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A novel mucosal vaccine platform for immunization against plague

Christina D'Arco

A Dissertation in the Program in Basic Medical Sciences
Submitted to the Faculty of the
Graduate School of Basic Medical Sciences
In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
at New York Medical College

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A novel mucosal vaccine platform for immunization against plague

Christina D'Arco

Paul M. Arnaboldi

Paul M. Arnaboldi, Ph.D.
Sponsor

Dana Mordue

Dana G. Mordue, Ph.D.
Committee Chair

Alison McCormick

Alison A. McCormick, Ph.D.
Committee Member

Chandra Shekhar Bakshi, DVM, Ph.D.
Committee Member

Raymond J Dattwyler

Raymond J. Dattwyler, MD
Committee Member

June 17, 2020

Date of Approval

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"Start by doing what is necessary; then do what's possible; suddenly you are doing the impossible."

– Saint Francis of Assisi

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Abbreviations

Abbr.	Definition
ADCP	antibody-dependent cellular phagocytosis
AM	alveolar macrophage(s)
Amp^r	ampicillin resistance
ANOVA	analysis of variance
APC	antigen-presenting cell(s)
BAL	bronchoalveolar fluid
BCR	B cell receptor
BEI	binary ethyleneimine
BMDC	bone-marrow derived dendritic cells of mice
Δcaf1A	mutation in the Caf1A protein of *Yersinia pestis*
CCR	C-C chemokine receptor
CD	cluster of differentiation/classification determinant (e.g. CD19)
cDC	conventional DC(s)
CDC	complement dependent cytotoxicity
CFU	colony forming units
CLN	cervical lymph node(s)
CP	coat protein
CpG	synthetic oligodeoxynucleotide containing an unmethylated motif of cysteine and guanine linked by a phosphodiester bond
CTL	cytotoxic T lymphocyte
DAMP	danger associated molecular pattern
DC	dendritic cell(s)
dLN	draining LN
$D488^+$	cell positive for a DyLight™ 488 labeled protein
EDC	1-ethyl-3-(-dimethylaminopropyl)-carbodiimide
ELISA	enzyme linked immunosorbent assay(s)
ELISPOT	enzyme linked immunospot (assay)
FcγR	Fc gamma receptor
F1	fraction 1 capsule antigen of *Yersinia pestis*
GC	germinal center
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTPase	hydrolase enzyme binding guanosine triphosphate (GTP)
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin (e.g. IL-17)
IL-1RA	interleukin-1 receptor antagonist
IM	interstitial macrophage(s)
i.m.	intramuscular / intramuscularly
i.n.	intranasal / intranasally
i.p.	intraperitoneal / intraperitoneally
Kan^r	kanamycin resistance
kDa	kilo Dalton

KWC	killed whole cell (vaccines)
LD50	lethal dose 50
LN	lymph node
MDR	multi-drug resistance
MHC Class I/II	major histocompatibility complex class I/II
MLN	mediastinal lymph node(s)
NALT	nasal-associated lymphoid tissue
NHP	nonhuman primates
NLR	NOD-like receptor
nm	nanometer
OD	optical density
OMV	outer-membrane vesicles
pg	picogram
Pgm	pigmentation locus of *Yersinia pestis*
Δpgm	mutation in the pigmentation locus of *Yersinia pestis*
PRR	pathogen recognition receptor
PAMP	pathogen associated molecular pattern
rF1	recombinant F1 protein
RLR	RIG-I-like receptors
RT	room temperature
rTMV	recombinant Tobacco Mosaic Virus
rV	recombinant LcrV protein
s.c.	subcutaneous / subcutaneously
SIgA	secretory IgA
SLO	secondary lymphoid organ(s)
STING	stimulator of interferon genes
TCR	T cell receptor
TD	T cell dependent
T_{FH}	T follicular helper cells
T_H	T helper cells
T_H1/2/17	T cell helper type 1/2/17 response
TI	T cell independent
TLR	toll-like receptor
TMV	Tobacco Mosaic Virus
TMV-F1	TMV covalently conjugated to F1 protein
TMV-F1+TMV-V	mixture of TMV-F1 and TMV-V (also abbr. as TMV-F1/V)
TMV-V	TMV covalently conjugated to LcrV protein
TNF-α	tumor necrosis factor alpha
T3SS	type 3 secretion system
µg	microgram
µL	microliter
µm	micrometer
V	LcrV/virulence antigen of *Yersinia pestis*
V-*L.p.*	*Lactobacillus plantarum* expressing V antigen of *Yersinia pestis*
VLP	virus-like particles
Yop	Yersinia outer membrane protein

Abstract

Yersinia pestis, the causative agent of plague, has killed millions throughout human history. Today, it remains a significant threat for use as a bioweapon. Naturally occurring antibiotic resistance has been observed in *Y. pestis* isolates, and resistant strains have been engineered for use in biological warfare. Vaccines remain our best means of protection against plague, as well as countless other global contagions.

We have developed a vaccine consisting of two *Y. pestis* virulence proteins, LcrV (V) and F1, conjugated to Tobacco Mosaic Virus (TMV), a safe, non-replicating plant virus that can be administered mucosally, providing complete protection against pneumonic plague, the deadliest form of the disease and the one most likely to be seen in a bioterror attack. The goal of this dissertation was to understand the mechanism(s) by which intranasal (i.n.) administration of TMV-F1 and TMV-V conjugates enhanced vaccine-specific immunity in a mouse model of pneumonic plague. I hypothesized that the recombinant TMV (rTMV) virion was enhancing the immune response to the associated subunit proteins through direct uptake of the virus by APCs and could induces responses analogous to VLP vaccines. The resulting high-density display of antigens on the virus acts as an immunostimulant when compared to their soluble forms, without the need for adjuvants.

My research was divided into three overarching aims: (1) APC uptake of TMV conjugates, (2) T and B cell responses from TMV conjugate immunization, and (3) use of TMV conjugates in a single dose plague vaccine and its efficacy in controlling inflammation and bacterial dissemination. Challenge experiments utilized BSL-2 *Y. pestis* strains with the chromosomal *pgm* locus deleted (CO92pgm−) or mutated (KIM5*caf1A*). We found TMV conjugation to F1 and V increased their uptake into splenic and mucosal B-cells, and

BMDCs *in vitro*, as well lung DCs *in vivo*, and was not due to any TMV-specific mechanism. We could not show that these vaccines were immunostimulants, as both conjugates failed to upregulate MHC II, CD80, or CD86 over that of unstimulated $CD11c^+$ DCs *in vitro.*

We also demonstrated that generation of antibody-based, but not T cell-based, immunity could be attributed to vaccination with TMV conjugates. Mice immunized i.n. with TMV-F1, specifically, predictably developed high F1-specific serum IgG titers after single and multiple administrations. When applied to an infection model using F1+ *Y. pestis* CO92pgm-, we observed that the protective memory response induced by TMV vaccination did not require $CD4^+$ or $CD8^+$ T cells. This protection is lost against F1- *Y. pestis*, where mice vaccinated with TMV-F1+TMV-V demonstrated poor protection.

Finally, TMV-F1+TMV-V vaccination showed significant reduction in histopathology, bacterial burden, and inflammatory cytokine production in the days following challenge with 100 LD50 *Y. pestis* CO92pgm-. Pneumonic challenge with this strain resulted in systemic dissemination of the bacteria in all groups, but only TMV-F1+TMV-V immunized mice rapidly cleared bacteria from the spleen and liver within 5 days. There was a direct correlation between pre-challenge serum F1 titers and protection in all immunized mice, strongly suggesting a role for anti-F1 antibody in the neutralization and/or opsonization of *Y. pestis* in this model. Furthermore, the TMV-F1+TMV-V i.n. vaccine was capable of mediating single-dose protection.

Taken together, my data indicates the usefulness of rTMV – an inactivated virus – as a carrier for VLP immunogenic presentation and mucosal delivery of whole protein antigens. This particulate vaccine has the ability to target antigens to DCs at mucosal surfaces, which elicits a potent antiviral-like immune response to the bound proteins in the form of

neutralizing IgG antibodies. T follicular helper (T_{FH}) cells may be required in the activation of naïve B cells and/or activation and maintenance of memory B cells in secondary lymphoid organs. Further work should identify where vaccine-specific IgG^+ memory B cells reside (lymph nodes or spleen) after i.n. immunization with the TMV conjugates, the duration of B cell memory in this model, and reasons for why TMV-V immunization cannot generate 100% protection against plague.

Chapter 1. Introduction

Dissertation Premise and Rationale

In 2016, our laboratory reported a study comparing two mucosal plague vaccines: a LcrV (V) antigen-expressing *Lactobacillus plantarum* (V-*L.p.*) live vectored vaccine, and a subunit vaccine, consisting of Tobacco Mosaic Virus (TMV) covalently conjugated to F1 antigen, the *Yersinia pestis* capsule protein, and LcrV (V) antigen, the tip protein of the Yersinia type 3 secretion system (T3SS) (Arnaboldi *et al.*, 2016). The virion is a recombinant TMV engineered to display a surface-exposed lysine on the CP, which allows for amine conjugation of proteins to its surface. Mice were orally and intranasally (i.n.)-immunized with V-*L.p.*, and i.n.-immunized with TMV-F1 and TMV-V. For either vaccine, no adjuvant was administered. In the case of i.n. immunization with V-*L.p.*, mice were primed and boosted twice (day 28 and 56). For i.n. immunization with the TMV conjugates, mice also were primed and boosted twice (day 21 and 35). All immunized mice were challenged on day 70 with an i.n. lethal dose (10 LD50) of the pigmentation-deficient (pgm−) *Y. pestis* CO92 defined mutant. This attenuated strain enables us to work with the organism in our Animal Biosafety Level (ABSL) 2 facility. Pgm− *Y. pestis* strains lack a 102 kb chromosomal fragment (*pgm* locus) encoding iron binding and transport functions, and its deletion attenuates virulence due to the bacterium's inability to efficiently acquire host iron (Buchrieser *et al.*, 1998; Fetherston and Perry, 1994). Although this strain is considered safe to most humans, we have verified that it maintains virulence in mice by the i.n. route.

Complete protection in our challenge experiments was only achieved using three vaccine strategies: a mixture of TMV-F1 and TMV-V, TMV-F1 alone, and intramuscularly

(i.m.)-injected rV and rF1 in alum (positive control). This was surprising given the robust humoral immune response generated by the i.n. V-*L.p.* vaccine; V-specific IgG1, IgG2b, IgG2a, and mucosal IgA were all generated prior to challenge. Still, all V-*L.p.* vaccinees succumbed to infection. Mice vaccinated with TMV-V also produced antigen-specific IgG. V-specific IgG1 was strongly generated by immunization with TMV-V, and titers were generally increased when TMV-F1 was co-administered with TMV-V. This pattern was also observed with F1-specific IgG1, IgG2a, and IgG2b. All completely protected mice shared one thing in common: an antibody response to the F1 protein. For these three groups, mean titers were always in the thousands to tens of thousands for anti-F1 IgG1 and IgG2b, and in the tens to hundreds for anti-F1 IgG2a. Higher V-specific titers, meanwhile, did not signify a better survival outcome. Although mice vaccinated with i.n. TMV-V demonstrated higher mean anti-V IgG1 and IgG2b titers compared with i.m. rF1+rV vaccination, only 60% of the TMV-V vaccinated group survived while all mice in the rF1+rV vaccinated group survived. Furthermore, conjugation to the TMV particle proved necessary for 100% survival, as the mice i.n.-immunized with rF1+rV or individual rF1 and rV proteins never achieved higher than 50% survival.

From these findings, we hypothesized that TMV was enhancing the immune response to the associated subunit proteins through direct uptake of the virus and/or simulation of anti-viral immunity. This mechanism of protection appeared to augment the humoral response to the associated proteins, as F1-specific and V-specific pre-challenge titers were frequently lower in mice i.n.-immunized with free recombinant protein compared to TMV-F1, TMV-V, or both conjugates. *L. plantarum* is a Gram-positive commensal organism, and so it is possible it does not induce the same level of activation as TMV – an RNA virus. Despite the

marked difference in protection between mice given i.n. V-*L.p.* and i.m. rV + alum (positive control), cytokine secretion was minimal and similar, as measured in *ex vivo* antigen recall assays. Other than IL-17, no V-*L.p.* vaccine-specific cytokines were observed. IgA production also proved nonessential for pneumonic plague protection. V-specific IgA was detected in BAL washes of V-*L.p.*-immunized mice and not in rV + alum-immunized mice, yet only the latter were protected from infection. The role of $CD4^+$ and $CD8^+$ T cells in vaccine-induced protection was not assessed in this study, and still needs to be investigated to rule out (or rule in) the possible contribution of cell-mediated immunity. The most interesting observation was the synergetic effect of combining TMV-F1 and TMV-V, as antigen-specific antibody was generally elevated in co-immunized mice compared to mice that received each conjugate individually. Since the only additive component was the virus, I speculated that TMV was responsible for this observed effect, although the mechanism was unclear at the time.

Overall, our experiments provided more questions than it did answers. The study did succeed in uncovering an effective mucosal plague vaccine. The difference in protection between the TMV-based and V-*L.p.* vaccine was clear-cut: the viral particle vaccine protected (100% survival) while the live vector did not (0% survival). However, vaccine-induced protection could not be discerned simply from antibody levels or any particular cytokine profile (e.g. T helper 1 (Th1) response). The most logical lead of this investigation, in my perspective, was to study the response to F1 antigen of *Y. pestis*, since this was not a component of the failed V-*L.p.* vaccine. Clearly, the protective effects of the TMV conjugate vaccine had something to do with a response to F1 but understanding this response would be a major focus of this doctoral thesis. Through this body of research, I sought to define the

immune correlates of protection of the TMV conjugates through studying innate and adaptive immune responses, as well as the efficacy and application of this mucosal vaccine using a mouse model of pneumonic plague.

Tobacco Mosaic Virus

1. Structure and Prevalence

According to the International Committee on Taxonomy of Viruses (ICTV), there are nearly 1,000 different types of plant viruses known to our planet (King *et al.*, 2012). These viruses typically have simple structures: single-stranded (ss) or double-stranded (ds) DNA or RNA packed neatly into a matrix of protein capsids. No plant virus, however, may be more well-known, widely encountered, or relevant to human consumption than the Tobacco Mosaic virus (TMV). TMV was the first virus discovered in 1898, after the advent of Louis Pasteur's Germ Theory and Robert Koch's identifications of disease-causing bacteria (Beijerinck, 1898). TMV is a positive (+) sensed ssRNA virus producing mosaic-like mottling on leaves of infected plants, but namely, *Nicotiana spp*., or tobacco plants (**Fig 1**). TMV belongs to the Tobamoviruses, a genus of closely related viruses that infect numerous plant species. TMV, specifically, damages 125 different plant species, including common crops like tomato, cucumber, and pepper plants, in addition to tobacco plants (Yang, Y. and Klessig, 1996). This has contributed to its prevalence among human, wildlife, and domesticated animal populations.

TMV is a rod-shaped virus with a capsid composed of 2,130 coat proteins (CP) molecules surrounding its single ssRNA genomic core (6.3-6.5 kb) (Goelet *et al.*, 1982; Hwang *et al.*, 1994) (**Fig 1**). The TMV genome encodes four open reading frames (ORFs),

and the CP is self-assembled into a helical structure constituting 16.3 proteins per helix turn, creating a hairpin loop structure of the RNA (Klug, 1999; Stryer *et al.*, 1988). The CP monomer (17kDa) is a small, compact protein, consisting of 158 amino acids which are assembled into four main α-helices, and virions are 300 nm in length and 18 nm in diameter (Klug, 1999). It is considered a highly thermo-stable virus, tolerating up to 120°F (50°C) (Ashkin and Dziedzic, 1987). Because of its stability at high temperatures, TMV resists manufacturing processes and is present in the tobacco of cigarettes, chewing tobacco, and cigars, and also plant-based food products. TMV was found viable in 53% of all major cigarette brands as shown by the development of lesions on leaves of *Nicotiana tabacum* Xanthi after inoculation (Balique *et al.*, 2012). Similar to bacterial infections, plant viruses are transmissible by contact, such as from contaminated farm tools and human hands. Plant viruses such as TMV cannot replicate in animal hosts, largely due to the lack of specific receptors for recognition and entry into host cells (Liu *et al.*, 2013). In addition, temperatures above 34°C dramatically reduce the replication of TMV in tobacco leaf disks, although the viral particle is still stable (Lebeurier and Hirth, 1966).

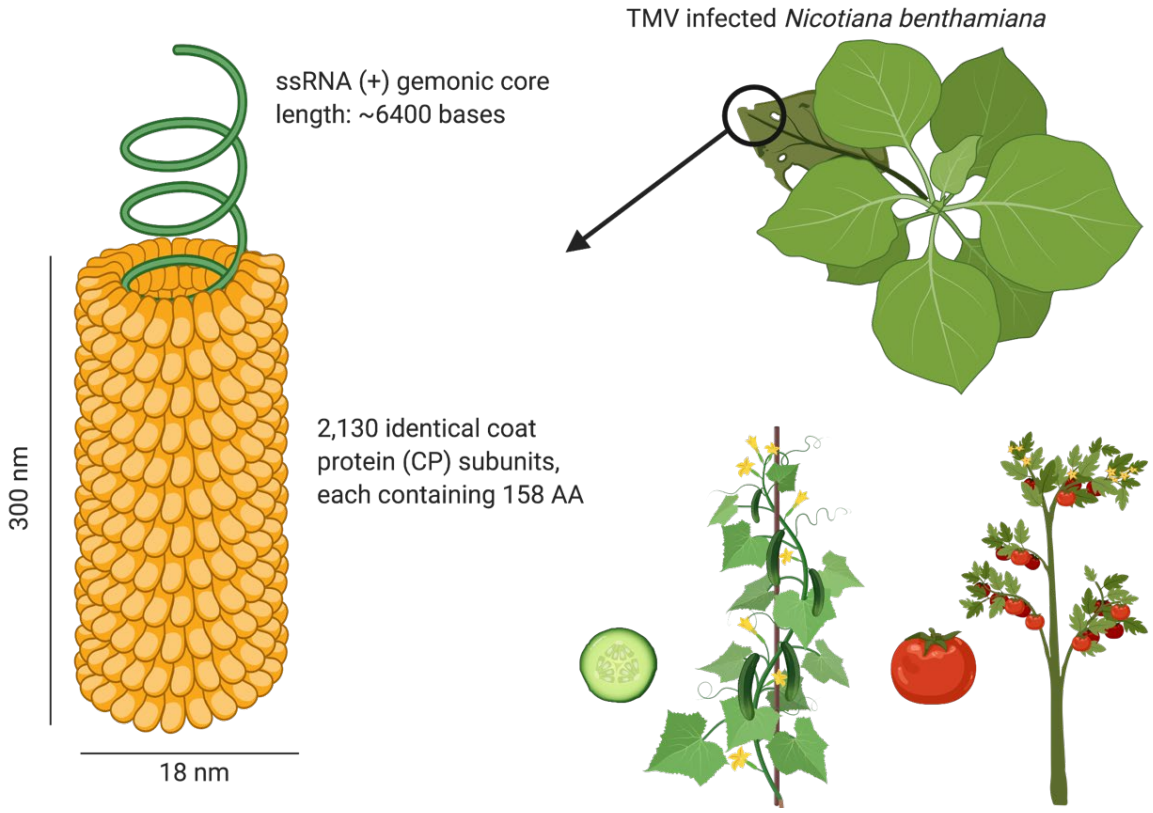

Fig 1. Structure and natural hosts of Tobacco Mosaic Virus (TMV). TMV is consists of 2,130 identical coat proteins surrounding a ssRNA (+) genome of about 6,400 bases. Virions are 300 nm in length and 18 nm in diameter. Here, an infected leaf of *Nicotiana benthamiana* (tobacco plant) is shown discolored, indicating TMV as the pathogen. TMV can infect common crops such as tomato and cucumber, which contributes to humans' ubiquitous exposure to this virus. TMV, tobacco mosaic virus; AA, amino acids; nm, nanometer; ssRNA, single-stranded RNA. Image created on BioRender.com.

Specific immune responses can be mounted against viral antigens, but it is generally thought such responses are not elicited by plant viruses due to their inability to replicate in mammalian cells, which thrive at 37°C (Mandal and Jain, 2010). Cytoplasmic or endosomal replication of viruses normally elicits pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively) that are sensed by animal host cell receptors (e.g. toll-like receptors, TLRs) that activate anti-viral signaling pathways. However, since plant viruses cannot replicate in these cells, these pathways are typically not activated, and the virus is perceived as innocuous (Balique *et al.*, 2015). Limited data is available regarding human immune responses elicited directly by TMV virions. Culturable TMV or TMV RNA have been recovered in the feces (Zhang *et al.*, 2006) and sputum (LeClair, 1967) of human patients but has yet to be linked to any disease in humans. A recent study detected serum antibodies to TMV within a range of ages, race/ethnicities, and tobacco use (smoke, smokeless, and non-users), but not gender (only males were studied). These anti-TMV antibodies were mainly of the IgG isotype, indicating class switching, and were detected in all subjects (Liu *et al.*, 2013). IgG1 was found to provide the dominant antibody response to TMV, and smokers of all ages and ethnicities had a significantly higher total IgG (IgG1, IgG3 and IgG4) against TMV than non-users (Liu *et al.*, 2013). Interestingly, anti-TMV IgG in smokeless tobacco users (i.e. chewing tobacco) did not significantly differ from those in non-users. Caucasians specifically showed no difference in anti-TMV IgG among the three subject groups, while African Americans did. Of note, Liu *et al.* compared absorbance of antibodies in a sandwich ELISA assay and did not report titers in this study. Interpreting these results as antibody "levels" is inaccurate and should instead be interpreted as avidity and affinity of anti-TMV antibodies. Fortunately, TMV-antibody interactions have been

extensively studied. Historically, the study of antibody binding kinetics to TMV made it possible to describe the neutralization of viruses by antibodies well before *in vivo* systems were used (Van Regenmortel *et al.*, 1993; Van Regenmortel, 1999). TMV is one of the best characterized plant viruses, and uniquely, almost all of its CP sequence has been shown to be antigenic through recognition by monoclonal antibodies (Holzem *et al.*, 2001).

2. Uses in Vaccination

TMV's physical and antigenic attributes, as well as its simple genome, made it an ideal candidate for study as a vaccine. In the last two decades, there has been significant progress in this field of research. TMV has been utilized as an epitope display system for numerous antigenic peptides, as well as biopharmaceutical protein production (Canizares *et al.*, 2005). One of the first published studies of peptide display on TMV reported the success of co-assembly of chimeric CP-malaria epitope particles, and concluded: "recombinant plant viruses have the potential to meet the need for scalable and cost effective production of subunit vaccines that can be easily stored and administered" (Turpen *et al.*, 1995). Beyond TMV's ability to serve as an epitope presentation system, it could also express exogenous proteins. In one study, TMV was engineered to produce the F1 and V virulence proteins from *Yersinia pestis*, that, when purified and administered to guinea pigs, were protective against aerosolized *Y. pestis* challenge (Santi *et al.*, 2006). The authors of that study concluded that a TMV-based expression system was practical to areas of biodefense by offering speed in production and scalability of vaccine proteins (Santi *et al.*, 2006).

Remarkably, a TMV-peptide virion was able to break B-cell tolerance and induce intentional production of autoreactive antibodies in vaccinated animals (Fitchen *et al.*, 1995).

This is not always easily achieved by vaccination. It was suggested that the ability of TMV to break immune tolerance is likely due to the "high-density, quasi-crystalline array" in which CP-peptide fusions are displayed on the virus, mimicking a PAMP (McCormick and Palmer, 2008). Koo *et al.* were the first to demonstrate that a TMV peptide-display vaccine for an infectious disease (murine hepatitis virus, MHV) could induce neutralizing antibodies that protected mice against MHV challenge (Koo *et al.*, 1999). It was later demonstrated TMV peptide-display vaccines did not have to be limited to one, or even two epitopes; their TMV virion presented three different human papillomavirus (HPV) epitopes that could induce cross-protective antibodies in a rabbit model of HPV (Palmer *et al.*, 2006). At this point, researchers anticipated that utility of TMV as a vaccine could be further developed. New focus was placed on the viral surface to allow addition of larger protein moieties. However, TMV CP surface residues naturally lack chemically reactive lysine and cysteine amino acids (McCormick and Palmer, 2008). Kenneth Palmer and colleagues at Large Scale Biology Corporation (Vacaville, CA) were the first to devise that simple addition of these amino acids to the N terminus, loop, or C terminus of the CP could provide a scaffold for surface conjugation (Smith *et al.*, 2006). After several screens, their team succeeded in creating a chemically stable virion with lysine as the surface-exposed residue – one that could participate in amine conjugation and covalently link whole proteins containing cysteine or lysine. Alison McCormick and Palmer's colleagues noted the bond stability is "important in inducing the maximum humoral immune response, possibly by generating a more stable semi-crystalline array of peptide antigen on the surface of TMV" (McCormick and Palmer, 2008). Their first test of whole antigen conjugation was to TMV-biotin; their model antigens were streptavidin (SA)-GFP and SA-COPV.L2 (L2 capsid of canine oral papillomavirus)

(Smith *et al.*, 2006). Mice and guinea pigs were immunized with SA-GFP and TMV-SA-GFP, and SA-COPV.L2 and TMV-SA-COPV.L2. In each case, the TMV-biotin-SA conjugates resulted in higher IgG titers in both species relative to the non-conjugated antigens (Smith *et al.*, 2006). Of note, GFP and the COPV L2 protein are 27kDa and 55kDa, respectively, and much larger than the 17kDa TMV CP, yet still successfully bound the virion. Continued observations of TMV-SA-GFP showed this model conjugate could also stimulate cellular immune responses, further verifying its status as an immunogen (McCormick and Palmer, 2008).

The feasibility of conjugating full-length antigens to TMV had now been realized. The discovery made by Smith *et al.* paved the way for several studies utilizing TMV as a vaccine delivery platform. Protein-TMV conjugate vaccines have now demonstrated protection against tularemia (Banik *et al.*, 2015; Mansour *et al.*, 2018; McCormick *et al.*, 2018), pneumonic plague (Arnaboldi *et al.*, 2016), and influenza H1N1 (Mallajosyula *et al.*, 2014) and H5N1 (Mallajosyula *et al.*, 2016) (**Table 1**). In these particular studies, the TMV-antigen conjugates were immunized by either subcutaneous (s.c.) or i.n. routes, and each time induced stronger antigen-specific IgG responses in mice compared to unbound proteins. Importantly, pre-exposing the mice to TMV did not reduce antigen-specific titers (Mallajosyula *et al.*, 2014). The adjuvant CpG was mixed with conjugates to boost Th1 responses for *Francisella tularensis*, an intercellular bacterial pathogen. Alum (Alhydrogel®) or a squalene-based oil-in-water emulsion (AddaVax™) were mixed with TMV-hemagglutinin (HA) conjugates. Each study offered unique observations, yet, this made it difficult to draw conclusive statements about the mechanism of protection. In the H1N1 study, AddaVax™ adjuvant was needed achieve 100% survival with the TMV-HA vaccine,

presumably by increasing total anti-HA IgG ((Mallajosyula *et al.*, 2014). In the study by Arnaboldi *et al*., co-administration of two TMV-protein conjugates (TMV-F1 + TMV-V) without any adjuvant augmented antigen-specific IgG titers compared to mice that received each conjugate individually. This mixture protected all mice from *Y. pestis* challenge, but TMV-F1 immunization also elicited 100% protection (Arnaboldi *et al.*, 2016). In this case, protection from TMV was antigen-specific and did not directly correlate with total IgG levels. T-cells were also thought to contribute to vaccine-induced protection. In the study by Mansour *et al*., immunization of IFN-γ-deficient mice with a tetravalent TMV conjugate mixture (TMV-DnaK + TMV-OmpA + TMV-Tul4 + TMV-SucB) showed IFN-γ was necessary for protection against lethal respiratory challenge with *F. tularensis* LVS (live vaccine strain) (Mansour *et al.*, 2018). In normal mice, the tetravalent conjugate mixture with CpG provided 100% protection after three doses (Mansour *et al.*, 2018). In support of these data, McCormick *et al*. had demonstrated previously that TMV conjugates to murine melanoma peptide epitopes stimulated IFN-γ-producing T-cells after vaccination and protected against tumors in mice (McCormick, Corbo, Wykoff-Clary, Palmer *et al.*, 2006; McCormick, Corbo, Wykoff-Clary, Nguyen *et al.*, 2006). Thus, TMV conjugate vaccines were capable of supporting both Th1 (IFN-γ-inducible) responses and antibody responses, but it was still not clear if both responses were always initiated after immunization. Was the virus itself an immunogen, skewing the immune response regardless of the antigen, or was it simply a scaffold for whatever antigen was bound to its surface?

3. Immune Targeting and Activation

The particulate nature of TMV is also thought to play a role in its immunogenicity. In early studies, dissembled TMV CP generated lower titers in comparison with intact virions, and TMV virions were more readily taken up by APCs and transported to secondary lymphoid organs and the spleen in rabbits (Loor, 1967; Marbrook and Matthews, 1966). The size of TMV makes it different from most other assembled virus-like particle (VLP) structures, which are between 10 and 100nm (Bachmann and Jennings, 2010). For this reason, immunized VLP either enter the lymphatic vessels and drain downstream of the immunization site or are taken up by antigen presenting cells (APCs). TMV at 300 nm is larger than most VLP and only suited for uptake and processing by APCs to reach the lymphatics (**Fig 2**). Unlike proteins, it is imperative that pathogens make contact with APCs early on in infection to mount an appropriate immune response. Professional APCs of the immune system (macrophages, dendritic cells, and B cells) are able to efficiently take up pathogens into the micrometer range (**Fig 2**) through phagocytosis or via receptor internalization (Bachmann and Jennings, 2010). Dendritic cells (DCs) uniquely can internalize, process, and present antigens through both major histocompatibility complexes (MHC) I and II and play the predominant role in priming naïve T-cells and initiating immune responses out of all APCs (Foged *et al.*, 2002). According to various studies, DCs can efficiently process 20-300nm particles by phagocytosis or macropinocytosis (Fifis *et al.*, 2004; Gamvrellis *et al.*, 2004; Woodrow *et al.*, 2012).

The particulate nature of VLP and high density of epitopes on their surface allow them to induce potent T-cell mediated immune responses through interaction with DCs (Chen and Lai, 2013). Studies led by McCormick have confirmed TMV's ability to be taken up by mammalian DCs both *in vivo* (following injection) and *in vitro*. Viable TMV was

capable of targeting DCs in murine lymph nodes (LN) and localizing in DC phagosomes, indicating the virus is indeed phagocytized (Kemnade *et al.*, 2014). Specifically, $CD11c^+$ DCs isolated from popliteal LN that had taken up TMV following s.c. footpad injection had increased surface expression of the T-cell costimulatory molecules CD54, CD40, CD86, MHC I, and MHC II, compared to DCs from PBS-injected mice (Kemnade *et al.*, 2014). McCormick *et al.* next tested if TMV could be taken up by splenocytes and LN cells *in vitro* (McCormick *et al.*, 2006). Indeed, over 90% of cells had taken up fluorescently labeled TMV particles in 1 hour *in vitro*, and DCs that had taken up TMV had increased expression of the CD86 surface maker, a prerequisite for naïve T-cell priming (McCormick *et al.*, 2006). It was suggested that this set of features– the particulate size, repetitive structure, and ssRNA – was responsible for the observed immune-activating effects of TMV as a VLP platform (McCormick and Palmer, 2008). Like mammalian viruses, the ssRNA may stimulate innate immunity in DCs via endosomal TLR7/8 recognition and downstream signaling. However, whether TMV RNA activates TLR7/8 has yet to be formally established. As of now, the immune-activating effects are largely attributed to that fact that particulates like TMV virions are taken up naturally by APCs based on its size and repetitive surface structure.

Table 1. List of protective vaccines comprising whole protein antigen-TMV conjugates.

Respiratory Pathogen	**Antigen(s)**	**Vaccine Route**	**Adjuvant(s)**	**Reference(s)**
Influenza (H1N1, H5N1)	HA	s.c.	Alhydrogel®, AddaVax™	Mallajosyula *et al.,* 2014; Mallajosyula *et al.,* 2016
Francisella tularensis (LVS)	DnaK, OmpA, Tul4, SucB	s.c., i.n.	CpG	Banik *et al.,* 2015; McCormick *et al.,* 2018; Mansour *et al.,* 2018
Yersinia pestis	LcrV, F1	i.n.	---	Arnaboldi *et al.,* 2016; D'Arco *et al.,* in review
Pseudomonas aeruginosa	PcrV	i.n.	di-cyclic GMP	Arnaboldi, unpublished

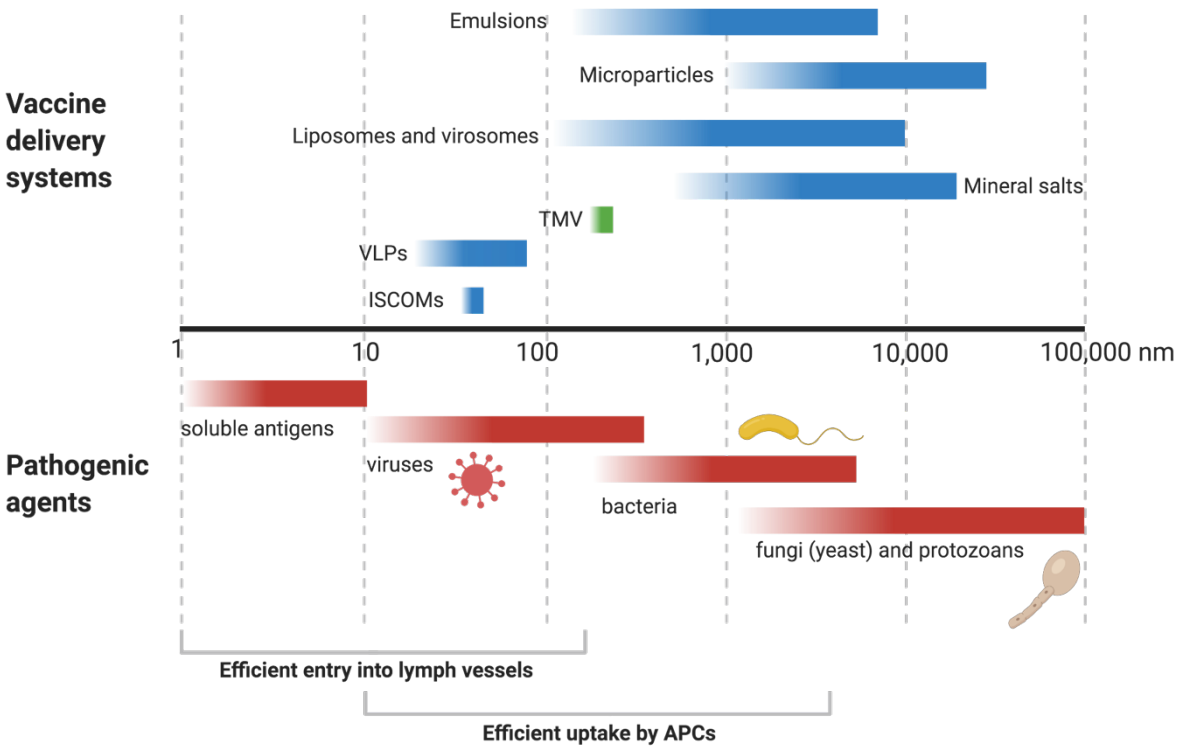

Fig 2. TMV as an APC-targeting delivery system. The size ranges of various vaccine delivery systems and different pathogenic agents are indicated on a nanometer (nm) log scale. The range of particle sizes that allow uptake by APCs and the size of macromolecules that enter the initial lymphatic vessels are shown. TMV (300nm) is in the particle size range ideal for APC uptake, while VLP fall into both ranges. ISCOMs, immune-stimulating complexes; VLP, virus-like particles; TMV, tobacco mosaic virus; APC, antigen-presenting cell. Image created on BioRender.com. Image and description adapted from: Bachmann M. and Jennings G. *Nat Rev Immunol*. 2010;10:787–796.

Yersinia pestis

1. The Disease and Historical Perspective

In 1894, Alexandre Yersin first described bacilli that were faintly Gram-negative bacteria with "rounded ends," cultured from buboes of cadavers during a plague outbreak in Hong Kong (Butler, 2014; Hawgood, 2008; Yersin, 1894). Yersin later inoculated guinea pigs with the bubo fluid, and all died within a few days (Yersin, 1894). The bacilli isolated from the guinea pigs resembled the initial inoculum, and thus, Yersin had discovered the agent of plague. Baron Shibasaburō Kitasato, a renowned Japanese physician-scientist and bacteriologist, also made the same observations in Hong Kong right before Yersin. Although his samples became contaminated, resulting in conflicting reports, it is believed Kitasato should be given credit for accurately isolating and characterizing the plague bacilli (Bibel and Chen, 1976). Yersin later made a major discovery – that rats were the pervasive source, as their lymph nodes also contained the bacilli (Solomon, 1995). Paul-Louis Simond eventually determined it was the fleas on infected rats that were the actual source of transmission to human hosts (Gross, 1995; Simond, 1898). *Yersinia pestis* was later formally given its name to honor its discoverer, Alexandre Yersin, although Kitasato is now acknowledged as a co-discoverer.

Y. pestis is most commonly transmitted to humans via a bite from a flea which previously fed on an infected rodent, resulting in bubonic plague (Perry and Fetherston, 1997). The distinctive clinical sign of this form of plague is a bubo, a gross enlargement of a lymph node that drains the site of the flea bite. If left untreated, bacteria within the buboes are left to multiply and disseminate throughout the lymphatic system, causing a bacteremia that can eventually lead to a fatal septicemia (Perry and Fetherston, 1997). Bubonic plague

has a mortality of ~60% in humans if left untreated. In rare cases, direct blood dissemination can occur, resulting in primary septicemic plague. Septicemic plague is often associated with a blackening of the hands and feet (necrosis) and is universally fatal if antibiotics are not given (Koirala, 2006). Thrombosis, hemorrhaging, and gangrenous necrosis of acral regions can also result from bubonic plague, if septicemia follows initial lymph node infection (**Fig 3**) (Demeure *et al.*, 2019). Primary septicemic plague accounts for roughly 10% of all cases of plague and is the least common form of the disease (Cornelis, 2000). Plague septicemia, whether primary or secondary, typically progresses to metastatic infection of organ systems and organ failure if left untreated.

Y. pestis was the notorious etiologic agent of the "Black Death" or "Black Plague" in the Middle Ages when approximately 30-60% of Europe's population succumbed to this disease during the mid-14th century (Butler, 2014; Perry and Fetherston, 1997). This Second Pandemic lasted 130 years, and smaller, recurrent outbreaks continued well into the 17th century (Riedel, 2005). Historical records indicate other plagues, both before and after the Black Death, decimated human populations. In the 6th century, the Plague of Justinian (First Pandemic) of Eurasia is suspected to have started in Constantinople (the epicenter) after ships from Egypt arrived carrying grain and infected rats (Wagner *et al.*, 2014). An estimated 25-50 million people died in North Africa, Europe, and central and southern Asia during two centuries of recurrence – an estimated 13-26% of the world population at the time (Riedel, 2005; Wagner *et al.*, 2014). Numbers vary wildly based on source, as much as 60% of the population of Europe may have died. Newly surfaced anthropological evidence suggests the Great Plague of Justinian contributed to the decline of the Roman Empire (Harbeck *et al.*, 2013). In the 19th century, plague returned to civilization with a vengeance, and episodes of

bubonic transmission arose and spread to all inhabited continents (except Australia), collectively known as the Third Pandemic. During this worldwide series of plagues, China and India saw a total of 12 million deaths (Riedel, 2005). The U.S. also saw the introduction of plague on its soil, which likely originated in the rats of steamships docked at ports along the Pacific coast. The city of San Francisco experienced a plague epidemic resulting in over 100 deaths at the turn of the 20th century (LINK, 1955). It was not until the mass production and distribution of antibiotics in the 1950s that the reign of the Third Pandemic finally ceased, or, at the very least, was suppressed.

Post-antibiotic discovery, sporadic outbreaks of plague continue to occur in many parts of the world, particularly in central Asia, central sub-Saharan Africa, and the island of Madagascar (Donaires *et al.*, 2010; Koirala, 2006; Randremanana *et al.*, 2019). The disease is endemic in rodent populations in much of Asia, parts of Africa, and the Americas. Plague remains endemic in prairie dogs in the western U.S. (Boone *et al.*, 2009; Richgels *et al.*, 2016). Approximately 5-15 human cases of plague are reported in the U.S. each year, usually in rural areas of the Southwest (New Mexico, California, Colorado, and Texas) where people accidentally come into contact with infected animals (CDC, 2019; Runfola *et al.*, 2015). The U.S. Centers for Disease Control and Prevention (CDC) has repeatedly assured the public that plague is not an emerging threat to the American population (CDC, 2019). Certainly, other countries report worse outbreaks. Madagascar experienced a large urban outbreak in 2017; 76% of the nearly 2,400 reported cases were confirmed, probable, or suspected pneumonic plague (Andrianaivoarimanana *et al.*, 2019; Nguyen *et al.*, 2018; Rabaan *et al.*, 2019; Randremanana *et al.*, 2019; WHO, 2017). Thus, although bubonic plague is

responsible for most cases of naturally occurring human plague, pneumonic transmission is often what drives modern-day outbreaks in concentrated or urban populations.

Primary pneumonic plague results from infection of the lungs with *Y. pestis*. While bubonic plague is transmitted via an insect bite, primary pneumonic plague is acquired through aerosols generated from the coughing of infected humans within 2 meters of proximity (Cornelis, 2000). This enables direct host-to-host transmission of the bacterium, and a low inoculum is needed to cause disease (Perry and Fetherston, 1997). Secondary pneumonic plague results from hematogenous spread of *Y. pestis* to the lungs, after bubonic or septicemic infection. Primary pneumonic plague, however, is the most virulent form, with death occurring within 48 hours of infection in the absence of treatment (100% mortality) (Koirala, 2006). This is due to a unique biphasic response in the lungs (**Fig 3**). During a long pre-inflammatory phase, *Y. pestis* bacteria replicate to high levels while suppressing and evading host immune defenses (Pechous *et al.*, 2013); 24-36 hours later, the pro-inflammatory phase occurs, characterized by extensive neutrophil influx and cytokine storms that severely damage lung tissue and prove too late to control the infection (Demeure *et al.*, 2019). Complicating matters, pneumonic plague can be confused with several other acute and community-acquired pneumonias, such as pneumococcal pneumonia, influenza viral pneumonia, as well as *Haemophilus influenza* pneumonia. In addition, diagnosis could be mistaken as pulmonary anthrax, tularemia, *Legionella pneumophila*, and Hantavirus syndrome (Cornelis, 2000). For an early and accurate diagnosis of plague, reasonable suspicion is required of the clinician, especially when biowarfare is suspected (Franz *et al.*, 1997). In cases where pneumonic plague is suspected, antibiotics must be administered within 24 hours of symptoms to prevent imminent death.

The extreme lethality of plague and history of weaponization have led to the assignment of Y. pestis as a Tier 1 Select Agent, and fears of its release as a bioterrorist weapon (Carus, 2015; Inglesby et al., 2000). During World War II, the Japanese army (Unit 731) is reported to have dropped plague-infected fleas in "flea bombs" over populated areas in China and Manchuria by aircraft (Eitzen and Takafuji, 1997; Riedel, 2004). Following World War II, the U.S., Great Britain, and the former Soviet Union (U.S.S.R.) established biowarfare programs during the Cold War, researching Y. pestis as a potential biological weapon and expanding on studies of Unit 731. These efforts focused on the study of plague in aerosolized form, thereby eliminating the need for flea vectors. In 1970, the World Health Organization (WHO) published a comprehensive report on the outcome of the possible use of biological weapons. The report predicted that the release of 50 kg of Y. pestis in aerosolized form over a city of 5 million could result in (worst case scenario) 150,000 cases of pneumonic plague, 80,000-100,000 cases of hospitalization, and 36,000 victims (WHO Group of Consultants, 1970). Furthermore, plague bacilli would remain viable in the air for 1 hour and up to a distance of 10 km (WHO Group of Consultants, 1970). The expert panel was concerned that in such a scenario, significant numbers of city inhabitants would likely flee, further spreading the agent. Unlike other biowarfare agents, plague is contagious and can be spread from person-to-person. Today, the possibility of plague being unleashed onto an unsuspecting city is still too nightmarish to comprehend but has yet to occur. *Y. pestis* can also be easily genetically manipulated to create strains with specific traits, such as engineering strains to be resistant to antibiotics commonly used to treat plague patients (streptomycin, gentamicin, tetracyclines, ciprofloxacin, and chloramphenicol) (Riedel, 2005).

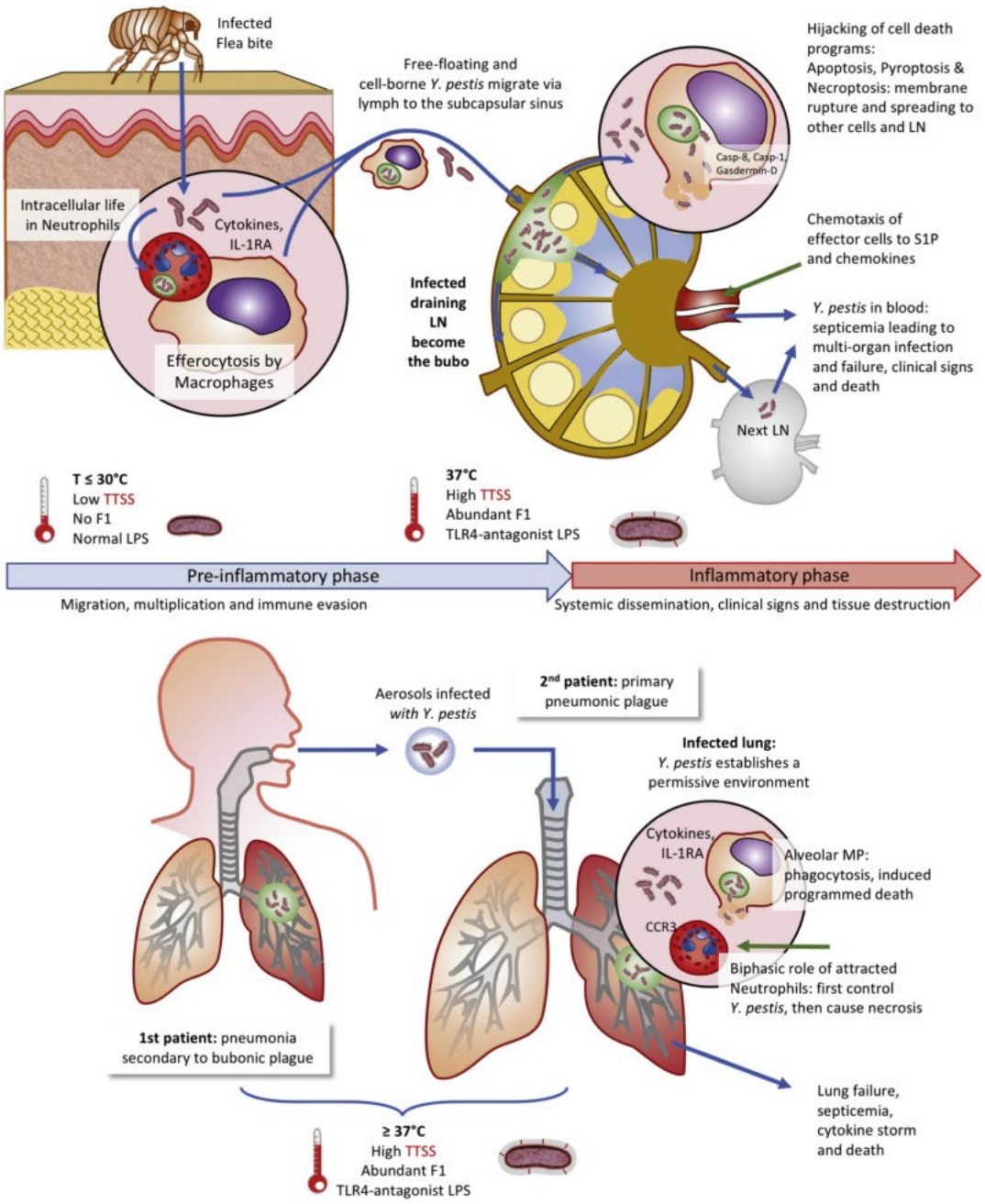

Fig. 3. Pathogenesis of bubonic and primary pneumonic plague. Schematic representation of *Y. pestis* transmission and migration in the human body during bubonic (upper panel) and pneumonic plague (lower panel). The effect of temperature on the regulation of bacterium virulence factors are indicated at various steps. Innate immunity host cells encountered by the pathogen and involved in immune evasion mechanisms are shown. Image and description credit: Demeure CE, et al. *Genes and Immunity.* 2019;20(5):357–370.

Multidrug-resistant *Y. pestis* isolates have been recovered from human plague patients, suggesting the bacterium continues to evolve mechanisms of survival in the mammalian host (Galimand *et al.*, 1997; Guiyoule *et al.*, 2001; Welch *et al.*, 2007). For several decades, researchers in government and academia have studied *Y. pestis* with the hope of developing preventative countermeasures to these scenarios, primarily in the form of vaccines.

2. Evolution and Virulence Factors

The pathogenicity of *Y. pestis* stems from its remarkable ability to overcome defense mechanisms of the mammalian immune system and quickly overwhelm the host. The rapid multiplication of the bacteria occurs mainly extracellularly, but can occur intracellularly initially, typically in macrophages (McNally *et al.*, 2016; Perry and Fetherston, 1997). The entrance of the organism into a mammalian host (37°C) induces the expression of several virulence factors (discussed below) that perturb or "paralyze" cells, enabling subversion of host immune responses. *Y. pestis* is highly similar on a genomic level to *Yersinia pseudotuberculosis*, which evolved from *Yersinia enterocolitica*, an infrequent enteric pathogen in humans (yersiniosis). However, a series of genetic gains and losses led to different mechanisms of disease and niche preference between *Y. pestis* and *Y. pseudotuberculosis* (Achtman *et al.*, 2004; Chain *et al.*, 2004; McNally *et al.*, 2016) that forever separated them in the Yersinia evolutionary tree.

It is now estimated that *Y. pestis* diverged from *Y. pseudotuberculosis* around 5700–6000 years ago, based on the recent recovery of *Y. pestis* DNA from the teeth of Neolithic peoples in Eurasia (Andrades Valtuena *et al.*, 2017; Rascovan *et al.*, 2019; Rasmussen *et al.*, 2015; Spyrou *et al.*, 2018). It is probable that *Y. pestis* existed prior to this in wild animal

populations. Its emergence was characterized by the acquisition of the two key plasmids: pFra/pMT1 and pPla/pPCP1. The acquisition of *ymt* (*Yersinia* murine toxin) in the pFra/pMT1 plasmid (Sun, Y. C. *et al.*, 2014) as well as inactivation of *ureD, rcsA, flhD, pde2* and *pde3* – all virulence-associated genes – enabled the transmissibility of *Y. pestis* by fleas (Chouikha and Hinnebusch, 2014). The aftermath of these changes gave rise to the ability to cause bubonic plague, which can be traced to the Bronze Age (Spyrou *et al.*, 2018). The plasminogen-activating protease, Pla, encoded by the pPla/pPCP1 plasmid, was carried by earlier lineages of *Y. pestis* and led to the ability to cause pneumonia. However, a random mutation in the *pla* gene, altering a single amino acid (I259 → I259T), enabled invasive and rapid extrapulmonary dissemination of the bacterium (Lathem *et al.*, 2007; Zimbler *et al.*, 2015). Taken together, this deadly repertoire – a set of exceptional virulence factors, the ability to infect lungs, and extreme invasiveness – made plague a death sentence. *Y. pestis* could multiply to high numbers since it was no longer restricted to the site of infection, thereby increasing its chances of transmission, and transforming a bacterium capable of causing local outbreaks into the mass killer we know today.

In primary pneumonic plague, it is believed the bacteria first infect macrophages – and then neutrophils – where they proliferate and produce factors in phagosomes in response to drops in [Ca^{++}] and pH (acidity) (McNally *et al.*, 2016). Within alveolar macrophages, *Y. pestis* persists in autophagosomes, and is capable of inhibiting phagosomal maturation by perturbing the host endosomal recycling pathway, allowing intracellular replication (Connor *et al.*, 2015; Connor *et al.*, 2018; Pujol *et al.*, 2009; Spinner *et al.*, 2014). Inflammasome-dependent IL-1β/IL-18 release occurs early after bacteria enter the lung but fails to cause inflammation due to the simultaneous release of anti-inflammatory IL-1RA blocking IL-1

receptors (Sivaraman *et al.*, 2015). *Y. pestis* can also directly evade phagocytosis by alveolar macrophages. Absence of the LpxL/HtrB acyltransferase in *Y. pestis* yields a tetra-acylated LPS, which prevents innate immune recognition by antagonizing TLR4 receptors (Montminy *et al.*, 2006). Fraction 1 (F1), the pFra/pMT1-encoded capsular antigen, prevents bacterial uptake by inhibiting adhesion (Liu et al 2006). The Psa fimbriae (pH 6 antigen) also inhibits phagocytosis by macrophages and mediates bacteria attachment to epithelial cells (Bao *et al.*, 2013). The Pla protease has been shown to cleave multiple host cell substrates *in vivo* and/or *in vitro* including plasminogen, plasminogen activator inhibitor 1, urokinase plasminogen activator, C3 complement, Fas ligand, as well functions as a critical adhesin to regulate activity of the T3SS (Lathem *et al.*, 2007; Zimbler *et al.*, 2015). The expression of such outer-membrane proteins is normally induced during transition from the flea midgut (26°C) to that of the mammalian host (37°C) (**Fig 3**). However, in primary pneumonic plague, there is no lag since many of these factors are already in abundance upon entering the lung. Indeed, the *Y. pestis* T3SS mediates activity in the lung as early as 6 hours following pneumonic infection (Pechous *et al.*, 2013).

The best characterized virulence factors of *Y. pestis* are associated with its T3SS, which is encoded in the pYV/pCD1 plasmid and shared with *Y. pseudotuberculosis* and *Y. enterocolitica*. Among them are the effector Yersinia outer membrane proteins (Yops), which are translocated via the T3SS directly into host cells. These effectors include YopH, YopE, YopT, YopJ, YpkA, YopM and YopK, which together inhibit Rho GTPases, remodel actin cytoskeleton (inhibiting phagocytes), dampen pro-inflammatory cytokines, and trigger apoptosis by multiple sophisticated mechanisms (Demeure *et al.*, 2019). Upon apoptosis, more virulent bacteria within phagocytes can be released into the lung environment. During

the pro-inflammatory stage of primary pneumonic plague, when bacterial burden has reached its peak, infiltrating neutrophils are responsible for the development of fulminant pneumonia and tissue necrosis. In this context, YopK modulates macrophage apoptosis and promotes progression of pneumonia (Peters *et al.*, 2013), while YopM "paralyzes" neutrophils, inhibiting their death in dense inflammatory lesions and preventing the release of their bactericidal content (Ratner *et al.*, 2016; Stasulli *et al.*, 2015). To overcome host nutritional immunity, *Y. pestis* secretes the siderophore yersiniabactin (Ybt) to acquire iron, which is essential in the pathogenesis of bubonic and pneumonic plague (Fetherston *et al.*, 2010). Ybt is also necessary for zinc uptake together with the ZnuABC transporter system (Bobrov *et al.*, 2017; Perry *et al.*, 2015). There are several other novel *Y. pestis* virulence factors that have been identified in recent years (Demeure *et al.*, 2019; Huang *et al.*, 2006; Sha *et al.*, 2016; Tidhar *et al.*, 2009), however, they will not be covered in detail in this review.

3. V and F1 Antigens

Despite numerous potential antigenic targets of *Y. pestis*, the most promising plague vaccines almost always target two proteins: F1 and LcrV. LcrV, also known as the virulence or "V" antigen, is the tip protein of the *Y. pestis* T3SS, and a pivotal component of virulence for all pathogenic Yersinia (Derewenda *et al.*, 2004). The pYV/pCD1 plasmid of *Y. pestis* contains at least five low-calcium response (*lcr*) genes, which appear to serve as regulators of V expression when extracellular [Ca^{++}] is extremely low or absent, and when temperatures are high (i.e. in a mammalian host). Insertion mutations made into *lcr* genes causes the loss of regulated Yop and V antigen expression as well as the loss of calcium dependence for growth (Goguen *et al.*, 1984). Contact of the bacteria with eukaryotic cells results in

secretion of V antigen (35-37kDa) into the surrounding medium, or into the cytosol of infected host cells (Fields and Straley, 1999). Once released, V antigen may function as a "diffusor" of host immunity, where it has been shown to suppress IFN-γ and TNF-α production (Nakajima *et al.*, 1995) via enhancement of IL-10 (Nedialkov *et al.*, 1997; Sing *et al.*, 2002), and inhibit chemotaxis of neutrophils (Visser *et al.*, 1995; Welkos *et al.*, 1998). V is indispensable for the targeting of Yops; upon contact with a host cell membrane, V undergoes conformational changes allowing a structure to form with translocator Yops (YopB and YopD) that mediates transfer of effector Yops into the host cell (Fields *et al.*, 1999; Pettersson *et al.*, 1999). This structure resembles the needle-like T3SS discovered in *Salmonella typhimurium* (Cornelis, 1998), and is highly conserved and nearly identical among *Salmonella spp., Pseudomonas spp*. *Shigella spp*., enteric hemorrhagic *Escherichia coli* (EHEC), and *Yersinia spp*. The combination of V antigen with F1 antigen (or as a fusion protein) has been shown to generate high levels of vaccine protection than either component individually (Amemiya *et al.*, 2009; Heath *et al.*, 1998; Jones *et al.*, 2006; Williamson *et al.*, 1997). Since V is also essential for *Y. pestis* virulence, it is frequently referred to as a protective antigen and continues to be a central target of almost all plague vaccines.

