at day 20 are shown in Fig 2. In general the mean bacterial loads in spleens at day 20
213 post-infection were lower than, but proportional to, the original challenge dose level. The
214 groups challenged with 10^6 CFU (DR1, DQ8) had high bacterial loads, although the mean
215 bacterial loads for the DQ6 mice (challenged with 10^5 CFU) did not differ significantly
216 from those for the DR1 or DQ8 mice, which had been challenged with ten-fold more
217 bacteria, suggesting that the DQ6 mice were slower to clear the infection.

218

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219 HLA transgenic mice were less susceptible to infection with *B. anthracis* STI strain than
220 the parent strain C57BL6 mice. HLA-DR1 mice were resistant to infection with a high-
221 level challenge (10^6 CFU). DQ6 strain mice were resistant to 10^5 CFU and relatively slow
222 to clear the infection. The order of susceptibility of mouse strains to *B. anthracis*
223 infection was determined to be: C57Bl6 > DR15 > DR4 > DQ6 > DQ8 > DR1.
224

225 ## *$CD4^+$ T cell responses to B. anthracis PA epitopes in HLA transgenic mice*

226 The greater immunogenetic complexity of HLA-outbred human populations makes it
227 considerably more challenging to define the restricting HLA molecule responsible for
228 antigen presentation; the HLA class II transgenic mouse models offer a reductionist
229 system in which to define HLA-restricted epitopes of relevance to humans carrying the
230 same alleles. Using these transgenic models in protein and peptide immunisation we were
231 able to build a comprehensive picture of immunodominant HLA class II restricted
232 epitopes derived from PA. Mice were immunised with the recombinant PA protein and
233 draining lymph node cells were restimulated with a peptide library spanning the PA
234 sequence (73 peptides in total, with some peptides overlapping the boundaries between
235 domains: domain 1 = PA 1-20 to PA 241-260; domain 2 = PA 251-270 to PA 471-490;
236 domain 3 = PA 491-510 to PA 581-600; domain 4 = PA 591-610 to PA 716-735,). After
237 immunisation with the recombinant protein of interest, all HLA transgenic mice
238 responded to the whole rPA (Fig 3), but the response to the individual peptides was found
239 to be HLA-specific.
240

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241 We investigated whether there might be any correlation between susceptibility of the
242 HLA transgenic lines to challenge and the breadth of T cell epitope recognition. Antigen-
243 specific T cell responses to all stimulatory peptides were further investigated by peptide
244 immunisation and screening (Figs S1, S2 and S3). In total, 6 HLA-DR1 restricted
245 epitopes were identified: PA 41-60, PA 361-380, PA 371-390, PA 561-580, PA 631-650,
246 and PA 681-700 (Fig 3A and Fig S3). In comparison 14 HLA-DQ8 restricted epitopes
247 were identified: PA 141-160, PA 151-170, PA 191-210, PA 201-220, PA 301-320, PA
248 331-350, PA 481-500, PA 491-510, PA 501-520, PA 591-610, PA 601-620, PA 651-670,
249 PA 711-730, and PA 716-735 (Fig 3B and Fig S1): and 15 HLA-DR4 restricted epitopes
250 were identified: PA 31-50, PA 51-70, PA 71-90, PA 121-140, PA 221-240, PA 321-340,
251 PA 331-350, PA 371-390, PA 451-470, PA 461-480, PA 591-610, PA 601-620, PA 641-
252 660, PA 681-700, and PA 711-730 (Fig 3C and Fig S2). Whilst some of these epitopes
253 were recognised by more than one HLA type (PA 331-350, PA 591-610, PA 601-620 and
254 PA 711-730 were constituents of both DR4 and DQ8 responses, while PA 371-390 and
255 PA 681-700 were recognised by both DR1 and DR4 alleles), no one epitope was found to
256 provoke a response in all 3 HLA alleles tested. Thus, it was noteworthy that HLA-DR1
257 transgenic mice, which were the least susceptible to anthrax challenge, responded to
258 fewer epitopes with a reduced repertoire of CD4+ T cell recognition than the other HLA
259 alleles screened.

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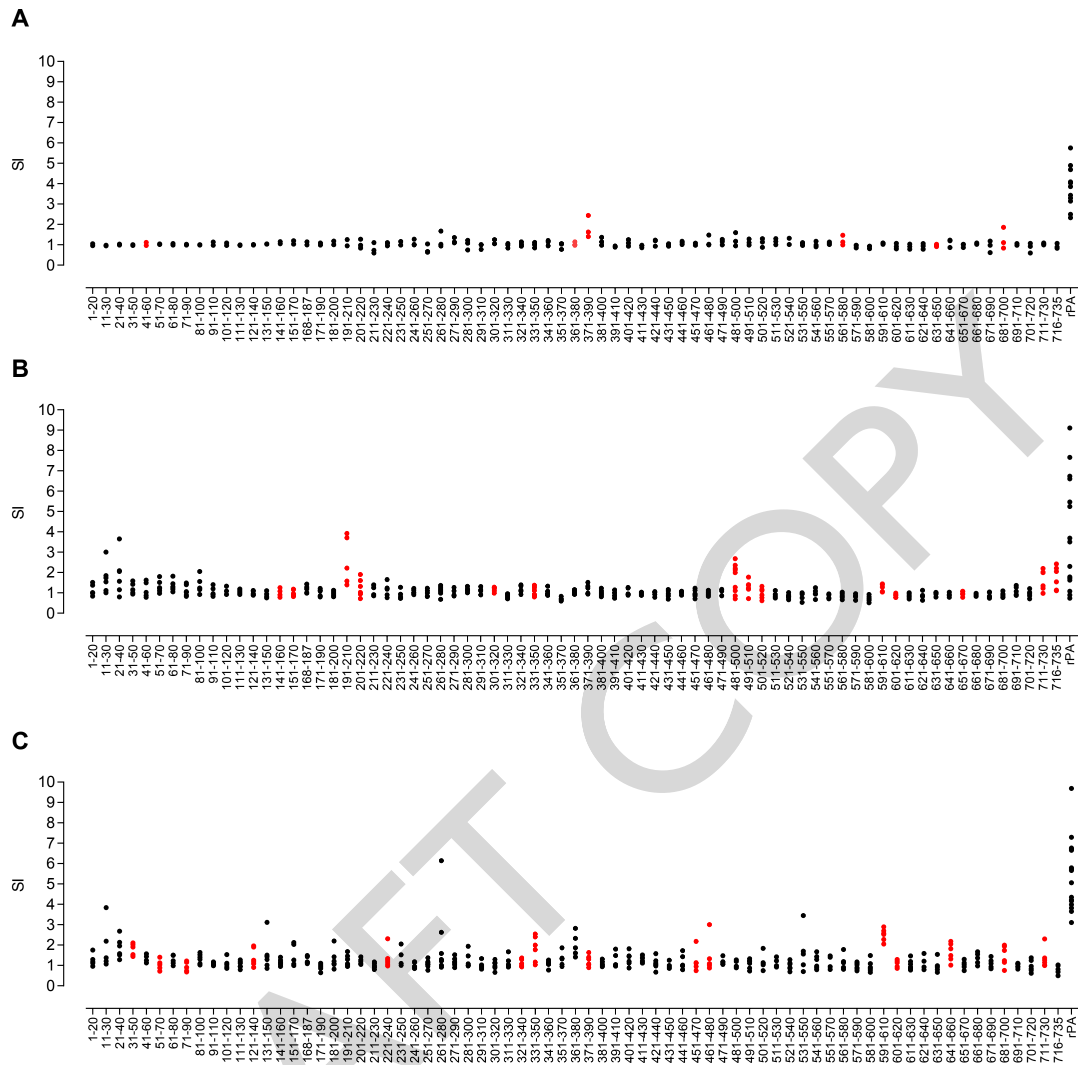

260

261 **Figure 3.**

262

263 ***The differential PA peptide binding across distinct HLA polymorphisms***

264 Overlapping 20-mer peptides that represented the whole PA protein sequence were
265 evaluated for binding affinity to seven common HLA-DR alleles and two common HLA-
266 DQ alleles (Table 1). The two epitopes that were recognised by multiple individuals from
267 the infected cohort (PA 168-187 and PA 651-670) showed a complete disparity in their

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268 HLA binding affinities. Whilst PA 168-187 was not recognised by any of the transgenic
269 lines and showed an exceptionally low binding affinity across all HLA-DR alleles tested,
270 PA 651-670 showed strong-to-moderate binding across all HLA-DR alleles, and bound
271 strongly to HLA-DQ8, which also correlated with a strong response seen in the
272 corresponding transgenic line. Overall, we were not able to identify a propensity towards
273 a strong HLA binding affinity in those epitopes that were a feature of the infected
274 response. In contrast, all but one (PA 501-520) of the seven epitopes identified in more
275 than 20% of the vaccinated cohort demonstrated high binding affinities for the HLA-DR
276 or DQ alleles carried by those individuals. This suggests that the binding affinity may be
277 a more important predictor of epitope hierarchy in the context of vaccination than
278 infection.

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Table 1. The PA peptides, identified in transgenic mouse strains and human cohorts, show relatively broad binding to common HLA-DR and HLA-DQ alleles.

