

Humoral and cellular immune correlates of protection against bubonic plague by a live *Yersinia pseudotuberculosis* vaccine

Christian E. Demeure, Anne Derbise, Chloé Guillas, Christiane Gerke, Simon Cauchemez, Elisabeth Carniel, Javier Pizarro-Cerdá

► To cite this version:

Christian E. Demeure, Anne Derbise, Chloé Guillas, Christiane Gerke, Simon Cauchemez, et al.. Humoral and cellular immune correlates of protection against bubonic plague by a live *Yersinia pseudotuberculosis* vaccine. *Vaccine*, Elsevier, 2019, 37 (1), pp.123-129. 10.1016/j.vaccine.2018.11.022 . pasteur-02067807

HAL Id: pasteur-02067807

<https://hal-pasteur.archives-ouvertes.fr/pasteur-02067807>

Submitted on 14 Mar 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Humoral and cellular immune correlates of protection against bubonic plague by a**
2 **live *Yersinia pseudotuberculosis* vaccine**

3 Christian E. Demeure^{1#}, Anne Derbise¹, Chloé Guillas¹, Christiane Gerke², Simon
4 Cauchemez^{3,4,5}, Elisabeth Carniel^{1,*} and Javier Pizarro-Cerdá¹.

5 Christian E. Demeure: cdemeure@pasteur.fr

6 Anne Derbise: anne.derbise@pasteur.fr

7 Chloé Guillas: chloe.guillas@pasteur.fr

8 Christiane Gerke : christiane.gerke@pasteur.fr

9 Simon Cauchemez: simon.cauchemez@pasteur.fr

10 Elisabeth Carniel: elisabeth.carniel@pasteur.fr

11 Javier Pizarro Cerda: javier.pizarro-cerda@pasteur.fr

12

13 ¹ Unité de Recherche *Yersinia*. Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex
14 15, France.

15 ² Vaccine Programs, Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15,
16 France.

17 ³ Unité de Modélisation Mathématique des Maladies Infectieuses, Institut Pasteur, 25-28
18 rue du Dr Roux, 75724 Paris Cedex 15, France.

19 ⁴ Centre National de la Recherche Scientifique, URA3012, 25-28 rue du Dr Roux, 75724
20 Paris Cedex 15, France

21 ⁵ Center of Bioinformatics, Biostatistics and Integrative Biology, 25-28 rue du Dr Roux,
22 75724 Paris Cedex 15, France

23

24 * Present address: Pasteur Center of Cameroon, BP 1274, Yaounde, Cameroon.

25

26 **Short title:** Correlates of protection for a live plague vaccine

27 #: Corresponding author: Dr C.E. Demeure,

28 Tel: (33-1)-45-68-84-48

29 Fax: (33-1)-40-61-30-01.

30

31 **Highlights:**

32

33 • IgG & cells triggered by a *Y. pseudotuberculosis* live vaccine each protect against
34 bubonic plague

35 • IFN γ production and IgG to F1, Yops or sonicated *Y. pestis* correlate to each other and
36 to protection

37 • Anti-F1 IgG is the easiest and statistically best predictor of protection, transposable to
38 humans

39

40 **Abstract:**

41 Immunization with the live-attenuated *Yersinia pseudotuberculosis* VTnF1 strain
42 producing a *Yersinia pestis* F1 pseudocapsule efficiently protects mice against bubonic and
43 pneumonic plague. In clinical trials, demonstration of a plague vaccine's efficacy in humans
44 will not be feasible, and correlates of protection will be needed to bridge the immune
45 response of protected animals to that of vaccinated humans. Using serum transfer and
46 vaccination of antibody-deficient μ MT mice, we established that both humoral and cellular
47 responses elicited by VTnF1 independently conferred protection against bubonic plague.
48 Thus, correlates were searched for in both responses, using blood only. Mice were
49 vaccinated with increasing doses of VTnF1 to provide a range of immune responses and
50 survival outcomes. The cellular response was evaluated using an *in vitro* IFN γ release
51 assay, and IFN γ levels were significantly associated with protection, although some
52 survivors were negative for IFN γ , so that IFN γ release is not a fully satisfactory correlate.
53 Abundant serum IgG against the F1 capsule, Yop injectable toxins, and also non-F1
54 *Y. pestis* antigens were found, but none against the LcrV antigen. All readouts correlated to
55 survival and to each other, confirming that vaccination triggered multiple protective
56 mechanisms developing in parallel. Anti-F1 IgG was the most stringent correlate of
57 protection, in both inbred BALB/c mice and outbred OF1 mice. This indicates that
58 antibodies (Ab) to F1 play a dominant role for protection even in the presence of Ab to
59 many other targets. Easy to measure, the anti-F1 IgG titer will be useful to evaluate the
60 immune response in other animal species and in clinical trials.

61

62 **Keywords:**

63 Plague, live vaccine, *Yersinia pestis*, *Yersinia pseudotuberculosis*, correlates of protection,
64 F1, IgG

65

66

67 **Introduction**

68 Plague is caused by *Y. pestis*, one of the deadliest infectious agents afflicting
69 humans. In nature, *Y. pestis* is mainly a zoonotic pathogen, infecting rodents in large
70 endemic territories throughout the world. Therefore, plague cannot be eradicated and has a
71 permanent capacity to pass to humans. Despite the availability of antibiotic treatments,
72 plague still has a high level of mortality, due mainly to the fast development of
73 pathogenesis. In addition, *Y. pestis* strains showing resistance to several antibiotics have
74 emerged [1, 2] and *Y. pestis* is a potential bioweapon [3]. To face this threat, the
75 development of safe and effective vaccination strategies is critical.

76 We previously reported that *Y. pseudotuberculosis* can be used as vaccine against
77 plague [4] because the two species share high-level genetic and antigenic identity,
78 including the same type III secretion system and injectable Yop toxins (*Yersinia* outer
79 proteins [5]). We constructed the *Y. pseudotuberculosis* strain VTnF1, highly and
80 irreversibly attenuated by deletion of three essential virulence factors (High Pathogenicity
81 Island, pH6/PsaA antigen and YopK toxin). In addition, insertion of the *caf* operon into the
82 chromosome allows production of a *Y. pestis* F1 pseudocapsule [4]. A single oral
83 vaccination with live VTnF1 confers protection against bubonic and pneumonic plague in
84 mice [4].

85 The licensure of a human vaccine requires evaluation of its efficacy in humans. For
86 plague, this is problematic because cases are too sporadic in the world for a field evaluation
87 and human challenge studies are not ethical. In such circumstances, demonstrating efficacy
88 in a well-understood animal model that resembles disease in humans can be considered for
89 approval under the “animal rule” in the USA [6, 7], or the “Extraordinary Use New Drugs
90 Pathway” in Canada [8]. In the EU, a recent perspective from the EMA stated that this does
91 not preclude ‘the possibility that animal models data in principle could have a critical role in
92 the assessment’ [9].

93 To bridge protection in animals to parameters evaluated during the clinical trials in
94 humans, the investigational compound's mechanism of action to prevent the toxic effects of
95 the pathogen need to be well understood. For vaccines, it should lead to the identification of
96 a relevant biomarker/correlate of protection in animals, presumed to also correlate with
97 protection in humans. Also, identifying correlates of protection for vaccine-induced immunity
98 is crucial during vaccine development [10], e.g. as it enables consistency assessment of
99 vaccine production for complex vaccines by identifying the essential antigen(s) for
100 quantification, and can be used for follow up of populations after immunization, including to
101 determine the need and/or frequency of boosters.