There are advantages to studying the F1 capsule protein in the context of *Y. pestis* pathogenesis, particularly as F1's protective mechanisms showcase the serological arm of adaptive immunity. Vaccination with F1, but not with V, has been shown to rapidly induce immunity within several days, in the form of antibodies (Levy *et al.*, 2011; Williamson *et al.*, 2007). It is thought the F1 capsule obstructs phagocytosis by acting at the level of receptor interaction with phagocytes (Du *et al.*, 2002) while potentially cloaking other surface features that would otherwise enable host recognition (Runco *et al.*, 2008). Therefore, blocking this

interaction remains a worthwhile and strategic goal of vaccination. Indeed, research has repeatedly supported protective efficacy of F1-specific antibodies in pneumonic and bubonic models of plague (Andrews *et al.*, 1996; Feodorova and Corbel, 2009; Feodorova and Motin, 2012; Heath *et al.*, 1998; Holtzman *et al.*, 2006; Levy *et al.*, 2011; Levy *et al.*, 2018; Okan *et al.*, 2010; Titball and Williamson, 2001; Titball and Williamson, 2004; Williamson *et al.*, 1996; Williamson and Oyston, 2013). Passive transfer of anti-F1 antibodies to naïve animals and generation of anti-F1 IgG following F1 immunization both lead to high survival rates in animal models of plague (Williamson and Oyston, 2013). The tendency for recombinant F1 (rF1) to form large molecular-weight polymers also aids in rapid recognition by innate-like B cells, permitting F1-specific antibody production within 3 days of immunization and complete protection from bubonic plague (Levy *et al.*, 2018). Additionally, these rF1 polymers can be easily purified from *E. coli* cultures expressing the *caf1* operon (Andrews *et al.*, 1996). The *caf1* operon also encodes for the transcriptional regulator Caf1R, the chaperon Caf1M, and the usher protein Caf1A (Galyov *et al.*, 1991). In the generation of the capsule, F1 monomers (15.5-17.5kDa) form polymers that are exported to the outer membrane of the bacteria, aided by Caf1M and Caf1A (Andrews *et al.*, 1996; Zavialov *et al.*, 2003). F1 capsule proteins have also been detected in outer membrane vesicles (OMV) of *Y. pestis,* which carry other virulence factors (Eddy *et al.*, 2014). Unlike V antigen, F1 is dispensable for virulence. Naturally occurring F1 mutant strains have been isolated and are capable of causing fatal disease similar to wild-type strains (Davis *et al.*, 1996; Friedlander *et al.*, 1995). In these cases, immunization with V alone can still generate protective immunity against both strains (Quenee *et al.*, 2008).

4. Immunity to Plague: Vaccines

Most early attempts at plague vaccines utilized killed whole cell (KWC) formulations. The Cutter vaccine was a formaldehyde-killed *Y. pestis* 195/P strain; it was believed to stimulate protection mainly against the F1 protein but is no longer in production (Friedlander *et al.*, 1995).The Commonwealth Serum Laboratories vaccine was a heat-killed suspension of *Y. pestis* that required multiple boosters to maintain protection. It was proven effective against bubonic plague in U.S. soldiers in the Vietnam War, but was ineffective against pneumonic plague, as was the Cutter vaccine (Meyer, 1970; SPECK and WOLOCHOW, 1957). Both KWC vaccines required an intensive prime-boost schedule to achieve long-term protective immunity (Bramwell *et al.*, 2005). Live vaccines were approached as a more immunogenic alternative. However, development of a suitable vaccine was challenged by the genomic instability of *Y. pestis* (Demeure *et al.*, 2017). Interest dwindled, largely owing to the unpredictability of the attenuation, as well as the tendency of the EV76 vaccine (an effective live *Y. pestis* strain developed by the former Soviet Union) to produce vaccine adverse reactions in humans and occasionally kill nonhuman primates (NHP) (Meyer, 1970; Verma and Tuteja, 2016). Studies in the Soviet Union had also shown that vaccinating baboons by the inhalation route with a live pgm− *Y. pestis* EV strain generated immunity, but s.c. injection of F1 still provided better protection against pneumonic plague (Anisimov *et al.*, 1995; Byvalov *et al.*, 1984). Pgm− *Y. pestis* strains were often more immunogenic than killed vaccines, but like EV76, frequently caused local and systemic reactions in animals and humans (Hallett *et al.*, 1973; Meyer, Smith *et al.*, 1974; Meyer, Cavanaugh *et al.*, 1974). Reactogenicity also varied with the animal and the route of inoculation, and several species of NHP were significantly more sensitive to pgm− *Y. pestis*

than guinea pigs (Meyer *et al.*, 1974). The *Y. pestis* CO92pgm− strain was shown to be virulent to mice and African green monkeys by aerosol route (Welkos *et al.*, 2002). Thus, the safety of such strains was deemed host-specific and was not recommended for use as a human vaccine.

The virulence of live strains needed to be reduced further. Efforts focused on "switching out" various immuno-suppressive elements of the bacterium. For instance, the replacement of the *Y. pestis* atypical tetra-acylated lipid A with the *E. coli* hexa-acylated lipid A resulted in an immunogenic, highly attenuated live vaccine construct, but still induced IL-10 (Sun, W. *et al.*, 2011). Another advantage of live vaccines are the contributions of T cell immunity for protection. Vaccination with live, replicating *Y. pestis* strains is one way to prime $CD4^+$ and $CD8^+$ T cells to respond to antigens distinct from those previously defined as targets for humoral immunity (e.g. F1 and V) (Smiley, 2008a; Sun *et al.*, 2011). Mice that were i.n. immunized with live *Y. pestis* KIM5 and KIM6 strains, indeed, generated T cells that could respond *in vitro* to a *Y. pestis* strain lacking F1, V, and all pCD1/pPCP-encoded proteins; however, 60% of mice receiving *Y. pestis* KIM5 as the initial vaccine administration failed to survive (Philipovskiy and Smiley, 2007). Concern about reversion to virulence continued to diminish enthusiasm for genetically manipulated (attenuated) virulent strains as vaccines. KWC vaccines did not generate timely immunity, while live vaccine strains posed too high of a risk. Researchers needed to strike a balance: a safe vaccine that also generated the precise "amount" of innate and adaptive immunity needed to eliminate *Y. pestis* successfully.

Because of the limitations of KWC and live attenuated vaccines, subunit vaccines were pursued against plague. Both F1 and V antigens were found to confer high levels of

protection, and thus success in experimental settings renewed interest in subunit formulations. Clinical trials of F1 and V subunit vaccines began around a decade ago (Quenee and Schneewind, 2009). Soluble F1 and V antigens make up the active components of RypVax™ (PharmAthene Inc.), a recombinant plague vaccine comprising separate rF1 and rV antigens produced in *E. coli*. Building on RypVax™ clinical data, the rF1-V fusion protein vaccine was developed by the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID) and its use is anticipated in 2021 by the U.S. Army (Demeure *et al.*, 2019). The rF1-V and RypVax™ vaccines are safe and have passed through Phase I and II clinical trials, but the results of the Phase II trials are not yet available. To abate V antigen's immuno-suppressive actions, a truncated version (recombinant V10) was also created; this was capable of maintaining protection against pneumonic plague infection in mice, guinea pigs, and macaques as well as the rF1-V vaccine (Cornelius *et al.*, 2008; DeBord *et al.*, 2006). However, both the rF1-V fusion and rV10 vaccines failed to protect African green monkeys against pneumonic plague as uniformly as Cynomolgus macaques, despite eliciting robust antibody responses (Pitt, 2004; Quenee *et al.*, 2011; Smiley, 2008). The inconsistent efficacy of these vaccines is speculated to be due to a deficiency in innate or cellular immunity, resulting in a lack of synergy between humoral and cell-mediated immune responses (Sun, W. and Singh, 2019). This synergistic effect is considered the optimal defense against pneumonic plague, since antibodies alone are not a reliable correlate of protection (Kummer *et al.*, 2008). African green monkeys (*Cercopitheus aethiops*) are evolutionarily closer to humans than Asian macaques (Ervin and Palmour, 2002). Thus, there is reasonable concern that the F1-V vaccines undergoing clinical trials may not protect

humans against pneumonic plague. Several groups are trying to enhance immunogenicity of subunit vaccines using different means.

Defining immune correlates of plague protection has proved challenging for over a century. On one hand, the antibody evidence was abundant. Yersin himself had showed that horse antiserum to plague could be used to treat plague-infected patients (Yersin, 1897), demonstrating that antibodies were highly protective. Similarly, sera from F1-immunized human volunteers can passively protect mice against bubonic plague (Meyer and Foster, 1948; Williamson *et al.*, 2005) as can anti-F1 monoclonal antibodies for bubonic and pneumonic plague (Anderson *et al.*, 1997). Immunization with V alone (Leary *et al.*, 1995) or in combination with F1 mainly works through robust production of antigen-specific IgG (Rocke *et al.*, 2008; Williamson *et al.*, 1999). Antibodies became consistently relied upon as correlates of vaccine-induced protection, but several studies soon cautioned against this. While the combination of F1 and V showed promise, any sole F1-based subunit vaccine would be ineffective against F1-negative *Y. pestis* strains (Feodorova and Corbel, 2009; Quenee *et al.*, 2008; Verma and Tuteja, 2016). Additionally, serological studies suggested that rV-based vaccines may fail to protect against *Y. pestis* strains expressing functional V variants (Anisimov *et al.*, 2004; Anisimov *et al.*, 2010; Roggenkamp *et al.*, 1997). Furthermore, it was found that only anti-V antibodies that promoted phagocytosis directly could neutralize pneumonic plague; simply blocking the T3SS tip was not enough (Eisele and Anderson, 2009). Complicating matters further, only a single V epitope generated antibodies capable of antibody-dependent cellular phagocytosis (ADCP) (Eisele and Anderson, 2009). It is now generally accepted that levels of anti-F1 and anti-V IgG titers are not a true correlate of protection. Rather, rapid antibody-mediated clearance of *Y. pestis*

during respiratory infection is dependent on the activation of phagocytes (macrophages and neutrophils), which can be assessed *in vitro* (Bashaw *et al.*, 2007). This lends further support to the notion that a coordinated effort between innate, humoral, and cellular immunity is needed to defend against pneumonic plague (Smiley, 2008b).

IFN-γ, TNF-α, nitric oxide synthase 2, and IL-17 have all been implicated in systemic host responses to pneumonic plague (Lin *et al.*, 2011; Nakajima and Brubaker, 1993; Parent *et al.*, 2005; Parent *et al.*, 2006; Szaba *et al.*, 2014), and play important roles in antibody-mediated protection generated by the rF1-V fusion protein vaccine (Lin *et al.*, 2010). Smiley and colleagues were the first to demonstrate that vaccination with live attenuated *Y. pestis* protected B-cell-deficient μMT mice against pneumonic infection in a T-cell dependent manner (Parent *et al.*, 2005; Parent *et al.*, 2006). Moreover, T cells from vaccinated μMT mice could be primed *in vitro* and adoptively transferred to naïve mice, conferring protection against lethal pneumonic plague (Parent *et al.*, 2005). Smiley's discoveries led subsequent research to pursue plague subunit vaccines that could potentially "balance" cellular and humoral immune responses. Adjuvants promoting Th1 cellular responses were explored. A recent study investigated the addition of SA-4-1BBL, an agonist of the CD137 costimulatory pathway, to the Alhydrogel® -adjuvanted rF1-V vaccine. Alhydrogel® is a common aluminum hydroxide-based adjuvant that tends to initiate T helper 2 (Th2)-like humoral responses (Brewer *et al.*, 1999). Interestingly, addition of SA-4-1BBL did not improve protection. Mice that received s.c. prime-boost with the added SA-4-1BBL were not as protected against pneumonic lethal infection compared with a prime-boost of just rF1-V + Alhydrogel® (75% vs. 90% survival, respectively), despite a robust antigen-specific IFN-γ response observed in SA-4-1BBL-administered mice (Bowen *et al.*, 2019). Generating

protective cell-mediated responses against plague using subunit vaccines has not been easy to accomplish (Smiley, 2008). Importantly, this study implicates there may be unknown or atypical factors that are critical to protection against pneumonic plague, and simply skewing the immune response one way or the other (e.g. Th1, Th2, Th17) through various adjuvants is not sufficient.

For mucosal (oral and nasal) plague vaccines, live attenuated vectors were an ideal choice as they can overcome the tolerogenic immune environment of the mucosa by mimicking a real infection (Neutra and Kozlowski, 2006; Wang *et al.*, 2015). Mucosal vaccines also have the added benefit of stimulating neutralizing secretory IgA (SIgA) responses, as well as the advantage of being easier and safer to administer than needle-based delivery (Garmory, Leary *et al.*, 2003; Levine, 2003). The first attempts to express the *caf* operon in a live attenuated *Salmonella enterica* serovar *Typhimurium* led to suboptimal expression and immunity (Oyston *et al.*, 1995; Titball *et al.*, 1997), and for V antigen, multiple doses were required for protection (Garmory, Griffin *et al.*, 2003). However, a *S. Typhimurium* vector containing two separate plasmids, one for each antigen, demonstrated improved and stable expression of F1 and V; orally immunized mice showed 88% protection against 1000 LD50 bubonic and 100 LD50 pneumonic plague challenges (Yang, X. *et al.*, 2007). Intranasal vaccination proved to be even more effective. Mice that were i.n. immunized twice with a F1- and V-expressing S. *Typhimurium* strain, and boosted once with V + alum, showed 100% protection against 37 LD50 and 177 LD50 pneumonic challenges (Galen *et al.*, 2015). Viral vectors were also explored. Ashok Chopra's group at the University of Texas Medical Branch successfully generated a replication-defective adenovirus (Ad5) vector expressing a fusion gene *YFV* (*ycsF*, *caf1,* and *lcrV*) (Sha *et al.*,

2016). Immunization with *Y. pestis* YcsF (Yop secretion protein F) has been previously shown to be protective against bubonic plague (Matson et al 2005). A mixed i.n.-i.m. prime-boost of mice and Cynomolgus macaques with the trivalent rAd5-YFV vaccine conferred 100% protection against aerosolized, fully virulent *Y. pestis* CO92pgm+, despite these animals having pre-existing adenoviral immunity (Sha *et al.*, 2016). Combinations of vaccines using this new heterologous-route prime-boost strategy might overcome current limitations of pneumonic plague vaccines. The most promising candidates appear to consist of a primary i.n. multivalent or live vectored vaccine dose, followed by an i.m. booster dose of the purified antigen(s) (Sun and Singh, 2019). However, live vectored vaccines are not safe for everyone and can cause opportunistic infections in certain individuals (e.g. in the immunocompromised and elderly). Even for replication-defective vectors, safety needs to be evaluated meticulously in preclinical and clinical studies. For this reason, subunit vaccines still remain the safest and most attractive approach for largescale vaccine production and stockpile.

In murine models, i.n. delivery of subunit plague vaccines has been explored using varying and creative formulations, including Protollin™ (Jones *et al.*, 2006), flagellin (Honko *et al.*, 2006; Mizel *et al.*, 2009), heat-labile enterotoxin (Glynn *et al.*, 2005), microspheres (Eyles *et al.*, 2000; Gupta *et al.*, 2010), lipid A mimetics (Airhart *et al.*, 2008), and more recently, double mutant heat-labile toxin (dmLT) (Heine *et al.*, 2019) and commensal gut bacteria-derived OMV (Carvalho *et al.*, 2019). These vaccines usually displayed good safety profiles and often produced higher levels of protection at mucosal surfaces compared to their parenteral equivalents. However, each of these formulations has been met with challenges, particularly, the need to administer booster doses to achieve

protection. Remarkably, Airhart *et al.* demonstrated 100% protection against 100 LD50 *Y. pestis* CO92pgm+ using a single dose i.n. synthetic lipid A adjuvanted F1 and V vaccine; however, this protection was not achieved until 150 days after immunization (Airhart *et al.*, 2008). They were able to achieve 100% protection in as early as 11 days post-immunization, but only after a prime-boost schedule. As of present, a single-dose i.n. F1 and V-based subunit vaccine has yet to achieve early complete protection against pneumonic plague.

Hypothesis and Specific Aims

The major goal of this work was to understand the mechanism(s) by which the TMV-F1 and TMV-V conjugates enhanced vaccine-specific immunity to a mouse model of plague. My overall hypothesis stated that: inactivated and rTMV was enhancing the immune response to the associated subunit proteins through direct uptake of the virus by APCs and may induce responses analogous to VLP vaccines. Like VLP, the resulting high-density display of protein antigens on the rTMV virion is more immunogenic compared to their soluble form, and no adjuvants are needed to boost immunity in our model. When administered i.n., this particulate vaccine has greater potential to stimulate B cells or DCs at mucosal surfaces and elicit a potent antigen-specific response to the bound proteins in the form of neutralizing antibodies and T-cell mediated responses (**Fig 4**).

To answer this central question, my research was divided into three overarching aims: **(1)** the innate response to TMV conjugates, **(2)** the resulting adaptive response from TMV conjugate immunization, and **(3)** the application of the conjugates to a single dose administration and its effects on bacterial dissemination and inflammation. In two of my three aims, TMV conjugates had to be studied in the context of infection, and so, challenge

experiments utilized BSL-2 *Y. pestis* strains with the chromosomal *pgm* locus deleted (CO92pgm−) or mutated (KIM5*caf1A*). Each aim below lists various hypotheses (sub-aims) separately or jointly investigated to address specific experimental questions. Each aim represents its own chapter in this dissertation, and follows the traditional scientific paper format (Introduction, Methods, Results, Discussion).

SPECIFIC AIM 1: Determine if TMV Conjugates Target Conjugated Antigens to Antigen Presenting Cells *in vitro* and *in vivo*

Aim 1a: Test the hypothesis that TMV promotes uptake into antigen-presenting cells in vitro (BMDCs) and ex vivo (B-cells)

Aim 1b: Test the hypothesis that TMV is an immunostimulant by upregulating costimulatory surface markers on dendritic cells in vitro

Aim 1c: Test the hypothesis that TMV targets conjugated protein antigens (F1 and V) to pulmonary dendritic cells in vivo after intranasal immunization

SPECIFIC AIM 2: Evaluating Efficacy and Correlates of Protection of a Single-Dose Intranasal TMV Conjugate Vaccine for Pneumonic Plague

Aim 2a: Test the hypothesis that a single intranasal administration of the vaccine is protective against high-dose pneumonic plague infection

Aim 2b: Test the hypothesis that single administration of the vaccine prevents or reduces dissemination of *Y. pestis* in the infected host

Aim 2c: Test the hypothesis that pre-challenge vaccine-specific antibody titers are a correlate of TMV vaccine-induced protection

Aim 2d: Test the hypothesis that the TMV vaccine induces a T_H1 or T_H17 specific response in the infected host

SPECIFIC AIM 3: Identify the Role of T Cell-Mediated and Antibody-Mediated Immune Responses of TMV Conjugates as an Intranasal VLP-like Vaccine

Aim 3a: Test the hypothesis that $CD4^+$ T cells and/or $CD8^+$ T cells are required for vaccine-mediated protection at the time of infection

Aim 3b: Test the hypothesis that intranasal vaccination induces both virus-specific and antigen-specific antibody responses

Aim 3c: Test the hypothesis that the antibody response to vaccination is rapid and localized to the lung after infection

Aim 3d: Test the hypothesis that vaccine-specific immunity is not limited to F1 immunity

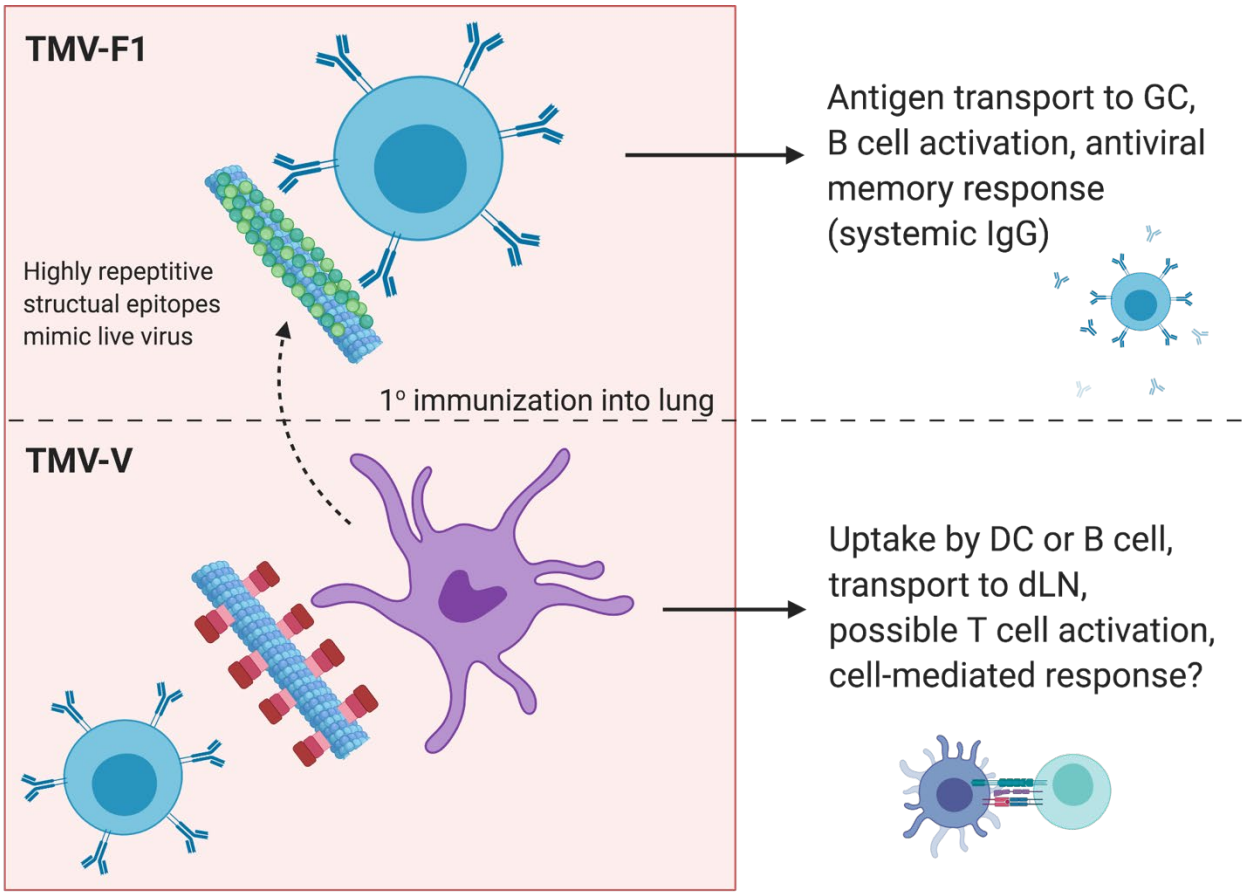

Fig 4. Potential APCs involved in the primary uptake of i.n.-administered TMV-F1 and TMV-V and the logical subsequent adaptive immune response. This image summarizes the general hypothesis**,** which is centered on the necessary uptake of TMV conjugate vaccines by APCs at the mucosal surface. Based on how the antigen is introduced (i.n. droplets), the anatomy of the mouse lymphatic system, and the data generated previously in our laboratory, the particulates are likely being transported by low-affinity B cells or DCs to a SLO, where naïve antibody production is stimulated in GC reactions, and where naïve T cells can be primed and activated. Since the F1 capsule is similar in size to TMV CP (17-20kDa), its display on the virion more closely resembles the surface structure of TMV and could explain why F1-specific IgG is almost always produced after single vaccination, while V-specific IgG is not. High V-specific IgG titers are generated, but only after multiple boosts of TMV-V. Image created on BioRender.com.

References

Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, Vogler AJ, Wagner DM, Allender CJ, Easterday WR, Chenal-Francisque V, Worsham P, Thomson NR, Parkhill J, Lindler LE, Carniel E, Keim P (2004). Microevolution and history of the plague bacillus, yersinia pestis. *Proceedings of the National Academy of Sciences of the United States of America.* 101: 17837-17842.

Airhart CL, Rohde HN, Hovde CJ, Bohach GA, Deobald CF, Lee SS, Minnich SA (2008). Lipid A mimetics are potent adjuvants for an intranasal pneumonic plague vaccine. *Vaccine.* 26: 5554-5561.

Amemiya K, Meyers JL, Rogers TE, Fast RL, Bassett AD, Worsham PL, Powell BS, Norris SL, Krieg AM, Adamovicz JJ (2009). CpG oligodeoxynucleotides augment the murine immune response to the yersinia pestis F1-V vaccine in bubonic and pneumonic models of plague. *Vaccine.* 27: 2220-2229.

Anderson GW, Worsham PL, Bolt CR, Andrews GP, Welkos SL, Friedlander AM, Burans JP (1997). Protection of mice from fatal bubonic and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of yersinia pestis. *The American Journal of Tropical Medicine and Hygiene.* 56: 471-473.

Andrades Valtuena A, Mittnik A, Key FM, Haak W, Allmae R, Belinskij A, Daubaras M, Feldman M, Jankauskas R, Jankovic I, Massy K, Novak M, Pfrengle S, Reinhold S, Slaus M, Spyrou MA, Szecsenyi-Nagy A, Torv M, Hansen S, Bos KI, Stockhammer PW, Herbig A, Krause J (2017). The stone age plague and its persistence in eurasia. *Current Biology : CB.* 27: 3683-3691.e8.

Andrews GP, Heath DG, Anderson GW, Welkos SL, Friedlander AM (1996). Fraction 1 capsular antigen (F1) purification from yersinia pestis CO92 and from an escherichia coli recombinant strain and efficacy against lethal plague challenge. *Infection and Immunity.* 64: 2180-2187.

Andrianaivoarimanana V, Piola P, Wagner DM, Rakotomanana F, Maheriniaina V, Andrianalimanana S, Chanteau S, Rahalison L, Ratsitorahina M, Rajerison M (2019). Trends of human plague, madagascar, 1998-2016. *Emerging Infectious Diseases.* 25: 220-228.

Anisimov AP, Dentovskaya SV, Panfertsev EA, Svetoch TE, Kopylov PK, Segelke BW, Zemla A, Telepnev MV, Motin VL (2010). Amino acid and structural variability of yersinia pestis LcrV protein. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases.* 10: 137-145.

Anisimov AP, Lindler LE, Pier GB (2004). Intraspecific diversity of yersinia pestis. *Clinical Microbiology Reviews.* 17: 434-464.

Anisimov AP, Nikiforov AK, Eremin SA, Drozdov IG (1995). Design of a yersinia pestis strain with increased protection. [Konstruirovanie shtamma Yersinia pestis s povyshennoi protektivnost'iu] *Biulleten' Eksperimental'Noi Biologii i Meditsiny.* 120: 532-534.

Arnaboldi PM, Sambir M, D'Arco C, Peters LA, Seegers JF, Mayer L, McCormick AA, Dattwyler RJ (2016). Intranasal delivery of a protein subunit vaccine using a tobacco mosaic virus platform protects against pneumonic plague. *Vaccine.* 34: 5768-5776.

Ashkin A and Dziedzic JM (1987). Optical trapping and manipulation of viruses and bacteria. *Science (New York, N.Y.).* 235: 1517-1520.

Bachmann MF and Jennings GT (2010). Vaccine delivery: A matter of size, geometry, kinetics and molecular patterns. *Nature Reviews.Immunology.* 10: 787-796.

Balique F, Colson P, Raoult D (2012). Tobacco mosaic virus in cigarettes and saliva of smokers. *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology.* 55: 374-376.

Balique F, Lecoq H, Raoult D, Colson P (2015). Can plant viruses cross the kingdom border and be pathogenic to humans? *Viruses.* 7: 2074-2098.

Banik S, Mansour AA, Suresh RV, Wykoff-Clary S, Malik M, McCormick AA, Bakshi CS (2015). Development of a multivalent subunit vaccine against tularemia using tobacco mosaic virus (TMV) based delivery system. *PloS One.* 10: e0130858.

Bao R, Nair MK, Tang WK, Esser L, Sadhukhan A, Holland RL, Xia D, Schifferli DM (2013). Structural basis for the specific recognition of dual receptors by the homopolymeric pH 6 antigen (psa) fimbriae of yersinia pestis. *Proceedings of the National Academy of Sciences of the United States of America.* 110: 1065-1070.

Bashaw J, Norris S, Weeks S, Trevino S, Adamovicz JJ, Welkos S (2007). Development of in vitro correlate assays of immunity to infection with yersinia pestis. *Clinical and Vaccine Immunology : CVI.* 14: 605-616.

Beijerinck MW (1898). *Concerning a contagium vivum fluidum as cause of the spot disease of tobacco leaves.* (Phylopathological Classics No. 7). St. Paul: American Phytopathological Society.

Bibel DJ and Chen TH (1976). Diagnosis of plaque: An analysis of the yersin-kitasato controversy. *Bacteriological Reviews.* 40: 633-651.

Bobrov AG, Kirillina O, Fosso MY, Fetherston JD, Miller MC, VanCleave TT, Burlison JA, Arnold WK, Lawrenz MB, Garneau-Tsodikova S, Perry RD (2017). Zinc transporters YbtX and ZnuABC are required for the virulence of yersinia pestis in bubonic and pneumonic plague in mice. *Metallomics : Integrated Biometal Science.* 9: 757-772.

Boone A, Kraft JP, Stapp P (2009). Scavenging by mammalian carnivores on prairie dog colonies: Implications for the spread of plague. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.).* 9: 185-190.

Bowen W, Batra L, Pulsifer AR, Yolcu ES, Lawrenz MB, Shirwan H (2019). Robust Th1 cellular and humoral responses generated by the yersinia pestis rF1-V subunit vaccine formulated to contain an agonist of the CD137 pathway do not translate into increased protection against pneumonic plague. *Vaccine.* 37: 5708-5716.

Bramwell VW, Eyles JE, Oya Alpar H (2005). Particulate delivery systems for biodefense subunit vaccines. *Advanced Drug Delivery Reviews.* 57: 1247-1265.

Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J (1999). Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *Journal of Immunology (Baltimore, Md.: 1950).* 163: 6448-6454.

Buchrieser C, Prentice M, Carniel E (1998). The 102-kilobase unstable region of yersinia pestis comprises a high-pathogenicity island linked to a pigmentation segment which undergoes internal rearrangement. *Journal of Bacteriology.* 180: 2321-2329.

Butler T (2014). Plague history: Yersin's discovery of the causative bacterium in 1894 enabled, in the subsequent century, scientific progress in understanding the disease and the development of treatments and vaccines. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases.* 20: 202-209.

Byvalov AA, Pautov VN, Chicherin I, Lebedinskii VA, Evtigneev VI (1984). Effectiveness of revaccinating hamadryas baboons with NISS live dried plague vaccine and fraction I of the plague microbe. [Effektivnost' revaktsinatsii pavianov gamadrilov chumnoi zhivoi sukhoi vaktsinoi NIIS i fraktsii I chumnogo mikroba] *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii.* (4): 74-76.

Canizares MC, Nicholson L, Lomonossoff GP (2005). Use of viral vectors for vaccine production in plants. *Immunology and Cell Biology.* 83: 263-270.

Carus WS (2015). The history of biological weapons use: What we know and what we don't. *Health Security.* 13: 219-255.

Carvalho AL, Miquel-Clopes A, Wegmann U, Jones E, Stentz R, Telatin A, Walker NJ, Butcher WA, Brown PJ, Holmes S, Dennis MJ, Williamson ED, Funnell SGP, Stock M, Carding SR (2019). Use of bioengineered human commensal gut bacteria-derived microvesicles for mucosal plague vaccine delivery and immunization. *Clinical and Experimental Immunology.* 196: 287-304.

CDC (2019). Plague. Retrieved from https://www.cdc.gov/plague/index.html

Chain PS, Carniel E, Larimer FW, Lamerdin J, Stoutland PO, Regala WM, Georgescu AM, Vergez LM, Land ML, Motin VL, Brubaker RR, Fowler J, Hinnebusch J, Marceau M, Medigue C, Simonet M, Chenal-Francisque V, Souza B, Dacheux D, Elliott JM, Derbise A, Hauser LJ, Garcia E (2004). Insights into the evolution of yersinia pestis through whole-genome comparison with yersinia pseudotuberculosis. *Proceedings of the National Academy of Sciences of the United States of America.* 101: 13826-13831.

Chen Q and Lai H (2013). Plant-derived virus-like particles as vaccines. *Human Vaccines & Immunotherapeutics.* 9: 26-49.

Chouikha I and Hinnebusch BJ (2014). Silencing urease: A key evolutionary step that facilitated the adaptation of yersinia pestis to the flea-borne transmission route. *Proceedings of the National Academy of Sciences of the United States of America.* 111: 18709-18714.

Connor MG, Pulsifer AR, Chung D, Rouchka EC, Ceresa BK, Lawrenz MB (2018). Yersinia pestis targets the host endosome recycling pathway during the biogenesis of the yersinia-containing vacuole to avoid killing by macrophages. *mBio.* 9: 10.1128/mBio.01800-17.

Connor MG, Pulsifer AR, Price CT, Abu Kwaik Y, Lawrenz MB (2015). Yersinia pestis requires host Rab1b for survival in macrophages. *PLoS Pathogens.* 11: e1005241.

Cornelis GR (1998). The yersinia deadly kiss. *Journal of Bacteriology.* 180: 5495-5504.

Cornelis GR (2000). Molecular and cell biology aspects of plague. *Proceedings of the National Academy of Sciences of the United States of America.* 97: 8778-8783.

Cornelius CA, Quenee LE, Overheim KA, Koster F, Brasel TL, Elli D, Ciletti NA, Schneewind O (2008). Immunization with recombinant V10 protects cynomolgus macaques from lethal pneumonic plague. *Infection and Immunity.* 76: 5588-5597.

Davis KJ, Fritz DL, Pitt ML, Welkos SL, Worsham PL, Friedlander AM (1996). Pathology of experimental pneumonic plague produced by fraction 1-positive and fraction 1-negative yersinia pestis in african green monkeys (cercopithecus aethiops). *Archives of Pathology & Laboratory Medicine.* 120: 156-163.

DeBord KL, Anderson DM, Marketon MM, Overheim KA, DePaolo RW, Ciletti NA, Jabri B, Schneewind O (2006). Immunogenicity and protective immunity against bubonic plague and pneumonic plague by immunization of mice with the recombinant V10 antigen, a variant of LcrV. *Infection and Immunity.* 74: 4910-4914.

Demeure CE, Derbise A, Carniel E (2017). Oral vaccination against plague using yersinia pseudotuberculosis. *Chemico-Biological Interactions.* 267: 89-95.

Demeure CE, Dussurget O, Mas Fiol G, Le Guern AS, Savin C, Pizarro-Cerda J (2019). Yersinia pestis and plague: An updated view on evolution, virulence determinants, immune subversion, vaccination, and diagnostics. *Genes and Immunity.* 20: 357-370.

Derewenda U, Mateja A, Devedjiev Y, Routzahn KM, Evdokimov AG, Derewenda ZS, Waugh DS (2004). The structure of yersinia pestis V-antigen, an essential virulence factor and mediator of immunity against plague. *Structure (London, England : 1993).* 12: 301-306.

Donaires LF, Cespedes M, Valencia P, Salas JC, Luna ME, Castaneda A, Peralta V, Cabezas C, Pachas PE (2010). Primary pneumonic plague with nosocomial transmission in la libertad, peru 2010. [Peste neumonica primaria con transmision intrahospitalaria en La Libertad, Peru 2010] *Revista Peruana De Medicina Experimental Y Salud Publica.* 27: 326-336.

Du Y, Rosqvist R, Forsberg A (2002). Role of fraction 1 antigen of yersinia pestis in inhibition of phagocytosis. *Infection and Immunity.* 70: 1453-1460.

Eddy JL, Gielda LM, Caulfield AJ, Rangel SM, Lathem WW (2014). Production of outer membrane vesicles by the plague pathogen yersinia pestis. *PloS One.* 9: e107002.

Eisele NA and Anderson DM (2009). Dual-function antibodies to yersinia pestis LcrV required for pulmonary clearance of plague. *Clinical and Vaccine Immunology : CVI.* 16: 1720-1727.

Eitzen EM, Jr and Takafuji ET (1997). Historical overview of biological warfare. In F. R. Sidell, E. T. Takafuji & D. R. Franz (Eds), *Medical aspects of chemical and biological warfare* (1st ed., pp. 415-423). Washington, DC: Office of the Surgeon General, Borden Institute, Walter Reed Army Medical Center.

Ervin F and Palmour R (2002). *Primates for 21st century biomedicine: The st. kitts vervet (chlorocebus aethiops, SK).* (Report No. 1). Washington, DC: National Academies Press. . (International Perspectives: The Future of Nonhuman Primate Resources)

Eyles JE, Williamson ED, Spiers ID, Stagg AJ, Jones SM, Alpar HO (2000). Generation of protective immune responses to plague by mucosal administration of microsphere coencapsulated recombinant subunits. *Journal of Controlled Release : Official Journal of the Controlled Release Society.* 63: 191-200.

Feodorova VA and Corbel MJ (2009). Prospects for new plague vaccines. *Expert Review of Vaccines.* 8: 1721-1738.

Feodorova VA and Motin VL (2012). Plague vaccines: Current developments and future perspectives. *Emerging Microbes & Infections.* 1: e36.

Fetherston JD, Kirillina O, Bobrov AG, Paulley JT, Perry RD (2010). The yersiniabactin transport system is critical for the pathogenesis of bubonic and pneumonic plague. *Infection and Immunity.* 78: 2045-2052.

Fetherston JD and Perry RD (1994). The pigmentation locus of yersinia pestis KIM6+ is flanked by an insertion sequence and includes the structural genes for pesticin sensitivity and HMWP2. *Molecular Microbiology.* 13: 697-708.

Fields KA, Nilles ML, Cowan C, Straley SC (1999). Virulence role of V antigen of yersinia pestis at the bacterial surface. *Infection and Immunity.* 67: 5395-5408.

Fields KA and Straley SC (1999). LcrV of yersinia pestis enters infected eukaryotic cells by a virulence plasmid-independent mechanism. *Infection and Immunity.* 67: 4801-4813.

Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, McKenzie IF, Plebanski M (2004). Size-dependent immunogenicity: Therapeutic and protective properties of nano-vaccines against tumors. *Journal of Immunology (Baltimore, Md.: 1950).* 173: 3148-3154.

Fitchen J, Beachy RN, Hein MB (1995). Plant virus expressing hybrid coat protein with added murine epitope elicits autoantibody response. *Vaccine.* 13: 1051-1057.

Foged C, Sundblad A, Hovgaard L (2002). Targeting vaccines to dendritic cells. *Pharmaceutical Research.* 19: 229-238.

Franz DR, Jahrling PB, Friedlander AM, McClain DJ, Hoover DL, Bryne WR, Pavlin JA, Christopher GW, Eitzen EM (1997). Clinical recognition and management of patients exposed to biological warfare agents. *Jama.* 278: 399-411.

Friedlander AM, Welkos SL, Worsham PL, Andrews GP, Heath DG, Anderson GW, Pitt ML, Estep J, Davis K (1995). Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of yersinia pestis. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America.* 21 Suppl 2: 178.

Galen JE, Wang JY, Carrasco JA, Lloyd SA, Mellado-Sanchez G, Diaz-McNair J, Franco O, Buskirk AD, Nataro JP, Pasetti MF (2015). A bivalent typhoid live vector vaccine expressing both chromosome- and plasmid-encoded yersinia pestis antigens fully protects against murine lethal pulmonary plague infection. *Infection and Immunity.* 83: 161-172.

Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E, Courvalin P (1997). Multidrug resistance in yersinia pestis mediated by a transferable plasmid. *The New England Journal of Medicine.* 337: 677-680.

Galyov EE, Karlishev AV, Chernovskaya TV, Dolgikh DA, Smirnov OY, Volkovoy KI, Abramov VM, Zav'yalov VP (1991). Expression of the envelope antigen F1 of yersinia

pestis is mediated by the product of caf1M gene having homology with the chaperone protein PapD of escherichia coli. *FEBS Letters.* 286: 79-82.

Gamvrellis A, Leong D, Hanley JC, Xiang SD, Mottram P, Plebanski M (2004). Vaccines that facilitate antigen entry into dendritic cells. *Immunology and Cell Biology.* 82: 506-516.

Garmory HS, Griffin KF, Brown KA, Titball RW (2003). Oral immunisation with live aroA attenuated salmonella enterica serovar typhimurium expressing the yersinia pestis V antigen protects mice against plague. *Vaccine.* 21: 3051-3057.

Garmory HS, Leary SE, Griffin KF, Williamson ED, Brown KA, Titball RW (2003). The use of live attenuated bacteria as a delivery system for heterologous antigens. *Journal of Drug Targeting.* 11: 471-479.

Glynn A, Roy CJ, Powell BS, Adamovicz JJ, Freytag LC, Clements JD (2005). Protection against aerosolized yersinia pestis challenge following homologous and heterologous prime-boost with recombinant plague antigens. *Infection and Immunity.* 73: 5256-5261.

Goelet P, Lomonossoff GP, Butler PJ, Akam ME, Gait MJ, Karn J (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proceedings of the National Academy of Sciences of the United States of America.* 79: 5818-5822.

Goguen JD, Yother J, Straley SC (1984). Genetic analysis of the low calcium response in yersinia pestis mu d1(ap lac) insertion mutants. *Journal of Bacteriology.* 160: 842-848.

Gross L (1995). How the plague bacillus and its transmission through fleas were discovered: Reminiscences from my years at the pasteur institute in paris. *Proceedings of the National Academy of Sciences of the United States of America.* 92: 7609-7611.

Guiyoule A, Gerbaud G, Buchrieser C, Galimand M, Rahalison L, Chanteau S, Courvalin P, Carniel E (2001). Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of yersinia pestis. *Emerging Infectious Diseases.* 7: 43-48.

Gupta G, Khan AA, Rao DN (2010). Cell-mediated immune response and th/th cytokine profile of B-T constructs of F1 and V antigen of yersinia pestis. *Scandinavian Journal of Immunology.* 71: 186-198.

Hallett AF, Isaacson M, Meyer KF (1973). Pathogenicity and immunogenic efficacy of a live attentuated plaque vaccine in vervet monkeys. *Infection and Immunity.* 8: 876-881.

Harbeck M, Seifert L, Hansch S, Wagner DM, Birdsell D, Parise KL, Wiechmann I, Grupe G, Thomas A, Keim P, Zoller L, Bramanti B, Riehm JM, Scholz HC (2013). Yersinia pestis DNA from skeletal remains from the 6(th) century AD reveals insights into justinianic plague. *PLoS Pathogens.* 9: e1003349.

Hawgood BJ (2008). Alexandre yersin (1863-1943): Discoverer of the plague bacillus, explorer and agronomist. *Journal of Medical Biography.* 16: 167-172.

Heath DG, Anderson GW, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, Friedlander AM (1998). Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. *Vaccine.* 16: 1131-1137.

Heine SJ, Franco-Mahecha OL, Sears KT, Drachenberg CB, van Roosmalen ML, Leenhouts K, Picking WL, Pasetti MF (2019). A combined YopB and LcrV subunit vaccine elicits protective immunity against yersinia infection in adult and infant mice. *Journal of Immunology (Baltimore, Md.: 1950).* 202: 2005-2016.

Holtzman T, Levy Y, Marcus D, Flashner Y, Mamroud E, Cohen S, Fass R (2006). Production and purification of high molecular weight oligomers of yersinia pestis F1 capsular antigen released by high cell density culture of recombinant escherichia coli cells carrying the caf1 operon. *Microbial Cell Factories.* 5: 98.

Holzem A, Nahring JM, Fischer R (2001). Rapid identification of a tobacco mosaic virus epitope by using a coat protein gene-fragment-pVIII fusion library. *The Journal of General Virology.* 82: 9-15.

Honko AN, Sriranganathan N, Lees CJ, Mizel SB (2006). Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with yersinia pestis. *Infection and Immunity.* 74: 1113-1120.

Huang XZ, Nikolich MP, Lindler LE (2006). Current trends in plague research: From genomics to virulence. *Clinical Medicine & Research.* 4: 189-199.

Hwang DJ, Roberts IM, Wilson TM (1994). Assembly of tobacco mosaic virus and TMV-like pseudovirus particles in escherichia coli. *Archives of Virology.Supplementum.* 9: 543-558.

Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Koerner JF, Layton M, McDade J, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Schoch-Spana M, Tonat K (2000). Plague as a biological weapon: Medical and public health management. working group on civilian biodefense. *Jama.* 283: 2281-2290.

Jones T, Adamovicz JJ, Cyr SL, Bolt CR, Bellerose N, Pitt LM, Lowell GH, Burt DS (2006). Intranasal protollin/F1-V vaccine elicits respiratory and serum antibody responses and protects mice against lethal aerosolized plague infection. *Vaccine.* 24: 1625-1632.

Kemnade JO, Seethammagari M, Collinson-Pautz M, Kaur H, Spencer DM, McCormick AA (2014). Tobacco mosaic virus efficiently targets DC uptake, activation and antigen-specific T cell responses in vivo. *Vaccine.* 32: 4228-4233.

King AMQ, Adams MJ, Carstens EB, Lefkowitz EJVirus taxonomy ninth report of the international committee on taxonomy of viruses. Paper presented at the *International Committee on Taxonomy of Viruses,* San Diego, CA. : 1327.

Klug A (1999). The tobacco mosaic virus particle: Structure and assembly. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences.* 354: 531-535.

Koirala J (2006). Plague: Disease, management, and recognition of act of terrorism. *Infectious Disease Clinics of North America.* 20: 273-87, viii.

Koo M, Bendahmane M, Lettieri GA, Paoletti AD, Lane TE, Fitchen JH, Buchmeier MJ, Beachy RN (1999). Protective immunity against murine hepatitis virus (MHV) induced by intranasal or subcutaneous administration of hybrids of tobacco mosaic virus that carries an MHV epitope. *Proceedings of the National Academy of Sciences of the United States of America.* 96: 7774-7779.

Kummer LW, Szaba FM, Parent MA, Adamovicz JJ, Hill J, Johnson LL, Smiley ST (2008). Antibodies and cytokines independently protect against pneumonic plague. *Vaccine.* 26: 6901-6907.

Lathem WW, Price PA, Miller VL, Goldman WE (2007). A plasminogen-activating protease specifically controls the development of primary pneumonic plague. *Science (New York, N.Y.).* 315: 509-513.

Leary SE, Williamson ED, Griffin KF, Russell P, Eley SM, Titball RW (1995). Active immunization with recombinant V antigen from yersinia pestis protects mice against plague. *Infection and Immunity.* 63: 2854-2858.

Lebeurier G and Hirth L (1966). Effect of elevated temperatures on the development of two strains of tobacco mosaic virus. *Virology.* 29: 385-395.

LeClair RA (1967). Recovery of culturable tobacco mosaic virus from sputum and thoracentesis fluids obtained from cigarette smokers with a history of pulmonary disease. *The American Review of Respiratory Disease.* 95: 510-511.

Levine MM (2003). Can needle-free administration of vaccines become the norm in global immunization? *Nature Medicine.* 9: 99-103.

Levy Y, Flashner Y, Tidhar A, Zauberman A, Aftalion M, Lazar S, Gur D, Shafferman A, Mamroud E (2011). T cells play an essential role in anti-F1 mediated rapid protection against bubonic plague. *Vaccine.* 29: 6866-6873.

Levy Y, Vagima Y, Tidhar A, Aftalion M, Gur D, Nili U, Chitlaru T, Zauberman A, Mamroud E (2018). Targeting of the yersinia pestis F1 capsular antigen by innate-like

B1b cells mediates a rapid protective response against bubonic plague. *NPJ Vaccines.* 3: 52-z. eCollection 2018.

Lin JS, Kummer LW, Szaba FM, Smiley ST (2011). IL-17 contributes to cell-mediated defense against pulmonary yersinia pestis infection. *Journal of Immunology (Baltimore, Md.: 1950).* 186: 1675-1684.

Lin JS, Park S, Adamovicz JJ, Hill J, Bliska JB, Cote CK, Perlin DS, Amemiya K, Smiley ST (2010). TNFalpha and IFNgamma contribute to F1/LcrV-targeted immune defense in mouse models of fully virulent pneumonic plague. *Vaccine.* 29: 357-362.

LINK VB (1955). A history of plague in united states of america. *Public Health Monograph.* 26: 1-120.

Liu R, Vaishnav RA, Roberts AM, Friedland RP (2013). Humans have antibodies against a plant virus: Evidence from tobacco mosaic virus. *PloS One.* 8: e60621.

Loor F (1967). Comparative immunogenicities of tobacco mosaic virus, protein subunits, and reaggregated protein subunits. *Virology.* 33: 215-220.

Mallajosyula JK, Hiatt E, Hume S, Johnson A, Jeevan T, Chikwamba R, Pogue GP, Bratcher B, Haydon H, Webby RJ, McCormick AA (2014). Single-dose monomeric HA subunit vaccine generates full protection from influenza challenge. *Human Vaccines & Immunotherapeutics.* 10: 586-595.

Mallajosyula JK, Jeevan T, Chikwamba R, Webby RJ, McCormick AA (2016). A single dose TMV-HA vaccine protects mice from H5N1 influenza challenge. *International Journal of Vaccine Research.* 1: 6.

Mandal B and Jain RK (2010). Can plant virus infect human being? *Indian Journal of Virology : An Official Organ of Indian Virological Society.* 21: 92-93.

Mansour AA, Banik S, Suresh RV, Kaur H, Malik M, McCormick AA, Bakshi CS (2018). An improved tobacco mosaic virus (TMV)-conjugated multiantigen subunit vaccine against respiratory tularemia. *Frontiers in Microbiology.* 9: 1195.

Marbrook J and Matthews RE (1966). The differential immunogenicity of plant viral protein and nucleoproteins. *Virology.* 28: 219-228.

McCormick AA, Corbo TA, Wykoff-Clary S, Nguyen LV, Smith ML, Palmer KE, Pogue GP (2006). TMV-peptide fusion vaccines induce cell-mediated immune responses and tumor protection in two murine models. *Vaccine.* 24: 6414-6423.

McCormick AA, Corbo TA, Wykoff-Clary S, Palmer KE, Pogue GP (2006). Chemical conjugate TMV-peptide bivalent fusion vaccines improve cellular immunity and tumor protection. *Bioconjugate Chemistry.* 17: 1330-1338.

McCormick AA and Palmer KE (2008). Genetically engineered tobacco mosaic virus as nanoparticle vaccines. *Expert Review of Vaccines.* 7: 33-41.

McCormick AA, Shakeel A, Yi C, Kaur H, Mansour AM, Bakshi CS (2018). Intranasal administration of a two-dose adjuvanted multi-antigen TMV-subunit conjugate vaccine fully protects mice against francisella tularensis LVS challenge. *PloS One.* 13: e0194614.

McNally A, Thomson NR, Reuter S, Wren BW (2016). 'Add, stir and reduce': Yersinia spp. as model bacteria for pathogen evolution. *Nature Reviews.Microbiology.* 14: 177-190.

Meyer KF (1970). *Effectiveness of live or killed plague vaccines in man.* (Bulletin No. 42).World Health Organization.

Meyer KF, Cavanaugh DC, Bartelloni PJ, Marshall JD (1974). Plague immunization. I. past and present trends. *The Journal of Infectious Diseases.* 129: Suppl:S13-8.

Meyer KF and Foster LE (1948). *Measurement of protective serum antibodies in human volunteers inoculated with plague prophylatics.* (Stanford Medical Bulletin No. 6). Stanford, CA: Stanford University.

Meyer KF, Smith G, Foster L, Brookman M, Sung M (1974). Live, attenuated yersinia pestis vaccine: Virulent in nonhuman primates, harmless to guinea pigs. *The Journal of Infectious Diseases.* 129: Suppl:S85-12.

Mizel SB, Graff AH, Sriranganathan N, Ervin S, Lees CJ, Lively MO, Hantgan RR, Thomas MJ, Wood J, Bell B (2009). Flagellin-F1-V fusion protein is an effective plague vaccine in mice and two species of nonhuman primates. *Clinical and Vaccine Immunology : CVI.* 16: 21-28.

Montminy SW, Khan N, McGrath S, Walkowicz MJ, Sharp F, Conlon JE, Fukase K, Kusumoto S, Sweet C, Miyake K, Akira S, Cotter RJ, Goguen JD, Lien E (2006). Virulence factors of yersinia pestis are overcome by a strong lipopolysaccharide response. *Nature Immunology.* 7: 1066-1073.

Nakajima R and Brubaker RR (1993). Association between virulence of yersinia pestis and suppression of gamma interferon and tumor necrosis factor alpha. *Infection and Immunity.* 61: 23-31.

Nakajima R, Motin VL, Brubaker RR (1995). Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization. *Infection and Immunity.* 63: 3021-3029.

Nedialkov YA, Motin VL, Brubaker RR (1997). Resistance to lipopolysaccharide mediated by the yersinia pestis V antigen-polyhistidine fusion peptide: Amplification of interleukin-10. *Infection and Immunity.* 65: 1196-1203.

Neutra MR and Kozlowski PA (2006). Mucosal vaccines: The promise and the challenge. *Nature Reviews.Immunology.* 6: 148-158.

Nguyen VK, Parra-Rojas C, Hernandez-Vargas EA (2018). The 2017 plague outbreak in madagascar: Data descriptions and epidemic modelling. *Epidemics.* 25: 20-25.

Okan NA, Mena P, Benach JL, Bliska JB, Karzai AW (2010). The smpB-ssrA mutant of yersinia pestis functions as a live attenuated vaccine to protect mice against pulmonary plague infection. *Infection and Immunity.* 78: 1284-1293.

Oyston PC, Williamson ED, Leary SE, Eley SM, Griffin KF, Titball RW (1995). Immunization with live recombinant salmonella typhimurium aroA producing F1 antigen protects against plague. *Infection and Immunity.* 63: 563-568.

Palmer KE, Benko A, Doucette SA, Cameron TI, Foster T, Hanley KM, McCormick AA, McCulloch M, Pogue GP, Smith ML, Christensen ND (2006). Protection of rabbits against cutaneous papillomavirus infection using recombinant tobacco mosaic virus containing L2 capsid epitopes. *Vaccine.* 24: 5516-5525.

Parent MA, Berggren KN, Kummer LW, Wilhelm LB, Szaba FM, Mullarky IK, Smiley ST (2005). Cell-mediated protection against pulmonary yersinia pestis infection. *Infection and Immunity.* 73: 7304-7310.

Parent MA, Wilhelm LB, Kummer LW, Szaba FM, Mullarky IK, Smiley ST (2006). Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary yersinia pestis infection. *Infection and Immunity.* 74: 3381-3386.

Pechous RD, Sivaraman V, Price PA, Stasulli NM, Goldman WE (2013). Early host cell targets of yersinia pestis during primary pneumonic plague. *PLoS Pathogens.* 9: e1003679.

Perry RD, Bobrov AG, Fetherston JD (2015). The role of transition metal transporters for iron, zinc, manganese, and copper in the pathogenesis of yersinia pestis. *Metallomics : Integrated Biometal Science.* 7: 965-978.

Perry RD and Fetherston JD (1997). Yersinia pestis--etiologic agent of plague. *Clinical Microbiology Reviews.* 10: 35-66.

Peters KN, Dhariwala MO, Hughes Hanks JM, Brown CR, Anderson DM (2013). Early apoptosis of macrophages modulated by injection of yersinia pestis YopK promotes progression of primary pneumonic plague. *PLoS Pathogens.* 9: e1003324.

Pettersson J, Holmstrom A, Hill J, Leary S, Frithz-Lindsten E, von Euler-Matell A, Carlsson E, Titball R, Forsberg A, Wolf-Watz H (1999). The V-antigen of yersinia is surface

exposed before target cell contact and involved in virulence protein translocation. *Molecular Microbiology.* 32: 961-976.

Philipovskiy AV and Smiley ST (2007). Vaccination with live yersinia pestis primes CD4 and CD8 T cells that synergistically protect against lethal pulmonary Y. pestis infection. *Infection and Immunity.* 75: 878-885.

PittNon-human primates as a model for pneumonic plague. Paper presented at the *Animals Models and Correlates of Protection for Plague Vaccines Workshop,*

Pujol C, Klein KA, Romanov GA, Palmer LE, Cirota C, Zhao Z, Bliska JB (2009). Yersinia pestis can reside in autophagosomes and avoid xenophagy in murine macrophages by preventing vacuole acidification. *Infection and Immunity.* 77: 2251-2261.

Quenee LE, Ciletti NA, Elli D, Hermanas TM, Schneewind O (2011). Prevention of pneumonic plague in mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or F1-V vaccines. *Vaccine.* 29: 6572-6583.

Quenee LE, Cornelius CA, Ciletti NA, Elli D, Schneewind O (2008). Yersinia pestis caf1 variants and the limits of plague vaccine protection. *Infection and Immunity.* 76: 2025-2036.

Quenee LE and Schneewind O (2009). Plague vaccines and the molecular basis of immunity against yersinia pestis. *Human Vaccines.* 5: 817-823.

Rabaan AA, Al-Ahmed SH, Alsuliman SA, Aldrazi FA, Alfouzan WA, Haque S (2019). The rise of pneumonic plague in madagascar: Current plague outbreak breaks usual seasonal mould. *Journal of Medical Microbiology.* 68: 292-302.

