Vaccine 37 (2019) 123–129

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

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

Christian E. Demeure [a,*], Anne Derbise [a], Chloé Guillas [a], Christiane Gerke [b], Simon Cauchemez [c,d,e], Elisabeth Carniel [a,1], Javier Pizarro-Cerdá [a]

[a] Unité de Recherche Yersinia, Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France
[b] Vaccine Programs, Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France
[c] Unité de Modélisation Mathématique des Maladies Infectieuses, Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France
[d] Centre National de la Recherche Scientifique, URA3012, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France
[e] Center of Bioinformatics, Biostatistics and Integrative Biology, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France

ARTICLE INFO

Article history:
Received 4 September 2018
Received in revised form 5 November 2018
Accepted 11 November 2018
Available online 19 November 2018

Keywords:
Plague
Live vaccine
Yersinia pestis
Yersinia pseudotuberculosis
Correlates of protection
F1
IgG

ABSTRACT

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

1. Introduction

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

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

The licensure of a human vaccine requires evaluation of its efficacy in humans. For plague, this is problematic because cases are

* Corresponding author.
E-mail addresses: cdemeure@pasteur.fr (C.E. Demeure), anne.derbise@pasteur.fr (A. Derbise), chloe.guillas@pasteur.fr (C. Guillas), christiane.gerke@pasteur.fr (C. Gerke), simon.cauchemez@pasteur.fr (S. Cauchemez), elisabeth.carniel@pasteur.fr (E. Carniel), javier.pizarro-cerda@pasteur.fr (J. Pizarro-Cerdá).
[1] Present address: Pasteur Center of Cameroon, BP 1274, Yaounde, Cameroon.

https://doi.org/10.1016/j.vaccine.2018.11.022
0264-410X/

too sporadic in the world for a field evaluation and human challenge studies are not ethical. In such circumstances, demonstrating efficacy in a well-understood animal model that resembles disease in humans can be considered for approval under the "animal rule" in the USA [6,7], or the "Extraordinary Use New Drugs Pathway" in Canada [8]. In the EU, a recent perspective from the EMA stated that this does not preclude 'the possibility that animal models data in principle could have a critical role in the assessment' [9].

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

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

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

In this study, we examined which humoral and/or cellular immunological readout correlated with protection in mice vaccinated with the VTnF1 strain, in order to determine parameters that can be further developed for evaluation in other animal models and use in future human trials.

2. Materials and methods

2.1. Bacterial strains

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

2.2. Animal vaccination and infection

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

Infections with *Y. pestis* were performed in a BSL3 animal facility as previously [4,19]. *Y. pestis* CO92 grown at 28 °C was resuspended in saline and bubonic plague was induced by injection of 10^3 CFU (i.e. $10^2 \times LD_{50}$, in 50 μl) subcutaneously (sc) in ventral skin. Animals clinical state and survival was monitored daily for 21 days.

2.3. Immuno-assays

IgG specific for *Yersinia* were quantified by ELISA as described previously [21]: microtiter plates (NUNC) were coated with either purified F1 antigen, or sonicated *Y. pestis* CO92Δ*caf* (containing all *Y. pestis* antigens except F1 [4]), or purified recombinant LcrV antigen, or purified Yops, all at 5 μg/ml in PBS, except LcrV: 1 μg/ml. The ELISA using Yops was initially developed for the serodiagnostic of infection by pathogenic Yersiniae [22]. The Yops solution used contains a mix of all the *Yersinia* Outer Proteins (Yops) produced in medium by bacteria grown at 37 °C in the absence of Calcium, as described by Michiels [23]. Sera diluted in PBS containing 0.1% BSA were incubated in coated plates. Bound IgG were detected by a mouse IgG-specific, HRPO–coupled, rat antibody (Bio-Rad. Antibody titers were calculated as the reciprocal of the lowest sample dilution giving a signal equal to two times the background.

2.4. Cell-mediated response of vaccinated animals

Blood collected on heparin (50 U) was centrifuged on a Lympholyte®-Mammal gradient (Cedarlane, manufacturer' protocol) to separate leukocytes from plasma and erythrocytes. Plasma was frozen for IgG evaluation, while leukocytes were resuspended in culture medium: RPMI 1640 + Glutamax™ (Invitrogen) containing 10% bovine serum (Lonza), Penicillin/Streptomycin (Life Sciences) and 10 mM ß-mercapto-ethanol. Leukocytes (10^6 cells) were restimulated *in vitro* using either sterile F1 antigen or sonicated *Y. pestis* CO92Δ*caf* (both at 5 μg/ml). Supernatants were collected after 48 h for cytokine measurements (IFNγ and IL-17 Duosets, R&D Systems).