PA peptide sequence	HLA transgenic strain responding to epitope after PA immunisation	Human cohort responding to epitope (>20% cohort responding)	Relative binding of HLA class II								
			DR1	DR3	DR4	DR7	DR11	DR13	DR15	DQ6	DQ8
^{21}GYYFSDLNFQAPMVVTSSTT^{40}	-	Vaccinee, Infected	23	60	0.3	22	53	>1908	118	ND	ND
^{31}APMVVTSSTTGDLSIPSSEL^{50}	DR4	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
^{41}GDLSIPSSELENIPSENQYF^{60}	DR1	Infected	3312	>728	46	424	>1288	>1288	134	ND	ND
^{51}ENIPSENQYFQSAIWSGFIK^{70}	DR4	Infected	3	600	1	2	2	>2733	55	ND	ND
^{61}QSAIWSGFIKVKKSDEYTFA^{80}	-	Infected	617	12	650	89	14	4	1	ND	ND
^{71}VKKSDEYTFATSADNHVTMW^{90}	DR4	Infected	11	8	1	2	118	>2733	45	ND	ND
^{91}VDDQEVINKASNSNKIRLEK^{110}	-	Infected	1333	283	992	36	>1357	245	164	ND	ND
^{121}QRENPTEKGLDFKLYWTDSQ^{140}	DR4	Infected	>2563	800	6	1549	>1357	>2733	119	ND	ND
^{141}NKKEVISSDNLQLPELKQKS^{160}	DQ8	Infected	131	26	48	28	849	>2733	1	>3054	>166
^{148}SDNLQLPELKQKSSNSRKKR^{167}	-	Infected	ND	ND	ND	ND	ND	ND	ND	ND	ND
^{151}LQLPELKQKSSNSRKKRSTS^{170}	DQ8	Infected	>6667	>667	>1788	>1543	701	177	>511	>3054	>166
^{161}SNSRKKRSTSAGPTVPDRDN^{180}	-	Infected	>6667	>667	>1788	>1543	>1336	>1908	>511	ND	ND
^{168}STSAGPTVPDRDNDGIPDSL^{187}	-	Infected	>6667	>667	>1788	>1543	>1336	>1908	>511	ND	ND
^{171}AGPTVPDRDNDGIPDSLEVE^{190}	-	Infected	149	211	10	1167	7	51	95	ND	ND
^{191}GYTVDVKNKRTFLSPWISNI^{210}	DQ8	Infected	216	0.2	190	11	231	0.5	15	2488	>166
^{201}TFLSPWISNIHEKKGLTKYK^{220}	DQ8	-	3162	>667	1400	873	327	306	14	3077	>166
^{221}SSPEKWSTASDPYSDFEKVT^{240}	DR4	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
^{241}GRIDKNVSPEARHPLVAAYP^{260}	DQ8	Vaccinee, Infected	2828	15	55	833	567	9	9	899	>166
^{261}IVHVDMENIILSKNEDQSTQ^{280}	-	Infected	>6667	>667	167	>1543	>1336	>1908	>511	ND	ND
^{281}NTDSETRTISKNTSTSRTHT^{300}	-	Infected	>6667	23	179	707	535	60	>511	ND	ND
^{301}SEVHGNAEVHASFFDIGGSV^{320}	DQ8	Vaccinee, Infected	>6667	>667	>1788	327	>1336	>1908	>511	3	7
^{321}SAGFSNSNSSTVAIDHSLSL^{340}	DR4	Infected	279	1	6	0.4	935	>1908	95	ND	ND
^{331}TVAIDHSLSLAGERTWAETM^{350}	DR4, DQ8	Infected	176	0.3	10	11	30	4	120	1056	0.1
^{361}NANIRYVNTGTAPIYNVLPT^{380}	DR1	Vaccinee, Infected	15	>728	0.4	1	12	>1288	3	ND	ND
^{371}TAPIYNVLPTTSLVLGKNQT^{390}	DR1, DR4	Infected	1	12	2	0.4	78	300	6	ND	ND
^{391}LATIKAKENQLSQILAPNNY^{410}	-	Infected	89	176	7	179	46	43	0.2	ND	ND
^{421}LNAQDDFSSTPITMNYNQFL^{440}	-	Infected	>6667	>667	1265	22	>1336	>1908	77	ND	ND
^{431}PITMNYNQFLELEKTKQLRL^{450}	-	Vaccinee, Infected	15	25	38	1	2	7	0.1	ND	ND
^{451}DTDQVYGNIATYNFENGRVR^{470}	DR4	Infected	200	>667	293	30	>1336	>1908	2	ND	ND
^{461}TYNFENGRVRVDTGSNWSEV^{480}	DR4	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
^{481}LPQIQETTARIIFNGKDLNL^{500}	DQ8	Infected	2	5	886	0.3	37	2	1	240	6
^{491}IIFNGKDLNLVERRIAAVNP^{510}	DQ8	-	3801	31	327	267	0.1	7	95	693	75
^{501}VERRIAAVNPSDPLETTKPD^{520}	DQ8	Vaccinee, Infected	721	75	69	55	>1288	>1288	145	2506	29
^{511}SDPLETTKPDMTLKEALKIA^{530}	-	Infected	211	10	1800	401	1000	10	37	ND	ND
^{521}MTLKEALKIAFGFNEPNGNL^{540}	-	Infected	1155	18	>1788	98	189	10	77	ND	ND
^{531}FGFNEPNGNLQYQGKDITEF^{550}	-	Infected	>6667	>667	207	>1543	1134	>1908	63	ND	ND
^{541}QYQGKDITEFDFNFDQQTSQ^{560}	-	Infected	>2563	25	306	>3365	>1357	>2733	44	ND	ND
^{551}DFNFDQQTSQNIKNQLAELN^{570}	-	Infected	249	82	239	267	>1336	250	200	ND	ND
^{561}NIKNQLAELNATNIYTVLDK^{580}	DR1	-	2	>728	76	8	137	64	8	ND	ND
^{571}ATNIYTVLDKIKLNAKMNIL^{590}	-	Infected	31	5	278	19	0.5	3	11	ND	ND
^{581}IKLNAKMNILIRDKRFHYDR^{600}	-	Infected	2160	0.1	500	378	6	0	4	ND	ND
^{591}IRDKRFHYDRNNIAVGADES^{610}	DR4, DQ8	-	25	1	0.2	4	4	25	2	1132	0.5
^{601}NNIAVGADESVVKEAHREVI^{620}	DR4, DQ8	-	4989	10	1183	750	732	16	122	2191	6
^{621}NSSTEGLLLNIDKDIRKILS^{640}	-	Infected	2236	1	414	80	53	3	77	ND	ND
^{631}IDKDIRKILSGYIVEIEDTE^{650}	DR1	Infected	5	775	510	1	72	1026	0.3	ND	ND
^{641}GYIVEIEDTEGLKEVINDRY^{660}	DR4	-	3162	82	21	65	433	1333	0.1	ND	ND
^{651}GLKEVINDRYDMLNISSLRQ^{670}	DQ8	Infected	7	10	6	22	46	15	6	>3054	1
^{661}DMLNISSLRQDGKTFIDFKK^{680}	-	Infected	200	2	30	27	33	4	27	ND	ND

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^{671}DGKTFIDFKKYNDKLPLYIS^{690}	-	Vaccinee, Infected	2494	100	>1788	138	14	25	4	ND	ND
^{681}YNDKLPLYISNPNYKVNVYA^{700}	DR1, DR4	Infected	1	4	1	2	2	30	1	ND	ND
^{691}NPNYKVNVYAVTKENTIINP^{710}	-	Infected	50	3	75	0.3	13	6	10	ND	ND
^{711}SENGDTSTNGIKKILIFSKK^{730}	DR4, DQ8	-	>6667	>667	>1788	133	3	217	3	>3054	>166
^{716}TSTNGIKKILIFSKKGYEIG^{735}	DQ8	-	183	1	849	35	0.4	0.4	1	>3054	>166

Binding affinities are expressed as relative values which were calculated as the ratio of the PA peptides IC50 to the IC50 of a reference peptide chosen as a high binder for each allele. High affinity values were interpreted as < 100. Means were calculated from at least three independent experiments. ND = Not Done.

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288 # Discussion

289 Human exposure to anthrax spores continues to be of considerable concern in diverse
290 spheres of clinical infectious disease; most commonly, exposure may occur naturally,
291 either after ingestion of infected animals or through contact with infected animal
292 products. Other routes of exposure could occur through deliberate release, acts of
293 bioterrorism, or injection of contaminated drugs by intravenous drug users [3, 27, 30]; in
294 these contexts, especially the threat of bioterrorist use, there has long been a perceived
295 need to have an effective anthrax vaccination programme available. Three major vaccines
296 have been in use in various parts of the world since the Cold War, with various
297 recombinant subunit vaccine candidates in trial for rollout [31, 32]. Interestingly
298 however, compared to many other bacterial pathogens, the immunology and
299 immunogenetics underpinning any clear understanding of correlates of protection (CoP)
300 are poorly delineated for anthrax [33]. Although vaccine development has focused largely
301 on the endpoint of PA-targeted neutralising antibody, this alone is unlikely to confer
302 sterilising immunity. At a general level, the CoP for effective AVA-vaccine-induced
303 protection of macaques from anthrax challenge are IgG titre and IFNγ^{+} T cell frequency
304 against PA [34]. Protection conferred by anthrax spores is entirely dependent on CD4+ T
305 cells [35].