Randremanana R, Andrianaivoarimanana V, Nikolay B, Ramasindrazana B, Paireau J, Ten Bosch QA, Rakotondramanga JM, Rahajandraibe S, Rahelinirina S, Rakotomanana F, Rakotoarimanana FM, Randriamampionona LB, Razafimbia V, De Dieu Randria, M J, Raberahona M, Mikaty G, Le Guern AS, Rakotonjanabelo LA, Ndiaye CF, Rasolofo V, Bertherat E, Ratsitorahina M, Cauchemez S, Baril L, Spiegel A, Rajerison M (2019). Epidemiological characteristics of an urban plague epidemic in madagascar, august-november, 2017: An outbreak report. *The Lancet.Infectious Diseases.* 19: 537-545.

Rascovan N, Sjogren KG, Kristiansen K, Nielsen R, Willerslev E, Desnues C, Rasmussen S (2019). Emergence and spread of basal lineages of yersinia pestis during the neolithic decline. *Cell.* 176: 295-305.e10.

Rasmussen S, Allentoft ME, Nielsen K, Orlando L, Sikora M, Sjogren KG, Pedersen AG, Schubert M, Van Dam A, Kapel CM, Nielsen HB, Brunak S, Avetisyan P, Epimakhov A, Khalyapin MV, Gnuni A, Kriiska A, Lasak I, Metspalu M, Moiseyev V, Gromov A, Pokutta D, Saag L, Varul L, Yepiskoposyan L, Sicheritz-Ponten T, Foley RA, Lahr MM,

Nielsen R, Kristiansen K, Willerslev E (2015). Early divergent strains of yersinia pestis in eurasia 5,000 years ago. *Cell.* 163: 571-582.

Ratner D, Orning MP, Proulx MK, Wang D, Gavrilin MA, Wewers MD, Alnemri ES, Johnson PF, Lee B, Mecsas J, Kayagaki N, Goguen JD, Lien E (2016). The yersinia pestis effector YopM inhibits pyrin inflammasome activation. *PLoS Pathogens.* 12: e1006035.

Richgels KL, Russell RE, Bron GM, Rocke TE (2016). Evaluation of yersinia pestis transmission pathways for sylvatic plague in prairie dog populations in the western U.S. *EcoHealth.* 13: 415-427.

Riedel S (2004). Biological warfare and bioterrorism: A historical review. *Proceedings (Baylor University.Medical Center).* 17: 400-406.

Riedel S (2005). Plague: From natural disease to bioterrorism. *Proceedings (Baylor University.Medical Center).* 18: 116-124.

Rocke TE, Smith S, Marinari P, Kreeger J, Enama JT, Powell BS (2008). Vaccination with F1-V fusion protein protects black-footed ferrets (mustela nigripes) against plague upon oral challenge with yersinia pestis. *Journal of Wildlife Diseases.* 44: 1-7.

Roggenkamp A, Geiger AM, Leitritz L, Kessler A, Heesemann J (1997). Passive immunity to infection with yersinia spp. mediated by anti-recombinant V antigen is dependent on polymorphism of V antigen. *Infection and Immunity.* 65: 446-451.

Runco LM, Myrczek S, Bliska JB, Thanassi DG (2008). Biogenesis of the fraction 1 capsule and analysis of the ultrastructure of yersinia pestis. *Journal of Bacteriology.* 190: 3381-3385.

Runfola JK, House J, Miller L, Colton L, Hite D, Hawley A, Mead P, Schriefer M, Petersen J, Casaceli C, Erlandson KM, Foster C, Pabilonia KL, Mason G, Douglas JM, Centers for Disease Control and Prevention, (CDC) (2015). Outbreak of human pneumonic plague with dog-to-human and possible human-to-human transmission--colorado, june-july 2014. *MMWR.Morbidity and Mortality Weekly Report.* 64: 429-434.

Santi L, Giritch A, Roy CJ, Marillonnet S, Klimyuk V, Gleba Y, Webb R, Arntzen CJ, Mason HS (2006). Protection conferred by recombinant yersinia pestis antigens produced by a rapid and highly scalable plant expression system. *Proceedings of the National Academy of Sciences of the United States of America.* 103: 861-866.

Sha J, Kirtley ML, Klages C, Erova TE, Telepnev M, Ponnusamy D, Fitts EC, Baze WB, Sivasubramani SK, Lawrence WS, Patrikeev I, Peel JE, Andersson JA, Kozlova EV, Tiner BL, Peterson JW, McWilliams D, Patel S, Rothe E, Motin VL, Chopra AK (2016). A replication-defective human type 5 adenovirus-based trivalent vaccine confers

complete protection against plague in mice and nonhuman primates. *Clinical and Vaccine Immunology : CVI.* 23: 586-600.

Simond P (1898). La propagation de la peste. *Annales De L'Institut Pasteur.* 12: 625-687.

Sing A, Rost D, Tvardovskaia N, Roggenkamp A, Wiedemann A, Kirschning CJ, Aepfelbacher M, Heesemann J (2002). Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *The Journal of Experimental Medicine.* 196: 1017-1024.

Sivaraman V, Pechous RD, Stasulli NM, Eichelberger KR, Miao EA, Goldman WE (2015). Yersinia pestis activates both IL-1beta and IL-1 receptor antagonist to modulate lung inflammation during pneumonic plague. *PLoS Pathogens.* 11: e1004688.

Smiley ST (2008a). Current challenges in the development of vaccines for pneumonic plague. *Expert Review of Vaccines.* 7: 209-221.

Smiley ST (2008b). Immune defense against pneumonic plague. *Immunological Reviews.* 225: 256-271.

Smith ML, Lindbo JA, Dillard-Telm S, Brosio PM, Lasnik AB, McCormick AA, Nguyen LV, Palmer KE (2006). Modified tobacco mosaic virus particles as scaffolds for display of protein antigens for vaccine applications. *Virology.* 348: 475-488.

Solomon T (1995). Alexandre yersin and the plague bacillus. *The Journal of Tropical Medicine and Hygiene.* 98: 209-212.

SPECK RS and WOLOCHOW H (1957). Studies on the experimental epidemiology of respiratory infections. VIII. experimental pneumonic plague in macacus rhesus. *The Journal of Infectious Diseases.* 100: 58-69.

Spinner JL, Winfree S, Starr T, Shannon JG, Nair V, Steele-Mortimer O, Hinnebusch BJ (2014). Yersinia pestis survival and replication within human neutrophil phagosomes and uptake of infected neutrophils by macrophages. *Journal of Leukocyte Biology.* 95: 389-398.

Spyrou MA, Tukhbatova RI, Wang CC, Valtuena AA, Lankapalli AK, Kondrashin VV, Tsybin VA, Khokhlov A, Kuhnert D, Herbig A, Bos KI, Krause J (2018). Analysis of 3800-year-old yersinia pestis genomes suggests bronze age origin for bubonic plague. *Nature Communications.* 9: 2234-9.

Stasulli NM, Eichelberger KR, Price PA, Pechous RD, Montgomery SA, Parker JS, Goldman WE (2015). Spatially distinct neutrophil responses within the inflammatory lesions of pneumonic plague. *mBio.* 6: 1530.

Stryer L, Berg J, Tymoczko J, Clarke N (1988). *Biochemistry* (3rd ed.) W. H. Freeman And Company.

Sun W and Singh AK (2019). Plague vaccine: Recent progress and prospects. *NPJ Vaccines.* 4: 11-9. eCollection 2019.

Sun W, Six D, Kuang X, Roland KL, Raetz CR, Curtiss R (2011). A live attenuated strain of yersinia pestis KIM as a vaccine against plague. *Vaccine.* 29: 2986-2998.

Sun YC, Jarrett CO, Bosio CF, Hinnebusch BJ (2014). Retracing the evolutionary path that led to flea-borne transmission of yersinia pestis. *Cell Host & Microbe.* 15: 578-586.

Szaba FM, Kummer LW, Duso DK, Koroleva EP, Tumanov AV, Cooper AM, Bliska JB, Smiley ST, Lin JS (2014). TNFalpha and IFNgamma but not perforin are critical for CD8 T cell-mediated protection against pulmonary yersinia pestis infection. *PLoS Pathogens.* 10: e1004142.

Tidhar A, Flashner Y, Cohen S, Levi Y, Zauberman A, Gur D, Aftalion M, Elhanany E, Zvi A, Shafferman A, Mamroud E (2009). The NlpD lipoprotein is a novel yersinia pestis virulence factor essential for the development of plague. *PloS One.* 4: e7023.

Titball RW, Howells AM, Oyston PC, Williamson ED (1997). Expression of the yersinia pestis capsular antigen (F1 antigen) on the surface of an aroA mutant of salmonella typhimurium induces high levels of protection against plague. *Infection and Immunity.* 65: 1926-1930.

Titball RW and Williamson ED (2001). Vaccination against bubonic and pneumonic plague. *Vaccine.* 19: 4175-4184.

Titball RW and Williamson ED (2004). Yersinia pestis (plague) vaccines. *Expert Opinion on Biological Therapy.* 4: 965-973.

Turpen TH, Reinl SJ, Charoenvit Y, Hoffman SL, Fallarme V, Grill LK (1995). Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Bio/Technology (Nature Publishing Company).* 13: 53-57.

Van Regenmortel MH (1999). The antigenicity of tobacco mosaic virus. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences.* 354: 559-568.

Van Regenmortel MH, Altschuh D, Zeder-Lutz G (1993). Tobacco mosaic virus: A model antigen to study virus-antibody interactions. *Biochimie.* 75: 731-739.

Verma SK and Tuteja U (2016). Plague vaccine development: Current research and future trends. *Frontiers in Immunology.* 7: 602.

Visser LG, Annema A, van Furth R (1995). Role of yops in inhibition of phagocytosis and killing of opsonized yersinia enterocolitica by human granulocytes. *Infection and Immunity.* 63: 2570-2575.

Wagner DM, Klunk J, Harbeck M, Devault A, Waglechner N, Sahl JW, Enk J, Birdsell DN, Kuch M, Lumibao C, Poinar D, Pearson T, Fourment M, Golding B, Riehm JM, Earn DJ, Dewitte S, Rouillard JM, Grupe G, Wiechmann I, Bliska JB, Keim PS, Scholz HC, Holmes EC, Poinar H (2014). Yersinia pestis and the plague of justinian 541-543 AD: A genomic analysis. *The Lancet.Infectious Diseases.* 14: 319-326.

Wang S, Liu H, Zhang X, Qian F (2015). Intranasal and oral vaccination with protein-based antigens: Advantages, challenges and formulation strategies. *Protein & Cell.* 6: 480-503.

Welch TJ, Fricke WF, McDermott PF, White DG, Rosso ML, Rasko DA, Mammel MK, Eppinger M, Rosovitz MJ, Wagner D, Rahalison L, Leclerc JE, Hinshaw JM, Lindler LE, Cebula TA, Carniel E, Ravel J (2007). Multiple antimicrobial resistance in plague: An emerging public health risk. *PloS One.* 2: e309.

Welkos S, Pitt MLM, Martinez M, Friedlander A, Vogel P, Tammariello R (2002). *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*

Welkos S, Friedlander A, McDowell D, Weeks J, Tobery S (1998). V antigen of yersinia pestis inhibits neutrophil chemotaxis. *Microbial Pathogenesis.* 24: 185-196.

WHO (2017). *Plague outbreak madagascar.* ().World Health Organization.

WHO Group of Consultants (1970). *Health aspects of chemical and biological weapons.* (Report No. 1). Geneva, Switzerland: United Nations.

Williamson ED, Eley SM, Stagg AJ, Green M, Russell P, Titball RW (1997). A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague. *Vaccine.* 15: 1079-1084.

Williamson ED, Flick-Smith HC, Lebutt C, Rowland CA, Jones SM, Waters EL, Gwyther RJ, Miller J, Packer PJ, Irving M (2005). Human immune response to a plague vaccine comprising recombinant F1 and V antigens. *Infection and Immunity.* 73: 3598-3608.

Williamson ED and Oyston PC (2013). Protecting against plague: Towards a next-generation vaccine. *Clinical and Experimental Immunology.* 172: 1-8.

Williamson ED, Sharp GJ, Eley SM, Vesey PM, Pepper TC, Titball RW, Alpar HO (1996). Local and systemic immune response to a microencapsulated sub-unit vaccine for plague. *Vaccine.* 14: 1613-1619.

Williamson ED, Stagg AJ, Eley SM, Taylor R, Green M, Jones SM, Titball RW (2007). Kinetics of the immune response to the (F1+V) vaccine in models of bubonic and pneumonic plague. *Vaccine.* 25: 1142-1148.

Williamson ED, Vesey PM, Gillhespy KJ, Eley SM, Green M, Titball RW (1999). An IgG1 titre to the F1 and V antigens correlates with protection against plague in the mouse model. *Clinical and Experimental Immunology.* 116: 107-114.

Woodrow KA, Bennett KM, Lo DD (2012). Mucosal vaccine design and delivery. *Annual Review of Biomedical Engineering.* 14: 17-46.

Yang X, Hinnebusch BJ, Trunkle T, Bosio CM, Suo Z, Tighe M, Harmsen A, Becker T, Crist K, Walters N, Avci R, Pascual DW (2007). Oral vaccination with salmonella simultaneously expressing yersinia pestis F1 and V antigens protects against bubonic and pneumonic plague. *Journal of Immunology (Baltimore, Md.: 1950).* 178: 1059-1067.

Yang Y and Klessig DF (1996). Isolation and characterization of a tobacco mosaic virus-inducible myb oncogene homolog from tobacco. *Proceedings of the National Academy of Sciences of the United States of America.* 93: 14972-14977.

Yersin A (1894). Le peste bubonique à hong kong. *Annales De L'Institut Pasteur.* 8: 662-667.

Yersin A (1897). Sur la peste bubonique. *Annales De L'Institut Pasteur.* 11: 81-93.

Zavialov AV, Berglund J, Pudney AF, Fooks LJ, Ibrahim TM, MacIntyre S, Knight SD (2003). Structure and biogenesis of the capsular F1 antigen from yersinia pestis: Preserved folding energy drives fiber formation. *Cell.* 113: 587-596.

Zhang T, Breitbart M, Lee WH, Run JQ, Wei CL, Soh SW, Hibberd ML, Liu ET, Rohwer F, Ruan Y (2006). RNA viral community in human feces: Prevalence of plant pathogenic viruses. *PLoS Biology.* 4: e3.

Zimbler DL, Schroeder JA, Eddy JL, Lathem WW (2015). Early emergence of yersinia pestis as a severe respiratory pathogen. *Nature Communications.* 6: 7487.

Chapter 2. Specific Aim 1

AIM 1:
Determine if TMV Conjugates Target Conjugated Antigens to Professional Antigen Presenting Cells *in vitro* and *in vivo*

Aim 1a: Test the hypothesis that TMV promotes uptake into antigen-presenting cells *in vitro* (BMDCs) and *ex vivo* (B cells)

Aim 1b: Test the hypothesis that TMV is an immunostimulant by upregulating costimulatory surface markers on dendritic cells *in vitro*

Aim 1c: Test the hypothesis that TMV targets conjugated protein antigens (F1 and V) to pulmonary dendritic cells *in vivo* after intranasal immunization

1.INTRODUCTION

Rationally designed vaccines are comprised of antigen(s) co-inoculated with adjuvant(s) that elicit predictable and protective immune responses against the targeted pathogen (Rueckert and Guzman, 2012). A properly formulated vaccine ensures the antigen is 1) protected from enzymes and degradation, 2) stable in a wide-pH range, and 3) taken up by antigen presenting cells (APCs). APC recognition is crucial to development of immunity as professional APCs (collectively macrophages, dendritic cells, and B cells) play a role in differentiating innocuous particles from microbial intruders via identification of danger- and pathogen-associated molecular patterns (DAMPS and PAMPs, respectively) through different receptor families, including toll-like receptors (TLRs), NOD-like receptors (NLR), RIG-I-like receptors (RLRs), DNA receptors (e.g. STING), C-type lectins, and scavenger receptors. Dendritic cells (DCs), the most "professional" of the APCs, have the capacity to internalize, process, and present antigens through both major histocompatibility complexes (MHC) I and II, and have the predominant role in initiating primary T-cell immune responses over macrophages and B cells (Foged *et al.*, 2002). Thus, targeting of vaccines to DCs, either parenterally or mucosally, has great potential to reduce antigen dose, limit side effects, and directly improve vaccine efficacy.

Mucosally, DCs are less ubiquitous than tissue macrophages, yet are nonetheless central to vaccine priming. In the lung, the interstitium, terminal bronchioles, and alveoli are dominantly patrolled by macrophages (>80%), a majority of which are alveolar macrophages (AMs). AMs have limited APC function (Kunda *et al.*, 2013), but can induce type I interferons after infection with certain RNA viruses (Kumagai *et al.*, 2007). CD11c is the marker used conventionally used to identify and distinguish DCs from other immune cells;

however, several subsets can be identified by expression of specific co-markers. In mice, the lung contains $CD11b^+$ conventional DCs (cDCs), $CD103^+$ cDCs, and plasmacytoid DCs, with $CD103^+$ cDCs in the epithelium and $CD11b^+$ cDCs in the lamina propria (Neyt and Lambrecht, 2013) . Additionally, $Ly6C^{hi}$ monocytes can be recruited to the lung, and have ability to take up antigen, increase their expression of MHC II, costimulatory molecules, and CCR7, and migrate to draining lymph nodes (dLN) in a CCR2-dependent manner (Teh *et al.*, 2019), much like cDCs. In the absence of inflammation, most pulmonary DC display an immature phenotype and mediate immune tolerance to exogenous and self-antigens (Lambrecht *et al.*, 2001). Direct detection of PAMPS or DAMPS after antigen internalization triggers DC maturation and migration to dLN, where they can prime naïve T cells. DCs have been the target of numerous mucosal vaccines to influenza (Khalil *et al.*, 2014; Wakim *et al.*, 2015), *Chlamydia trachomatis* (Stary *et al.*, 2015), HIV (Ruane *et al.*, 2016), *Streptococcus pneumoniae* (Kataoka *et al.*, 2017), *Mycobacterium tuberculosis* (Griffiths *et al.*, 2016), and *Yersinia pestis* (Do *et al.*, 2008; Do *et al.*, 2012). These vaccines succeeded in inducing protective $CD4^+$ and $CD8^+$ T cell responses, enhancing the amplitude and durability of vaccine-induced immune responses in the mucosal tissue.

We previously reported that complete protection against lethal pneumonic challenge with *Y. pestis* CO92pgm- could be achieved with an intranasally (i.n.)-administered vaccine comprised of F1 and LcrV (V), the capsule protein and the tip protein of the *Y. pestis* type III secretion system (T3SS), respectively, covalently linked to recombinant Tobacco Mosaic Virus (rTMV) (Arnaboldi *et al.*, 2016). TMV is a single-strand (+) RNA plant virus, consisting of 2,130 copies of a single coat protein (CP) surrounding the RNA core. It is considered safe to humans because it cannot replicate in mammalian cells (Smith *et al.*,

2006). The CP was genetically modified to express a surface-exposed lysine residue that allows for covalent conjugation of proteins, converting TMV into a highly versatile antigen presentation platform (McCormick and Palmer, 2008; Smith *et al.*, 2006). Viable TMV (not chemically inactivated or irradiated) has been shown to target to and upregulate costimulatory molecules on DCs both *in vitro* and *in vivo* (Kemnade *et al.*, 2014; McCormick *et al.*, 2006) . Interactions between plant viruses and immune cells has been documented (Balique *et al.*, 2013; Gonzalez *et al.*, 2009; Lacasse *et al.*, 2008) (Gonzalez *et al.*, 2009; Lacasse *et al.*, 2008) however, there is limited evidence of their direct interactions with mucosal DCs *in vivo*. The rTMV used in our vaccine studies (Banik *et al.*, 2015; Mallajosyula *et al.*, 2014; Mallajosyula *et al.*, 2016; Mansour *et al.*, 2018; McCormick *et al.*, 2006; McCormick *et al.*, 2018) still contains its RNA core (albeit inactivated). Due to this, we hypothesize that rTMV is analogous to a virus-like particle (VLP) vaccine, with capacity to be both an immunostimulant and DC-targeting particulate. In this current study, we extend previous observations regarding the targeting and immune activating abilities of TMV using our TMV-conjugated *Y. pestis* vaccine. We compared uptake and activation of APCs by our *Y. pestis* vaccine conjugates (TMV-F1 and TMV-V) to TMV alone as well as their respective recombinant protein antigens (rF1 and rV) both *in vitro* and *in vivo.*

2.MATERIALS AND METHODS

2.1 Recombinant proteins, TMV, and fluorescence labeling

Cloning, expression, and purification of rF1 and rLcrV (rV), and preparation of TMV-F1 and TMV-V conjugate vaccines were performed as described previously (Arnaboldi *et al.*, 2016). Purified TMV and TMV conjugates were supplied by Alison McCormick. The TMV

was inactivated via binary ethyleneimine (BEI) prior to the antigen conjugation process (EDC mediated amine conjugation) and has no replication capacity in plant or mammalian cells. Each purified protein and conjugate were fluorescently labeled with DyLight™ 488 NHS Ester amine-reactive dye according to the manufacturer's protocol (ThermoFisher Scientific). Labeled proteins were aliquoted separately and stored at -20°C. A chromogenic LAL (limulus amebocyte lysate) endotoxin assay was also performed to assess LPS contamination from recombinant proteins, as well as TMV and conjugates, according to manufacturer's kit protocol (GenScript).

2.2 Mice

Male and female C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME), and maintained by the husbandry staff of the Department of Comparative Medicine at New York Medical College. All experiments were conducted with the approval of the New York Medical College Institutional Animal Care and Use Committee (IACUC). All mice were utilized between 6-25 weeks of age. For protocols that required anesthetization, mice were anesthetized using 200µL of ketamine/xylazine cocktail (IP injection).

2.3 BMDC isolation, cell culture, and *in vitro* uptake studies

Murine bone marrow-derived dendritic cells (BMDCs) were prepared and maintained as previously described (Dong *et al.*, 2016) from adult C57Bl/6J mice, with minor modifications to the protocol. Bone marrow progenitor cells were obtained by flushing the femoral bones and cultured in sterile Petri dishes at 37°C in 10mL BMDC media: Advanced RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 20mM

HEPES (Gibco), 2mM L-glutamine (GlutaMAX, Gibco), 50 U/mL penicillin and streptomycin (Pen Strep, Gibco), 55 μM 2-mercaptoethanol (Gibco), and 20 ng/mL recombinant mouse GM-CSF and 10ng/mL recombinant mouse IL-4 (R&D Systems). RBCs were allowed to die in culture. On Day 3, 10mL fresh BMDC media was added (doubling volume). On Day 6, a half media change was performed with fresh BMDC media containing 20 ng/mL GM-CSF only (no IL-4). Non-adherent and loosely adherent cells were harvested from days 7-15 as previously described (Dong *et al.*, 2016). BMDC generation was assessed by flow cytometry as described below. $5x10^5$ BMDCs/well were seeded in a 12-well cell culture plate in 2mL of minimal media: Advanced RPMI 1640 medium supplemented with 10% FBS and 50 U/mL penicillin and streptomycin. Wells were treated separately with the following final concentrations of DyLight™ 488-labeled proteins: 0.5 μg/mL TMV, 0.5 μg/mL rF1, 0.5 μg/mL rV, 1 μg/mL TMV-F1, and 1 μg/mL TMV-V. The concentration of TMV conjugates was double that of recombinant protein to ensure molar equivalency of rF1 and rV between the groups, since TMV conjugates contain equivalent concentrations of TMV and recombinant protein. Cells were incubated at 37°C for 1, 4, 24, 48, and 72 hours and collected into Eppendorf tubes. Cells were centrifuged at 350xg for 5 min, washed with 500μL PBS, and replicate samples were processed for flow cytometry or immunofluorescence microscopy as described below. As a positive control for BMDC activation additional wells treated with 1 μg/mL LPS (Sigma-Aldrich).

2.4 Immunofluorescence of BMDCs

Antigen stimulated and control BMDCs, described above, were treated with 4% paraformaldehyde (PFA) in PBS for 15-20 min at RT. Cells were centrifuged at 350xg for 5

min and pellets were resuspended in 400µL of 1% BSA in PBS. 60-100µL of each sample was pipetted into a StatSpin cassette, and cells were spun onto glass microscope slides (Fisher Scientific) using a StatSpin Cytofuge Cytocentrifuge (Beckman Coulter). Slides were fixed in 200µL 2.5% PFA in PBS for 10 min and washed by quickly pipetting 1mL of PBS over the slide (3x). Slides were blocked with 500µL of 1% BSA in PBS for 45 min in the dark at RT. 200µL of the Alexa Fluor 594 anti-mouse CD11c primary antibody (N418; BioLegend) or Alexa Fluor 594 Armenian hamster IgG isotype control antibody (HTK888; BioLegend) was added to each slide (diluted 1:100 in 1% BSA in PBS) for 1 hour in the dark at RT. Slides were washed again 3x with PBS for 5 min each and allowed to dry. 1-2 drops of DAPI-Flouromount-G (Southern Biotech) was applied to each slide and immediately covered with a micro cover glass (No. 1, VWR). Slides were stored in the dark at 4°C until ready for imaging. Imaging was performed on a Revolve microscope (Echo Inc.).

2.5 B lymphocyte isolation, cell culture, and *ex vivo* uptake studies

For mucosal B cell isolation, lungs from two naïve adult C57Bl/6J mice were removed and processed as described below (see **2.7 Isolation of lung cells**). For splenic B - cells, spleens were removed aseptically from two naïve adult C57Bl/6J mice and placed on ice in sterile PBS. Single cell suspensions were generated by pressing the organ through a sterile 40µm cell strainer (Fisher Scientific) with a sterile syringe plunger. Splenocytes were pelleted by centrifugation at 350xg for 5 min at 4°C, and resuspended and incubated in 0.5mL of filtered red cell lysis buffer (0.8% ammonium chloride) for 5 min in a 37°C bead bath. The cells were centrifuged again and resuspended in sterile PBS containing 2%FBS and 1mM EDTA. B lymphocytes were isolated using the EasySEP™ Mouse B Cell Isolation Kit

protocol (STEMCELL Technologies). $5x10^5$ purified B lymphocytes were seeded in a 12-well cell culture plate in 2mL of primary lymphocyte media: Advanced RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco), 1% HEPES (Gibco), 1% penicillin streptomycin L-glutamine (Pen Strep Glutamine, Gibco), and 55 μM 2-mercaptoethanol (Gibco). B cells were rested for 1 hour and then treated with DyLight™ 488-labeled proteins (as above).

2.6 Intranasal vaccination and *in vivo* uptake studies

C57Bl/6J mice (8-25 weeks old) were anesthetized in order to preform i.n. immunization with either: 10μg of TMV-V+10μg of TMV-F1 (total of 20 μg), 5μg rV+5μg rF1 (total of 10μg), or 10ug of TMV in 40μL volume of saline. Control mice received no i.n. immunization. Mice were vaccinated with 10μg of TMV-conjugate and 5 μg of recombinant protein to ensure molar equivalency of rF1 and rV between the two vaccines. Mice were not vaccinated separately with TMV-F1/rF1 and TMV-V/rV to reduce the number of animals needed for study. Working concentrations of vaccines were prepared from freezer stocks on the day of immunization. A total of four mice were used for each time point and included an even number of males and females (e.g. rF1+rV-male, TMV-F1+TMV-V-female, TMV-male, control-female). A table providing the sexes used for each timepoint is supplied in Appendix I (Appendix I Table S1).

2.7 Isolation of lung cells

Mice were sacrificed and whole lungs were harvested 1, 2, 4, 24, 48, and 96 hours after i.n. immunization with labeled vaccine proteins. Before harvest, lungs were gently perfused via the right ventricle with 5–10mL of PBS to remove leucocytes in the blood from

the pulmonary circulation. Lungs from each mouse were placed in sterile gentleMACS C tubes (Miltenyi Biotec) and single cell suspensions were prepared using the mouse Lung Dissociation Kit and a gentleMACS Dissociator, according to the manufacturer's protocol (Miltenyi Biotec). Lung cell suspension was strained through a 70µm cell strainer (Fisher Scientific) into a 50mL conical tube and flushed with 15mL of PEB buffer (1x PBS, 0.5% BSA, 2mM EDTA) (the strainer was pre-soaked with 1 mL PEB to assist straining) and then centrifuged at 300xg for 10 min at 4°C. The supernatant was discarded, and cell pellets were resuspended in 0.5mL of red cell lysis buffer and incubated in a 37°C bead bath for 4 min. After, the cells were centrifuged again at 300xg for 10 min at 4°C. The supernatant was carefully pipetted off and discarded and pellet was gently resuspended in 2-3 mL of cold PEB buffer. Cells were strained for a second time through a cell strainer snap cap test tube (BD Falcon) and kept on ice to ensure preservation of surface markers for flow cytometry. Cell concentration and viability were determined using Trypan Blue solution (0.4%, Gibco) staining and a hemocytometer.

2.8 Flow cytometry

$5x10^5$ - $1x10^6$ cells/well were placed in a 96-well V-bottom plate. Cells were centrifuged at 350xg for 5 min at 4°C and washed twice with PBS. Cells were stained with Fixable Viability Dye eFluor™ 450 for 30 min on ice in the dark (1:1000) (eBioscience). Cells were then centrifuged and washed once with staining buffer (1x PBS, 0.1% sodium azide, 2% BSA). Cells were incubated with Fc receptor block (anti-mouse CD16/CD32, clone93; eBioscience) for 20 min in staining buffer and stained with fluorochrome-conjugated primary anti-mouse antibodies for 30min at RT (see Table 1). Antibodies were

titrated to determine optimal concentrations prior to use (data not shown). After staining, cells were washed twice with staining buffer and fixed in 2% PFA. Extra wells containing untreated lung/BMDC/B cells were stained with individual antibodies to calculate compensation. Cells that were treated with DyLight™ 488-labeled proteins (*in vitro* or *in vivo*) acted as compensation control for FITC. All marker and isotype control antibodies were manufactured by eBioscience unless noted below in Table 1.

In vitro uptake studies: Antibodies used for BMDC activation (marker name followed by clone in parenthesis): anti-CD11c APC-eFluor 780 (N418), anti-MHC Class II (1-A/I-E) PE-Cyanine7 (M5/114.15.12), anti-CD86 (B7-2) APC (GL1), anti-CD80 (B7-1) PerCP-eFluor 710 (16-10A1). B cells: anti-CD19 APC e-Fluor 780 (1D3), and appropriate isotype control antibodies. Four wells were stained per sample: staining mix, isotype controls, anti-CD11c plus isotype controls for other markers, and an unstained well. Samples were acquired using a MACSQuant flow cytometer and analyzed using Flowlogic™ software (Miltenyi Biotec).

In vivo uptake studies: Data was acquired using three different flow cytometers over the course of the study due to equipment malfunction, and unexpected changes in core facility availability. Three wells were created per sample: mix, anti-CD45 (see table below) plus isotype controls for other markers, and an unstained well. Antibody mixes are described below and were modified based on the channels available on different cytometers. For the Miltenyi MACSQuant, different mixes had to be used for B cells and DCs. An extra well containing lung cells was stained with CD45 isotype control (rat IgG2bk PerCP/Cy5.5 (BioLegend) or APC (eBioscience)) to confirm CD45 gating. All samples were analyzed using Flowlogic™ software (Miltenyi Biotec).

Table 1: List of primary antibody mixes used for each flow cytometer.

Flow Cytometer	Primary anti-mouse antibody mixes
Miltenyi Biotec MACSQuant (7 channel)	Mix 1: anti-CD11c APC-eFluor 780 (N418), anti-MHC Class II (1-A/I-E) PE-Cyanine7 (M5/114.15.12), anti-SiglecF (CD170) PE (1RNM44N), anti-CD45 PerCP/Cyanine5.5 (30-F11, BioLegend), anti-CD11b APC (M1/70)
	Mix 2: anti-CD19 APC e-Fluor 780 (1D3), anti-CD4 APC (GK1.5), anti-CD45 PerCP/Cyanine5.5 (30-F11, BioLegend), anti-CD40 PE (1C10)
Beckman Coulter MoFlo XDP (11 channel)	Fixable Viability Dye eFluor™ 455UV (1:1000, before antibodies). anti-CD11c APC-eFluor 780 (N418), anti-MHC Class II (1-A/I-E) PE-Cyanine7 (M5/114.15.12), anti-SiglecF (CD170) PE (1RNM44N), anti-CD45 PerCP/Cyanine5.5 (30-F11, BioLegend), anti-CD11b PE-eFluor 610 (M1/70), anti-CD4 APC (GK1.5), anti-CD19 Alexa Fluor 700 (1D3),
BD FACS Celesta (12 channel)	anti-CD11c APC-eFluor 780 (N418), anti-MHC Class II (1-A/I-E) Super Bright 645 (M5/114.15.12), anti-SiglecF (CD170) PE (1RNM44N), anti-CD45 APC (30-F11), anti-CD11b PE-eFluor 610 (M1/70), anti-CD4 PerCP-Cyanine5.5 (RM4-5), anti-CD19 Alexa Fluor 700 (1D3)

2.9 Statistical analysis

Means between two groups were compared by unpaired t test. For comparisons of three or more groups in relation to time, data was analyzed by a repeated measures two-way ANOVA, with Tukey's multiple comparisons test as the post-hoc analysis per time point. Values for significance were set at $p<0.05$. All values are expressed as the mean ± SD or SEM, where indicated. Statistical analysis was performed using GraphPad Prism 7 software.

3.RESULTS

Inhaled antigens are taken up largely by AMs, but TMV-conjugates are taken up at a higher rate by lung DCs compared to unconjugated rF1+rV or TMV

It has been demonstrated that TMV is taken up by DCs and induces upregulation of costimulatory molecules on the DC surface in dLN following subcutaneous (s.c.) injection (Kemnade *et al.*, 2014). And when administered intratracheally to mice, TMV was taken up by macrophages obtained from bronchoalveolar fluid (BAL), presumably AMs (Balique *et al.*, 2013). However, this study by Balique *et al.* did not examine other cell populations, and it has yet to be shown if TMV induces uptake by lung DCs. To gain further insight into the *in vivo* uptake of TMV and TMV-conjugated vaccines following mucosal administration, we analyzed the uptake of DyLight™ 488 (D488)-labeled TMV, TMV conjugates, and soluble proteins in the lung 1, 2, 4, 24, 48, 96h after i.n. immunization. We evaluated TMV-F1+TMV-V together and rF1+rV together, as *Y. pestis* vaccine formulations commonly include both antigens.

Following i.n. immunization there was no significant difference in the overall uptake of the fluorescently labelled antigens in terms of the percent total $D488^+$ staining cells in the

lung (either $CD45^+$ or CD45-) at any time point (Appendix I Fig S1). Interestingly, unconjugated TMV also was almost exclusively taken up by $CD45^+$ cells at all time points (Appendix I Fig S1C), unlike the conjugates and proteins, where a small percentage always reached CD45- cells (presumably pneumocytes) (Appendix I Fig S1A, S1B). Uptake of the TMV conjugates by $CD45^+$ cells was consistently higher than uptake of unconjugated TMV, though these differences were not statistically significant (Fig 1A, 1B). Uptake of rF1+rV was also higher than unconjugated TMV but only at later time points (ns, Fig 1A, 1B). Uptake of TMVF1+TMV-V generally higher (but not statistically different) than uptake of rF1+rV, but only very early (1h and 2h); uptake was similar in $CD45^+$ cells at later time points. Interestingly, TMVF1+TMV-V and TMV both exhibited smoother and more consistent uptake in $CD45^+$ cells over the 4 days (though TMV was always <6%), whereas rF1+rV uptake was spiked and inconsistent (Fig 1A). A striking percentage of $CD11c^+$ cells took up antigen in the lungs. It is important to note that while $CD11c^+$ cells represent around 20% of total lung $CD45^+$ cells (Yu *et al.*, 2016), CD11c is a marker for more than just DCs in the lung (see below). Similarly, uptake of both TMVF1+TMV-V and rF1+rV was consistently higher than uptake of unconjugated TMV at all timepoints assessed, but this did not reach statistical significance (Fig 1C). Only a small percentage of $CD19^+$ cells took up fluorescent antigen in the lung, no differences significant or otherwise were observed between the groups (Fig 1D). In summation, roughly 4-8% of leucocytes were associated with all antigens (Fig 1A), yet these fractions still represented 20-50% of the $CD11c^+$ population (Fig 1C). Therefore, this data suggests all i.n. immunized antigens – TMV, TMV-F1+TMV-V, and rF1+rV – were mostly taken up into $CD11c^+$ cells.

Several non-DC myeloid populations express CD11c, including AMs, some interstitial macrophages (IMs), and resident Ly6C^{lo} monocytes. Therefore, further analysis was needed to determine which of these cells were participating in antigen uptake. The gating strategy and cell markers used for flow cytometric analysis is supplied in Appendix I (Appendix I Fig S2). AMs (CD11c^{+} SiglecF^{+} SSC^{hi}) represent around 70% of the total CD11c^{+} cells in the lung (Yu *et al.*, 2016), and accordingly accounted for >50% of D488^{+} cells for every antigen assessed (Fig 2A). TMV showed the highest mean uptake in AMs at all time points, which accounted for >80% of the D488^{+} cells. Uptake of TMV by AMs was substantially higher than both TMVF1+TMV-V and rF1+rV at all time points except for 96h, while uptake of TMVF1+TMV-V by AMs was consistently lower than it was for rF1+rV (Fig 2A). These were reproducible differences that were statistically significant by two-way ANOVA (p=0.001), however, the post-hoc analysis did not reveal significant differences in pairwise comparisons per time point. DCs were defined as CD11c^{+} MHC II^{+} and SSC^{lo} to help differentiate from SSC^{hi} macrophages that are also CD11c^{+} MHC II^{+} (Yu *et al.*, 2016) . At each time point, particularly early time points, TMV-conjugated vaccine was taken up by a higher percentage of DCs than either unconjugated recombinant protein or unconjugated TMV (Fig 2B). As early as 1h and 2h post-immunization with TMV-F1+TMV-V, the mean percentage of D488^{+} DCs was more than double that of rF1+rV (9.6% and 6.6% vs. 4.2% and 2.1%, 1h and 2h TMV-F1+TMV-V vs. rF1+rV respectively) (Fig 2B). DCs continued to account for a higher percentage of D488^{+} cells in TMVF1+TMV-V-immunized mice for the remaining time points (Fig 2B). However, despite consistent, reproducible differences, the values did not reach statistically significance by two-way ANOVA likely because of variability inherent to this experiment and technique. IMs (CD11b^{+} SSC^{hi}), APCs that

contribute to tissue homeostasis in the lung, could also represent a target for i.n.-administered vaccine proteins. IMs comprise a heterogenous $CD11b^+$ population, and in our analysis, >90% of IMs were CD11c- and MHC II- (Appendix I Fig S3). There were no significant differences between the antigens in regard to uptake into IMs, as analyzed by two-way ANOVA (Fig 2C).

We also discovered a population of $CD11c^+$ MHCII- CD11b- cells that were taking up fluorescently labelled antigen, which we identified as probable tissue-resident DC precursors (pre-DC) (Suda *et al.*, 1998; von Garnier *et al.*, 2017; Wang *et al.*, 2006). Interestingly, both TMV-F1+TMV-V and rF1+rV were consistently taken up by substantially more pre-DC than unconjugated TMV at all but the final time point (Fig 2D), however, these differences did not reach statistical significance by two-way ANOVA. The percentage of $D488^+$ antigens that were associated with pre-DCs (0-40%) was roughly double of that seen with cDCs (0-20%) for all antigens. Uptake of antigen in CD11c- $CD11b^+$ monocytes (presumably Ly6C+ monocytes) was highly variable, and there were no significant differences between the antigens in regard to uptake into this cell population (Fig 2E). $CD19^+$ B cells demonstrated similar uptake of antigen at all time points (Fig 2F). $CD4^+$ T cells represented another insignificant target of inhaled antigens; less than 1% of lung $CD4^+$ cells participated in uptake of all proteins from 1-96h post-immunization (data not shown). Overall, these data supported that i.n.-administered antigens are largely taken up by AMs in the lungs, but TMV-F1+TMV-V was associated with a higher percentage of DCs compared with the unconjugated rF1+rV and TMV, especially in the first 2 hours post-immunization. We also looked at activation markers on $CD11c^+$ cells in the lung post-immunization, but preliminary data showed there was no difference in expression of MHC II and CD86 of

antigen-immunized mice compared to unimmunized control mice (data not shown). Therefore, we moved to an *in vitro* system.

TMV conjugation promotes uptake of F1 and V antigens in BMDCs and B cells *in vitro*, but does not induce costimulatory molecules

Kemnade and colleagues were the first to demonstrate uptake of TMV into mouse and human DCs (Kemnade *et al.*, 2014). However, these experiments utilized live TMV without conjugated antigen. One other *in vitro* study on TMV solely focused on macrophages, where they found live TMV localized in the cytoplasm of murine bone-marrow derived macrophages (BMDMs) and persisted for over two weeks, but not replicating (Balique *et al.*, 2013). We sought to evaluate the uptake of TMV into three other APCs: bone-marrow derived DCs (BMDCs), splenic B cells, and lung mucosal B cells. In our studies, TMV was inactivated and conjugated separately to F1 and V antigens. This allowed us to examine vaccine-APC interactions under more reproducible conditions than is possible in the mouse.

When conjugated to TMV, uptake of V antigen by BMDCs was significantly increased at 4, 24, 48, and 72 hours (h), compared to uptake of unconjugated protein, with the largest difference being at 72h (TMV-V 78.8% ± 2.2% vs rV 58.4% ± 1.9% of BMDC population) (Fig 3A). Uptake of TMV-F1, conversely, was not significantly different from rF1 over the time course (Fig 3A). The F1 capsule is known to possess anti-phagocytic properties, and therefore, coating the TMV particle in F1 proteins could theoretically inhibit uptake into phagocytic cells. This was further supported by the fact that TMV uptake in BMDCs was significantly higher than TMV-F1 at all time points, and uptake was parallel to

that of TMV-V (Fig 3A). Microscopically, TMV could be seen internalized by $CD11c^+$ BMDCs as early as 1h of incubation, as well as by CD11c- macrophages/monocytes in culture (Fig 3B). For both splenic and mucosal B cells, the percentage of cells participating in uptake (<3%) was much smaller compared to that of BMDCs (20-80%). Since B cells internalize antigen through the B cell receptor or other surface receptors, this indicated there is likely no PRR specific for TMV on B cells, and there are only few B cells with BCRs capable of binding TMV. Unlike with BMDCs, uptake of TMV-V and rV antigen in splenic B cells was not significantly different at any timepoint, although TMV-V was associated with a greater percentage of B cells (≥1%) at all timepoints (Fig 3C). On the other hand, uptake of TMV-F1 was significantly higher than that of rF1 beginning at 24h (TMV-F1 0.77% ± 0.03% vs rF1 0.24% ± 0.05%), and the difference increased at each subsequent timepoint (Fig 3C). In lung B cells, the reverse was observed, uptake of TMV-F1 and rF1 antigen was not significantly different at any timepoint, although TMV-F1 was associated with a greater percentage of B cells at most timepoints (Fig 3D). However, uptake of TMV-V was significantly higher compared to rV at all time points (Fig 3D). Uptake of unconjugated TMV negligible in both splenic and lung B cells (Fig 3C, 3D). This suggests that the uptake of antigens by B cells is utterly independent of the antigenicity of TMV. Enhancement of TMV conjugates compared to protein antigens in both BMDCs *in vitro* and B cells *ex vivo* is likely due to increased particle size, and not directly related to properties of the virus itself.

It has been previously shown that $CD11c^+$ DCs isolated from dLN that had taken up fluorescently-labeled TMV following s.c. footpad injection had increased surface expression of the T cell costimulatory molecules CD54, CD40, CD86, MHC I, and MHC II compared to DCs from PBS-injected mice (Kemnade *et al.*, 2014). This suggested that TMV uptake

induced the immune-activating potential of these cells; however, this was a live, non-inactivated TMV (Kemnade *et al.*, 2014). We therefore asked whether inactivated TMV conjugated to either F1 or V antigen, could also trigger DC activation. LPS, a TLR4 agonist, significantly increased the mean fluorescence intensity (MFI) of all costimulatory molecules compared to unstimulated BMDCs, as expected (Fig 3E, 3F, 3G). MFI increased from 24 to 72h before decreasing at 144h. There was no significant difference in the surface expression of any costimulatory molecule following incubation of BMDCs with any antigen at any time point assessed, compared to unstimulated cells (Fig 3E, 3F, 3G). We also assessed possible *E. coli* endotoxin (LPS) contamination in recombinant proteins, as LPS upregulates costimulatory molecules in APCs and would interfere with the interpretation of this data. Purified TMV contained no endotoxin, as expected, and rF1 and rV had similar levels of endotoxin as molar equivalent concentrations of their respective TMV conjugates (Appendix I Fig S4). This indicated that any inadvertent effects resulting from endotoxin-contaminated proteins would also be seen by their respective TMV conjugates, and the effects would be similar. Despite the detectable contamination, we did not observe any differences in activation compared to unstimulated BMDCs, as already stated. Altogether, these data suggested that neither inactivated TMV itself, or as part of a conjugate, directly stimulates the maturation of DCs *in vitro*, supporting our *in vivo* findings.

4. DISCUSSION

DCs are professional APCs that are present throughout the respiratory tract and play a vital role in the initiation of immune responses, whether those responses may be allergen-based, or directed at replicating viral, bacterial, or fungal pathogens. In healthy lungs, the

majority of DC are immature and mediate immune tolerance to various innocuous antigens in both mice and humans (Kuipers and Lambrecht, 2004). Exposure of antigens to the lung, therefore, often leads to induction of tolerance rather than immunization. Effective respiratory vaccines have to overcome this tolerance so that a protective immune response can be generated. The addition of an adjuvant is key in a mucosal vaccination setting. Vaccine delivery systems, while less potent than toxoid-based adjuvants, have the advantage of facilitating the uptake of soluble antigens into APCs with low risk of damaging mucus tissue. These systems include nano- and micro-particles, liposomes, particulates (e.g. VLPs), and emulsions (Kunda *et al.*, 2013). Based on the findings of the current study, we propose that TMV can be classified as such an adjuvant. We reported 100% protection against lethal pneumonic plague challenge could be achieved with i.n. administration of TMV-F1+TMV-V, but not rF1+rV, and that TMV-F1- and/or TMV-V-immunized mice had greater production of antigen-specific antibodies compared to mice immunized with rF1 and/or rV (Arnaboldi *et al.*, 2016). Although the success of the vaccine was apparent, the mechanism of how TMV enhances vaccine-specific immunity had not been identified.

To define cell populations involved in uptake of vaccine antigens *in vivo*, we analyzed the uptake of fluorescently labeled TMV, TMV-F1+TMV-V, and rF1+rV in the lung for 1-96h post-immunization. Viable TMV has been known to persist in lung macrophages when administered to mice intratracheally (Balique *et al.*, 2013). In the current study, TMV was primarily found in AMs (>80% of the $D488^{+}$ cells were AMs). AMs represent a significant macrophage population in the lung: 14% of all $CD45^{+}$ cells (Yu *et al.*, 2016). It is unclear why TMV is preferentially associated with AMs. TMV is relatively small at 300nm, compared to a bacterium or fungus, yet is stable enough to endure the harsh

environment of phagocytes, and that of the surrounding mucosa. Preliminary data showed TMV and our TMV conjugate vaccine was detected in lungs at 10 days post- i.n. administration (data not shown). We think this stability could be its most important feature, as this would greatly increase likelihood of APC detection. All antigens – TMV, TMV-F1+TMV-V, and rF1+rV – were mostly taken up into $CD11c^+$ cells, a majority of which were AMs. TMV-F1+TMV-V was the only group consistently taken up by less AMs and more DCs compared to unconjugated protein and TMV alone (Fig 3). This suggests that TMVF1+TMV-V conjugates can overcome the immune tolerant threshold mediated by AMs, the first line of defense against invading microorganisms, to reach a greater number of lung DCs. However, further studies would need to determine the fate of these TMV-F1+TMV-V^+ DCs in dLN, the type of DC ($CD11b^+$ or $CD103^+$), and whether they are capable of stimulating antigen-specific $CD4^+$ T cell proliferation in secondary lymphoid organs (SLO).

We also observed that lung-resident DC precursors represented a significant target of inhaled antigens. These DC precursors can generate into fully functional $CD11c^+$ $CD11b^+$ DCs but are not considered APCs themselves due to lack of MHC II and costimulatory molecules (Wang *et al.*, 2006). Both TMVF1+TMV-V and rF1+rV were consistently taken up by a higher percentage of pre-DC compared to TMV, although the biological relevance of this is questionable given that pre-DCs are poor APCs and their purpose is to replenish the resident lung DC population. The percentage of $D488^+$ pre-DCs was roughly double that of $D488^+$ cDCs for all antigens, which is interesting given that pre-DCs make up a similar cell percentage as cDCs in the naïve mouse lung (6% and 5% of $CD45^+$, respectively) (Wang *et al.*, 2006; Yu *et al.*, 2016). IMs were also associated with all vaccine proteins, and uptake of D488+ proteins by IMs was the highest at days post-immunization, as opposed to hours.

Most studies on murine IMs have focused on their immunoregulatory nature, and human and murine IMs have been shown to express IL-10 at steady state (Schyns *et al.*, 2018). Therefore, it is unlikely that IMs are key players in vaccine-induced protection. B cells, which act as APCs for follicular DCs in germinal centers (GC) to enable GC class switching and affinity maturation, were also a smaller but significant target of the TMV-F1+TMV-V conjugates. From this, we can infer that B cells do bind the conjugates, but there are likely not high numbers of TMV-, V-, and F1-specific B cells in the naïve lung, as mentioned previously. *In vivo*, B cell data is difficult to interpret without the use of fluorescently labeled tetramers, which can identify rare antigen-specific B cells. It is also possible these proteins were interacting with low-affinity B cells generated against other antigens with cross-reactive epitopes. This process could be occurring beyond 4 days, and thus beyond the scope of these experiments. Further studies will need to look at conjugates individually (TMV-F1 vs TMV-V) and investigate conjugate uptake into B cell responses in the lung mucosa.

Considering the prior report of viable TMV activating DCs *in vivo* (Kemnade *et al.*, 2014), we hypothesized that TMV had the potential to be both an immunostimulant and stable vaccine delivery system targeted to DCs. To test this hypothesis, we cultured BMDCs in the presence of fluorescently labeled proteins (TMV, TMV-F1, TMV-V, rF1, rV) and analyzed their uptake by flow cytometry. While TMV-V showed significantly greater uptake into BMDCs compared to rV, the same was not true for TMV-F1. The F1 capsule is known to possess anti-phagocytic properties (Runco *et al.*, 2008). Therefore, coating the TMV particle in F1 proteins could theoretically create the same protective effect exerted by the bacterial capsule. This was further supported by the fact that TMV uptake in BMDCs was significantly higher than TMV-F1 at all time points, but no different compared to TMV-V.

This suggests addition of F1 to the TMV particle could limit its uptake capacity into DCs *in vivo*, potentially dampening anti-F1 $CD4^+$ T cell responses.

Uptake of TMV-V and TMV-F1 into lung B cells *ex vivo* was generally higher than both rV and rF1 proteins. Mucosa-associated B lymphocytes largely represent effector or memory cells (Allie *et al.*, 2019). Thus, we did not expect there to be a high number of TMV-, V-, and F1-specific B cells in the naïve lung, since these antigens are not encountered there normally in mice. For splenic B cells, uptake of TMV-F1 was significantly higher than rF1, and this difference increased with each day of incubation. TMV alone was barely taken up by splenic B cells, indicating there are more splenic B cells that are cross-reactive with F1 than TMV. Overall this suggests that the uptake of antigens by B cells is utterly independent of the virus and is likely due to the random presence of B cells with BCRs capable of binding to F1 or V with high avidity. BCR interaction would also explain why such a low percentage of B cells (<3%) were involved in uptake; BCR binding must precede internalization of the antigen. A spleen also contains a higher proportion of naïve B cells. If presented at a high frequency in SLO, our data suggests there is potential for direct or indirect (BCR crosslinking) activation of naïve B cells by TMV-F1.

Mature APCs upregulate the expression of MHC class II and costimulatory molecules before or as they drain to dLN where they then interact with antigen-specific B or T-cells to trigger immune responses. *In vivo*, viable TMV is known to induce costimulatory molecules on $CD11c^+$ DCs from dLN (Kemnade *et al.*, 2014). We investigated whether TMV, if conjugated to F1 and V antigen, could also trigger DC activation. However, we could not show that TMV conjugates are immunostimulants. Incubation of TMV conjugates *in vitro* failed to upregulate MHC II, CD80, or CD86 over that of unstimulated BMDCs, while LPS

alone did. This may be because the TMV RNA is altered via BEI prior to the antigen conjugation process, which could negate any agonist interactions with PRRs (e.g. endosomal TLR7 ligation). We looked at activation markers on $D488^+$ $CD11c^+$ lung cells *in vivo*, post-i.n. immunization, but preliminary data showed there was no differences in expression of MHC II and CD86 compared to unimmunized mice.

In summary, although inactive TMV conjugates do not appear to induce DC activation, TMV conjugation to the whole protein antigens increased their uptake into splenic and mucosal B cells, BMDCs, as well lung DCs *in vivo*. This process seemed dependent on the vaccine particulate's size and characteristics, and not due to any TMV-specific mechanism. While AMs were the primary cell of uptake in the lung for TMV, TMV-F1+TMV-V, and rF1+rV, the dual vaccine conjugates were consistently taken up by less AMs and more DCs compared to unconjugated protein and TMV alone. Our data suggests that rTMV is an efficient vaccine delivery platform by targeting antigens to APCs, even in the mucosal respiratory tract where overcoming immune tolerance is challenging (Neutra and Kozlowski, 2006). TMV conjugation to protein antigens can increase their uptake into murine DCs, indicating potential for antigen-specific T-cell proliferation and long-term vaccine-induced protection.

References

Allie SR, Bradley JE, Mudunuru U, Schultz MD, Graf BA, Lund FE, Randall TD (2019). The establishment of resident memory B cells in the lung requires local antigen encounter. *Nature Immunology.* 20: 97-108.

Arnaboldi PM, Sambir M, D'Arco C, Peters LA, Seegers JF, Mayer L, McCormick AA, Dattwyler RJ (2016). Intranasal delivery of a protein subunit vaccine using a tobacco mosaic virus platform protects against pneumonic plague. *Vaccine.* 34: 5768-5776.

Balique F, Colson P, Barry AO, Nappez C, Ferretti A, Moussawi KA, Ngounga T, Lepidi H, Ghigo E, Mege JL, Lecoq H, Raoult D (2013). Tobacco mosaic virus in the lungs of mice following intra-tracheal inoculation. *PloS One.* 8: e54993.

Banik S, Mansour AA, Suresh RV, Wykoff-Clary S, Malik M, McCormick AA, Bakshi CS (2015). Development of a multivalent subunit vaccine against tularemia using tobacco mosaic virus (TMV) based delivery system. *PloS One.* 10: e0130858.

Do Y, Didierlaurent AM, Ryu S, Koh H, Park CG, Park S, Perlin DS, Powell BS, Steinman RM (2012). Induction of pulmonary mucosal immune responses with a protein vaccine targeted to the DEC-205/CD205 receptor. *Vaccine.* 30: 6359-6367.

Do Y, Park CG, Kang YS, Park SH, Lynch RM, Lee H, Powell BS, Steinman RM (2008). Broad T cell immunity to the LcrV virulence protein is induced by targeted delivery to DEC-205/CD205-positive mouse dendritic cells. *European Journal of Immunology.* 38: 20-29.

Dong Y, Arif AA, Poon GF, Hardman B, Dosanjh M, Johnson P (2016). Generation and identification of GM-CSF derived alveolar-like macrophages and dendritic cells from mouse bone marrow. *Journal of Visualized Experiments : JoVE.* (112). doi: 10.3791/54194.

Foged C, Sundblad A, Hovgaard L (2002). Targeting vaccines to dendritic cells. *Pharmaceutical Research.* 19: 229-238.

Gonzalez MJ, Plummer EM, Rae CS, Manchester M (2009). Interaction of cowpea mosaic virus (CPMV) nanoparticles with antigen presenting cells in vitro and in vivo. *PloS One.* 4: e7981.

Griffiths KL, Ahmed M, Das S, Gopal R, Horne W, Connell TD, Moynihan KD, Kolls JK, Irvine DJ, Artyomov MN, Rangel-Moreno J, Khader SA (2016). Targeting dendritic cells to accelerate T-cell activation overcomes a bottleneck in tuberculosis vaccine efficacy. *Nature Communications.* 7: 13894.

Kataoka K, Fukuyama Y, Briles DE, Miyake T, Fujihashi K (2017). Dendritic cell-targeting DNA-based nasal adjuvants for protective mucosal immunity to streptococcus pneumoniae. *Microbiology and Immunology.* 61: 195-205.

Kemnade JO, Seethammagari M, Collinson-Pautz M, Kaur H, Spencer DM, McCormick AA (2014). Tobacco mosaic virus efficiently targets DC uptake, activation and antigen-specific T cell responses in vivo. *Vaccine.* 32: 4228-4233.

Khalil SM, Tonkin DR, Snead AT, Parks GD, Johnston RE, White LJ (2014). An alphavirus-based adjuvant enhances serum and mucosal antibodies, T cells, and protective immunity to influenza virus in neonatal mice. *Journal of Virology.* 88: 9182-9196.

