

THE INNATE IMMUNE RESPONSE MAY BE IMPORTANT FOR SURVIVING PLAGUE IN WILD GUNNISON'S PRAIRIE DOGS

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ABSTRACT: Prairie dogs (*Cynomys* spp.) are highly susceptible to *Yersinia pestis*, with $\geq 99\%$ mortality reported from multiple studies of plague epizootics. A colony of Gunnison's prairie dogs (*Cynomys gunnisoni*) in the Aubrey Valley (AV) of northern Arizona appears to have survived several regional epizootics of plague, whereas nearby colonies have been severely affected by *Y. pestis*. To examine potential mechanisms accounting for survival in the AV colony, we conducted a laboratory *Y. pestis* challenge experiment on 60 wild-caught prairie dogs from AV and from a nearby, large colony with frequent past outbreaks of plague, Espee ($n=30$ per colony). Test animals were challenged subcutaneously with the fully virulent *Y. pestis* strain CO92 at three doses: 50, 5,000, and 50,000 colony-forming units (cfu); this range is lethal in black-tailed prairie dogs (*Cynomys ludovicianus*). Contrary to our expectations, only 40% of the animals died. Although mortality trended higher in the Espee colony (50%) compared with AV (30%), the differences among infectious doses were not statistically significant. Only 39% of the survivors developed moderate to high antibody levels to *Y. pestis*, indicating that mechanisms other than humoral immunity are important in resistance to plague. The ratio of neutrophils to lymphocytes was not correlated with plague survival in this study. However, several immune proteins with roles in innate immunity (VCAM-1, CXCL-1, and vWF) were upregulated during plague infection and warrant further inquiry into their role for protection against this disease. These results suggest plague resistance exists in wild populations of the Gunnison's prairie dog and provide important directions for future studies.

Key words: Aubrey Valley, innate immunity, plague resistance, prairie dog, *Yersinia pestis*.

INTRODUCTION

Yersinia pestis, the etiologic agent of plague, is a recently emerged pathogen of rodents that has spread widely around the world (Gage and Kosoy, 2005; Keim and Wagner, 2009; Morelli et al., 2010). More than 200 mammalian species are susceptible to plague (Pollitzer, 1960), and *Y. pestis* is known to be transmitted by at least 260 flea vectors (Serzhan and Ageyev, 2000). Significant research effort has been directed at elucidating specific host mechanisms underlying resistance to *Y. pestis* (see reviews by Perry and Fetherston, 1997; Smiley, 2008a; Bergsbaken and Cookson, 2009), yet no single

model adequately predicts which individuals will survive infection. Although the complexity of plague's virulence arsenal is recognized, most published challenge studies have focused on individual virulence components rather than investigating the multiple immune pathways that come into play during progression of plague. Furthermore, most studies are conducted on inbred lines of laboratory rodents, which lack the immune system variation found in wild rodent populations.

Plague became ecologically established in the western United States after inadvertently being introduced to North America around 1900 (Link, 1955). Since that time, it has been a major cause of

population decline in the four species of prairie dogs (*Cynomys* spp.) in the western United States (Hoogland et al., 2004), with mortality levels near 100% in affected colonies (Cully et al., 1997; Cully and Williams, 2001). In recent decades, plague outbreaks have decimated colonies of Gunnison's prairie dog (*C. gunnisoni*) across northern Arizona (Girard et al., 2004; Wagner et al., 2006), with one notable exception, the Aubrey Valley (AV). The AV colony is unique among Gunnison's prairie dog colonies in Arizona for several reasons. It occupies a larger area compared with other studied colonies in Arizona, which tend to occupy ≤ 100 ha (Wagner et al., 2006). The geographic extent of the AV colony has varied less than 10% during the past 40 yr, whereas other colonies have varied much more through time (Van Pelt, 1995; Wagner et al., 2006). Finally, the AV colony does not appear to experience plague-caused die-offs, which have resulted in numerous local extinctions of Gunnison's prairie dog colonies elsewhere in Arizona (Girard et al., 2004; Wagner et al., 2006). Because of these qualities, the AV colony is the only Gunnison's prairie dog colony able to successfully support black-footed ferrets (*Mustela nigripes*), which require large, stable prairie dog populations for their survival (Biggins et al., 1993). It is not known why Gunnison's prairie dogs in AV do not experience die-offs like other colonies in the area. The AV is predicted to be an area of plague occurrence in published spatial models (Eisen et al., 2007; Nakazawa et al., 2007), and epizootics of plague have affected a neighboring colony at Seligman, Arizona, 6 km away (Wagner et al., 2006). No natural barriers to the spread of plague appear to exist between these two prairie dog areas.

