Comprehensive characterization of toxins defines progression and stages in a non-human primate model of inhalation anthrax

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Abstract

Inhalation anthrax has three clinical stages: early-prodromal, intermediate-progressive and late-fulminant. The toxins produced during infection exert pathologic effects leading to death, but they have not been comprehensively characterized throughout the course of infection. Mass spectrometry methods for anthrax toxins, total-protective antigen (PA), total-lethal factor (LF), total-edema factor (EF), and toxin complexes, lethal toxin and edema toxin were used to characterize the stages of inhalation anthrax in 23 cynomolgus macaques. The target aerosol dose was 200 LD_{50} *B. anthracis* Ames spores. 22 animals died during the study. Different patterns of toxemia and bacteremia were observed in 11 animals with the shortest survival times (fast progression), the 11 animals with longer survival (slow progression), and the one survivor. Toxemia and bacteremia were predominantly triphasic with an early rise (phase-1), a plateau/decline (phase-2), and a final rapid rise (phase-3). The patterns were consistent for all toxins. The end-of-phase-1 LF was higher in fast progression [median(lower quartile–upper quartile)] of [195(57.4–326)-ng/mL], than in slow progression [23.8(15.6–26.3)-ng/mL] (p=0.0001), or the surviving animal [11.1-ng/mL]. End-of-phase-1 EF was also higher in fast [22.2(2.7–42.8)-ng/mL] than slow progression [0.17(0.064–0.066)-ng/mL] (p=0.0005), or the surviving animal [0.040-ng/mL]. Animals with slow progression and lower end-of-phase-1 toxemia, had an extended plateau/decline (≥24-hours), with low variability of PA, LF, and LTx across all animals. Its characterization revealed an upper threshold; a limit for exiting phase-2 and entering the critical phase-3, 342-ng/mL (PA), 35.8-ng/mL (LF), and 1.10-ng/mL (EF). The thresholds were exceeded early in animals with fast progression (38.3±7.4-hours) and later in slow progression (78.7±14.1-hours). Once the threshold was passed toxin levels rose rapidly, differences in toxemia were reduced, and the duration to terminal was rapid and similar; 21.0±7.3-hours for fast and 20.4±7.3-hours for slow. This first comprehensive evaluation of anthrax toxins defined all stages, providing insights into disease progression.

Abbreviations

Total lethal factor (LF), total edema factor (EF), total protective antigen (PA), full length 83-KDa PA (PA83), active 63-KDa PA (PA63), lethal toxin (LTx), edema toxin (ETx). Non-human primate (NHP). Large unstained cells (LUC), neutrophil lymphocyte ratio (NL Ratio), white blood cells (WBC), red blood cells (RBC), corpuscular hemoglobin concentration mean (CHCM), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV), mean corpuscular volume (MCV), red cell distribution width (RDW).

Author Summary

The comprehensive analysis of all major anthrax toxins and bacteremia in a non-human primate model of inhalation anthrax revealed a triphasic kinetics of toxemia that aligns with the three clinical stages, early-prodromal, intermediate-progressive and late-fulminant. End of phase-1 toxin levels may predict the subsequent speed of progression. Phase-2 toxemia helped define critical thresholds representing the entry to phase-3. Exceeding these thresholds was associated with a short remaining survival time (about 21 hours). This first comprehensive characterization of toxemia provides knowledge and guidance for better management of anthrax.

Introduction

Bacillus anthracis is a gram positive, spore-forming bacterium and the causative agent of anthrax (1). Anthrax can take many forms, cutaneous, gastrointestinal, injection, and the most lethal, inhalation. As such, *B. anthracis* is a U.S. Category A priority pathogen, and Tier 1 select agent (www.selectagents.gov), with the potential for aerosol dispersion and mass casualties (2). Historically, the fatality rate of untreated inhalation anthrax ranged from 89%-100% (3-6). In 2001, anthrax spores were intentionally distributed through the U.S. mail. Even with the use of antimicrobial agents and modern intensive medical support, the fatality rate was 45% (7-9). The high rate of fatality is due to the production of two binary toxins that disable host's immune response, cause endothelial barrier dysfunction, edema, and hemorrhage (10). Even after the clearance of bacilli by antimicrobial treatment, the toxins persists for days, causing further damage and death (11). A better understanding of the levels of anthrax toxins during the stages of infection provides insights into their role in pathogenicity.

The two major *B. anthracis* toxins consist of binary combinations of protective antigen (PA), which binds enzymatic factors, lethal factor (LF) and edema factor (EF), forming active toxin complexes lethal toxin (LTx) and edema toxin (ETx), respectively (12). PA is secreted as an 83-kDa protein (PA83) that binds cell surface receptors TEM8 and CMG2 (13, 14). There it is hydrolyzed by furin-like proteases to 63 kDa (PA63), which causes it to form heptamers/octamers that readily bind LF and EF, forming the LTx and ETx complexes (15-18). PA83 is also hydrolyzed by serum proteases, forming PA63, and ultimately,

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70 LTx and ETx (19-23). LF, a 90-kDa zinc-dependent endoproteinase, hydrolyzes and inactivates several
71 members of the mitogen-activated protein kinase kinase (MAPKK) family of proteins that are central to
72 various cell signaling responses (24). EF, an 89-kDa calcium and calmodulin dependent adenylate
73 cyclase, has a high rate of catalysis of ATP to cyclic AMP (25). The accumulation of cyclic AMP
74 contributes locally to edema, but also activates protein kinase A and exchange protein, both of which
75 control a variety of cellular functions. In early stages of infection, LTx and ETx synergistically suppress
76 innate immune responses, allowing the infection to take hold, bacteria to proliferate, and toxins to
77 accumulate (10, 26-28). In later stages the toxins contribute to disruption of endothelial barrier
78 integrity, causing edema, pleural effusions, hemorrhage, and hemodynamic instability (29-31). After a
79 certain point, the damage becomes insurmountable and largely resistant to standard procedures of
80 cardiovascular support (32). It is not yet known what toxin levels correlate with these stages and their
81 clinical impact.

82 Clinically, inhalation anthrax has been described as having three-stages: 1) the early-prodromal
83 stage, 2) intermediate-progressive stage, and 3) late-fulminant stage (33, 34). The first stage presents
84 with non-specific symptoms, often resembling the flu. The cure rate is highest in this early stage, but
85 since anthrax is rare, a low index of suspicion at clinical presentation can delay appropriate treatment. In
86 the second stage, non-specific symptoms continue but pathology and the clinical signs progress. The
87 potential for survival is still high in this stage but requires aggressive medical intervention. Onset of the
88 third stage is rapid with respiratory failure, shock, and often meningitis. The likelihood of survival is low
89 in the third stage. This was most evident during the anthrax letter attacks of 2001. All 11 cases with
90 inhalation anthrax received aggressive medical intervention. However, it was the stage at presentation
91 that was associated with outcome. The six that presented during stage-2 survived, while the five
92 presenting in stage-3 did not (7, 8, 34). Novel treatments such as antitoxins developed in more recent
93 years may have helped to improve outcome (35). However, more needs to be learned about the
94 progression of anthrax to help discern stages.

95 Of the animal models for inhalation anthrax, non-human primates (NHP), both rhesus macaques and
96 cynomolgus macaques have been extensively studied for anthrax pathology, therapeutics, and vaccines
97 (36-43). However, of the many toxins and biomarkers of anthrax, only PA and bacteremia have been
98 characterized in cynomolgus macaques (42). We have developed and validated sensitive and specific
99 mass spectrometry (MS) methods for all major anthrax toxin proteins and their toxin complexes. They
100 include methods for total-LF (LF+LTx) (44), LTx (PA-LF) (22), total-EF (EF+ETx), ETx (PA-EF) (23), and PA
101 (total PA, full length PA83, and PA63) (45). Application of the methods in five rhesus macaques showed a
102 triphasic kinetics of toxemia (22, 23, 45, 46). These were too few and limited in scope to draw major
103 conclusions about staging. Here, these MS methods were applied to measure and characterize anthrax
104 toxins over the course of infection in the cynomolgus macaque NHP model. This study provides the first

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comprehensive assessment of all the major anthrax toxins during infection which, along with traditionally measured bacteremia, addresses the complexities of anthrax progression, yielding insights that may improve medical management and public health outcomes during an exposure event.

Results

1. Cynomolgus macaque study outcomes

Exposures and clinical outcomes. The study included 23 cynomolgus macaques, 12 males and 11 females, with a mean±standard deviation weight of 4.0±0.61 kg at challenge (Table 1). The weight for females was 3.7±0.48 kg and males was 4.3±0.61 kg. The NHP's were randomized and challenged in two groups. Group-1 included 10 animals with two non-challenged control, for 8 challenged in group-1. The two controls did not develop disease or detectable toxemia/bacteremia. Group-2 included the two control animals from group-1 which were challenged with 13 others, for 15 animals. The collective exposure dose of 221(±29.2) Ames LD_{50} and that of both group-1 (218±32 LD_{50}) and group-2 (223±29 LD_{50}) were similar. There were 22 non-survivors, ordered by time to death or euthanasia which ranged from 48.5-201.9 hours-h (Table 1 and S1 Fig, survival curve). One animal survived to the end-of-study. When adjusted by weight, the Ames LD_{50} exposure dose (LD_{50}/kg) tended to be higher in animals that had shorter survival times with some notable exceptions. The animal with the shortest survival time (48.5 hours) dose (48 LD_{50}/kg) was similar to the animal that survived (49 LD_{50}/kg), and lower than the animal with the latest time to death (60 LD_{50}/kg). The mean±standard deviation spore dose/kg of the 11 animals with shorter survival times was 61.6±11.7 LD_{50}/kg and for the 11 non-survivors with longer survival was 51.9±11.0 LD_{50}/kg. These differences did not meet the alpha level of 0.05 for significance ($p=0.0583$). As can be seen in Table 1, a longer time to death does not correlate with lower doses.

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135 **Table 1. Cynomolgus macaques, challenge, symptoms, and survival time.**

	Animal ID	Gender	Weight (Kg) Day 0	Ames LD_{50}	LD_{50} per kg	Symptom Onset-h	First Symptom	Hours to Death (Days)	Manner of Death	Pathology Cause of Death
1	C58282[1]	M	4.6	219	**48**	29.1	HP	48.5 (2.0)	Euth	Sepsis
2	C59621	F	3.2	230	72	47.4	N/Eu	48.9 (2.0)	FD	Sepsis
3	C60763	M	3.3	200	61	54.7	FD	54.7 (2.3)	FD	Sepsis
4	C58176	M	4.2	280	67	38.2	W	55.2 (2.3)	FD	Sepsis
5	C58003[+]	F	4.2	232	55	40.3	HP	57.3 (2.4)	FD	Sepsis
6	C59656	F	3.7	206	56	40.9	HP	57.9 (2.4)	FD	Sepsis
7	C58214[1]	F	3.4	246	72	28.5	HP	58.4 (2.4)	FD	Sepsis
8	C60732	M	3.1	258	83	35.8	HP	59.0 (2.5)	Euth	Sepsis
9	C59669	F	3.4	223	66	56.3	HP, L	64.8 (2.7)	Euth	Sepsis
10	C57161[1]	F	3.7	203	55	41.5	HP	66.9 (2.8)	FD	Sepsis
11	C58170[+]	M	4.8	208	43	61.4	HP	74.1 (3.1)	Euth	Sepsis
12	C59619	F	3.6	270	75	35.3	W, HP, V	76.4 (3.2)	FD	Sepsis
13	C58135	M	4.0	186	47	34.9	HP	90.4 (3.8)	Euth	Sepsis
14	C57793	M	4.1	187	46	67.0	FA	91.1 (3.8)	FD	Sepsis
15	C58167	M	4.9	215	44	37.3	HP, W	91.7 (3.8)	FD	Sepsis
16	C57998[1]	F	3.5	199	57	77.2	L, W	100.8 (4.2)	FD	Sepsis
17	C59617	F	4.9	203	41	88.1	HP	105.1 (4.4)	FD	Meningitis / Sepsis
18	C58159[1]	M	4.2	188	45	30.2	HP	109.0 (4.5)	Euth	Meningitis
19	C58122[1]	M	4.8	181	38	35.6	DI	115.1 (4.8)	FD	Sepsis
20	C57997[1]	F	3.9	234	60	53.9	W	125.9 (5.2)	FD	Sepsis
21	C58156[1]	M	4.7	274	58	60.7	HP	140.1 (5.8)	FD	Meningitis
22	C59618	F	3.4	202	60	57.0	HP	201.9 (8.4)	Euth	Sepsis
23	C58174	M	4.9	239	**49**	108.4	FA	336.6 (14)	EOS	NA
	Mean±SD		4.0±0.61	221±29.2	56.4±12.0	50.4±20.2		86.1±37.1		

136 23 animals were challenged in two groups. [1]First group challenged included 8 challenged animals and 2 non-
137 challenged controls. The second group challenged included 15 animals, among these were the 2 controls from the
138 first group[+]. SD-standard deviation. LD_{50}/Kg in bold font show similar exposure doses for the animal with shortest
139 survival and the surviving CM. *Survivor sacrificed at the end-of-study (EOS), 14 days. Animals were observed
140 every 6 hours for symptoms and criteria for euthanasia. Symptom onset is the time from challenge to first
141 symptom. Symptoms included hunched posture (HP), wheezing (W), lethargic (L), vomiting (V), facial edema (FA)
142 and diarrhea (DI), or none (N) until euthanized (E), found dead (FD).