Kuipers H and Lambrecht BN (2004). The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma. *Current Opinion in Immunology.* 16: 702-708.

Kumagai Y, Takeuchi O, Kato H, Kumar H, Matsui K, Morii E, Aozasa K, Kawai T, Akira S (2007). Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. *Immunity.* 27: 240-252.

Kunda NK, Somavarapu S, Gordon SB, Hutcheon GA, Saleem IY (2013). Nanocarriers targeting dendritic cells for pulmonary vaccine delivery. *Pharmaceutical Research.* 30: 325-341.

Lacasse P, Denis J, Lapointe R, Leclerc D, Lamarre A (2008). Novel plant virus-based vaccine induces protective cytotoxic T-lymphocyte-mediated antiviral immunity through dendritic cell maturation. *Journal of Virology.* 82: 785-794.

Lambrecht BN, Prins JB, Hoogsteden HC (2001). Lung dendritic cells and host immunity to infection. *The European Respiratory Journal.* 18: 692-704.

Mallajosyula JK, Hiatt E, Hume S, Johnson A, Jeevan T, Chikwamba R, Pogue GP, Bratcher B, Haydon H, Webby RJ, McCormick AA (2014). Single-dose monomeric HA subunit vaccine generates full protection from influenza challenge. *Human Vaccines & Immunotherapeutics.* 10: 586-595.

Mallajosyula JK, Jeevan T, Chikwamba R, Webby RJ, McCormick AA (2016). A single dose TMV-HA vaccine protects mice from H5N1 influenza challenge. *International Journal of Vaccine Research.* 1: 6.

Mansour AA, Banik S, Suresh RV, Kaur H, Malik M, McCormick AA, Bakshi CS (2018). An improved tobacco mosaic virus (TMV)-conjugated multiantigen subunit vaccine against respiratory tularemia. *Frontiers in Microbiology.* 9: 1195.

McCormick AA, Corbo TA, Wykoff-Clary S, Nguyen LV, Smith ML, Palmer KE, Pogue GP (2006). TMV-peptide fusion vaccines induce cell-mediated immune responses and tumor protection in two murine models. *Vaccine.* 24: 6414-6423.

McCormick AA and Palmer KE (2008). Genetically engineered tobacco mosaic virus as nanoparticle vaccines. *Expert Review of Vaccines.* 7: 33-41.

McCormick AA, Shakeel A, Yi C, Kaur H, Mansour AM, Bakshi CS (2018). Intranasal administration of a two-dose adjuvanted multi-antigen TMV-subunit conjugate vaccine fully protects mice against francisella tularensis LVS challenge. *PloS One.* 13: e0194614.

Neutra MR and Kozlowski PA (2006). Mucosal vaccines: The promise and the challenge. *Nature Reviews.Immunology.* 6: 148-158.

Neyt K and Lambrecht BN (2013). The role of lung dendritic cell subsets in immunity to respiratory viruses. *Immunological Reviews.* 255: 57-67.

Ruane D, Do Y, Brane L, Garg A, Bozzacco L, Kraus T, Caskey M, Salazar A, Trumpheller C, Mehandru S (2016). A dendritic cell targeted vaccine induces long-term HIV-specific immunity within the gastrointestinal tract. *Mucosal Immunology.* 9: 1340-1352.

Rueckert C and Guzman CA (2012). Vaccines: From empirical development to rational design. *PLoS Pathogens.* 8: e1003001.

Runco LM, Myrczek S, Bliska JB, Thanassi DG (2008). Biogenesis of the fraction 1 capsule and analysis of the ultrastructure of yersinia pestis. *Journal of Bacteriology.* 190: 3381-3385.

Schyns J, Bureau F, Marichal T (2018). Lung interstitial macrophages: Past, present, and future. *Journal of Immunology Research.* 2018: 5160794.

Smith ML, Lindbo JA, Dillard-Telm S, Brosio PM, Lasnik AB, McCormick AA, Nguyen LV, Palmer KE (2006). Modified tobacco mosaic virus particles as scaffolds for display of protein antigens for vaccine applications. *Virology.* 348: 475-488.

Stary G, Olive A, Radovic-Moreno AF, Gondek D, Alvarez D, Basto PA, Perro M, Vrbanac VD, Tager AM, Shi J, Yethon JA, Farokhzad OC, Langer R, Starnbach MN, von Andrian UH (2015). VACCINES. A mucosal vaccine against chlamydia trachomatis generates two waves of protective memory T cells. *Science (New York, N.Y.).* 348: aaa8205.

Suda T, McCarthy K, Vu Q, McCormack J, Schneeberger EE (1998). Dendritic cell precursors are enriched in the vascular compartment of the lung. *American Journal of Respiratory Cell and Molecular Biology.* 19: 728-737.

Teh YC, Ding JL, Ng LG, Chong SZ (2019). Capturing the fantastic voyage of monocytes through time and space. *Frontiers in Immunology.* 10: 834.

von Garnier C, Blank F, Rothen-Rutishauser B, Goethert JR, Holt PG, Stumbles PA, Strickland DH (2017). Identification and characterization of a dendritic cell precursor in parenchymal lung tissue. *American Journal of Respiratory Cell and Molecular Biology.* 56: 353-361.

Wakim LM, Smith J, Caminschi I, Lahoud MH, Villadangos JA (2015). Antibody-targeted vaccination to lung dendritic cells generates tissue-resident memory CD8 T cells that are highly protective against influenza virus infection. *Mucosal Immunology.* 8: 1060-1071.

Wang H, Peters N, Laza-Stanca V, Nawroly N, Johnston SL, Schwarze J (2006). Local CD11c+ MHC class II- precursors generate lung dendritic cells during respiratory viral infection, but are depleted in the process. *Journal of Immunology (Baltimore, Md.: 1950).* 177: 2536-2542.

Yu YR, O'Koren EG, Hotten DF, Kan MJ, Kopin D, Nelson ER, Que L, Gunn MD (2016). A protocol for the comprehensive flow cytometric analysis of immune cells in normal and inflamed murine non-lymphoid tissues. *PloS One.* 11: e0150606.

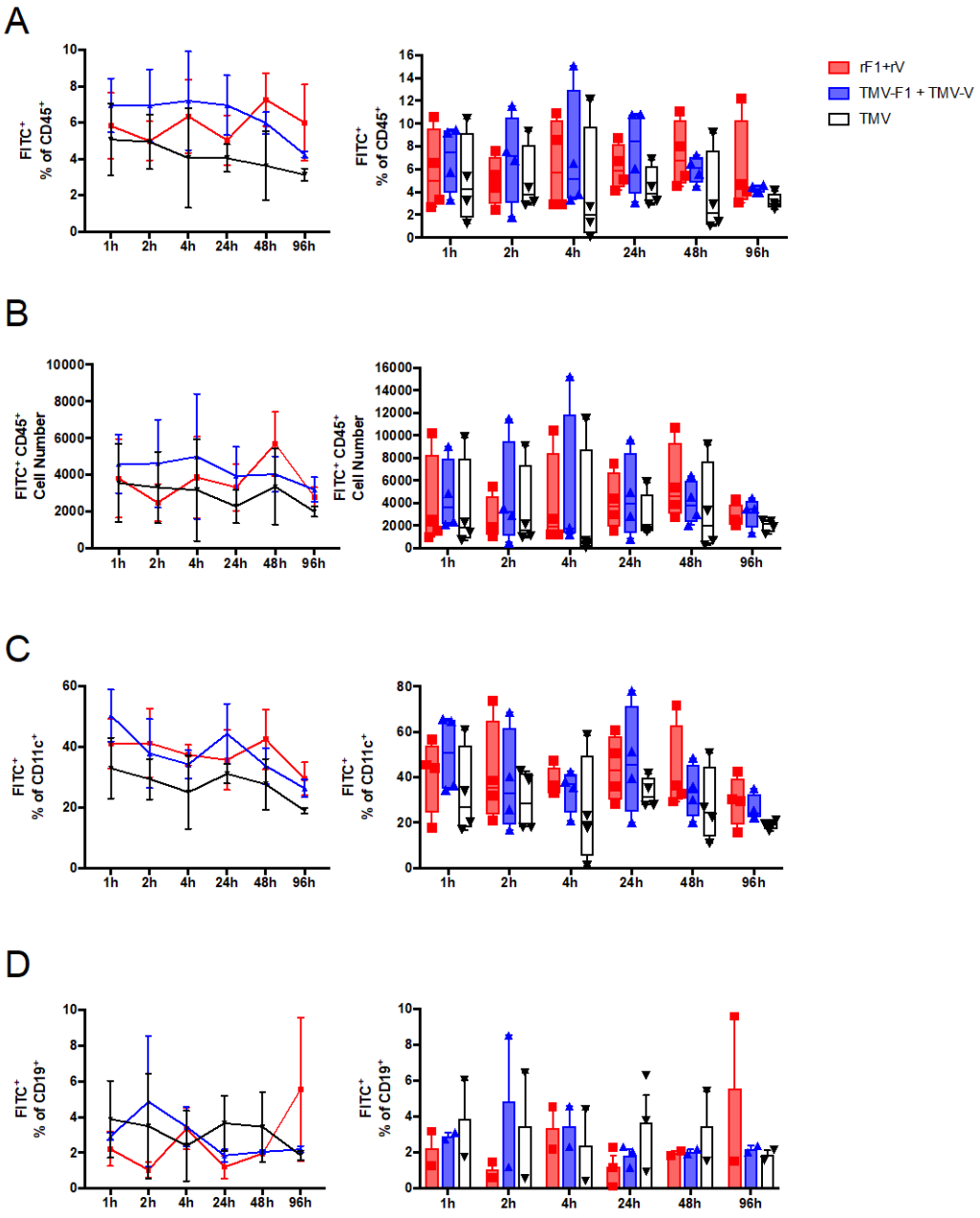

Fig 1. Protein antigens, TMV conjugates, and TMV are taken up by <16% of lung CD45$^+$ cells *in vivo*, a majority of which are CD11c$^+$. Uptake of DyLight™ 488-labeled proteins (FITC$^+$) proteins into the lung after 1, 2, 4, 24, 48, and 96 hours post- i.n. immunization, shown as the percent of CD45$^+$ cells (A) and the absolute number of FITC$^+$ CD45$^+$ cells (B). Uptake of FITC$^+$ proteins, as a percent of the total CD11c$^+$ cells (C) and total CD19$^+$ cells (D) in the lung, was also measured by flow cytometry. For each panel, a line and bar graph of the same data are shown to highlight overall trends (line) and variation between the groups (bars). For each graph, significance was analyzed by repeated-measures two-way ANOVA, with Tukey's multiple comparisons test, however, at no time point were the groups statistically different from one another. All errors bars represent mean±SEM.

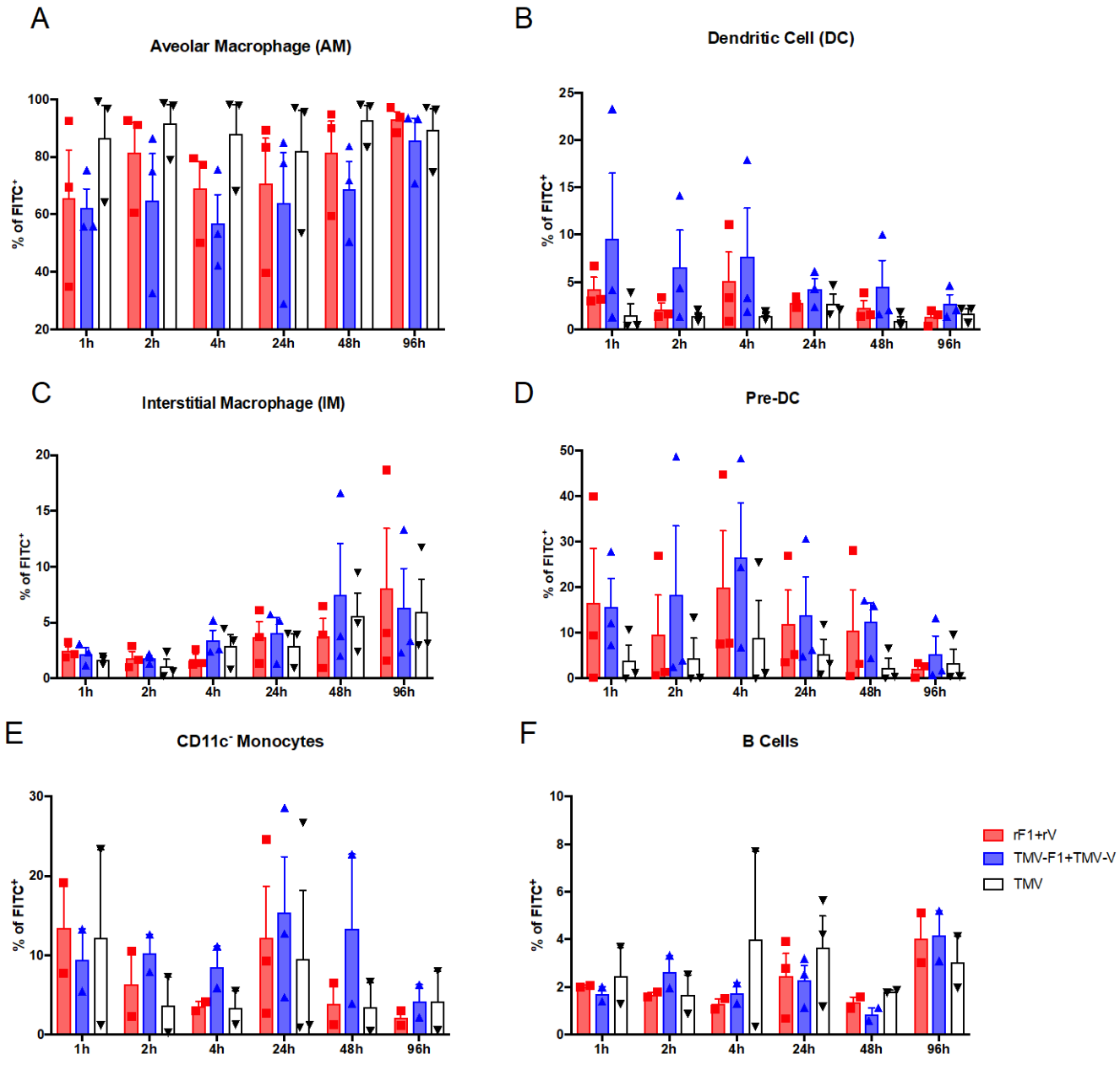

Fig 2. Protein antigens, TMV conjugates, and TMV are mostly taken up by AMs, but conjugates are associated with more lung DCs at earlier time points than proteins and TMV. Uptake of DyLight™ 488-labeled ($FITC^+$) proteins into different lung immune cell types after 1, 2, 4, 24, 48, and 96h post- i.n. immunization. Percent of the $FITC^+$ cells that represented AMs (A), conventional DCs (B), IMs (C), pre-DC (D), CD11c- monocytes (E), and $CD19^+$ B cells (F) were measured by flow cytometry. For each graph, differences between the groups were analyzed by a repeated-measures two-way ANOVA, with Tukey's multiple comparisons test, however, at no time point were the groups statistically different from one another. All errors bars represent mean±SEM.

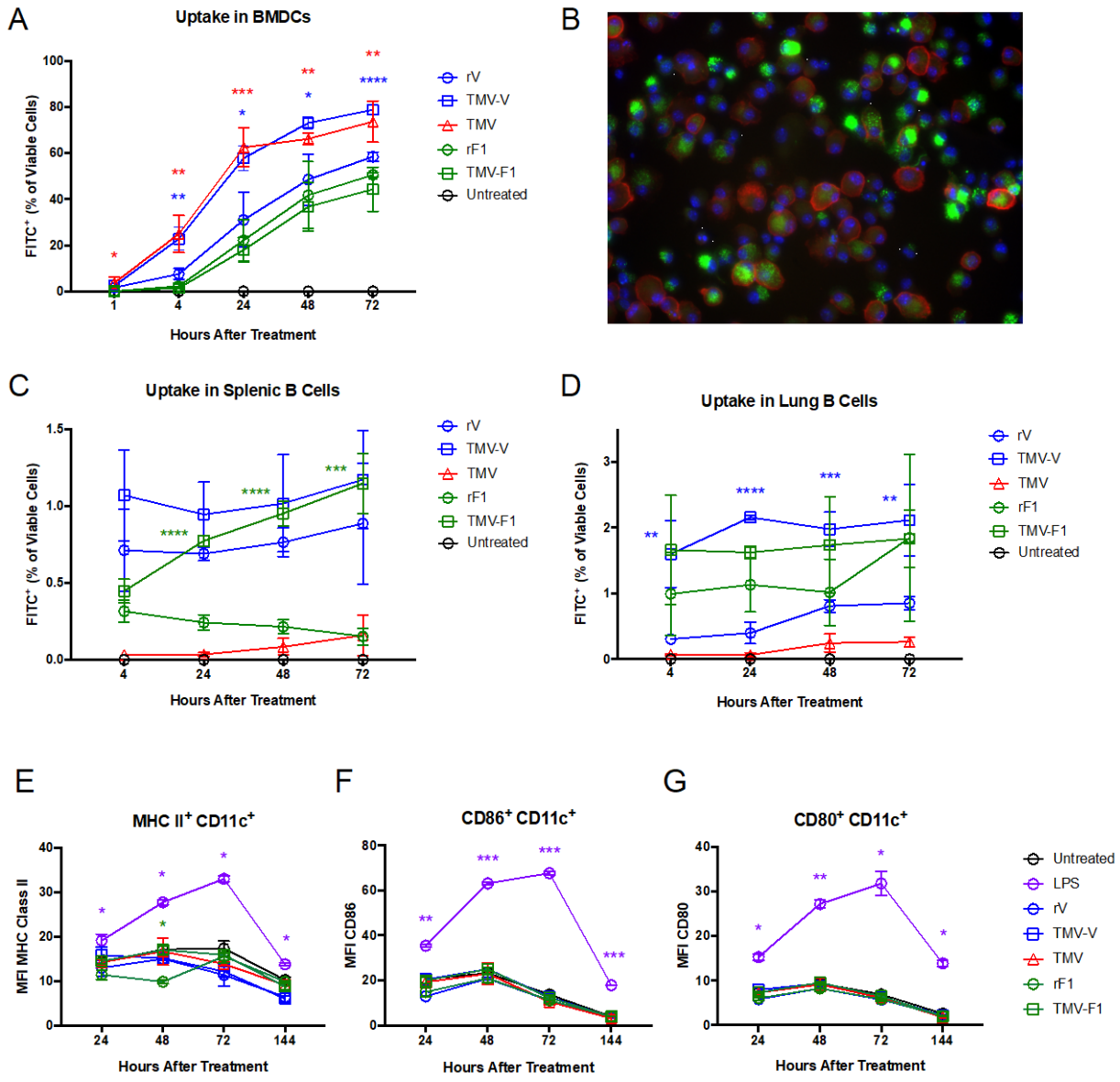

Fig 3. TMV conjugation promotes uptake of F1 or V antigens in BMDCs and B cells *in vitro* but does not induce costimulatory molecules on BMDCs. (A) Uptake of DyLight™488-labeled ($FITC^+$) proteins into BMDCs *in vitro* as measured by flow cytometry. Blue and red asterisks indicate statistical significance for TMV-V vs rV and TMV vs TMV-F1, respectively. (B) Image of $CD11c^+$ BMDCs (red) after 1h incubation with 488-labeled TMV (green) with DAPI-stained nuclei (20x). Uptake of $FITC^+$ proteins into splenic B cells (C) and lung B cells (D) *ex vivo* as measured by flow cytometry. Green and blue asterisks indicate statistical significance for TMV-F1 vs rF1 and TMV-V vs rV, respectively. Mean fluorescence intensity (MFI) of surface MHC II (E), CD86 (F), and CD80 (G) on $CD11c^+$ BMDCs *in vitro* as measured by flow cytometry. Purple and green asterisks indicate statistical significance for LPS vs untreated cells (control) and TMV-F1 vs rF1, respectively. For each graph, significance was analyzed by multiple t tests. **** $p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$. All errors bars represent mean±SD.

Chapter 3. Specific Aim 2

AIM 2:
Evaluating Efficacy and Correlates of Protection of a Single-Dose Intranasal TMV Conjugate Vaccine for Pneumonic Plague

Aim 2a: Test the hypothesis that a single intranasal administration of TMV vaccine is protective against high-dose pneumonic plague infection

Aim 2b: Test the hypothesis that single administration of TMV vaccine prevents or reduces dissemination of *Y. pestis* in the infected host

Aim 2c: Test the hypothesis that pre-challenge antigen-specific titers are a correlate of TMV vaccine-induced protection

Aim 2d: Test the hypothesis that the TMV vaccine induces a T_H1 or T_H17 specific response in the infected host

1.INTRODUCTION

Yersinia pestis is the causative agent of plague, an acute febrile illness with a high mortality if left untreated. Most natural infections occur through the bite of infected fleas, resulting in bubonic plague, where lymphohematogenous dissemination of the bacteria typically results in infection, and characteristic swelling, of draining lymph nodes (termed buboes). In rare cases, direct blood dissemination can occur resulting septicemic plague, which is often associated with a blackening of the hands and feet (necrosis) and a very high mortality rate if treatment is not administered rapidly (Perry and Fetherston, 1997). In addition, *Y. pestis* can disseminate to the lung and be transmitted via inhalation of infectious aerosol droplets, which enables person-to-person transmission. Pneumonic plague is one of the deadliest diseases known to mankind; death is almost certain if antibiotic treatment is delayed past the first 24 hours. Plague is still endemic in many regions of the world, particularly in central Africa and Madagascar where outbreaks are not uncommon. Madagascar experienced a large urban outbreak in 2017, with 76% of the nearly 2,400 reported cases were confirmed, probable, or suspected pneumonic plague (Andrianaivoarimanana *et al.*, 2019; Nguyen *et al.*, 2018; Rabaan *et al.*, 2019; Randremanana *et al.*, 2019; WHO, 2017). Among confirmed pneumonic cases, the fatality rate was 25%, and over 80% of confirmed or probable pneumonic cases were concentrated in two major cities (Randremanana *et al.*, 2019). Despite sensitivity of *Yersinia ssp.* to a range of antibiotics, the recent increases in the number of worldwide annual cases, findings of multi-drug resistant (MDR) strains from clinical isolates, and potential for local outbreaks to evolve into a pandemic – whether natural or intentional – have prompted global efforts to develop

effective plague vaccines. Despite decades of ongoing research, an FDA-approved vaccine does not currently exist.

Most current efforts are focused on developing recombinant subunit vaccines against pneumonic plague. Top vaccine candidates include the well-characterized F1 and LcrV (V) antigens, the *Y. pestis* capsule protein and the tip protein of the type III secretion system (T3SS), respectively. Despite its success in small animal models (Anderson *et al.*, 1998; Bowen *et al.*, 2019; Heath *et al.*, 1998; Jones *et al.*, 2000; Williamson *et al.*, 1995; Williamson *et al.*, 1996; Williamson *et al.*, 1997), intramuscular (i.m.) administration of F1/V generated mixed results in NHP models of pneumonic plague (Smiley, 2008). Over the last few decades, numerous approaches have sought to improve the efficacy of the F1/V-vaccines (Demeure *et al.*, 2019; Sun and Singh, 2019; Titball and Williamson, 2004) to generate rapid mucosal and systemic immunity against plague.

We previously reported that complete protection against lethal pneumonic challenge with *Y. pestis* CO92pgm- could be achieved using three doses (prime + two boosts) of an intranasal (i.n.) vaccine comprised of V and F1 covalently linked to recombinant Tobacco Mosaic Virus (TMV) (Arnaboldi *et al.*, 2016). TMV is a single-strand RNA virus that cannot replicate in mammalian cells. Despite this, TMV has adjuvant-like properties, targeting antigen to dendritic cells, upregulating MHC and co-stimulatory molecules, and capable of inducing antigen-specific $CD8^+$ T cells *in vivo* in mice (Kemnade *et al.*, 2014). Due to ubiquitous exposure, most, if not all humans have serum antibodies to the virus, regardless of their tobacco use (Liu *et al.*, 2013). In addition to our own studies, others have demonstrated that TMV-based vaccines also induce protective immunity against tularemia

(Banik *et al.*, 2015)(Mansour *et al.*, 2018) , as well as influenza H1N1 (Mallajosyula *et al.*, 2014) and H5N1 (Mallajosyula *et al.*, 2016).

Although the 3-dose vaccine regimen was protective, it would be impractical to use in the setting of an outbreak. In the present study, we tested i.n. administration of a single dose of TMV-F1+TMV-V (abbr. TMV-F1/V), with no other adjuvant, 5 weeks prior to challenge with a pigmentation locus-deficient (pgm-) strain of Y. pestis (CO92pgm−). Mice immunized with the dual antigen TMV conjugate mixture rapidly cleared bacteria from distal tissues and reduced inflammatory cytokine production compared to mice immunized with unconjugated rF1+rV. Our study showed that a single dose of vaccine provided protection to mice that was superior to unconjugated protein (rF1+rV), irrespective of comparable F1 antibody titers prior to challenge.

2.MATERIALS AND METHODS

2.1 Bacterial strains, recombinant protein, and TMV

Yersinia pestis CO92pgm− was a generous gift of Dr. James Bliska (Stonybrook University, NY), and was cultivated as previously described (Arnaboldi *et al.*, 2016). Cloning, expression, and purification of rF1 and rLcrV (rV), and preparation of TMV-F1 and TMV-V conjugate vaccines were performed as described previously (Arnaboldi *et al.*, 2016). Briefly, rF1 and rLcrV (rV) proteins were conjugated independently to rTMV by EDC mediated amine conjugation to generateTMV-F1 and TMV-V. Conjugation efficacy was measured by SDS-PAGE, with little or no free rF or rV measurable by using the same reaction conditions. The TMV is inactivated via binary ethyleneimine (BEI) prior to the antigen conjugation process (EDC mediated amine conjugation) and has no replication

capacity in plant or mammalian cells. The TMV-F1 and TMV-V conjugates used in this study were from the same lot.

2.2 Mice and intranasal vaccination

Adult (6-week-old) male and female C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, Maine), and maintained by the husbandry staff of the Department of Comparative Medicine at New York Medical College. All experiments were conducted with the approval of the New York Medical College Institutional Animal Care and Use Committee (IACUC). All mice were vaccinated at 6 weeks old and challenged at 11 weeks of age. Equal numbers of male and female mice were tested in all groups. Mice were anesthetized using ketamine/xylazine cocktail in order to preform i.n. vaccination with either: 10μg of TMV-V+10μg of TMV-F1 (total of 20 μg) or 5μg rV+5μg rF1 (total of 10μg) in 40μL volume of saline. TMV-conjugated vaccines contain equivalent concentrations of TMV and recombinant protein (5μg TMV+5μg protein). Mice were vaccinated with 10μg of TMV-conjugate and 5 μg of recombinant protein to ensure molar equivalency of the *Y. pestis* antigens between the two vaccines. Control mice were unvaccinated. We have previously observed that TMV-vaccination alone does not impart protection to mice (Banik *et al.*, 2015). Therefore, we did not include a TMV-alone vaccine group to minimize the number of animals needed for study. Working concentrations of vaccines were prepared from freezer stocks on the day of immunization.

2.3 Bacterial Challenge

All mice were challenged on day 35 post-immunization with *Y. pestis* CO92pgm− as previously described (Arnaboldi *et al.*, 2016). Briefly, *Y. pestis* CO92pgm− was grown to an OD_{620} of 0.9 in heart infusion broth supplemented with 2.5mM $CaCl_2$ and 0.2% Xylose at 37°C in a shaking incubator (225 RPM). Culture medium was removed by centrifugation and the bacterial pellet was washed 3× with sterile saline. Bacteria were resuspended in normal saline to an OD_{620} of 1.1, assuming a concentration of $1x10^9$ CFU. The LD50 of *Y. pestis* CO92pgm− in C57Bl/6J mice was previously determined to be $2.5x10^4$ CFU (Arnaboldi *et al.*, 2016). Bacteria were diluted accordingly, and anesthetized mice were intranasally (i.n.) administered 10 LD50 or 100 LD50 in 50µL saline in a class II biosafety cabinet. 10-fold dilutions of the bacteria were streaked on HI agar and incubated for 2 days at 28°C to confirm the number of bacterial CFU administered for each challenge. After challenge (D0), mice were weighed and checked daily for signs of morbidity (ruffled fur, hunched posture, reduced activity, weight loss). Mice that lost more than 20% of their pre-challenge body weight, or were severely moribund, were euthanized. Survival was monitored for 21 days, and mice were euthanized according to approved IACUC guidelines.

2.4 Tissue collection

In a separate experiment, mice that were immunized and challenged as described above (2.3) were euthanized daily for the first 5 days following challenge and lungs, spleen, liver, and blood were collected (n=6/group). The left lobe of the lung, half of the spleen and ~150mg of liver tissue were placed in 1.5ml centrifuge tubes filled with ~100uL of ceria stabilized zirconium oxide beads (2mm diameter, Next Advance, Inc.) and 0.5mL of sterile PBS on ice. The tissues were homogenized using a BioSpec Mini-Beadbeater™. Six 10-

fold dilutions of the homogenates were generated in sterile PBS, and 10uL of each dilution was pipetted onto replicate HI plates (6 dilutions per petri dish). Plates were incubated for 2 days at 28°C. CFUs were calculated as described previously (Wang, N. *et al.*, 2011). Counts per dilution were averaged from plates per mouse. Briefly, calculations were performed using the formula: CFU/tissue= CFU/100uL x dilution factor x 0.5mL. This number was then divided by 0.5 for the spleen and lung, and 0.2 for the liver (the percentage of the total organ mass used for homogenization). Therefore, if no CFUs were recovered on HI plates, these values were assigned for the indicated mouse. For blood plating, ~500uL of whole blood was collected, and dilutions were plated as described above. Because whole tissues were not processed, the limit of detection for each tissue was calculated as follows: lung 10 CFUs/organ, liver 25 CFUs/organ, spleen 10 CFUs/organ, blood 100 CFUs/mL. Serum was collected from the remaining blood by centrifugation at 2500xg for 15 min and stored at -20°C. Remainder of tissue homogenates were centrifuged at 10,000rpm x 10 min, and the supernatants were aliquoted and stored at -80°C.

2.5 Histology

Formalin fixed lung, liver, and spleen tissue was processed, paraffin-embedded, and stained with hematoxylin and eosin (H&E). For quantification of pathology, tissues were scored in a blinded fashion for severity of inflammation and necrosis (0-4) (see Appendix II Table S1 for scoring). Imaging of the sections was performed on a Revolve microscope (ECHO, San Diego, CA). Images were collected at 4x magnification, with exception of naïve lung (2x).

2.6 Antibody measurements

Blood was collected via retroorbital bleed twice prior to challenge, at days 14-18 and 28-32 post-immunization. Sera were collected by centrifugation at 2500xg for 15 min and stored at -20°C. 96 well ELISA plates (Nunc MaxiSorp™ flat bottom or Immulon® 2 HB Flat Bottom MicroTiter™) were coated with rV or rF1 (2 μg/ml) in 0.1M Carbonate Buffer, pH 9.4 overnight at 4°C. Plates were blocked with 1% BSA in PBS for 1h at RT, and washed 3x with 0.05% Tween in PBS. Sera were diluted in blocking buffer and were incubated for 1h at RT. Plates were washed 3x again, and HRP-labeled goat anti-mouse Ig heavy and light (H+L) chain or IgG2b (Southern Biotech) diluted 1:5000 in blocking buffer was added to each well for 1h at RT. The plates were washed and developed with TMB substrate (KPL) for 30 min at RT. The reaction was stopped by addition of 2N sulfuric acid, and absorbance was read at 450nm and 570nm on a SpectraMax Plus 384 microplate reader (Molecular Devices). Absorbance was plotted vs. reciprocal serum dilution and 50% maximal binding titers were calculated using GraphPad Prism 7.0 software.

2.7 Cytokine measurements

Cytokine concentrations in lung and spleen supernatant from tissue homogenates were evaluated with the Invitrogen/eBioscience uncoated ELISA kits according to manufacturer's instructions: TNF-α (cat # 88-7324-77), IL-17 (cat # 88-7371-77), and IFN-γ (cat # 88-7314-77). All homogenates were diluted in assay diluent. Samples were analyzed in duplicate.

2.8 Statistical analyses

Statistical analyses were performed using Prism 7.0 GraphPad software. Survival curves were compared using a log-rank (Mantel-Cox) test. Differences in means were analyzed using an unpaired student's t-test or one-way ANOVA. Multiple t tests were used to analyze mean differences between ELISA serum dilutions. Linear correlations were analyzed by Pearson's correlation coefficient. P values of <0.05 were considered statistically significant.

3.RESULTS

A single i.n. administration of TMV-F1+TMV-V enhances survival and bacterial clearance compared to recombinant protein alone

We previously demonstrated that conjugating V and F1 to a rTMV vaccine delivery platform imparts complete protection against lethal challenge with 10 LD50 of *Y. pestis* CO92pgm- after a minimum of three doses (Arnaboldi *et al.*, 2016) If this vaccine were to be used as a countermeasure for a potential biological attack, fewer doses would be optimal. In the present study, we evaluated a single dose of TMV-F1/V against challenge with both 10 and 100 LD50 Y. pestis CO92pgm−.

A single i.n. dose of TMV-F1+TMV-V imparted a mean survival of 88% (23/26 mice) over two studies following i.n. challenge with 100 LD50 of *Y. pestis* CO92pgm−. This was significantly higher than the survival rate of mice immunized with rF1+rV- (11.5%, 3/26) or unimmunized mice (11.5%, 3/26) (Fig 1A). Similar results were obtained when a challenge dose of 10 LD50 was utilized (Appendix II Fig S1). Importantly, conjugation of V and F1 to TMV was required for protection as mice immunized with rF1+rV mixed with TMV demonstrated similar protection to mice immunized with rF1+rV alone (Appendix II

Fig S1). Mice vaccinated with TMV-V+TMV-F1 showed initial weight loss. However, weight loss was significantly reduced in TMV conjugate-immunized mice compared to unimmunized mice as early as day 3 (D3) post-infection (PI) and was reduced compared to mice immunized with rF1+rV, though this difference was not statistically significant (Fig 1B). Morbidity, defined as the subjective condition and activity level of the mice, was also reduced compared to mice that received rF1+rV. Three unimmunized mice, all female, and three rF1+rV immunized mice, two females and one male survived the infection. Three TMV-conjugate immunized mice, two males and one female, succumbed to the infection. Although three unimmunized mice managed to survive infection, these mice took longer to recover compared to rF1+rV- and TMV-conjugate immunized mice, as evidenced by a delay in returning to pre-challenge weight (Appendix II Fig S2). It has been reported that up to 50% of naïve C57Bl/6J mice can survive a similar i.n. dose of a nonpigmented *Y. pestis* strain (Olson and Anderson, 2019), however, the reason for this is not fully understood, as we have observed lower doses (e.g. 10 LD50) remain fully lethal (Appendix II Fig S1).

Lungs, spleens, and livers were collected from mice during the first five days of infection to determine the kinetics of bacterial clearance. Bacteremia was also assessed using peripheral blood. In the lungs, the mean bacterial burden decreased in TMV-F1+TMV-V immunized mice daily; the same trend was not observed in unimmunized or rF1+rV immunized mice (Fig 2A). In the liver and spleen, bacterial burden peaked at D3 PI in TMV-F1+TMV-V immunized mice, and bacteria were cleared by D5 (Fig 2B, 2C). In rF1+rV immunized mice and unimmunized mice, bacterial burden peaked a day later (D4), and also began to decrease by D5 (Fig 2B, 2C); however, most of these mice had succumbed to infection by D5 (Fig 1A). Bacterial burdens were significantly higher in rF1+rV immunized

mice D4-D5 in the spleen, D4 in the liver, and D4 in the lung compared to TMV-F1+TMV-V immunized mice. Bacterial burdens were significantly higher in unimmunized mice at D3-D4 in liver, D2-D5 in the spleen (D3 p=0.05), and D3-D4 in the lungs compared to TMV-F1+TMV-V immunized mice. Bacterial burdens were similar in all rF1+rV immunized and unimmunized mice. In peripheral blood, *Y. pestis* was not detected in any TMV-F1/V immunized mice, however, bacteria were found in the bloodstream of the other groups of mice beginning on D3 PI (Fig 2D). Statistical analyses were not performed since bacteremia was only detected in a few mice.

We performed a histological analysis of lung, liver, and spleen tissue obtained from the same mice used for bacterial burden studies. Inflammation was scored in a blinded fashion for severity of tissue necrosis, and edema (Fig 3A, Appendix II Table S2) in comparison to a naïve mouse (Fig 3B). Differences in disease severity were most apparent at D5 PI (Fig 3C-E). On this day, unimmunized mice showed signs of severe inflammation, as evidence by the loss of rich red pulp in the spleen, inflammatory foci in the liver, and dense neutrophilic infiltrates and edema throughout the lung tissue (Fig 3C). rF1+rV immunized mice demonstrated similar disease severity; lung tissue was marked by edema, perivascular inflammation, and loss of the alveolar architecture; the liver contained several inflammatory foci; and spleen germinal centers (GC) were distorted by large neutrophilic lesions (Fig 3D). TMV-F1/V immunized mice showed signs of moderate inflammation at D5 in the lung, but not necrosis, and overall inflammation was significantly reduced in the spleen and liver compared to the other groups (Fig 3A, 3E). A time course of D1-D4 histology for each tissue is presented in Appendix II figures S3-S5. At no point was lung tissue severity of TMV-F1/V immunized mice significantly different from the other groups (Appendix II Fig

S3, Fig 3A). By D4 and D5, disease severity in TMV-F1/V immunized mice was significantly reduced in the spleen (Appendix II Fig S4, Fig 3A) and liver (Appendix II Fig S5, Fig 3A), as compared to either unimmunized or rF1+rV immunized mice. This would indicate that, here, clearance of bacteria first occurs systemically, while clearance and tissue repair in the lung occurs more slowly, and likely extends beyond 5 days.

A stronger F1-specific humoral response is associated with survival

Serum antibody production was limited following single dose immunization. Only antibodies against F1 were detected at 2- and 4-weeks post-immunization (Fig 4A). V-antigen-specific antibody titers were below the limits of detection in both TMV-F1+TMV-V and rF1+rV immunized mice. V-antigen specific antibodies were not detected by ELISA in serum or tissue homogenates even after infection, indicating anti-V antibodies may not be necessary for survival in the presence of anti-F1 antibodies (data not shown). Significantly higher mean total F1 antibody titers were observed in mice immunized with TMV-F1+TMV-V compared to mice vaccinated with rF1+rV at both week 2 and 4 post-vaccination, with a statistically greater difference seen at week 4 (Fig 4A). Interestingly, this was not due to an increase in F1 titers among TMV-F1+TMV-V immunized mice, but rather a decline in these titers in rF1+rV immunized females from the second to fourth week. At week 2, there was a higher mean F1 titer in female mice immunized with rF1+rV as compared to males ($p=0.05$), however, antibody titers diminished from week 2 to 4 and this was no longer apparent. F1 antibody titers were similar between male and female mice immunized with TMV-F1+TMV-V at either timepoint (Fig 4A). Mice that survived infection had significantly higher week 2 F1 titers compared to mice that died (Fig 4B). Interestingly, a small proportion of surviving

TMV-F1+TMV-V immunized mice produced little anti-F1 antibody (titer <50), while surviving rF1+rV immunized mice all had relatively high F1 titers prior to infection. Correspondingly, there was a positive moderate correlation between this F1 titer and percent body weight for rF1+rV immunized (r^2=0.5214, $p<0.01$) and TMV-F1+TMV-V immunized mice (r^2=0.411, $p<0.01$) at D3 PI (Fig 4C). This was the timepoint when differences in weight loss between groups reached statistical significance (Fig 1B). Together this data showed that TMV-F1+TMV-Vimmunized mice had increased F1 titers prior to challenge, and higher F1 titers were moderately associated with survival.

We also evaluated antibody isotype levels D1-D5 PI. TMV-F1+TMV-V immunized mice demonstrated a rapid increase in anti-F1 IgG2b antibody within the first 5 days PI (Fig 5A-E). This increase was not observed in unimmunized mice or mice immunized with rF1+rV. Up until D2 PI, there was no difference in serum F1-specific IgG2b (Fig 5A-B); however, beginning D3 PI, significant differences could be seen between TMV-F1+TMV-V immunized mice and unimmunized mice (Fig5C-E). We also detected anti-F1 IgG1, but not IgG2a, in serum at D5 PI. Absorbance curves for IgG1 at D5 were virtually identical to D1 IgG2b (Fig 5A) and group differences were not significant (data not shown). Overall the data showed a strong trend between the quantity of pre-challenge F1-specific antibody and better survival outcomes. TMV-F1+TMV-V immunized mice exhibited faster anti-F1 IgG2b production upon infection relative to the other groups, likely indicating a larger pool of F1-specific memory B cells generated by immunization.

TMV-F1+TMV-V vaccinated mice have reduced IFN-γ, TNF-α, and IL-17 cytokines after challenge

In a pilot study examining cytokine expression in the lungs of vaccinated and unvaccinated mice in the first 72h after infection we observed a very large induction of inflammatory cytokines and chemokines. Strikingly, inflammatory cytokine and chemokine expression was reduced in mice vaccinated with TMV-F1+TMV-V compared to unvaccinated mice with rF1-rV vaccinated mice falling between (data not shown). We extended this observation by assessing production of IFN-γ, IL-17, and TNF-α in lung, spleen, and serum of mice after infection (Fig 6 and 7). IFN-γ production peaked at D2 PI in lungs of all three groups of mice but was reduced in TMV-F1+TMV-V immunized mice compared to rF1+rV immunized and unimmunized mice (Fig 6A, n.s.). IFN-γ levels progressively decreased in the lungs of all mice after D2 and were virtually undetectable by D5 PI (Fig 6A). In the spleen, IFN-γ peaked at D3 in unimmunized mice, decreasing thereafter, but levels remained elevated at D5; by comparison, IFN-γ levels were reduced in rF1+rV-vaccinated mice (n.s.) and were virtually undetectable in most TMV-F1+TMV-V immunized mice (Fig 6B). TNF-α was elevated at D1 in lung tissue of TMV-F1+TMV-V immunized and unimmunized mice, compared to rF1+rV- immunized mice (Fig 6C). In the spleen, TNF-α levels were similar between treatment groups at all timepoints assessed (Fig 6D), perhaps moderately reduced in TV-conjugate immunized mice at D4 compared to the other groups; however, total TNF-α levels in the spleen were at or below the average levels observed in naïve untreated mice (dotted line, Fig 6D). Very low concentrations of IL-17A were found in all tissues, compared to mean levels found in naïve unvaccinated, uninfected mice (dashed lines), with no discernable differences between immunization groups (Fig 6E-F).

Serum levels of IFN-γ and TNF-α were also elevated only in unimmunized and rF1+rV immunized mice beginning on D3 PI, highly suggesting the onset of sepsis in those mice. IFN-γ levels were highest at D3 PI and decreased steadily through D5; by comparison; IFN-γ was negligible in all but one TMV-F1+TMV-V immunized mouse (Fig 7A). Serum TNF-α levels were elevated in unimmunized mice at D3, D4, and D5 without decreasing, and at D4 in rF1+rV immunized mice, although this was not significantly different from the TMV-F1+TMV-V immunized group, where levels were undetectable in most mice (Fig 7B). These data suggest that the TMV-F1+TMV-V vaccine induces immunity marked by a highly regulated inflammatory environment immediately following challenge. In our model, the absence of a hyper-inflammatory environment appears more helpful than harmful and may actually be sparing the animals from tissue destruction, sepsis, and ultimately death.

4.DISCUSSION

Y. pestis remains a significant public health threat due to the potential for the natural or intentional spread of antibiotic resistant strains. Even in the presence of appropriate antibiotic treatment, mortality remains high. Stockpiled antibiotics will be of little use against MDR *Y. pestis*, necessitating the development of a preventative vaccine. Previously explored killed whole cell (KWC) and live attenuated vaccines for plague were discontinued in the U.S. because they either did not provide long-term protection, had negative side-effects, or were not protective against pneumonic plague (Smiley, 2008; Wang, X. *et al.*, 2013). A protein subunit vaccine consisting of F1+V was very effective at protecting mice from pneumonic plague but produced mixed results when tested in Cynomolgus macaques and African green monkeys, raising doubt that this formulation would produce widespread

protection in humans (Smiley, 2008). Additionally, parenteral administration of protein antigens induces only limited protection at mucosal surfaces, whereas, at least experimentally, mucosal introduction of such antigens provides strong immunity at both mucosal surfaces and systemically (Srivastava *et al.*, 2015; Woodrow *et al.*, 2012) .

We previously demonstrated that conjugating F1 and V to TMV induced complete protection against lethal challenge with i.n. dosing of 10 LD50 *Y. pestis* CO92pgm- after a minimum of three vaccine doses (Arnaboldi *et al.*, 2016). That study was limited to evaluating protection and antibody production in response to vaccination with TMV. In the current study, we evaluated against one vaccine dose and further extend our previous findings by evaluating dissemination, histopathology, the kinetics of bacterial clearance, and cytokine production. In the present study we found that a single i.n. administration of the TMV-conjugate vaccine protected 88% of mice challenged with 100 LD50 and 100% of mice challenged i.n. with 10 LD50 of *Y. pestis* CO92pgm-. rF1+rV immunized mice challenged with 10 LD50 of bacteria exhibited 40% protection while less than 10% of mice challenged with 100 LD50 survived. This suggests that with higher-dose challenges, rF1+rV i.n. immunization alone cannot control the infection any better than unvaccinated mice, supporting the addition of an adjuvant or carrier to improve the mucosal response to vaccination. Furthermore, we demonstrated for the first time that conjugation of the proteins to TMV was critical for vaccine protection, as co-administration of unconjugated rF1+rV mixed with TMV could not protect mice any better than those vaccinated with rF1+rV alone.

Some reports have suggested that pigmentation locus deficient (pgm-) strains of *Y. pestis* do not cause pneumonia when administered i.n., in contrast to fully virulent strains of *Y. pestis* (Galvan *et al.*, 2010; Lee-Lewis and Anderson, 2010). In the present study we find

that i.n. administration of *Y. pestis* CO92pgm- seeds the lung causing the development of pneumonia that also rapidly disseminates to other tissues, including the spleen and the lung resulting in fulminant bacteremia by D3 PI. In TMV-F1+TMV-V mice, systemic distribution of the bacteria is cleared by D5 and is associated with a significant decline in bacterial numbers in the lung. Surviving mice fully clear the infection. It is not clear why this discrepancy in the literature exists; however, differences among strains of *Y. pestis* used (KIM vs. CO92) (Galvan *et al.*, 2010; Lee-Lewis and Anderson, 2010), as well as differences in the volume of the i.n. dose may contribute to discordant outcomes (Olson and Anderson, 2019) .

Interestingly, following a single i.n. vaccination, antibodies were only detected against the F1 portion of the vaccine. In our previous study, we did observe serum antibodies to V (Arnaboldi *et al.*, 2016) , though the data here suggest multiple immunizations are required for this. Overall, antibody titers were low, with mean F1 titers between 1:100 and 1:125. F1 titers correlated with both a reduction in weight loss and survival. In addition, there was a rapid increase in anti-F1 antibody within the first five days of infection in TMV immunized mice compared to rF1+rV immunized mice, presumably from memory B cell populations. Despite this, it is not clear if pre-challenge antibody is protective in this model, or if it simply serves as an indication that a protective immune response has developed. Passive transfer of anti-F1 antibodies to naïve animals and generation of anti-F1 IgG following F1 immunization both lead to high survival rates in animal models of plague (Williamson and Oyston, 2013). The tendency for rF1 to form large molecular-weight polymers also aids in rapid recognition by innate-like B1b cells, resulting in a protective anti-

F1 antibody response in just 3 days (Levy *et al.*, 2018). However, despite a well-documented role for anti-F1 antibodies in protection from plague in mice, primate challenge demonstrated discordant survival results between Cynomolgus macaques (protected) and African green monkeys (poorly protected), which could not be explained by differences in antibody levels (Bashaw *et al.*, 2007; Pitt, 2004; Smiley, 2008). Thus, it is not clear if antibody plays a significant role in protection from pneumonic plague or is only a component of a more complex process. Since pneumonic plague in African green monkeys rapidly progresses to septicemia in as early as 3 days (Layton *et al.*, 2011), and F1/V-based vaccines could not fully protect this species against aerosolized *Y. pestis* in multiple trials (Smiley, 2008), further study is needed to determine this. Furthermore, F1 is not required for virulence, and F1- strains are still lethal and found in nature (Quenee *et al.*, 2008). This has led other researchers to suggest that mucosal and serum anti-V antibodies are the best correlates for survival against pneumonic plague in mice, as V antigen is a critical virulence factor (Reed and Martinez, 2006). Further study is needed to determine which components of host immunity are critical for rapid protection.

Recently, Bowen *et al.* reported a sex bias in protection against fully virulent pneumonic plague following s.c. immunization with rF1-V in Alhydrogel®. This vaccine better protected female mice (C57Bl/6J and BALB/c) against fully virulent pneumonic plague, irrespective of rF1-V-specific titers, which were comparable between male and female mice (Bowen *et al.*, 2019). In our study, males and females were equally protected when immunized with either TMV-F1+TMV-V or rF1+rV, though of the total unimmunized and rF1+rV immunized survivors (n=6), almost all were female (5/6). There were also higher F1 titers in our rF1+rV vaccinated females at 2 weeks following vaccination as compared to

males (n.s., p=0.05). Nonetheless, these data reinforce the importance of using both sexes in plague vaccine studies, as this can reveal disparities that would otherwise be hidden in all-female experiments.

Several studies have implicated T_H1 and T_H17 cell-mediated immunity in protection in mouse models of pneumonic plague. IFN-γ, TNF-α, and IL-17 have all been proposed to play critical roles in vaccine-mediated protection (Lin *et al.*, 2011; Nakajima and Brubaker, 1993; Parent *et al.*, 2006). In the present study we found that the majority of TMV-F1+TMV-V immunized mice showed diminished levels of IFN-γ in the lung, spleen, and blood, compared to unimmunized and rF1+rV unimmunized mice. This suggests the presence of a more highly regulated inflammatory response in mice that survive infection, particularly as bacteria disseminated from the lungs in all mice during the first 3 days of infection regardless of prior vaccination. The role of antigen-specific T cells in vaccine-mediated immunity to plague needs further study.

In conclusion, TMV-F1+TMV-V is capable of mediating single-dose protection against high-dose pneumonic challenge of *Y. pestis* CO92pgm-. Protection moderately correlated with F1 serum antibody titers. TMV-F1+TMV-V immunized mice were able to rapidly clear disseminated bacteria from the spleen and liver in the presence of a well-regulated inflammatory response. Though further study is needed to determine whether this protection is conferred against fully lethal strains of *Y. pestis* bacteria, these results warrant further investigation into TMV conjugate vaccines for severe respiratory infections.

References

Anderson GW, Heath DG, Bolt CR, Welkos SL, Friedlander AM (1998). Short- and long-term efficacy of single-dose subunit vaccines against yersinia pestis in mice. *The American Journal of Tropical Medicine and Hygiene.* 58: 793-799.

Andrianaivoarimanana V, Piola P, Wagner DM, Rakotomanana F, Maheriniaina V, Andrianalimanana S, Chanteau S, Rahalison L, Ratsitorahina M, Rajerison M (2019). Trends of human plague, madagascar, 1998-2016. *Emerging Infectious Diseases.* 25: 220-228.

Arnaboldi PM, Sambir M, D'Arco C, Peters LA, Seegers JF, Mayer L, McCormick AA, Dattwyler RJ (2016). Intranasal delivery of a protein subunit vaccine using a tobacco mosaic virus platform protects against pneumonic plague. *Vaccine.* 34: 5768-5776.

Banik S, Mansour AA, Suresh RV, Wykoff-Clary S, Malik M, McCormick AA, Bakshi CS (2015). Development of a multivalent subunit vaccine against tularemia using tobacco mosaic virus (TMV) based delivery system. *PloS One.* 10: e0130858.

Bashaw J, Norris S, Weeks S, Trevino S, Adamovicz JJ, Welkos S (2007). Development of in vitro correlate assays of immunity to infection with yersinia pestis. *Clinical and Vaccine Immunology : CVI.* 14: 605-616.

Bowen W, Batra L, Pulsifer AR, Yolcu ES, Lawrenz MB, Shirwan H (2019). Robust Th1 cellular and humoral responses generated by the yersinia pestis rF1-V subunit vaccine formulated to contain an agonist of the CD137 pathway do not translate into increased protection against pneumonic plague. *Vaccine.* 37: 5708-5716.

Demeure CE, Dussurget O, Mas Fiol G, Le Guern AS, Savin C, Pizarro-Cerda J (2019). Yersinia pestis and plague: An updated view on evolution, virulence determinants, immune subversion, vaccination, and diagnostics. *Genes and Immunity.* 20: 357-370.

Galvan EM, Nair MK, Chen H, Del Piero F, Schifferli DM (2010). Biosafety level 2 model of pneumonic plague and protection studies with F1 and psa. *Infection and Immunity.* 78: 3443-3453.

Heath DG, Anderson GW, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, Friedlander AM (1998). Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. *Vaccine.* 16: 1131-1137.

Jones SM, Day F, Stagg AJ, Williamson ED (2000). Protection conferred by a fully recombinant sub-unit vaccine against yersinia pestis in male and female mice of four inbred strains. *Vaccine.* 19: 358-366.

Kemnade JO, Seethammagari M, Collinson-Pautz M, Kaur H, Spencer DM, McCormick AA (2014). Tobacco mosaic virus efficiently targets DC uptake, activation and antigen-specific T cell responses in vivo. *Vaccine.* 32: 4228-4233.

Layton RC, Brasel T, Gigliotti A, Barr E, Storch S, Myers L, Hobbs C, Koster F (2011). Primary pneumonic plague in the african green monkey as a model for treatment efficacy evaluation. *Journal of Medical Primatology.* 40: 6-17.

Lee-Lewis H and Anderson DM (2010). Absence of inflammation and pneumonia during infection with nonpigmented yersinia pestis reveals a new role for the pgm locus in pathogenesis. *Infection and Immunity.* 78: 220-230.

Levy Y, Vagima Y, Tidhar A, Aftalion M, Gur D, Nili U, Chitlaru T, Zauberman A, Mamroud E (2018). Targeting of the yersinia pestis F1 capsular antigen by innate-like B1b cells mediates a rapid protective response against bubonic plague. *NPJ Vaccines.* 3: 52-z. eCollection 2018.

Lin JS, Kummer LW, Szaba FM, Smiley ST (2011). IL-17 contributes to cell-mediated defense against pulmonary yersinia pestis infection. *Journal of Immunology (Baltimore, Md.: 1950).* 186: 1675-1684.

Liu R, Vaishnav RA, Roberts AM, Friedland RP (2013). Humans have antibodies against a plant virus: Evidence from tobacco mosaic virus. *PloS One.* 8: e60621.

Mallajosyula JK, Hiatt E, Hume S, Johnson A, Jeevan T, Chikwamba R, Pogue GP, Bratcher B, Haydon H, Webby RJ, McCormick AA (2014). Single-dose monomeric HA subunit vaccine generates full protection from influenza challenge. *Human Vaccines & Immunotherapeutics.* 10: 586-595.

Mallajosyula JK, Jeevan T, Chikwamba R, Webby RJ, McCormick AA (2016). A single dose TMV-HA vaccine protects mice from H5N1 influenza challenge. *International Journal of Vaccine Research.* 1: 6.

Mansour AA, Banik S, Suresh RV, Kaur H, Malik M, McCormick AA, Bakshi CS (2018). An improved tobacco mosaic virus (TMV)-conjugated multiantigen subunit vaccine against respiratory tularemia. *Frontiers in Microbiology.* 9: 1195.

Nakajima R and Brubaker RR (1993). Association between virulence of yersinia pestis and suppression of gamma interferon and tumor necrosis factor alpha. *Infection and Immunity.* 61: 23-31.

Nguyen VK, Parra-Rojas C, Hernandez-Vargas EA (2018). The 2017 plague outbreak in madagascar: Data descriptions and epidemic modelling. *Epidemics.* 25: 20-25.

Olson RM and Anderson DM (2019). Shift from primary pneumonic to secondary septicemic plague by decreasing the volume of intranasal challenge with yersinia pestis in the murine model. *PloS One.* 14: e0217440.

Parent MA, Wilhelm LB, Kummer LW, Szaba FM, Mullarky IK, Smiley ST (2006). Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary yersinia pestis infection. *Infection and Immunity.* 74: 3381-3386.

Perry RD and Fetherston JD (1997). Yersinia pestis--etiologic agent of plague. *Clinical Microbiology Reviews.* 10: 35-66.

PittNon-human primates as a model for pneumonic plague. Paper presented at the *Animals Models and Correlates of Protection for Plague Vaccines Workshop,*

Quenee LE, Cornelius CA, Ciletti NA, Elli D, Schneewind O (2008). Yersinia pestis caf1 variants and the limits of plague vaccine protection. *Infection and Immunity.* 76: 2025-2036.

Rabaan AA, Al-Ahmed SH, Alsuliman SA, Aldrazi FA, Alfouzan WA, Haque S (2019). The rise of pneumonic plague in madagascar: Current plague outbreak breaks usual seasonal mould. *Journal of Medical Microbiology.* 68: 292-302.