306

307 In seeking an improved understanding of the interaction between *B. anthracis* and
308 protection by the human immune system, a key question has been the impact of
309 immunogenetic heterogeneity at the population level [36]; work in mouse models has
310 suggested that, as expected, both MHC and non-MHC polymorphisms are influencing

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311 these factors [37]. With respect to human vaccination, there is evidence of reduced
312 immune responsiveness to PA in individuals with the DRB1*1501/DQB1*0602
313 haplotype [38]. In light of the importance of anti-PA immunity for protection and the
314 relatively high frequency of this haplotype in many human populations, there is cause for
315 concern in relation to vaccine efficacy and vaccine confidence. The situation is
316 reminiscent of hepatitis B virus and MMR vaccinations, both of which demonstrate the
317 profound influence of HLA polymorphism [39, 40].
318
319 Our aim here has been to shed light on the role of HLA class II alleles in PA epitope
320 presentation to the immune system and thus on disease outcome after anthrax challenge.
321 A key experiment in this regard was to compare the impact of STI challenge on survival
322 and the control of bacterial load in mice, all on a C57BL/6 background and lacking
323 expression of endogenous murine MHC class II heterodimers but differing in expression
324 of specific HLA-DR or HLA-DQ alleles. The background C57BL/6 strain is considered
325 one that mounts a low antibody response to anthrax PA and LF [37]. We found that HLA-
326 DR15 transgenics (expressing the HLA-DRB1*1501 allele) were the most susceptible to
327 challenge, echoing the results of human AVA HLA-DRB1*1501^{+} vaccinees [38]. It is
328 particularly noteworthy that the effects of HLA class II alleles must be differentially
329 effective in CD4+ T cell-mediated control of bacterial dissemination during the first 4 to
330 6 days after challenge, the very earliest days of detectable priming of an adaptive immune
331 response. Nuanced differences in the potency and frequency of the initial CD4+ T cell
332 responses have the potential to favourably impact survival by driving cellular responses
333 to intracellular infection and generation of an initial neutralising antibody response. Such

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334 differences in susceptibility due to HLA polymorphisms are unlikely to have imposed
335 evolutionary selection pressure in anthrax-exposed human populations. The pathogen is
336 rarely transmitted from human-to-human, outbreaks tend to be of a limited nature (such
337 as a local community consuming the same contaminated livestock), and most cases are
338 not fatal. The greater concern relates to potential gaps in the efficacy of large-scale
339 vaccination programmes for biodefense purposes, such as in the US military.
340
341 We looked at mechanisms underpinning HLA differences in susceptibility, starting with
342 mapping of CD4+ T cell epitopes from PA. Our key findings were that natural infection
343 elicits a considerably broader CD4+ epitope response than AVP vaccination and at least
344 in the setting of natural infection, this is a very epitope-rich sequence, with epitopes
345 spanning the entire length of the protein. It is well-established that in communities where
346 environmental exposure to anthrax is relatively common, such as among goat-herders,
347 symptomatic exposure confers lifelong protection from re-infection [25]. Differences in
348 antigen processing and generation of epitopes for HLA class II binding between the AVP
349 subunit vaccine components and live infection of APC might in some respects have been
350 predictable, except that earlier studies of dendritic cells treated with lethal toxin showed a
351 complete loss of the ability to effectively stimulate peptide-specific CD4+ T cells [41].
352 The PA sequence contains a number of regions with potential broad-ranging
353 immunogenicity in terms of high-affinity binding to the majority of HLA class II alleles
354 tested: 5 of the PA peptides analysed are relatively unusual in their capacity to bind very
355 diverse HLA class II heterodimers at high affinity; PA191-210, 331-350, 481-500, 591-
356 610 and 711-730. The 191-200 PA epitope overlaps one that we have previously

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357 identified at the CD4+ T cell level as being strongly recognised in the memory T cell
358 response of a 60-year old intravenous drug-user who survived injection of anthrax-
359 contaminated heroin [27]. This collection of epitopes would be excellent candidates for a
360 highly immunogenic, widely applicable, epitope-string vaccine. Importantly, the fact that
361 all bind HLA-DRB1*1501 with high or very high affinity makes it likely that the 'low-
362 responder' status of HLA-DRB1*1501 vaccinees would be overcome by an approach
363 focused on these epitopes. However, HLA class II-related differences in susceptibility to
364 anthrax challenge cannot be a simple question of relative availability of high-affinity
365 HLA class II-binding PA epitopes to activate the CD4+ T cell repertoire: the most
366 susceptible HLA allele that we identified, HLA-DRB1*1501, can present at least as many
367 PA epitopes as can the least susceptible allele, HLA-DRB1*0101. It is also important to
368 stress that, while the HLA transgenic mice used to define immunodominant PA epitopes
369 offer a useful reductionist system, the immune responses seen in this system may not
370 fully recapitulate the effect of the individual HLA polymorphisms in a complete immune
371 system. This may give a partial explanation for the divergence in epitopes identified in
372 the HLA transgenics and those found in the human cohorts.
373
374 In summary, we draw two important conclusions from this comprehensive analysis of T
375 cell recognition of anthrax PA. The first is that PA is an unexpectedly epitope-rich
376 antigen, whether considered from a perspective of HLA class II binding or of CD4+ T
377 cell recognition. The second key point, and one that offers an important note of caution to
378 vaccinologists and to those planning biodefense strategies, is that there are likely to be
379 major differences in both vaccine efficacy and anthrax severity imposed by HLA

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380 polymorphism within the population. These factors underscore the importance of
381 considering immunological and vaccination strategies that can overcome such
382 differences.
383

384 # Materials and Methods

385 ## *Ethics Statement*

386 Human blood samples from Kayseri (Turkey) were obtained with full review and
387 approval by The Ethics Committee of the Faculty of Medicine, Erciyes University.
388 Human vaccinees based at DSTL, Porton Down, participated in the context of a study
389 protocol approved by the CBD IEC (Chemical and Biological Defence Independent
390 Ethics Committee). Written informed consent was obtained from all human volunteers.
391 All mouse experiments were performed under the control of UK Home Office legislation
392 in accordance with the terms of the Project License (70/5994) granted for this work under
393 the Animals (Scientific Procedures) Act 1986, having also received formal approval of
394 the document through the Imperial College Ethical Review Process (ERP) Committee.

395

396 ## *HLA class II transgenic mice*

397 HLA class II transgenic mice carrying genomic constructs for HLA-DRA1*0101/HLA-
398 DRB1*0101 (HLA-DR1), HLA-DRA1*0101/HLA-DRB1*0401 (HLA-DR4), HLA-
399 DRA1*0101/HLA-DRB1*1501 (HLA-DR15) and HLA-DQA1*0301-DQB1*0302
400 (HLA-DQ8), crossed for more than six generations to C57BL/6 H2-Ab^{00} mice, were as

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401 described previously [42-46]. All experiments were performed in accordance with the
402 Animals (Scientific Procedures) Act 1986 and were approved by local ethical review
403 panel.
404

405 ### *Live B. anthracis challenge*

406 Preliminary data indicated that there was a divergence in the susceptibility of mouse
407 strains to anthrax challenge. Therefore, naïve mice were challenged with *B. anthracis* STI
408 strain by the intraperitoneal route at one of two dose levels: 11 HLA-DR1 and 10 HLA-
409 DQ8 mice were challenged with 10^6 colony forming units (CFU) while 9 HLA-DR15, 10
410 HLA-DR4, 8 HLA-DQ6 and 10 C57Bl6 were challenged with 10^4 CFU per mouse. The
411 animals were monitored for 20 days post-infection, after which all survivors were
412 sacrificed and their spleens were removed and homogenised in 1 mL of PBS before
413 plating out onto L-agar plates. Colonies were counted after 24 hours of culture at 37°C,
414 and the mean bacterial count per spleen was determined.
415

416 ### *Expression and purification of PA antigens*

417 Good Manufacturing Practice grade rPA was provided by Avecia Vaccines (Billingham,
418 UK) and had endotoxin levels of < 1 EU/mg. Individual domains of PA and peptides
419 were expressed in E. coli and purified as previously described [47]. All proteins and
420 peptides were resuspended in DMSO at 25 mg/mL.
421

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422 ***PA epitope mapping in transgenic mice***

423 Mice were immunised in one hind footpad with 50 µL of 12.5 µg recombinant full-length
424 PA, PA peptides, or a control of PBS, emulsified in an equal volume of TiterMax Gold
425 (Sigma-Aldrich, USA) by syringe extrusion. After 10 days, immunised draining popliteal
426 lymph nodes were removed and disaggregated into single-cell suspensions by filtration
427 through 0.7 µm cell strainers. Lymph node cell responses were recalled *in vitro* with 25
428 µg/mL of either rPA, truncated PA domains comprising the PA protein, or the
429 overlapping 20-mer peptides covering the full-length PA sequence. This produced a
430 CD4+ T cell epitope map of the entire PA protein sequence. To confirm the
431 immunodominant epitopes identified by this large-scale mapping, mice were then
432 immunised subcutaneously with 12.5 µg of the individual PA peptides in TitreMax
433 adjuvant. After 10 days the lymph node cells were challenged *in vitro* with 25 µg/mL of
434 the recombinant full-length PA and the immunising and two flanking PA peptides.

435

436 Quantification of murine antigen-specific INFγ levels was carried out by ELISpot
437 (Diaclone, USA) analysis of T cell populations directly *ex vivo*. Ninety-six-well
438 hydrophobic polyvinylidene difluoride membrane-bottomed plates (MAIP S 45;
439 Millipore, USA) were pre-wetted with 70% ethanol. The plates were washed twice with
440 PBS, then coated with anti-INFγ monoclonal antibody at 4°C overnight. After blocking
441 with 2% skimmed milk, plates were washed with PBS, and 100 µL/well of antigen was
442 added in triplicate to appropriate wells. For each assay, a medium-only negative control
443 and a positive control of staphylococcal enterotoxin B (SEB 25 ng/mL) were included.
444 Wells were seeded with 2 x 10^6 cells/mL in HL-1 medium (supplemented with 1% L-

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445 glutamine, 1% penicillin/streptomycin, and 2.5% β-mercaptoethanol) and plates were
446 incubated for 72 hours at 37 °C with 5% CO_2. The plate contents were then discarded and
447 plates were incubated with PBS/Tween 20 (0.1%) for 10 minutes at 4°C. Plates were then
448 washed twice with PBS/Tween 20 (0.1%) and incubated with biotinylated anti-INFγ
449 monoclonal antibody. Plates were again washed twice with PBS/Tween 20 (0.1%), and
450 then incubated with streptavidin-alkaline phosphatase conjugate. After a wash with
451 PBS/Tween 20 (0.1%), plates were treated with 5-bromo-4-chloro-3-indolyl phosphate
452 and nitro blue tetrazolium (BCIP/NBT) and spot formation was monitored visually. The
453 plate contents were then discarded and plates were washed with water, then air-dried and
454 incubated overnight at 4°C to enhance spot clarity. Spots were counted using an
455 automated ELISpot reader (AID), and results expressed as delta spot-forming cells per
456 10^6 cells (ΔSFC/10^6 which is calculated as SFC/10^6 of stimulated cells minus SFC/10^6 of
457 negative control cells). The results were considered positive if the ΔSFC/10^6 was more
458 than two standard deviations above the negative control.
459