2.5. Transfer of immune serum and vaccination of antibody-deficient μMT mice

To evaluate the protective value of antibodies, OF1 mice received either normal or immune serum (500 μl) intraperitoneally (ip) two hours before a bubonic plague challenge (CO92, 10^3 CFU sc). To evaluate the protective value of the cellular immune response, μMT mice (unable to produce antibodies [20]) and immune-competent B6 littermates were vaccinated. Because VTnF1 was harmful for immuno-deficient μMT mice (Fig. S1), vaccinated and control mice received immune serum (100 μl ip) at the time of vaccination, according to Parent et al. [12]. Specific antibodies in blood were evaluated by ELISA every four weeks, and mice were challenged when antibodies were undetectable in μMT mice.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.11.022.

2.6. Statistical analysis

We studied the association between IgG titers (log_{10} transformed) and the probability to survive a plague challenge. Univariate logistic regressions were used to compute the odds ratio for

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

3. Results

3.1. VTnF1 vaccine-induced protection is mediated by both humoral and cellular immunity

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

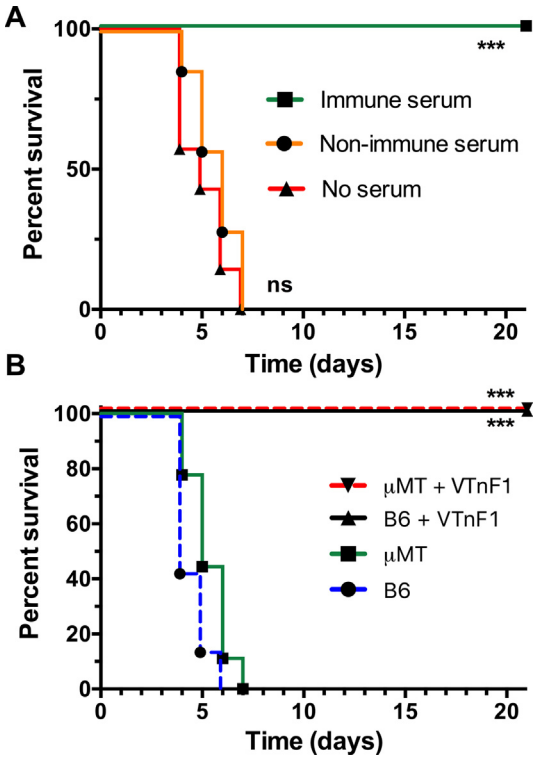

Fig. 1. Cellular and humoral immunity independently protect VTnF1-vaccinated mice against plague. A: Groups of seven OF1 mice received either no serum, or serum from normal mice (non-immune serum), or immune serum obtained from mice vaccinated orally with one dose of VTnF1 (10^8 CFU). Immediately after, mice were infected subcutaneously to evaluate resistance to bubonic plague (10^3 *Y. pestis* CO92 sc). B: Groups of five μMT mice (B cell-deficient) or wild type C57BL/6 mice were vaccinated or not with VTnF1 (10^8 CFU) immediately after intraperitoneal injection (100 μl) of immune serum. After 2 months to allow clearance of antibodies, mice were infected as in A. Survival significance was evaluated by the Fisher Exact test, using the "no serum" (A) or "no vaccine" (B) condition as reference. ns: not significant, **: $p < 0.01$, ***: $p < 0.0001$.

3.2. Evaluation of humoral parameters as correlates of protection against plague

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

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

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

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

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 10^3 *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.