Field sampling during and after outbreaks of plague in prairie dogs suggest that some, but not all, survivors develop antibodies to *Y. pestis* (Cully et al., 1997; Pauli et al., 2006). However, in most rodents, plague begins to cause death

within a few days of infection, which is an insufficient time for the development of protective immunoglobulin (Ig) M or IgG antibodies. *Yersinia pestis* possesses multiple virulence mechanisms that are mainly directed at the host's innate immune system (Heesemann et al., 2006; Bubeck et al., 2007). This suggests that earlier immune responses (e.g., inflammation and the acute-phase response) may underlie critical differences between surviving individuals and those that succumb to plague. Supporting this hypothesis, researchers found that certain black-tailed prairie dogs can survive a high dosage (50,000 colony forming units [CFU]) of subcutaneous plague challenge without a detectable antibody response to the surface antigen F1 (Rocke et al., 2012).

We investigated immune responses to *Y. pestis* in the Gunnison's prairie dog, an important natural host of this pathogen. Our study is the first, to our knowledge, to use wild *C. gunnisoni* in a laboratory challenge experiment and provides a critical starting point for methodically evaluating mechanisms of resistance and immune upregulation. We hypothesized that AV prairie dogs are more resistant to plague than prairie dogs from other colonies in northern Arizona because historic evidence suggests AV prairie dogs may have survived past plague epizootics in this region. To test this, we trapped wild prairie dogs from AV and a colony known to have experienced plague outbreaks (Espee Ranch) and challenged them in the laboratory with a fully virulent strain of *Y. pestis*, CO92. Our study highlights the importance of innate immunity and reveals an unexpected level of plague resistance in wild prairie dogs from both populations.

MATERIALS AND METHODS

Field collections

We live-trapped 60 Gunnison's prairie dogs, 30 from AV (35°28'59"N, 113°05'57"W) and 30 from Espee Ranch (35°50'28"N, 112°32'37"W). The two sites are 66 km apart and are

demographically independent. We balanced age (J=juvenile, A=adult) and sex categories (F=female, M=male) between samples as much as possible (AV with 8 JF, 12 JM, 5 AF, and 5 AM; Espee with 7 JF, 12 JM, 5 AF, and 6 AM). After initial capture, animals were treated with a commercially available spray (Jeffers® flea and tick mist, Dothan, Alabama, USA; active ingredients: 0.15% pyrethrins, 1% piperonyl butoxide, 0.5% *N*-octyl bicycloheptane dicarboximide, and 0.5% di-*n*-propyl isocinchomerone) for external parasite control and were quarantined for 3-wk at Northern Arizona University. Each animal was housed separately in cages measuring 61 cm×61 cm×41 cm (length, width, height), which were lined with timothy hay, and provided with a 12-cm-diameter polyvinyl chloride (PVC) pipe for enrichment shelter. Because prairie dogs are social animals, all animals were housed with visual and auditory contact with conspecifics. Water and high-fiber prairie dog pellets (Exotic Nutrition, Newport News, Virginia, USA) were provided ad libitum, and fresh vegetables (cob corn and carrots) were provided once daily. After transport to the US Geological Survey National Wildlife Health Center (USGS-NWHC), study animals were given a second antiparasitic treatment (Revolution [selamectin], 6 mg/kg applied topically, Pfizer Animal Health, New York, New York, USA) and were housed together by population in two large isolation rooms (16.7 m² each). Bedding material (Beta Chips, Northeastern Products Corp., Warrensburg, New York, USA) covered the floor and custom-made stainless steel nest boxes with connecting lengths of PVC pipe were used for shelter. Alfalfa-based pellets (approximately 50 g/animal per day) and fresh vegetables (broccoli, carrot, green beans, and sweet potato chunks) were provided once daily. Water was available ad libitum. All collections and captive protocols were approved by the Arizona Game and Fish (AZGF) Department, the Institutional Animal Care and Use Committee (IACUC) at Northern Arizona University (NAU), and the IACUC at USGS-NWHC.