Randremanana R, Andrianaivoarimanana V, Nikolay B, Ramasindrazana B, Paireau J, Ten Bosch QA, Rakotondramanga JM, Rahajandraibe S, Rahelinirina S, Rakotomanana F, Rakotoarimanana FM, Randriamampionona LB, Razafimbia V, De Dieu Randria, M J, Raberahona M, Mikaty G, Le Guern AS, Rakotonjanabelo LA, Ndiaye CF, Rasolofo V, Bertherat E, Ratsitorahina M, Cauchemez S, Baril L, Spiegel A, Rajerison M (2019). Epidemiological characteristics of an urban plague epidemic in madagascar, august-november, 2017: An outbreak report. *The Lancet.Infectious Diseases.* 19: 537-545.

Reed DS and Martinez MJ (2006). Respiratory immunity is an important component of protection elicited by subunit vaccination against pneumonic plague. *Vaccine.* 24: 2283-2289.

Smiley ST (2008). Current challenges in the development of vaccines for pneumonic plague. *Expert Review of Vaccines.* 7: 209-221.

Srivastava A, Gowda DV, Madhunapantula SV, Shinde CG, Iyer M (2015). Mucosal vaccines: A paradigm shift in the development of mucosal adjuvants and delivery vehicles. *APMIS : Acta Pathologica, Microbiologica, Et Immunologica Scandinavica.* 123: 275-288.

Sun W and Singh AK (2019). Plague vaccine: Recent progress and prospects. *NPJ Vaccines.* 4: 11-9. eCollection 2019.

Titball RW and Williamson ED (2004). Yersinia pestis (plague) vaccines. *Expert Opinion on Biological Therapy.* 4: 965-973.

Wang N, Strugnell R, Wijburg O, Brodnicki T (2011). Measuring bacterial load and immune responses in mice infected with listeria monocytogenes. *Journal of Visualized Experiments : JoVE.* (54). pii: 3076. doi: 10.3791/3076.

Wang X, Zhang X, Zhou D, Yang R (2013). Live-attenuated yersinia pestis vaccines. *Expert Review of Vaccines.* 12: 677-686.

WHO (2017). *Plague outbreak madagascar.* ().World Health Organization.

Williamson ED, Eley SM, Griffin KF, Green M, Russell P, Leary SE, Oyston PC, Easterbrook T, Reddin KM, Robinson A (1995). A new improved sub-unit vaccine for plague: The basis of protection. *FEMS Immunology and Medical Microbiology.* 12: 223-230.

Williamson ED, Eley SM, Stagg AJ, Green M, Russell P, Titball RW (1997). A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague. *Vaccine.* 15: 1079-1084.

Williamson ED and Oyston PC (2013). Protecting against plague: Towards a next-generation vaccine. *Clinical and Experimental Immunology.* 172: 1-8.

Williamson ED, Sharp GJ, Eley SM, Vesey PM, Pepper TC, Titball RW, Alpar HO (1996). Local and systemic immune response to a microencapsulated sub-unit vaccine for plague. *Vaccine.* 14: 1613-1619.

Woodrow KA, Bennett KM, Lo DD (2012). Mucosal vaccine design and delivery. *Annual Review of Biomedical Engineering.* 14: 17-46.

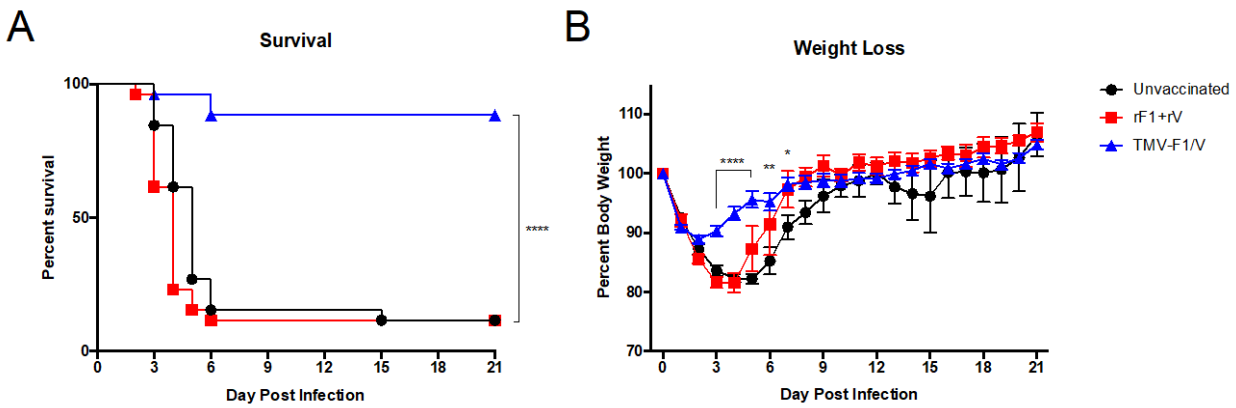

Fig 1. Single TMV-F1+TMV-V i.n. immunization is protective against pneumonic plague challenge. 26 mice (13 males, 13 females) were i.n. immunized with either TMV-F1+TMV-V, rF1+rV, or not immunized (controls) and monitored for 21 days post-infection with 100 LD50 *Y. pestis* CO92pgm-. (A) Kaplan-Meier curve showing comparison of survival between TMV-F1+TMV-V -immunized mice and the other groups. Asterisks indicate statistical significance by the Mantel-Cox log-rank test. (B) Average percent body weight (from initial weight on day of challenge). Asterisks indicated statistical significance between TMV-F1+TMV-V- and un-immunized groups by unpaired student's t test for each day post-infection (DPI). Data pooled from two separate experiments. **** $p<0.0001$, **$p<0.01$, *$p<0.05$. All errors bars represent mean±SEM.

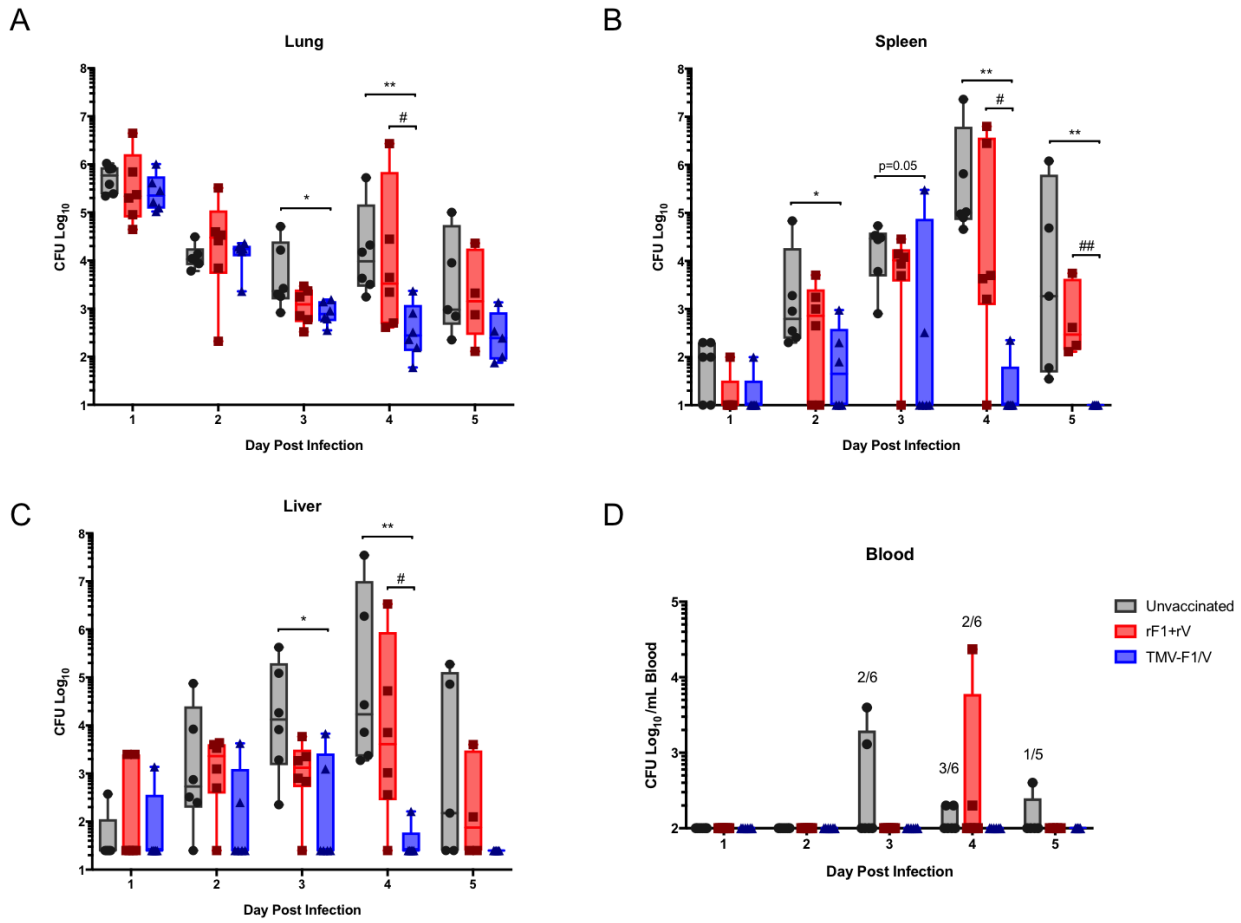

Fig 2. TMV-F1+TMV-V immunized mice experience a decrease in bacterial burden in lung, spleen, and liver, and no sepsis, in the 5 days post-infection. Bacterial burden was quantified from the lungs, spleens, and livers of infected mice for days 1-5 following challenge. Individual mice are indicated by their respective symbol (n=6/group) for lung (A), spleen (B), and liver (C). For septicemic burden, the number of mice/group where CFUs were retrievable from peripheral blood is shown as fraction (D). Asterisks and pound symbols indicate statistical significance by Mann-Whitney test for each DPI. *= TMV-F1+TMV-V vs. unvaccinated, #= TMV-F1+TMV-V vs. rF1+rV. Data pooled from two separate experiments. **p<0.01, *p<0.05. All errors bars represent min to max values, with lines indicating the median.

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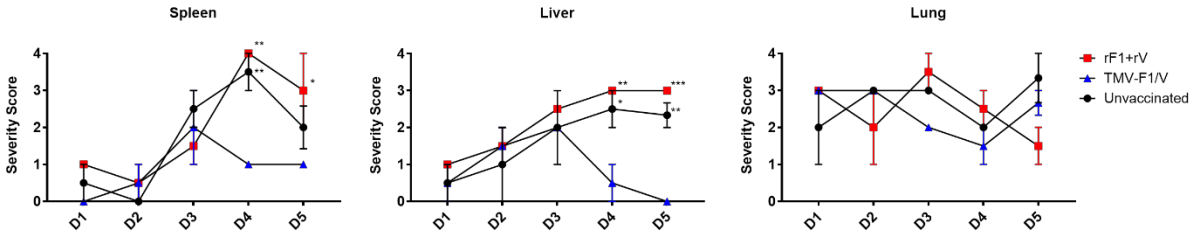
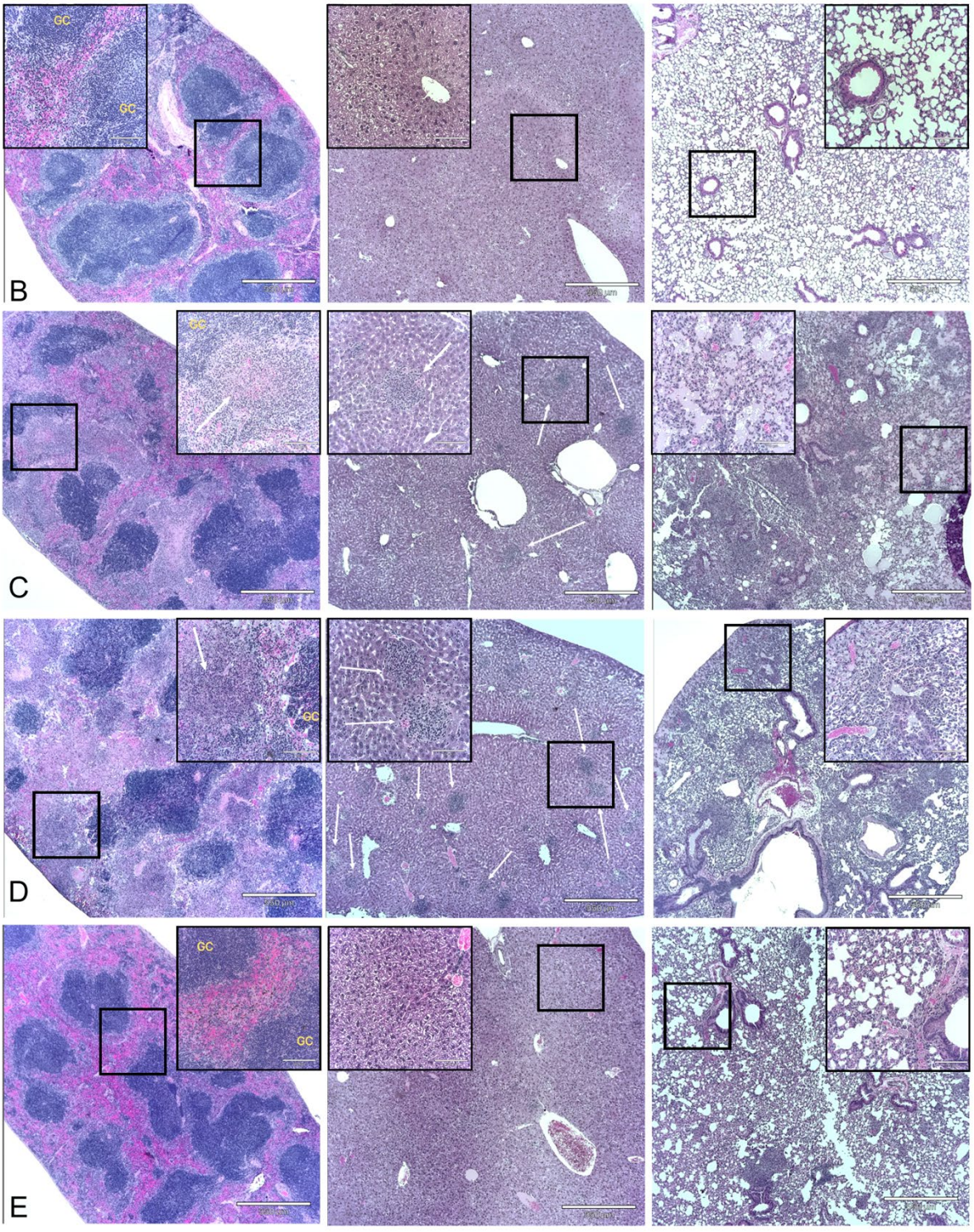


Fig 3. Comparison of tissue inflammation in the lung, spleen, and liver of TMV-F1+TMV-V immunized, unimmunized, rF1+rV-immunized mice at D3 post-infection. Representative spleen (left), liver (center), and lung (right) sections were collected from mice challenged with 100 LD50 Y. pestis CO92pgm-. Formalin-fixed tissues were sectioned and stained with hematoxylin and eosin (H&E). Pathological severity scoring (0-4) ± SEM vs. day post-infection is shown in (A). No errors bars indicate mice for that group received the same score. Asterisks symbols in (A) indicate statistical significance by two-way ANOVA between the indicated group and TMV-F1/V, *<0.05, **<0.01, and ***<0.001.

Fig 3. **Continued on next page:** Images represent sections from a (B) naïve (uninfected), (C) unvaccinated, (D) rF1+rV vaccinated, and (E) TMV-F1/V vaccinated mouse sacrificed at D5 PI. Arrows indicate neutrophilic foci. The inlays (20x) represent the black boxed region in each larger image (4x). Scale bar represents 550µm in all 4x images and 110µm in all 20x images.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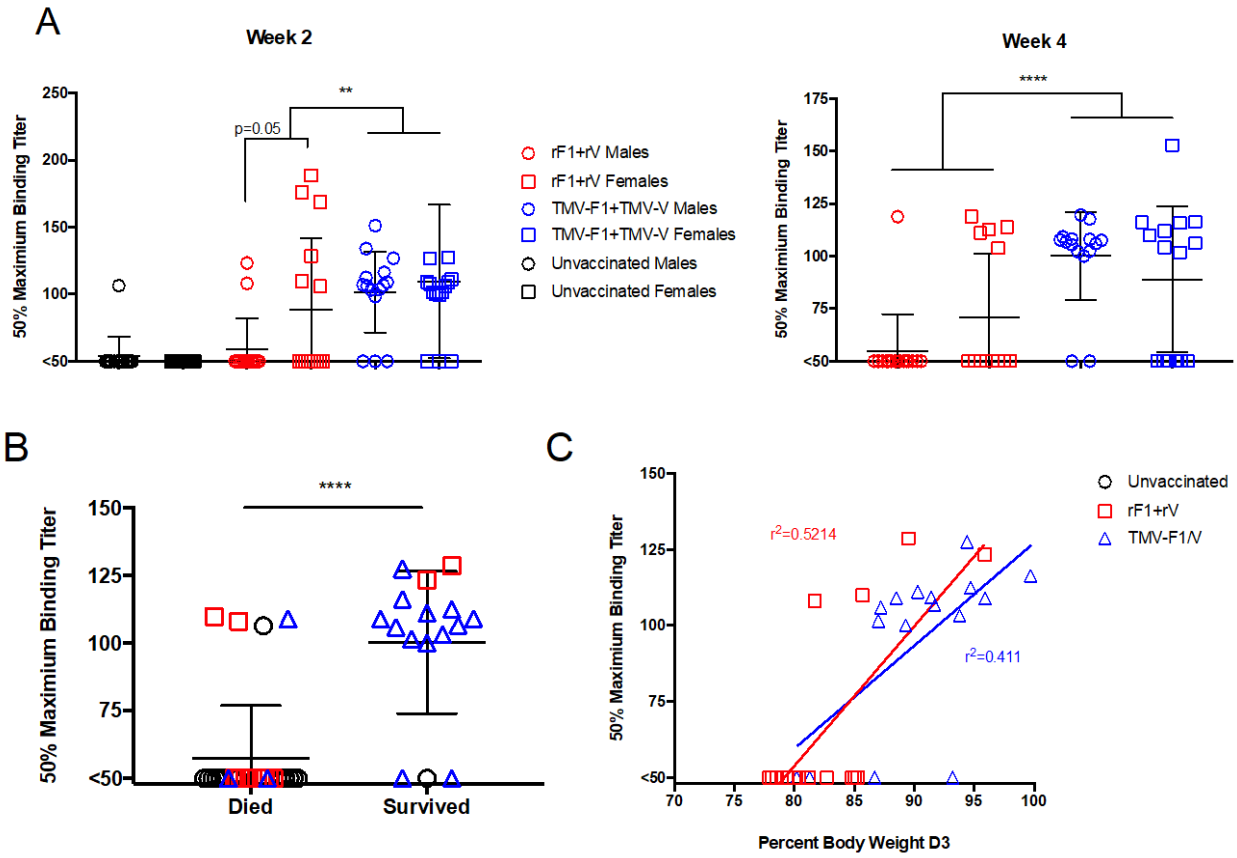


Fig 4. TMV-F1+TMV-V immunized mice have increased anti-F1 titers prior to challenge, and higher F1-specific titers are correlated with survival for both vaccines. (A) Serum F1-specific antibody titers (total Ig) week 2 post-immunization for unvaccinated, rF1+rV-vaccinated, and TMV-F1+TMV-V-vaccinated mice, and serum total F1-specific titers for these groups week 4 post-immunization. Unvaccinated mice sera were not collected week 4, as past experiments have shown they remain unchanged. (B) Comparison of the week 2 total serum F1-specific titers between the deceased and surviving mice. (C) Correlation of week 2 serum F1-specific titers and percent body weight D3 post-infection. Pearson r correlation was significant for rF1+rV-immunized mice ($p<0.01$, $r^2=0.5214$), and for TMV-F1+TMV-V-immunized mice ($p<0.01$, $r^2=0.411$). Asterisks indicated statistical significance compared by unpaired student's t test between indicated groups. Data is representative of two experiments. Individual mice are indicated by their respective symbol. ****$p<0.0001$, **$p<0.01$. All errors bars represent mean±SD.

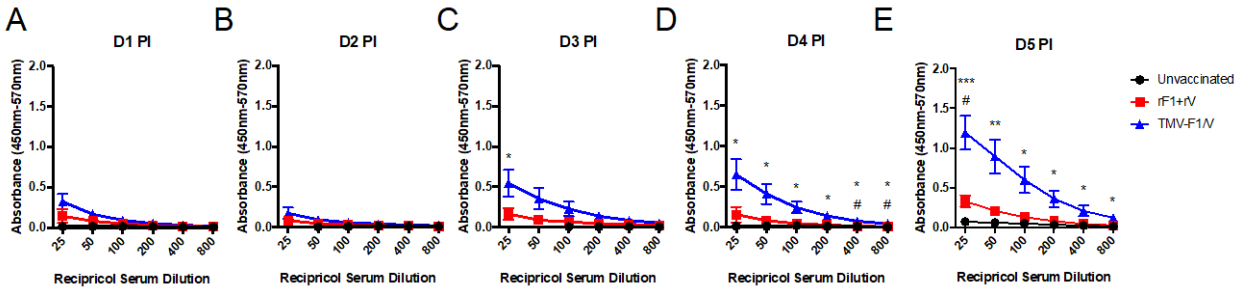


Fig 5. F1-specific IgG increases rapidly in response to plague infection in TMV-F1+TMV-V immunized mice. Comparison of ELISA curves measuring serum IgG2b. Sera was obtained from mouse peripheral blood collected 24HPI (A), 48HPI (B), 72HPI (C), 96HPI (D), and 120HPI (E) for each experimental group. Asterisks indicated statistical significance compared by multiple t tests. *= TMV-F1+TMV-V vs. unvaccinated, #= TMV-F1+TMV-V vs. rF1+rV. *** $p<0.001$, **$p<0.01$, *$p<0.05$. All errors bars represent mean±SEM.

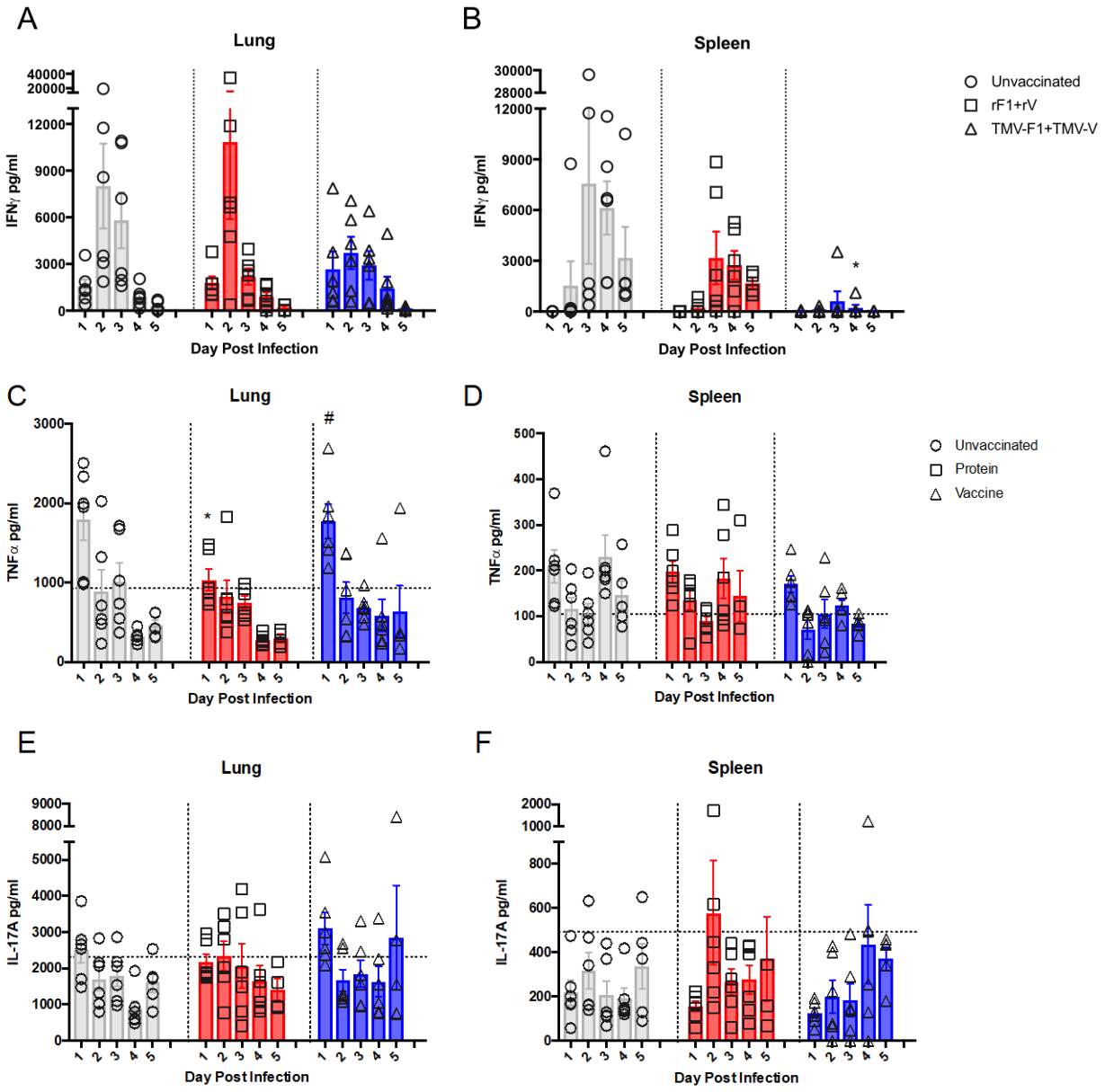


Fig 6. TMV-F1+TMV-V i.n. vaccine does not induce Th1 or Th17 cytokine response post-infection. Tissue cytokine levels of the lung and spleen D1-D5 post-challenge for (A-B) IFN-γ, (C-D) TNF-α, and (E-F) IL-17A. Dashed lines represent the average tissue cytokine measurement of naïve male and female mice (n=10). Asterisks indicated statistical significance compared by one-way ANOVA for each time point. Individual mice are indicated by their respective symbol. *= vs. unvaccinated, #= vs. rF1+rV. *$p<0.05$. All error bars represent mean±SEM.

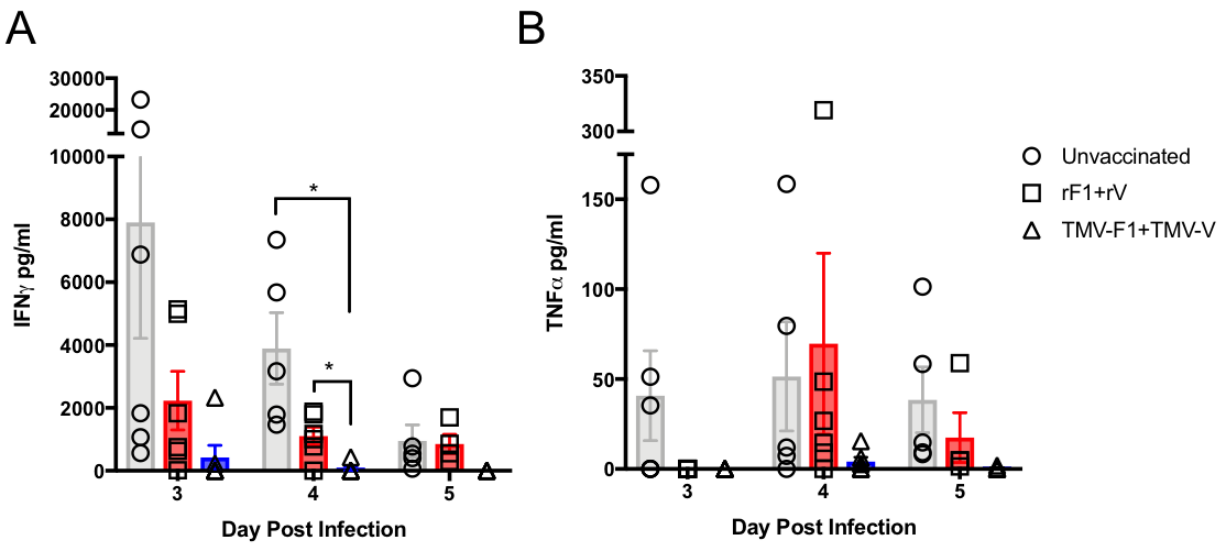


Fig 7. Single immunization with TMV-F1+TMV-V vaccine protects against secondary plague sepsis. Serum cytokine levels obtained from mouse peripheral blood collected D3-D5 post-infection for IFN-γ (A) and TNF-α (B). Cytokine levels were minimal and similar for D1-D2 post-infection, and so are not shown. Asterisks indicated statistical significance compared by one-way ANOVA for each time point. Individual mice are indicated by their respective symbol. *$p<0.05$. All errors bars represent mean±SEM.

Chapter 4. Specific Aim 3

AIM 3:
Identify the Role of T Cell-Mediated and Antibody-Mediated Immune Responses of TMV Conjugates as an Intranasal VLP-like Vaccine

Aim 2a: Test the hypothesis that $CD4^+$ T cells and/or $CD8^+$ T cells are required for protection from TMV vaccine at the time of infection

Aim 2b: Test the hypothesis that intranasal immunization with TMV induces both virus-specific and antigen-specific antibody responses

Aim 2c: Test the hypothesis the TMV vaccine induces a rapid and localized mucosal antibody response after lung infection

Aim 3d: Test the hypothesis that vaccine-specific immunity is not limited to F1 immunity

1.INTRODUCTION

Most infectious pathogens enter the body through mucosal surfaces. Optimal protection is usually triggered by local secretory IgA (SIgA) production at the mucosal barrier but can include other isotypes such as IgG (Chen, K. *et al.*, 2020). Intranasal (i.n.), oral, vaginal, and rectal vaccines typically achieve higher levels of neutralizing antibody responses at mucosal surfaces than intramuscular (i.m.) or subcutaneous (s.c.) administered vaccines (Neutra and Kozlowski, 2006) and have the added advantage of being needle-free (Levine, 2003). However, such vaccines must also possess features that can get around mucosal tolerance. Many subunit vaccines routinely fail to induce mucosal immunity. With the exception of toxins, most soluble proteins are ignored or even induce immunosuppression through tolerogenic dendritic cells (DCs), induction of regulatory T cells (T_{reg}) cells, as well as inducing anergy or clonal deletion of responsive T cells at mucosal barriers (Weiner, 2001). Successful experimental mucosal vaccines have thus been described as possessing the following: (1) they are particulate, (2) they directly stimulate innate immunity (e.g. TLR activation), or, (3) they target underlying antigen-presenting cells (APCs) in the mucosa (Neutra and Kozlowski, 2006) . Virus-like particles (VLP) are unique antigen carriers that fulfill all of these requirements.

VLP are devoid of an intact virus genome, and thus incapable of infection and replication; but their repetitive immunogenic surface structure still enables the retention of cell uptake and immune processing pathways normally associated with live viruses (Al-Barwani *et al.*, 2014). VLP vaccines are often described as possessing the safety of a subunit vaccine, but with efficacy and outcomes that can be comparable to live attenuated or live vectored vaccines (Bessa *et al.*, 2008). One of these outcomes is the induction of mucosal

immunity. VLP vaccines approved for clinical use have utilized various administration routes, including mucosal vaccination (Herzog, 2014). I.n. vaccination with VLP in mice has shown protection against norovirus (Tamminen *et al.*, 2016), respiratory syncytial virus (RSV) (Jiao *et al.*, 2017), and hepatitis B (Huang *et al.*, 2006). Specifically, i.n. immunization with VLP has been shown to be protective by inducing high systemic (IgG) and mucosal antibody (SIgA) titers in mouse models without the need for adjuvants (Balmelli *et al.*, 1998; Sedlik *et al.*, 1999).

The success of human VLP vaccine programes, such as Gardasil® and Cervarix™ for human papillomavirus, have encouraged the development of novel VLP platforms. Recombinant VLP, also termed chimeric VLP, was explored as a "new generation" VLP that went beyond the basic, first-generation capsid VLP vaccines resembling the parent virus. Genetic fusion and chemical conjugation are two experimental approaches used to create chimeric virus particles. Chemical conjugation has two advantages over a genetically engineered virus: (1) diverse sizes and types of whole antigens can be displayed, and (2) the antigen-virus binding site can be manipulated to maximize exposure of the conjugated antigen (Chen, Q. and Lai, 2013). These features prove crucial for pathogens with antigenic variations, as larger proteins are more potent than short peptides in provoking neutralizing antibodies that recognize a broad range of liner and conformational epitopes on the pathogen (Chen and Lai, 2013; Jennings and Bachmann, 2008).

Antigens can be linked to viruses or virus particles through either covalent or noncovalent chemical bonds. We use an inactivated and recombinant plant virus, Tobacco Mosaic Virus (rTMV), that enables covalent linkage of whole proteins. Our rTMV virion has been genetically engineered to display a lysine on the coat protein (CP) that allows for amine

conjugation of exogenous proteins containing lysine or cysteine (McCormick and Palmer, 2008; Smith *et al.*, 2006). We reported complete protection in a pneumonic plague mouse model using an i.n. vaccine comprised of *Yersinia pestis* virulence proteins, LcrV (V) and F1, separately linked to rTMV (Arnaboldi *et al.*, 2016). In this aim, I sought to identify the humoral and cellular mechanisms that support rTMV's classification as a mucosal vaccine that enables high-density, VLP display of protein antigens. These include the conjugates' ability to generate TMV-specific as well as antigen-specific high titer antibodies in circulation. It also includes its potential contribution of $CD8^+$ cytotoxic T cell (CTL) or $CD4^+$ T cell responses to plague defense. Our findings suggest that rTMV represents an efficient vaccine delivery platform for the generation of IgG antibody-based, but not T cell-based, immunity that can be administered i.n. safely without the need for adjuvants.

2.MATERIALS AND METHODS

2.1 Bacterial strains

Yersinia pestis CO92pgm− was a gift of Dr. James Bliska (Stonybrook University, NY), and was cultivated as previously described (Arnaboldi *et al.*, 2016). The *Y. pestis* KIM5*caf1A* (Δ*pgm* Δc*af1A,* Amp^r Kan^r) F1 negative (F1-) mutant strain was also a gift of Dr. Bliska and was cultivated as previously described, with the exception that cultures were incubated shaking at 37°C overnight instead of 26°C (Ivanov *et al.*, 2008). All cultures were revived from frozen glycerol stocks.

2.2 Recombinant proteins and TMV

Cloning, expression, and purification of rF1 and rLcrV (rV), and preparation of TMV-F1 and TMV-V conjugate vaccines were performed as described previously (Arnaboldi *et al.*, 2016) . Purified TMV and TMV conjugates were created and supplied by Alison McCormick. The TMV was inactivated via binary ethyleneimine (BEI) prior to the antigen conjugation process (EDC mediated amine conjugation) and has no replication capacity in plant or mammalian cells.

2.3 Mice and intranasal vaccination

Adult (6-week-old) male and female C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, Maine), and maintained by the husbandry staff of the Department of Comparative Medicine at New York Medical College. All experiments were conducted with the approval of the New York Medical College Institutional Animal Care and Use Committee (IACUC). All mice were vaccinated at 6-8 weeks. Equal numbers of male and female mice were tested in all groups. Mice were anesthetized using ketamine/xylazine cocktail in order to perform i.n. vaccination with the following in 40µL volume of saline: 10µg of TMV-V+10µg of TMV-F1 (total of 20 µg), 5µg rV+5µg rF1 (total of 10µg), 5µg rV, or 10µg of TMV-V. TMV-conjugated vaccines always equivalent concentrations of TMV and recombinant protein (5µg TMV+5µg protein). Mice were vaccinated with 10µg of TMV-conjugate and 5 µg of recombinant protein to ensure molar equivalency of the antigens between the two vaccines. All control mice were unvaccinated. For the challenge with *Y. pestis* KIM5*caf1A,* mice were boosted i.n. with the indicated vaccines 21 days after the prime vaccination.

2.3 Bacterial Challenge

Mice were challenged on day 35 post-immunization with *Y. pestis* CO92pgm−. Briefly, *Y. pestis* CO92pgm− was grown to an OD620 of 0.9 in heart infusion broth supplemented with 2.5mM CaCl2 and 0.2% Xylose at 37°C in a shaking incubator (225 RPM). Culture medium was removed by centrifugation and the bacterial pellet was washed 3x with sterile saline. Bacteria were resuspended in saline to an OD620 of 1.1, assuming a concentration of $1x10^9$ CFU. The LD50 of *Y. pestis* CO92pgm− in C57Bl/6J mice was previously determined to be $2.5x10^4$ CFU (Arnaboldi *et al.*, 2016). Bacteria were diluted accordingly, and anesthetized mice were i.n. administered 10 or 100 LD50 in 50µL saline in a class II biosafety cabinet. 10-fold dilutions of the bacteria were streaked on HI agar and incubated for 2 days at 28°C to confirm the number of bacterial CFU administered for each challenge. After challenge (D0), mice were weighed and checked daily for signs of morbidity (ruffled fur, hunched posture, reduced activity, weight loss). Mice that lost more than 20% of their pre-challenge body weight, or were severely moribund, were euthanized. Survival was determined at 21 days, and mice were euthanized according to approved IACUC guidelines.

The i.n. LD50 of *Y. pestis* KIM5*caf1A* F1- strain was determined to be $1.1\text{-}1.5x10^6$ CFU in our laboratory (data not shown). For this experiment, mice were challenged i.n. with $2.58x10^7$ CFU in 50µL saline 15 days after the boost. Determination of bacterial CFU and mouse morbidity were made as described above, with the exception that survival was determined at 14 days.

2.4 $CD4^+$ and $CD8^+$ T cell depletion

Mice were injected i.p. with either anti- mouse CD4 (clone GK1.5; BioXCell) or rat IgG2bk isotype control antibody (clone LTF-2; BioXCell) 2 days prior to challenge and weekly thereafter. In a separate experiment, mice were injected i.p. with anti-mouse CD8α (clone YTS 169.4; BioXCell) or the isotype control described above (clone LTF-2; BioXCell) 2 days prior to challenge and weekly thereafter. The dose for all antibody injections was 100µg in 200µL PBS per mouse.

2.5 Lung collection

Mice that were immunized and challenged with 100 LD50 *Y. pestis* CO92pgm− as described above (see 2.3) were euthanized daily for the first 5 days following challenge to collect infected organs. The left lobe of the lung was placed in 1.5ml centrifuge tubes filled with ~100uL of ceria stabilized zirconium oxide beads (2mm diameter, Next Advance, Inc.) and 0.5mL of sterile PBS on ice. The tissues were homogenized using a BioSpec Mini-BeadbeaterTM, were centrifuged at 10,000rpm x 10 min, and supernatants were aliquoted and stored at -80°C. In a separate experiment, the same was done for all survivors of a 10 LD50 *Y. pestis* CO92pgm− challenge.

2.6 Antibody measurements

All serum antibodies were determined from blood samples collected via retroorbital bleed week 2 or 3 post-immunization. Sera was allowed to sit at RT for at least 30 min before being centrifuged at 2500xg for 15 min and stored at -20°C. Antibodies were also determined from lung tissue homogenate supernatant that had been stored at -80°C (see 2.4). 96 well ELISA plates (Nunc MaxiSorp™ flat bottom or Immulon® 2 HB Flat Bottom MicroTiter™)

were coated with rV or rF1 (2 μg/ml) in 0.1M Carbonate Buffer, pH 9.4 overnight at 4°C. Plates were blocked with 1% BSA in PBS for 1h at RT, and washed 3x with 0.05% Tween in PBS. Sera or supernatant were diluted in blocking buffer and were incubated for 1h at RT. Plates were washed 3x again, and HRP-labeled goat anti-mouse Ig heavy and light (H+L) chain, IgM, IgG1, IgG2a, IgG2b, IgG3, IgE, or IgA (Southern Biotech) diluted 1:5000 in blocking buffer was added to each well for 1h at RT. The plates were washed and developed with TMB substrate (KPL) for 30 min at RT. The reaction was stopped by addition of 2N sulfuric acid, and absorbance was read at 450nm and 570nm on a SpectraMax Plus 384 microplate reader (Molecular Devices). Absorbance was plotted vs. reciprocal serum dilution and 50% maximal binding titers were calculated using Prism 7.0 (GraphPad). Samples were analyzed in duplicate.

Antibody specific for TMV was measured using a modified sandwich ELISA protocol. Anti-TMV coating antibody reagent (Agdia®, Catalog# SRA 57400), was applied to 96 well ELISA plates overnight according to the manufacturer's protocol. The next day, plates were washed 3x with 0.05% Tween in PBS, blocked with 1% BSA in PBS for 1h at RT, and then washed 3x again. The protocol continued as described above for sera and supernatant samples.

2.7 Statistical analysis

Unpaired student's t test was used to analyze means between two groups. Multiple t tests were used to analyze differences between groups at various time points or dilutions (e.g. body weight, ELISA dilutions). One-way ANOVA was used to compare means of three or

more groups, with Tukey's multiple comparisons test as the post-hoc analysis. Statistical analyses were performed using GraphPad Prism 7 software.

3.RESULTS

$CD4^+$ and $CD8^+$ T cells are not required for vaccine-induced protection against lethal pneumonic plague infection

Arnaboldi *et al.* previously showed that i.n. immunization with TMV-F1 and TMV-V conjugates effectively protected against pneumonic plague most likely through augmentation of an F1-specific humoral response (Arnaboldi *et al.*, 2016). However, the role of $CD4^+$ and $CD8^+$ T cells in vaccine-induced protection was not assessed in that study. Using antibody-mediated depletion of $CD4^+$ and $CD8^+$ T cells, we sought to determine if these two populations were critical for protection mediated by TMV-F1+TMV-V. Groups of 12 mice (6 males + 6 females) were i.n. immunized once with TMV-F1+TMV-V and challenged i.n. with 9-15 LD50 *Y. pestis* CO92 pgm- 5 weeks later. Unimmunized mice were used as a control.

For the first experiment, we evaluated the contribution of $CD4^+$ T cells to TMV vaccine-induced protection. In each group, half (3 males + 3 females) of mice were injected intraperitoneally (i.p.) with anti-CD4 antibody (clone GK1.5), and other half with a "mock" non-specific isotype control antibody 2 days prior to challenge (Fig 1A). Prior to conducting this experiment, we had confirmed the anti-CD4 mediated antibody depletion was effective at 2 days post-depletion and lasted until day 7 post-depletion in the peripheral blood, lung, and spleen (Appendix III Fig S1). At the one-week time point, mice were injected weekly to maintain CD4+ T cell depletion. As predicted, all non-immunized mice succumbed to

pneumonic plague infection by day 6, while 83% of all TMV-F1+TMV-V-immunized mice were protected (Fig 1B). For both groups of immunized mice (mock and CD4-depleted), only one mouse succumbed to infection in each group (5 out of 6 mice, 83% survival) (Fig 1B). The presence of $CD4^+$ T cells did, however, increase the mean time to death by 2 days compared to mice lacking $CD4^+$ T cells (Fig 1B). Both of these groups also showed no significant weight loss relative to each other over the 21 days post-challenge (Fig 1C). It may be important to note that two TMV-F1+TMV-V-immunized mock females accidentally became pregnant and had to be separated from their offspring just prior to challenge while nursing. These two females never returned to their initial body weight, and we assumed is what kept the average weight of-immunized mock mice consistently below that of the CD4-depleted immunized mice (Fig 1C). There was no significant difference in the pre-challenge total F1-specific immunoglobulin (Ig) serum titers between deceased and surviving immunized mice, indicating that F1-specific antibody levels were not a reliable correlate of vaccine-induced protection (Fig 1D). Although all immunized mice did generate an anti-F1 humoral response from single vaccination, this did not assure protection from plague infection in the presence or absence of $CD4^+$ T cells. Total V-specific antibody titers were below the limit of detection (<20) at week 3 post-challenge, and therefore, are not reported here (data not shown). Focusing solely on the survivors' weight loss, it is evident that a single-vaccination of TMV-F1+TMV-V effectively kept these mice from experiencing morbidity post-challenge; mice were able to maintain their initial body weight on the day of infection (Fig 1E). For the reason explained above, the immunized mock group contained two surviving females which kept the average weight below that of the CD4-depleted immunized group. It is assumed the percent body weight of these groups would have been

comparable if these females were of normal weight on day of challenge. Overall, we can conclude from these data that for 3 weeks post-infection, $CD4^+$ T cells do not play a crucial role in the protection from plague that is generated by a single i.n. administration of the TMV-F1+TMV-V vaccine.

For the second experiment, we evaluated the contribution of $CD8^+$ T cells to TMV vaccine-induced protection. As with the $CD4^+$ T cell-depletion experiment, in each group (immunized or control), half of mice (3 males + 3 females) were injected i.p. with anti-CD8α antibody (clone YTS 169.4), while the other half were treated with the "mock" isotype control antibody 2 days prior to challenge, and weekly for the 21 days post-challenge (Fig 2A). Prior to conducting this experiment, we again confirmed the anti-CD8α mediated depletion was effective at 2 days depletion and 7 days post-depletion in the peripheral blood, lung, and spleen (Appendix III Fig S2). To keep consistent with the $CD4^+$ depletion experiment, we continued weekly antibody injections to maintain depletion of $CD8^+$ T cells. The survival results obtained from this experiment were identical to the $CD4^+$ T cell-depletion experiment. All non-immunized mice succumbed to pneumonic plague by day 6, while 83% of all immunized mice were protected (Fig 2B). For both groups of immunized mice (mock and CD8-depleted), 5 out of 6 mice survived infection (83% survival) (Fig 2B). The presence of $CD8^+$ T cells increased the mean time to death by 4 days compared to mice lacking $CD8^+$ T cells (Fig 1B). Both of these groups also showed no significant body weight loss relative to each other over the 21 days post-challenge (Fig 2C). There was no significant difference in the pre-challenge total Ig F1-specific serum titers of between the deceased and surviving immunized mice, although deceased mice did have a lower mean titer (Fig 2D). Of note, one immunized mouse did not produce a detectable anti-F1 titer prior to challenge and

died from infection (Fig 2D). Surviving mice also showed no morbidity and maintained their initial weight on the day of infection (Fig 2E). Total V-specific antibody titers were below the limit of detection (<20) at week 3 post-challenge, and therefore, are not reported here (data not shown). Interestingly, the absence of $CD8^+$ T cells in recovering survivors appeared to elevate the mean percent body weight above that of survivors with $CD8^+$ T cells, although this difference was not statistically significant at any day post-infection (Fig 2E). Again, we can conclude that, like $CD4^+$ T cells, $CD8^+$ CTL do not play a crucial role in TMV-F1+TMV-V vaccine-induced protection for pneumonic plague and sepsis.

TMV-F1+ TMV-V i.n. immunization auguments serum IgG against TMV and F1+V

One question that had not yet been addressed until this point was if immunization with the TMV conjugates was inducing TMV-specific IgG production. Using archived pre-challenge serum from our published challenge and immunization study (Arnaboldi *et al.,* 2016), we wanted to identify the isotypes of the anti-TMV antibody response – if there was, in fact, a response. This serum was collected on 56 days after the prime immunization (day 0) and after the second and final boost (day 35) from TMV-F1+TMV-V i.n.-immunized mice.

We found that i.n. immunization with TMV conjugates did generate an anti-TMV antibody response in mice, and this response was dominated by IgG2b, followed by IgM and IgG3 (Appendix III Fig S3). We adapted a commercial TMV sandwich ELISA in order to quantify serological levels of TMV-specific antibody in our mice. We first asked if there was any possible sex bias in the anti-TMV antibody response. Upon immunization with TMV-F1+TMV-V, female mice generally had higher anti-TMV IgG2b and IgG3 serum titers; however, these were not significantly different than that of immunized males (Appendix III

Fig S4B). A prime immunization and two boosters of TMV-F1+TMV-V produced an anti-TMV IgG2b serum titer of nearly 1:200,000 (Fig 3A) and an anti-TMV IgG3 serum titer of about 1:15,000 (Fig 3B). In each case, this titer was significantly greater than that of mice immunized with recombinant proteins only (rF1+rV) and unimmunized mice (Fig 3A, 3B). We also examined anti-TMV antibody in other naïve mice and found titers were below our detection limits (data not shown). In our 2016 study, F1- and V-specific IgG1 was elevated in response to the three vaccine doses; however, anti-TMV IgG1 was not elevated in response to these vaccinations, nor was IgG2a (Appendix III FigS3). The more interesting observation was that these same predominant IgG subclasses (IgG2b and IgG3) were also specific for F1 and V – the antigens conjugated to TMV. For this ELISA, the plate was coated with both antigens (rF1+rV) since it was previously shown the sera contained both F1-specific and V-specific IgG (Arnaboldi *et al.*, 2016). A prime immunization and two boosts of TMV-F1+TMV-V produced an F1/V-specific IgG2b serum titer of about 1:6,000 (Fig 3C) and an F1/V-specific IgG3 serum titer of 1:600 (Fig 3D). Both of these titers were, again, significantly greater than that of mice i.n. immunized with recombinant free antigens and unimmunized mice (Fig 3C, 3D). Interestingly, unimmunized mice had a higher – but not statistically different – mean F1/V-specific IgG3 serum titer than mice immunized with rF1 + rV (Fig 3D). Overall, the data suggests that the same IgG subclasses directed against TMV (IgG2b, IgG3) are also directed against the proteins bound to TMV.

F1-specific IgG generated by i.n. TMV-F1+TMV-V is not localized to the lung

Now that we had a better understanding of antigen-specific antibody enhancement induced by our vaccine, we next wanted to see where this response was occurring. We have

shown that vaccination with TMV-F1+TMV-V induced F1-specific IgG production in circulation by the third day following i.n. challenge with *Y. pestis*, implying a vaccine-induced central memory B cell pool (see Aim 2). However, i.n. vaccination and the induction of SIgA production is also an important outcome for vaccines against pathogens that infect through mucosal surfaces. Mediastinal lymph nodes (MLN) drain the lung in mice and humans and are usually the site where mucosal immune responses (IgG or SIgA) are initiated against antigens reaching the lung (Bessa *et al.*, 2008).

We evaluated the F1-specific IgG2b antibody levels in the lung post-infection to signify a local mucosal immune response. Mice had been immunized once with a mixture of rF1+rV, TMV-F1+TMV-V, or not immunized at all (control) 5 weeks prior to i.n. challenge with 100 LD50 of *Y. pestis* CO92pgm−. Little IgG2b was detected in lung homogenates for 5 days post-challenge in any of the three groups, indicating this was likely residual serum from circulation (Fig 4A-E). It was not until day 5 post-infection (120HPI) that F1-specific IgG2b levels could be seen increasing for only TMV-F1+TMV-V-immunized mice, but this was not a statistically significant difference (Fig 4E). This was in contrast to anti-F1 IgG2b serum levels, where TMV-F1+TMV-V-immunized mice were producing significantly more antibody than unimmunized mice as early as day 3 post-infection (72HPI) (see Aim 2). F1-specific IgA and IgG2b were detected in the lungs of surviving mice from a separate challenge experiment (10 LD50 of *Y. pestis* CO92pgm−) (Appendix III Fig S5A, S5B). In this experiment, mice received a single-dose vaccine prior to challenge. The majority of TMV-F1+TMV-V-immunized survivors did produce F1-specifc IgA titers (7 out of 9 mice), however, it was also detected in both surviving rF1+rV-immunized mice, and in one mouse immunized with an unconjugated mix (TMV+rF1+rV) (Appendix III Fig S5B). Since these

lung samples were collected 21 days after infection, it was not possible to determine how soon these IgA or IgG2b responses were initiated in the lung. TMV-specific IgG2b titers of roughly 1:160 were detected in all mice except those immunized with rF1+rV (Appendix III Fig S5C), as predicted. On the other hand, anti-TMV IgA in the lung was not high enough to report a titer, indicating that there is not a strong localized IgA response upon i.n. administration of TMV (Appendix III Fig S5D). Anti-F1 IgG2a levels were also extremely low in all immunized groups, indicating that F1-specific IgG2a is not critical in protection against pneumonic plague using our model vaccines (Appendix III Fig S5E). Likewise, V-specific IgG2a, IgG2b, and IgA titers were below the limit of detection (<50) in these lung samples, and therefore, are not reported here (data not shown). Overall, this data suggests that i.n. administration of the TMV conjugate vaccine does not induce a localized F1-specific IgG response in the lung immediately after infection with *Y. pestis*.

Unconjugated rV antigen provides optimal protection against lethal pneumonic infection with F1- *Y. pestis*

Until this point, our investigation of TMV conjugate vaccine-induced immunity has focused on the humoral response to the F1 capsule, since this is evidently the immunodominant antigen for this vaccine. Next, we wanted to evaluate the role of V-specific antibody in protection against a *Y. pestis* strain lacking F1, where contribution of F1-specific immunity is nullified. We selected the *Y. pestis* KIM5*caf1A* mutant strain (*Δpgm Δcaf1*) for i.n. challenge to see if the enhanced production of anti-V responses in co-immunized mice (TMV-F1+TMV-V) (Arnaboldi *et al.*, 2016) translated to enhanced protection against lethal pneumonic plague.

Groups of 10 mice, each containing males and females, were i.n. immunized and boosted once with rV, TMV-V, rF1+rV, TMV-F1+TMV-V, or not immunized (control) and challenged i.n. with 20 LD50 *Y. pestis* KIM5*caf1A* (~2.5x10^7 CFU/mouse) 15 days after the boost (Fig 5A). Survival was assessed at day 14 post-challenge. Interestingly, mice immunized and boosted with rV alone all failed to survive i.n. challenge with F1-wild-type *Y. pestis* CO92pgm- strain (Arnaboldi *et al.*, 2016). However, in the present study, mice immunized and boosted with rV were the best protected against the F1- strain (55% survival), followed by immunization with rF1+rV (37% survival), TMV-F1+TMV-V (30% survival), and lastly, TMV-V (12% survival) (Fig 5B). All unimmunized mice succumbed to infection by day 3 (Fig 5B). Combining TMV-F1 and TMV-V into a co-administered vaccine provided better protection than vaccination with TMV-V (Fig 5B). However, the free recombinant protein, rV, or a mixture of the recombinant proteins, rF1+rV, still provided better protection than the TMV conjugates (Fig 5B). All groups showed a decline to about 80% of their initial body weight in the first 3 days of infection; however, mice that recovered eventually regained 90-100% of their initial weight (Fig 5C). There were no significant differences in percent body weight loss between groups over this time period (Fig 5C). The rapid plunge in body weight was also seen in survivors, although, a majority of these mice began regaining their weight by day 3 (Fig 5D). Finally, the potential contribution of V- and F1-specific antibody responses to protection was assessed. None of the vaccinated groups produced detectable F1-specific titers (<50) other than TMV-F1+TMV-V-vaccinated mice, as predicted (Fig 5E). Surprisingly, none of the groups produced detectable V-specific titers (<50) other than two TMV-V vaccinated mice (Fig 5E). These two mice (1 male and 1 female) did not survive infection, whereas the one TMV-V male that did survive (1 out of 8

mice) did not produce anti-V antibodies prior to challenge. Therefore, although co-immunization of TMV-F1+TMV-V protected mice better than TMV-V immunization against F1- *Y. pestis*, their survival was not associated with a greater V-specific antibody response.

4.DISCUSSION

For mucosal vaccines, in particular, live vaccines are frequently preferred over subunit, killed, or particulate vaccines as they can circumvent the tolerogenic environment of mucosal tissues by creating a benign infection (Neutra and Kozlowski, 2006; Wang *et al.*, 2015). VLPs are a unique category of non-replicating vaccines that can balance safety and potency significantly better than live vaccines. While the induction of high titers is typical of VLP vaccines, it is not always sufficient for protection. The induction of CTL responses can be an added benefit for specific pathogens, including *Y. pestis* (Parent *et al.*, 2005; Smiley, 2007; Smiley, 2008). A potent cell-mediated response to VLP vaccines is dependent upon DC cross-presentation of VLP-derived antigens on MHC Class I, which then trigger CTL through interaction with the TCR (Donadson *et al.*, 2018). In the literature, viable TMV has been shown to target and activate DCs *in vitro* and *in vivo* (Kemnade *et al.*, 2014; McCormick *et al.*, 2006), and my own observations have shown that the TMV conjugates are also capable of targeting to DCs *in vitro* and *in vivo* (see Aim 1). DC-targeting inherently increases chances of naïve T cell activation in secondary lymphoid organs (SLO), and thus, contribution of cell-mediated immunity.

The data presented here demonstrated that neither $CD4^+$ T cells nor $CD8^+$ CTL are required for vaccine-induced protection against pneumonic plague. With the exception of mice that succumbed to the disease, all TMV-F1+TMV-V-immunized mice showed no

evident weight loss or other outward signs of illness post-challenge, even as $CD4^+$ and $CD8^+$ T cells were continually depleted until day of sacrifice. This was surprising, given that TMV conjugates were shown to target DCs. This data shows that a humoral immune response is sufficient for protection. Further work might wish to experiment with adjuvants in help TMV conjugates activate DCs as well as viable TMV can *in vivo* to induce CTL responses (Kemnade *et al.*, 2014). Other studies have showed that i.n. administration of VLP did not induce strong effector CTL responses (Bessa *et al.*, 2008; Bessa *et al.*, 2012), providing some support of our findings. Adoptive transfer of $CD4^+$ and $CD8^+$ T cells from TMV-F1 and/or TMV-V vaccinated mice to naïve mice remains the best method to confirm T cells are sufficient for vaccine-mediated protection against plague.