460 For assessment of peptide-specific T cell proliferation, murine lymphocytes were
461 resuspended at 3.5x10^6 cells/mL in supplemented HL-1 media (Lonza, UK) (1% L-
462 glutamine, 1% penicillin/streptomycin, 2.5% β-mercaptoethanol) and 100 µL/well was
463 plated out in triplicate in 96-well Costar tissue culture plates (Corning Incorporated,
464 USA). The cells were stimulated with 100 µL/well of appropriate antigen, positive
465 controls of 5 µg/mL Con A (Sigma-Aldrich, USA) or 25 ng/mL of SEB (Sigma-Aldrich,
466 USA) or negative controls of medium with cells. Plates were incubated at 37°C with 5%
467 CO_2 for 5 days. Eight hours before harvesting, 1 µCi/well of [^{3}H]-thymidine (GE

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468 Healthcare, UK) was added. The cells were harvested onto fiberglass filtermats
469 (PerkinElmer, USA) using a Harvester 96 cell harvester (Tomtec, USA) and counted on a
470 Wallac Betaplate scintillation counter (EG&G Instruments, Netherlands). Results were
471 expressed as either delta counts per minute (ΔCPM which is calculated as CPM of
472 stimulated cells minus CPM of negative control cells) or stimulation index (SI which is
473 calculated as CPM of stimulated cells divided by CPM of negative control cells). An SI
474 of ≥ 2.5 was considered to indicate a positive proliferation response.
475

476 ***PA epitope mapping with human donor PBMC samples***

477 Lymphocytes were isolated from human peripheral blood samples and stimulated as
478 described previously [25]. In brief, sodium-heparinised blood was collected with full
479 informed consent (Ericyes University Ethical Committee) from nine Turkish patients
480 treated for cutaneous anthrax infection within the last eight years and 10 volunteers
481 routinely vaccinated every 12 months for a minimum of five years with the UK AVP
482 vaccine (UK Department of Health under approval by the Convention on Biological
483 Diversity Independent Ethics Committee for the UK Ministry of Defence). Peripheral
484 blood mononuclear cells (PBMC) were isolated from the blood by centrifugation at 800g
485 for 30 minutes in Accuspin tubes (Sigma, UK) cells were then removed from the
486 interface and washed twice in AIM-V serum free media. Cells were counted for viability
487 and resuspended at $2x10^6$ cells/mL.
488

489 Human T cell INFγ levels were quantified by ELISpot (Diaclone, France) as previously
490 described [25]. In brief, the peptide library was prepared in a matrix comprising six

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491 peptides per pool, so that each peptide occurred in two pools but no peptides occurred
492 together in multiple pools. This allowed the determination of responses to individual
493 peptides. The in-well concentration of each peptide was 25 µg/mL and total peptide
494 concentration per well was 150 µg/mL. After addition of antigen to the wells the plates
495 were frozen at -80 °C until use. Wells were seeded with human PBMCs at 2 x 10^5
496 cells/well (range: 1.6 x 10^5 to 2.1 x 10^5 cells/well) in AIM-V media (Gibco, UK) and
497 plates were incubated for 72 hours at 37 °C with 5% CO2. The plate contents were then
498 discarded and plates were washed with PBS-Tween 20 (0.1%) and incubated with
499 biotinylated anti-INFγ, then washed again before streptavidin-alkaline-phosphatase
500 conjugate was added. After a final wash, plates were developed by addition of
501 BCIP/NBT. Spots were counted using an automated ELISpot reader (AID), and results
502 were expressed as ΔSFC/10^6. The results were considered positive if the ΔSFC/106 was
503 more than two standard deviations above the negative control and ≥ 50 spots.
504

505 ### *HLA-peptide binding assay*

506 Competitive ELISAs were used to determine the relative binding affinity of PA peptides
507 to HLA-DR molecules as previously described [48, 49]. Briefly, the HLA-DR molecules
508 were immunopurified from homozygous EBV-transformed lymphoblastoid B cell lines
509 by affinity chromatography. The HLA-DR molecules were diluted in HLA binding buffer
510 and incubated for 24 to 72 hours with an appropriate biotinylated reporter peptide, and a
511 serial dilution of the competitor PA peptides. Controls of unlabelled reporter peptides
512 were used as reference peptides to assess the validity of each experiment. 50 µL of HLA
513 binding neutralisation buffer was added to each well and the resulting supernatants were

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514 incubated for 2 hours at room temperature in ELISA plates (Nunc, Denmark) previously
515 coated with 10 μg/mL of the monoclonal antibody L243. Bound biotinylated peptide was
516 detected by addition of streptavidin-alkaline phosphatase conjugate (GE Healthcare,
517 France) and 4-methylumbelliferyl phosphate substrate (Sigma-Aldrich, France). Emitted
518 fluorescence was measured at 450 nm post-excitation at 365 nM on a SpectraMax Gemini
519 fluorometer (Molecular Devices, France). The PA peptide concentration that prevented
520 binding of 50% of the labeled peptide (IC_{50}) was evaluated, and data expressed as relative
521 binding affinity (ratio of IC_{50} of the PA competitor peptide to the IC_{50} of the reference
522 peptide that binds strongly to the HLA-DR molecule). Sequences of the reference
523 peptides and their IC50 values were as follows: HA 306–318 (PKYVKQNTLKLAT) for
524 DRB1*0101 (4 nM), DRB1*0401 (8 nM), DRB1*1101 (7 nM), YKL
525 (AAYAAAKAAALAA) for DRB1*0701 (3 nM), A3 152–166
526 (EAEQLRAYLDGTGVE) for DRB1*1501 (48 nM), MT 2–16 (AKTIAYDEEARRGLE)
527 for DRB1*0301 (100 nM), B1 21–36 (TERVRLVTRHIYNREE) for DRB1*1301 (37
528 nM), DQB45–57 (ADVEVYRAVTPLGPPD) for DQ8 (100 nM) and INS1–15A
529 (FVNQHLAGSHLVEAL) for DQ6 (100nM). Strong binding affinity was defined in this
530 study as a relative activity <100.

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532 Author Contributions

533 Conceived and designed the experiments: SA RJI KKC EDW LB SS JHR BM RJB
534 DMA. Performed the experiments: SA RJI KKC HD EDW JHR BM SJM. Analysed the
535 data: SA RJI KKC JHR BM. Contributed reagents/materials/analysis tools: MD GM YO
536 LB SJM TG HD. Wrote the paper: SA RJI RJB DMA. All authors listed have made a

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537 substantial, direct, and intellectual contribution to the manuscript and approved it for
538 publication.
539

540 Conflict of Interest Statement

541 DMA has received payment in a role as scientific consultant to the anthrax vaccine
542 programme at Pfenex Inc. San Diego. The authors declare that this relationship had no
543 role in the study design, data collection and analysis, decision to publish, or preparation
544 of the manuscript.

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547

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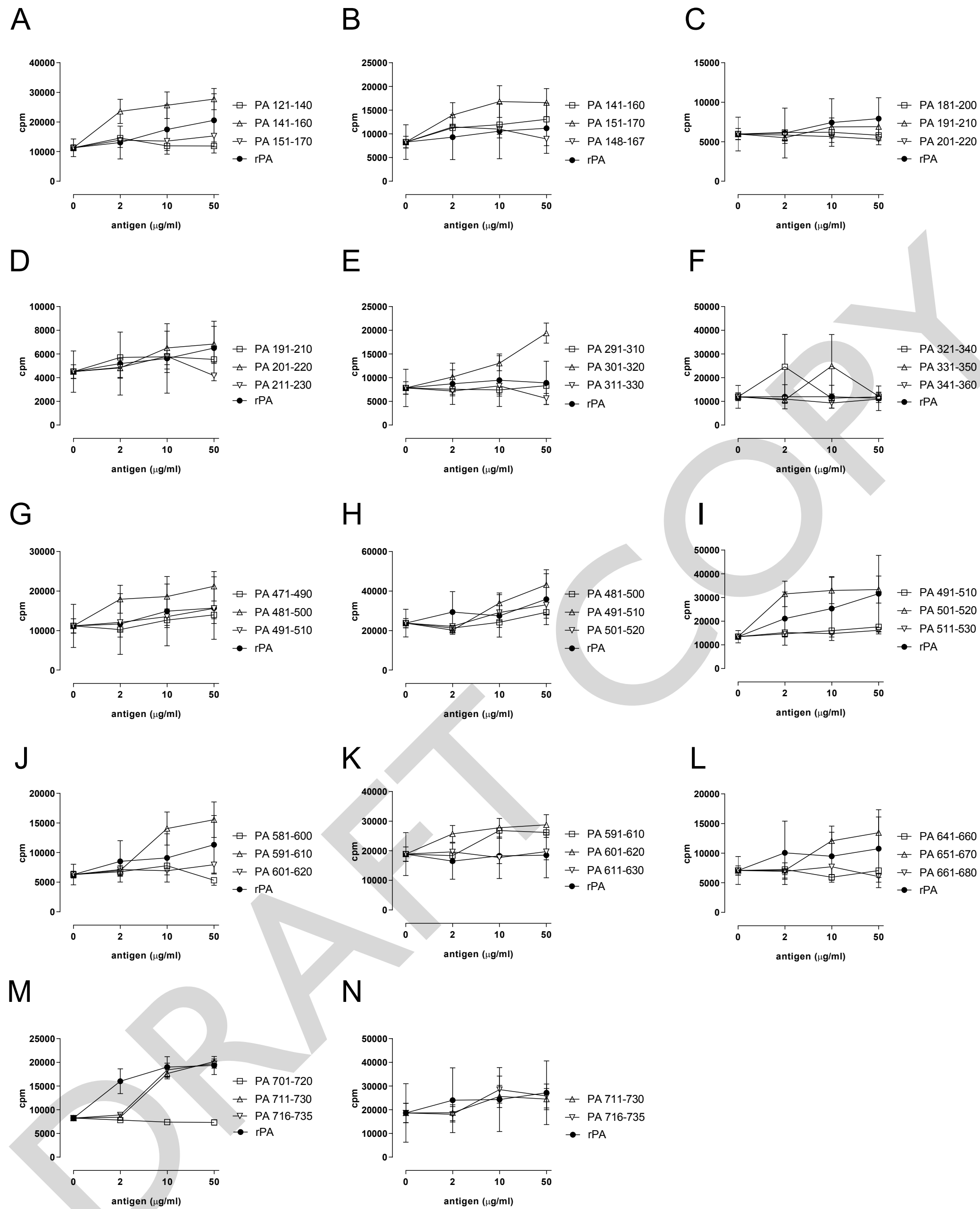