3.3. Evaluation of vaccine-induced cellular memory against plague

In vitro stimulated splenocytes from VTnF1 -vaccinated mice produce IFNγ, revealing a type 1 cellular response (Th1) [4]. Here, the IFNγ release assay was applied to blood leukocytes restimulated with either F1 or sonicated *Y. pestis* CO92Δ*caf* (all *Y. pestis* antigens except F1). Purified F1 failed to induce any IFNγ production, whereas the sonicated *Y. pestis* induced IFNγ in the majority of samples (Fig. 3A). Unspecific stimulation was unlikely, because cells from naïve mice did not respond. All mice positive for IFNγ

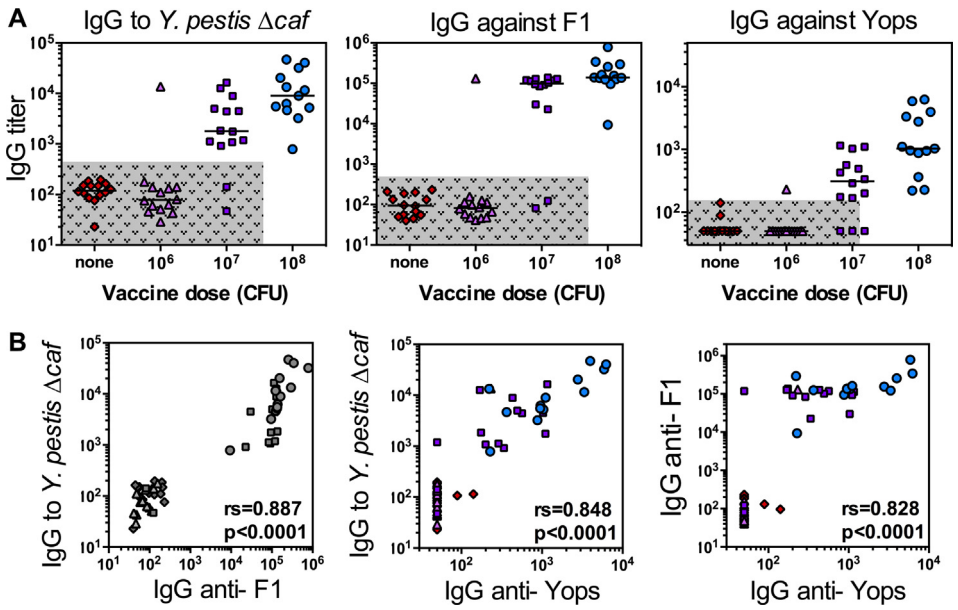

Fig. 2. Humoral immune response induced by vaccination against different targets. (A) Groups of 14 BALB/c mice were vaccinated orally with one dose of VTnF1 containing 10^6 (triangles), 10^7 (squares) or 10^8 (circles) CFU, or were not vaccinated (diamonds). Blood was taken 3 weeks later and serum IgG specific for sonicated *Y. pestis* Δ*caf* antigens, purified F1 or purified Yops were measured by ELISA. Horizontal lines indicate the median value for each dose. Four weeks after vaccination, mice were infected subcutaneously to evaluate resistance to bubonic plague by sc injection of 10^3 *Y. pestis* CO92. The grey region indicates animals which did not survive. (B) Statistical correlation between IgG levels measured in (A) was determined using Spearman's rank correlation test. The corresponding "rs" coefficient and significance (p value) are indicated.

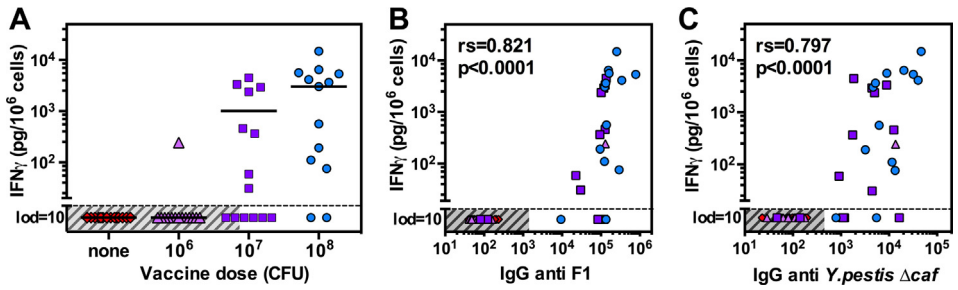

Fig. 3. Cellular immune response of vaccinated mice and its correlation with the humoral response. Groups of 14 BALB/c mice were vaccinated ig with different doses of the VTnF1 strain (10^6: triangles; 10^7: squares; 10^8: circles), or were not vaccinated (7 mice: diamonds). Blood was collected 3 weeks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

survived plague, although IFNγ was not detected in all protected individuals, yielding a 69% confidence (18 mice/26 survivors; Fig. 3A). IFNγ correlated significantly with anti-F1 IgG (Fig. 3B) as well as with anti-*Y. pestis* Δ*caf* IgG (Fig. 3C), and both increased according to the vaccine dose.