Prechallenge samples

Before challenge, ≤300 μL of blood was drawn from the medial saphenous vein of each animal. Sera were tested for antibodies to two *Y. pestis* antigens (F1 and LcrV) by enzyme-linked immunosorbent assay (ELISA) (Mencher et al., 2004). F1 is a capsular antigen specific to *Y. pestis*; LcrV (V antigen) is produced by *Y. pestis* and two other pathogenic *Yersinia* species: *Y. pseudotuberculosis* and *Y. enterocolitica*. We submitted 50–100 μL of serum from a subset of

animals (10 survivors and 10 deaths) for testing with RodentMAP™ v2.0 (Rules-Based Medicine, Austin, Texas, USA). This fluorescent ELISA multiplex quantifies 59 blood proteins (mainly cytokines) that may be expressed during pathogen infection. Although the platform was designed for laboratory mice, a subset of biomarkers (including IgA) has been shown to work in *C. gunnisoni* (Busch et al., 2011).

Yersinia pestis culture and infection

After a 3 wk acclimation period, we challenged 60 prairie dogs with a highly virulent wild-type *Y. pestis* strain, CO92 (Doll et al., 1994) originally provided by the US Army Medical Research Institute of Infectious Diseases (Fort Detrick, Frederick, Maryland, USA). The preparation of the CO92 challenge inoculum has been described (Osorio et al., 2003). Animals from each population were randomly placed into three treatment groups. A stock aliquot of the *Y. pestis* challenge inoculum was diluted in phosphate-buffered saline to provide 50, 5,000, and 50,000 cfu, which corresponds to 2.5, 250, and 2,500 mouse 50% lethal dose (LD₅₀), respectively. A volume of 0.2 mL of the low, medium, or high dilution was administered to each individual by subcutaneous injection in the right hip region; all individuals were inoculated on the same day. Dilutions of the challenge inocula were plated on blood agar to confirm the dosages, and concurrent intradermal inoculation of mice (Swiss Webster, Harlan Sprague Dawley) was conducted to confirm the expected virulence of the stock inoculum (20 mouse LD₅₀/mL). We chose a wide range of infectious doses because the LD₅₀ for plague is unknown in Gunnison's prairie dogs. The quantity of *Y. pestis* delivered in a flea bite may vary from a few bacteria up to 24,000 cfu (Burroughs, 1947; Eisen et al., 2008); we chose a dosage range that should encompass the various doses prairie dogs may experience during natural infection.

Study animals were monitored for 28 days, 2–3 times daily, for signs of illness. Moribund animals (exhibiting labored breathing, lethargy, or anorexia) were immediately euthanized, and these, as well as animals found dead, were necropsied. Blood samples for serum and white blood cell counts (described below) were drawn just before euthanasia from animals found moribund. The study continued until 30 days postinoculation (DPI). All animals alive at that time were considered survivors and were euthanized 33–40 DPI.

Postchallenge measurements

Postchallenge blood samples from survivors were used for white-blood cell counts, cytokine

analysis, and serology. Spleen, liver, and lung from all 60 prairie dogs were used to isolate *Y. pestis*. Frozen organs were partially thawed and cut in half, and the internal tissue surface was smeared onto 6% sheep's blood agar. Plates were incubated for 2 days at 28 C and checked for growth. Each successful *Y. pestis* culture was restreaked to isolate colonies. A sample of each pure culture was preserved in a glycerol stock at -80 C and used to extract DNA using the Qiagen (Valencia, California, USA) DNeasy Blood and Tissue Kit. All cultures were validated as *Y. pestis* using two qPCR assays; one targets the chromosomal *3a* gene and the other targets the *pla* gene on plasmid pPCPI (Hinnebusch and Schwan, 1993; Stevenson et al., 2003).

Statistical analysis

A list of the statistical tests we used on each experimental group is provided in Table 1. To compare survivorship during plague challenge, we sorted study animals into relevant categories (population, age, and infectious dose) and performed χ^2 tests of independence on the number of surviving versus dead subjects in each group. We used a Kaplan-Meier analysis of survival times (log-rank χ^2) as a complementary analysis.

Postchallenge antibody data from surviving animals in each dosage group (50–5,000–50,000) were analyzed with Spearman's rank correlation. We counted postchallenge samples of white blood cells (WBCs) from blood smears and compared the ratio of neutrophils to other WBCs using a Wilcoxon ranked-sums test by dosage group. RodentMAP cytokine data were analyzed with nonmetric multidimensional scaling (NMDS) ordination (Busch et al., 2011). A multivariate one-way analysis of similarity (ANOSIM) (Clarke, 1993) was used to test for any difference between the prechallenge cytokine profiles from a subset of animals that died ($n=10$) versus survived ($n=10$). The 10 survivors were also sampled for postchallenge cytokine expression to compare against prechallenge levels (paired analysis). We tested prechallenge versus postchallenge cytokine profiles using a nonparametric, multiresponse blocked permutation (MRBP) procedure (Mielke, 1984, 1991) in PC-ORD 4.25 software (McCune and Mefford, 1999). All ELISA biomarker measurements were converted to picograms per milliliter units and transformed into a percentage of the maximum value for each biomarker. We included only biomarkers that provided robust data in prairie dogs and are important for host bacterial defense (see (Busch et al., 2011)). We generated multivar-