102 When modified or killed *Y. pestis* have been used as vaccine, both antibodies (Ab) and
103 the cellular arm of immunity contributed to protection [11-13]. The recently developed F1-V
104 recombinant vaccines are based on a combination of F1 and LcrV antigens and induced
105 high levels of Ab against these targets in mice or non-human primates (NHP). Ab against
106 these antigens are protective [14-16] and their level predicts protection [15, 17]. Whereas
107 measuring Ab as correlate for a vaccine consisting of two purified antigens seems
108 straightforward, the definition of a correlate is more complicated for live vaccines due to
109 their complex composition and the humoral and cellular immune mechanisms triggered.

110 Correlates must be relatively easy to measure, so serum antibodies have caught most of
111 the attention. However, the T cell response may also contribute to protective immunity
112 against *Y. pestis* [11]. As compared to assays measuring antibodies, those measuring
113 cellular responses are more challenging due to logistic and technical limitations [18]. They
114 include the complexity of the assays, the fragility and diversity of living cells, and the
115 absence of standardization of T cell functions. For any test, the biomarker must be
116 measured using the only biological material safely available in humans: the blood.

117 In this study, we examined which humoral and/or cellular immunological readout
118 correlated with protection in mice vaccinated with the VTnF1 strain, in order to determine

119 parameters that can be further developed for evaluation in other animal models and use in
120 future human trials.

121

122 **Materials and Methods**

123 **Bacterial strains**

124 *Y. pestis* and *Y. pseudotuberculosis* isolates and their derivatives have previously
125 been described [4, 19].

126 **Animal vaccination and infection**

127 Institut Pasteur animal facilities are accredited by the French Ministry of Agriculture
128 (N° B 75 15-01; May 22nd, 2008), in compliance with the French and European regulations
129 on care and protection of the Laboratory Animals (EC Directive 86/609, French Law 2001-
130 486; June 6, 2001). The research protocol was approved by the Institut Pasteur Ethics
131 Committee for Animal Experimentation and the French Ministry of Research (N° 2013-
132 0038). Seven-week-old female OF1, BALB/c and C57BL/6 (B6) mice were from Charles
133 River France. μ MT (C57BL/6 μ mt^{-/-}) mice [20] were bred in the Institute. Vaccination
134 consisted in a single dose of VTnF1 (200 μ l) inoculated intragastrically (ig). Blood was
135 collected 3 weeks after vaccination to obtain immune serum (IS).

136 Infections with *Y. pestis* were performed in a BSL3 animal facility as previously [4,
137 19]. *Y. pestis* CO92 grown at 28°C was re-suspended in saline and bubonic plague was
138 induced by injection of 10³ CFU (i.e. 10²xLD₅₀, in 50 μ l) subcutaneously (sc) in ventral skin.
139 Animals clinical state and survival was monitored daily for 21 days.

140 **Immuno-assays**

141 IgG specific for *Yersinia* were quantified by ELISA as described previously [21]:
142 microtiter plates (NUNC) were coated with either purified F1 antigen, or sonicated *Y. pestis*
143 CO92 Δ caf (containing all *Y. pestis* antigens except F1 [4]), or purified recombinant LcrV
144 antigen, or purified Yops, all at 5 μ g/ml in PBS, except LcrV: 1 μ g/ml. The ELISA using
145 Yops was initially developed for the sero-diagnostic of infection by pathogenic *Yersinia*

146 [22]. The Yops solution used contains a mix of all the *Yersinia* Outer Proteins (Yops)
147 produced in medium by bacteria grown at 37°C in the absence of Calcium, as described by
148 Michiels [23]. Sera diluted in PBS containing 0.1% BSA were incubated in coated plates.
149 Bound IgG were detected by a mouse IgG-specific, HRPO-coupled, rat antibody (Bio-Rad.
150 Antibody titers were calculated as the reciprocal of the lowest sample dilution giving a
151 signal equal to two times the background.

152 **Cell-mediated response of vaccinated animals**

153 Blood collected on heparin (50 U) was centrifuged on a Lympholyte®-Mammal
154 gradient (Cedarlane, manufacturer' protocol) to separate leukocytes from plasma and
155 erythrocytes. Plasma was frozen for IgG evaluation, while leukocytes were re-suspended in
156 culture medium: RPMI 1640 + Glutamax™ (Invitrogen) containing 10% bovine serum
157 (Lonza), Penicillin /Streptomycin (Life Sciences) and 10 mM β-mercapto-ethanol.
158 Leukocytes (10⁶ cells) were restimulated *in vitro* using either sterile F1 antigen or sonicated
159 *Y. pestis* CO92Δ*caf* (both at 5 μg/ml). Supernatants were collected after 48 h for cytokine
160 measurements (IFNγ and IL-17 Duosets, R&D Systems).

161 **Transfer of immune serum and vaccination of antibody-deficient μMT mice**

162 To evaluate the protective value of antibodies, OF1 mice received either normal or
163 immune serum (500 μl) intraperitoneally (ip) two hours before a bubonic plague challenge
164 (CO92, 10³ CFU sc). To evaluate the protective value of the cellular immune response,
165 μMT mice (unable to produce antibodies [20]) and immune-competent B6 littermates were
166 vaccinated. Because VTnF1 was harmful for immuno-deficient μMT mice (Fig S1),
167 vaccinated and control mice received immune serum (100 μl ip) at the time of vaccination,
168 according to Parent *et al.* [12]. Specific antibodies in blood were evaluated by ELISA every
169 four weeks, and mice were challenged when antibodies were undetectable in μMT mice.

170 **Statistical analysis**

171 We studied the association between IgG titers (log₁₀ transformed) and the probability
172 to survive a plague challenge. Univariate logistic regressions were used to compute the

173 odds ratio for survival to the plague challenge for each 10-fold increase in IgG titers (R software
174 3.3.2). The Akaike's Information Criterion (AIC) was calculated to compare logistic
175 regression models with the best fitting model, having the lowest AIC. The significance level
176 chosen for all analysis was $p < 0.05$ (two-tailed). The Prism software (GraphPad) was used
177 for other statistics: the Fisher exact test to compare survival rates, the unpaired, two-tailed
178 Mann-Whitney test to compare groups for antibody titers and cytokines levels, and the two-
179 tailed Spearman's rank test for correlation analyses.

180

181 **RESULTS**

182 **VTnF1 vaccine-induced protection is mediated by both humoral and cellular** 183 **immunity**

184 We previously reported that oral vaccination with the VTnF1 strain protected against
185 bubonic plague and induced the production of both specific antibodies and memory cells
186 [4]. To determine which immunological effectors conferred protection, the contribution of
187 antibodies was examined by passive serum transfer. Serum from VTnF1-vaccinated mice
188 or naïve mice (pools from 14 mice) was injected to naïve mice 2 hours prior to infection.
189 Whereas mice with non-immune serum or no serum died, those with immune serum
190 survived (Fig. 1A), showing that VTnF1-specific antibodies alone protected against plague.
191 To evaluate the importance of cellular immunity, μ MT mice (unable to produce antibodies)
192 were vaccinated. Because these mice are too immuno-compromised to tolerate the live
193 vaccine (Fig.S1), immune serum was injected to μ MT mice and B6 littermates at the time of
194 vaccination [12], allowing 100% survival. When IgG were no longer detected in blood of
195 μ MT mice (> 2 months), animals were challenged with *Y. pestis*. All vaccinated μ MT and B6
196 mice survived, whereas unvaccinated mice (having received and eliminated the antibodies)
197 died (Fig.1B), showing that VTnF1-induced cell-mediated immunity alone protected against
198 plague.

199

200 **Evaluation of humoral parameters as correlates of protection against plague**

201 To mimic the heterogeneity of a vaccinated population, groups of mice were vaccinated
202 with suboptimal (no vaccine, 10^6 CFU, 10^7 CFU) to optimal (10^8 CFU) doses. A dose-
203 dependent acquisition of protection was observed in both outbred (OF1) and inbred BALB/c
204 or C57BL/6 (B6) mice (Table 1).