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

3.4. Logistic regression analysis of serological immune readouts

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

Because the human population is genetically diverse, the possibility to predict protection was examined in outbred OF1 mice. IgG levels were more heterogeneous (Fig. S3), but each IgG titer was significantly associated with survival as revealed using univariate logistic regressions (Fig. 4B, Fig. S2B, S2D), with $p < 0.001$ (Table 2). Based on the AIC, the anti-F1 IgG titer had the strongest association with survival to bubonic plague. For each 10-fold increase in anti-F1 IgG titer, the odd ratio for survival to the plague challenge was 3.5 (95% confidence interval: 2.1, 7.0) (Table 2; Fig. 4B).

4. Discussion

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

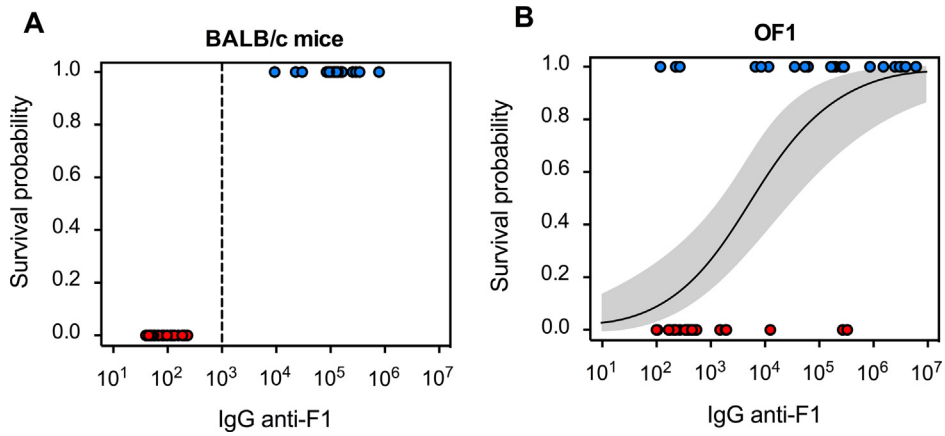

Fig. 4. Survival as a function of anti-F1 IgG titers in BALB/c mice and OF1 mice. Groups of 14 BALB/c (A) or OF1 (B) mice were vaccinated ig with different doses of the VTnF1 strain (10^6 to 10^8 or none, as in Fig. 3). Blood was collected 3 weeks after vaccination to collect serum and measure titers of IgG recognizing F1. Four weeks after vaccination, mice received an sc injection of 10^3 *Y. pestis* CO92, and mice were followed for 21 days. For each individual mouse, the IgG titer is plotted against survival (blue, noted 1) or death (red, noted 0). For BALB/c mice (A), anti-F1 IgG titers perfectly separated mice who died (IgG anti-F1 titers $\leq 10^3$) from those who survived (IgG anti-F1 titers $> 10^3$) so that the logistic model could not be fitted to the data. For OF1 mice (B), the predicted survival probability obtained with the logistic regression (black line) is plotted along with the 95% confidence interval (grey area). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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

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

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.

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

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

In addition to anti-F1 Ab, our study also shows a significant association between survival and serum IgG Ab directed to Yops or to *Yersinia* antigens (*Y. pestis* sonicate, including Yops but not F1). These Ab therefore are likely to contribute to protection. Importantly, immunization with VTnF1 protected mice also against F1-negative *Y. pestis* strains [4]. While in the present study, challenge experiments were performed with the F1-positive *Y. pestis* strain, Ab responses against Yops or *Yersinia* antigens might be essential for protection against F1-negative *Y. pestis* strains and will be subject to future evaluations.

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

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

F1 triggered a very low IFNγ production by blood leukocytes. In a previous study, splenocytes produced 30 times less IFNγ in response to F1 than to sonicated *Y. pestis*, composed of multiple targets [4]. In agreement, plague patients have a low frequency of F1-reactive T

cells [37]. Furthermore, despite the contribution of IL-17 to plague survival in mice [26], IL-17 was hardly detectable in the blood cultures, probably due to a low Th17 frequency. Thus, these parameters could not be established as correlate of protection.