143 *Symptom onset*. The onset of symptoms has been represented by an increase in body temperature
144 from normal or baseline (47). Body temperatures were monitored by telemetry before and after
145 challenge. However, the increase in body temperature was not a consistent indicator of symptoms. It
146 was present in only 12 animals for the shoulder transponder and 11 animals for the hip. When present,
147 the elevated temperatures were brief, even transient in most, except in the shoulder of three animals
148 with the longest survival time (S2 Fig).

149 Symptoms were also monitored visually by veterinarians, every 6 hours after challenge. The first
150 clinical sign(s) and the time of occurrence (symptom onset) was recorded (Table 1). The time from
151 challenge to symptom onset ranged from 28.5 hours at the earliest, to 108.4 hours at the latest, in the
152 survivor. Mean±standard deviation time to symptom onset was 50.4±20.2 hours. The most common first
153 clinical sign was hunched posture, present in 15 animals and the least common was diarrhea and

154 vomiting, present in only one each. Four animals had two or more symptoms at onset, while two had no
155 clinical signs recorded before early death/euthanasia at 48.9- and 54.7-hours, suggesting the
156 progression from normal behavior to death or moribund occurred in less than 6 hours.

157 *Cause of death*. Terminal microscopic and gross pathology, to be described later, provided the final
158 determination of the cause of death as being due to either sepsis, for which bacteremia was found in
159 multiple organs, or meningitis, for which whole brain pathology was primary and bacteremia was limited
160 to lymphatic organs (Table 1). Only three animals, all with longer survival times, showed whole brain
161 pathology; two of these were considered meningitis alone (pathology details below).

162 2. Anthrax toxemia and bacteremia

163 2.1 Pre-symptom onset detection of anthrax biomarkers

164 The limits of detection for all the anthrax toxin methods are summarized in Table 2. Total-LF and -EF
165 methods detect and measure the enzymatic activity which gives lower detection limits, whereas the PA
166 method detects and measures the specific trypsin-digest peptide fragments of PA itself which has a
167 higher detection limit. Figure 1 compares earliest detection of total toxins LF, EF, and PA, and
168 bacteremia relative to symptom onset. This showed that LF was the first biomarker detected and it
169 preceded symptom onset in all exposed animals. EF was the next biomarker detected and was first
170 observed at the same time as LF in 9 of the animals, after LF but still before symptoms in 13 animals, and
171 at symptom onset in one animal. PA and bacteremia were detected later with bacteremia detection
172 preceding PA detection in 12 animals and coinciding in 11 animals. Bacteremia and PA detection was
173 before symptom onset in 18 and 16 animals, respectively.

174 **Table 2. Anthrax toxin mass spectrometry (MS) 3-step methods summary.**

Analyte	Pre-MS Sample Prep	LOD (ng/mL)	pmol/mL
LF Total	IP-catalysis	0.0027	NA
LTx	IP-catalysis	0.0075	NA
EF Total	IP-catalysis	0.00002	NA
ETx	IP-catalysis	0.00002	NA
PA Total	IP-tryp digest	1.87	0.0225
PA83	IP-tryp digest	1.22	0.0147

175 Immunoprecipitation (IP), limit of detection (LOD), tryptic (tryp).

176

177 **Figure 1. First detection of anthrax biomarkers.** First detection of biomarkers relative to symptom onset
178 in 23 cynomolgus macaques with inhalation anthrax. Pre-symptom onset period (gray bar) and post-
179 symptom onset period (blue bar) out to 144 hours, for total lethal factor (LF), total edema factor (EF),
180 total protective antigen (PA), and confirmed bacteremia. Detection limits for LF (0.0027 ng/mL), EF
181 (0.00002 ng/mL), PA (1.87 ng/mL) and confirmed bacteremia (reported to 25 cfu/mL). Non-confirmed
182 results of '+' excluded here but included in Table 2.

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Table 3 includes the summary for pre-symptom onset detection for total toxins, toxin complexes, LTx and ETx, and full length PA83 for 1) the mean time to first detection, 2) the time that first detection occurred before symptom onset, and 3) the percent of animals positive for each biomarker at each early time point. It showed that LF was detected earliest, well before symptoms, and most frequently, in 100% of animals by 24 hours. EF was detected second earliest and most frequently, in 65% by 24 hours and 100% by 30 hours. Detection of bacteremia followed. Toxin complexes, LTx and ETx, were detected on average about 9 hours after their total-LF and total-EF counterparts and may reflect the accumulation and activation of PA83 to PA63, binding LF and EF in circulation. Total PA was detected later, even though PA was present earlier in the form of LTx and ETx. Detection limits are lower for the two enzymatic methods, allowing detection of lower levels earlier. Full length PA83 was the last to test positive and never reached 100% positivity at any time point for available samples. Later results show that PA83, when detectable, was only a small fraction of the total PA measured. Therefore, most of the PA was in the PA63 form throughout the entire course of infection.

Table 3. Pre-symptom onset detection.

Biomarker	Time first detected (h)	Time detected before symptoms (h)	Percent positive at post-challenge time				
			18h	24h	30h	36h	48h
Total LF	20.9±3.1	29.6±20.5	52.2	100.0	100	100	100
Total EF	24.5±4.4	25.9±19.8	21.7	65.2	100	100	100
Bact NC (+)	30.0±7.0	20.4±19.1	17.4	26.1	52.2	100	100
LTx	30.5±4.4	19.9±19.5	0	22.7	100	100	100
ETx	33.4±7.6	17.0±22.6	0	21.7	52.2	73.9	100
Bact Confirmed	34.2±6.1	16.2±19.0	0	17.4	30.4	87.0	100
Total PA	38.1±7.4	12.3±19.7	0	8.70	26.1	50.0	100
PA83 (n=21)	59.9±39.1	-11.2±36.2	0	0	4.3	18.2	40.9

First appearance of anthrax toxins and bacteremia. Mean time (hours-h) each biomarker was first detected post-challenge and the mean time (h) the first detected occurred before symptom onset in each animal. The percent positive for each biomarker of 23 animals at all early time points (prior to the mean symptom onset at 50.4 hours). Bacteremia was quantified by serial dilution, plating in triplicate and colony counting which has an LOD of 100 cfu/mL. Only bacteremia reported ≥100 cfu/mL were confirmed. Results below this were reported as + for two of three plates with less than 10 colonies. Inclusion of the non-confirmed (NC) + results were included for Bact NC (+).

2.2 Biomarker levels at symptom onset

Toxin levels measured at the time points closest to symptom onset were determined for each animal (Table 4). LF, LTx and EF were positive at symptom onset in all 23 animals (100%). Although the detection of LTx in all animals at symptom onset indicates that PA is present (as PA-LF), total PA, with a higher detection limit, was not detected in four animals in which the LTx levels were below 0.26 ng/mL. Total PA was detectable in 19 of the 23 animals (83%) at symptom onset, one of which had lower LTx of 0.893 ng/mL. ETx and bacteremia were detectable in 20 and 21 of 23 animals, respectively. PA83 was

214 only positive in 7 animals (30.4%). Median symptom onset levels for all 23 animals were in the mid-low
215 ng/mL for PA, LF, and LTx and sub-ng/mL for EF and ETx. The median for bacteremia was 1.3E+04
216 cfu/mL. Lower and upper quartiles for all 23 animals, demonstrate a broad range which was due to
217 either or both the variable rates of progression and the different stages during which symptoms were
218 first observed. As related to progression, median symptom onset levels were higher in 11 animals that
219 died at earlier time points. The median symptom onset levels of PA, LF, LTx and bacteremia were 2.3-,
220 10-, 14-, and 22-fold higher and EF and ETx were 91- and 136-fold higher, respectively, for the 11
221 animals with the shortest survival times, than the 11 animals with longer survival times. As related to
222 time of symptom onset, the four animals with earliest symptoms (28.5–34.9 h) had the lowest LF, LTx
223 and EF levels at symptom onset. PA was <LOD in all four of these animals, ETx was <LOD in three of
224 these animals, and bacteremia was negative in two and a low positive (+) in one. In these four animals
225 with earliest symptoms, LF ranged from 0.076–1.3 ng/mL. The next sample, 6 hours later, showed that
226 LF increased 5- to 13-fold to 0.538–6.4 ng/mL. However, PA and ETx remained <LOD, 6-hours later, in
227 the two animals with LF levels below 1 ng/mL.

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228 **Table 4. Symptom onset biomarker levels.**

Animal ID	Hours to term	Symptom Onset-h	Sample time-h	Bact (next/term*)	Total-LF (next)	LTx	Total-EF	ETx (next)	Total-PA (next)	PA83
1) C58282	48.5	29.1	30	pos (5.3E+02)	0.235 (2.99)	0.103	0.00068	<LOD (0.0045)	<LOD (9.11)	<LOD
2) C59621	48.9	47.4	42˜	2.9E+06 (1.3E+09*)	57.8 (NA)	23.7	20.7	1.87	333 (NA)	67.4
3) C60763	54.7	54.7	48˜	5.4E+06 (6.2E+08*)	366 (NA)	203	74.7	15.7	2222 (NA)	259.4
4) C58176	55.2	38.2	42	9.1E+04 (3.1E+06)	190 (NA)	63.9	40.6	10.3	199 (NA)	47.3
5) C58003	57.3	40.3	42	5.9E+06 (2.6E+05)	220 (NA)	97.8	6.89	2.0	1143 (NA)	<LOD
6) C59656	57.9	40.9	42	7.0E+03 (3.6E+06)	8.5 (75.2)	0.893	0.059	0.00082	13.6 (731)	<LOD
7) C58214	58.4	28.5	30	neg (3.0E+02)	0.136 (0.839)	0.058	0.0005	<LOD (<LOD)	<LOD (<LOD)	<LOD
8) C60732	59.0	35.8	36	7.4E+06 (2.9E+03)	313 (557)	165	39.2	7.1	1189 (693)	<LOD
9) C59669	64.8	56.3	60	1.9E+05 (4.0E+08*)	200 (NA)	149	5.62	0.872	282 (NA)	<LOD
10) C57161	66.9	41.5	48	7.2E+06 (1.1E+06)	366 (741)	245	53.4	15	2221 (2303)	516
11) C58170	74.1	61.4	60˜	6.3E+04 (8.3E+08*)	189 (NA)	139	0.194	0.034	428 (NA)	<LOD
Mean-h Median Fast	58.7±7.6	43.1±10.8	41.3±9.9	1.9E+05 (3.5E+04–5.7E+06)	190 (33.2–267)	97.8 (12.3–157)	6.9 (0.13–40)	1.9 (0.017–8.7)	333 (106–1166)	ND
12) C59619	76.4	35.3	36	1.8E+05 (1.5E+04)	32.5 (37.4)	12.5	0.661	0.278	368 (367)	36.2
13) C58135	90.4	34.9	36	2.5E+02 (5.3E+03)	1.30 (6.4)	0.256	0.0053	0.00035	<LOD (7.21)	<LOD
14) C57793	91.1	67.0	72	9.2E+04 (1.2E+08)	33.3 (7996)	15.8	0.933	0.272	275 (34638)	<LOD
15) C58167	91.7	37.3	42	8.5E+03 (7.1E+03)	18.9 (19.4)	6.49	0.38	0.082	129 (65.0)	<LOD
16) C57998	100.8	77.2	84	2.9E+04 (3.1E+07)	22.6 (1754)	9.59	0.459	0.035	144 (15503)	5.565
17) C59617	105.1	88.1	84	9.6E+03 (5.9E+04)	93.8 (178)	64.1	1.95	0.511	599 (813)	25.4
18) C58159	109.0	30.2	30	neg (pos)	0.076 (0.538)	0.029	0.00056	<LOD (<LOD)	<LOD (<LOD)	<LOD
19) C58122	115.1	35.6	36	8.4E+03 (3.6E+03)	5.33 (22.0)	1.29	0.044	0.00042	18.7 (152)	<LOD
20) C57997	125.9	53.9	60	3.7E+02 (5.3E+02)	15.5 (10.4)	6.97	0.048	0.014	200 (96.7)	<LOD
21) C58156	140.1	60.7	60	1.3E+04 (2.6E+02)	19.8 (11.7)	8.68	0.076	0.014	172 (137)	<LOD
22) C59618	201.9	57.0	60	9.2E+02 (pos)	8.51 (6.3)	1.05	0.021	0.00065	25.7 (28.3)	<LOD
Mean-h Median Slow	113±34.4	52.5±19.5	54.5±19.8	8.5E+03 (6.5E+02–2.1E+04)	18.9 (6.9–27.6)	7.0 (1.2–11.0)	0.076 (0.033–0.56)	0.014 (0.00054–0.18)	144 (22.2–238)	ND
23) C58174	EOS	108.4	108	3.4E+02 (neg)	7.3 (4.2)	1.6	0.041	0.0058	43.8	<LOD
Mean-h 22-NS Median All 23	86.1±37.1	47.8±16.1	48.9±17.4	1.3E+04 (6.5E+02–1.9E+05)	22.6 (7.9–190)	9.6 (1.2–81.0)	0.38 (0.043–6.3)	0.035 (0.00074–1.4)	199 (22.2–398)	