205 To fit with immunogenicity assays in clinical trials, all immune response analyses were
206 performed with blood, the simplest sample for analysis of the human response. Serum IgG
207 against F1, Yops and a preparation of *Y. pestis* antigens other than F1 were quantified. F1
208 is specific for *Y. pestis*, whereas LcrV and Yops are common to pathogenic *Yersiniae*. Both
209 are protective targets abundant *in vivo* [24, 25]. IgG against F1, Yops and non-F1 *Y. pestis*
210 antigens could be evidenced (Fig. 2), but no anti-LcrV IgG above the 50 pg/ml limit of
211 detection of the ELISA, determined using a monoclonal Ab (not shown). Comparable
212 results were obtained with inbred BALB/c or outbred OF1 mice. Despite intra-group
213 variability of IgG levels in all groups, the dose of vaccine clearly dictated the magnitude of
214 the antibody response (Fig. 2 A). In addition, IgG levels against a given target significantly
215 correlated with the levels against the other antigens (Fig. 2 B).

216

217 **Evaluation of vaccine-induced cellular memory against plague**

218 *In vitro* stimulated splenocytes from VTnF1 -vaccinated mice produce IFN γ , revealing a
219 type 1 cellular response (Th1) [4]. Here, the IFN γ release assay was applied to blood
220 leukocytes restimulated with either F1 or sonicated *Y. pestis* CO92 Δ caf (all *Y. pestis*
221 antigens except F1). Purified F1 failed to induce any IFN γ production, whereas the
222 sonicated *Y. pestis* induced IFN γ in the majority of samples (Fig. 3A). Unspecific stimulation
223 was unlikely, because cells from naïve mice did not respond. All mice positive for IFN γ
224 survived plague, although IFN γ was not detected in all protected individuals, yielding a 69%
225 confidence (18 mice / 26 survivors; Fig. 3A). IFN γ correlated significantly with anti-F1 IgG

226 (Fig. 3B) as well as with anti-*Y. pestis* Δcaf IgG (Fig. 3C), and both increased according to
227 the vaccine dose.

228 IL-17 plays a protective role against plague [26], and is produced by splenocytes from
229 VTnF1-vaccinated mice [4]. However, blood leukocyte cultures yielded only very low
230 amounts (not shown) and thus was not further investigated.

231

232 **Logistic regression analysis of serological immune readouts**

233 In BALB/c mice (naïve and vaccinated using all doses), the three serum IgG titers
234 (against F1, Yops & sonicated *Y. pestis*) were strongly associated with survival. IgG anti-F1
235 and IgG anti-*Yersinia* titers perfectly separated mice that survived and those that died. All
236 mice with IgG anti-F1 $\leq 10^3$ and all those with IgG anti-*Yersinia* $\leq 3 \times 10^2$ died while all others
237 survived (Fig. 2A & 4A, Fig. S2A). IgG anti-Yops titers also almost perfectly separated the
238 mice by outcome: most protected mice (28/29) had IgG anti-Yops above 150, and no one
239 which died (Fig. S2B).

240 Because the human population is genetically diverse, the possibility to predict
241 protection was examined in outbred OF1 mice. IgG levels were more heterogeneous (Fig.
242 S3), but each IgG titer was significantly associated with survival as revealed using
243 univariate logistic regressions (Fig. 4B, Fig. S2B,2D), with $p < 0.001$ (Table 2). Based on the
244 AIC, the anti-F1 IgG titer had the strongest association with survival to bubonic plague. For
245 each 10-fold increase in anti-F1 IgG titer, the odd ratio for survival to the plague challenge
246 was 3.5 (95% confidence interval: 2.1, 7.0) (Table 2; Fig. 4B).

247

248

249 **Discussion**

250 Identifying correlates of protection is of high interest during vaccine development, to
251 identify key antigenic components for consistency evaluation of vaccine production, to

252 evaluate the responses of individuals and populations after vaccination, and, as in the case
253 for a plague vaccine for which efficacy trials are not ethical, to establish parameters for
254 evaluation [10]. In order to prepare for future evaluation of the immune response in other
255 animal models and particularly in volunteers in clinical trials, the present work aimed at
256 identifying a correlate of protection in mice vaccinated with VTnF1, which could be
257 measured using blood. In addition to measuring an immune parameter with robust
258 statistical correlation to protection, the test had to be as simple as possible to perform.

259 We found that both humoral and cellular responses elicited by VTnF1 had a protective
260 capacity, so that parameters measuring each could correlate to protection. Among the
261 analyzed readouts, the anti-F1 IgG titer was the most significant predictor, and therefore
262 represented the best correlate of protection of our study. In agreement, the only mouse
263 (1/14 OF1) not protected 6 months after vaccination in a previous study [4] had
264 progressively lost its anti-F1 IgG after vaccination (Fig S4). While it has previously been
265 reported that anti-F1 Ab were good correlates of protection in mice and NHP, for F1 + LcrV
266 vaccines [17], F1-V fusion protein [16, 27, 28], or F1-producing live *Salmonella* or Poxvirus
267 vectors [29, 30], our results highlight the fact that Ab against F1 play a dominant role for
268 protection even in the presence of Ab to many other targets. This supports the conclusion
269 that F1 is a major target for protective immunity. The function of these anti-F1 antibodies
270 against *Y. pestis* remains to be evaluated. Because F1 is the principal component of the
271 *Y. pestis* pseudocapsule, easily accessible, Ab may opsonize and aggregate bacteria,
272 facilitating engulfment and destruction by phagocytes. Such functions may guide the
273 development of functional assays [31].

274 In addition to anti-F1 Ab, our study also shows a significant association between
275 survival and serum IgG Ab directed to Yops or to *Yersinia* antigens (*Y. pestis* sonicate,
276 including Yops but not F1). These Ab therefore are likely to contribute to protection.
277 Importantly, immunization with VTnF1 protected mice also against F1-negative plague
278 strains [4]. While in the present study, challenge experiments were performed with the F1-

279 positive *Y. pestis* strain, Ab responses against Yops or *Yersinia* antigens might be essential
280 for protection against F1-negative *Y. pestis* strains and will be subject to future evaluations.

281 No significant serum IgG against LcrV could be observed in VTnF1-vaccinated mice.
282 While it is known that V is immunogenic when given with adjuvant [32, 33], and is produced
283 by our vaccine strain, our finding is in agreement with previous reports that almost no Ab
284 against V were produced by murine or human plague survivors, and only low amounts by
285 mice vaccinated with various attenuated *Y. pestis* strains [32-36], whereas Ab to F1 were
286 abundant [34, 37]. V produced by live *Yersinia* strains therefore appears poorly
287 immunogenic. This could results from the ability of V to induce tolerogenic dendritic cells
288 [38], which could prevent the development of V-specific T cells. This function could be
289 active in live strains, but overcome by the adjuvant's effects in molecular vaccines such as
290 F1V.

291 Besides the humoral response, cellular immune responses alone were also sufficient
292 for protection as demonstrated by the survival of μ MT mice lacking Ab (the present work
293 and [12, 13]). This was in line with the expectation that in contrast to molecular vaccines
294 which principally prime humoral immunity and type 2 T cells [39, 40], vaccines comprised of
295 replicating agents (live attenuated organisms, for example BCG) [41]. Also, it has been
296 shown that Ab and T cells collaborate to protect against *Y. pestis* [12, 13]. IFN γ , released
297 by type 1 T cells, activates macrophages and dendritic cells against bacteria, and its
298 injection to mice during plague induced survival [42]. Therefore, an IFN γ -release assay
299 could have predicted protection against plague. The cellular response induced by VTnF1
300 indeed involved IFN γ production and thus included Th1 cells. However, IFN γ was detected
301 in most protected individuals, but not in all of them. IFN γ levels and anti-F1 IgG were
302 correlated, indicating that humoral and cellular mechanisms developed in parallel. Thus,
303 IFN γ release is a statistically significant but not fully satisfying correlate of protection.