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

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

In conclusion, our results show that measurement of anti-F1 IgG titer is a correlate of protection against bubonic plague after immunization with the VTnF1 candidate vaccine. A human anti-F1 IgG ELISA test already has been used as a sero-diagnostic tool in plague patients in Madagascar [49]. A similar assay has been used in trials of the F1-V in NHP [50,51] and a bridge ELISA for anti-F1 Ab established to compare antibody levels in different species was used for evaluation of the rF1V vaccine to bridge results obtained in mice, NHP and humans [16,52]. Because all species do not respond equally to vaccination [53], these tests will have to be adapted and evaluated for further assessment of the VTnF1 vaccine in the second animal species tested and in future clinical trials to confirm the value of this correlate in humans.

Acknowledgements

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

Conflicts of interests

Authors declare no conflict of interests.

References

[1] Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E, et al. Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. N Engl J Med 1997;337:677–80.
[2] Guiyoule A, Gerbaud G, Buchrieser C, Galimand M, Rahalison L, Chanteau S, et al. Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of *Yersinia pestis*. Emerg Infect Dis 2001;7:43–8.
[3] Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Plague as a biological weapon – medical and public health management. J Am Med Assoc 2000;283:2281–90.
[4] Derbise A, Hanada Y, Khalife M, Carniel E, Demeure CE. Complete protection against pneumonic and bubonic plague after a single oral vaccination. PLoS Negl Trop Dis 2015;9:e0004162.
[5] Cornelis GR. The *Yersinia* YSC-YOP 'type III' weaponry. Nat Rev Mol Cell Biol 2002;3:742–52.
[6] Food Drug Administration CfDER. 21CFR601 – Biologics – Part 601: Licencing. Subpart H: Approval of biological products when human efficacy studies are not ethical or feasible. In: FDA, editor. FDA Maryland; 2017.
[7] Food Drug Administration CfDER. 21CFR314 – Drugs for Human Use – Part 314: Applications for FDA approval to market a new Drug – Subpart I: Approval of New Drugs when Human Efficacy Studies are not Ethical or Feasible. In: FDA, editor. FDA Maryland; 2017.
[8] Office of Regulatory Affairs. Guidance Document – Submission and Information Requirements for Extraordinary Use New Drugs (EUNDs). In: Health-Canada, editor. Ottawa; 2014.
[9] Cavaleri M, Thomson A, Salmonson T, Hemmings RJ. A viewpoint on European Medicines Agency experience with investigational medicinal products for Ebola. Clin Trials 2016;13:101–4.
[10] Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol: CVI 2010;17:1055–65.
[11] Kummer LW, Szaba FM, Parent MA, Adamovicz JJ, Hill J, Johnson LL, et al. Antibodies and cytokines independently protect against pneumonic plague. Vaccine 2008;26:6901–7.
[12] Parent MA, Berggren KN, Kummer LW, Wilhelm LB, Szaba FM, Mullarky IK, et al. Cell-mediated protection against pulmonary Yersinia pestis infection. Infect Immun 2005;73:7304–10.
[13] Smiley ST. Current challenges in the development of vaccines for pneumonic plague. Expert Rev Vaccines 2008;7:209–21.