229 Biomarker levels at the sample time (hours-h) closest to symptom onset for toxins (ng/mL) and bacteremia (cfu/mL), in cynomolgus macaques (CM) with
230 inhalation anthrax. ˜ Last available sample was used when symptom onset sample was not available. CM listed by survival time (h) from challenge to terminal
231 (term). Symptom onset is the time from challenge to first observed symptom. Mean±standard deviation-SD for hours (survivor excluded) and median and
232 interquartile range (IQR) presented as median (lower–upper IQR) for all biomarker levels, except PA83. For calculations, values <LOD were included at ½ the
233 LOD per method (Table 2) and for bacteremia (Bact) a '+' for low positive (pos) was 6 cfu/mL and '0' negative (neg) was 3 cfu/mL [35]. CM with earliest
234 symptom onset (underlined) had low/negative toxemia. Measurements for the next time point were included for <LOD ETx, bacteremia, LF and PA levels 6-12
235 hours later; *bacteremia when the next measurement was at terminal time. CM were analyzed collectively (all, n=23) and separately for fast (n=12) and slow
236 progression (n=11). End of study (EOS). Not determined (ND).

2.3 Kinetics of toxemia and bacteremia

Variable Kinetics of LF and bacteremia. During anthrax, vegetative *B. anthracis* and the toxins are found in circulation at varying levels at varying times. Bacteremia and LF represent the two sides of anthrax, infection, and intoxication. Bacteremia and LF graphed over time in all 23 animals demonstrate the variability of progression and the differences in these two biomarkers (Fig 2A,B). Most, but not all, of the animals appeared to have a triphasic kinetics of bacteremia and LF, with an initial rapid increase (phase-1), a plateau or decrease (phase-2), and a rapid increase leading to terminal stage (phase-3). The kinetics of bacteremia and LF were dramatically different in the 11 animals that progressed faster and died earlier compared to the 11 with longer survival times and slower progression. Specifically, early levels of bacteremia and LF appeared to be much higher in animals with faster progression and shorter survival than in the 11 animals with longer survival, and the one animal that survived. Overall, the kinetic trends, rises and declines, were similar between bacteremia and LF, but the phase-2 declines were more dramatic, declining for bacteremia compared to a plateau for LF.

Figure 2. Kinetics of bacteremia and lethal factor in 23 cynomolgus macaques with variable progression. Bacteremia (Bact) in (cfu/mL) (A) and total lethal factor (LF) in (ng/mL) (B) over the course of infection in 23 cynomolgus macaques with inhalation anthrax. 11 animals had higher early bacteremia/toxemia and shorter survival (fast progression) (red/orange/purple lines), and 11 animals had lower early toxemia and longer survival (slow progression) (aqua/green lines), and one survivor with low early toxemia (blue line, square symbols). Most animals had triphasic kinetics consisting of an initial rise (phase-1), plateau or decline (phase-2), and final rise to terminal (phase-3). Bacteremia and LF in 7 animals with fast triphasic kinetics (C) and (E) and 4 animals with monophasic kinetics, a single rapid rise, (D) and (F), respectively. Bacteremia and LF in 11 animals with slow progression and survivor, (G) and (H), respectively. The last time point for 22) C59618 that died at 8.4 days (8.4D) and the survivor, 23) C58174, which declined to less than the limit of detection (LOD) at the end-of-study (EOS, 14-days/336 hours) are not to scale. For bacteremia (bact), non-quantifiable results of low positive were assigned a value of 6 and negative at 3 cfu/mL. Bacteremia and LF below LOD (x-symbol). Boxes represent the broad range of bacteremia in phase-2 (G) and narrow range of LF levels during phase-2 (H).

Disease progression. So, with higher early bacteremia and LF for the 11 animals with shorter survival time (faster progressing) and lower levels for the 11 animals with longer survival times (slower progressing), these early biomarker levels may distinguish subsequent progression. Separating these two groups, we show that of the 11 faster progressing animals, 7 had a triphasic kinetics of bacteremia and LF (Fig 2C, E), and 4 had monophasic progression, where bacteremia and LF appeared to have a single stage that increased directly from detection to terminal time (stage-3), unbroken by a plateau/decline (Fig 2D, F). The speed of progression meant that terminal plasma samples were not obtained for LF (or any toxin measurement) (Fig 2E, F). However, terminal measurements for bacteremia were available and provided evidence of the final rise to terminal (Fig 2 C, D). In animals with fast progression, end-of-phase-1 bacteremia and LF levels were significantly higher than in slow progression and the phase 2 plateaus were short in length, only 6 to 12 hours, compared to 24 hours and longer for animals with slow progression.

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275 In the 11 non-survivors with slower progression, almost all had a triphasic kinetics of bacteremia and
276 LF (Fig 2 G,H). In phase-1, LF increased to lower levels at the end-of-phase-1 (Fig 2H). In these animals,
277 phase-2 provided a stable feature, with LF levels that varied by less than one order of magnitude from
278 lowest at 4.8 ng/mL to highest at 41.5 ng/mL across all animals and time points (boxed area) (Fig 2H).
279 Conversely, phase-2 bacteremia varied over 4-orders of magnitude, from 1.76E+05 cfu/mL to low '+' and
280 was even non-detectable at several time points in the animal that survived to the end-of-study (Fig 2G).
281 For other toxins, phase-2 for PA and LTx had less variability (similar to LF) and EF and ETx had more
282 variability (similar to bacteremia) (S3 Fig). The final phase-3 rise in LF and bacteremia was rapid and to
283 high levels for most animals. Those that did not reach high terminal bacteremia of 10^7-10^9 cfu/mL, or
284 high LF, were either euthanized, for which timing may vary, or were determined to have died from
285 meningitis rather than sepsis.
286 The progression of toxemia and bacteremia for the one animal that survived to the end of the study
287 and one animal that survived to 8.4 days were similar for bacteremia out to 144 hours and LF out to 108
288 hours. Both animals had the lowest but positive bacteremia in phase-2, but the survivor had some
289 negative points later (Fig 2G). The survivor resisted the pressures of toxemia and infection, whereas in
290 the non-survivor, resistance was overcome.
291 *All toxins and bacteremia: kinetics in individual animals*. A complete list of all toxin levels for each
292 animal at each time point is included in a separate file (S1 File) and bacteremia is included in S2 File. All
293 toxins and bacteremia were graphed together for all 23 individual animals, numbered by survival time
294 (Fig 3). Due to the speed of progression and hematological changes in the latest hours of anthrax,
295 terminal plasma samples for toxin measurements were not available in 17 animals, all 11 with short
296 survival and 6 with longer survival (Fig 3, animals 1-16, 19). However, whole blood for bacteremia was
297 collected at terminal stage, allowing visualization of kinetic trends in the final hours of progression.

298 **Figure 3. Anthrax toxins and bacteremia during inhalation anthrax in individual cynomolgus macaques.**
299 Total PA, PA83, LF, EF, LTx and ETx (ng/mL) and bacteremia (cfu/mL) in 11 animals with fast progression (numbered
300 1-11), 11 with slow progression (12-22), and the survivor (23). Phase-1 (P1), phase-2 (P2) and phase-3 (P3) are
301 indicated when present. Blue vertical dashed lines indicate the point separating the phases; the end-of-P1 (EOP1)
302 that ends the P1 rise and begins the P2 plateau and the end-of-P2 (EOP2) that ends P2 and begins P3. Black arrows
303 indicate the time of symptom onset. Survival time-days (D), hours (h). Black rectangles at the end-of-phase-1 show
304 how toxin levels are convergent (lower differences between highest toxin PA and lowest toxin ETx) in animals with
305 shorter survival or divergent (higher differences between PA and ETx) in animals with longer survival. Red
306 rectangles in phase-3 in animals with longer survival show convergent levels at terminal infection. Though units for
307 toxins (ng/mL) and bacteremia (cfu/mL) are not directly comparable, both were graphed on a similar scale (10-
308 orders of magnitude) for comparison of dynamic changes, orders of magnitude. Aqua horizontal dashed line at 1
309 ng/mL toxin concentration and 1.0E+05 cfu/mL bacteremia. Dynamic changes of bacteremia trended most closely
310 with EF and ETx. Low positive culture (+) graphed at 6 cfu/mL and negative culture (-) at 3 cfu/mL. Less than the
311 limit of detection (<LOD).

312 The toxin/bacteremia profiles of the 11 animals with shortest survival (fast progression) included
313 four with monophasic progression with an initial rise in phase-1 and a continued rise in bacteremia at

314 terminal stage (Fig 3, animals 1, 2, 6, 7). Animal 1 only had toxins measured out to 36 hours post-
315 challenge and therefore, didn't reach levels as high as the other three monophasic animals with toxins
316 measured at 42 hours (animal 2) and 48 hours (animals 6, 7). Terminal stage bacteremia measured after
317 the last toxin samples, reached high levels of 10^8 to more than 10^9 cfu/mL. In the other seven animals
318 with fast progression, toxin levels only showed the phase-1 increase and a short phase-2 plateau/decline
319 (6 to 12 hours). However, a third stage was evident with the phase-3 increase in bacteremia, also to high
320 levels (animals 3-5, 8-11).

321 Kinetic profiles for the 11 non-survivors with longer survival (slow progression) were triphasic with
322 one exception discussed below. Three phases were observed for all toxins, even in the six animals
323 missing terminal toxemia (Fig 3, animals 12-16, 19). These 6 showed the phase-1 increase, the phase-2
324 plateau, and the final rise in phase-3 approaching terminal. The changes in bacteremia in the final few
325 hours showed a continued rise in four (animals 12, 13, 15, 19), and a decline in two (animals 14, 16). For
326 these two, changes in the internal environment, hypoxia, or other conditions near the terminal stage
327 may have inhibited further replication of the bacilli. Animal 20 had a terminal low positive (+)
328 bacteremia in the presence of high toxemia and was excluded. It was presumed to be a failed test since
329 all other animals with high toxemia had high bacteremia.