304 F1 triggered a very low IFN γ production by blood leukocytes. In a previous study,
305 splenocytes produced 30 times less IFN γ in response to F1 than to sonicated *Y. pestis*,

306 composed of multiple targets [4]. In agreement, plague patients have a low frequency of F1-
307 reactive T cells [37]. Furthermore, despite the contribution of IL-17 to plague survival in
308 mice [26], IL-17 was hardly detectable in the blood cultures, probably due to a low Th17
309 frequency. Thus, these parameters could not be established as correlate of protection.

310 Live vaccines are known to induce strong humoral and type 1 cellular immunity [41],
311 with the counterpart that they should not be given to people who are immunosuppressed
312 (either due to drug treatment or underlying illness). Public health institutions and
313 professionals are conscientious about this [43, 44]. Because our vaccine strain is not well
314 tolerated by immunodepressed mice, we currently characterize a new, completely avirulent
315 sub-strain derived from VTnF1, which does not present this risk. The promising results will
316 be the topic of a forthcoming publication.

317 A future step in the development of our vaccine strains will be the evaluation in
318 primates. In the past, the F1V vaccine failed to protect African Green Monkeys in spite of
319 high anti-F1 antibody titers [45], and this was ascribed to the presence of non-protective
320 antibodies [27]. Whether these antibodies will also be seen in primates vaccinated with
321 VTnF1 will be important to determine, because it is not known whether non-protective
322 antibodies appear to the same extent when antigens are part of a live bacteria or injected
323 in a pure form associated with an adjuvant. In addition, care must be taken not to over-
324 interpret results obtained in the mouse, because various species previously tested (mice,
325 guinea pigs, primates) have shown great differences in their susceptibility to attenuated
326 strains vaccines and in their responsiveness to vaccines and given antigens [46-48]. In
327 primates and humans, IFN γ should also be measured in addition to antibodies to evaluate
328 the role of cellular immunity in protection and to determine which correlate is the best.

329 In conclusion, our results show that measurement of anti-F1 IgG titer is a correlate of
330 protection against bubonic plague after immunization with the VTnF1 candidate vaccine. A
331 human anti-F1 IgG ELISA test already has been used as a sero-diagnostic tool in plague
332 patients in Madagascar [49]. A similar assay has been used in trials of the F1-V in NHP [50,

333 51] and a bridge ELISA for anti-F1 Ab established to compare antibody levels in different
334 species was used for evaluation of the rF1V vaccine to bridge results obtained in mice,
335 NHP and humans [16, 52]. Because all species do not respond equally to vaccination [53],
336 these tests will have to be adapted and evaluated for further assessment of the VTnF1
337 vaccine in the second animal species tested and in future clinical trials to confirm the value
338 of this correlate in humans.
339

340 **Acknowledgements**

341 The project was supported by an ANR Emergence grant (ANR-12-EMMA-0011-01) and an
342 Institut Pasteur Accelerating Preclinical Candidates - GPF- Vaccinology 2015 grant
343 (GPFVacc2-08). The authors wish to warmly thank Pierre Goossens for precious advice
344 and support, Henrik Salje for help with statistical analysis, H. Saklani for advice regarding
345 the cell culture protocols and Stéphanie Simon (CEA, Saclay, France) for the kind gift of
346 recombinant LcrV and a mouse monoclonal anti-LcrV.

347

348 **Conflicts of interests :**

349 Authors declare no conflict of interests.

350

351 **Figure captions**

352 Figure 1: Cellular and humoral immunity independently protect VTnF1-vaccinated mice
353 against plague.

354 A : Groups of seven OF1 mice received either no serum, or serum from normal mice (non-
355 immune serum), or immune serum obtained from mice vaccinated orally with one dose of
356 VTnF1 (10^8 CFU). Immediately after, mice were infected subcutaneously to evaluate
357 resistance to bubonic plague (10^3 *Y. pestis* CO92 sc). B : Groups of five μ MT mice (B cell-
358 deficient) or wild type C57BL/6 mice were vaccinated or not with VTnF1 (10^8 CFU)
359 immediately after intraperitoneal injection (100 μ l) of immune serum. After 2 months to allow
360 clearance of antibodies, mice were infected as in A. Survival significance was evaluated by
361 the Fisher Exact test, using the “no serum“ (A) or “no vaccine“ (B) condition as reference.
362 ns: not significant, **: $p < 0.01$, ***: $p < 0.0001$

363

364 Figure 2: Humoral immune response induced by vaccination against different targets.

365 (A) Groups of 14 BALB/c mice were vaccinated orally with one dose of VTnF1 containing
366 10^6 (triangles), 10^7 (squares) or 10^8 (circles) CFU, or were not vaccinated (diamonds).

367 Blood was taken 3 weeks later and serum IgG specific for sonicated *Y. pestis* Δcaf
368 antigens, purified F1 or purified Yops were measured by ELISA. Horizontal lines indicate
369 the median value for each dose. Four weeks after vaccination, mice were infected
370 subcutaneously to evaluate resistance to bubonic plague by sc injection of 10^3 *Y. pestis*
371 CO92. The grey region indicates animals which did not survive. (B) Statistical correlation
372 between IgG levels measured in (A) was determined using Spearman's rank correlation
373 test. The corresponding "rs" coefficient and significance (p value) are indicated.

374

375 Figure 3: Cellular immune response of vaccinated mice and its correlation with the humoral
376 response.

377 Groups of 14 BALB/c mice were vaccinated ig with different doses of the VTnF1 strain (10^6 :
378 triangles; 10^7 : squares; 10^8 : circles), or were not vaccinated (7 mice: diamonds). Blood was
379 collected 3 weeks after vaccination to collect plasma and perform a leukocytes restimulation
380 assay using a sonicated *Y. pestis* Δcaf as stimulus. IFN γ was measured by ELISA in 48h
381 culture supernatants. A: Production of IFN γ according to the vaccine dose. The limit of detection
382 (lod) is indicated by a dotted line. B, C: Correlation in the same blood samples between IFN γ
383 levels and IgG recognizing F1 (B) or the sonicated *Y. pestis* Δcaf (C), determined as in Fig. 2.
384 Correlations were estimated using Spearman's rank test, which rs coefficient and
385 corresponding p value are given for each plot. Four weeks after vaccination, mice received an
386 sc injection of 10^3 *Y. pestis* CO92, and survival was followed 21 days. In all plots, the grey area
387 indicates mice which did not survive the challenge.

388

389 Figure 4: Survival as a function of anti-F1 IgG titers in BALB/c mice and OF1 mice.

390 Groups of 14 BALB/c (A) or OF1 (B) mice were vaccinated ig with different doses of the VTnF1
391 strain (10^6 to 10^8 or none, as in Figure 3). Blood was collected 3 weeks after vaccination to
392 collect serum and measure titers of IgG recognizing F1. Four weeks after vaccination, mice
393 received an sc injection of 10^3 *Y. pestis* CO92, and mice were followed for 21 days. For each
394 individual mouse, the IgG titer is plotted against survival (blue, noted 1) or death (red, noted 0).

395 For BALB/c mice (A), anti-F1 IgG titers perfectly separated mice who died (IgG anti-F1
396 titers $\leq 10^3$) from those who survived (IgG anti-F1 titers $> 10^3$) so that the logistic model could
397 not be fitted to the data. For OF1 mice (B), the predicted survival probability obtained with
398 the logistic regression (black line) is plotted along with the 95% confidence interval (grey
399 area).