[14] Zauberman A, Cohen S, Levy Y, Halperin G, Lazar S, Velan B, et al. Neutralization of *Yersinia pestis*-mediated macrophage cytotoxicity by anti-LcrV antibodies and its correlation with protective immunity in a mouse model of bubonic plague. Vaccine 2008;26:1616–25.
[15] Little SF, Webster WM, Wilhelm H, Fisher D, Norris SL, Powell BS, et al. Quantitative anti-F1 and anti-V IgG ELISAs as serological correlates of protection against plague in female Swiss Webster mice. Vaccine 2010;28:934–9.
[16] Fellows P, Price J, Martin S, Metcalfe K, Krile R, Barnewall R, et al. Characterization of a cynomolgus macaque model of pneumonic plague for evaluation of vaccine efficacy. Clin Vaccine Immunol: CVI 2015;22:1070–8.
[17] Williamson ED, Vesey PM, Gillhespy KJ, Eley SM, Green M, Titball RW. An IgG1 titre to the F1 and V antigens correlates with protection against plague in the mouse model. Clin Exp Immunol 1999;116:107–14.
[18] Salerno-Goncalves R, Sztein MB. Cell-mediated immunity and the challenges for vaccine development. Trends Microbiol 2006;14:536–42.
[19] Derbise A, Cerdà Marín A, Ave P, Blisnick T, Huerre M, Carniel E, et al. An encapsulated *Yersinia pseudotuberculosis* is a highly efficient vaccine against pneumonic plague. PLoS NTD 2012;6:e1528.
[20] Kitamura D, Rajewsky K. Targeted disruption of mu chain membrane exon causes loss of heavy-chain allelic exclusion. Nature 1992;356:154–6.
[21] Chanteau S, Rahalison L, Ralafiarisoa L, Foulon J, Ratsitorahina M, Ratsifasoamanana L, et al. Development and testing of a rapid diagnostic test for bubonic and pneumonic plague. Lancet 2003;361:211–6.
[22] Benoit C, Guiyoule A, Carniel E. Sérodiagnostic des infections humaines à *Yersinia* pathogènes. Presse Méd 1996;25:1627–30.
[23] Michiels T, Wattiau P, Brasseur R, Ruysschaert JM, Cornelis G. Secretion of Yop proteins by Yersiniae. Infect Immun 1990;58:2840–9.
[24] Lawton WD, Erdman RL, Surgalla MJ. Biosynthesis and purification of V and W antigens in *Pasteurella pestis*. J Immunol 1963;91:179–84.
[25] Flashner Y, Fisher M, Tidhar A, Mechaly A, Gur D, Halperin G, et al. The search for early markers of plague: evidence for accumulation of soluble Yersinia pestis LcrV in bubonic and pneumonic mouse models of disease. FEMS Immunol Med Microbiol 2010;59:197–206.
[26] Lin JS, Kummer LW, Szaba FM, Smiley ST. IL-17 contributes to cell-mediated defense against pulmonary *Yersinia pestis* infection. J Immunol 2011;186:1675–84.
[27] Bashaw J, Norris S, Weeks S, Trevino S, Adamovicz JJ, Welkos S. Development of in vitro correlate assays of immunity to infection with Yersinia pestis. Clin Vaccine Immunol: CVI 2007;14:605–16.
[28] Williamson ED. Acellular vaccines against plague. In: Carniel E, Hinnebusch B, editors. Yersinia, systems biology and control. Hethersett: Horizon Scientific Press; 2012. p. 123–42.
[29] Morton M, Garmory HS, Perkins SD, O'Dowd AM, Griffin KF, Turner AK, et al. A *Salmonella enterica* serovar Typhi vaccine expressing *Yersinia pestis* F1 antigen on its surface provides protection against plague in mice. Vaccine 2004;22:2524–32.
[30] Mencher JS, Smith SR, Powell TD, Stinchcomb DT, Osorio JE, Rocke TE. Protection of black-tailed prairie dogs (Cynomys ludovicianus) against plague after voluntary consumption of baits containing recombinant raccoon poxvirus vaccine. Infect Immun 2004;72:5502–5.