330 Overall, trends were consistent in all animals. The detection of PA83 was sporadic indicating that
331 PA63 was the predominant form of PA circulating in cynomolgus macaques. The kinetic trends were
332 similar for bacteremia and all toxins, LF, LTx, EF, ETx, and PA. However, phase-2 declines in EF and ETx
333 were more dramatic, similar to declines in bacteremia, whereas PA, LF, and LTx tended to plateau rather
334 than decline in phase-2. The similar trends occurred whether the kinetics were monophasic with one
335 continuous rise, or triphasic, with the rise-plateau/decline-rise. All toxins followed the same general
336 pattern, with two exceptions. Animal 11 had a mixed triphasic kinetics with phase-2 declines for EF, ETx
337 and bacteremia, while PA, LF, and LTx levels did not appear to plateau but continued to rise, albeit more
338 slowly than in phase-1 (Fig 3, 11). Animal 21 had a long phase-2 plateau to terminal for all toxins, while
339 only bacteremia started to rise in phase-3. The pathological cause of death in animal 21 was meningitis
340 rather than sepsis.

341 *Potential relationship with survival time.* As seen above, LF and bacteremia rose to higher levels
342 early in the 11 animals with fast progression. As ordered by survival time, there appeared to be an
343 association between early toxin levels and subsequent survival time. Early toxin levels and bacteremia,
344 for example, at the end-of-phase-1 (black box), were higher in animals with shorter survival and lower in
345 those that survived longer. Using the horizontal line at 1 ng/mL toxin concentration and 1.0E+05 cfu/mL
346 bacteremia as a gauge, you can see that at the end-of-phase-1 (first rise), toxins and bacteremia trended
347 lower with longer survival. Another trend was the magnitude and length of the phase-2 plateau, with

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348 higher concentrations and shorter duration in animals with short survival and lower concentrations and
349 longer duration with longer survival. This indicated that early toxin levels appeared to be associated with
350 subsequent survival time. This association was explored in detail below.

351 *Relationship of toxins to each other*. In every animal, total PA, once positive, was the most abundant
352 toxin, with PA>LF>LTx>EF>ETx (Fig 3). In animals with longer survival, the ranges of these toxins during
353 phase-2 were more divergent, with 4- to 5-orders of magnitude between the concentrations of PA
354 (highest) and ETx (lowest) and most divergent in animals 16-23 (Fig 3, black boxes). In phase-3, all toxins
355 increased rapidly and converged (Fig 3, red boxes). In contrast, in animals with shorter survival, toxins
356 reached higher levels and converged much earlier, approaching or at the end-of-phase-1 (Fig 3, animals
357 2-11). Lower toxin levels were associated with overall divergent toxin levels and high toxin ratios and
358 higher toxemia with convergent toxin levels and lower toxin ratios.

359 2.4 Characterization of toxins, bacteremia, and toxin ratios stratified by phase 360 as defined by toxemic profiles.

361 2.4.1 Phase-1.

362 *Toxemia in the early Incubation period*. The earliest time points at 18- and 24-hours post-challenge
363 were collected before earliest symptoms at 29 hours post-challenge. LF was positive in 12 animals by 18
364 hours post-challenge and in all 23 by 24 hours. Combined 18-to-24-hour median (interquartile range-
365 IQR) LF was 0.025 (0.013–0.115) ng/mL (n=35). EF was positive in 5 animals at 18 hours and 16 at 24
366 hours, at 0.00021 (0.0014-0.00007) ng/mL (n=21). Other toxins and bacteremia were not detected at
367 these early time points in sufficient numbers for assessment. These early phase-1 levels were similar for
368 animals with fast and slow progression (Fig 4C, Phase-1).

369 **Figure 4. Stage-dependent biomarker levels.** Lethal factor levels in animals with fast and slow progression, and
370 survivor, at the end-of-phase-1 (A) and 48 hours (B). Box plot is for median and quartiles, error bars for minimum
371 and maximums, excluding outliers, median levels and p-values included for Wilcoxon rank sum test. (C) LF, EF
372 levels (upper panel), and LF/EF ratio (lower panel), during earliest detection in phase-1 (P1), at the end-of-phase-1
373 (EOP1), subsequent phase-2 (P2), and phase-3 (P3) for fast progression (left panel) and slow progression (right
374 panel). Lines connect the medians and error bars give quartiles for each stage. Red dashed line represents the LF
375 threshold and blue line the EF threshold for phase-2. P-values included for testing differences in toxin levels and
376 toxin ratios between phases. Black font includes all parameters unless another color font is shown for a specific
377 parameter. Black rectangle shows similarity of low LF/EF ratios at the EOP1 for fast progression and P3 for slow
378 progression.

379 *End-of-phase-1*.The end-of-phase-1, also the beginning of the phase-2 plateau, was readily apparent
380 in all but the four animals with monophasic progression (Fig 3). In these four, the phase-1 rise continues
381 to terminal bacteremia, corresponding to the late-fulminant stage-3 (phase-3 without an observable
382 phase-2). By the end-of-phase-1, all toxins and bacteremia, except for the unprocessed PA83, were
383 positive in all animals. The timing to reach the end-of- phase-1 ranged from 30 to 60 hours post-
384 challenge. Levels at the end-of-phase-1 were higher in the fast progression group, than in the slow

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385 progression group, and the survivor (Table 5 and Fig 4A). The differences were also observed at a
386 specific time point near the end-of-phase-1, at 48h (Fig 4B). For either feature, the kinetically defined
387 end-of-phase-1, or a timed collection at 48 h, higher toxemia at this early stage was associated with a
388 faster course of infection and a shorter survival time. In addition to LF, end-of-phase-1 levels of all toxins
389 and bacteremia were significantly higher in fast progression compared to slow (Table 5). Higher toxemia
390 at the end-of-phase-1 in fast progression had lower toxin ratios and lower toxemia had higher ratios.
391 Differences between end-of-phase-1 ratios were significant for LF/EF and PA/ETx (Table 5). LF/LTx and
392 EF/ETx trended lower for fast progression but the differences were not significant. PA/LF did not fit the
393 trend and was similar for fast and slow progression at the end-of-phase-1. Individual animal
394 measurements at the end-of phase-1 (or within 12 hours when later toxin samples were not available)
395 for all toxins (including PA83), toxin ratios, and bacteremia, are included in S1 Table.

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Table 5. Stage-dependent biomarker levels.

	End-of-Phase-1 (Beginning-of-Phase-2)			All of Phase-2				Phase-3 (≤12 hours from terminal)	
Parameter	Fast (n=10)[10]	Slow (n=11)[11]	Survived (n=1)[1]	Fast (n=7)[15]	Slow (n=11)[52]	Survived (n=1)[10]	Slow Phase-2 Threshold# (n=11)[52]	Fast (n=9)[9] 7.2(6.8–10.2)-hours	Slow (n=10)[11] 4.4(0–7.1)-hours
Total-PA (ng/mL)	620 (328–1447)	133 (54.2–200)[c]	33.9	326 (199–1189)	137 (67.4–205)[a]	34.6 (26.2–41.3)	342	509 (308–1477)	8547 (813–34638)[d]
Total LF (ng/mL)	138 (54.9–326)	23.8 (15.6–26.3)[b]	11.1	200 (98.1–366)	20.2 (13.9–26.1)[a]	6.7 (5.6–9.8)	35.8	200 (66.5–337)	1754 (171–7996)[f (ns)]
Lethal Toxin (ng/mL)	54.7 (19.5–188)	8.6 (4.6–10.3)[b]	3.25	139 (50.9–245)	7.38 4.51–10.3)[a]	1.3 (0.89–2.0)	18.9	97.8 (25.7–192)	1799 (92.9–7991)[e (ns)]
Total EF (ng/mL)	22.2 (2.7–42.8)	0.17 (0.064–0.66)[a]	0.040	2.68 (1.8–34.9)	0.172 (0.042–0.47)[a]	0.025 (0.0074–0.040)	1.10	20.7 (5.6–37.75)	201 (6.5–797)[e (ns)]
Edema Toxin (ng/mL)	3.4 (0.51–9.1)	0.0057 (0.0020–0.15)[b]	0.0023	0.663 (0.294–7.1)	0.035 (0.0048–0.095)[a]	0.0028 (0.0013–0.0046)	0.312	2 (0.62–11.9)	54.8 (2.9–272)[d]
Bact (cfu/mL)	4.8E+06 (1.2E+06–7.6E+06)	1.1E+04 (5.6E+03–6.2E+04[a]	6.4E+03	2.6E+05 (6.3E+04–7.2E+06)	4.9E+03 (7.1E+02–1.4E+04)[a]	2.4E+02 (5.3E+00–1.2E+03)	1.6E+05	1.3E+06 (2.2E+05–3.4E+06) 0h-Term (n=11) 6.3E+08 (4.0E+08–8.8E+08)	6.7E+06 (3.9E+05–9.7E+07)[f (ns)] 0h-Term (n=10) 4.4E+07 (1.2E+06–2.1E+08)[c]
PA/LF Ratio	4.8 (2.9–7.1)	5.6 (3.4–7.9)[g (ns)]	3.1	2.9 (1.4–3.8)	7.0 (4.4–8.8)[a]	5.3 (2.9–6.9)	2.5	3.1 (1.4–7.6)	4.3 (3.4–6.3)[f (ns)]
LF/LTx Ratio	2.4 (1.8–2.9)	2.7 (2.5–5.2)[f (ns)]	3.4	1.8 (2.4–1.4)	2.6 (2.3–3.8)[b]	5.0 (4.4–6.6)	1.7	2.2 (1.5–2.7)	1.20 (1.0–1.8)[d]
LF/EF Ratio	9.0 (4.4–18.2)	103 (49.2–270)[b]	278	31.9 (12.7–74.6)	127 (50.8–400)[b]	350 (234–761)	21.4	10.1 (3.7–33.8)	11.0 (8.6–22.8)[g (ns)]
EF/ETx Ratio	5.4 (4.2–12.3)	16.0 (6.0–30.7)[e (ns)]	17.4	4.5 (3.6–5.7)	5.5 (3.7–11.7)[f (ns)]	9.9 (6.8–16.7)	2.7	4.8 (3.5–8.8)	3.7 (2.6–4.3)[e (ns)]
PA/ETx Ratio	190 (148–615)	19,733 (2144–31,667)[c]	14739	323 (167–986)	4565 (1383–23,958)[a]	13,467 (8967–35804)	506	171 (118–733)	198 (86.6–338)[g (ns)]

Triphasic kinetics of toxemia is defined by LF levels that increase to the end-of-phase-1, a plateau/decline in phase-2, and a final rapid increase in phase-3. Median (lower quartile–upper quartile) are given for fast (n=11) and slow (n=11) progression groups, and the animal that survived (animal 23, C58174) at the end-of-phase-1, all points making up phase-2, and phase-3 (available measurements within 12 hours of terminal). The survivor did not have phase-3. Toxin ratios were included for PA/LF, LF/EF, LF/LTx, EF/ETx and PA/ETx (showing the span from highest- to lowest-level toxin). PA83, not consistently positive, was excluded. Of 23 animals, all but one animal (fast) had an end-of-phase-1 sample. Phase-2 was evaluated as an entire period. Numbers of animals included in each measurement (n) with the superscripts for the number of measurements eg (n=10)[10] included in the statistics for each parameter and phase of infection. With several points per animal for phase-2 with slow progression (n=11)[52], except for ETx (n=11)[51]. The limits of phase-2 for slow progression, the upper 95th percentile (%) for toxins and bacteremia and lower 95th percentile for toxin ratios, was included for evaluation as a threshold for exiting phase-2 (critical threshold for entry to phase-3). P-values are from Wilcoxon Rank Sum Test in JMP, for differences between fast vs slow progression. Letter superscripts indicate p-values where, a (p≤0.0001), b (p≤0.001), c (p≤0.01), d (p≤0.05), e (p≤0.1), f (p≤0.5), g (p≤1.0). Alpha level set at 0.05 indicating significant differences were observed for superscripts a-d, but e-g were not significant (ns).