iate pairwise distances using Bray-Curtis dissimilarity in DECODA v3 (Minchin, 1999). A Monte Carlo test of 100 randomizations was used to determine the probability that the observed stress value of the final solution could occur by chance alone. Using DECODA, we set up 100 runs of real data with random starting configurations, with up to 250 iterations per run and a stability criterion of 0.0001. After identifying the best ordination solution, we plotted the ranked distances of all individuals on an NMDS graph. Next, we calculated the maximum correlation (R_{\max}) of each biomarker to the ordination solution using up to 10,000 random permutations in DECODA. We fit vectors for significant biomarkers onto a two-dimensional NMDS graph (Kantvilas and Minchin, 1989) to interpret biologically relevant patterns of immune expression.

RESULTS

Survival after *Y. pestis* challenge

Contrary to our expectation of 90–99% mortality, only 24 of 60 prairie dogs (40%) died following challenge. Animals from both colonies succumbed, with 15 deaths from Espee (50%) and nine from AV (30%). Although there tended to be higher survival in AV, as predicted by our hypothesis, the difference was not statistically significant (Fig. 1a; $\chi^2=2.5$, $df=1$, $P=0.113$). We pooled animals from both populations into two groups, survivors and dead, to achieve greater statistical power for further testing. Subsequent tests of these groups did not result in any statistically significant patterns by age (Fig. 1c; $\chi^2=2.06$, $df=1$, $P=0.151$) or infectious dose (Fig. 1e; $\chi^2=2.9$, $df=2$, $P=0.232$). Kaplan-Meier analysis failed to identify significant differences in survival times in any experimental treatment (Fig. 1b, d, f).

Prairie dogs in each common room were able to freely interact with each other. Cannibalism occurred on plague-infected carcasses before they could be removed, but only in the Espee room (six carcasses). This behavior took place on days 8–16. In most cases, the upper chest cavity had been opened, with evidence of feeding on lungs and heart. This would

TABLE 1. Data collected following laboratory challenge of Gunnison's prairie dogs (*Cynomys gunnisoni*) from two Arizona, USA, populations (Aubrey Valley [AV] and Espee) with *Yersinia pestis*: relevant comparison groups and statistical analyses for these comparisons.^a

Data	Prechallenge samples ²	Postchallenge samples ²	Comparisons	Analysis	Reported
Survival counts and times (DPI)	NA	Yes	Survived/died: AV vs. Espee	χ^2 test of independence and Kaplan-Meier log-rank χ^2	Figure 1a, b
			Survived/died: Adults vs. juveniles	χ^2 test of independence and Kaplan-Meier log-rank χ^2	Figure 1c, d
			Survived/died: three doses (50/5,000/50,000 cfu)	χ^2 test of independence and Kaplan-Meier log-rank χ^2	Figure 1e, f
Antibodies in sera	Yes	Yes	Prechallenge: F1 and V titers (all prairie dogs)	Proportion of total	Text
			Postchallenge F1 and V titers: survivors only (grouped by cfu treatment)	Spearman's rank correlation	Figure 2
Cytokines in sera	Yes	Yes	Prechallenge cytokines: survivors vs. dead	ANOSIM	Text
			Prechallenge vs. postchallenge cytokines: survivors only (paired analysis)	MRBP and NMDS ordination	Table 2 and Figure 3
Neutrophil ratio	No	Yes	Postchallenge: survivors vs. dead	Mann-Whitney <i>U</i>	Text
			Postchallenge: AV vs. Espee (survivors only)	Mann-Whitney <i>U</i>	Text
Organ damage	No	Yes	Postchallenge: gross evaluation of damage (survivors and dead)	Proportion of total	Text

^a DPI = days postinfection; NA = not applicable; cfu = colony-forming units; F1 = *Y. pestis* F1 capsular antigen; V = *Yersinia* spp. LcrV antigen; ANOSIM = analysis of similarity; MRBP = multiresponse, blocked, permutation procedure; NMDS = nonmetric, multidimensional scaling.

likely have increased the challenge dose for the cannibalistic individuals because we verified that *Y. pestis* was present in major organs of all animals that died (see below). Interestingly, the last two Espee animals to die had been cannibalized, but the unknown cannibal(s) survived to the end of the experiment, despite likely exposure to additional *Y. pestis*.