400

401

402 **Supplemental material**

403 Figure S1: Survival of antibody-deficient μ MT mice to vaccination with VTnF1.

404 μ MT mice (circles) and C57BL/6 mice (same background lineage as μ MT; squares)

405 received one oral dose of VTnF1 (10^8 CFU ig; 7 mice/ group) and survival was recorded for
406 21 days.

407

408 Figure S2: Survival probability as a function of anti-*Yersinia* and anti-Yops IgG titers in
409 BALB/c and OF1 mice.

410 BALB/c mice (A, B) and OF1 mice (C, D) were vaccinated as in Figure 4, and IgG titers
411 against *Yersinia* antigens (A, C) and Yops (B, D) were plotted against the survival (blue,
412 noted 1) or death (red, noted 0) observed during the plague challenge. For OF1 mice, the
413 predicted survival probability could be calculated by logistic regression (black line) and is
414 plotted along with the 95% confidence interval (grey area). For BALB/c mice, the titers
415 almost perfectly separated mice that died from those that survived so that the logistic model
416 could not be fitted to the data.

417

418 Figure S3: Dose-dependent humoral immune response induced by vaccination in outbred
419 OF1 mice

420 Groups of 14 mice were vaccinated or not with one dose of VTnF1 consisting in 10^6 , 10^7 or 10^8
421 CFU ig, and blood was taken 3 weeks later to evaluate seric IgG specific for *Y. pestis* antigens
422 other than F1 or purified F1 or Yops by ELISA. Horizontal lines indicate the median value for
423 each dose of vaccine.

424

425 Figure S4: Relationship between long-term evolution of the anti-F1 IgG titer and the outcome of
426 a bubonic plaque challenge

427 These results were previously reported in [4] and were re-analyzed and plotted here. OF1
428 mice were vaccinated once with VTnF1 (10^8 CFU ig) and sera were collected before and
429 every month after up to 6 months. Animals were then infected with *Y. pestis* (CO92, 10^3
430 CFU sc) and indicated is the outcome after 3 weeks. Anti-F1 serum IgG titers were
431 determined by Elisa. Shown are individual evolution curves of 14 mice.

432

433

434 **References**

- 435 [1] Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E, et al.
436 Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. New England
437 Journal of Medicine. 1997;337:677-80.
- 438 [2] Guiyoule A, Gerbaud G, Buchrieser C, Galimand M, Rahalison L, Chanteau S, et al.
439 Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of *Yersinia*
440 *pestis*. Emerging Infectious Diseases. 2001;7:43-8.
- 441 [3] Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Plague
442 as a biological weapon - Medical and public health management. Journal of the American
443 Medical Association. 2000;283:2281-90.
- 444 [4] Derbise A, Hanada Y, Khalife M, Carniel E, Demeure CE. Complete Protection against
445 Pneumonic and Bubonic Plague after a Single Oral Vaccination. PLoS Negl Trop Dis.
446 2015;9:e0004162.
- 447 [5] Cornelis GR. The *Yersinia* YSC-YOP 'type III' weaponry. Nature Reviews Molecular Cell
448 Biology. 2002;3:742-52.
- 449 [6] Food Drug Administration CfDER. 21CFR601 - Biologics -Part 601: Licencing. Subpart
450 H: Approval of Biological Products when Human Efficacy Studies are not Ethical or Feasible.
451 In: FDA, editor. FDA Maryland2017.
- 452 [7] Food Drug Administration CfDER. 21CFR314 - Drugs for Human Use - Part 314:
453 Applications for FDA approval to market a new Drug - Subpart I: Approval of New Drugs
454 when Human Efficacy Studies are not Ethical or Feasible. In: FDA, editor. FDA
455 Maryland2017.
- 456 [8] Office of Regulatory Affairs. Guidance Document - Submission and Information
457 Requirements for Extraordinary Use New Drugs (EUNDS). In: Health-Canada., editor.
458 Ottawa2014.
- 459 [9] Cavaleri M, Thomson A, Salmonson T, Hemmings RJ. A viewpoint on European
460 Medicines Agency experience with investigational medicinal products for Ebola. Clinical
461 Trials. 2016;13:101-4.
- 462 [10] Plotkin SA. Correlates of protection induced by vaccination. Clinical and vaccine
463 immunology : CVI. 2010;17:1055-65.
- 464 [11] Kummer LW, Szaba FM, Parent MA, Adamovicz JJ, Hill J, Johnson LL, et al.
465 Antibodies and cytokines independently protect against pneumonic plague. Vaccine.
466 2008;26:6901-7.
- 467 [12] Parent MA, Berggren KN, Kummer LW, Wilhelm LB, Szaba FM, Mullarky IK, et al.
468 Cell-mediated protection against pulmonary *Yersinia pestis* infection. Infect Immun.
469 2005;73:7304-10.
- 470 [13] Smiley ST. Current challenges in the development of vaccines for pneumonic plague.
471 Expert review of vaccines. 2008;7:209-21.
- 472 [14] Zauberman A, Cohen S, Levy Y, Halperin G, Lazar S, Velan B, et al. Neutralization of
473 *Yersinia pestis*-mediated macrophage cytotoxicity by anti-LcrV antibodies and its correlation
474 with protective immunity in a mouse model of bubonic plague. Vaccine. 2008;26:1616-25.
- 475 [15] Little SF, Webster WM, Wilhelm H, Fisher D, Norris SL, Powell BS, et al. Quantitative
476 anti-F1 and anti-V IgG ELISAs as serological correlates of protection against plague in
477 female Swiss Webster mice. Vaccine. 2010;28:934-9.
- 478 [16] Fellows P, Price J, Martin S, Metcalfe K, Krile R, Barnewall R, et al. Characterization of
479 a Cynomolgus Macaque Model of Pneumonic Plague for Evaluation of Vaccine Efficacy.
480 Clinical and vaccine immunology : CVI. 2015;22:1070-8.

481 [17] Williamson ED, Vesey PM, Gillhespy KJ, Eley SM, Green M, Titball RW. An IgG1 titre
482 to the F1 and V antigens correlates with protection against plague in the mouse model.
483 *Clinical and Experimental Immunology*. 1999;116:107-14.

484 [18] Salerno-Goncalves R, Sztejn MB. Cell-mediated immunity and the challenges for
485 vaccine development. *Trends Microbiol*. 2006;14:536-42.

486 [19] Derbise A, Cerdà Marín A, Ave P, Blisnick T, Huerre M, Carniel E, et al. An
487 encapsulated *Yersinia pseudotuberculosis* is a highly efficient vaccine against pneumonic
488 plague. *PLoS NTD*. 2012;6:e1528.

489 [20] Kitamura D, Rajewsky K. Targeted disruption of mu chain membrane exon causes loss
490 of heavy-chain allelic exclusion. *Nature*. 1992;356:154-6.

491 [21] Chanteau S, Rahalison L, Ralafiarisoa L, Foulon J, Ratsitorahina M, Ratsifasoamanana
492 L, et al. Development and testing of a rapid diagnostic test for bubonic and pneumonic
493 plague. *Lancet*. 2003;361:211-6.

494 [22] Benoit C, Guiyoule A, Carniel E. Sérodiagnostic des infections humaines à *Yersinia*
495 pathogènes. *Presse Méd*. 1996;25:1627-30.

496 [23] Michiels T, Wattiau P, Brasseur R, Ruyschaert JM, Cornelis G. Secretion of Yop
497 Proteins by *Yersiniae*. *Infection and Immunity*. 1990;58:2840-9.

498 [24] Lawton WD, Erdman RL, Surgalla MJ. Biosynthesis and purification of V and W
499 antigens in *Pasteurella pestis*. *Journal of Immunology*. 1963;91:179-84.