407 **2.4.2 Phase-2.**

408 The phase-2 plateau/decline varies in levels and length, ranging from 6 to 12 hours in faster
409 progressing anthrax to more than four days in animals with slower progression (Fig 2 and 3). The end-of-
410 phase-1 is also the beginning of phase-2. Therefore, the differences at the end-of-phase-1 continued
411 during phase-2, though only briefly in fast-progression, with significantly higher levels of all toxins and
412 bacteremia for the 7 animals with phase-2 in fast progression, compared to phase-2 in the 11 animals
413 with slow progression (Table 5). Phase-2 toxin ratios were lower in fast progression compared to slow
414 progression. The differences in all ratios were significant except for EF/ETx. In the one survivor (animal
415 23), phase-2 toxin levels and bacteremia were even lower and toxin ratios higher (except for PA/LF),
416 than in animals with slow progression.

417 *Phase-2 critical thresholds for entry to phase-3*. In slow progression, phase-2 LF levels varied less
418 than one order of magnitude for the entire phase-2 period in all 11 animals (Fig 2H). This represents a
419 period of relative stability that seems to be sustained only over a narrow range of LF. As such, it also
420 represents a period where relatively small increases in LF may move infection to the final stage 3.
421 Median, interquartile ranges and upper (95%) and lower (5%) confidence intervals characterize this
422 stable period in slow progression. The upper 95% interval of phase-2 toxemia is like an upper confidence
423 limit. Numbers above this limit fail the criteria for being classified or included in the phase-2 plateau (for
424 these animals and this study). Therefore, the upper limit represents a potential threshold for phase-2,
425 above which an animal has exited phase-2 and entered phase-3. For toxin ratios, the lower 95%
426 confidence limit applies. The thresholds for all toxins and ratios are included (Table 5) and were used to
427 determine the timing of entry to phase-3 below.

428 **2.4.3 Phase-3.**

429 Stage-3, late-fulminant anthrax, is the stage where cure is the most unlikely. The remaining survival
430 time after reaching this stage can be as short as a few hours and usually no longer than one day (48).
431 Phase-3, as defined by toxin kinetics, is the final rise during the final hours of terminal infection as seen
432 for each animal (Fig 3). Both toxemic phase-3 and clinical stage-3 represent the final hours of survival.
433 Characterization of phase-3 requires consideration of two main points. One point is the speed of this
434 stage which resulted in relatively few samples collected for toxin measurements and few at terminal
435 times. Therefore, to characterize phase-3 for toxins which lack terminal samples, we chose to include
436 any measurements for time points collected within 12 hours of terminal time. The second point is that
437 for animals with fast progression, the kinetic features are compressed within the last 12-to-24 hours (Fig
438 3, animals 1-11). As such, for animals with fast progression, points within 12 hours of terminal may
439 include those at the end-of-phase-1 or phase-2. Bacteremia, which did have terminal measurements,
440 was characterized two ways; 1) with the same time points as available for toxins and 2) with only the
441 final terminal time points (0 hours before terminal).

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With the limitations described above, phase-3 toxemia was compared between animals with fast and slow progression (Table 5 and Figure 4C). Available phase-3 samples for fast progression were collected at 7.2 (6.8–10.2) hours before terminal and earlier than those for slow progression at 4.4 (0–7.1) hours before terminal. Phase-3 toxin levels and bacteremia were higher for animals with slow progression than fast progression for all points within 12 hours of terminal, but the differences were not significant except for PA and ETx (Table 5). Of the toxin ratios only LF/LTx was significantly lower in slow progression than fast progression. With rapid changes during this stage, these differences may simply be an artifact of the differences in collection times. To support this, we compared bacteremia at final terminal collection times, which was significantly higher in animals with fast progression than in those with slow progression (Table 5). Of the significant changes in ratios, LF/LTx had the lowest ratio in phase-3, approaching 1.0, indicating that LTx nearly equals total LF and 100% existing in complex with PA63.

Overall, for stage progression, all toxins and bacteremia increase from first detection to the terminal stage, whereas all toxin ratios (except PA/LF) declined from earlier stages to terminal phase-3. PA/LF was the only toxin ratio to deviate from this pattern. PA/LF did not change consistently through all the stages. These trends, increasing toxemia and declining ratios through the phases may be used to define early and intermediate stages, with the phase-2 thresholds indicating the transition to late-stage anthrax.

Comprehensive staging with anthrax progression. Figure 4C summarizes four aspects of these three phases, early phase-1, the end-of-phase-1, subsequent phase-2, and phase-3. LF, EF, and LF/EF ratios are representative of differences between phases and between fast and slow progression. In early phase-1, LF and EF levels, and LF/EF ratios, were similar between fast and slow progression with low levels and high ratios. From early phase-1 to the end-of-phase-1, there were significant increases in LF and EF levels and decreases in LF/EF ratios in both groups, but to higher levels and lower ratios in animals with fast progression. At the end-of-phase-1, most points in animals with fast progression were above the phase-2 thresholds indicated for LF and EF, whereas all points in animals with slow progression were at or below the thresholds. LF/EF ratios at the end-of-phase-1 in fast progression were low and similar to those at phase-3 in slow progression. For fast progression, LF levels did not change from the end-of-phase-1, through phase-2, and phase-3. Thresholds were already exceeded and only a short time followed. EF levels declined significantly from the end-of-phase-1 to phase-2, then increased from phase-2 to phase-3. The changes in EF between these phases also resulted in inverse changes in the LF/EF ratio. For slow progression LF and EF levels increased from early phase-1 to the end-of-phase-1, and LF/EF ratios declined significantly. There were no significant changes between the end-of-phase-1 and phase-2, whereas between phase-2 and phase-3 LF and EF levels increased, and LF/EF ratios declined significantly.

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2.5 Relationship of toxemia and time.

2.5.1 Time to reach phase-2 threshold and time from threshold to terminal.

The relationship between phase-2 toxin levels and the time to reach the toxin thresholds was calculated for each animal for all toxins and bacteremia. The survivor never even reached the phase-2 mean so was excluded. Animals with fast progression reached the thresholds at 38.3±7.4 hours and those with slow progression at 78.7±14.1 hours (Fig 5A). Thus, on average, the threshold was reached in half the time in the 11 animals that had shortest survival times compared to the 11 animals with longer survival times. Most of the animals in the fast progression group exceeded the phase-2 threshold by the end-of-phase-1 and levels of most toxins remained above it throughout their short phase 2 plateaus (when present). After reaching the threshold, the time to terminal was remarkably similar for all animals, with 21.0±7.3 hours for fast and 20.4±7.3 hours for slow progression. These calculations were consistent for all biomarkers. This suggests that the time to reach critical toxin thresholds is variable but once it is exceeded, the course is rapid and similar.

Figure 5. Relationship of toxemia and time. (A) Time in hours (h) from exposure to reach the phase-2 (P2) threshold and time from threshold to terminal. The 95th percentile of phase-2 for all toxins and bacteremia in animals with slow progression represent an upper threshold for exiting phase-2 and entry to phase-3 (Table 5). The time to reach this threshold was calculated for individual animals and means for the number (n) animals for fast and slow progression. Error bars represent the standard deviation (SD). The overall mean±SD for all toxins and bacteremia combined for fast and slow progression is included above the bars. (B) Relationship between LF levels and survival time (days). Mean $\log_{10}$ concentrations and standard deviations as error bars for total LF over the time post-challenge (hours-h) for animals that died/euthanized at 1.9-3.0 days (n=10), 3.1 to 4.0 days (n=5), 4.1 to 5 days (n=4) and 5.1 to 6 days (n=2), and for one animal at 8.4 days and survivor to end-of-study (End). Final time points for the C59618 at 8.4 days and for C58174 the survivor at end-of-study (14 days) were included together (End). Horizontal lines shown for median phase-2 LF (dashed grey) and LF phase-2 threshold (red dotted line). The black rectangle for LF at 60 h showed mean $\log_{10}$ LF graphed for mean subsequent survival time (60-h to terminal) (inset), fitted with a power regression, indicating the inverse relationship of early LF levels with subsequent survival time.

2.5.2 Relationship between LF levels and survival time.

Our results for the fast and slow progression groups showed that animals with faster progression reached critical thresholds of toxin levels earlier and died sooner than those with slow progression. However, the time-to-death for animals with slow progression varied from 3.8- to 14-days. The relationship between LF levels and the time-dependent interval to death was demonstrated by grouping animals by survival time (Fig 5B). LF levels in animals surviving 1.9- to 3.0-days (n=10) reached the LF threshold first. Those surviving 3.1 to 4.0 days (n=5) reached the threshold second and the pattern continued with fewer animals and longer survival times reaching critical levels later. LF levels in the animal with the longest survival remained well below the phase-2 median until 108 hours, and in the animal that survived to the end of the study, LF levels remained below the phase-2 median throughout

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514 the entire course of infection. At 60 hours there was a negative relationship between LF levels and
515 subsequent survival time. This relationship will be characterized in detail in the future. The pattern
516 observed was strongest for LF but was present for all toxins and bacteremia (S4 Fig). Separation by
517 survival time showed the relationship between toxin levels and survival time and the reliability with
518 which exceeding the phase-2 threshold was followed by a short interval to death.

519 **2.5.3 Long progression of inhalation anthrax.**

520 The toxemia profiles for animal 23 that survived to the end of the study and animal 22 that survived
521 8.4 days were very similar with nearly identical for LF in these two animals out to 108 hours, both below
522 the means for phase-2 (Figure 5B). Toxins were even lower in the non-survivor at 96 hours. By 108- to
523 120-hours toxins start to diverge. In the non-survivor. EF and ETx approached the phase-2 threshold at
524 120 hours, and LF by 144 hours (S4 Fig). PA and bacteremia remained below the mean in both animals
525 out to 144 hours (the last scheduled sample before terminal). In the final sample, all toxins and
526 bacteremia were non-detectable for the survivor and all toxin levels were high for the non-survivor.

527 3. Early hematology associated with time and progression.

528 Samples were collected for hematological parameters through 72 hours in challenge group-1 and
529 144 hours in challenge group-2, prohibiting full comparison of hematology in all animals beyond 72
530 hours. Comparison of hematology was further limited to 48h because only two of 11 animals with fast
531 progression had later measurements, prohibiting comparisons beyond 48h. All or most animals had
532 measurements on day -7, 24-, 36- and 48-hours post-challenge, so these four time points were assessed
533 for both changes with time and differences between fast (high early toxemia) and slow (low early
534 toxemia) progression groups and the survivor. There were no combined effects of time and progression
535 on early hematology. All hematology measurements are included in S3 File.

536 *Changes in early hematology with time.* Changes during early infection were significant ($p \leq 0.05$) for
537 many hematological parameters (Figure 6). P-values for time overall (TO) are included. There were
538 significant increases through 48 hours in parameters associated with innate immunity, neutrophils,
539 neutrophil%, neutrophil/lymphocyte (NL) ratio, NL% ratio, and white blood cells (WBC). Monocytes,
540 monocytes%, large unstained cells, basophils, basophil%, eosinophils and eosinophil percentage
541 increased at 24 hours and/or 36 hours, then declined at 36 hours and/or 48 hours. Lymphocytes and
542 lymphocyte percentage declined from 36 hours to 48 hours. Parameters associated with red cells and
543 oxygenation, hemoglobin, hematocrit, and red blood cells (RBC) declined significantly through 48 hours.
544 These changes were observed for both fast and slow progression. Other parameters did not change
545 significantly over time.

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546 **Figure 6. Early hematology during inhalation anthrax.** Means and 95% confidence intervals graphed from Day -7
547 to 48 hours(h) post-challenge for fast progression (n=11, n=9 at 48 hours), slow progression (n=11), and survivor
548 (n=1). Differences between animals with fast and slow progression at individual time points were determined by
549 one-way ANOVA. P-values ≤0.10 were included in black font and p-values ≤0.05 in red font. All means, confidence
550 intervals and p-values are included in Table 6. JMP fit model was used to test the effect of hematology with time
551 (day -7, 24 hours-h, 36h and 48h) and progression (fast vs slow). P-value for time overall (TO) and progression
552 overall (PO) is given. Basophils (Baso), eosinophils (Eos) large unstained cells (LUC), monocytes (Mono), neutrophils
553 (Neu), lymphocytes (Lymph), neutrophil lymphocyte ratio (NL Ratio), white blood cells (WBC), red blood cells
554 (RBC), hemoglobin (Hgb), hematocrit (Hct), corpuscular hemoglobin concentration mean (CHCM), mean
555 corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), mean
556 platelet volume (MPV), mean corpuscular volume (MCV), red cell distribution width (RDW).