Plate counts and mouse inoculation confirmed the dosage and virulence of the CO92 strain used in this study. We also resequenced the entire genome of the CO92 culture from this study using an Illumina Genome Analyzer II (Illumina, San Diego, California, USA) with paired-

end 50–base pair (bp) reads from 500-bp insert libraries. This yielded an average of 58× sequence coverage at each nucleotide position, which was aligned against the annotated CO92 genome in GenBank (Accession NC003143; [Parkhill et al., 2001]) using SeqMan NGEN (DNASTar, Madison, Wisconsin, USA). All virulence genes in the chromosome and the three virulence plasmids (pCD1, pPCP1, pMT1) were identified and confirmed to be free of single nucleotide polymorphisms (SNPs) or indels. These results, combined with 100% mortality in the control mice, provide strong evidence that all virulence factors in our CO92 culture were intact.

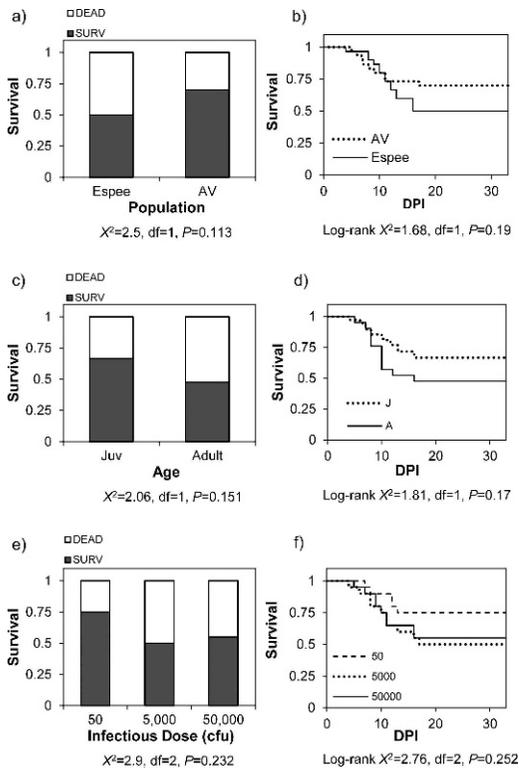


FIGURE 1. Gunnison prairie dog (*Cynomys gunnisoni*) mortality from *Yersinia pestis* challenge across experimental treatments. Statistical comparisons include (a) survival proportions in Espee versus Aubrey Valley (AV), Arizona, populations with χ^2 test of independence; (b) Kaplan-Meier survival plot for up to 30 days postinfection (DPI) with log-rank χ^2 ; (c) survival proportions in juvenile versus adult prairie dogs with χ^2 test of independence; (d) Kaplan-Meier survival plot with log-rank χ^2 ; (e) survival proportions across three infectious dose treatments (colony-forming units) with χ^2 test of independence; (f) Kaplan-Meier survival plot with log-rank χ^2 .

Antibody responses

Of the 36 survivors, 61% produced either a weak (1:640 dilution) response to F1 ($n=6$), or an undetectable response ($n=16$); the remaining 39% had variable titers, with one as high as 1:163,840. Although postchallenge F1 titers were variable (Fig. 2), they were positively correlated with the infection dosage (Spearman's $\rho=0.71, P<0.0001$). Only one prairie dog had a detectable response to the V antigen (1:640); that animal was

from the 50,000-cfu treatment. Before the challenge, four prairie dogs from AV were found to carry levels of F1 antibody above background, all at the 1:640 dilution. One of those individuals died, and another survived with a background F1 antibody level ($<1:160$). The remaining two survived and had strong F1 responses (1:10,240 and 1:40,960). The antibody levels and fate of these four prairie dogs reflected the overall patterns observed in this study and removing them had only a minor effect on the Spearman's rank correlation ($\rho=0.69, P<0.0002$).

WBC and cytokine responses

We found no statistical difference in the postchallenge ratio of neutrophils to lymphocytes in animals that survived vs. died (Mann-Whitney U -test = $-0.321, P=0.748$). No significant differences were found in levels of other WBCs, including macrophages, eosinophils, and basophils. However, the mean percentage of neutrophils was significantly higher in AV survivors (42.6%, SE 2.9%) than in Espee survivors (32.3%; Mann-Whitney $U = -2.458, P=0.007$).