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754 **Supporting Figure 1.**

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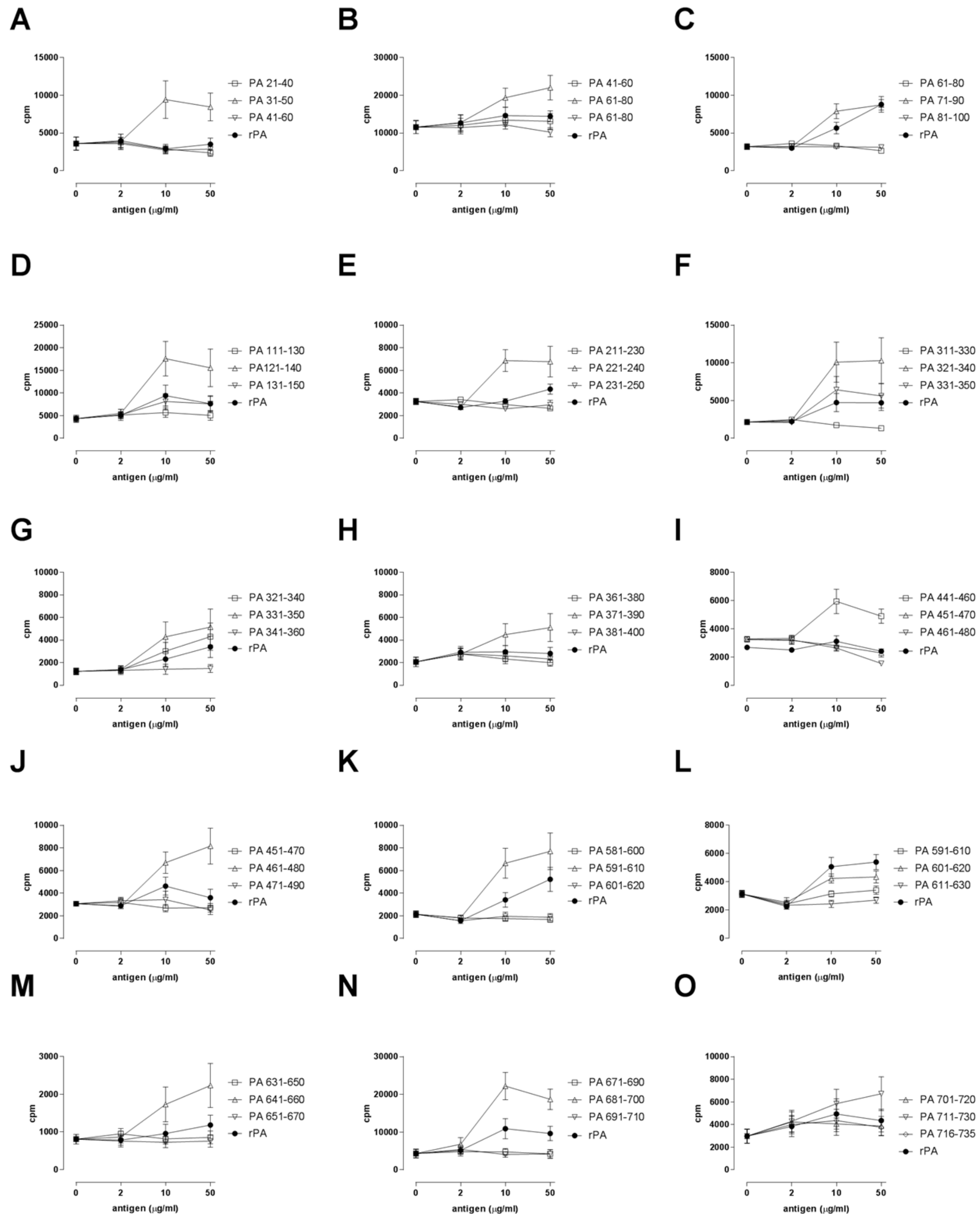

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756 **Supporting Figure 2.**
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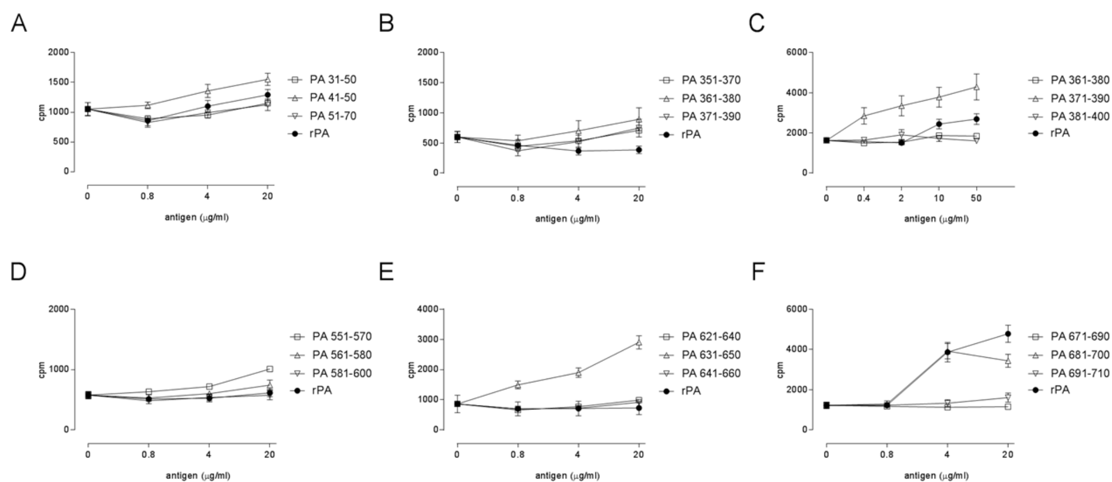

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760 **Supporting Figure 3.**
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762 **Supporting Table 1. CD4+ T cell responses to B. anthracis PA epitopes in AVP vaccinees.**

Human cohorts	HLA class II						T cell response to anthrax PA domain I-IV epitopes, SFC/10^6 cells																									
	HLA-DRB1*		HLA-DRB3*/4*/5*		HLA-DQB1*		11-30	21-40	41-60	61-80	81-100	141-160	161-180	191-210	221-240	241-260	261-280	301-320	321-340	361-380	391-410	421-440	431-450	491-510	501-520	521-540	561-580	601-620	621-640	631-650	641-660	671-690
AVP vaccinee 1	11	15	51	52	6	7	0	0	0	0	0	0	219	266	0	0	0	0	0	190	217	0	284	0	0	0	0	0	0	242	242	313
AVP vaccinee 2	11	15	51	52	6	7	0	891	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	819	0	0	0	0	0	0	0
AVP vaccinee 3	11	13	52	-	6	7	1247	1177	1057	977	895	933	0	0	1159	1077	485	681	169	521	0	1123	1199	1109	1133	857	821	1015	1077	0	0	1079
AVP vaccinee 4	15	7	51	53	2	6	0	0	0	0	0	0	0	0	0	519	0	309	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AVP vaccinee 5	103	17	52	-	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AVP vaccinee 6	1	13	52	-	5	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AVP vaccinee 7	11	15	51	52	6	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AVP vaccinee 8	1	-	-	-	5	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AVP vaccinee 9	4	12	52	53	7	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AVP vaccinee 10	7	15	51	53	2	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Supporting Table 2. CD4+ T cell responses to B. anthracis PA epitopes in anthrax-recovered patients.