557 *Differences between early hematology with progression.* Considering the speed with which some
558 animals hit the late stage, differences at any one or more early time points may influence progression.
559 Differences in hematology parameters between animals with fast progression and slow progression
560 were determined at individual time points (Table 6) and overall. P-values for significant parameters at
561 individual time points, and all p-values for progression overall (PO) for the entire period are included (Fig
562 6). For progression overall, monocyte% was significantly higher in slow progression than fast progression
563 (Fig 6). Conversely, red cell distribution width (RDW), corpuscular hemoglobin concentration mean
564 (CHCM), and mean corpuscular hemoglobin concentration (MCHC) were higher in fast progression than
565 slow, approaching significance ($p \leq 0.1$) for the entire early period ($p \leq 0.0921$) (Fig 6). These parameters
566 and others were significantly higher at individual time points (Table 6 and Fig 6). Innate immune
567 parameters were higher in animals with fast progression than slow; specifically, monocyte% on day -7,
568 and monocyte count and white blood cells at 24 hours. Platelets were also higher in slow progression,
569 approaching significance at 24 hours and 48 hours. CHCM was significantly higher in fast progression
570 than slow progression at 24- and 48 hours and approached significance at 36 hours. RDW was
571 significantly higher in fast progression than slow progression and 36 and 48 hours and approached
572 significance at day -7 and 24 hours. Compared to all non-surviving animals, with both fast and slow
573 progression, the survivor had higher lymphocytes, lower NL ratios, among the highest WBC count,
574 exceptionally high RBC, hemoglobin, and hematocrit, higher MCV, and low MCHC and CHCM (Fig 6 and
575 Table 6).

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576 **Table 6. Early hematology in cynomolgus macaques with inhalation anthrax.**

	Day -7				24 hours				36 hours				48 hours			
Parameter	Fast (n=11)	Slow (n=11)	Surv	p-value	Fast (n=11)	Slow (n=11)	Surv	p-value	Fast (n=11)	Slow (n=11)	Surv	p-value	Fast (n=9)	Slow (n=11)	Surv	p-value
Baso (10e3/µL)	0.046 (0.024–0.067)	0.054 (0.032–0.075)	0.080	0.576	0.092 (0.023–0.161)	0.155 (0.085–0.224)	0.090	0.1965	0.082 (0.057–0.106)	0.089 (0.065–0.114)	0.15	0.6657	0.083 (0.039–0.128)	0.093 (0.052–0.133)	0.09	0.7476
Baso%	0.373 (0.258–0.488)	0.409 (0.294–0.524)	0.60	0.647	0.764 (0.243–1.28)	1.03 (0.507–1.55)	0.50	0.4639	0.446 (0.377–0.514)	0.509 (0.441–0.577)	0.60	0.185	0.367 (0.270–0.464)	0.364 (0.276–0.451)	0.30	0.9617
Eos (10e3/µL)	0.117 (0.0595–0.175)	0.188 (0.130–0.246)	0.260	0.0851	0.184 (0.100–0.267)	0.186 (0.102–0.269)	0.35	0.9747	0.309 (0.116–0.502)	0.306 (0.114–0.499)	0.38	0.9835	0.104 (0.023–0.186)	0.082 (0.008–0.155)	0.06	0.6689
Eos%	1.06 (0.424–1.70)	1.77 (1.13–2.41)	1.70	0.118	1.45 (0.817–2.07)	1.25 (0.626–1.88)	2.00	0.659	2.13 (0.693–3.56)	1.95 (0.521–3.39)	1.7	0.8608	0.656 (0.042–1.27)	0.473 (-0.082–1.03)	0.20	0.6479
LUC (10e3/µL)	0.036 (0.017–0.054)	0.050 (0.031–0.069)	0.040	0.270	0.045 (0.025–0.064)	0.062 (0.043–0.081)	0.070	0.1995	0.060 (0.036–0.084)	0.081 (0.057–0.105)	0.12	0.2132	0.046 (0.020–0.071)	0.060 (0.037–0.083)	0.02	0.3847
LUC%	0.327 (0.212–0.443)	0.409 (0.294–0.524)	0.30	0.307	0.355 (0.242–0.467)	0.409 (0.297–0.521)	0.40	0.4816	0.382 (0.231–0.532)	0.491 (0.340–0.641)	0.5	0.2978	0.233 (0.053–0.414)	0.318 (0.155–0.481)	0.10	0.4735
Lymph (10e3/µL)	5.01 (3.48–6.54)	5.11 (3.57–6.64)	9.46	0.930	5.99 (4.52–7.47)	6.22 (4.74–7.69)	9.35	0.8239	6.05 (4.29–7.81)	6.53 (4.77–8.29)	10.2	0.6928	3.75 (2.41–5.09)	3.75 (2.54–4.97)	5.35	0.9948
Lymph%	43.2 (33.5–53.0)	42.0 (32.2–51.8)	63.3	0.857	46.4 (38.6–54.2)	41.3 (33.5–49.2)	53.4	0.3501	38.8 (27.6–50.1)	39.3 (28.0–50.6)	45	0.9532	21.9 (12.8–31.0)	19.2 (10.9–27.5)	18.1	0.6521
Mono (10e3/µL)	0.346 (0.181–0.512)	0.561 (0.396–0.726)	0.350	0.0701	0.497 (0.359–0.635)	0.696 (0.558–0.835)	0.480	**0.0462**	0.660 (0.483–0.837)	0.663 (0.486–0.840)	0.83	0.9821	0.538 (0.268–0.807)	0.766 (0.523–1.01)	0.51	0.2028
Mono%	2.90 (2.00–3.79)	4.41 (3.52–5.30)	2.40	**0.0216**	3.84 (3.09–4.59)	4.63 (3.88–5.38)	2.70	0.1352	4.07 (2.97–5.18)	3.89 (2.78–5.00)	3.6	0.8112	2.82 (1.79–3.85)	3.25 (2.31–4.18)	1.7	0.5299
Neu (10e3/µL)	6.09 (4.45–7.73)	6.11 (4.47–7.76)	4.76	0.982	5.97 (4.65–7.29)	7.59 (6.27–8.91)	7.15	0.0851	10.4 (5.62–15.2)	10.1 (5.33–14.9)	11.0	0.9281	17.2 (9.29–25.1)	19.1 (11.9–26.2)	23.53	0.7187
Neu%	52.1 (41.6–62.6)	51.0 (40.4–61.5)	31.8	0.874	47.2 (39.0–55.5)	51.4 (43.1–59.6)	40.9	0.4651	54.1 (41.3–67.0)	53.9 (41.0–66.7)	48.6	0.9762	74.0 (63.8–84.3)	76.4 (67.1–85.7)	79.6	0.7216
NL Ratio	1.46 (0.769–2.14)	1.63 (0.947–2.32)	0.503	0.707	1.17 (0.066–1.67)	1.49 (0.986–1.99)	0.765	0.3542	2.76 (0.19–5.33)	2.80 (0.231–5.37)	1.08	0.9813	7.48 (-1.32–16.3)	9.74 (1.78–17.7)	4.398	0.6937
NL% Ratio	1.45 (0.767–2.14)	1.63 (0.947–2.32)	0.502	0.704	1.17 (0.663–1.67)	1.49 (0.986–1.99)	0.766	0.355	2.76 (0.198–5.31)	2.79 (0.236–5.35)	1.08	0.9828	7.48 (-1.44–16.4)	9.81 (1.75–17.9)	4.398	0.6883
PLT (10e3/µL)	404 (351–457)	452 (399–505)	418	0.192	415 (356–475)	489 (430–549)	421	0.0826	382 (311–453)	451 (380–522)	370	0.1687	364 (302–426)	440 (384–497)	357	0.0718
MPV (femtoL)	8.77 (7.81–9.74)	8.42 (7.46–9.38)	8.10	0.593	8.92 (8.10–9.74)	8.74 (7.92–9.55)	8.00	0.746	9.1 (8.09–10.1)	8.76 (7.76–9.77)	9.0	0.6273	9.27 (8.34–10.2)	8.55 (7.71–9.38)	8.1	0.2407
WBC (10e3/µL)	11.6 (9.69–13.6)	12.1 (10.1–14.0)	15.0	0.753	12.8 (11.3–14.3)	14.9 (13.4–16.4)	17.5	**0.046**	17.6 (13.6–21.6)	17.8 (13.8–21.8)	22.7	0.9387	21.7 (14.1–29.3)	23.8 (16.9–30.7)	29.56	0.6736
CHCM (g/dL)	32.1 (31.4–32.8)	31.4 (30.7–32.1)	30.5	0.199	32.5 (32.0–33.0)	31.7 (31.2–32.2)	30.8	**0.0448**	32.1 (31.6–32.6)	31.4 (30.9–31.9)	30.8	0.0772	32.6 (32.1–33.0)	31.9 (31.5–43.2)	31.4	**0.0455**
Hbg (g/dL)	12.8 (12.3–13.3)	12.4 (11.9–12.9)	14.4	0.294	12.1 (11.6–12.6)	12.0 (11.5–12.5)	14.1	0.753	11.9 (11.4–12.3)	11.9 (11.4–12.3)	13.7	0.9775	11.4 (10.7–12.2)	11.7 (11.0–12.4)	13.3	0.5534
Hct%	40.6 (38.6–42.7)	40.2 (38.1–42.2)	48.0	0.755	38.7 (36.9–40.5)	38.9 (37.1–40.7)	47.6	0.8791	37.9 (36.2–39.6)	38.4 (36.7–40.1)	45.9	0.6802	36.1 (33.5–38.8)	37.6 (35.1–40.0)	44.2	0.4188
MCH (pg)	24.3 (23.6–24.9)	23.7 (23.0–24.3)	24.0	0.178	23.8 (23.1–24.5)	23.4 (22.7–24.1)	23.5	0.379	24.1 (23.5–24.7)	23.7 (23.0–24.3)	23.7	0.3269	23.8 (23.1–24.5)	23.5 (22.9–24.1)	23.4	0.4909
MCHC (g/dL)	31.5 (31.0–32.0)	30.9 (30.3–31.4)	30.1	0.109	31.3 (30.7–32.0)	30.9 (30.3–31.6)	29.7	0.3892	31.3 (30.8–31.8)	30.9 (30.4–31.4)	29.9	0.2525	31.6 (31.2–31.9)	31.2 (30.8–31.5)	30.2	0.1435
MCV (femtoL)	77.1 (75.2–79.1)	76.7 (74.7–78.6)	79.9	0.744	76.1 (74.1–78.0)	75.7 (73.7–77.6)	79.2	0.7752	77 (75.4–78.6)	76.6 (75.0–78.2)	79.3	0.6938	75.4 (73.4–77.3)	75.4 (73.6–77.1)	77.6	0.9842
RBC (10e6/µL)	5.27 (5.04–5.50)	5.24 (5.01–5.47)	6.01	0.833	5.09 (4.88–5.31)	5.14 (4.92–5.35)	6.01	0.7655	4.93 (4.72–5.14)	5.02 (4.81–5.23)	5.8	0.5467	4.8 (4.45–5.14)	4.98 (4.67–5.30)	5.7	0.4047
RDW%	14.1 (13.7–14.6)	13.6 (13.2–14.1)	14.5	0.0879	14.1 (13.7–14.5)	13.6 (13.1–14.0)	14.0	0.0933	14.1 (13.6–14.5)	13.4 (13.0–13.9)	14.0	**0.0366**	14.5 (14.0–14.9)	13.6 (13.2–13.9)	13.8	**0.0039**

577 Means and 95% confidence intervals from Oneway ANOVA, assuming equal variances, for 11 animals with fast progression, 11 animals with slow progression,
578 and one animal that survived to the end-of-study, on on day -7, 24-, 36-, and 48-hours post-challenge. P-values ≤0.10 are underlined and p-values ≤0.05 are in
579 bold font. Basophils (Baso), eosinophils (Eos) large unstained cells (LUC), monocytes (Mono), neutrophils (Neu), lymphocytes (Lymph), neutrophil lymphocyte
580 ratio (NL Ratio), white blood cells (WBC), red blood cells (RBC), hemoglobin (Hgb), hematocrit (Hct), corpuscular hemoglobin concentration mean (CHCM),
581 mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), mean platelet volume (MPV), mean corpuscular
582 volume (MCV), red cell distribution width (RDW).