Prechallenge expression of 10 immune proteins did not differ between animals that survived or succumbed to *Y. pestis* (one-way ANOSIM, $R = -0.061, P=0.908$). This suggests that the baseline immune state of these study animals was similar and not inherently protective. We did not collect sera midstudy, except as individual animals became moribund. As such, we cannot evaluate differential cytokine expression early in the infection. However, we found a strong upregulation of immune proteins between the prechallenge and 33–40 DPI samples in survivors (MRBP, $A=0.22, P=0.001$). This difference is clearly observed in the NMDS ordination, which shows complete separation between the prechallenge and postchallenge groups (Fig. 3). Changes in expression were observed in each of the 10 prairie dogs, with strong upregulation in VCAM-1, vWF, and Myo (Table 2). Other immune proteins showed smaller changes (CXCL-1, Hap,

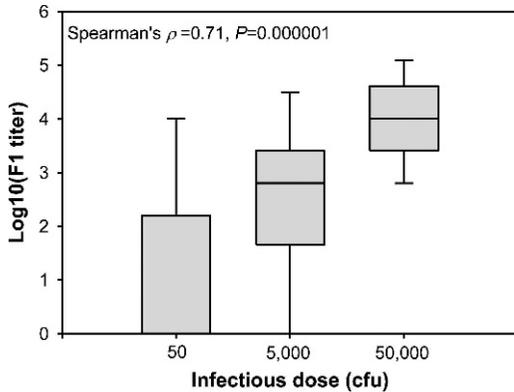


FIGURE 2. F1 antibody response of 36 Gunnison's prairie dogs (*Cynomys gunnisoni*) surviving *Yersinia pestis* challenge. Box and whisker plots show the median, 25–75% quartiles (grey shaded boxes), and 5–95% bounds (error bars). Prairie dogs with no F1 antibody were found in the 50 colony-forming unit (cfu; $n=12$) and 5,000 cfu ($n=4$) groups. Two individuals in the 50,000 cfu group had very low F1 levels (1:640).

IgA, GST- α , MIP-1 α , SGOT), and only Fib was downregulated postchallenge.

Yersinia pestis cultures

The individuals that succumbed to plague exhibited massive necrosis of major organs and intestinal ulceration (data not shown). We successfully cultured *Y. pestis* from multiple organs (spleen, liver, and lung) of all 24 prairie dogs that died but were unable to recover *Y. pestis* from the organs of any of the 36 survivors. Thus, despite variation in time to death (4–17 DPI), *Y. pestis* had successfully spread to the spleen, liver, and lung tissues of all animals that succumbed to challenge. The lack of *Y. pestis* in survivors indicates that *Y. pestis* was likely cleared before the time of euthanasia (33–40 DPI) and provides an upper estimate for the time needed to clear plague infections in *C. gunnisoni*.

DISCUSSION

Past studies of plague events in sylvatic prairie dog colonies consistently reported a death rate of 90–100% (Cully et al., 1997; Cully and Williams, 2001; Pauli et al.,

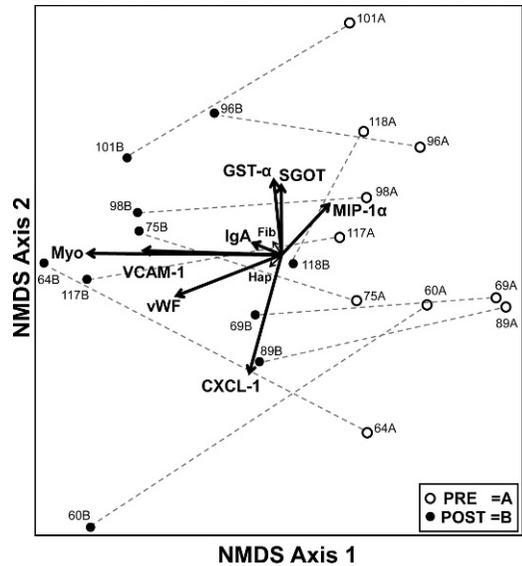


FIGURE 3. Nonmetric multidimensional scaling (NMDS) plot of 10 immune factors sampled prechallenge and postchallenge from 10 Gunnison's prairie dogs (*Cynomys gunnisoni*) that survived challenge with *Yersinia pestis*. Prechallenge samples (group A) are shown as open circles; postchallenge samples (group B) are shown as closed circles. Vector lengths of each biomarker are proportional to their R_{\max} value; biomarkers and R_{\max} values are the same as in Table 2. The dashed lines track each paired sample (prechallenge and postchallenge) for individual prairie dogs.