Specific immune responses are mounted against replicating viruses, but it is generally thought such responses are not elicited by plant viruses (Mandal and Jain, 2010). Effective VLP vaccines induce the production of antiviral antibodies specific for the virus surface proteins, which are neutralizing and protective by nature (Chen and Lai, 2013; Donaldson *et al.*, 2018). In the present study, we have confirmed that i.n. exposure to whole, inactivated TMV at vaccine-comparable doses generates systemic anti-TMV IgG2b titers. This response is magnitudes higher compared to mice not immunized with TMV, and unexposed mice appear to have no anti-TMV antibody (but they likely still do) (Fig 3). Most viruses and VLP consist of structurally identical capsid proteins arranged in a repetitive quasi-crystalline pattern; this structure naturally crosslinks BCRs and promotes MHCII presentation of peptides to T cells (Donaldson *et al.*, 2018). If foreign epitopes or proteins are displayed on the virus in this high-density display (genetically or chemically), immune responses can also be directed at those epitopes. Indeed, our data shows that the dominant IgG subclasses

directed against TMV (IgG2b, IgG3) are also directed against the CP-bound proteins: F1 and V. Like IgG2a, IgG2b fixes complement and binds to all activating Fcγ receptors (FcγR) (Collins, 2016). IgG1, on the other hand, does not fix complement and only binds well to the inhibitory FcγRIIb (Nimmerjahn and Ravetch, 2005), and so its contribution to protective immunity in mice is still unclear and was not examined here. F1/V-specific IgG3 is likely not critical for plague defense in mice, as this isotype does not exhibit high-affinity for FcγR (Bruhns and Jonsson, 2015; Collins, 2016). Also, the fact that unvaccinated mice had a higher IgG3 titer compared to rF1+rV vaccinated mice suggested binding to F1/V in these samples is nonspecific and perhaps due to contamination from *E. coli,* since LPS alone is sufficient to induce isotype switching to IgG3 (Deenick *et al.*, 1999).

I.n. administration of TMV-F1 can completely protect mice against the F1 wild-type *Y. pestis* CO92pgm- strain as well as mice given i.n. TMV-F1+TMV-V, which can likely be attributed to a strong antibody response against F1 in both groups (Arnaboldi *et al.*, 2016). However, the role of V antigen in vaccine-induced protection was still unclear. The best way to evaluate this role, we believed, was to use a *Y. pestis* strain lacking F1, where we could confirm contribution of V-specific adaptive immunity from the conjugate vaccine (TMV-F1+TMV-V) in the absence of the immunodominant F1. The KIM5*caf1A* mutant of *Y. pestis* (*Δpgm Δcaf1*) was selected; this strain cannot assemble the F1 capsule on its surface due to the lack of the Caf1A protein, which is required for secretion of F1 (Andrews *et al.*, 1996; Ivanov *et al.*, 2008; Zavialov *et al.*, 2003). Interestingly, mice i.n. immunized with rV, a mix of rF1 and rV (rF1+rV), or TMV-F1+TMV-V had no measurable V-specific serum titer prior to challenge, yet all of these groups fared better in survival against pneumonic plague than mice immunized with TMV-V alone, in which two mice did produce anti-V antibodies. Both

of those mice (a male and female) succumbed to infection while the one surviving TMV-V-immunized male had no titer prior to infection. The number of mice sampled in this experiment is small, and some mice were lost after the boost due to issues with the i.n. immunization, and therefore, should be repeated. The data thus far suggests that V-specific immunity may be T cell-mediated and not antibody-dependent. All immunized mice drastically lost body weight in the first 2-3 days after challenge, but then eventually rebounded if they survived. Activation of T memory cells in this case could be delayed in comparison to the rapid recall responses seen with F1-based humoral immunity (see Aim 2). This also involved a different *Y. pestis* strain (KIM5) and the lethal inoculation dose required two orders of magnitude of bacteria (~$2.5x10^7$ CFU) higher than that established for *Y. pestis* CO92pgm- (~$2.5x10^5$). This could be why dramatic weight loss was not observed in any of our 10 LD50 *Y. pestis* CO92 challenges. The data also clearly indicated that, for *Y. pestis* strains lacking F1, linking V to TMV for i.n. vaccination does not offer any benefit over V alone. Further experiments should seek to understand how rV as a vaccine could contribute to cell-mediated immunity better than TMV-V.

Another interesting observation was that the mixed TMV-F1+TMV-V vaccine offered better protection (30% survival) compared to TMV-V (12% survival). This finding echoes those of our laboratory, which reported that mean F1- and V-specific titers were elevated in mice co-immunized with TMV-F1 and TMV-V, compared to mice that received TMV-F1 or TMV-V alone (Arnaboldi *et al.*, 2016). Addition of TMV-F1 did not detract from the immune response to V, and vice versa. This is seen in cases, here, where antibody may not be critical to survival. In the aforementioned study, i.n. co-immunization of rF1 and rV did not increase V-specific IgG1, IgG2a, or IgG2b titers compared with i.n. rV

immunization alone (Arnaboldi *et al.*, 2016). Therefore, I hypothesized that the added presence of TMV was likely responsible for the enhanced anti-V response. These results encourage the addition of viable TMV as an adjuvant to the TMV-V vaccine. Our rTMV is replication-defective; the RNA is inactivated by BEI and cannot be translated, but is still intact. Since it is unclear how this inactivation affects RNA interaction with PRRs, addition of active TMV (with replication-capable RNA) is worth exploring. Active RNA may boost the antiviral-like response to TMV, and thus the anti-V response, since these are one-in-the-same during VLP-driven immunity. Further experiments looking at V-specific immunity may wish to add viable TMV to bolster $CD4^+$ T helper or CTL cell responses, since our data suggests V-specific antibodies are not a correlate of protection of TMV-V.

In summary, we show that TMV conjugates are a potent and safe chimeric mucosal vaccine that initiates VLP-like immune responses. Any mouse immunized i.n. with TMV-F1, specifically, usually developed high F1-specific serum IgG titers after single or multiple administrations. When applied to an infection model, we find that this response is protective against pneumonic infection with a *Y. pestis* strain expressing the F1 capsule protein, and this memory recall response is T-cell independent. This protection is lost against F1- *Y. pestis*, even when the TMV-V vaccine is also administered. Taken together, our results indicate the potential usefulness of rTMV as a carrier for VLP immunogenic presentation and mucosal delivery of whole protein antigens.

References

Al-Barwani F, Donaldson B, Pelham SJ, Young SL, Ward VK (2014). Antigen delivery by virus-like particles for immunotherapeutic vaccination. *Therapeutic Delivery.* 5: 1223-1240.

Andrews GP, Heath DG, Anderson GW, Welkos SL, Friedlander AM (1996). Fraction 1 capsular antigen (F1) purification from yersinia pestis CO92 and from an escherichia coli recombinant strain and efficacy against lethal plague challenge. *Infection and Immunity.* 64: 2180-2187.

Arnaboldi PM, Sambir M, D'Arco C, Peters LA, Seegers JF, Mayer L, McCormick AA, Dattwyler RJ (2016). Intranasal delivery of a protein subunit vaccine using a tobacco mosaic virus platform protects against pneumonic plague. *Vaccine.* 34: 5768-5776.

Balmelli C, Roden R, Potts A, Schiller J, De Grandi P, Nardelli-Haefliger D (1998). Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. *Journal of Virology.* 72: 8220-8229.

Bessa J, Schmitz N, Hinton HJ, Schwarz K, Jegerlehner A, Bachmann MF (2008). Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: Implications for vaccine design. *European Journal of Immunology.* 38: 114-126.

Bessa J, Zabel F, Link A, Jegerlehner A, Hinton HJ, Schmitz N, Bauer M, Kundig TM, Saudan P, Bachmann MF (2012). Low-affinity B cells transport viral particles from the lung to the spleen to initiate antibody responses. *Proceedings of the National Academy of Sciences of the United States of America.* 109: 20566-20571.

Bruhns P and Jonsson F (2015). Mouse and human FcR effector functions. *Immunological Reviews.* 268: 25-51.

Chen K, Magri G, Grasset EK, Cerutti A (2020). Rethinking mucosal antibody responses: IgM, IgG and IgD join IgA. *Nature Reviews.Immunology.*

Chen Q and Lai H (2013). Plant-derived virus-like particles as vaccines. *Human Vaccines & Immunotherapeutics.* 9: 26-49.

Collins AM (2016). IgG subclass co-expression brings harmony to the quartet model of murine IgG function. *Immunology and Cell Biology.* 94: 949-954.

Deenick EK, Hasbold J, Hodgkin PD (1999). Switching to IgG3, IgG2b, and IgA is division linked and independent, revealing a stochastic framework for describing differentiation. *Journal of Immunology.* 163: 4707-4714.

Donaldson B, Lateef Z, Walker GF, Young SL, Ward VK (2018). Virus-like particle vaccines: Immunology and formulation for clinical translation. *Expert Review of Vaccines.* 17: 833-849.

Herzog C (2014). Influence of parenteral administration routes and additional factors on vaccine safety and immunogenicity: A review of recent literature. *Expert Review of Vaccines.* 13: 399-415.

Huang Z, Santi L, LePore K, Kilbourne J, Arntzen CJ, Mason HS (2006). Rapid, high-level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice. *Vaccine.* 24: 2506-2513.

Ivanov MI, Noel BL, Rampersaud R, Mena P, Benach JL, Bliska JB (2008). Vaccination of mice with a yop translocon complex elicits antibodies that are protective against infection with F1- yersinia pestis. *Infection and Immunity.* 76: 5181-5190.

Jennings GT and Bachmann MF (2008). The coming of age of virus-like particle vaccines. *Biological Chemistry.* 389: 521-536.

Jiao YY, Fu YH, Yan YF, Hua Y, Ma Y, Zhang XJ, Song JD, Peng XL, Huang J, Hong T, He JS (2017). A single intranasal administration of virus-like particle vaccine induces an efficient protection for mice against human respiratory syncytial virus. *Antiviral Research.* 144: 57-69.

Kemnade JO, Seethammagari M, Collinson-Pautz M, Kaur H, Spencer DM, McCormick AA (2014). Tobacco mosaic virus efficiently targets DC uptake, activation and antigen-specific T cell responses in vivo. *Vaccine.* 32: 4228-4233.

Levine MM (2003). Can needle-free administration of vaccines become the norm in global immunization? *Nature Medicine.* 9: 99-103.

Mandal B and Jain RK (2010). Can plant virus infect human being? *Indian Journal of Virology : An Official Organ of Indian Virological Society.* 21: 92-93.

McCormick AA, Corbo TA, Wykoff-Clary S, Palmer KE, Pogue GP (2006). Chemical conjugate TMV-peptide bivalent fusion vaccines improve cellular immunity and tumor protection. *Bioconjugate Chemistry.* 17: 1330-1338.

McCormick AA and Palmer KE (2008). Genetically engineered tobacco mosaic virus as nanoparticle vaccines. *Expert Review of Vaccines.* 7: 33-41.

Neutra MR and Kozlowski PA (2006). Mucosal vaccines: The promise and the challenge. *Nature Reviews.Immunology.* 6: 148-158.

Nimmerjahn F and Ravetch JV (2005). Divergent immunoglobulin g subclass activity through selective fc receptor binding. *Science (New York, N.Y.).* 310: 1510-1512.

Parent MA, Berggren KN, Kummer LW, Wilhelm LB, Szaba FM, Mullarky IK, Smiley ST (2005). Cell-mediated protection against pulmonary yersinia pestis infection. *Infection and Immunity.* 73: 7304-7310.

Sedlik C, Dridi A, Deriaud E, Saron MF, Rueda P, Sarraseca J, Casal JI, Leclerc C (1999). Intranasal delivery of recombinant parvovirus-like particles elicits cytotoxic T-cell and neutralizing antibody responses. *Journal of Virology.* 73: 2739-2744.

Smiley ST (2007). Cell-mediated defense against yersinia pestis infection. *Advances in Experimental Medicine and Biology.* 603: 376-386.

Smiley ST (2008). Current challenges in the development of vaccines for pneumonic plague. *Expert Review of Vaccines.* 7: 209-221.

Smith ML, Lindbo JA, Dillard-Telm S, Brosio PM, Lasnik AB, McCormick AA, Nguyen LV, Palmer KE (2006). Modified tobacco mosaic virus particles as scaffolds for display of protein antigens for vaccine applications. *Virology.* 348: 475-488.

Tamminen K, Malm M, Vesikari T, Blazevic V (2016). Mucosal antibodies induced by intranasal but not intramuscular immunization block norovirus GII.4 virus-like particle receptor binding. *Viral Immunology.* 29: 315-319.

Wang S, Liu H, Zhang X, Qian F (2015). Intranasal and oral vaccination with protein-based antigens: Advantages, challenges and formulation strategies. *Protein & Cell.* 6: 480-503.

Weiner HL (2001). The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. *Nature Immunology.* 2: 671-672.

Zavialov AV, Berglund J, Pudney AF, Fooks LJ, Ibrahim TM, MacIntyre S, Knight SD (2003). Structure and biogenesis of the capsular F1 antigen from yersinia pestis: Preserved folding energy drives fiber formation. *Cell.* 113: 587-596.

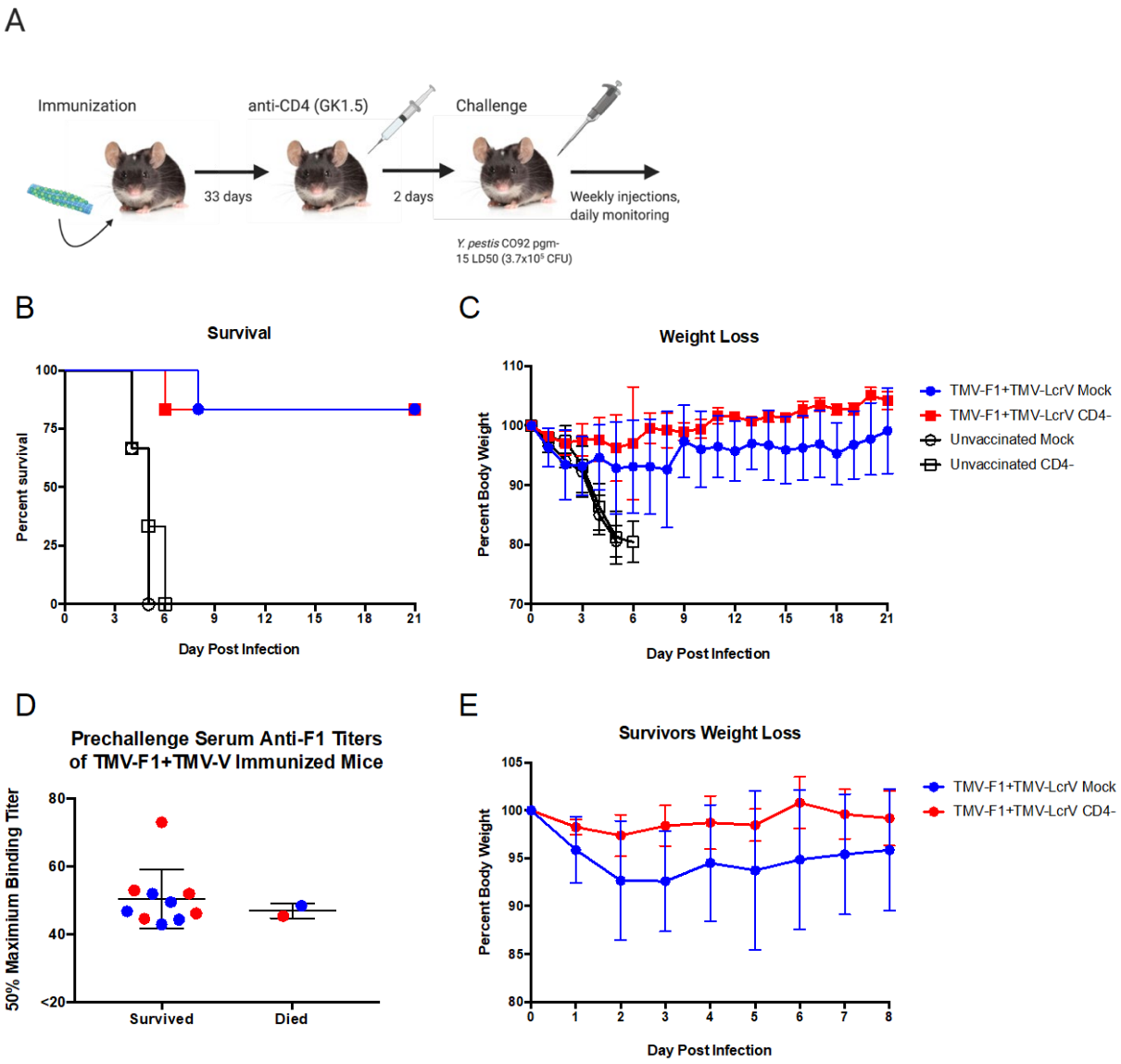


Fig 1. CD4⁺ T cell depletion prior to lethal pneumonic infection with *Y. pestis* CO92pgm- does not affect survival in immunized mice. 12 mice were i.n. immunized once with TMV-F1+TMV-V or not immunized (control), challenged i.n. with *Y. pestis* 5 weeks later, and monitored for 21 days post-infection. In each group, half of mice were injected IP with anti-CD4 antibody, and other half with rat IgG2bk isotype antibody, 2 days prior to challenge and weekly thereafter. (A) Immunization and challenge timeline. (B) Kaplan-Meier curve showing comparison of survival. Statistical significance was analyzed by the Mantel-Cox log-rank test ($p<0.0001$). (C) Average percent body weight (from initial weight on day of challenge). (D) Comparison of the pre-challenge total serum F1-specific titers between deceased and surviving TMV-F1+TMV-V-immunized mice. Unpaired t test analysis revealed no significant difference between the two groups. (E) Survivors' weight loss during the critical period of infection (D1-D8). Multiple t test analysis for each day post-infection revealed no significant difference between the two groups. All errors bars represent mean±SD.

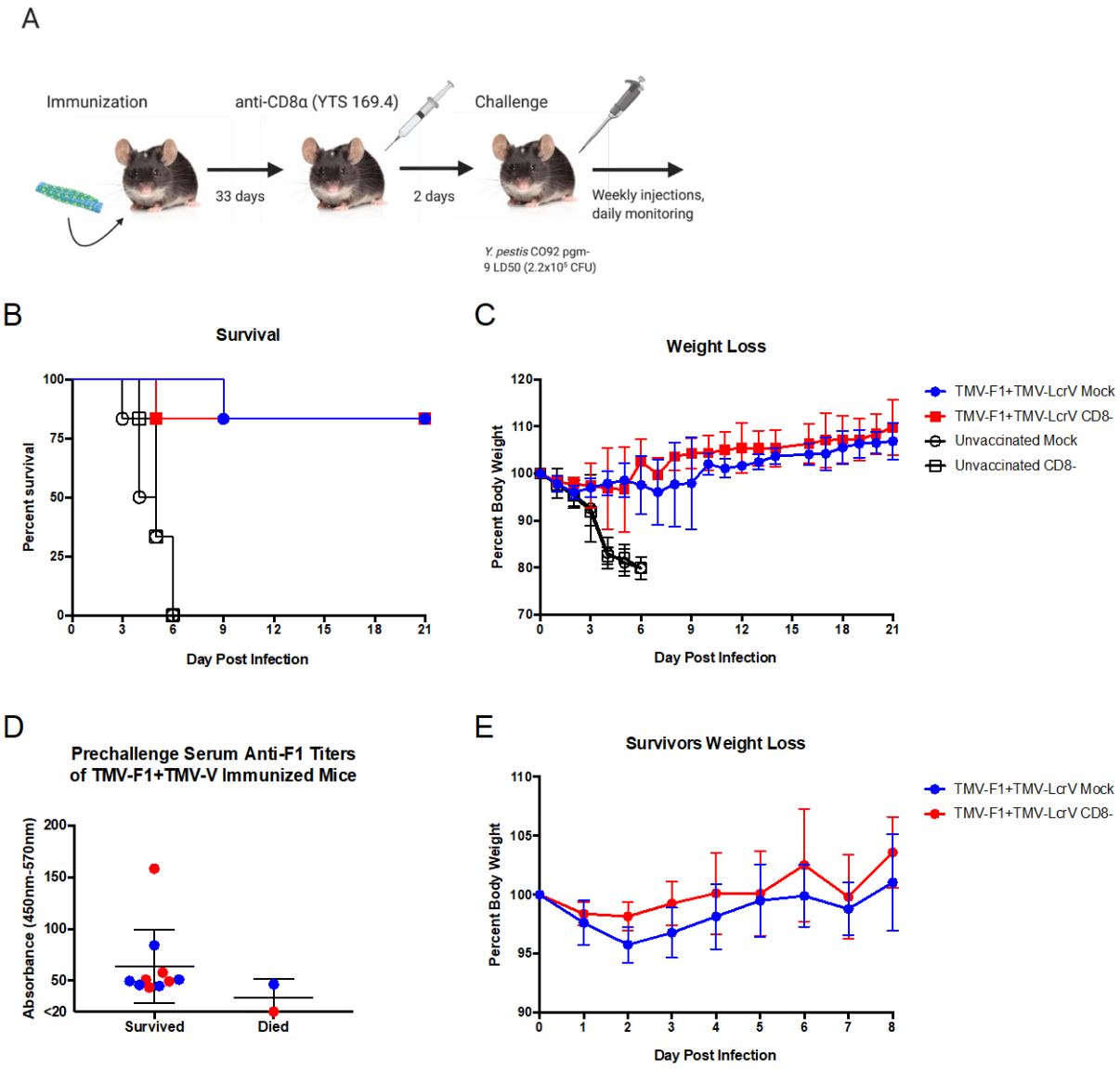


Fig 2. CD8$^+$ T cell depletion prior to lethal pneumonic infection with *Y. pestis* CO92pgm- does not affect survival in immunized mice. 12 mice were i.n. immunized once with TMV-F1+TMV-V or not immunized (control), challenged i.n. with *Y. pestis* 5 weeks later, and monitored for 21 days post-infection. In each group, half of mice were injected IP with anti-CD8α antibody, and other half with rat IgG2bk isotype antibody, 2 days prior to challenge and weekly thereafter. (A) Immunization and challenge timeline. (B) Kaplan-Meier curve showing comparison of survival. Statistical significance was analyzed by the Mantel-Cox log-rank test ($p<0.0001$). (C) Average percent body weight (from initial weight on day of challenge). (D) Comparison of the pre-challenge total serum F1-specific titers between deceased and surviving TMV-F1+TMV-V-immunized mice. Unpaired t test analysis revealed no significant difference between the two groups. (E) Survivors' weight loss during the critical period of infection (D1-D8). Multiple t test analysis for each day post-infection revealed no significant difference between the two groups. All errors bars represent mean±SD.

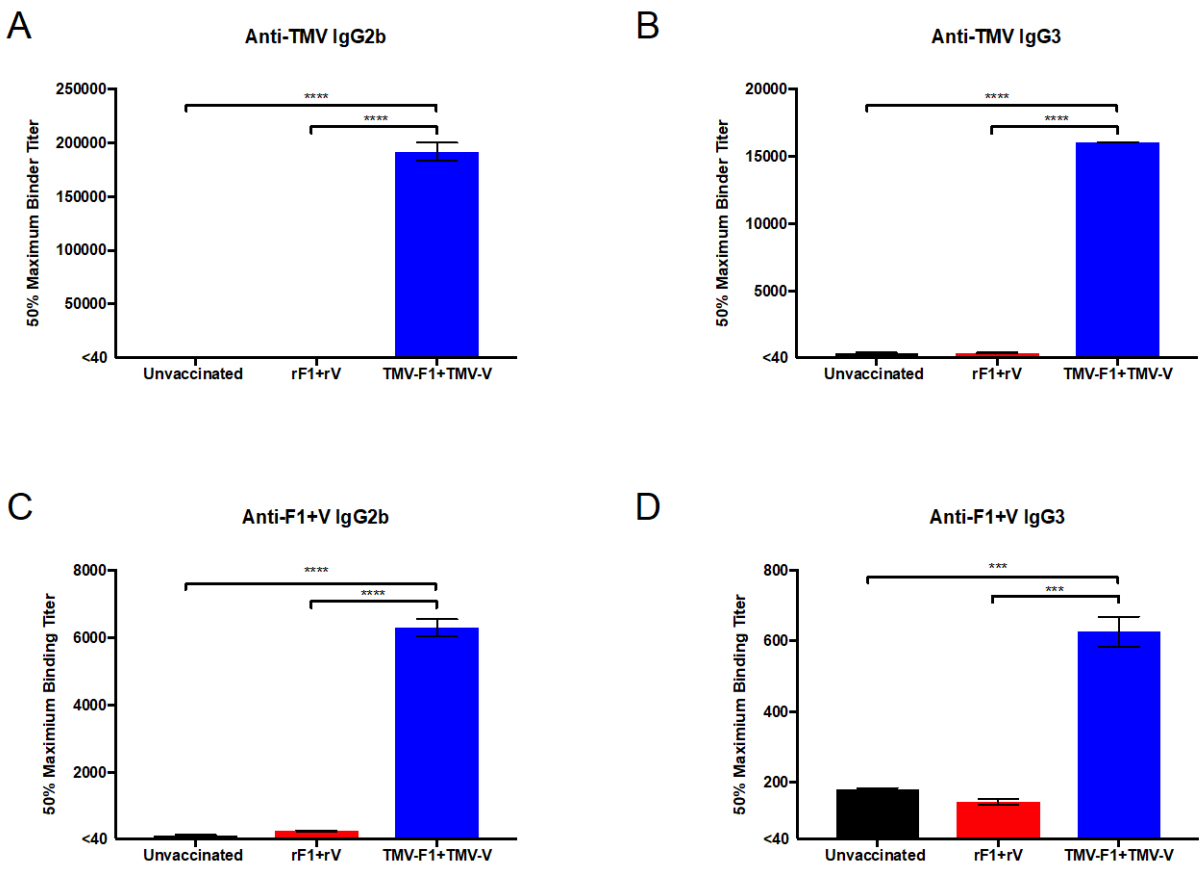


Fig 3. I.n. immunization with TMV-F1+TMV-V generates robust serum IgG response of the same subclasses (IgG2b, IgG3) against TMV and conjugated antigens. Serum antibody titers for anti-TMV IgG2b (A), anti-TMV IgG3 (B), anti-F1+V IgG2b (C), and anti-F1+V IgG3 (D) following i.n. vaccinations with rF1+rV, TMV-F1+TMV-V, or no vaccine (control). For all groups, serum was collected on day 56 post-prime immunization (day 0) and after the second and final boost (day 35). One-way ANOVA analysis revealed TMV-F1+TMV-V immunized mice had a significant increase in IgG2b and IgG3 titers for both TMV and F1+ V antigens compared to unvaccinated and rF1+rV-immunized mice. ****$p<0.0001$, ***$p<0.001$. n=4 mice/group. All errors bars represent mean±SD.

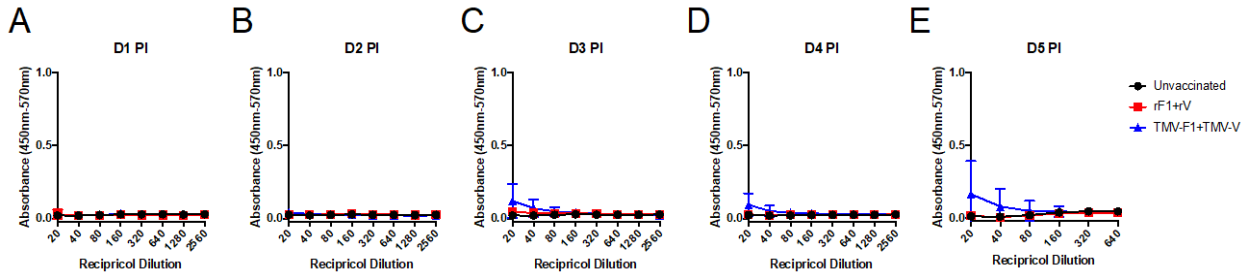


Fig 4. F1-specific IgG2b production is not localized to lung following infection. Comparison of ELISA curves for F1-specific IgG2b collected from lung homogenates 24 (A), 48 (B), 72 (C), 96 (D), and 120 (E) hours post-infection (HPI) with i.n. dose of 100 LD50 *Y. pestis* CO92pgm-. Multiple t test analysis for each dilution revealed no significant difference between the groups. n=6 mice/group. All errors bars represent mean±SD.

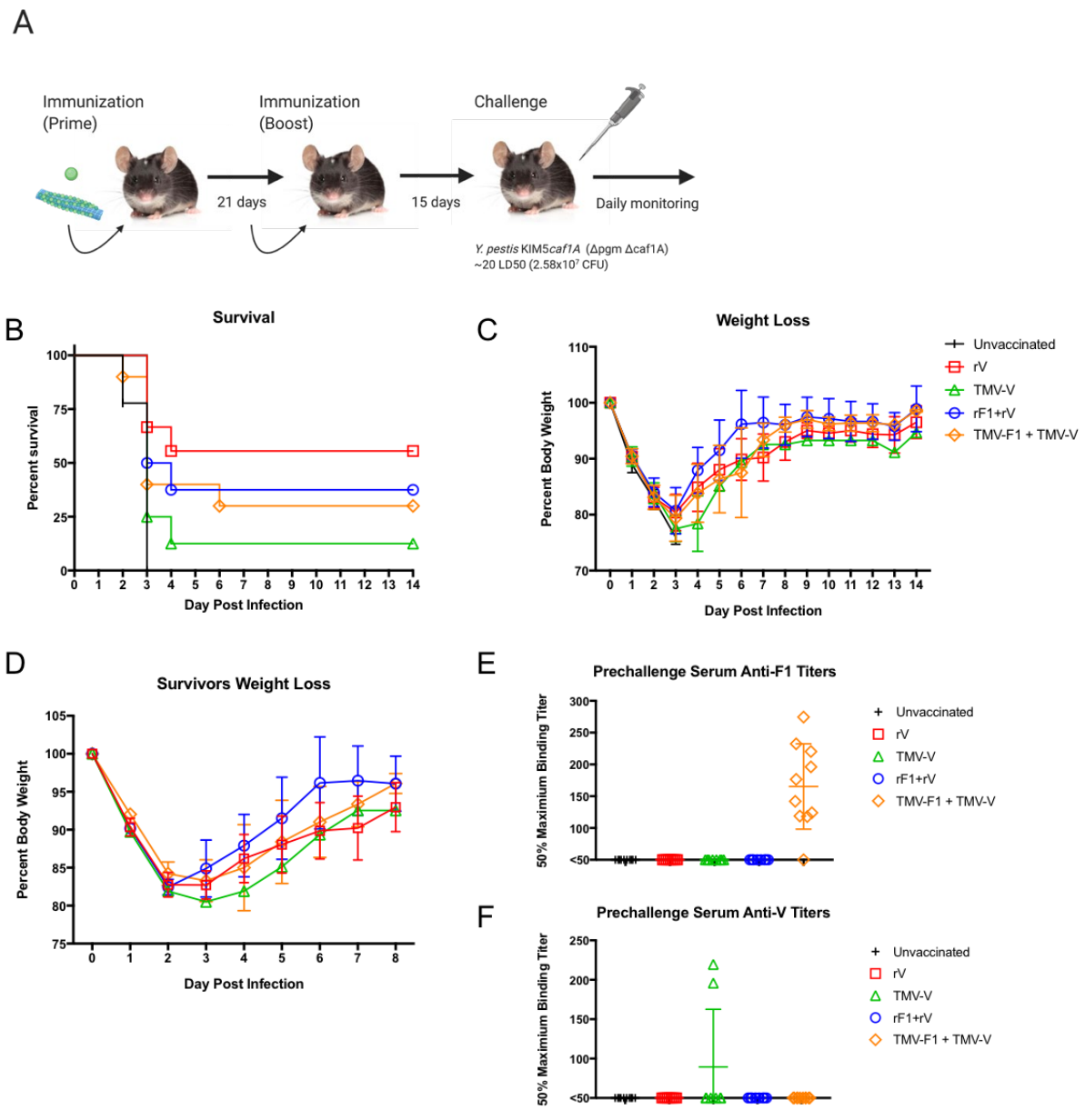


Fig 5. I.n. immunization and boost with rV provides the best protection against lethal pneumonic infection with a *Y. pestis* F1-mutant strain. 10 mice (5 males, 5 females) were i.n. immunized and boosted once with rV (red), TMV-V (green), rF1+rV (blue), TMV-F1+TMV-V (orange), or not immunized (black), and challenged i.n. with 20 LD50 *Y. pestis* KIM5*caf1A*. (A) Immunization and challenge timeline. (B) Kaplan-Meier curve showing comparison of survival. Statistical significance was analyzed by the Mantel-Cox log-rank test ($p<0.05$). (C) Average percent body weight (from initial weight on day of challenge). (D) Survivors' weight loss during the critical period of infection (D1-D8). Multiple t test analysis for each day post-infection revealed no significant differences between groups. Pre-challenge total serum F1-specific titers (E) and V-specific titers (F) for all groups, collected 4 days before challenge. Individual mice are indicated by their respective symbol. All errors bars represent mean±SD.

Chapter 5. Overall Discussion

The implementation of global vaccination in the 20th century is undoubtedly one of humanity's greatest medical achievements. Communicable diseases that once ravaged communities – smallpox, polio, diphtheria, pertussis, measles, rubella, mumps, hepatitis B, *Haemophilus influenzae* type b, tuberculosis, rotavirus, tetanus, meningococcal disease – are now considered vaccine-preventable diseases. The success of global vaccination programmes and eradication of smallpox in 1977 inspired the idea of disease eradication – an idealistic concept that a selected disease could be eradicated from all human populations through global cooperation (CDC, 1999). The discovery of antibiotics was yet another breakthrough in the fight against infectious diseases in the 20th century. However, overuse of these medicines in livestock, fish farming, and the clinic have contributed to multi-drug resistant (MDR) bacteria. The emergence of MDR bacterial infections has underscored the importance of disease prevention using vaccines.

In the event of a biological attack, antibiotics cannot be relied upon as the first, or even last, line of defense. Weaponized plague (*Yersinia pestis*) bacteria, for instance, would be expected to be MDR. Thus, routine antibiotics could not be used, and the bioweapon would successfully infect a densely populated city, aided by human-to-human transmission of airborne *Y. pestis*. To envision such a scenario is unsettling. Currently, there is no FDA-approved vaccine against plague for mass vaccination to protect the general public, although one is anticipated for use by the U.S. Army in 2021 (Demeure *et al.*, 2019). Vaccination would not only protect civilians before a bioterror attack, but it would also be a way to minimize casualties and deter future attacks. Since the anthrax attacks of September 2001,

our nation has prioritized and supported academic and government-led research of multi-biothreat vaccines, for example, a dual vaccine for *Bacillus anthracis* and *Y. pestis* (Tao *et al.*, 2017), or a trivalent vaccine for *B. anthracis*, *Y. pestis*, and *Francisella tularensis* (Jia *et al.*, 2018).

Whatever the target pathogen(s) may be, vaccines for biothreats should aim to have four key features: (1) broadly neutralizing ability against multiple or predicted antigenic variants, (2) capacity to induce rapid or sufficient protection after a primary dose, (3) capacity to generate long-lived memory in the host, and (4) above all, a good safety profile. Subunit vaccines containing pure recombinant antigens are the best option in regard to safety. Many laboratories utilize non-replicating vector platforms engineered to express antigens, or a fusion protein of two or more antigens. While this approach is feasible in small-scale laboratory experiments, such vectors could prove laborious to produce on a massive scale. For use in vaccines, fusion proteins – despite the advantage of multivalence – run the risk of concealing neutralizing epitopes that are accessible when individual antigens are administered. Singular proteins have better success at provoking antibodies against a wider range of linear and conformational epitopes, and antigen-specific responses can be further teased out experimentally.

Additionally, subunit vaccines typically require adjuvants to increase antigen-specific immunogenicity and maximize effectiveness. For instance, in the study by Tao *et al.,* their subunit vaccine against anthrax and plague required the use of Alhydrogel® for protection (Tao *et al.*, 2017). This vaccine could never be administered mucosally to people, as alum induces a strong proinflammatory immune response that will damage vital mucosal tissue. Non-replicating vectors and purified antigens commonly stimulate responses dominated by

antibodies; however, antibodies may not always provide optimal protection (Savelkoul *et al.*, 2015). This was the major limitation of KWC plague vaccines used on soldiers during the Vietnam War, where high antibody titers were effective against bubonic plague, but ineffective against pneumonic plague. A growing body of evidence supports that cell-mediated immunity, in cooperation with humoral immunity, is the best defense against pneumonic plague infection in NHP, and this method of protection likely extends to humans (Smiley, 2008). While this is not impossible with subunit vaccination, it has nonetheless proved challenging.

In the early-mid 2000s, McCormick, Palmer, and colleagues at Large Scale Biology Corp. created a novel recombinant virus delivery vehicle capable of binding whole protein antigens (McCormick and Palmer, 2008; Smith *et al.*, 2006). Our own version of this platform has been made to separately "carry" *Y. pestis* virulence proteins, LcrV (V) and F1 (TMV-V and TMV-F1, respectively). The proteins become covalently bound to the rTMV CP; they are not expressed by the virion. The unique structure of rTMV-protein conjugates gives it subunit, whole-inactivated virus, and VLP vaccine classification: it is an inactivated natural virus that carries subunit proteins in a high-density, repetitive display (similar to a viral capsid). It is worth noting that several discoveries were made with TMV-F1 and TMV-V prior to this dissertation. Most importantly, it was shown that the conjugates could be safely administered i.n. while retaining immunogenicity in mice. Additionally, co-vaccination of the TMV conjugates did not detract from the immune response to either antigen. In fact, F1- and V-specific titers were consistently elevated in mice co-immunized with TMV-F1+TMV-V, compared to mice that received TMV-F1 or TMV-V alone (with exception of anti-V IgG2b, IgG2a), as discussed previously (Arnaboldi *et al.*, 2016). It was

first thought by my lab that the combination of F1 and V was responsible for this effect, as is often reported with F1/V based vaccines (Amemiya *et al.*, 2009; Heath *et al.*, 1998; Jones *et al.*, 2006; Williamson *et al.*, 1997) . However, in our model, i.n. vaccination with rF1+rV did not significantly increase V- or F1-specific titers compared with i.n. rF1 or rV alone. Therefore, I hypothesized that the TMV was directly responsible for the enhancement of vaccine-specific immunity and protection in these mice. It was unclear at the time how TMV conjugates were accomplishing this. The initial hypothesis proposed by my PI stated that increased uptake of the TMV conjugates into DCs promoted T cell-mediated responses over that of the unbound antigens. However, this had yet to be formally established.

This dissertation made several new discoveries that have built upon the initial work of my lab and support further development of this unique carrier platform. A single i.n. dose of TMV-F1+TMV-V was capable of mediating 100% protection against 10 LD50 *Y. pestis* CO92pgm-, and physical linkage of proteins to rTMV (i.e. conjugation) was necessary for protection. When applied to a high-dose infection (100 LD50), a single i.n. dose of TMV-F1+TMV-V was capable of mediating 88% protection against this *Y. pestis* strain by the i.n. route, while i.n. immunization with rF1+rV protected <12% of mice. TMV conjugate immunization additionally showed reduction in histopathology, bacterial burden, and IFN-γ, TNF-α, and IL-17 tissue levels in the days following challenge. Pneumonic challenge resulted in systemic dissemination of *Y. pestis* bacteria in all groups, but only TMV-F1+TMV-V immunized mice rapidly cleared bacteria from the spleen and liver 4-5 days into infection. Additionally, I found that TMV conjugation to F1 and V increased their uptake into splenic and mucosal B-cells, and BMDCs *in vitro*, as well lung DCs *in vivo*. Neither TMV-F1 nor TMV-V were classic immunostimulants, as they did not upregulate

costimulatory markers (MHC II, CD80, CD86) over that of unstimulated $CD11c^+$ DCs. Finally, I demonstrated that generation of antibody-based, but not T cell-based, immunity could be attributed to vaccination with TMV conjugates. Mice administered with TMV-F1 i.n. predictably produced anti-F1 IgG after a single vaccine dose. When applied to an infection model of F1+ *Y. pestis*, we observed that the protective memory response induced by TMV vaccination did not require $CD4^+$ or $CD8^+$ T cells. This protection is lost against F1- *Y. pestis* KIM5*caf1A*, where mice vaccinated with TMV-F1+TMV-V demonstrated poor protection (30% survival).

In summary, my research has uncovered mechanisms by which rTMV platform is enhancing vaccine-specific immunity and protection against plague through i.n. vaccination. I have demonstrated the usefulness of rTMV as a carrier for VLP immunogenic presentation of some, but perhaps not all, antigenic proteins. Although these studies have revealed a considerable amount of information about the immune response to TMV and conjugates, we are left with a number of questions.

1.How important is the F1 antibody response induced by TMV-F1 vaccination?

A major finding of this dissertation was that higher F1-specific serum antibody levels correlated significantly with the weight of mice challenged i.n. with *Y. pestis*, regardless of the i.n. vaccine used: TMV-F1+TMV-V (conjugates) or rF1+rV (proteins) (Aim 2, Fig 4). This suggested to us that a higher F1 titer prior to challenge signified a better chance of survival. Although my Aim 2 results showed that TMV-F1+TMV-V immunized mice had increased F1 titers compared to the other groups, higher F1-specific titers were only moderately associated with percent body weight at D3 PI, and pre-challenge titers were not

required for survival. In fact, conjugate-vaccinated mice survived challenge despite a low F1 titer (<50); and conversely, two protein-vaccinated mice and one conjugated-vaccinated mouse died despite an F1 titer of 100-125 (comparable to titers of survivors) (Aim 2, Fig 4). Interestingly, only a small proportion of surviving TMV-F1+TMV-V immunized mice produced little anti-F1 antibody, while all surviving rF1+rV immunized mice had relatively high anti-F1 titers prior to infection.

When comparing the F1 titers of only TMV-F+TMV-V vaccinated mice in Aim 3, we found no significant difference in the pre-challenge total F1 serum antibody titers between the deceased and surviving mice (Aim 3, Fig 1 and 2). T cells were depleted weeks after blood collection and should have had no effect on immune responses to immunization. Although this finding seems contradictory to that of the Aim 2 challenge, these analyses answered separate questions. The above Pearson's' correlation asked if F1 antibody levels correlated with the overall health and recovery of mice, to which the answer was yes. In the newer sets of experiments, since no unimmunized mice survived, the titer comparison specifically asked if the quantity of F1 antibodies (titer) could explain the discordant outcomes from TMV conjugate immunization. Here, survivors had a mean serum F1 titer of at least 50 prior to challenge, however, the titers of the decreased mice were only slightly less. So, while it can be said that F1 antibody is predictably generated in response to TMV-F1+TMV-V vaccination, the antibody level *itself* is not a determinant of vaccine-mediated protection. It is more likely that the quality – not quantity – of the antibody response is correlative of protection. Further work should utilize *in vitro* neutralization, ADCP, and CDC assays to evaluate functionality of vaccine-specific antibodies. Does antibody function (neutralization, opsonization, cytolysis) improve with boosts? What host factors may

negatively influence antibody production from vaccination? These questions need to be addressed. Therefore, although we found a significant correlation between post-infection recovery and serum F1 titers, the results obtained by the later set of challenge experiments show that the correlation may not be biologically relevant. Further work needs to elucidate what other factors are critical for vaccine-mediated protection and how to better characterize and predict vaccine efficacy.

2.What is the contribution of DCs vs. B cells to TMV-F1-mediated immunity?

Although my work has shown that TMV-F1+TMV-V were increasingly taken up DCs in the lung, compared to TMV and unconjugated rF1+rV, other results of my dissertation do not support a strong role for DCs in vaccine-mediated immunity. Both conjugates failed to upregulate costimulatory markers on DCs *in vitro*. These molecules are a major requirement for activation of naïve T cells, and thus, T cell-mediated responses. I also failed to see the same effect *in vivo* with CD86 and MHC II. It is still possible that if MLNs were isolated, instead of the lungs, costimulatory molecules would be upregulated on DCs that internalized the conjugates, although this would not explain why we did not see the effect *in vitro*. As previously mentioned, the TMV as part of the conjugate is inactivated and has lost the ability to replicate. Further experimentation is needed to determine if BEI inactivation interferes in the RNA's interaction with PRRs in DCs, although the literature provides evidence for T cell activation when viable (non-inactivated) TMV is used in immunization strategies (Kemnade *et al.*, 2014)

Results of Aim 1 also showed no significant difference in uptake of TMV-F1 and F1 into DCs (Aim 1, Fig 3), yet the primary immune responses found and investigated in this

dissertation were against F1. Coating the TMV virion in F1 proteins could limit its uptake by phagocytosis, as F1 protein naturally accomplishes this in *Y. pestis* as part of the bacterial capsule. DCs cannot initiate anti-F1 T cell responses if they are unable to first process the antigen. Yet, a structure that prevents uptake by phagocytes could simultaneously promote endocytosis by B cells, on account of the specialization of professional APCs (Bachmann *et al.*, 1993). Unlike DCs, repetitive protein displays naturally crosslink BCRs on B cells and promotes MHCII presentation of peptides to T cells (Donaldson *et al.*, 2018). Aim 1 demonstrated that uptake of antigens by B cells is independent of the virus and is likely due to the random presence of BCRs capable of binding to F1 or V antigens. For splenic B cells, uptake of TMV-F1 was significantly higher than rF1, and this difference increased with each day of incubation, indicating potential for activation of naïve B cells by TMV-F1, but not soluble rF1 (Aim 1, Fig 3). However, due to the specificity of antigen internalization, few naïve B cells participate in uptake of novel antigens. The lung showed high variability in uptake of all antigens into B cells that made it difficult to infer significant uptake (Aim 1, Fig 1 and 2). The variation can be attributed to at least two sources: (1) the vast repertoire of BCR that can exist between individual mice, and (2), the nature of i.n. instillation, as inhaled material deposits asymmetrically in both lungs and inoculums can vary in size/volume between subjects, as opposed to uniform aerosol inhalation (Mizgerd and Skerrett, 2008). Despite these limitations, B cells may represent the primary APC involved in uptake of TMV-F1, and further *in vivo* studies should seek to improve experimental conditions where B cell uptake of this conjugate can be more accurately measured.

If B cells are key APCs, what is their fate? Since lungs were not perfused when sampled, the presence of F1-specific IgG2b in the lung post-infection was likely due to

transudation from circulation and not local production (Aim 3, Fig 4). Upon re-exposure to antigen, memory B cells immediately differentiate into plasma cells in a manner that is faster, stronger, and more specific than the naïve response (Palm and Henry, 2019). Some also retreat to SLO to undergo further affinity maturation and proliferate to create more memory cells. Although it seems critical in pneumonic plague infection that memory B cells would home to the lung, to maximize their chances of encountering antigen mucosally, our data suggests F1-specific IgG^+ memory B cells do not reside there. Rather, the spleen, cervical LN (CLN), MLN, or nasal-associated mucosal tissue (NALT) are the likely sites of germinal center (GC) responses generated by i.n. immunization with TMV-F1 (**Fig 1**). One study in mice identified low-affinity lung B cells as the key APCs that shuttle inhaled VLP from the lung to the spleen via the circulation to initiate GC antibody responses (Bessa *et al.*, 2012). The marginal zone of the spleen serves as a major reservoir of memory B cells in mice, as does the subcapsular sinus of LNs (Tangye and Tarlinton, 2009). Going forward, F1-specific IgG^+ memory B cells should be isolated from TMV-F1 immunized mice. If identification of such memory cells is possible, future experiments can adoptively transfer B cells from TMV-F1 vaccinated mice to naïve mice to demonstrate conferred protection against plague.

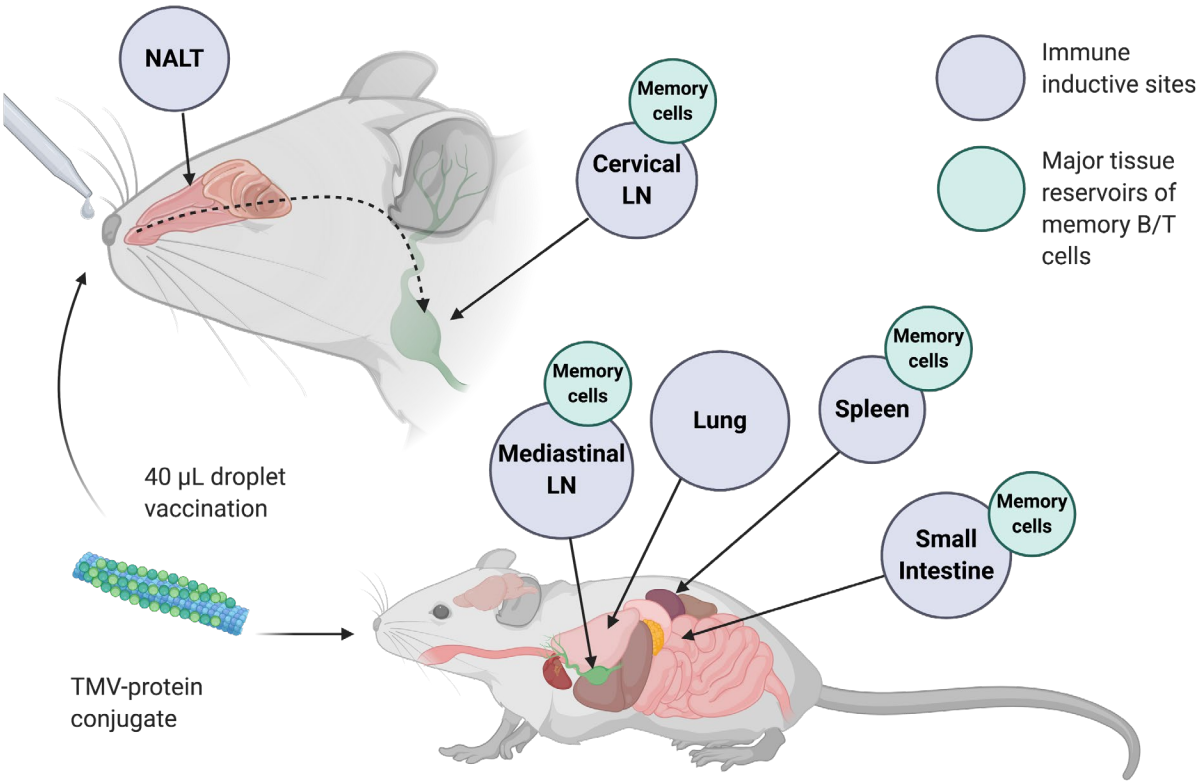


Fig 1. Potential immune inductive sites and memory T and B cell locations generated by i.n. immunization in mice. Various SLO and organized lymphoid tissues could serve as sites of immune responses to i.n. immunization. The aqueous droplet inhaled by the mouse may stimulate an immune response to TMV conjugates in the NALT, if volume is retained in the nasal tissue. In this case, antigen-specific and TMV-specific memory cells would likely be stored in the downstream cervical LNs (CLN). Due to the large droplet volume used (40µL), the inhaled TMV conjugates are also absorbed in the lungs, in which case downstream mediastinal LNs (MLN) would serve as the site of immune induction and memory cells. If the TMV conjugates reach circulation, the spleen could also serve as additional inductive site and pool of antigen-specific memory cells. There is also a chance a small volume of the conjugates bypasses the trachea and enters the gastrointestinal tract, where there could be generation of intestine-resident memory cells if immune responses were initiated there. Image created on BioRender.com.

3.What is the role of T cells in vaccine-mediated immunity, if these cells are not required for protection against plague?

One of the major findings of this dissertation was that the predominant IgG subclass in the humoral response to TMV (IgG2b) mirrored the response to F1 and V. Conjugation of these proteins to TMV significantly increased both viral titers and antigen-specific IgG2b titers compared to mice given rF1+rV i.n. (Aim 3, Fig 3). This suggests that the TMV conjugate is enhancing humoral responses by exploiting a phenomenon known as linked recognition, where B-cells internalize the highly repetitive TMV surface structure, degrade and process the whole conjugate, and present both virial and antigen peptides to cognate T_H cells. The cognate interaction between T follicular helper (T_{FH}) cells and B cells is necessary for T cell-dependent (TD) Ig class switching and high-affinity antibodies (Shinomiya *et al.*, 1989). T_H1 or T_H17 cellular responses were not required to eliminate the bacteria (Aim 3, Fig 1 and 2). Therefore, the main contribution of T cells in our vaccine model is likely in the "priming phase," helping B cells make specific antibody to TMV CP and *Y. pestis* proteins, as opposed to the "effector phase," where T cells would be directly or indirectly killing infected cells.

T_{FH}-secreted IL-21 and ligation of CD40L to CD40 promotes differentiation of B cells into long-lived memory cells and plasma cells – a hallmark of TD B cell responses. Generation of T_{FH} cells is thought to be driven by DCs *in vivo,* where signals downregulate CCR7 on $CD4^+$ T cells allowing them to migrate to B cell follicles (Fazilleau *et al.*, 2009). In GC, cognate activated B cells are also thought to provide similar signals that drive the full development of T_{FH} cells, such as secretion of IL-6 and IL-27 (Ma *et al.*, 2012). However, naïve antigen-specific B cells are rare *in vivo*, as previously mentioned, and are not available

as APCs for $CD4^+$ T cells as readily as DCs are (Kolenbrander *et al.*, 2018). Thus, DCs play a principal role in initiating potent T_{FH} responses. T cell-independent (TI) activation of B cells can also occur and requires synchronized BCR and TLR signaling. It has been shown that TLR7 stimulation by a ssRNA-containing VLP (Qβ-VLP) can overcome the requirement of T_{FH}-derived IL-21 to generate IgG responses in GC (Bessa *et al.*, 2010). rTMV – while a whole virus and not a VLP –contains intact ssRNA as well. The conjugated protein antigens (rF1 and rV) are additionally LPS-contaminated due to expression in *E. coli* (Appendix I, Fig S4). It would be interesting to see whether TLR7 and/or TLR4 signaling in B cells with TMV conjugates translated to a TI response *in vivo*. TD responses usually follow initial TI responses to viral proteins and contribute to affinity maturation and memory B cell maintenance. Despite the robust TI response to Qβ-VLP i.p. immunization, T cells were ultimately needed to induce long-lived Qβ-specific memory IgG^+ B cells that were detected in the spleen for >1 year (Liao *et al.*, 2017). Thus, $CD4^+$ T_{FH} cells would be expected to play a key role in the generation of long-term TMV (vaccine)-specific memory; although, we found that $CD4^+$ T cells may not be required for re-activation of memory B cells from a single vaccination (Aim 3, Fig 1). Adoptive transfer of T cells from TMV-F1 and/or TMV-V vaccinated mice to naïve mice can confirm if T cells are still sufficient for vaccine-mediated protection against plague.

4.How are mucosal tissue and SIgA contributing to vaccine-mediated immunity?

The nasal route represents the best method of delivery for non-replicating chimeric plant virus particles, as i.n. delivery of these vaccines has been shown to optimally induce both mucosal and systemic IgA and IgG responses in mice, without the requirement of

adjuvants (Brennan *et al.*, 1999). Humoral immune responses at mucosal surfaces have historically focused on SIgA. With i.n. vaccination, SIgA production could take place in the lung, MLN, and also at distal mucosal sites such as the intestinal and vaginal tracts. Unfortunately, the lungs collected in the 5 days post-infection were not perfused, so it could not be determined if there were F1-specific IgA^+ B cells in the lung mucosa. Other mucosal sites (e.g. intestine) were not assessed for vaccine-specific memory cells or Ig in our model. Antigen-specific SIgA can be difficult to detect and usually requires specialized techniques because there is so little antigen-specific IgA compared to the total IgA content.

Possible contribution of the NALT to immunity could not be determined in the course of this project. Groups have pointed to the NALT as the major site for priming B cells following i.n. infection or immunization (Brandtzaeg, 1999; Kiyono and Fukuyama, 2004; Liang *et al.*, 2001; Zuercher *et al.*, 2002). However, the NALT in mice lacks memory cells and afferent lymphatics, and thus, is an irrelevant source of antibody production. Prior work in our laboratory has shown B cells of the NALT are positive for fluorescently labeled TMV upon i.n. immunization within hours (data not shown), but these experiments did not clarify the fate of these B cells, nor the requirement of NALT to vaccine-induced protection. My work attempted this but the NALT ablation surgical procedure was not successful in our mice, and so the results of that challenge study were scraped. Our VLP-like vaccine is administered i.n. as droplets, which can be retained in both the upper and lower respiratory tracts of the mouse depending on the volume used (Mizgerd and Skerrett, 2008) . However, a large droplet, such as the 40µL volume used in our studies, is mostly inhaled to trigger B-cell responses preferentially in MLN or lower respiratory tract (Bessa *et al.*, 2008). I.n. immunization may also lead to induction of B cell responses in the CLN which drain the nose

and upper airways. However, observations comparing the number of VLP-specific B cells in the CLN and MLN have shown that the murine lower respiratory tract is the major site draining particulate vaccines for the induction of antibody responses in MLN (Bessa *et al.*, 2008). Further investigation is needed to identify where antigen-specific memory B cells reside (in LNs or spleen) after i.n. immunization with TMV conjugates. The use of ELISPOT (*ex vivo*), BCR transgenic mice (*in vivo*), multiphoton microscopy (*in vivo*), or fluorescently labeled antigen tetramers and anti-idiotypic antibodies in flow cytometry (*ex vivo*) are some potential methods for identification of antigen-specific B cells in mice.

References

Amemiya K, Meyers JL, Rogers TE, Fast RL, Bassett AD, Worsham PL, Powell BS, Norris SL, Krieg AM, Adamovicz JJ (2009). CpG oligodeoxynucleotides augment the murine immune response to the yersinia pestis F1-V vaccine in bubonic and pneumonic models of plague. *Vaccine.* 27: 2220-2229.