500 [25] Flashner Y, Fisher M, Tidhar A, Mechaly A, Gur D, Halperin G, et al. The search for
501 early markers of plague: evidence for accumulation of soluble *Yersinia pestis* LcrV in
502 bubonic and pneumonic mouse models of disease. *FEMS Immunol Med Microbiol*.
503 2010;59:197-206.

504 [26] Lin JS, Kummer LW, Szaba FM, Smiley ST. IL-17 contributes to cell-mediated defense
505 against pulmonary *Yersinia pestis* infection. *J Immunol*. 2011;186:1675-84.

506 [27] Bashaw J, Norris S, Weeks S, Trevino S, Adamovicz JJ, Welkos S. Development of in
507 vitro correlate assays of immunity to infection with *Yersinia pestis*. *Clinical and vaccine*
508 *immunology : CVI*. 2007;14:605-16.

509 [28] Williamson ED. Acellular vaccines against plague. In: Carniel E, Hinnebusch B, editors.
510 *Yersinia, Systems Biology and Control*. Hethersett: Horizon Scientific Press; 2012. p. 123-42.

511 [29] Morton M, Garmory HS, Perkins SD, O'Dowd AM, Griffin KF, Turner AK, et al. A
512 *Salmonella enterica* serovar Typhi vaccine expressing *Yersinia pestis* F1 antigen on its
513 surface provides protection against plague in mice. *Vaccine*. 2004;22:2524-32.

514 [30] Mencher JS, Smith SR, Powell TD, Stinchcomb DT, Osorio JE, Rocke TE. Protection of
515 black-tailed prairie dogs (*Cynomys ludovicianus*) against plague after voluntary consumption
516 of baits containing recombinant raccoon poxvirus vaccine. *Infect Immun*. 2004;72:5502-5.

517 [31] Anderson GW, Worsham PL, Bolt CR, Andrews GP, Welkos SL, Friedlander AM, et al.
518 Protection of mice from fatal bubonic and pneumonic plague by passive immunization with
519 monoclonal antibodies against the F1 protein of *Yersinia pestis*. *American Journal of Tropical*
520 *Medicine and Hygiene*. 1997;56:471-3.

521 [32] Williamson ED, Eley SM, Griffin KF, Green M, Russell P, Leary SE, et al. A new
522 improved sub-unit vaccine for plague: the basis of protection. *FEMS Immunol Med*
523 *Microbiol*. 1995;12:223-30.

524 [33] Qiu Y, Liu Y, Qi Z, Wang W, Kou Z, Zhang Q, et al. Comparison of immunological
525 responses of plague vaccines F1+rV270 and EV76 in Chinese-origin rhesus macaque, *Macaca*
526 *mulatta*. *Scand J Immunol*. 2010;72:425-33.

527 [34] Quenee LE, Cornelius CA, Ciletti NA, Elli D, Schneewind O. *Yersinia pestis* *caf1*
528 variants and the limits of plague vaccine protection. *Infection and Immunity*. 2008;76:2025-
529 36.

530 [35] Braciale VL, Nash M, Sinha N, Zudina IV, Motin VL. Correlates of Immunity Elicited
531 by Live *Yersinia pestis* Vaccine. In: V. St. Georgiev KAW, J.J.McGowan, editor. National
532 Institutes of Allergy and Infectious Diseases, NIH Volume 1, Frontiers in Research. Totawa,
533 NJ.: Humana Press; 2008. p. 473-80.

534 [36] Sun W, Sanapala S, Henderson JC, Sam S, Olinzock J, Trent MS, et al. LcrV delivered
535 via type III secretion system of live attenuated *Yersinia pseudotuberculosis* enhances
536 immunogenicity against pneumonic plague. *Infect Immun*. 2014;82:4390-404.

537 [37] Li B, Zhou DS, Wang ZY, Song ZZ, Wang H, Li M, et al. Antibody profiling in plague
538 patients by protein microarray. *Microbes and Infection*. 2008;10:45-51.

539 [38] DePaolo RW, Tang FM, Kim I, Han M, Levin N, Ciletti N, et al. Toll-Like Receptor 6
540 Drives Differentiation of Tolerogenic Dendritic Cells and Contributes to LcrV-Mediated
541 Plague Pathogenesis. *Cell Host and Microbe*. 2008;4:350-61.

542 [39] Heath DG, Anderson GW, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, et al.
543 Protection Against Experimental Bubonic and Pneumonic Plague By a Recombinant Capsular
544 F1-V Antigen Fusion Protein Vaccine. *Vaccine*. 1998;16:1131-7.

545 [40] Meyer KF, Cavanaugh DC, Bartelloni PJ, Marshall JD, Jr. Plague immunization. I. Past
546 and present trends. *Journal of Infectious Diseases*. 1974;129:S13-8.

547 [41] Levine MM, Sztein MB. Vaccine development strategies for improving immunization:
548 the role of modern immunology. *Nat Immunol*. 2004;5:460-4.

549 [42] Nakajima R, Brubaker RR. Association between virulence of *Yersinia pestis* and
550 suppression of gamma interferon and tumor necrosis factor alpha. *Infection and Immunity*.
551 1993;61:23-31.

552 [43] Public Health England. Live attenuated vaccines: avoid use in those who are clinically
553 immunosuppressed. In: GOV.UK, editor. Vigilance, safety alerts and guidance 2016.

554 [44] Lopez A, Mariette X, Bachelez H, Belot A, Bonnotte B, Hachulla E, et al. Vaccination
555 recommendations for the adult immunosuppressed patient: A systematic review and
556 comprehensive field synopsis. *J Autoimmun*. 2017;80:10-27.

557 [45] Pitt L. Nonhuman Primates as a Model for Pneumonic Plague. . Public workshop on
558 animal models and correlates of protection for plague vaccines Gaithersburg, Maryland,
559 USA. 2004.

560 [46] Girard G. Immunity in Plague. Acquisitions Supplied by 30 Years of Work on the
561 "Pasteurella Pestis Ev" (Girard and Robic) Strain. *Biol Med (Paris)*. 1963;52:631-731.

562 [47] Hallett AF, Isaacson M, Meyer KF. Pathogenicity and immunogenic efficacy of a live
563 attenuated plague vaccine in vervet monkeys. *Infect Immun*. 1973;8:876-81.

564 [48] Welkos S, Pitt ML, Martinez M, Friedlander A, Vogel P, Tammariello R. Determination
565 of the virulence of the pigmentation-deficient and pigmentation-/plasminogen activator-
566 deficient strains of *Yersinia pestis* in non-human primate and mouse models of pneumonic
567 plague. *Vaccine*. 2002;20:2206-14.

568 [49] Rasoamanana B, Leroy F, Boisier P, Rasolomaharo M, Buchy P, Carniel E, et al. Field
569 evaluation of an immunoglobulin G anti-F1 enzyme-linked immunosorbent assay for
570 serodiagnosis of human plague in Madagascar. *Clin Diagn Lab Immunol*. 1997;4:587-91.

571 [50] Quenee LE, Ciletti NA, Elli D, Hermanas TM, Schneewind O. Prevention of pneumonic
572 plague in mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or
573 F1-V vaccines. *Vaccine*. 2011;29:6572-83.

574 [51] Chichester JA, Musiyhuk K, Farrance CE, Mett V, Lyons J, Mett V, et al. A single
575 component two-valent LcrV-F1 vaccine protects non-human primates against pneumonic
576 plague. *Vaccine*. 2009;27:3471-4.

577 [52] Fellows P, Adamovicz J, Hartings J, Sherwood R, Mega W, Brasel T, et al. Protection in
578 mice passively immunized with serum from cynomolgus macaques and humans vaccinated
579 with recombinant plague vaccine (rF1V). *Vaccine*. 2010;28:7748-56.