2006). Since 1938 (Eskey and Haas, 1940), Gunnison's prairie dog colonies throughout northern Arizona have experienced plague epizootics that often result in the extirpation of local colonies (Wagner et al., 2006). In contrast, our laboratory challenge experiment yielded a mortality rate of just 40% in wild-caught Gunnison's prairie dogs. This suggests that a considerable level of plague resistance exists in this species, even within populations other than AV. Mortality in these Gunnison's prairie dogs was similar to the postchallenge mortality of wild black-tailed prairie dogs sampled from Texas and Colorado and was considerably lower than that observed in black-tailed prairie dogs from South Dakota. Those groups experienced mortality levels of 40, 50, and 99%, respectively, when challenged with the same doses and

TABLE 2. Ten immune system biomarkers sampled from 10 survivors before and after challenge with *Yersinia pestis* and measured using the RodentMAP v2.0 enzyme-linked immunosorbent assay panel. Biomarkers are grouped according to function, which is listed in the immune pathways column.^a

Abbreviation	Biomarker	Immune pathway	PRE mean (SE)	POST mean (SE)	R_{\max}	P
Fib	Fibrinogen	Clotting	$4.5 \times 10^8 (\pm 3.9 \times 10^8)$	$2.9 \times 10^8 (\pm 0.9 \times 10^8)$	0.23	0.658
vWF	von Willebrand Factor	Clotting	$1.8 \times 10^4 (\pm 0.7 \times 10^4)$	$1.8 \times 10^5 (\pm 0.6 \times 10^5)$	0.74	0.001*
Hap	Haptoglobin	Acute phase	$6.9 \times 10^7 (\pm 0.6 \times 10^7)$	$6.5 \times 10^7 (\pm 0.3 \times 10^7)$	0.06	0.971
SGOT	Serum glutamic-oxaloacetic transaminase	Acute phase	$2.4 \times 10^7 (\pm 0.9 \times 10^7)$	$3.3 \times 10^7 (\pm 0.6 \times 10^7)$	0.59	0.027
Myo	Myoglobin	Damage	$7.7 \times 10^4 (\pm 2.4 \times 10^4)$	$5.9 \times 10^6 (\pm 0.9 \times 10^6)$	0.90	0.001*
CXCL1	Growth stimulatory activity protein	Innate	$1.5 \times 10^3 (\pm 0.4 \times 10^3)$	$2.3 \times 10^3 (\pm 0.4 \times 10^3)$	0.75	0.001*
GST- α	Glutathione S-Transferase α	Innate	210 (± 134)	168 (± 100)	0.55	0.039
MIP-1 α	Macrophage inflammatory protein-1 α	Innate	$1.1 \times 10^3 (\pm 66)$	$0.9 \times 10^3 (\pm 127)$	0.61	0.024
VCAM-1	Vascular endothelial cell growth factor	Innate	0 ^b (NA)	$4.4 \times 10^3 (\pm 1.5 \times 10^3)$	0.78	0.001*
IgA	Immunoglobulin A	Adaptive	$3.5 \times 10^6 (\pm 0.9 \times 10^6)$	$6.1 \times 10^6 (\pm 1.4 \times 10^6)$	0.38	0.254

^a NA = not applicable; PRE = sampled before challenge with *Y. pestis*; POST = sampled after challenge with *Y. pestis*; R_{\max} = maximum correlation value of a marker to the two-dimensional nonmetric multidimensional scaling (NMDS) analysis (Fig. 3); P = P -value of each R_{\max} using 10,000 randomizations.

^b VCAM-1 was not detected in any pre-challenge samples.

* Statistically significant after Bonferroni correction ($\alpha=0.005$).

the same stock inoculum of *Y. pestis* CO92 (Rocke et al., 2012). In both prairie dog species, survivors were able to clear infectious doses of up to 5×10^4 cfu. Other cases of plague immunity are known from wild rodents, including black rats (*Rattus rattus*), California voles (*Microtus californicus*), gerbils (*Meriones* sp.), northern grasshopper mice (*Onychomys leucogaster*), North American deer mice (*Peromyscus maniculatus*), African multimammate mice (*Mastomys* sp.), and Asian marmots (*Marmota* sp.) (Hubbert and Goldenberg, 1970; Isaacson et al., 1983; Thomas et al., 1988; Gage and Kosoy, 2005; Tollenaere et al., 2008).