Arnaboldi PM, Sambir M, D'Arco C, Peters LA, Seegers JF, Mayer L, McCormick AA, Dattwyler RJ (2016). Intranasal delivery of a protein subunit vaccine using a tobacco mosaic virus platform protects against pneumonic plague. *Vaccine.* 34: 5768-5776.

Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, Zinkernagel RM (1993). The influence of antigen organization on B cell responsiveness. *Science (New York, N.Y.).* 262: 1448-1451.

Bessa J, Kopf M, Bachmann MF (2010). Cutting edge: IL-21 and TLR signaling regulate germinal center responses in a B cell-intrinsic manner. *Journal of Immunology (Baltimore, Md.: 1950).* 184: 4615-4619.

Bessa J, Schmitz N, Hinton HJ, Schwarz K, Jegerlehner A, Bachmann MF (2008). Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: Implications for vaccine design. *European Journal of Immunology.* 38: 114-126.

Bessa J, Zabel F, Link A, Jegerlehner A, Hinton HJ, Schmitz N, Bauer M, Kundig TM, Saudan P, Bachmann MF (2012). Low-affinity B cells transport viral particles from the lung to the spleen to initiate antibody responses. *Proceedings of the National Academy of Sciences of the United States of America.* 109: 20566-20571.

Brandtzaeg P (1999). Regionalized immune function of tonsils and adenoids. *Immunology Today.* 20: 383-384.

Brennan FR, Bellaby T, Helliwell SM, Jones TD, Kamstrup S, Dalsgaard K, Flock JI, Hamilton WD (1999). Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice. *Journal of Virology.* 73: 930-938.

CDC (1999). Achievements in public health, 1900-1999: Control of infectious diseases . *MMWR Weekly.* 48: 621-629.

Demeure CE, Dussurget O, Mas Fiol G, Le Guern AS, Savin C, Pizarro-Cerda J (2019). Yersinia pestis and plague: An updated view on evolution, virulence determinants, immune subversion, vaccination, and diagnostics. *Genes and Immunity.* 20: 357-370.

Donaldson B, Lateef Z, Walker GF, Young SL, Ward VK (2018). Virus-like particle vaccines: Immunology and formulation for clinical translation. *Expert Review of Vaccines.* 17: 833-849.

Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG (2009). Follicular helper T cells: Lineage and location. *Immunity.* 30: 324-335.

Heath DG, Anderson GW, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, Friedlander AM (1998). Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. *Vaccine.* 16: 1131-1137.

Jia Q, Bowen R, Dillon BJ, Maslesa-Galic S, Chang BT, Kaidi AC, Horwitz MA (2018). Single vector platform vaccine protects against lethal respiratory challenge with tier 1 select agents of anthrax, plague, and tularemia. *Scientific Reports.* 8: 7009-y.

Jones T, Adamovicz JJ, Cyr SL, Bolt CR, Bellerose N, Pitt LM, Lowell GH, Burt DS (2006). Intranasal protollin/F1-V vaccine elicits respiratory and serum antibody responses and protects mice against lethal aerosolized plague infection. *Vaccine.* 24: 1625-1632.

Kemnade JO, Seethammagari M, Collinson-Pautz M, Kaur H, Spencer DM, McCormick AA (2014). Tobacco mosaic virus efficiently targets DC uptake, activation and antigen-specific T cell responses in vivo. *Vaccine.* 32: 4228-4233.

Kiyono H and Fukuyama S (2004). NALT- versus peyer's-patch-mediated mucosal immunity. *Nature Reviews.Immunology.* 4: 699-710.

Kolenbrander A, Grewe B, Nemazee D, Uberla K, Temchura V (2018). Generation of T follicular helper cells in vitro: Requirement for B-cell receptor cross-linking and cognate B- and T-cell interaction. *Immunology.* 153: 214-224.

Liang B, Hyland L, Hou S (2001). Nasal-associated lymphoid tissue is a site of long-term virus-specific antibody production following respiratory virus infection of mice. *Journal of Virology.* 75: 5416-5420.

Liao W, Hua Z, Liu C, Lin L, Chen R, Hou B (2017). Characterization of T-dependent and T-independent B cell responses to a virus-like particle. *Journal of Immunology (Baltimore, Md.: 1950).* 198: 3846-3856.

Ma CS, Deenick EK, Batten M, Tangye SG (2012). The origins, function, and regulation of T follicular helper cells. *The Journal of Experimental Medicine.* 209: 1241-1253.

McCormick AA and Palmer KE (2008). Genetically engineered tobacco mosaic virus as nanoparticle vaccines. *Expert Review of Vaccines.* 7: 33-41.

Mizgerd JP and Skerrett SJ (2008). Animal models of human pneumonia. *American Journal of Physiology.Lung Cellular and Molecular Physiology.* 294: 387.

Palm AE and Henry C (2019). Remembrance of things past: Long-term B cell memory after infection and vaccination. *Frontiers in Immunology.* 10: 1787.

Savelkoul HF, Ferro VA, Strioga MM, Schijns VE (2015). Choice and design of adjuvants for parenteral and mucosal vaccines. *Vaccines.* 3: 148-171.

Shinomiya N, Kuratsuji T, Yata J (1989). The role of T cells in immunoglobulin class switching of specific antibody production system in vitro in humans. *Cellular Immunology.* 118: 239-249.

Smiley ST (2008). Current challenges in the development of vaccines for pneumonic plague. *Expert Review of Vaccines.* 7: 209-221.

Smith ML, Lindbo JA, Dillard-Telm S, Brosio PM, Lasnik AB, McCormick AA, Nguyen LV, Palmer KE (2006). Modified tobacco mosaic virus particles as scaffolds for display of protein antigens for vaccine applications. *Virology.* 348: 475-488.

Tangye SG and Tarlinton DM (2009). Memory B cells: Effectors of long-lived immune responses. *European Journal of Immunology.* 39: 2065-2075.

Tao P, Mahalingam M, Zhu J, Moayeri M, Kirtley ML, Fitts EC, Andersson JA, Lawrence WS, Leppla SH, Chopra AK, Rao VB (2017). A bivalent anthrax-plague vaccine that can protect against two tier-1 bioterror pathogens, bacillus anthracis and yersinia pestis. *Frontiers in Immunology.* 8: 687.

Williamson ED, Eley SM, Stagg AJ, Green M, Russell P, Titball RW (1997). A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague. *Vaccine.* 15: 1079-1084.

Zuercher AW, Coffin SE, Thurnheer MC, Fundova P, Cebra JJ (2002). Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. *Journal of Immunology (Baltimore, Md.: 1950).* 168: 1796-1803.

Chapter 6. Appendices

Supportive Data and Analyses

Appendix I.

Table S1. Total number of randomly selected male and female mice used per time point for *in vivo* replicate experiments.

Immunization Group	**1h**	**2h**	**4h**	**24h**	**48h**	**96h**
Control (unimmunized)	2 males 1 female	1 male 2 females	1 male 2 females	1 male 2 females	2 males 1 female	2 males 1 female
rF1+rV	1 male 2 females	2 males 1 female	2 males 1 female	2 males 1 female	2 males 1 female	1 male 2 females
TMV-F1+TMV-V	1 male 2 females	1 male 2 females	2 males 1 female	1 male 2 females	1 male 2 females	1 male 2 females
TMV	2 males 1 female	2 males 1 female	1 male 2 females	2 males 1 female	1 male 2 females	2 males 1 female

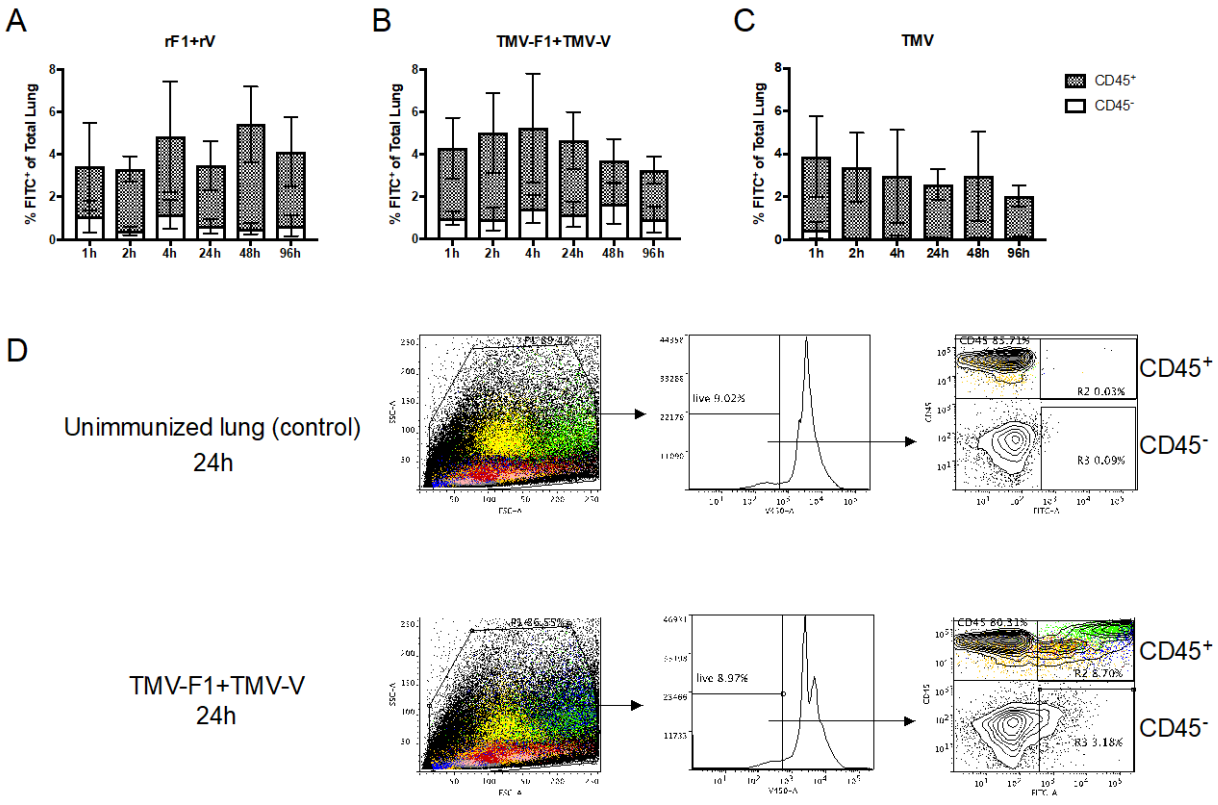
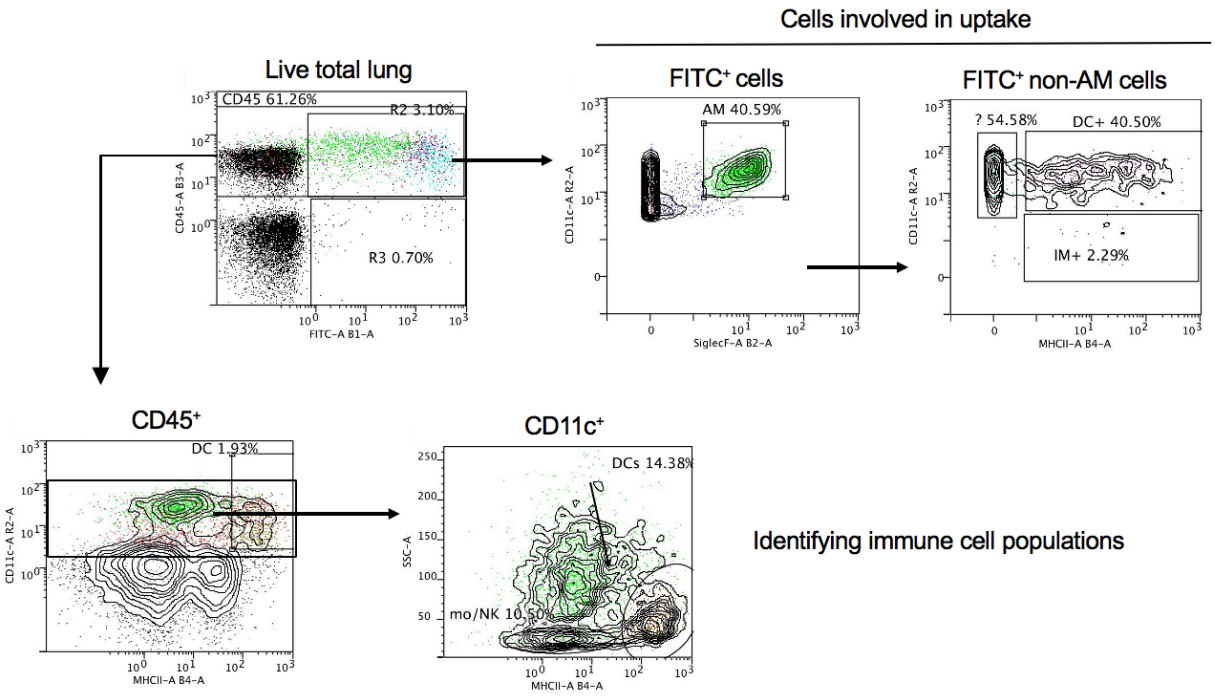


Fig S1. General uptake of i.n.-administered protein antigens, TMV conjugates, and TMV represents <8% of total viable lung cells. Uptake of 488-labeled proteins ($FITC^+$) proteins into different immune cell populations in the murine lung after 1, 2, 4, 24, 48, and 96 hours post- i.n. immunization. Uptake of $FITC^+$ rF1+rV proteins (A), TMV-F1+TMV-V conjugates (B), and unconjugated TMV (C) into leucocytes ($CD45^+$) and non-leucocytes (CD45-) are shown as the percent of total viable lung cells. CD45- bars (white) are superimposed onto $CD45^+$ bars (grey). (D) Flow cytometry analysis and gating for general lung uptake. Example plots shown: a control and TMV-F1+TMV-V-immunized lung sacrificed at 24-hour time point. Significance between all three immunization groups was analyzed in one graph (not shown) by repeated-measures two-way ANOVA with Tukey's multiple comparisons test, however, at no time point were the groups statistically different from one another. All errors bars represent mean±SEM.



Cell type	Surface markers and cell scatter
Alveolar macrophage (AM)	CD11c+ SiglecF+ SSChi MHCII- CD11b-
Dendritic cell (DC)	CD11c+ MHCII+ SSC^{lo} CD11b+/- SiglecF-
Interstitial macrophage (IM)	CD11b+ CD11c+/- SSC^{hi} MHCII+/- SiglecF-
Monocyte (Ly6C+ presumed)	CD11c- CD11b+/- MHCII+/- SSCl^{o} SiglecF-
B cell	CD19+ SSC^{lo}
DC precursor (pre-DC)	CD11c+ CD11b- MHCII- SSC^{lo} SiglecF-

Fig S2. Gating strategy and cell markers used for analysis of FITC^{+} protein uptake into lung CD45^{+} cells *in vivo*. Flow cytometry analysis and gating used to define CD45^{+} cell populations, as well as cells associated with FITC^{+} proteins. FITC^{+} cells could be clearly separated into AMs and non-AMs. Boolean gates were created (e.g. DC gate + FITC^{+} gate = FITC^{+} DCs) to accurately determine all CD45^{+} cell populations involve in uptake, using Flowlogic™ analysis software. The table lists the surface markers and scatter used to identify the various cell populations used in this study. SSC^{hi} was defined as >50 and SSC^{lo} as ≤50.

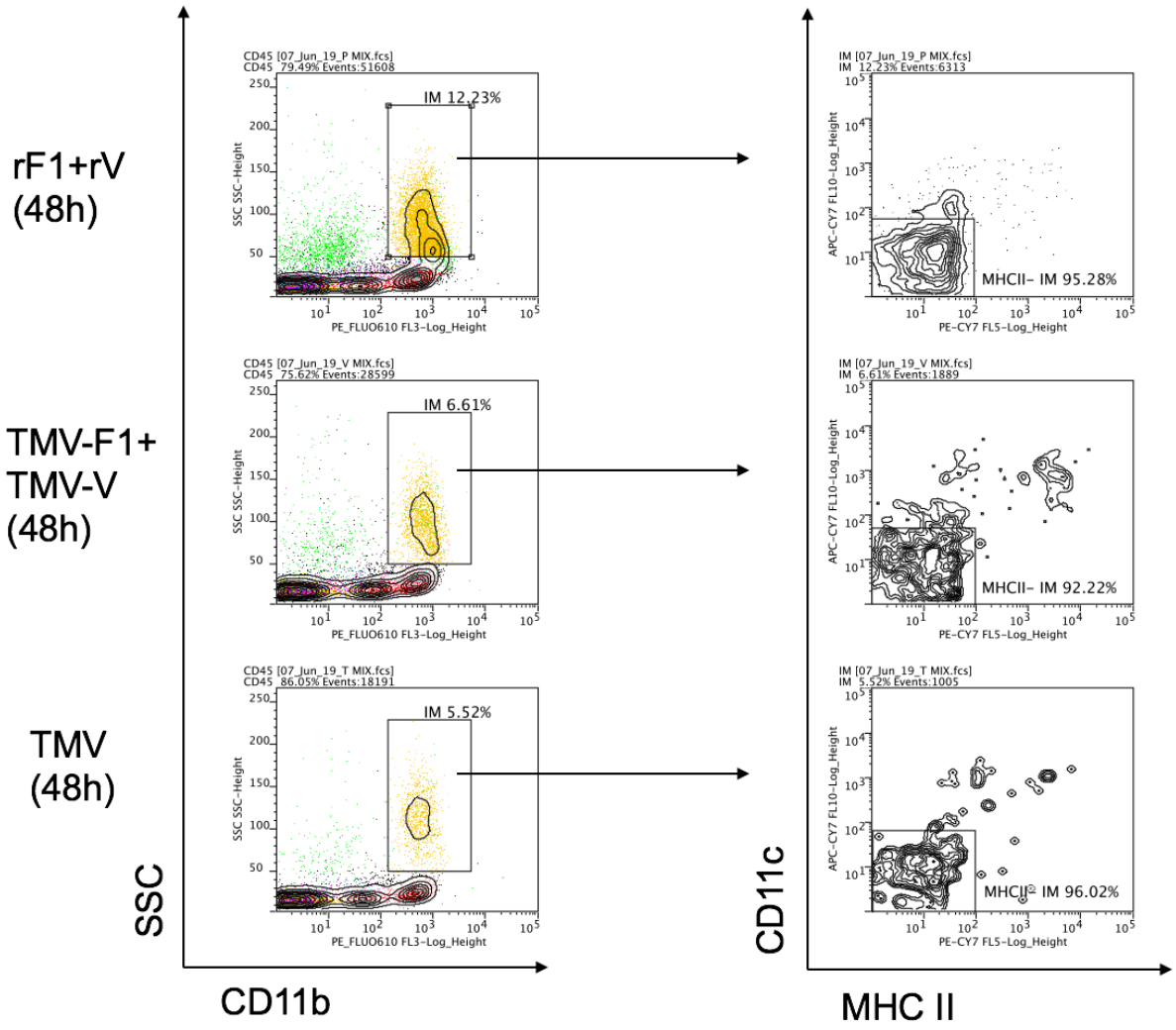


Fig S3. Most interstitial macrophages (IMs) (CD11b^{+} SSC^{hi}) are CD11c- MHC II-. Flow cytometry analysis and gating used to define IMs *in vivo*. CD11b^{+} IMs can represent a heterogenous population of CD11c- MHCII-, CD11c^{+} MHC II^{lo}, and CD11c^{+} MHC II^{hi} cells. Example plots shown: rF1+rV, TMV-F1+TMV-V, and TMV immunized lung sacrificed at 48-hour time point.

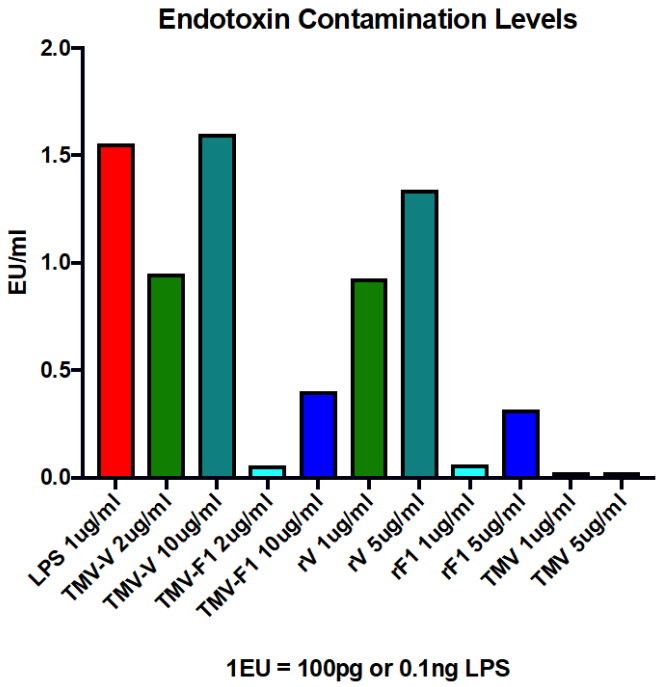


Fig S4. rF1 and rV contain similar levels of endotoxin as corresponding TMV conjugates. A chromogenic LAL endotoxin assay was performed to assess endotoxin (LPS) contamination from rF1 and rV antigens, and TMV-F1 and TMV-V conjugates. The concentration of TMV conjugates was double that of the recombinant protein to ensure molar equivalency of rF1 and rV between the groups, since TMV conjugates contain equivalent concentrations of TMV and recombinant protein. Molar equivalent pairs of TMV conjugate and corresponding protein are color-matched (e.g. TMV-V 10µg/mL and rV 5µg/mL are both teal). Purified TMV contained no endotoxin.

Appendix II.

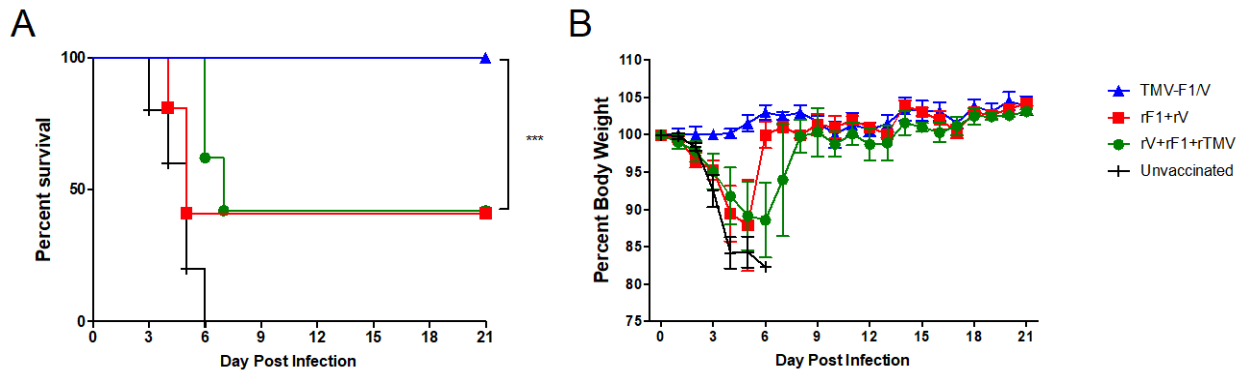


Fig S1. Conjugation to recombinant TMV (rTMV) for protection against pulmonary challenge with 10 LD_{50} *Y. pestis* CO92pgm-. Mice were i.n. vaccinated once with either TMV-F1/V, rF1+rV, rF1+rV+rTMV, or not vaccinated (controls) and monitored for 21 days post-infection (n=5 mice/group, mix of male/female). (A) Kaplan-Meier curves showing comparison of survival. Asterisks indicate statistical significance by the Mantel-Cox log-rank test. (B) Average percent body weight (from initial weight on day of challenge). *** $p<0.001$. All errors bars represent mean±SEM.

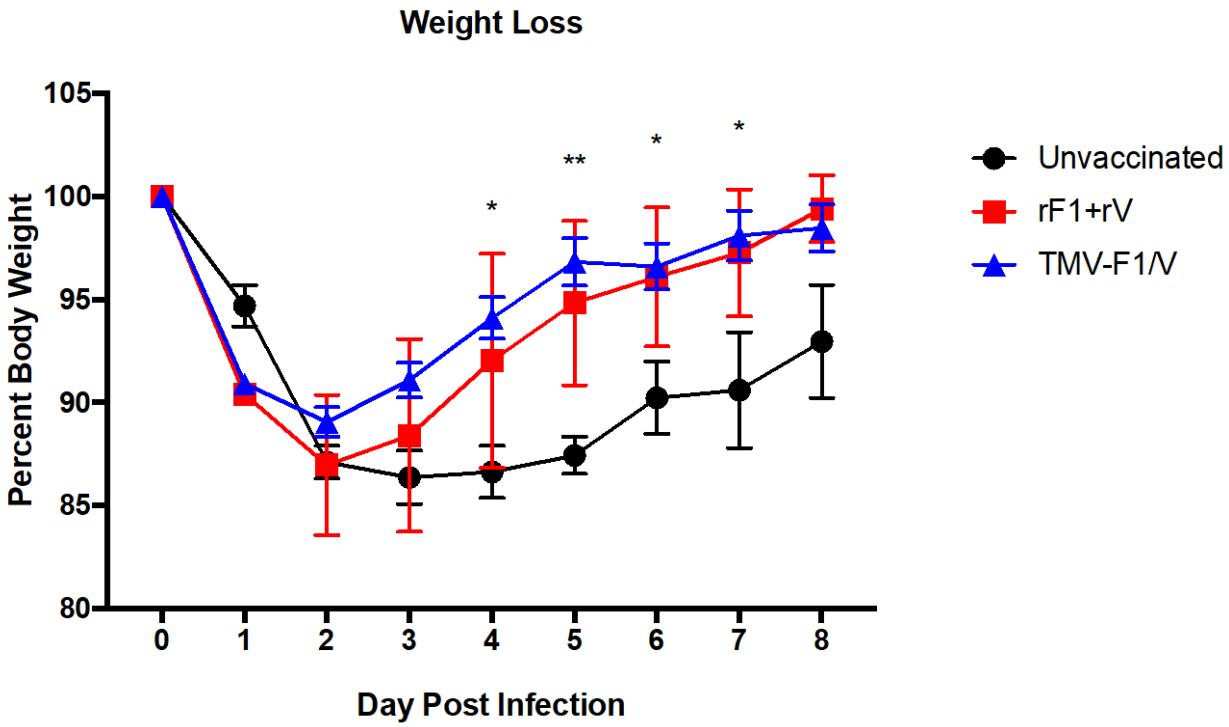


Fig S2. **Survivors' weight loss during the critical period of infection (D1-D8).** Asterisks indicated statistical significance between TMV-F1+TMV-V vaccinated and unvaccinated mice, compared by unpaired student's t test for each day post-infection. Data is pooled from two separate experiments. Unvaccinated n=3, rF1+rV n=3, TMV-F1/V n=23. **p<0.01, *p<0.05. All errors bars represent mean±SEM.

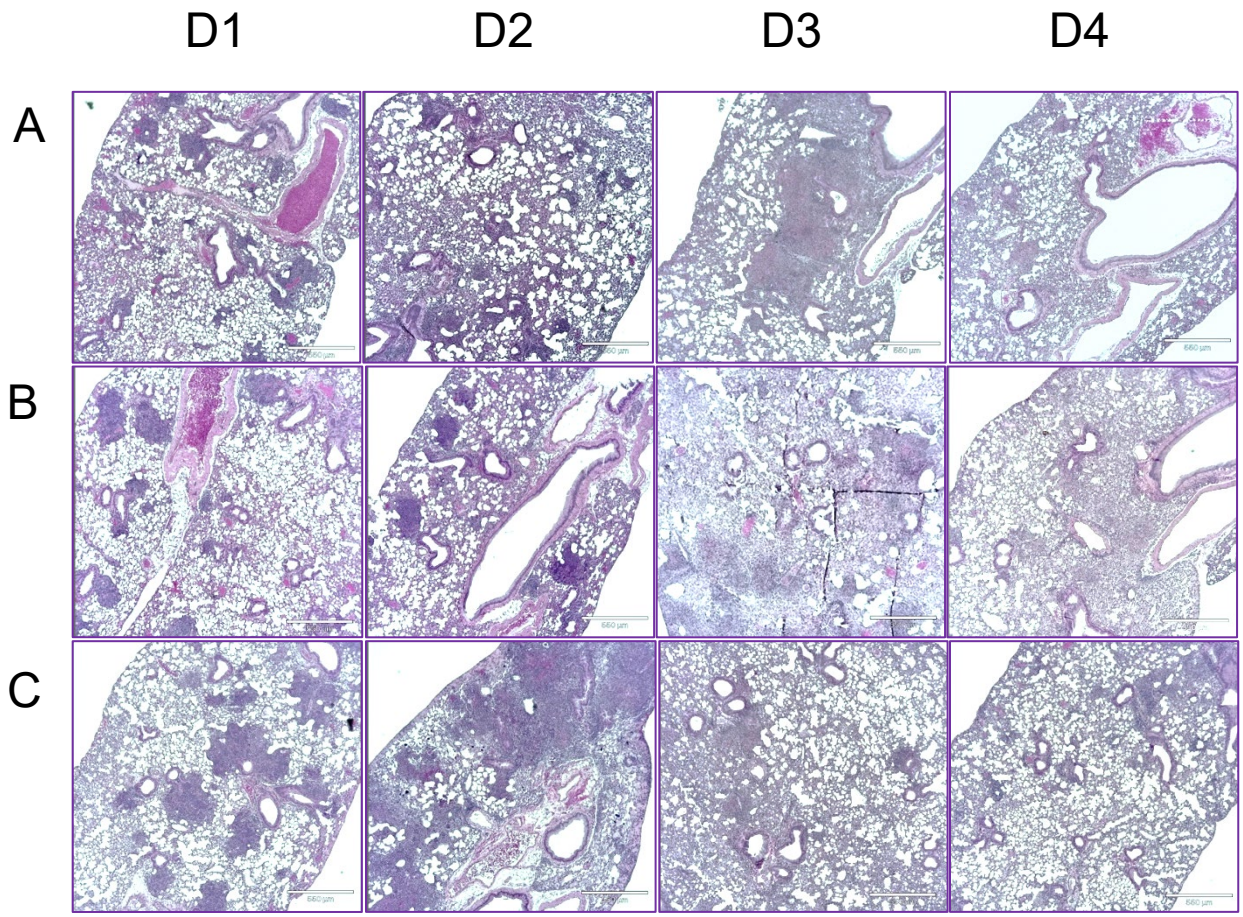


Fig S3. Representative lung sections D1-D4 post-infection with 100 LD_{50} *Y. pestis* CO92*pgm-*. Formalin-fixed tissues were sectioned and stained with hematoxylin and eosin (H&E). Images represent sections from a (A) unvaccinated, (B) rF1+rV-immunized, and (C) TMV-F1/V-immunized mouse sacrificed at D1-D4 post-infection (left to right). Scale bar represents 550µm; all images taken at 4x magnification.

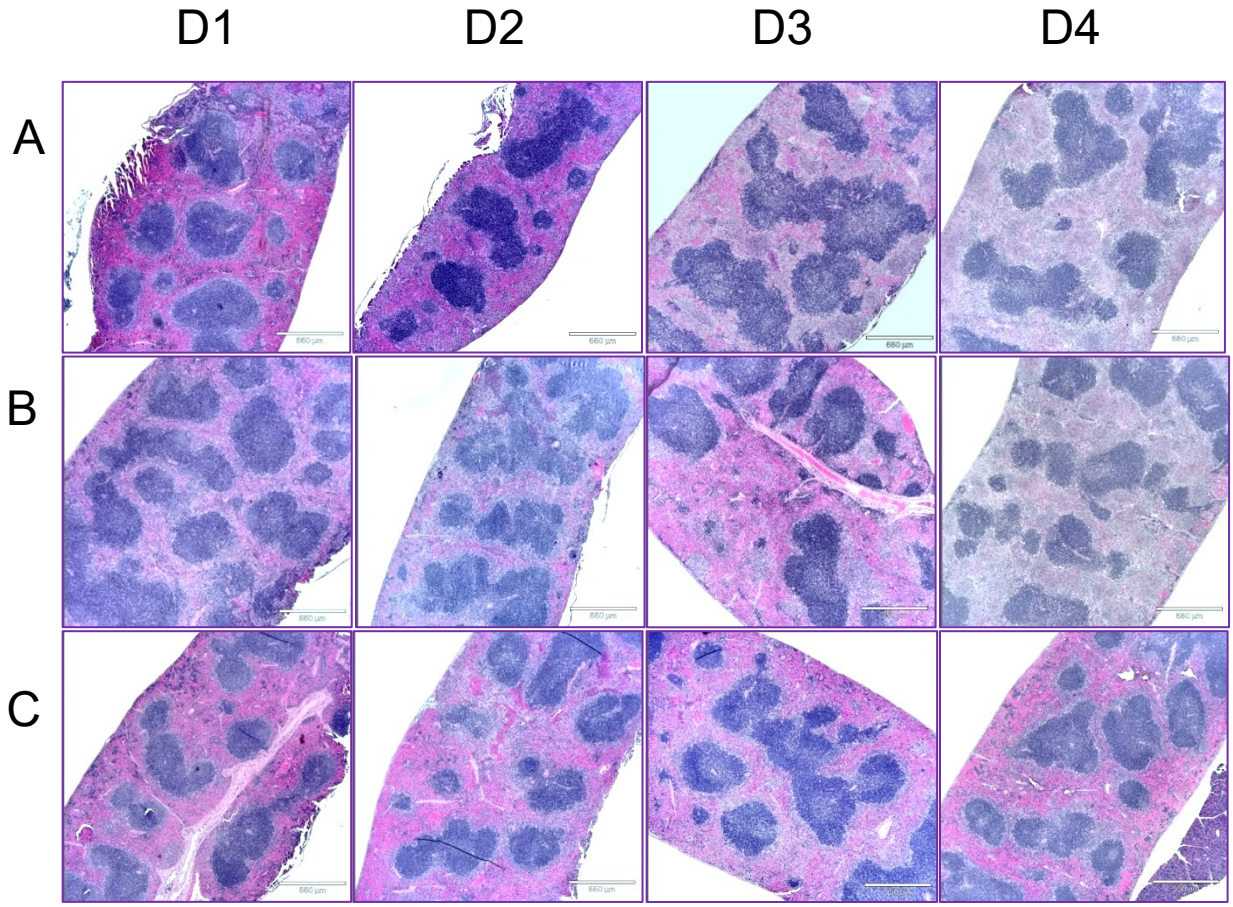


Fig S4. Representative spleen sections D1-D4 post-infection with 100 LD_{50} *Y. pestis* CO92*pgm-*. Formalin-fixed tissues were sectioned and stained with hematoxylin and eosin (H&E). Images represent sections from a (A) unvaccinated, (B) rF1+rV-immunized, and (C) TMV-F1/V-immunized mouse sacrificed at D1-D4 post-infection (left to right). Scale bar represents 550µm; all images taken at 4x magnification.

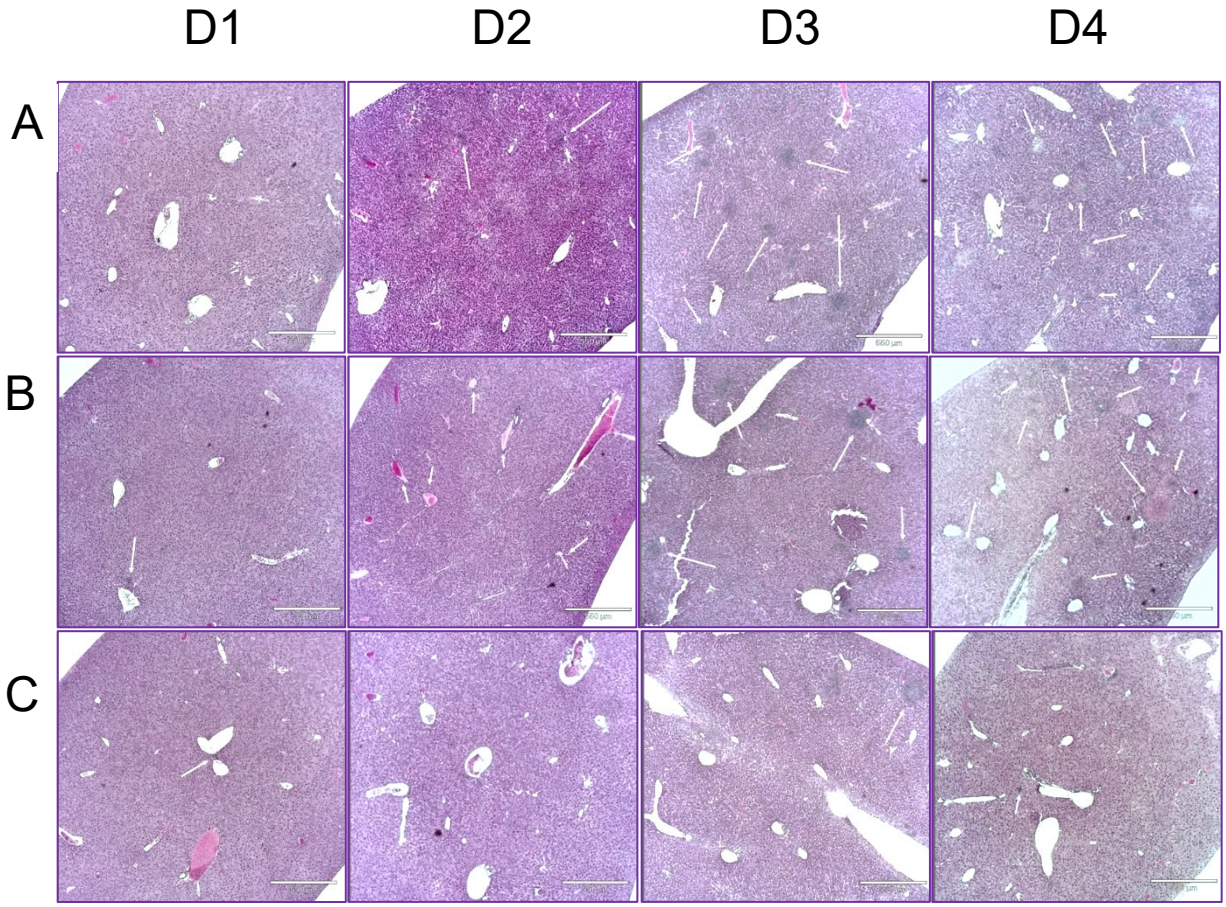


Fig S5. Representative liver sections D1-D4 post-infection with 100 LD_{50} *Y. pestis* CO92*pgm-*. Formalin-fixed tissues were sectioned and stained with hematoxylin and eosin (H&E). Images represent sections from a (A) unvaccinated, (B) rF1+rV-immunized, and (C) TMV-F1/V-immunized mouse sacrificed at D1-D4 post-infection (left to right). Arrows indicate locations of inflammation. Scale bar represents 550µm; all images taken at 4x magnification.

Table S2: Histopathology Scoring System for Tissue Sections

Severity Score	Spleen	Lung	Liver
0	Vibrant red pulp, neat germinal center (GC) boundaries, GCs stain rich blue-purple	Largely eosinophilic (pink) staining, alveoli are clear, no perivascular lymphocytes	Light pink-purple staining, sinusoids are clear and free of lymphocytes, hepatocytes appear "foamy"
1	Some disorganization of GCs, number of lymphocytes is increased, red pulp still present	Some lymphocyte infiltration, 1 or 2 inflammatory foci, lung architecture and alveoli remain intact	Some lymphocyte infiltration (dark purple staining is apparent), hepatocyte morphology and sinusoids appear undisturbed
2	Disorganization of GCs, neutrophil/granulocyte infiltration, some red pulp is still present	Neutrophil infiltration, increased numbers of lymphocytes, 3-5 inflammatory foci, alveoli remain intact, edema possible	Neutrophil infiltration in small clusters, some sinusoids are closed and/or surrounded by lymphocytes, edema possible
3	Most GCs have amalgamated, large neutrophilic lesions, necrosis, loss of most or all red pulp	Neutrophil infiltration, increased numbers of lymphocytes, >5 large inflammatory foci, areas of alveoli still visible, edema in some areas	Multiple neutrophilic foci (>5) are evident, some necrosis, edema, many sinusoids are closed and/or surrounded by lymphocytes, hepatocytes no longer appear "foamy"
4	Over 80% has necrotic and/or inflammatory lesions, red pulp has disappeared completely	Over 80% is inflamed, architecture and alveoli severely altered, edema, necrosis, inflammatory foci are too large to distinguish	Over 80% has necrotic and/or inflammatory lesions. ***This was not observed in this study.***

Appendix III.

Day 2 Post-Depletion Day 7 Post-Depletion

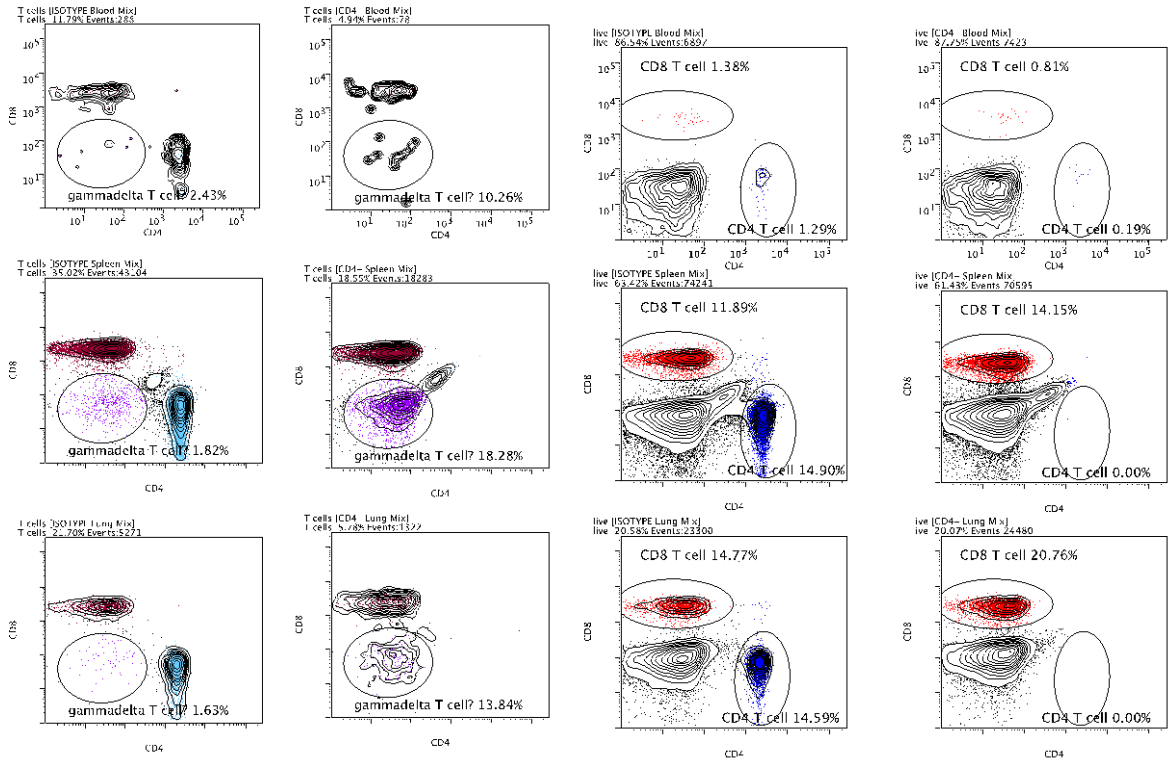


Fig S1. Confirmation of anti-CD4 antibody (GK1.5) depletion 2- and 7-days post-injection. Mice were injected intraperitoneally (IP) with a 100ug dose of anti-mouse anti-CD4 antibody (clone GK1.5) in 200ul volume of PBS. Peripheral blood, whole spleen, and whole lung were collected and processed by flow cytometry for markers CD3 and CD4. Plots that indicate "ISOTYPE" were mice given a non-specific rat IgG2bk isotype control antibody at the same time and same dose/volume as the anti-CD4 antibody. Plots that indicate "CD4-" are mice given the anti-CD4 antibody.

Day 2 Post-Depletion Day 7 Post-Depletion

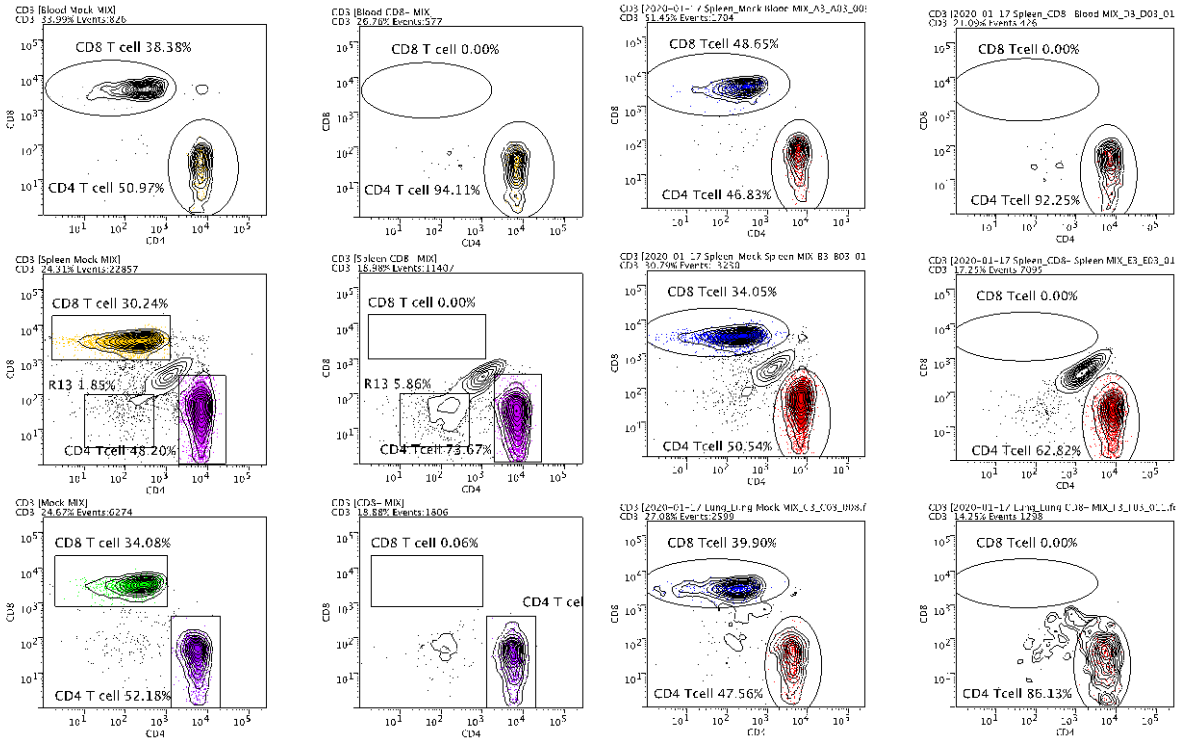


Fig S2. Confirmation of anti-CD8α antibody (YTS 169.4) depletion 2- and 7-days post-injection. Mice were injected intraperitoneally (IP) with a 100ug dose of anti-mouse anti-CD8α antibody (clone YTS 169.4) in 200ul volume of PBS. Peripheral blood, whole spleen, and whole lung were collected and processed by flow cytometry for markers CD3 and CD8. Plots that indicate "Mock" were mice given a non-specific rat IgG2bk isotype control antibody at the same time and same dose/volume as the anti-CD8 antibody. Plots that indicate "CD8-" are mice given the anti-CD8 antibody.

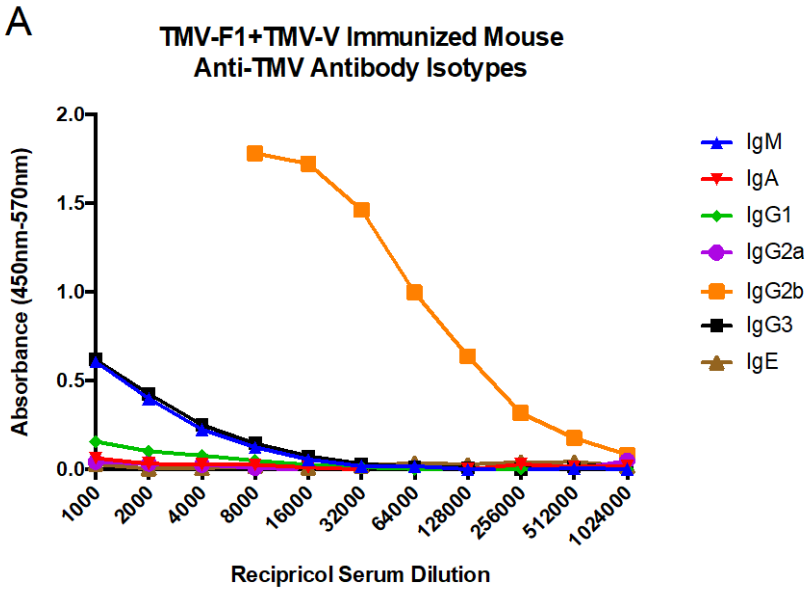


Fig S3. I.n. immunization with TMV conjugates generates anti-TMV antibody consisting of IgG2b, IgG3, and IgM isotypes. (A) ELISA curves showing serum absorbance for detection of anti-TMV antibody isotypes. Serum was collected on day 56 post-prime immunization (day 0) and after the second and final boost (day 35) from a TMV-F1+TMV-V-immunized mouse.

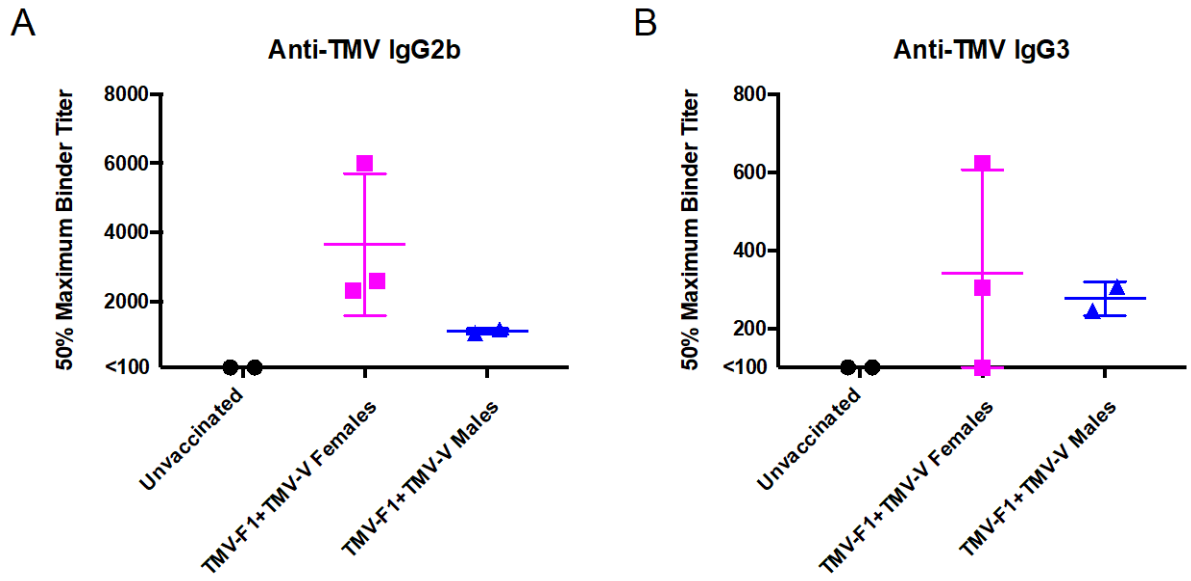


Fig S4. Single i.n. immunization with TMV conjugates generates similar anti-TMV IgG titers in females and males, while naïve mice lack a detectable titer. Pilot experiment showing pre-challenge anti-TMV titers (week 2 post-immunization) of the isotype IgG2b (A) and IgG3 (B) in TMV-F1+TMV-V-vaccinated and unvaccinated (naïve) mice. One-way ANOVA analysis revealed no significant difference between titers for panels (A) and (B). Unvaccinated (control) n=2; TMV-F1+TMV-V females n=3; TMV-F1+TMV-V males n=2. All errors bars represent mean±SD.

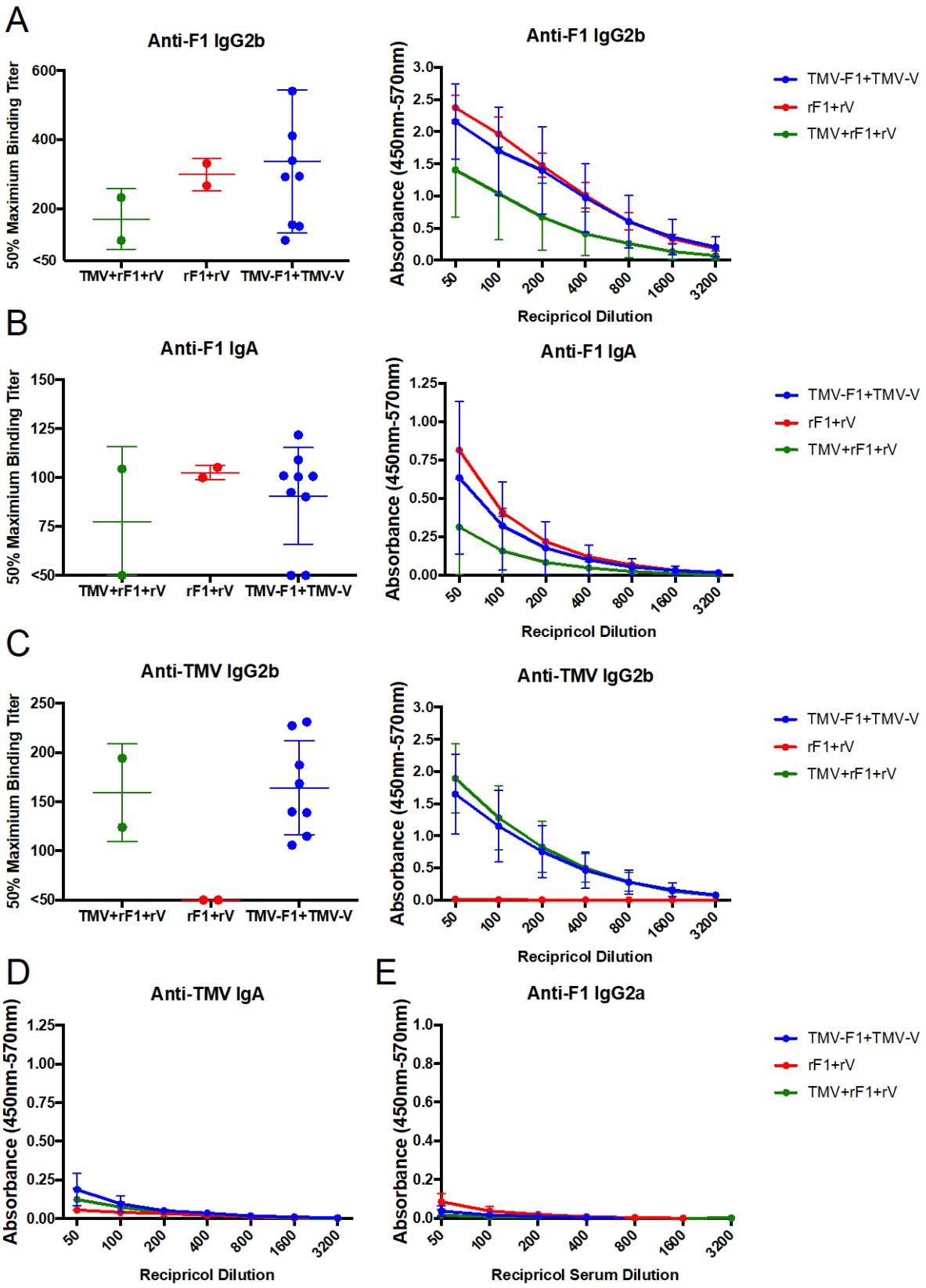

A
Anti-F1 IgG2b
50% Maximium Binding Titer
600
400
200
<50
TMV+rF1+rV
rF1+rV
TMV-F1+TMV-V
Anti-F1 IgG2b
Absorbance (450nm-570nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50
100
200
400
800
1600
3200
Recipricol Dilution
TMV-F1+TMV-V
rF1+rV
TMV+rF1+rV
B
Anti-F1 IgA
50% Maximium Binding Titer
150
125
100
75
<50
TMV+rF1+rV
rF1+rV
TMV-F1+TMV-V
Anti-F1 IgA
Absorbance (450nm-570nm)
1.25
1.00
0.75
0.50
0.25
0.00
50
100
200
400
800
1600
3200
Recipricol Dilution
TMV-F1+TMV-V
rF1+rV
TMV+rF1+rV
C
Anti-TMV IgG2b
50% Maximium Binding Titer
250
200
150
100
<50
TMV+rF1+rV
rF1+rV
TMV-F1+TMV-V
Anti-TMV IgG2b
Absorbance (450nm-570nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50
100
200
400
800
1600
3200
Recipricol Dilution
TMV-F1+TMV-V
rF1+rV
TMV+rF1+rV
D
Anti-TMV IgA
Absorbance (450nm-570nm)
1.25
1.00
0.75
0.50
0.25
0.00
50
100
200
400
800
1600
3200
Recipricol Dilution
E
Anti-F1 IgG2a
Absorbance (450nm-570nm)
1.0
0.8
0.6
0.4
0.2
0.0
50
100
200
400
800
1600
3200
Recipricol Serum Dilution
TMV-F1+TMV-V
rF1+rV
TMV+rF1+rV

Fig S5. Titers of survivors show anti-F1 IgG2b and IgA, but not IgG2a, are generated in response to lethal pneumonic plague infection, regardless of vaccine. Post-challenge lung homogenate antibody titers for F1-specific IgG2b (A) and IgA (B), and TMV-specific IgG2b (C) following single i.n. vaccination with rF1+rV (red), TMV-F1+TMV-V (blue), or TMV+rF1+rV (green). Titers were determined from respective ELISA curves (right-hand panel) for survivors 21 days after i.n. challenge with 10 LD50 *Y. pestis* CO92pgm-. Titers were below the limit of detection (<50) for TMV-specific IgA (D) and F1-specific IgG2a (E), so only ELISA curves are shown. One-way ANOVA analysis revealed no significant differences between titers shown in (A) and (B). Unpaired t test revealed no significant differences in (C). Note: TMV-specific IgG2b was not detected in rF1+rV-immunized mice, as expected, and was excluded. All errors bars represent mean±SD.