583 These results suggest that hematological differences may make for a more or less robust innate
584 immune response (defense) or render the animal more or less susceptible to the destruction of red cells
585 and associated physiologic consequences of anthrax (resilience). Collectively, the results suggest that
586 higher levels of innate immune parameters (such as monocytes and WBC) at early time points (such as
587 day -7 and 24 hours), were associated with slower progression and lower levels with fast progression.
588 Conversely, higher levels of parameters associated with disparately sized red blood cells, or hemolytic
589 anemia (CHCM, MCHC and RDW) favored fast progression and lower ones slow.

590 # 4. Pathology

591 Assessment of terminal stage pathology can provide evidence of the impact of the toxemia and
592 bacteremia measured during infection. Animals underwent complete necropsy, and gross and
593 microscopic observations were recorded. Microscopic observations were assigned a semi-quantitative
594 severity score as follows: minimal for a minimally detectable lesion (Grade 1), mild for an easily
595 visualized lesion (Grade 2), moderate for a lesion affecting larger areas of the tissue (Grade 3) and
596 marked for lesions affecting maximum areas of the tissue (Grade 4). Microscopic observations attributed
597 to anthrax in this study included the presence of bacteria and other pathologic changes, such as fibrin,
598 hemorrhage, necrosis, and inflammation (Table 7).

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Table 7. Microscopic and gross pathology summary.

		Microscopic Tissue Bacteria									Microscopic Pathology																																			Gross Pathology		
		Bacteria in all organs									Brain				Kidney			Liver				Lung					Bronchial Lymph Nodes					Mandibular Lymph Nodes				Mesenteric Lymph Nodes					Spleen				Thy-mus			
	Animal ID	Brain	Kidney	Liver	Lung	BLN	MnLN	MsLN	Spleen	Total Bact	Fibrin	Inflammation	Hemorrhage	Necrosis	Inflammation	Hemorrhage	Necrosis	Inflammation	Hemorrhage	Necrosis	Leukocytosis	Fibrin	Inflammation	Hemorrhage	Necrosis	Edema	Fibrin	Inflammation	Hemorrhage	Necrosis	Edema	Fibrin	Inflammation	Hemorrhage	Necrosis	Fibrin	Inflammation	Hemorrhage	Necrosis	Edema	Fibrin	Inflammation	Hemorrhage	Necrosis	Edema	Gross lesion codes	Gross lesion incidence	Total Path
Fast Progression (n=11)	1) C58282	1	1	0	1	1	0	0	2	6	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	4	0	1	0	0	2	0	0	0	0	0	0	0	0	0	1	2	0	1	-		0	13
	2) C59261	2	2	2	2	2	0	0	4	14	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	1	-		0	5
	3) C60763	1	2	2	2	2	1	1	3	14	0	0	0	0	0	0	0	0	0	0	2	0	1	1	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	1	0	2	0	0	2	-		0	13
	4) C58176	1	2	2	1	1	1	1	4	13	0	0	0	0	0	0	0	0	0	0	2	0	1	1	0	2	0	1	4	3	0	0	0	0	0	0	0	0	0	0	0	2	1	2	-		0	19
	5) C58003	1	1	2	2	1	1	1	3	12	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	2	0	0	0	1	0	0	0	1	0	0	2	0	0	2	-		0	10
	6) C59656	1	1	2	2	2	2	1	3	14	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	2	1	0	0	0	0	0	1	0	0	0	2	0	0	2	-		0	12
	7) C58214	1	1	1	2	1	0	1	2	9	0	0	0	0	0	0	0	0	0	0	2	0	1	1	0	0	0	1	3	3	0	0	0	0	1	0	0	0	1	0	0	1	0	2	-		0	16
	8) C60732	1	1	1	2	1	1	1	3	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	2	-		0	7
	9) C59669	0	2	1	2	1	1	2	4	13	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	2	-		0	6
	10) C57161	1	2	2	0	1	1	2	2	11	0	0	1	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0	1	0	0	-		0	8
	11) C58170	0	2	2	3	1	0	0	4	12	0	0	0	0	0	0	1	0	0	0	1	0	3	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	3	0	2	-	A	1	14
	% affected	**81.8**	**100**	**91.7**	**91.7**	**100**	**66.7**	**75.0**	**100**		0	0	9.1	0	9.1	0	18.2	0	0	0	**90.9**	0	**63.6**	27.3	0	**45.5**	0	27.3	**36.4**	**72.7**	27.3	0	0	9.1	9.1	0	9.1	9.1	36.4	0	45.5	**63.6**	9.1	**90.9**	-		9.1	
	Mean Fast	0.91	1.5	1.5	1.7	1.3	0.7	0.9	**3.1**	11.7	0	0	0.09	0	0.09	0	0.18	0	0	0	**1.4**	0	0.82	0.27	0	0.91	0	0.27	**0.91**	**1.5**	0.45	0	0	0.09	0.09	0	0.09	0.09	0.36	0	0.73	**1.1**	0.09	**1.6**	-		0.1	**11.2**
	Std Dev	0.54	0.52	0.69	0.79	0.47	0.65	0.70	0.83	2.5			0.30	0.0	0.30		0.40				0.67		0.87	0.47		1.3		0.47	1.4	1.1	0.82			0.30	0.30		0.30	0.30	0.50	0.00	0.90	1.0	0.30	0.67	-		0.3	4.4
Slow Progression (n=11)	12) C59619	1	2	2	2	2	2	1	3	15	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	2	2	0	0	0	2	0	0	0	0	0	0	0	0	1	4	B	1	16
	13) C58135	0	2	1	3	1	2	1	4	14	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	1	1	0	-		0	8
	14) C57793	0	1	2	1	1	1	1	4	11	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	2	0	0	0	2	0	0	2	1	2	-		0	13
	15) C58167	0	2	1	3	2	1	2	4	15	0	0	0	1	0	0	1	0	0	0	2	0	3	2	0	2	0	0	2	2	2	0	0	0	0	0	0	0	2	0	2	0	0	2	-		0	23
	16) C57998	1	1	2	2	1	0	0	2	9	0	0	0	0	0	0	0	0	0	1	1	0	1	2	0	0	1	0	2	4	0	0	0	0	0	0	0	0	0	0	0	2	1	0	-		0	15
	17) C59617	2	1	2	3	2	2	1	4	17	0	2	2	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	3	0	2	1	1	3	0	0	0	0	0	2	1	0	0	-	C	1	22
	18) C58159	2	0	0	0	0	1	1	1	5	2	2	2	0	1	0	0	0	0	0	1	1	0	0	0	2	0	2	1	2	2	0	0	0	0	0	0	0	0	0	0	2	0	0	-	C	1	21
	19) C58122	0	1	2	2	1	1	1	1	9	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	1	1	2	2	1	1	0	0	0	0	0	0	1	1	3	0	2	-		0	19
	20) C57997	0	1	1	1	1	1	1	1	7	0	0	0	0	0	0	1	0	0	1	2	1	1	1	0	1	0	2	4	4	0	0	1	0	2	1	0	0	0	0	0	1	0	0	-		0	23
	21) C58156	1	0	0	0	0	0	1	0	2	1	3	2	0	0	0	0	2	2	2	2	1	2	2	0	0	0	1	2	2	0	0	1	1	0	0	0	0	0	0	0	3	0	2	-	C, D	2	33
	22) C59618	0	1	2	2	1	1	2	2	11	0	0	0	0	0	0	0	2	0	0	2	1	0	0	0	0	0	1	4	4	0	0	0	1	0	0	1	0	3	0	1	2	0	0	-	A,E,F	3	25
	% affected	**45.5**	**81.8**	**81.8**	**81.8**	**81.8**	**81.8**	**90.9**	**90.9**		**18.2**	**27.3**	**27.3**	9.1	9.1	0.0	36.4	**27.3**	9.1	**45.5**	**90.9**	**36.4**	**63.6**	45.5	0	**54.5**	9.1	45.5	**90.9**	***100***	36.4	18.2	**36.4**	27.3	**36.4**	9.1	9.1	0.0	27.3	9.1	36.4	**81.8**	27.3	**45.5**	N/A		45.5	
	Mean Slow	0.64	1.1	1.4	1.7	1.1	1.1	1.1	**2.4**	10.5	0.27	0.6	0.5	0.1	0.1	0.0	0.4	0.5	0.2	0.5	**1.3**	0.4	0.9	0.7	0.0	0.7	0.1	0.6	**1.7**	**2.5**	0.7	0.3	0.4	0.3	0.8	0.1	0.1	0.0	0.6	0.1	0.5	**1.5**	0.3	**0.8**	N/A		0.7	**19.8**
	Std Dev	0.81	0.70	0.81	1.10	0.70	0.70	0.54	1.5	4.6	0.65	1.12	0.93	0.3	0.3	0.0	0.5	0.8	0.6	0.7	0.6	0.5	0.9	0.9	0.0	0.8	0.3	0.8	1.3	1.0	1.0	0.6	0.5	0.5	1.2	0.3	0.3	0.0	1.1	0.3	0.8	1.0	0.5	1.0	N/A		1.0	6.7

Microscopic observations of bacteria (left) and other anthrax-related pathology were recorded for each animal. Organs evaluated microscopically in all animals included brain, kidney, liver, lung, spleen, bronchial lymph nodes, mandibular lymph nodes, and mesenteric lymph nodes. Thymus was evaluated in animal 20) C57997 due to the presence of a gross lesion. Parameters scored semi-quantitatively included bacteria and other microscopic pathology, including fibrin, hemorrhage, inflammation, necrosis, and edema. Severity scores were minimal (grade 1, yellow), mild (grade 2, orange), moderate (grade 3, red) or marked (grade 4, dark red). A pathology score of 0 was included for microscopically normal tissues. The percent (%) of animals affected (incidence), and the mean severity scores with standard deviations (Std Dev) were calculated by group for fast and slow progression. A total score for bacteria (Total Bacteria) and a total score for other pathology findings (Total Pathology) was calculated for each animal. Group mean and Std Dev for Total Bacteria and Total Pathology scores were calculated for fast and slow progression groups. The single surviving animal 23 (C58174) did not have any pathologies consistent with anthrax and is not included. **Bold** font indicates a high percentage (%) of animals affected and/or the highest one or two mean scores. **Bold underlined** fonts indicate parameters where the % affected or means were notably different between fast and slow progression. Cell type-specific necrosis involved lymphocytes in lymph nodes, lymphoid follicles in spleen, hepatocytes in liver, and renal tubules in kidney. Gross pathology findings (far right column) were coded as: A) 10-15 milliliters red fluid (ascites), B) 10 mL clear fluid in thymus, C) dark or red discoloration of meninges, brain, D) multiple dark liver foci, E) multiple pale liver foci, F) multiple enlarged, dark bronchial lymph nodes.

611 *Bacteremia and sepsis.* Based on histopathological evaluation, both fast and slow progression animals
612 had a high incidence (67 – 100%) of bacteria in organs and associated vascular spaces (Table 7). Overall,
613 the spleen represented the greatest bacterial reservoir, with 21 animals affected and a severity grade of
614 3 or above in many of the animals (8 among fast progression and 5 among slow progression animals).
615 Thus, the highest group mean severity scores for bacteria were in the spleen, averaging 3.1 in the fast
616 progression group and 2.4 in the slow progression group, followed by a mean severity score of 1.7 in the
617 lungs in both groups. The only notable difference in bacterial tissue distribution between fast and slow
618 progression animals was the presence of bacteria in brain in 81.8% of animals with fast progression and
619 only 45.5% of animals with slow progression.