Humoral immunity, at least an IgG-mediated response, does not appear to be the most important factor for plague resistance in Gunnison's prairie dog. Although antibody production was corre-

lated with infectious dose, it was not required for survival. Variation in antibody production among individuals was high (Fig. 2), and more than 60% of the survivors cleared the infection without producing detectable antibodies. An even greater proportion of black-tailed prairie dogs (76%) survived identical infection levels without an antibody response (Rocke et al., 2012). This is consistent with early plague challenge studies of other ground squirrels (Williams et al., 1979; Quan et al., 1985), which rarely documented an antibody response after controlled infection. Although the adaptive immune response has long been a focus in models of host immunity to plague (e.g., Chen and Meyer, 1974; Friedlander et al., 1995), most *Y. pestis* virulence mechanisms are targeted at the innate immune system; the race to survive

is often won or lost well before adaptive immunity can begin to protect the host (Haiko et al., 2009; Demeure et al., 2012). Even individuals that have been immunized may have insufficient time to mount an effective anamnestic antibody response against plague's rapid pathogenesis (Rocke et al., 2008). We found four AV animals that carried detectable levels of F1 antibody before challenge. This introduces the possibility that these individuals had been exposed to plague in the wild. If so, their prechallenge antibody levels did not predict their fate: one of the four died, one survived with a decreased level of serum antibodies, and only two produced increased titers during infection. The lack of a strong antibody response in our study may suggest that plague challenge does not necessarily lead to protective immunity in prairie dogs, and survivors could remain at risk following future exposures. It also suggests that other immune pathways are more important for survival in prairie dogs, such as the early innate or T-cell responses, which is consistent with previous work (Lukaszewski et al., 2005; Rocke et al., 2010).

In other studies, neutrophils played a key role in the innate response to plague infection (Bubeck et al., 2007). However, we did not find any difference in the postchallenge ratio of neutrophils in moribund versus surviving prairie dogs. We also did not find any difference in prechallenge cytokine and IgA profiles between individuals that survived versus died. However, a shift in immune expression was observed between the prechallenge and postchallenge samples of survivors (Fig. 3). The strongest upregulation occurred in VEGF-1 (>4,000-fold increase), an important innate cytokine that increases the permeability of vascular cell walls and attracts lymphocytes to sites of infection (Table 2). These innate cytokines and coagulation factors clearly demonstrate a shift in response to *Y. pestis* infection and will be priority targets to assay in future challenge experiments.

Because the level of plague resistance we observed in prairie dogs was unexpected, we validated the virulence of our *Y. pestis* culture by resequencing the CO92 genome. A comparison with the annotated CO92 genome (Parkhill et al., 2001) unambiguously shows that all three virulence plasmids were present. Furthermore, we did not find any SNPs in coding regions or virulence genes. The genome sequence and mouse virulence testing provide key evidence that the CO92 isolate used in this study was fully virulent and that survival in prairie dogs was the result of differences in immune upregulation.

Transmission route is an important consideration during plague epizootics and may lead to the higher mortality rates observed in wild prairie dogs. Laboratory challenge studies (including ours) are typically single-dose experiments that mimic the delivery of *Y. pestis* in a single flea bite, and studies that incorporate multiple infection events over a short time series may provide a more accurate simulation of flea-based transmission. An unexpected transmission route may have occurred in the Espee animals: ingestion of infected organs. Cannibalism and carrion feeding is well described in prairie dogs (Hoogland, 2005) as well as grasshopper mice (*Onychomys* spp.) and may be an important transmission mechanism in wild rodent populations (Thomas et al., 1989). Maintaining plague solely through the ingestion route has been demonstrated in grasshopper mice in a laboratory setting (Rust et al., 1972). Because *Y. pestis* can use these different infection routes, the transmission landscape in nature is complex, and future inquiries should reflect this complexity by simulating transmission from multiple infection times and routes.

It is obvious from our studies and others that mechanisms other than humoral immunity—including a robust innate response or T-cell response—are more important for surviving plague infection in prairie dogs. Our results support the investigation of early immune responses to develop a

more-complete resistance model (Smiley, 2008b). Gunnison's and black-tailed prairie dogs are becoming very useful species in this regard. Whereas ground squirrels in central Asia have long been important plague hosts (Gage and Kosoy, 2005), North American ground squirrels, including prairie dogs, have experienced plague only recently in their evolutionary history. The natural immune system variation found in prairie dogs may provide useful insights for models of plague immunity in wild rodents.

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