580 [53] Feodorova VA, Sayapina LV, Motin VL. Assessment of Live Plague Vaccine
581 Candidates. In: S. T, editor. Vaccine Design Methods in Molecular Biology. New York, NY:
582 Humana Press; 2016.
583

Mouse lineage	Percent survival; proportion surviving (live /total); significance			
	Naives	10 ⁶ CFU	10 ⁷ CFU	10 ⁸ CFU
OF1	0%	23%	67%	100%
	(14/14)	(3/13)	(14/21)	(14/14)
		ns	***	***
BALB/c	0%	7%	86%	100%
	(0/13)	(1/14)	(12/14)	(13/13)
		ns	***	***
C57BL/6	0%	nt	nt	100%
	(0/12)			(12/12)

Table 1: Protection of various mouse strains against bubonic plague provided by vaccination with graded doses of VTnF1.

OF1 (outbred), C57BL/6 or BALB/c (inbred) mice (groups of 12-21 from 2 experiments) were orally vaccinated with graded doses of VTnF1 (as indicated), and were exposed four weeks later to bubonic plague (sc injection of 10³ *Y. pestis* CO92, fatal for naïve mice). Survival was followed for 21 days, and significance of protection was calculated using the Fisher exact test. nt: not tested. ns: not significant, *: p<0.05, ***: p<0.0001 against naïve mice.

	OR (95% CI)	p-value	AIC
IgG anti-F1	3.5 (2.1, 7.0)	<0.001	49.4
IgG anti-<i>Yersinia</i>	7.9 (3.1, 26.9)	<0.001	54.3
IgG anti-Yops	7.8 (2.9, 29.5)	<0.001	58.0

Table 2: Odds ratio for survival to the plaque challenge for each 10-fold increase in IgG anti-F1, IgG anti-*Yersinia* and IgG anti-Yops titers, in OF1 mice.

Odds ratio (OR) and their 95% confidence intervals (95% CI) were computed with univariate logistic regressions. The Akaike Information Criterion (AIC) is also provided for model comparison. The best fitting model is the one with the smallest AIC.

Figure 1

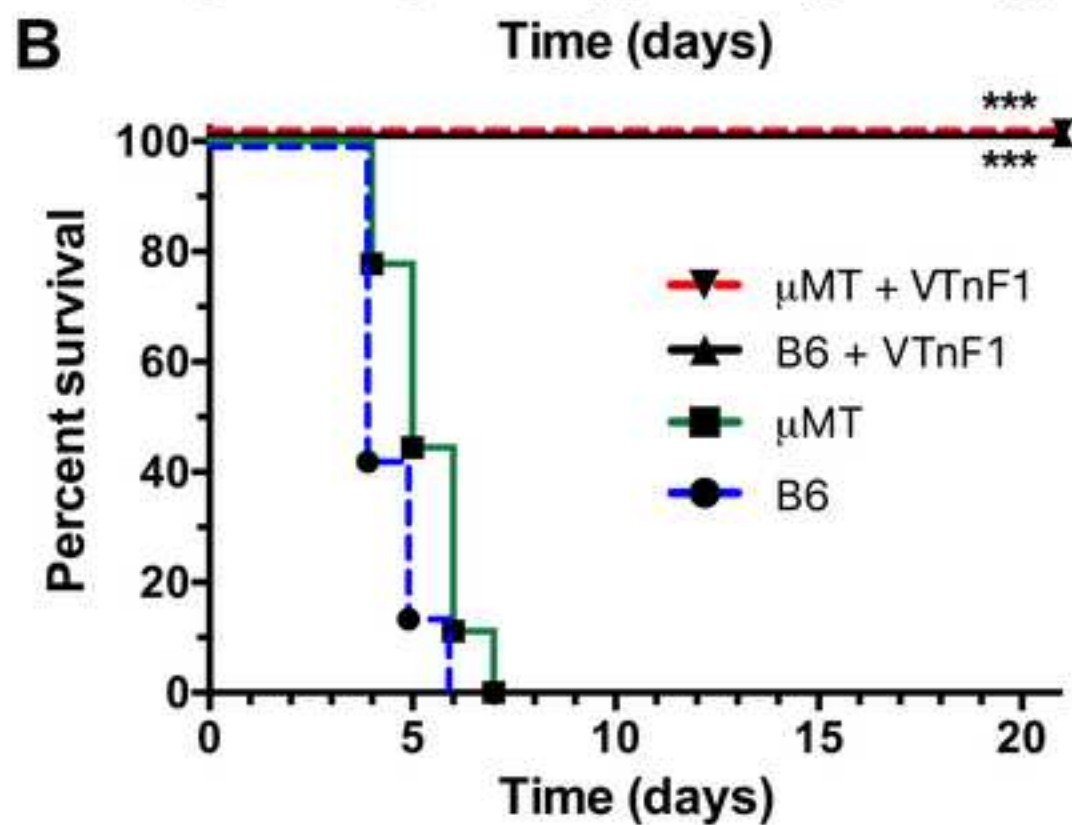
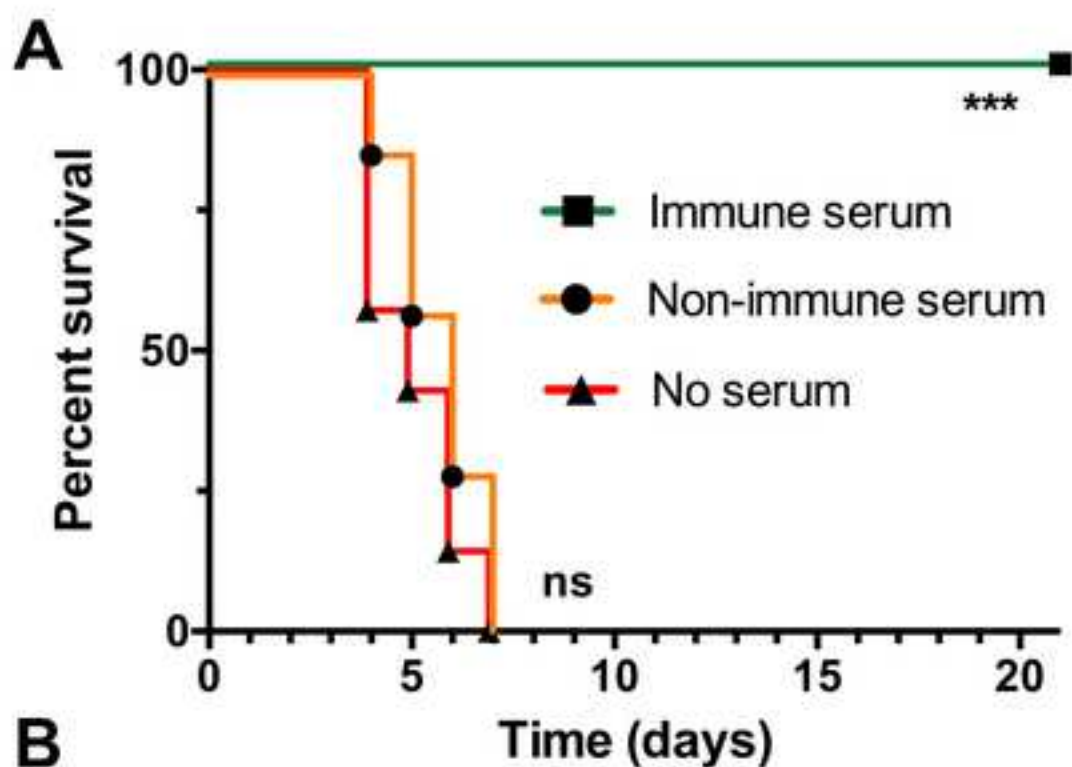