Human cohorts	HLA class II						T cell response to anthrax PA domain I-IV epitopes, SFC/10^6 cells														
	HLA-DRB1*		HLA-DRB3*/4*/5*		HLA-DQB1*		1-20	11-30	21-40	31-50	41-60	51-70	61-80	71-90	81-100	91-110	101-120	121-140	131-150	141-160	151-170
Infected donor 1	11	13	52	-	6	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 2	4	-	53	-	8	-	299	330	301	263	224	0	203	0	0	0	0	0	304	299	417
Infected donor 3	4	14	52	53	5	8	0	0	0	0	0	0	0	486	0	454	0	0	0	0	0
Infected donor 4	15	-	51	-	6	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 5	8	11	52	-	7	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	977
Infected donor 6	11	13	52	-	6	7	0	0	0	0	0	0	0	0	0	0	0	581	0	0	0
Infected donor 7	4	14	52	53	5	-	0	0	1017	0	971	723	1193	957	1225	949	793	451	0	505	691
Infected donor 8	1	16	51	-	5	-	0	0	0	0	759	784	0	0	0	0	0	0	0	0	0
Infected donor 9	15	13	51	52	6	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Human cohorts	T cell response to anthrax PA domain I-IV epitopes, SFC/106 cells																
	148-167	168-187	161-180	171-190	181-200	191-210	211-230	221-240	231-250	241-260	261-280	271-290	281-300	301-320	311-330	321-340	331-350
Infected donor 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 2	337	383	349	268	0	229	229	246	0	304	0	0	0	176	0	0	0
Infected donor 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 6	0	0	0	0	0	0	0	0	0	0	0	0	641	0	0	0	477
Infected donor 7	1013	919	1251	915	911	921	0	0	1117	711	757	1063	759	649	799	805	685
Infected donor 8	0	813	0	0	0	0	0	0	0	0	723	0	0	0	0	212	0
Infected donor 9	0	263	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Human cohorts	T cell response to anthrax PA domain I-IV epitopes, SFC/106 cells																
	341-360	351-370	361-380	371-390	381-400	391-410	401-420	411-430	421-440	431-450	441-460	451-470	461-480	471-490	481-500	491-510	501-520
Infected donor 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 2	0	263	325	304	0	263	215	268	323	299	0	232	179	222	0	325	289
Infected donor 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 6	0	0	0	0	0	0	0	0	0	599	801	0	0	0	577	0	0
Infected donor 7	891	0	747	735	855	1199	0	0	859	1039	0	899	0	0	807	0	661
Infected donor 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Human cohorts	T cell response to anthrax PA domain I-IV epitopes, SFC/106 cells																
	511-530	521-540	531-550	541-560	551-570	561-580	571-590	581-600	591-610	601-620	611-630	621-640	631-650	641-660	651-670	661-680	671-690
Infected donor 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 2	241	234	0	306	0	0	0	0	232	234	301	311	414	357	270	256	222
Infected donor 3	0	0	0	0	0	0	396	0	0	0	0	0	0	0	0	0	0
Infected donor 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 5	1061	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected donor 6	0	0	603	0	0	0	495	779	0	0	0	0	539	0	0	0	545
Infected donor 7	419	815	995	1391	881	1085	983	619	0	0	0	621	1043	0	893	705	993
Infected donor 8	0	617	846	0	0	0	0	0	0	0	0	0	0	0	823	0	0
Infected donor 9	0	0	0	0	596	0	0	0	0	0	0	0	0	0	474	0	0

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	T cell response to anthrax PA domain I-IV epitopes, SFC/106 cells		
Human cohorts	681-700	691-710	716-735
Infected donor 1	0	0	0
Infected donor 2	0	210	0
Infected donor 3	532	0	0
Infected donor 4	0	0	0
Infected donor 5	0	0	0
Infected donor 6	905	0	0
Infected donor 7	929	1121	869
Infected donor 8	0	0	0
Infected donor 9	0	0	0

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776 **Supporting Table 3. Differential susceptibility of HLA class II transgenic mice to anthrax infection.**

HLA	*B. anthracis* STI challenge dose (CFU)	Number of mice challenged	Number of challenge survivors	Mean time to death (days)	Bacterial load in spleens within observation period post-infection mean CFU/spleen (± SEM)	Bacterial load in spleens of survivors at day 20 mean CFU/spleen (± SEM)	Estimated LD_{50} (CFU)
C57Bl6	10^5	10	4	4.5 (±0.22)	$1.0x10^3$ (±$0.32x10^3$) (n=6)	91 (±18)	10^5
DQ6	10^5	8	8	-	-	255 (±39)	>10^5
DR4	10^5	10	8	7 (±3)	$1.27x10^3$ (n=1)	22 (±13)	>10^5
DR15	10^5	9	5	5.75 (±0.48)	$0.85x10^3$ (±0.21 $x10^3$) (n=4)	67 (±32)	10^5
DQ8	10^6	10	8	6	$2.17x10^3$ (±$0.67x10^3$) (n=2)	896 (±263)	>10^6
DR1	10^6	10	10	-	-	411 (±93)	>10^6

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Figure Captions

Figure 1. Heat map of CD4+ T cell epitope responses to anthrax PA domain I-IV peptides in human donors.
Heat map representation of the epitope mapping results observed for positive CD4+ T cell IFNγ ELIspot responses in the human donor cohorts, comprising a total of 9 donors in the cutaneous anthrax (Kayseri) group and 10 donors in the AVP vaccinees (UK) group. Peptides were considered positive for the carriage of a CD4+ T cell epitope if the response was >50 SFC/10^6 PBMCs and 2SD above negative control, and the stimulation index (peptide response/negative control response) value was ≥ 1.5. The domains were defined as described previously; domain 1 = PA 1-20 to PA 241-260; domain 2 = PA 251-270 to PA 471-490; domain 3 = PA 491-510 to PA 581-600; domain 4 = PA 591-610 to PA 716-735, with some peptides overlapping the boundaries between domains) [50]. The colour bar at the right indicates the percentage of donors responding to a given epitope, with shading from white (0%) to dark blue (50%).

Figure 2. Differential susceptibility of HLA class II transgenic mice to anthrax infection.
Groups of naïve HLA transgenic (DR1 n=11, DQ8 n=10, DR15 n=9, DR4 n=10, DQ6 n=8) or C57Bl6 (n=10) mice were challenged with either 10^5 (C and D) or 10^6 (A and B) CFU *B. anthracis* STI strain, in order to compare susceptibility. Mice were challenged intraperitoneally and their survival observed for 20 days post-infection. Percentage survival, together with mean splenic bacterial counts per HLA type, is shown for mice succumbing within the observation period (days 1 to 19) and for survivors culled at day 20. Statistical comparison of mean bacterial loads by mouse strain (D) indicated that higher bacterial loads were seen in DQ6 in comparison to; C57BL/6 (** p=0.0093), DR4 (**** p<0.0001) and DR15 (** p=0.0014), (One-way ANOVA, Tukey's multiple comparisons).

Figure 3. T-cell responses to PA peptides in whole rPA-immunised HLA-DR and HLA-DQ transgenic mice.
Groups of HLA transgenic mice were immunised with the whole rPA protein in adjuvant, and the proliferative responses of draining lymph node cells to overlapping synthetic peptides representing the complete PA sequence were determined. Scatter plots show responses of individual mice transgenic for (A) HLA-DR1 (n=3 for each peptide data point, and n=11 for the rPA data point), B) HLA-DQ8 (n=6 for each peptide data point, n=18 for the rPA data point) and C) HLA-DR4 (n=6 for each peptide data point, and n=17 for the rPA data point). Data is presented as the SI calculated as the mean CPM of triplicate wells in the presence of peptide divided by the mean CPM in the absence of antigen. Values twice the mean CPM in the absence of antigen were considered positive responses. Confirmed epitopes are highlighted in red.

Supporting Figure 1. Fine specificity mapping of previously identified HLA-DQ8 restricted T cell epitopes.

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851 HLA-DQ8 transgenics were immunised with the previously identified PA peptide in
852 adjuvant. The proliferative responses of draining lymph node cells were measured in
853 response to the indicated concentrations of whole PA protein, domains I-IV of the protein
854 and the immunising and flanking peptides. The responses are shown as the stimulation
855 index calculated as the mean CPM of triplicate wells in the presence of peptide divided
856 by the mean CPM in the absence of antigen. Values twice the mean CPM in the absence
857 of antigen were considered positive responses (n=3 for each data point).
858

859 **Supporting Figure 2. Fine specificity mapping of previously identified HLA-DR4**
860 **restricted T cell epitopes.**
861 HLA-DR4 transgenics were immunised with the previously identified PA peptide in
862 adjuvant. The proliferative responses of draining lymph node cells were measured in
863 response to the indicated concentrations of whole PA protein, domains I-IV of the protein
864 and the immunising and flanking peptides. The responses are shown as the stimulation
865 index calculated as the mean CPM of triplicate wells in the presence of peptide divided
866 by the mean CPM in the absence of antigen. Values twice the mean CPM in the absence
867 of antigen were considered positive responses (n=3 for each data point).
868

869 **Supporting Figure 3. Fine specificity mapping of previously identified HLA-DR1**
870 **restricted T cell epitopes.**
871 HLA-DR1 transgenics were immunised with the previously identified PA peptide in
872 adjuvant. The proliferative responses of draining lymph node cells were measured in
873 response to the indicated concentrations of whole PA protein, domains I-IV of the protein
874 and the immunising and flanking peptides. The responses are shown as the stimulation
875 index calculated as the mean CPM of triplicate wells in the presence of peptide divided
876 by the mean CPM in the absence of antigen. Values twice the mean CPM in the absence
877 of antigen were considered positive responses (n=3 for each data point).
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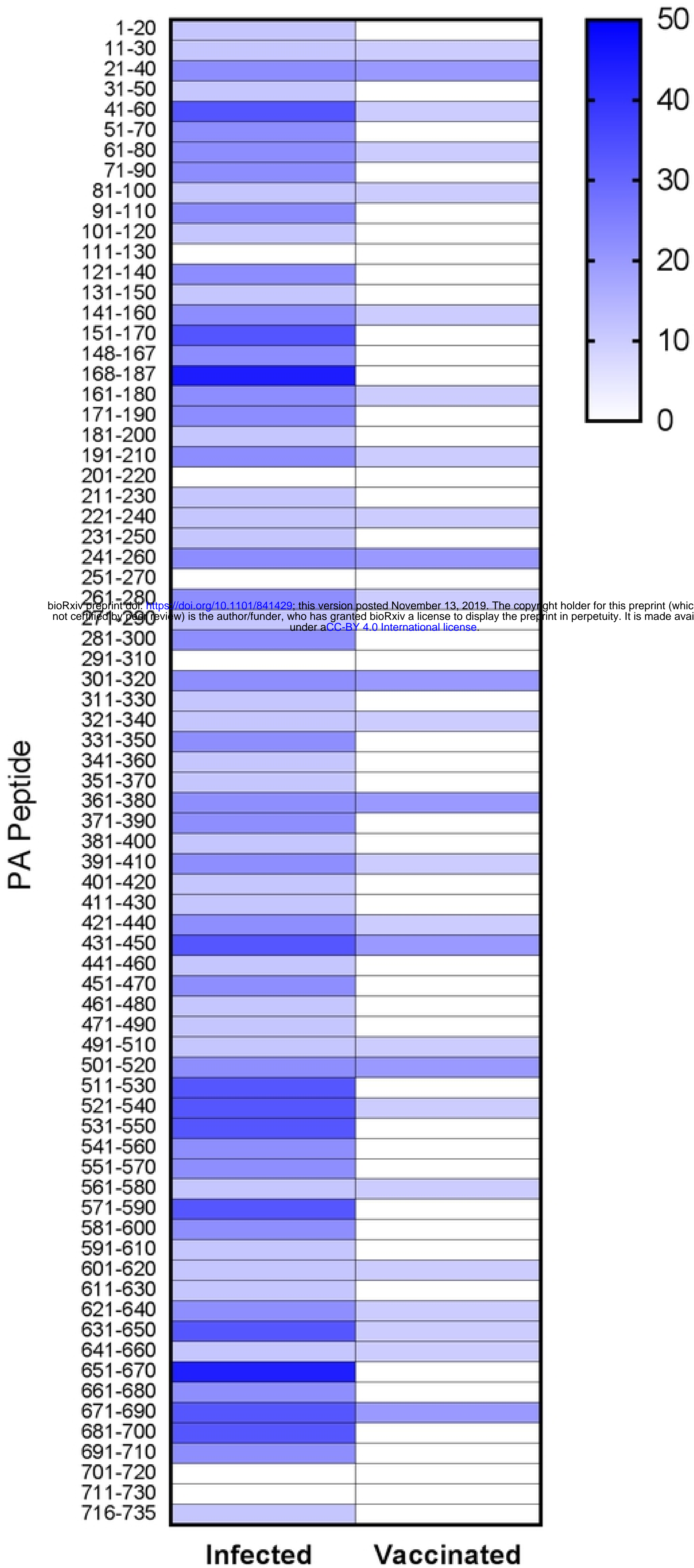