[31] Anderson GW, Worsham PL, Bolt CR, Andrews GP, Welkos SL, Friedlander AM, et al. Protection of mice from fatal bubonic and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of *Yersinia pestis*. Am J Trop Med Hyg 1997;56:471–3.
[32] Williamson ED, Eley SM, Griffin KF, Green M, Russell P, Leary SE, et al. A new improved sub-unit vaccine for plague: the basis of protection. FEMS Immunol Med Microbiol 1995;12:223–30.
[33] Qiu Y, Liu Y, Qi Z, Wang W, Kou Z, Zhang Q, et al. Comparison of immunological responses of plague vaccines F1+rV270 and EV76 in Chinese-origin rhesus macaque, Macaca mulatta. Scand J Immunol 2010;72:425–33.
[34] Quenee LE, Cornelius CA, Ciletti NA, Elli D, Schneewind O. *Yersinia pestis* caf1 variants and the limits of plague vaccine protection. Infect Immun 2008;76:2025–36.
[35] Braciale VL, Nash M, Sinha N, Zudina IV, Motin VL. Correlates of Immunity Elicited by Live *Yersinia pestis* Vaccine. In: Georgiev V St, Western KA, McGowan JJ, editors. National Institutes of allergy and infectious diseases, NIH volume 1, frontiers in research. Totawa, NJ: Humana Press; 2008. p. 473–80.
[36] Sun W, Sanapala S, Henderson JC, Sam S, Olinzock J, Trent MS, et al. LcrV delivered via type III secretion system of live attenuated *Yersinia pseudotuberculosis* enhances immunogenicity against pneumonic plague. Infect Immun 2014;82:4390–404.
[37] Li B, Zhou DS, Wang ZY, Song ZZ, Wang H, Li M, et al. Antibody profiling in plague patients by protein microarray. Microbes Infect 2008;10:45–51.
[38] DePaolo RW, Tang FM, Kim I, Han M, Levin N, Ciletti N, et al. Toll-like receptor 6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis. Cell Host Microbe 2008;4:350–61.
[39] Heath DG, Anderson GW, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, et al. Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. Vaccine 1998;16:1131–7.
[40] Meyer KF, Cavanaugh DC, Bartelloni PJ, Marshall Jr JD. Plague immunization. I. Past and present trends. J Infect Dis 1974;129:S13–8.
[41] Levine MM, Sztein MB. Vaccine development strategies for improving immunization: the role of modern immunology. Nat Immunol 2004;5:460–4.
[42] Nakajima R, Brubaker RR. Association between virulence of *Yersinia pestis* and suppression of gamma interferon and tumor necrosis factor alpha. Infect Immun 1993;61:23–31.
[43] Public Health England. Live attenuated vaccines: avoid use in those who are clinically immunosuppressed. In: GOV.UK, editor. Vigilance, safety alerts and guidance; 2016.
[44] Lopez A, Mariette X, Bachelez H, Belot A, Bonnotte B, Hachulla E, et al. Vaccination recommendations for the adult immunosuppressed patient: A systematic review and comprehensive field synopsis. J Autoimmun 2017;80:10–27.
[45] Pitt L. Nonhuman Primates as a Model for Pneumonic Plague. Public workshop on animal models and correlates of protection for plague vaccines Gaithersburg, Maryland, USA; 2004.
[46] Girard G. Immunity in plague. Acquisitions supplied by 30 years of work on the "Pasteurella Pestis Ev" (Girard and Robic) Strain. Biol Med (Paris) 1963;52:631–731.
[47] Hallett AF, Isaacson M, Meyer KF. Pathogenicity and immunogenic efficacy of a live attentuated plaque vaccine in vervet monkeys. Infect Immun 1973;8:876–81.
[48] Welkos S, Pitt ML, Martinez M, Friedlander A, Vogel P, Tammariello R. Determination of the virulence of the pigmentation-deficient and pigmentation-/plasminogen activator-deficient strains of Yersinia pestis in non-human primate and mouse models of pneumonic plague. Vaccine 2002;20:2206–14.
[49] Rasoamanana B, Leroy F, Boisier P, Rasolomaharo M, Buchy P, Carniel E, et al. Field evaluation of an immunoglobulin G anti-F1 enzyme-linked immunosorbent assay for serodiagnosis of human plague in Madagascar. Clin Diagn Lab Immunol 1997;4:587–91.
[50] Quenee LE, Ciletti NA, Elli D, Hermanas TM, Schneewind O. Prevention of pneumonic plague in mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or F1-V vaccines. Vaccine 2011;29:6572–83.
[51] Chichester JA, Musiychuk K, Farrance CE, Mett V, Lyons J, Mett V, et al. A single component two-valent LcrV-F1 vaccine protects non-human primates against pneumonic plague. Vaccine 2009;27:3471–4.
[52] Fellows P, Adamovicz J, Hartings J, Sherwood R, Mega W, Brasel T, et al. Protection in mice passively immunized with serum from cynomolgus macaques and humans vaccinated with recombinant plague vaccine (rF1V). Vaccine 2010;28:7748–56.
[53] Feodorova VA, Sayapina LV, Motin VL. Assessment of live plague vaccine candidates. In: Thomas S, editor. Vaccine design methods in molecular biology. New York, NY: Humana Press; 2016.