Figure 2

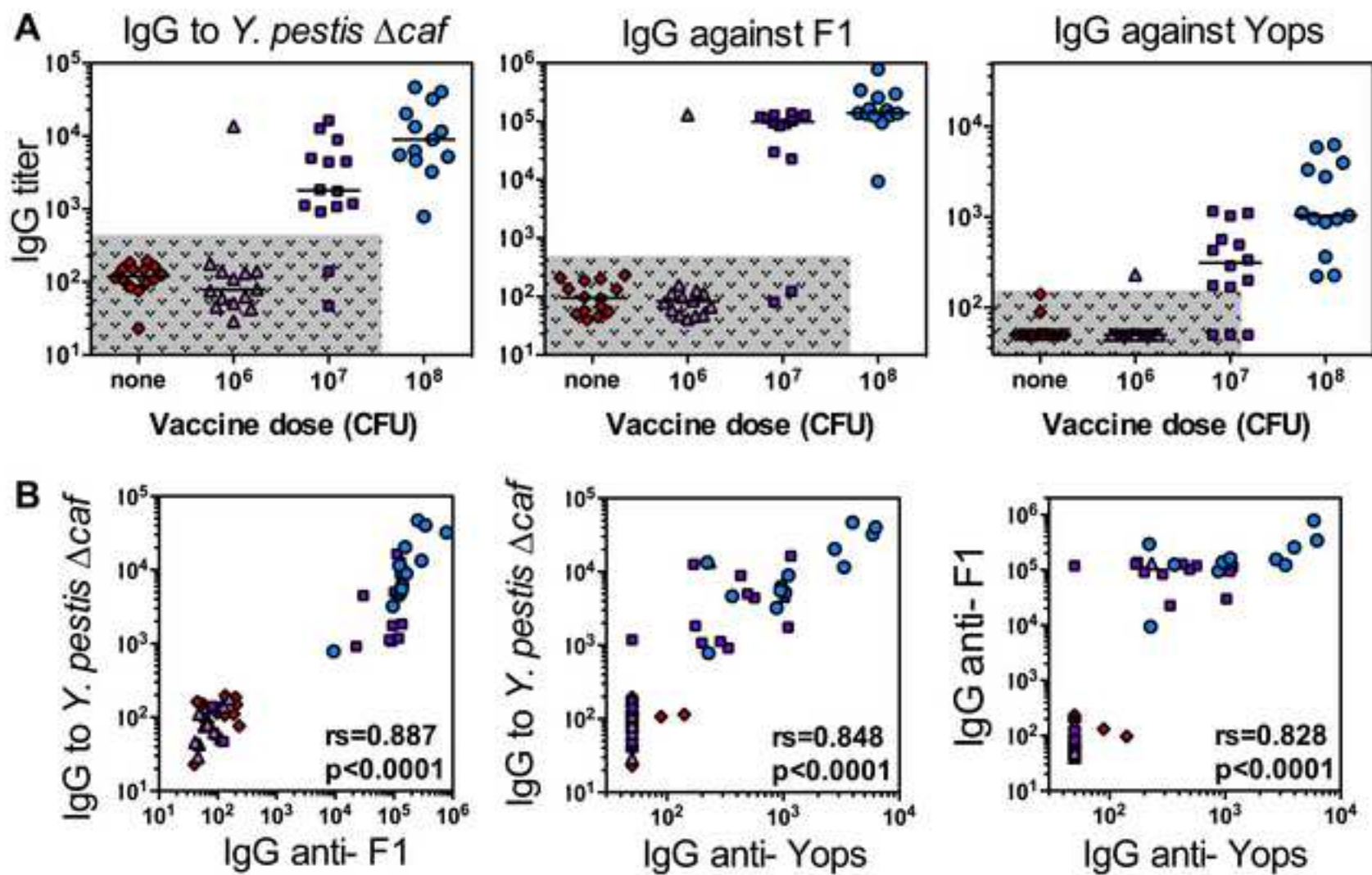


Figure 4

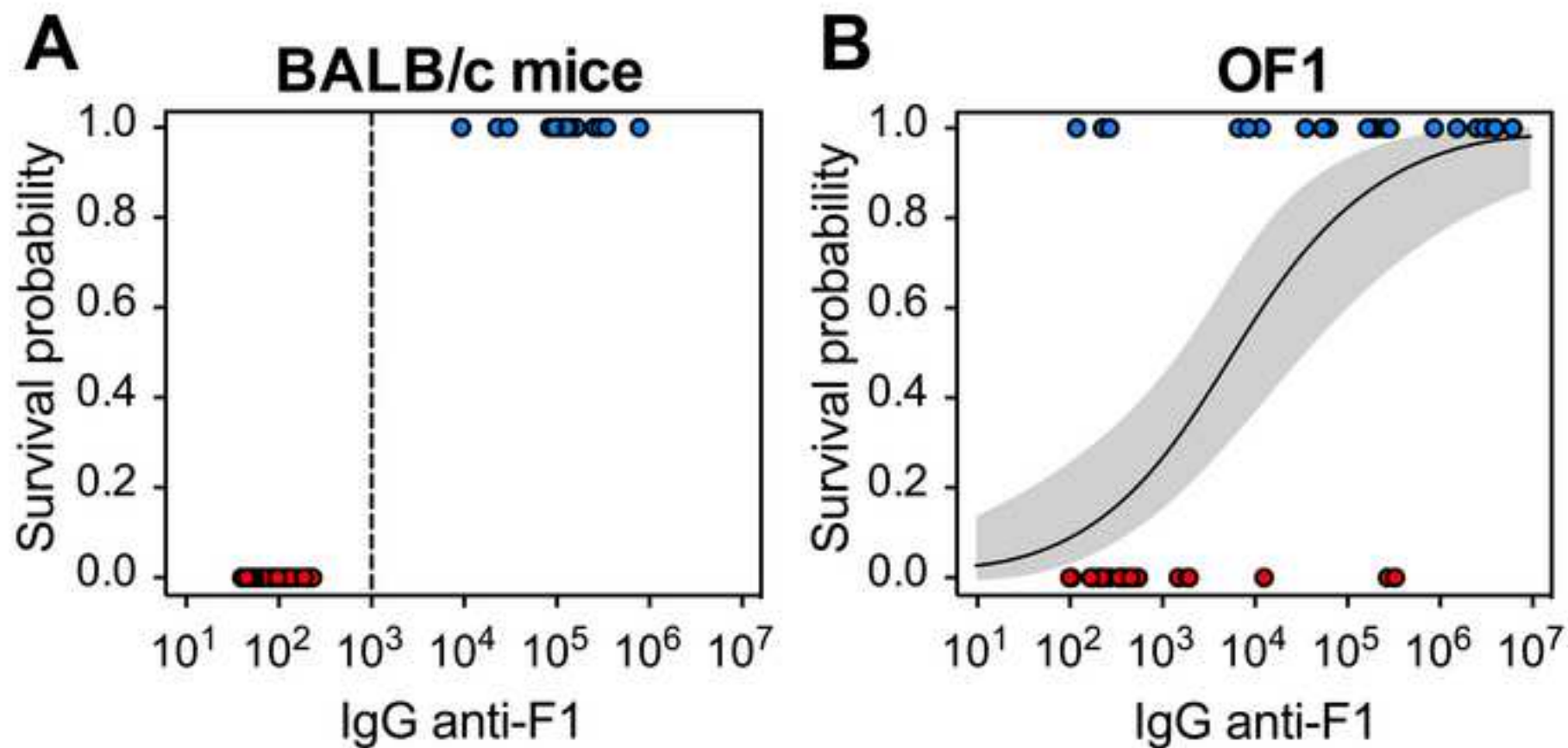


Figure 3