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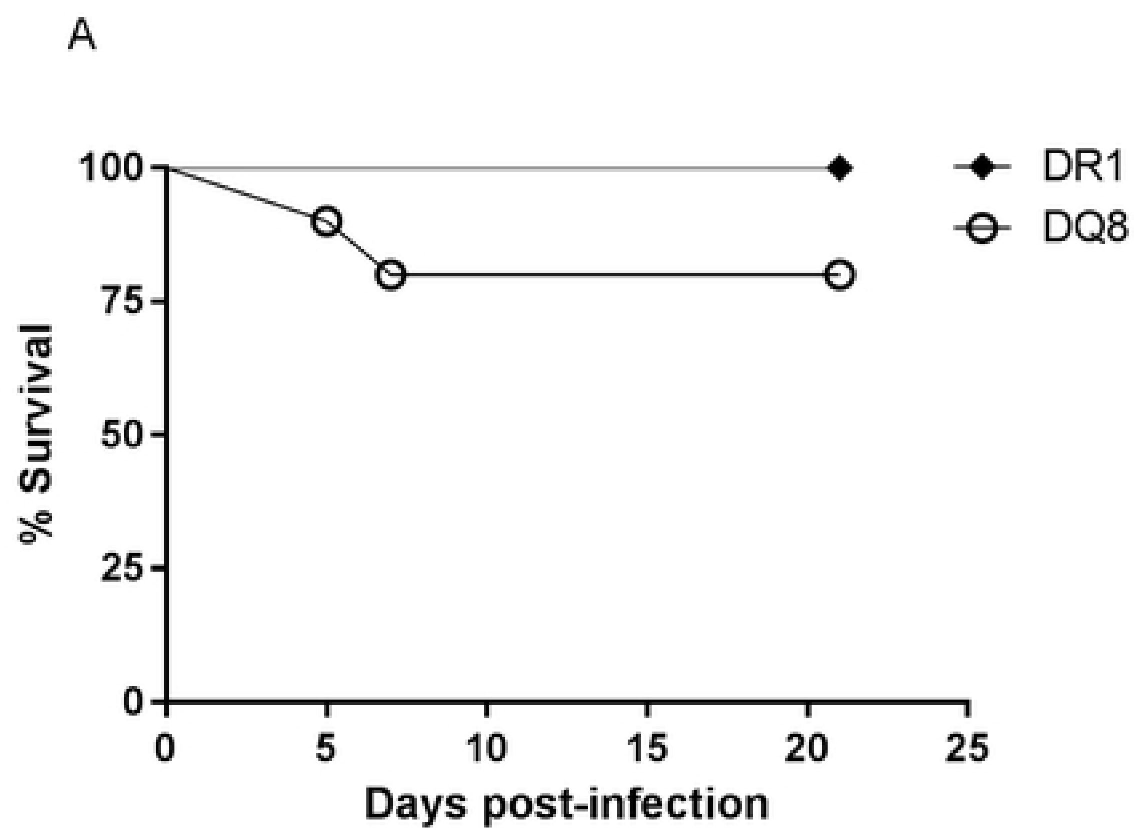
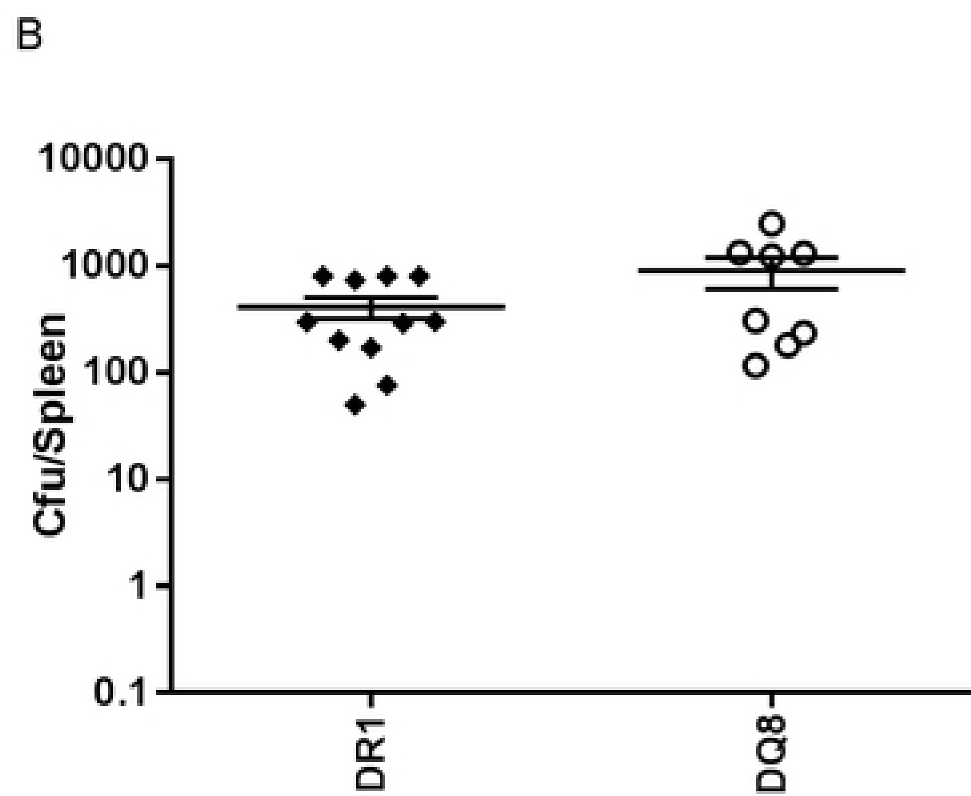
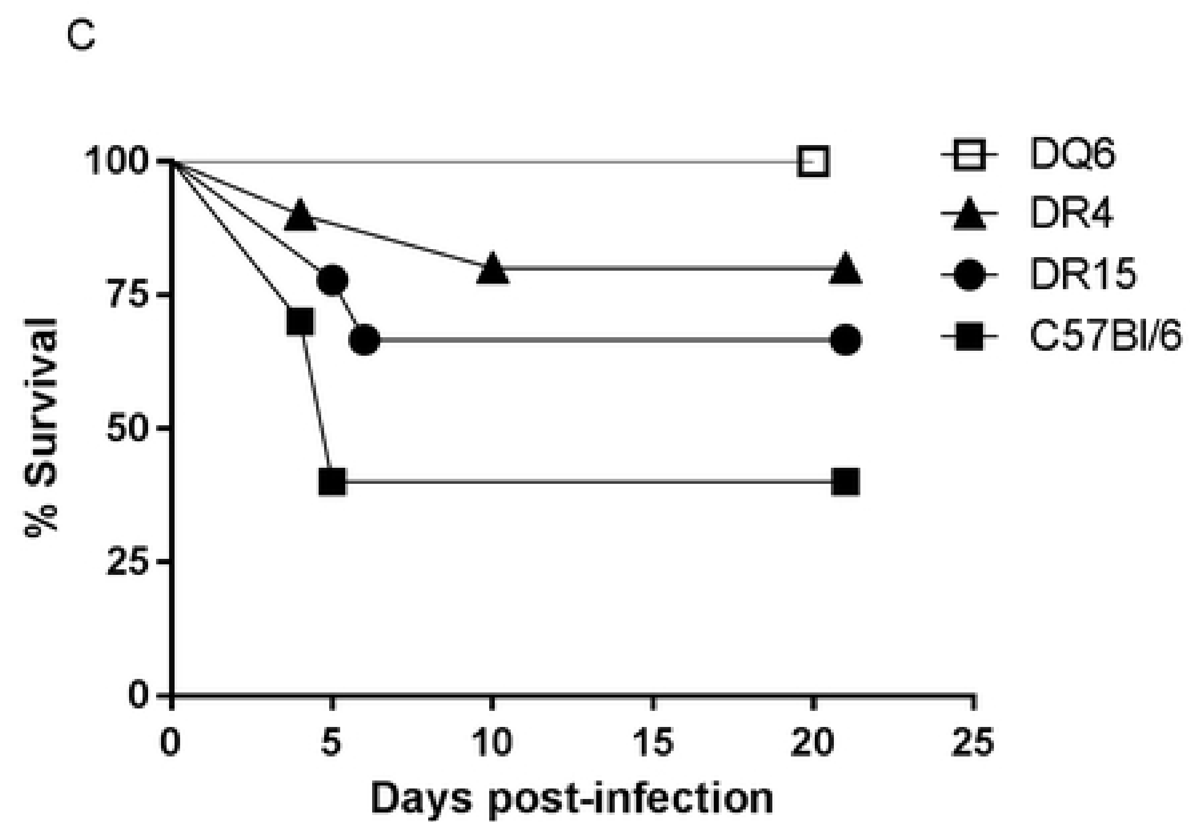
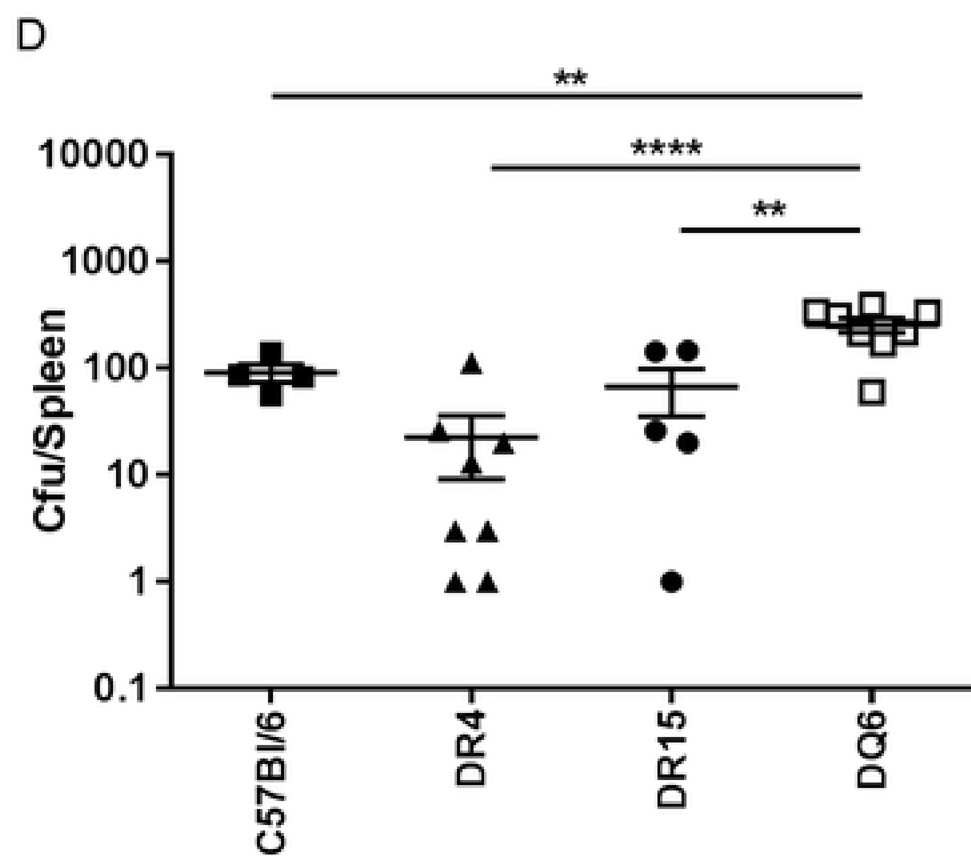
Figure