Chapter 7. Bibliography

Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, Vogler AJ, Wagner DM, Allender CJ, Easterday WR, Chenal-Francisque V, Worsham P, Thomson NR, Parkhill J, Lindler LE, Carniel E, Keim P (2004). Microevolution and history of the plague bacillus, yersinia pestis. *Proceedings of the National Academy of Sciences of the United States of America.* 101: 17837-17842.

Airhart CL, Rohde HN, Hovde CJ, Bohach GA, Deobald CF, Lee SS, Minnich SA (2008). Lipid A mimetics are potent adjuvants for an intranasal pneumonic plague vaccine. *Vaccine.* 26: 5554-5561.

Al-Barwani F, Donaldson B, Pelham SJ, Young SL, Ward VK (2014). Antigen delivery by virus-like particles for immunotherapeutic vaccination. *Therapeutic Delivery.* 5: 1223-1240.

Alexandre Yersin BW (2010). Etymologia: Yersinia. *Emerging Infectious Diseases.* 16: 496.

Amemiya K, Meyers JL, Rogers TE, Fast RL, Bassett AD, Worsham PL, Powell BS, Norris SL, Krieg AM, Adamovicz JJ (2009). CpG oligodeoxynucleotides augment the murine immune response to the yersinia pestis F1-V vaccine in bubonic and pneumonic models of plague. *Vaccine.* 27: 2220-2229.

Anderson GW, Heath DG, Bolt CR, Welkos SL, Friedlander AM (1998). Short- and long-term efficacy of single-dose subunit vaccines against yersinia pestis in mice. *The American Journal of Tropical Medicine and Hygiene.* 58: 793-799.

Anderson GW, Worsham PL, Bolt CR, Andrews GP, Welkos SL, Friedlander AM, Burans JP (1997). Protection of mice from fatal bubonic and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of yersinia pestis. *The American Journal of Tropical Medicine and Hygiene.* 56: 471-473.

Andrades Valtuena A, Mittnik A, Key FM, Haak W, Allmae R, Belinskij A, Daubaras M, Feldman M, Jankauskas R, Jankovic I, Massy K, Novak M, Pfrengle S, Reinhold S, Slaus M, Spyrou MA, Szecsenyi-Nagy A, Torv M, Hansen S, Bos KI, Stockhammer PW, Herbig A, Krause J (2017). The stone age plague and its persistence in eurasia. *Current Biology : CB.* 27: 3683-3691.e8.

Andrews GP, Heath DG, Anderson GW, Welkos SL, Friedlander AM (1996). Fraction 1 capsular antigen (F1) purification from yersinia pestis CO92 and from an escherichia coli recombinant strain and efficacy against lethal plague challenge. *Infection and Immunity.* 64: 2180-2187.

Andrianaivoarimanana V, Piola P, Wagner DM, Rakotomanana F, Maheriniaina V, Andrianalimanana S, Chanteau S, Rahalison L, Ratsitorahina M, Rajerison M (2019). Trends of human plague, madagascar, 1998-2016. *Emerging Infectious Diseases.* 25: 220-228.

Anisimov AP, Dentovskaya SV, Panfertsev EA, Svetoch TE, Kopylov PK, Segelke BW, Zemla A, Telepnev MV, Motin VL (2010). Amino acid and structural variability of yersinia pestis LcrV protein. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases.* 10: 137-145.

Anisimov AP, Lindler LE, Pier GB (2004). Intraspecific diversity of yersinia pestis. *Clinical Microbiology Reviews.* 17: 434-464.

Anisimov AP, Nikiforov AK, Eremin SA, Drozdov IG (1995). Design of a yersinia pestis strain with increased protection. [Konstruirovanie shtamma Yersinia pestis s povyshennoi protektivnost'iu] *Biulleten' Eksperimental'Noi Biologii i Meditsiny.* 120: 532-534.

Arnaboldi PM, Sambir M, D'Arco C, Peters LA, Seegers JF, Mayer L, McCormick AA, Dattwyler RJ (2016). Intranasal delivery of a protein subunit vaccine using a tobacco mosaic virus platform protects against pneumonic plague. *Vaccine.* 34: 5768-5776.

Ashkin A and Dziedzic JM (1987). Optical trapping and manipulation of viruses and bacteria. *Science (New York, N.Y.).* 235: 1517-1520.

Bachmann MF and Jennings GT (2010). Vaccine delivery: A matter of size, geometry, kinetics and molecular patterns. *Nature Reviews.Immunology.* 10: 787-796.

Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, Zinkernagel RM (1993). The influence of antigen organization on B cell responsiveness. *Science (New York, N.Y.).* 262: 1448-1451.

Balique F, Colson P, Raoult D (2012). Tobacco mosaic virus in cigarettes and saliva of smokers. *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology.* 55: 374-376.

Balique F, Lecoq H, Raoult D, Colson P (2015). Can plant viruses cross the kingdom border and be pathogenic to humans? *Viruses.* 7: 2074-2098.

Balmelli C, Demotz S, Acha-Orbea H, De Grandi P, Nardelli-Haefliger D (2002). Trachea, lung, and tracheobronchial lymph nodes are the major sites where antigen-presenting cells are detected after nasal vaccination of mice with human papillomavirus type 16 virus-like particles. *Journal of Virology.* 76: 12596-12602.

Balmelli C, Roden R, Potts A, Schiller J, De Grandi P, Nardelli-Haefliger D (1998). Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. *Journal of Virology.* 72: 8220-8229.

Banik S, Mansour AA, Suresh RV, Wykoff-Clary S, Malik M, McCormick AA, Bakshi CS (2015). Development of a multivalent subunit vaccine against tularemia using tobacco mosaic virus (TMV) based delivery system. *PloS One.* 10: e0130858.

Bao R, Nair MK, Tang WK, Esser L, Sadhukhan A, Holland RL, Xia D, Schifferli DM (2013). Structural basis for the specific recognition of dual receptors by the homopolymeric pH 6 antigen (psa) fimbriae of yersinia pestis. *Proceedings of the National Academy of Sciences of the United States of America.* 110: 1065-1070.

Bashaw J, Norris S, Weeks S, Trevino S, Adamovicz JJ, Welkos S (2007). Development of in vitro correlate assays of immunity to infection with yersinia pestis. *Clinical and Vaccine Immunology : CVI.* 14: 605-616.

Beijerinck MW (1898). *Concerning a contagium vivum fluidum as cause of the spot disease of tobacco leaves.* (Phylopathological Classics No. 7). St. Paul: American Phytopathological Society.

Bessa J, Kopf M, Bachmann MF (2010). Cutting edge: IL-21 and TLR signaling regulate germinal center responses in a B cell-intrinsic manner. *Journal of Immunology (Baltimore, Md.: 1950).* 184: 4615-4619.

Bessa J, Schmitz N, Hinton HJ, Schwarz K, Jegerlehner A, Bachmann MF (2008). Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: Implications for vaccine design. *European Journal of Immunology.* 38: 114-126.

Bessa J, Zabel F, Link A, Jegerlehner A, Hinton HJ, Schmitz N, Bauer M, Kundig TM, Saudan P, Bachmann MF (2012). Low-affinity B cells transport viral particles from the lung to the spleen to initiate antibody responses. *Proceedings of the National Academy of Sciences of the United States of America.* 109: 20566-20571.

Bibel DJ and Chen TH (1976). Diagnosis of plaque: An analysis of the yersin-kitasato controversy. *Bacteriological Reviews.* 40: 633-651.

Bobrov AG, Kirillina O, Fosso MY, Fetherston JD, Miller MC, VanCleave TT, Burlison JA, Arnold WK, Lawrenz MB, Garneau-Tsodikova S, Perry RD (2017). Zinc transporters YbtX and ZnuABC are required for the virulence of yersinia pestis in bubonic and pneumonic plague in mice. *Metallomics : Integrated Biometal Science.* 9: 757-772.

Boone A, Kraft JP, Stapp P (2009). Scavenging by mammalian carnivores on prairie dog colonies: Implications for the spread of plague. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.).* 9: 185-190.

Bowen W, Batra L, Pulsifer AR, Yolcu ES, Lawrenz MB, Shirwan H (2019). Robust Th1 cellular and humoral responses generated by the yersinia pestis rF1-V subunit vaccine formulated to contain an agonist of the CD137 pathway do not translate into increased protection against pneumonic plague. *Vaccine.* 37: 5708-5716.

Bramwell VW, Eyles JE, Oya Alpar H (2005). Particulate delivery systems for biodefense subunit vaccines. *Advanced Drug Delivery Reviews.* 57: 1247-1265.

Brandtzaeg P (1999). Regionalized immune function of tonsils and adenoids. *Immunology Today.* 20: 383-384.

Brennan FR, Bellaby T, Helliwell SM, Jones TD, Kamstrup S, Dalsgaard K, Flock JI, Hamilton WD (1999). Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice. *Journal of Virology.* 73: 930-938.

Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J (1999). Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *Journal of Immunology (Baltimore, Md.: 1950).* 163: 6448-6454.

Buchrieser C, Prentice M, Carniel E (1998). The 102-kilobase unstable region of yersinia pestis comprises a high-pathogenicity island linked to a pigmentation segment which undergoes internal rearrangement. *Journal of Bacteriology.* 180: 2321-2329.

Butler T (2014). Plague history: Yersin's discovery of the causative bacterium in 1894 enabled, in the subsequent century, scientific progress in understanding the disease and the development of treatments and vaccines. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases.* 20: 202-209.

Byvalov AA, Pautov VN, Chicherin I, Lebedinskii VA, Evtigneev VI (1984). Effectiveness of revaccinating hamadryas baboons with NISS live dried plague vaccine and fraction I of the plague microbe. [Effektivnost' revaktsinatsii pavianov gamadrilov chumnoi zhivoi sukhoi vaktsinoi NIIS i fraktsii I chumnogo mikroba] *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii.* (4): 74-76.

Canizares MC, Nicholson L, Lomonossoff GP (2005). Use of viral vectors for vaccine production in plants. *Immunology and Cell Biology.* 83: 263-270.

Carus WS (2015). The history of biological weapons use: What we know and what we don't. *Health Security.* 13: 219-255.

Carvalho AL, Miquel-Clopes A, Wegmann U, Jones E, Stentz R, Telatin A, Walker NJ, Butcher WA, Brown PJ, Holmes S, Dennis MJ, Williamson ED, Funnell SGP, Stock M, Carding SR (2019). Use of bioengineered human commensal gut bacteria-derived microvesicles for mucosal plague vaccine delivery and immunization. *Clinical and Experimental Immunology.* 196: 287-304.

CDC (1999). Achievements in public health, 1900-1999: Control of infectious diseases. *MMWR Weekly.* 48: 621-629.

CDC (2019). Plague. Retrieved from https://www.cdc.gov/plague/index.html

Chain PS, Carniel E, Larimer FW, Lamerdin J, Stoutland PO, Regala WM, Georgescu AM, Vergez LM, Land ML, Motin VL, Brubaker RR, Fowler J, Hinnebusch J, Marceau M, Medigue C, Simonet M, Chenal-Francisque V, Souza B, Dacheux D, Elliott JM, Derbise A, Hauser LJ, Garcia E (2004). Insights into the evolution of yersinia pestis through whole-genome comparison with yersinia pseudotuberculosis. *Proceedings of the National Academy of Sciences of the United States of America.* 101: 13826-13831.

Chen K, Magri G, Grasset EK, Cerutti A (2020). Rethinking mucosal antibody responses: IgM, IgG and IgD join IgA. *Nature Reviews.Immunology.*

Chen Q and Lai H (2013). Plant-derived virus-like particles as vaccines.*Human Vaccines & Immunotherapeutics.* 9: 26-49.

Chouikha I and Hinnebusch BJ (2014). Silencing urease: A key evolutionary step that facilitated the adaptation of yersinia pestis to the flea-borne transmission route. *Proceedings of the National Academy of Sciences of the United States of America.* 111: 18709-18714.

Collins AM (2016). IgG subclass co-expression brings harmony to the quartet model of murine IgG function. *Immunology and Cell Biology.* 94: 949-954.

Connor MG, Pulsifer AR, Chung D, Rouchka EC, Ceresa BK, Lawrenz MB (2018). Yersinia pestis targets the host endosome recycling pathway during the biogenesis of the yersinia-containing vacuole to avoid killing by macrophages. *mBio.* 9: 10.1128/mBio.01800-17.

Connor MG, Pulsifer AR, Price CT, Abu Kwaik Y, Lawrenz MB (2015). Yersinia pestis requires host Rab1b for survival in macrophages.*PLoS Pathogens.* 11: e1005241.

Cornelis GR (1998). The yersinia deadly kiss. *Journal of Bacteriology.* 180: 5495-5504.

Cornelis GR (2000). Molecular and cell biology aspects of plague. *Proceedings of the National Academy of Sciences of the United States of America.* 97: 8778-8783.

Cornelius CA, Quenee LE, Overheim KA, Koster F, Brasel TL, Elli D, Ciletti NA, Schneewind O (2008). Immunization with recombinant V10 protects cynomolgus macaques from lethal pneumonic plague.*Infection and Immunity.* 76: 5588-5597.

Davis KJ, Fritz DL, Pitt ML, Welkos SL, Worsham PL, Friedlander AM (1996). Pathology of experimental pneumonic plague produced by fraction 1-positive and fraction 1-negative yersinia pestis in african green monkeys (cercopithecus aethiops). *Archives of Pathology & Laboratory Medicine.* 120: 156-163.

DeBord KL, Anderson DM, Marketon MM, Overheim KA, DePaolo RW, Ciletti NA, Jabri B, Schneewind O (2006). Immunogenicity and protective immunity against bubonic plague and pneumonic plague by immunization of mice with the recombinant V10 antigen, a variant of LcrV. *Infection and Immunity.* 74: 4910-4914.

Deenick EK, Hasbold J, Hodgkin PD (1999). Switching to IgG3, IgG2b, and IgA is division linked and independent, revealing a stochastic framework for describing differentiation. *Journal of Immunology.* 163: 4707-4714.

Demeure CE, Derbise A, Carniel E (2017). Oral vaccination against plague using yersinia pseudotuberculosis. *Chemico-Biological Interactions.* 267: 89-95.

Demeure CE, Dussurget O, Mas Fiol G, Le Guern AS, Savin C, Pizarro-Cerda J (2019). Yersinia pestis and plague: An updated view on evolution, virulence determinants, immune subversion, vaccination, and diagnostics. *Genes and Immunity.* 20: 357-370.

Derewenda U, Mateja A, Devedjiev Y, Routzahn KM, Evdokimov AG, Derewenda ZS, Waugh DS (2004). The structure of yersinia pestis V-antigen, an essential virulence factor and mediator of immunity against plague. *Structure (London, England : 1993).* 12: 301-306.

Donaires LF, Cespedes M, Valencia P, Salas JC, Luna ME, Castaneda A, Peralta V, Cabezas C, Pachas PE (2010). Primary pneumonic plague with nosocomial transmission in la libertad, peru 2010. [Peste neumonica primaria con transmision intrahospitalaria en La Libertad, Peru 2010] *Revista Peruana De Medicina Experimental Y Salud Publica.* 27: 326-336.

Donaldson B, Lateef Z, Walker GF, Young SL, Ward VK (2018). Virus-like particle vaccines: Immunology and formulation for clinical translation. *Expert Review of Vaccines.* 17: 833-849.

Drancourt M and Raoult D (2016). Molecular history of plague. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases.* 22: 911-915.

Du Y, Rosqvist R, Forsberg A (2002). Role of fraction 1 antigen of yersinia pestis in inhibition of phagocytosis. *Infection and Immunity.* 70: 1453-1460.

Eddy JL, Gielda LM, Caulfield AJ, Rangel SM, Lathem WW (2014). Production of outer membrane vesicles by the plague pathogen yersinia pestis. *PloS One.* 9: e107002.

Eisele NA and Anderson DM (2009). Dual-function antibodies to yersinia pestis LcrV required for pulmonary clearance of plague.*Clinical and Vaccine Immunology : CVI.* 16: 1720-1727.

Eitzen EM, Jr and Takafuji ET (1997). Historical overview of biological warfare. In F. R. Sidell, E. T. Takafuji & D. R. Franz (Eds), *Medical aspects of chemical and biological warfare* (1st ed., pp. 415-423). Washington, DC: Office of the Surgeon General, Borden Institute, Walter Reed Army Medical Center.

Ervin F and Palmour R (2002). *Primates for 21st century biomedicine: The st. kitts vervet (chlorocebus aethiops, SK).* (Report No. 1). Washington, DC: National Academies Press. . (International Perspectives: The Future of Nonhuman Primate Resources)

Eyles JE, Williamson ED, Spiers ID, Stagg AJ, Jones SM, Alpar HO (2000). Generation of protective immune responses to plague by mucosal administration of microsphere coencapsulated recombinant subunits. *Journal of Controlled Release : Official Journal of the Controlled Release Society.* 63: 191-200.

Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG (2009). Follicular helper T cells: Lineage and location. *Immunity.* 30: 324-335.

Feodorova VA and Corbel MJ (2009). Prospects for new plague vaccines. *Expert Review of Vaccines.* 8: 1721-1738.

Feodorova VA and Motin VL (2012). Plague vaccines: Current developments and future perspectives. *Emerging Microbes & Infections.* 1: e36.

Fetherston JD, Kirillina O, Bobrov AG, Paulley JT, Perry RD (2010). The yersiniabactin transport system is critical for the pathogenesis of bubonic and pneumonic plague. *Infection and Immunity.* 78: 2045-2052.

Fetherston JD and Perry RD (1994). The pigmentation locus of yersinia pestis KIM6+ is flanked by an insertion sequence and includes the structural genes for pesticin sensitivity and HMWP2. *Molecular Microbiology.* 13: 697-708.

Fields KA, Nilles ML, Cowan C, Straley SC (1999). Virulence role of V antigen of yersinia pestis at the bacterial surface. *Infection and Immunity.* 67: 5395-5408.

Fields KA and Straley SC (1999). LcrV of yersinia pestis enters infected eukaryotic cells by a virulence plasmid-independent mechanism.*Infection and Immunity.* 67: 4801-4813.

Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, McKenzie IF, Plebanski M (2004). Size-dependent immunogenicity: Therapeutic and protective properties of nano-vaccines against tumors. *Journal of Immunology (Baltimore, Md.: 1950).* 173: 3148-3154.

Fitchen J, Beachy RN, Hein MB (1995). Plant virus expressing hybrid coat protein with added murine epitope elicits autoantibody response. *Vaccine.* 13: 1051-1057.

Franz DR, Jahrling PB, Friedlander AM, McClain DJ, Hoover DL, Bryne WR, Pavlin JA, Christopher GW, Eitzen EM (1997). Clinical recognition and management of patients exposed to biological warfare agents. *Jama.* 278: 399-411.

Friedlander AM, Welkos SL, Worsham PL, Andrews GP, Heath DG, Anderson GW, Pitt ML, Estep J, Davis K (1995). Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of yersinia pestis. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America.* 21 Suppl 2: 178.

Galen JE, Wang JY, Carrasco JA, Lloyd SA, Mellado-Sanchez G, Diaz-McNair J, Franco O, Buskirk AD, Nataro JP, Pasetti MF (2015). A bivalent typhoid live vector vaccine expressing both chromosome- and plasmid-encoded yersinia pestis antigens fully protects against murine lethal pulmonary plague infection. *Infection and Immunity.* 83: 161-172.

Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E, Courvalin P (1997). Multidrug resistance in yersinia pestis mediated by a transferable plasmid. *The New England Journal of Medicine.* 337: 677-680.

Galyov EE, Karlishev AV, Chernovskaya TV, Dolgikh DA, Smirnov OY, Volkovoy KI, Abramov VM, Zav'yalov VP (1991). Expression of the envelope antigen F1 of yersinia pestis is mediated by the product of caf1M gene having homology with the chaperone protein PapD of escherichia coli. *FEBS Letters.* 286: 79-82.

Gamvrellis A, Leong D, Hanley JC, Xiang SD, Mottram P, Plebanski M (2004). Vaccines that facilitate antigen entry into dendritic cells.*Immunology and Cell Biology.* 82: 506-516.

Garmory HS, Griffin KF, Brown KA, Titball RW (2003). Oral immunisation with live aroA attenuated salmonella enterica serovar typhimurium expressing the yersinia pestis V antigen protects mice against plague. *Vaccine.* 21: 3051-3057.

Garmory HS, Leary SE, Griffin KF, Williamson ED, Brown KA, Titball RW (2003). The use of live attenuated bacteria as a delivery system for heterologous antigens. *Journal of Drug Targeting.* 11: 471-479.

Glynn A, Roy CJ, Powell BS, Adamovicz JJ, Freytag LC, Clements JD (2005). Protection against aerosolized yersinia pestis challenge following homologous and heterologous prime-boost with recombinant plague antigens. *Infection and Immunity.* 73: 5256-5261.

Goelet P, Lomonossoff GP, Butler PJ, Akam ME, Gait MJ, Karn J (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proceedings of the National Academy of Sciences of the United States of America.* 79: 5818-5822.

Goguen JD, Yother J, Straley SC (1984). Genetic analysis of the low calcium response in yersinia pestis mu d1(ap lac) insertion mutants. *Journal of Bacteriology.* 160: 842-848.

Gross L (1995). How the plague bacillus and its transmission through fleas were discovered: Reminiscences from my years at the pasteur institute in paris. *Proceedings of the National Academy of Sciences of the United States of America.* 92: 7609-7611.

Guiyoule A, Gerbaud G, Buchrieser C, Galimand M, Rahalison L, Chanteau S, Courvalin P, Carniel E (2001). Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of yersinia pestis. *Emerging Infectious Diseases.* 7: 43-48.

Hallett AF, Isaacson M, Meyer KF (1973). Pathogenicity and immunogenic efficacy of a live attentuated plaque vaccine in vervet monkeys. *Infection and Immunity.* 8: 876-881.

Hamzabegovic F, Goll JB, Hooper WF, Frey S, Gelber CE, Abate G (2020). Flagellin adjuvanted F1/V subunit plague vaccine induces T cell and functional antibody responses with unique gene signatures. *NPJ Vaccines.* 5: 6-y. eCollection 2020.

Harbeck M, Seifert L, Hansch S, Wagner DM, Birdsell D, Parise KL, Wiechmann I, Grupe G, Thomas A, Keim P, Zoller L, Bramanti B, Riehm JM, Scholz HC (2013). Yersinia pestis DNA from skeletal remains from the 6(th) century AD reveals insights into justinianic plague. *PLoS Pathogens.* 9: e1003349.

Hawgood BJ (2008). Alexandre yersin (1863-1943): Discoverer of the plague bacillus, explorer and agronomist. *Journal of Medical Biography.* 16: 167-172.

Heath DG, Anderson GW, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, Friedlander AM (1998). Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. *Vaccine.* 16: 1131-1137.

Hebeis, Klenovsek, Rohwer, Ritter, Schneider, Mach, Winkler (2004). Activation of virus-specific memory B cells in the absence of T cell help. *The Journal of Experimental Medicine.* 199: 593-602.

Heine SJ, Franco-Mahecha OL, Sears KT, Drachenberg CB, van Roosmalen ML, Leenhouts K, Picking WL, Pasetti MF (2019). A combined YopB and LcrV subunit vaccine elicits protective immunity against yersinia infection in adult and infant mice. *Journal of Immunology (Baltimore, Md.: 1950).* 202: 2005-2016.

Herzog C (2014). Influence of parenteral administration routes and additional factors on vaccine safety and immunogenicity: A review of recent literature. *Expert Review of Vaccines.* 13: 399-415.

Holtzman T, Levy Y, Marcus D, Flashner Y, Mamroud E, Cohen S, Fass R (2006). Production and purification of high molecular weight oligomers of yersinia pestis F1 capsular antigen released by high cell density culture of recombinant escherichia coli cells carrying the caf1 operon. *Microbial Cell Factories.* 5: 98.

Holzem A, Nahring JM, Fischer R (2001). Rapid identification of a tobacco mosaic virus epitope by using a coat protein gene-fragment-pVIII fusion library. *The Journal of General Virology.* 82: 9-15.

Honko AN, Sriranganathan N, Lees CJ, Mizel SB (2006). Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with yersinia pestis. *Infection and Immunity.* 74: 1113-1120.

Huang XZ, Nikolich MP, Lindler LE (2006). Current trends in plague research: From genomics to virulence. *Clinical Medicine & Research.* 4: 189-199.

Huang Z, Santi L, LePore K, Kilbourne J, Arntzen CJ, Mason HS (2006). Rapid, high-level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice. *Vaccine.* 24: 2506-2513.

Hwang DJ, Roberts IM, Wilson TM (1994). Assembly of tobacco mosaic virus and TMV-like pseudovirus particles in escherichia coli.*Archives of Virology.Supplementum.* 9: 543-558.

Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Koerner JF, Layton M, McDade J, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Schoch-Spana M, Tonat K (2000). Plague as a biological weapon: Medical and public health management. working group on civilian biodefense. *Jama.* 283: 2281-2290.

Ivanov MI, Noel BL, Rampersaud R, Mena P, Benach JL, Bliska JB (2008). Vaccination of mice with a yop translocon complex elicits antibodies that are protective against infection with F1- yersinia pestis. *Infection and Immunity.* 76: 5181-5190.

Jennings GT and Bachmann MF (2008). The coming of age of virus-like particle vaccines. *Biological Chemistry.* 389: 521-536.

Jia Q, Bowen R, Dillon BJ, Maslesa-Galic S, Chang BT, Kaidi AC, Horwitz MA (2018). Single vector platform vaccine protects against lethal respiratory challenge with tier 1 select agents of anthrax, plague, and tularemia. *Scientific Reports.* 8: 7009-y.

Jiao YY, Fu YH, Yan YF, Hua Y, Ma Y, Zhang XJ, Song JD, Peng XL, Huang J, Hong T, He JS (2017). A single intranasal administration of virus-like particle vaccine induces an efficient protection for mice against human respiratory syncytial virus. *Antiviral Research.* 144: 57-69.

Jones SM, Day F, Stagg AJ, Williamson ED (2000). Protection conferred by a fully recombinant sub-unit vaccine against yersinia pestis in male and female mice of four inbred strains. *Vaccine.* 19: 358-366.

Jones T, Adamovicz JJ, Cyr SL, Bolt CR, Bellerose N, Pitt LM, Lowell GH, Burt DS (2006). Intranasal protollin/F1-V vaccine elicits respiratory and serum antibody responses and protects mice against lethal aerosolized plague infection. *Vaccine.* 24: 1625-1632.

Kemnade JO, Seethammagari M, Collinson-Pautz M, Kaur H, Spencer DM, McCormick AA (2014). Tobacco mosaic virus efficiently targets DC uptake, activation and antigen-specific T cell responses in vivo. *Vaccine.* 32: 4228-4233.

King AMQ, Adams MJ, Carstens EB, Lefkowitz EJVirus taxonomy ninth report of the international committee on taxonomy of viruses. Paper presented at the *International Committee on Taxonomy of Viruses,* San Diego, CA. : 1327.

Kiyono H and Fukuyama S (2004). NALT- versus peyer's-patch-mediated mucosal immunity. *Nature Reviews.Immunology.* 4: 699-710.

Klug A (1999). The tobacco mosaic virus particle: Structure and assembly. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences.* 354: 531-535.

Koirala J (2006). Plague: Disease, management, and recognition of act of terrorism. *Infectious Disease Clinics of North America.* 20: 273-87, viii.

Kolenbrander A, Grewe B, Nemazee D, Uberla K, Temchura V (2018). Generation of T follicular helper cells in vitro: Requirement for B-cell receptor cross-linking and cognate B- and T-cell interaction.*Immunology.* 153: 214-224.

Koo M, Bendahmane M, Lettieri GA, Paoletti AD, Lane TE, Fitchen JH, Buchmeier MJ, Beachy RN (1999). Protective immunity against murine hepatitis virus (MHV) induced by intranasal or subcutaneous administration of hybrids of tobacco mosaic virus that carries an MHV epitope. *Proceedings of the National Academy of Sciences of the United States of America.* 96: 7774-7779.

Kummer LW, Szaba FM, Parent MA, Adamovicz JJ, Hill J, Johnson LL, Smiley ST (2008). Antibodies and cytokines independently protect against pneumonic plague. *Vaccine.* 26: 6901-6907.

Lathem WW, Price PA, Miller VL, Goldman WE (2007). A plasminogen-activating protease specifically controls the development of primary pneumonic plague. *Science (New York, N.Y.).* 315: 509-513.

Leary SE, Williamson ED, Griffin KF, Russell P, Eley SM, Titball RW (1995). Active immunization with recombinant V antigen from yersinia pestis protects mice against plague. *Infection and Immunity.* 63: 2854-2858.

Lebeurier G and Hirth L (1966). Effect of elevated temperatures on the development of two strains of tobacco mosaic virus. *Virology.* 29: 385-395.

LeClair RA (1967). Recovery of culturable tobacco mosaic virus from sputum and thoracentesis fluids obtained from cigarette smokers with a history of pulmonary disease. *The American Review of Respiratory Disease.* 95: 510-511.

Lee-Lewis H and Anderson DM (2010). Absence of inflammation and pneumonia during infection with nonpigmented yersinia pestis reveals a new role for the pgm locus in pathogenesis. *Infection and Immunity.* 78: 220-230.

Levine MM (2003). Can needle-free administration of vaccines become the norm in global immunization? *Nature Medicine.* 9: 99-103.

Levy Y, Flashner Y, Tidhar A, Zauberman A, Aftalion M, Lazar S, Gur D, Shafferman A, Mamroud E (2011). T cells play an essential role in anti-F1 mediated rapid protection against bubonic plague. *Vaccine.* 29: 6866-6873.

Levy Y, Vagima Y, Tidhar A, Aftalion M, Gur D, Nili U, Chitlaru T, Zauberman A, Mamroud E (2018). Targeting of the yersinia pestis F1 capsular antigen by innate-like B1b cells mediates a rapid protective response against bubonic plague. *NPJ Vaccines.* 3: 52-z. eCollection 2018.

Liang B, Hyland L, Hou S (2001). Nasal-associated lymphoid tissue is a site of long-term virus-specific antibody production following respiratory virus infection of mice. *Journal of Virology.* 75: 5416-5420.

Liao W, Hua Z, Liu C, Lin L, Chen R, Hou B (2017). Characterization of T-dependent and T-independent B cell responses to a virus-like particle. *Journal of Immunology (Baltimore, Md.: 1950).* 198: 3846-3856.

Lin JS, Kummer LW, Szaba FM, Smiley ST (2011). IL-17 contributes to cell-mediated defense against pulmonary yersinia pestis infection. *Journal of Immunology (Baltimore, Md.: 1950).* 186: 1675-1684.

Lin JS, Park S, Adamovicz JJ, Hill J, Bliska JB, Cote CK, Perlin DS, Amemiya K, Smiley ST (2010). TNFalpha and IFNgamma contribute to F1/LcrV-targeted immune defense in mouse models of fully virulent pneumonic plague. *Vaccine.* 29: 357-362.

LINK VB (1955). A history of plague in united states of america. *Public Health Monograph.* 26: 1-120.

Liu F, Chen H, Galvan EM, Lasaro MA, Schifferli DM (2006). Effects of psa and F1 on the adhesive and invasive interactions of yersinia pestis with human respiratory tract epithelial cells. *Infection and Immunity.* 74: 5636-5644.

Liu R, Vaishnav RA, Roberts AM, Friedland RP (2013). Humans have antibodies against a plant virus: Evidence from tobacco mosaic virus. *PloS One.* 8: e60621.

Loor F (1967). Comparative immunogenicities of tobacco mosaic virus, protein subunits, and reaggregated protein subunits. *Virology.* 33: 215-220.

Ma CS, Deenick EK, Batten M, Tangye SG (2012). The origins, function, and regulation of T follicular helper cells. *The Journal of Experimental Medicine.* 209: 1241-1253.

Mallajosyula JK, Hiatt E, Hume S, Johnson A, Jeevan T, Chikwamba R, Pogue GP, Bratcher B, Haydon H, Webby RJ, McCormick AA (2014). Single-dose monomeric HA subunit vaccine generates full protection from influenza challenge. *Human Vaccines & Immunotherapeutics.* 10: 586-595.

Mandal B and Jain RK (2010). Can plant virus infect human being? *Indian Journal of Virology : An Official Organ of Indian Virological Society.* 21: 92-93.

Marbrook J and Matthews RE (1966). The differential immunogenicity of plant viral protein and nucleoproteins. *Virology.* 28: 219-228.

Matson JS, Durick KA, Bradley DS, Nilles ML (2005). Immunization of mice with YscF provides protection from yersinia pestis infections. *BMC Microbiology.* 5: 38-38.

McCormick AA, Corbo TA, Wykoff-Clary S, Palmer KE, Pogue GP (2006). Chemical conjugate TMV-peptide bivalent fusion vaccines improve cellular immunity and tumor protection. *Bioconjugate Chemistry.* 17: 1330-1338.

McIntyre TM, Klinman DR, Rothman P, Lugo M, Dasch JR, Mond JJ, Snapper CM (1993). Transforming growth factor beta 1 selectivity stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells. *The Journal of Experimental Medicine.* 177: 1031-1037.

McNally A, Thomson NR, Reuter S, Wren BW (2016). 'Add, stir and reduce': Yersinia spp. as model bacteria for pathogen evolution. *Nature Reviews.Microbiology.* 14: 177-190.

Meyer KF (1970). *Effectiveness of live or killed plague vaccines in man.* (Bulletin No. 42).World Health Organization.

Meyer KF, Cavanaugh DC, Bartelloni PJ, Marshall JD (1974). Plague immunization. I. past and present trends. *The Journal of Infectious Diseases.* 129: Suppl:S13-8.

Meyer KF and Foster LE (1948). *Measurement of protective serum antibodies in human volunteers inoculated with plague prophylatics.*(Stanford Medical Bulletin No. 6). Stanford, CA: Stanford University.

Meyer KF, Smith G, Foster L, Brookman M, Sung M (1974). Live, attenuated yersinia pestis vaccine: Virulent in nonhuman primates, harmless to guinea pigs. *The Journal of Infectious Diseases.* 129: Suppl:S85-12.

Mizgerd JP and Skerrett SJ (2008). Animal models of human pneumonia. *American Journal of Physiology.Lung Cellular and Molecular Physiology.* 294: 387.

Montminy SW, Khan N, McGrath S, Walkowicz MJ, Sharp F, Conlon JE, Fukase K, Kusumoto S, Sweet C, Miyake K, Akira S, Cotter RJ, Goguen JD, Lien E (2006). Virulence factors of yersinia pestis are overcome by a strong lipopolysaccharide response. *Nature Immunology.* 7: 1066-1073.

Nakajima R and Brubaker RR (1993). Association between virulence of yersinia pestis and suppression of gamma interferon and tumor necrosis factor alpha. *Infection and Immunity.* 61: 23-31.

Nakajima R, Motin VL, Brubaker RR (1995). Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization. *Infection and Immunity.* 63: 3021-3029.

Nedialkov YA, Motin VL, Brubaker RR (1997). Resistance to lipopolysaccharide mediated by the yersinia pestis V antigen-polyhistidine fusion peptide: Amplification of interleukin-10. *Infection and Immunity.* 65: 1196-1203.

Nguyen VK, Parra-Rojas C, Hernandez-Vargas EA (2018). The 2017 plague outbreak in madagascar: Data descriptions and epidemic modelling. *Epidemics.* 25: 20-25.

Nimmerjahn F and Ravetch JV (2005). Divergent immunoglobulin g subclass activity through selective fc receptor binding. *Science (New York, N.Y.).* 310: 1510-1512.

Nyirenda SS, Hang'ombe BM, Mulenga E, Kilonzo BS (2017). Serological and PCR investigation of yersinia pestis in potential reservoir hosts from a plague outbreak focus in zambia. *BMC Research Notes.* 10: 345-9.

Okan NA, Mena P, Benach JL, Bliska JB, Karzai AW (2010). The smpB-ssrA mutant of yersinia pestis functions as a live attenuated vaccine to protect mice against pulmonary plague infection. *Infection and Immunity.* 78: 1284-1293.

Olson RM and Anderson DM (2019). Shift from primary pneumonic to secondary septicemic plague by decreasing the volume of intranasal challenge with yersinia pestis in the murine model. *PloS One.* 14: e0217440.

Oyston PC, Williamson ED, Leary SE, Eley SM, Griffin KF, Titball RW (1995). Immunization with live recombinant salmonella typhimurium aroA producing F1 antigen protects against plague. *Infection and Immunity.* 63: 563-568.

Palm AE and Henry C (2019). Remembrance of things past: Long-term B cell memory after infection and vaccination. *Frontiers in Immunology.* 10: 1787.

Palmer KE, Benko A, Doucette SA, Cameron TI, Foster T, Hanley KM, McCormick AA, McCulloch M, Pogue GP, Smith ML, Christensen ND (2006). Protection of rabbits against cutaneous papillomavirus infection using recombinant tobacco mosaic virus containing L2 capsid epitopes. *Vaccine.* 24: 5516-5525.

Parent MA, Berggren KN, Kummer LW, Wilhelm LB, Szaba FM, Mullarky IK, Smiley ST (2005). Cell-mediated protection against pulmonary yersinia pestis infection. *Infection and Immunity.* 73: 7304-7310.

Parent MA, Wilhelm LB, Kummer LW, Szaba FM, Mullarky IK, Smiley ST (2006). Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary yersinia pestis infection. *Infection and Immunity.* 74: 3381-3386.

Pechous RD, Sivaraman V, Price PA, Stasulli NM, Goldman WE (2013). Early host cell targets of yersinia pestis during primary pneumonic plague. *PLoS Pathogens.* 9: e1003679.

Perry RD, Bobrov AG, Fetherston JD (2015). The role of transition metal transporters for iron, zinc, manganese, and copper in the pathogenesis of yersinia pestis. *Metallomics : Integrated Biometal Science.* 7: 965-978.

Perry RD and Fetherston JD (1997). Yersinia pestis--etiologic agent of plague. *Clinical Microbiology Reviews.* 10: 35-66.

Peters KN, Dhariwala MO, Hughes Hanks JM, Brown CR, Anderson DM (2013). Early apoptosis of macrophages modulated by injection of yersinia pestis YopK promotes progression of primary pneumonic plague. *PLoS Pathogens.* 9: e1003324.

Pettersson J, Holmstrom A, Hill J, Leary S, Frithz-Lindsten E, von Euler-Matell A, Carlsson E, Titball R, Forsberg A, Wolf-Watz H (1999). The V-antigen of yersinia is surface exposed before target cell contact and involved in virulence protein translocation. *Molecular Microbiology.* 32: 961-976.

Philipovskiy AV and Smiley ST (2007). Vaccination with live yersinia pestis primes CD4 and CD8 T cells that synergistically protect against lethal pulmonary Y. pestis infection. *Infection and Immunity.* 75: 878-885.

Pribul PK, Harker J, Wang B, Wang H, Tregoning JS, Schwarze J, Openshaw PJ (2008). Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development. *Journal of Virology.* 82: 4441-4448.

Pujol C, Klein KA, Romanov GA, Palmer LE, Cirota C, Zhao Z, Bliska JB (2009). Yersinia pestis can reside in autophagosomes and avoid xenophagy in murine macrophages by preventing vacuole acidification. *Infection and Immunity.* 77: 2251-2261.

Quenee LE, Ciletti NA, Elli D, Hermanas TM, Schneewind O (2011). Prevention of pneumonic plague in mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or F1-V vaccines.*Vaccine.* 29: 6572-6583.

Quenee LE and Schneewind O (2009). Plague vaccines and the molecular basis of immunity against yersinia pestis. *Human Vaccines.* 5: 817-823.

Rabaan AA, Al-Ahmed SH, Alsuliman SA, Aldrazi FA, Alfouzan WA, Haque S (2019). The rise of pneumonic plague in madagascar: Current plague outbreak breaks usual seasonal mould. *Journal of Medical Microbiology.* 68: 292-302.

Ramasindrazana B, Andrianaivoarimanana V, Rakotondramanga JM, Birdsell DN, Ratsitorahina M, Rajerison M (2017). Pneumonic plague transmission, moramanga, madagascar, 2015. *Emerging Infectious Diseases.* 23: 521-524.

Randremanana R, Andrianaivoarimanana V, Nikolay B, Ramasindrazana B, Paireau J, Ten Bosch QA, Rakotondramanga JM, Rahajandraibe S, Rahelinirina S, Rakotomanana F, Rakotoarimanana FM, Randriamampionona LB, Razafimbia V, De Dieu Randria, M J, Raberahona M, Mikaty G, Le Guern AS, Rakotonjanabelo LA, Ndiaye CF, Rasolofo V, Bertherat E, Ratsitorahina M, Cauchemez S, Baril L, Spiegel A, Rajerison M (2019). Epidemiological characteristics of an urban plague epidemic in madagascar, august-november, 2017: An outbreak report. *The Lancet.Infectious Diseases.* 19: 537-545.

Rascovan N, Sjogren KG, Kristiansen K, Nielsen R, Willerslev E, Desnues C, Rasmussen S (2019). Emergence and spread of basal lineages of yersinia pestis during the neolithic decline. *Cell.* 176: 295-305.e10.

Rasmussen S, Allentoft ME, Nielsen K, Orlando L, Sikora M, Sjogren KG, Pedersen AG, Schubert M, Van Dam A, Kapel CM, Nielsen HB, Brunak S, Avetisyan P, Epimakhov A, Khalyapin MV, Gnuni A, Kriiska A, Lasak I, Metspalu M, Moiseyev V, Gromov A, Pokutta D, Saag L, Varul L, Yepiskoposyan L, Sicheritz-Ponten T, Foley RA, Lahr MM, Nielsen R, Kristiansen K, Willerslev E (2015). Early divergent strains of yersinia pestis in eurasia 5,000 years ago. *Cell.* 163: 571-582.

Ratner D, Orning MP, Proulx MK, Wang D, Gavrilin MA, Wewers MD, Alnemri ES, Johnson PF, Lee B, Mecsas J, Kayagaki N, Goguen JD, Lien E (2016). The yersinia pestis effector YopM inhibits pyrin inflammasome activation. *PLoS Pathogens.* 12: e1006035.

Richgels KL, Russell RE, Bron GM, Rocke TE (2016). Evaluation of yersinia pestis transmission pathways for sylvatic plague in prairie dog populations in the western U.S. *EcoHealth.* 13: 415-427.

Riedel S (2004). Biological warfare and bioterrorism: A historical review. *Proceedings (Baylor University.Medical Center).* 17: 400-406.

Riedel S (2005). Plague: From natural disease to bioterrorism. *Proceedings (Baylor University.Medical Center).* 18: 116-124.

Rocke TE, Smith S, Marinari P, Kreeger J, Enama JT, Powell BS (2008). Vaccination with F1-V fusion protein protects black-footed ferrets (mustela nigripes) against plague upon oral challenge with yersinia pestis. *Journal of Wildlife Diseases.* 44: 1-7.

Roggenkamp A, Geiger AM, Leitritz L, Kessler A, Heesemann J (1997). Passive immunity to infection with yersinia spp. mediated by anti-recombinant V antigen is dependent on polymorphism of V antigen. *Infection and Immunity.* 65: 446-451.

Runfola JK, House J, Miller L, Colton L, Hite D, Hawley A, Mead P, Schriefer M, Petersen J, Casaceli C, Erlandson KM, Foster C, Pabilonia KL, Mason G, Douglas JM, Centers for Disease Control and Prevention, (CDC) (2015). Outbreak of human pneumonic plague with dog-to-human and possible human-to-human transmission--colorado, june-july 2014. *MMWR.Morbidity and Mortality Weekly Report.* 64: 429-434.

Santi L, Giritch A, Roy CJ, Marillonnet S, Klimyuk V, Gleba Y, Webb R, Arntzen CJ, Mason HS (2006). Protection conferred by recombinant yersinia pestis antigens produced by a rapid and highly scalable plant expression system. *Proceedings of the National Academy of Sciences of the United States of America.* 103: 861-866.

Savelkoul HF, Ferro VA, Strioga MM, Schijns VE (2015). Choice and design of adjuvants for parenteral and mucosal vaccines. *Vaccines.* 3: 148-171.

Sedlik C, Dridi A, Deriaud E, Saron MF, Rueda P, Sarraseca J, Casal JI, Leclerc C (1999). Intranasal delivery of recombinant parvovirus-like particles elicits cytotoxic T-cell and neutralizing antibody responses. *Journal of Virology.* 73: 2739-2744.

Sha J, Kirtley ML, Klages C, Erova TE, Telepnev M, Ponnusamy D, Fitts EC, Baze WB, Sivasubramani SK, Lawrence WS, Patrikeev I, Peel JE, Andersson JA, Kozlova EV, Tiner BL, Peterson JW, McWilliams D, Patel S, Rothe E, Motin VL, Chopra AK (2016). A replication-defective human type 5 adenovirus-based trivalent vaccine confers complete protection against plague in mice and nonhuman primates. *Clinical and Vaccine Immunology : CVI.* 23: 586-600.

Sha J, Kirtley ML, van Lier CJ, Wang S, Erova TE, Kozlova EV, Cao A, Cong Y, Fitts EC, Rosenzweig JA, Chopra AK (2013). Deletion of the braun lipoprotein-encoding gene and altering the function of lipopolysaccharide attenuate the plague bacterium. *Infection and Immunity.* 81: 815-828.

Shinomiya N, Kuratsuji T, Yata J (1989). The role of T cells in immunoglobulin class switching of specific antibody production system in vitro in humans. *Cellular Immunology.* 118: 239-249.

Simond P (1898). La propagation de la peste. *Annales De L'Institut Pasteur.* 12: 625-687.

Sing A, Rost D, Tvardovskaia N, Roggenkamp A, Wiedemann A, Kirschning CJ, Aepfelbacher M, Heesemann J (2002). Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *The Journal of Experimental Medicine.* 196: 1017-1024.

Sivaraman V, Pechous RD, Stasulli NM, Eichelberger KR, Miao EA, Goldman WE (2015). Yersinia pestis activates both IL-1beta and IL-1 receptor antagonist to modulate lung inflammation during pneumonic plague. *PLoS Pathogens.* 11: e1004688.

Smiley ST (2007). Cell-mediated defense against yersinia pestis infection. *Advances in Experimental Medicine and Biology.* 603: 376-386.

Smiley ST (2008a). Current challenges in the development of vaccines for pneumonic plague. *Expert Review of Vaccines.* 7: 209-221.

Smiley ST (2008b). Immune defense against pneumonic plague. *Immunological Reviews.* 225: 256-271.

Solomon T (1995). Alexandre yersin and the plague bacillus. *The Journal of Tropical Medicine and Hygiene.* 98: 209-212.

SPECK RS and WOLOCHOW H (1957). Studies on the experimental epidemiology of respiratory infections. VIII. experimental pneumonic plague in macacus rhesus. *The Journal of Infectious Diseases.* 100: 58-69.

Spinner JL, Winfree S, Starr T, Shannon JG, Nair V, Steele-Mortimer O, Hinnebusch BJ (2014). Yersinia pestis survival and replication within human neutrophil phagosomes and uptake of infected neutrophils by macrophages. *Journal of Leukocyte Biology.* 95: 389-398.

Spyrou MA, Tukhbatova RI, Wang CC, Valtuena AA, Lankapalli AK, Kondrashin VV, Tsybin VA, Khokhlov A, Kuhnert D, Herbig A, Bos KI, Krause J (2018). Analysis of

3800-year-old yersinia pestis genomes suggests bronze age origin for bubonic plague. *Nature Communications.* 9: 2234-9.

Stasulli NM, Eichelberger KR, Price PA, Pechous RD, Montgomery SA, Parker JS, Goldman WE (2015). Spatially distinct neutrophil responses within the inflammatory lesions of pneumonic plague.*mBio.* 6: 1530.

Stryer L, Berg J, Tymoczko J, Clarke N (1988). *Biochemistry* (3rd ed.) W. H. Freeman And Company.

Sun W and Singh AK (2019). Plague vaccine: Recent progress and prospects. *NPJ Vaccines.* 4: 11-9. eCollection 2019.

Sun W, Six D, Kuang X, Roland KL, Raetz CR, Curtiss R (2011). A live attenuated strain of yersinia pestis KIM as a vaccine against plague. *Vaccine.* 29: 2986-2998.

Sun YC, Jarrett CO, Bosio CF, Hinnebusch BJ (2014). Retracing the evolutionary path that led to flea-borne transmission of yersinia pestis. *Cell Host & Microbe.* 15: 578-586.

Szomolanyi-Tsuda E, Le QP, Garcea RL, Welsh RM (1998). T-cell-independent immunoglobulin G responses in vivo are elicited by live-virus infection but not by immunization with viral proteins or virus-like particles. *Journal of Virology.* 72: 6665-6670.

Tamminen K, Malm M, Vesikari T, Blazevic V (2016). Mucosal antibodies induced by intranasal but not intramuscular immunization block norovirus GII.4 virus-like particle receptor binding. *Viral Immunology.* 29: 315-319.

Tangye SG and Tarlinton DM (2009). Memory B cells: Effectors of long-lived immune responses. *European Journal of Immunology.* 39: 2065-2075.

Tao P, Mahalingam M, Zhu J, Moayeri M, Kirtley ML, Fitts EC, Andersson JA, Lawrence WS, Leppla SH, Chopra AK, Rao VB (2017). A bivalent anthrax-plague vaccine that can protect against two tier-1 bioterror pathogens, bacillus anthracis and yersinia pestis. *Frontiers in Immunology.* 8: 687.

Tidhar A, Flashner Y, Cohen S, Levi Y, Zauberman A, Gur D, Aftalion M, Elhanany E, Zvi A, Shafferman A, Mamroud E (2009). The NlpD lipoprotein is a novel yersinia pestis virulence factor essential for the development of plague. *PloS One.* 4: e7023.

Titball RW, Howells AM, Oyston PC, Williamson ED (1997). Expression of the yersinia pestis capsular antigen (F1 antigen) on the surface of an aroA mutant of salmonella typhimurium induces high levels of protection against plague. *Infection and Immunity.* 65: 1926-1930.

Titball RW and Williamson ED (2001). Vaccination against bubonic and pneumonic plague. *Vaccine.* 19: 4175-4184.

Titball RW and Williamson ED (2004). Yersinia pestis (plague) vaccines. *Expert Opinion on Biological Therapy.* 4: 965-973.

Turpen TH, Reinl SJ, Charoenvit Y, Hoffman SL, Fallarme V, Grill LK (1995). Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Bio/Technology (Nature Publishing Company).* 13: 53-57.

Van Regenmortel MH (1999). The antigenicity of tobacco mosaic virus. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences.* 354: 559-568.

Van Regenmortel MH, Altschuh D, Zeder-Lutz G (1993). Tobacco mosaic virus: A model antigen to study virus-antibody interactions.*Biochimie.* 75: 731-739.

Verma SK and Tuteja U (2016). Plague vaccine development: Current research and future trends. *Frontiers in Immunology.* 7: 602.

Visser LG, Annema A, van Furth R (1995). Role of yops in inhibition of phagocytosis and killing of opsonized yersinia enterocolitica by human granulocytes. *Infection and Immunity.* 63: 2570-2575.

Wagner DM, Klunk J, Harbeck M, Devault A, Waglechner N, Sahl JW, Enk J, Birdsell DN, Kuch M, Lumibao C, Poinar D, Pearson T, Fourment M, Golding B, Riehm JM, Earn DJ, Dewitte S, Rouillard JM, Grupe G, Wiechmann I, Bliska JB, Keim PS, Scholz HC, Holmes EC, Poinar H (2014). Yersinia pestis and the plague of justinian 541-543 AD: A genomic analysis. *The Lancet.Infectious Diseases.* 14: 319-326.

Wang N, Strugnell R, Wijburg O, Brodnicki T (2011). Measuring bacterial load and immune responses in mice infected with listeria monocytogenes. *Journal of Visualized Experiments : JoVE.* (54). pii: 3076. doi: 10.3791/3076.

Wang S, Liu H, Zhang X, Qian F (2015). Intranasal and oral vaccination with protein-based antigens: Advantages, challenges and formulation strategies. *Protein & Cell.* 6: 480-503.

Weiner HL (2001). The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. *Nature Immunology.* 2: 671-672.

Welch TJ, Fricke WF, McDermott PF, White DG, Rosso ML, Rasko DA, Mammel MK, Eppinger M, Rosovitz MJ, Wagner D, Rahalison L, Leclerc JE, Hinshaw JM, Lindler LE, Cebula TA, Carniel E, Ravel J (2007). Multiple antimicrobial resistance in plague: An emerging public health risk. *PloS One.* 2: e309.

Welkos S, Pitt MLM, Martinez M, Friedlander A, Vogel P, Tammariello R (2002). *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*

Welkos S, Friedlander A, McDowell D, Weeks J, Tobery S (1998). V antigen of yersinia pestis inhibits neutrophil chemotaxis. *Microbial Pathogenesis.* 24: 185-196.

WHO Group of Consultants (1970). *Health aspects of chemical and biological weapons.* (Report No. 1). Geneva, Switzerland: United Nations.

Williamson ED, Eley SM, Griffin KF, Green M, Russell P, Leary SE, Oyston PC, Easterbrook T, Reddin KM, Robinson A (1995). A new improved sub-unit vaccine for plague: The basis of protection. *FEMS Immunology and Medical Microbiology.* 12: 223-230.

Williamson ED, Eley SM, Stagg AJ, Green M, Russell P, Titball RW (1997). A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague. *Vaccine.* 15: 1079-1084.

Williamson ED, Flick-Smith HC, Lebutt C, Rowland CA, Jones SM, Waters EL, Gwyther RJ, Miller J, Packer PJ, Irving M (2005). Human immune response to a plague vaccine comprising recombinant F1 and V antigens. *Infection and Immunity.* 73: 3598-3608.

Williamson ED and Oyston PC (2013). Protecting against plague: Towards a next-generation vaccine. *Clinical and Experimental Immunology.* 172: 1-8.

Williamson ED, Sharp GJ, Eley SM, Vesey PM, Pepper TC, Titball RW, Alpar HO (1996). Local and systemic immune response to a microencapsulated sub-unit vaccine for plague. *Vaccine.* 14: 1613-1619.

Williamson ED, Stagg AJ, Eley SM, Taylor R, Green M, Jones SM, Titball RW (2007). Kinetics of the immune response to the (F1+V) vaccine in models of bubonic and pneumonic plague. *Vaccine.* 25: 1142-1148.

Williamson ED, Vesey PM, Gillhespy KJ, Eley SM, Green M, Titball RW (1999). An IgG1 titre to the F1 and V antigens correlates with protection against plague in the mouse model. *Clinical and Experimental Immunology.* 116: 107-114.

Yang X, Hinnebusch BJ, Trunkle T, Bosio CM, Suo Z, Tighe M, Harmsen A, Becker T, Crist K, Walters N, Avci R, Pascual DW (2007). Oral vaccination with salmonella simultaneously expressing yersinia pestis F1 and V antigens protects against bubonic and pneumonic plague. *Journal of Immunology (Baltimore, Md.: 1950).* 178: 1059-1067.

Yang Y and Klessig DF (1996). Isolation and characterization of a tobacco mosaic virus-inducible myb oncogene homolog from tobacco. *Proceedings of the National Academy of Sciences of the United States of America.* 93: 14972-14977.

Yersin A (1894). Le peste bubonique à hong kong. *Annales De L'Institut Pasteur.* 8: 662-667.

Yersin A (1897). Sur la peste bubonique. *Annales De L'Institut Pasteur.* 11: 81-93.

Zavialov AV, Berglund J, Pudney AF, Fooks LJ, Ibrahim TM, MacIntyre S, Knight SD (2003). Structure and biogenesis of the capsular F1 antigen from yersinia pestis: Preserved folding energy drives fiber formation. *Cell.* 113: 587-596.

Zhang T, Breitbart M, Lee WH, Run JQ, Wei CL, Soh SW, Hibberd ML, Liu ET, Rohwer F, Ruan Y (2006). RNA viral community in human feces: Prevalence of plant pathogenic viruses. *PLoS Biology.* 4: e3.

Zietz BP and Dunkelberg H (2004). The history of the plague and the research on the causative agent yersinia pestis. *International Journal of Hygiene and Environmental Health.* 207: 165-178.

Zimbler DL, Eddy JL, Schroeder JA, Lathem WW (2015). Inactivation of peroxiredoxin 6 by the pla protease of yersinia pestis. *Infection and Immunity.* 84: 365-374.

Zimbler DL, Schroeder JA, Eddy JL, Lathem WW (2015). Early emergence of yersinia pestis as a severe respiratory pathogen. *Nature Communications.* 6: 7487.

Zuercher AW, Coffin SE, Thurnheer MC, Fundova P, Cebra JJ (2002). Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. *Journal of Immunology (Baltimore, Md.: 1950).* 168: 1796-1803.