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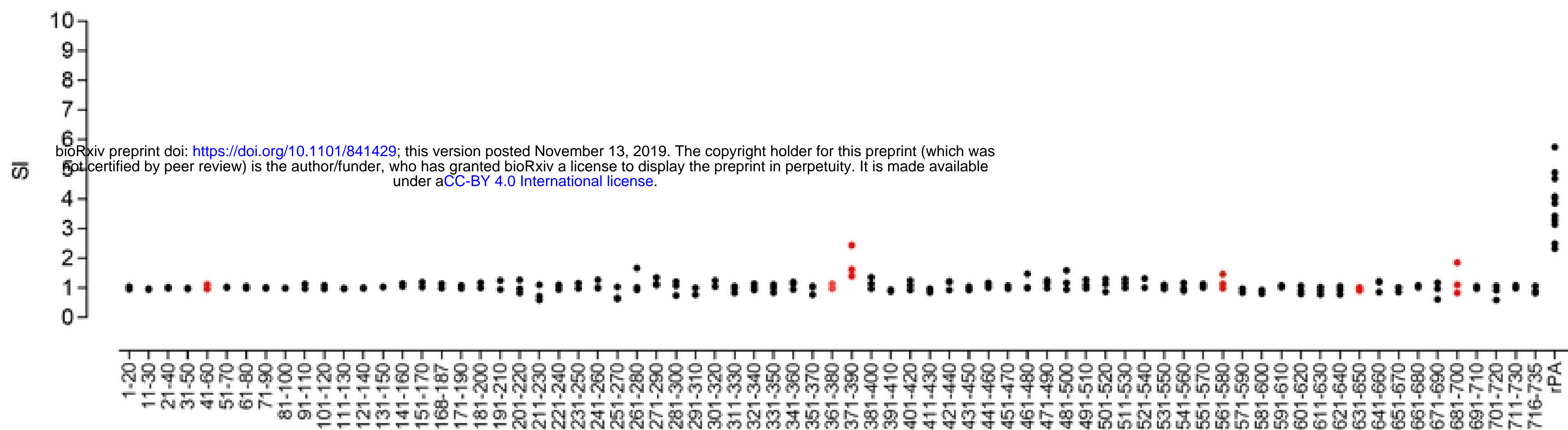

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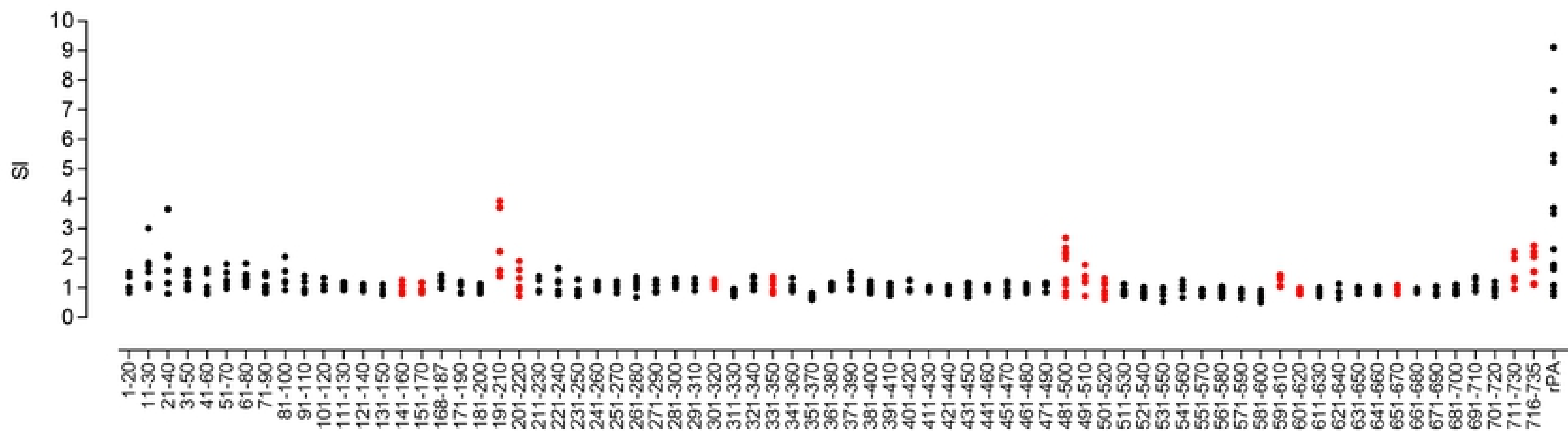

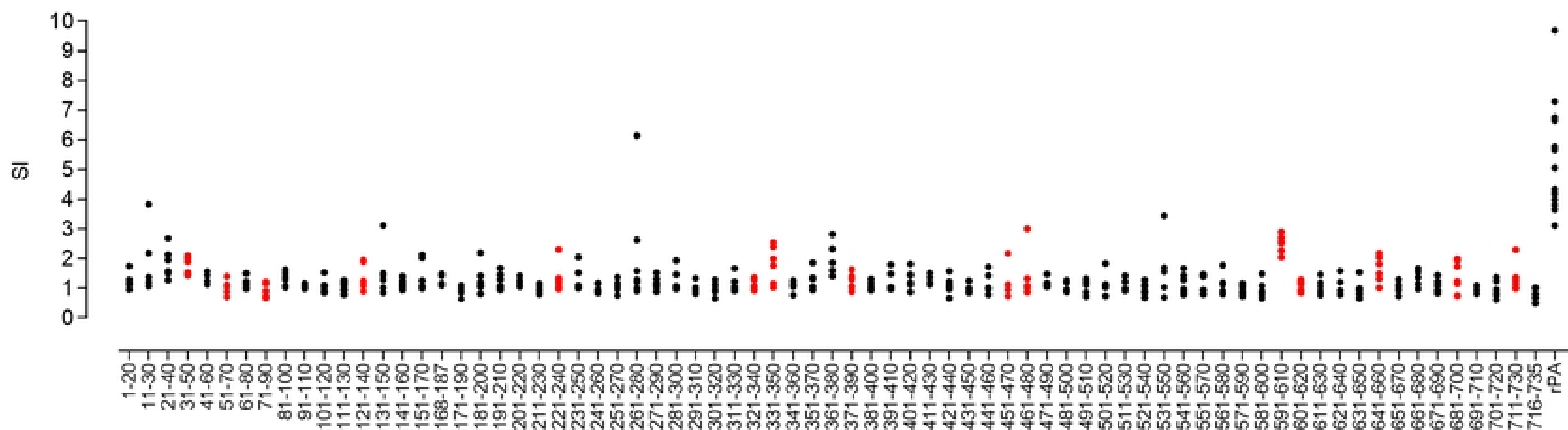

Figure