620 All but three of 23 animals in this study presented with features of fatal sepsis, as defined by *B.*
621 *anthracis* dissemination to non-lymphoid organs including kidneys, liver, and lungs. Of the three animals
622 without apparent sepsis, one (animal 23, C58174) survived *B. anthracis* challenge with no bacteria visible
623 in any tissue. Two others, animals 18 (C58159) and 21 (C58156), were in the slow progression group and
624 only had bacteria visible in the brain and lymphoid organs. Both animals had macroscopic observations of
625 dark or red meninges at necropsy that correlated with microscopic fibrin, inflammation, and/or
626 hemorrhage. Additionally, seizures were observed in animal 18 prior to euthanasia, and terminal
627 bacteremia measurements in animal 18 and terminal toxin measurements in animal 21 were lower than
628 those seen in animals with sepsis (Fig 3, animal 18, 21). Together, these findings supported that death in
629 these two animals was a consequence of meningitis rather than sepsis. A third animal in the slow
630 progression group, animal 17 (C59617), also had macroscopic and microscopic evidence of meningitis but
631 with concurrent sepsis, disseminated bacteria to all organs, and high terminal toxemia (Fig 3, animal 17).

632 *Histopathology*. Among the tissues collected and processed for histopathological analysis, liver, lung,
633 bronchial lymph nodes and spleen had highest incidence of anthrax-related microscopic lesions, including
634 edema, fibrin, inflammation, hemorrhage, and necrosis (Table 7). The incidence and severity of
635 microscopic changes in the kidney, liver, and lung were comparable between fast and slow progression
636 animals. Lesions in other organs often had higher incidence and/or severity among slow progression than
637 fast progression animals, including brain fibrin, inflammation, hemorrhage, and necrosis; liver
638 inflammation, hemorrhage, and hepatocellular necrosis; bronchial lymph node edema, inflammation,
639 hemorrhage, and lymphocyte necrosis; mandibular lymph node fibrin, inflammation, hemorrhage, and
640 lymphocyte necrosis; mesenteric lymph node lymphocyte necrosis; and splenic inflammation. Among fast
641 progression animals, a higher incidence and mean severity of splenic lymphoid necrosis was noted as
642 compared to slow progression animals.

643 Despite most fast progression animals having bacteria visible in the brain, there was little associated
644 tissue change (fibrin, inflammation, or necrosis) and only one animal with minimal hemorrhage. As

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645 previously noted, tissue changes in the brain of animals with slow progression were primarily limited to
646 the three animals with meningitis, and meningitis was not seen in any animal that died less than 105
647 hours (4.4 days) post-challenge.

648 To facilitate global comparison between fast and slow progression animals, Total Bacteria scores
649 were calculated for each animal by adding the individual microscopic severity scores for bacteria in all
650 examined organs. Total Pathology scores were calculated for each animal by adding the individual
651 microscopic severity scores for all anthrax-related lesions (except for bacteria) in all examined organs as
652 well as the incidence of any anthrax-related gross lesions. Group mean (±standard deviation) Total
653 Bacteria scores were 11.7±2.5 for fast progression and 10.5±4.6 for slow progression. Conversely, group
654 mean Total Pathology scores were 11.2±4.4 for fast progression and 19.8±6.7 for slow progression,
655 demonstrating higher pathology overall for animals with longer survival times and thus longer toxin
656 exposure.

657 The single animal surviving to end-of-study (day 14) had non-specific pathology that included minimal
658 chronic active inflammation in the lung and pale liver nodules, thought to be from a prior unrelated
659 infection, with no microscopic evidence of anthrax.

660 Discussion

661 *Anthrax progression.* Historically, clinical inhalation anthrax was described as a biphasic disease, with
662 two stages of 'illness', early-prodromal and late-fulminant (3, 48). The first stage lasted several days and
663 was followed by either direct progression to the late-fulminant stage or a period of improvement before
664 entry to the late stage. During a 1957 outbreak, Plotkin et al described five inhalation anthrax cases
665 where after initial symptoms, one patient continued to work and two of the patients' conditions were
666 briefly 'improved' (48). However, after onset of the final stage in four patients, they only survived 7 to 25
667 hours. The 2001 inhalation anthrax cases allowed refinement of the clinical description to include three
668 stages: 1) the early-prodromal, 2) intermediate-progressive, and 3) the late-fulminant stage (7, 33, 34).
669 The kinetics of anthrax toxins determined previously in five rhesus macaques showed that PA, LF, LTx, EF,
670 and the poly-glutamic acid capsule kinetics were triphasic, with a rise, a plateau (or decline), and a final
671 rise: potentially comparable to the three human clinical stages of anthrax (22, 23, 45, 46). This study,
672 with 23 cynomolgus macaques, allowed the first determination of all major anthrax toxins collectively,
673 along with quantitative bacteremia, through all stages of anthrax progression. The larger numbers of
674 animals (cynomolgus macaques) in this study yielded variations in progression compared to that seen in
675 rhesus macaques.

676 Two different patterns of toxemia emerged in the cynomolgus macaques in this study. In 11 animals
677 the toxin levels and bacteremia reached higher levels early, at 30-to-48 hours, compared to the other 12

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678 animals in the study, which had much lower levels during this period. The 11 animals with high early
679 toxemia/bacteremia happened to have the fastest disease progression and earliest deaths. In contrast,
680 the animals with lower early toxemia survived longer. Thus, the kinetics defined two distinct survival
681 groups, one with high early toxemia and fast progression and one with lower early toxemia and slow
682 progression. This allowed characterization and comparison of both types of infection progression in a
683 way that has not been done before. Some differences might be related to the higher spore dose per body
684 weight in fast progression, but the differences were not statically significant. Furthermore, there was no
685 significant correlation between LD_{50}/kg and time-to-death, or LD50/kg and early toxin levels or
686 bacteremia (data not shown). Nonetheless, there was a higher average LD_{50}/kg for animals with fast
687 progression. This prompts consideration, if feasible, for tailoring animal study design to target the spore
688 dose according to an animals' body weight.

689 Animals with slow progression exhibited a rise/plateau/rise, a triphasic kinetics, similar to the five
690 rhesus macaques described previously. The triphasic kinetics of slow progression can be aligned with the
691 3 clinical stages (Fig 7), similar to that suggested previously (49). Phase-1 begins with early toxemia, LF
692 and EF, appearing during the incubation period, before symptom onset. In the later part of phase-1,
693 other toxins, LTx, ETx, PA, and bacteremia appear, along with early symptom onset in some animals.
694 Toxins continue to rise to intermediate levels at the end-of-phase-1 (Table 5, median levels of 23.8 ng/mL
695 for LF). This period is analogous to the early-prodromal clinical stage. The end-of-phase-1 begins phase-2
696 where toxins levels plateau (PA/LF/LTx) or decline (EF/ETx/bacteremia) for an extended period,
697 analogous to the intermediate-progressive stage 2. LF levels throughout phase-2 were stable, ranging less
698 than one order of magnitude over all time points and animals as described. Characterizing the entire
699 stable phase-2 period allowed determination of the upper 95% confidence limits of this range,
700 establishing a phase-2 threshold, above which phase-2 has ended and phase-3 commenced. After the
701 plateau, toxins started to rise again and animals reached the phase-2 threshold (35.8 ng/mL for LF),
702 marking entry to phase-3 with rapid accumulation of toxemia and rapid demise, corresponding with the
703 late-fulminant stage.

704 **Figure 7. Proposed relationship of toxemia-defined progression with clinical staging.** The triphasic (TP) kinetics of
705 anthrax toxemia with slow progression in non-human primates mirrors the clinical staging algorithm. A brief
706 incubation period is followed by 1) the early-prodromal stage 1 with the onset of non-specific symptoms which
707 coincides with the early rise in toxemia to the end-of-phase-1 (EOP1). This is followed by 2) the intermediate-
708 progressive stage which coincides with the plateau/decline in toxemia of variable duration that continues to the
709 end-of-phase-2 (EOP2) when the critical toxemic thresholds of phase-2 are exceeded. Exceeding the threshold(s)
710 coincides with entry to 3) the late-fulminant stage with a rapid decline in clinical stability and rapid rise in
711 toxemia/bacteremia. In fast progression this occurs over a compressed timeline during which the phase-2 plateau
712 may be observed (fast triphasic-TP) or may not be observed (fast monophasic-MP) kinetics. The dependence of
713 progression on toxemia can be seen with the shorter time-to-threshold for fast progression than slow and the
714 similar time from threshold-to-terminal for both. Progression may be reoriented from an apparent time/clinical-
715 dependent staging to suggest possible toxin/clinical-dependent staging.

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716 For fast progression, the kinetics was predominantly triphasic with a rapid rise, shorter phase-2
717 plateau, and final rapid rise. A few animals appeared to be monophasic, with the phase-2 plateau
718 skipped, or not discernable within the early 6-hour collection times of this study. The kinetics of all
719 animals with fast progression occurred within a compressed time frame (Fig 7). As such, they reached
720 higher levels at the end-of-phase-1 (Table 5, 138 ng/mL for LF), already above the phase-2 threshold of
721 35.8 ng/mL for LF and for all toxins and bacteremia, indicating early entry to phase-3. In monophasic
722 progression the end-of-phase-1 and end-of-phase-3 are virtually synonymous. Passing the critical phase-2
723 toxin thresholds early led to rapid death, even before symptoms were observed in two of the animals.

724 An early study by Smith and Keppie identified a bacteremia threshold above which antibiotic
725 treatment failed (11). This bacteremia threshold represents a point of no return beyond which an animal
726 will not recover with antimicrobial treatment. In clinical anthrax the late-fulminant stage 3 has the lowest
727 cure rate, 97% fatality for a century of inhalation anthrax cases (5). Thus, it is analogous to the point of no
728 return. Stage-3 is analogous to phase-3 in this cynomolgus macaque model, indicating the phase-2
729 bacteremia/toxemia thresholds for entry to phase-3 may also predict stage-3 based treatment failures.
730 When toxemia went above the phase-2 threshold, toxins levels rapidly increased leading to the terminal
731 phase. The mean time to reach the thresholds and enter phase-3 was on average, twice as long in slow
732 progression as fast. In contrast, the mean time from threshold to terminal was nearly identical for both
733 fast and slow progression at 20-21 hours, consistent with the duration of stage 3 described in clinical
734 cases (3, 7, 48, 50). The findings support reorientation of the progression scheme from a time-dependent
735 to a toxin-dependent one (Fig 7). Animals that rapidly pass the critical threshold in phase-1 show a
736 compression (or elimination) of the intermediate-progressive phase-2. The 11 animals with the shortest
737 survival times had an accelerated progression due to early high toxemia. Evaluation of toxemia by
738 survival time supports the critical phase-2 threshold of toxin levels in the cynomolgus macaque model,
739 since no matter the time post-challenge, once the threshold is passed, terminal stage-3 follows rapidly
740 and within about 21 hours (Fig 5).

741 The fast progression observed in almost half of the animals is a challenging problem since it leaves
742 little time for successful treatment after symptoms. A rapid course has been suggested by evidence from
743 the Sverdlovsk outbreak for which dates of both symptom onset and death were available for 50 cases
744 (51). The time from onset to death was on the same day for one case and within one day for 8 cases,
745 consistent with the faster progression in this study. For all other cases, the time from symptom onset and
746 death were 2 days and longer, which is more consistent with slow progression in our cynomolgus
747 macaque study. A systematic review for a century of inhalation anthrax reported four cases with 1.5- to
748 1.6-days following initial symptoms (5). Though the incubation periods and exposure doses are not
749 certain, the rapid death following onset suggest the possibility of rapid progression in humans following
750 inhalation of *B. anthracis* spores, especially the weaponized spores in the Sverdlovsk cases.

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751 *Other factors.* Although, the average spore dose per kg was higher for fast progression than slow,
752 there was no correlation of LD_{50}/kg with early toxemia (data not shown). Vasconcelos *et al.* also observed
753 'a tendency' for cynomolgus macaques receiving higher spore doses to die more quickly than those that
754 received lower doses (37). Early hematology showed trends related to time or progression. There were
755 time-dependent changes over the first 48 hours, increases in innate immune cells, such as white blood
756 cells and neutrophils, and declines in red blood cells, hemoglobin, and hematocrit. These occurred in
757 both fast and slow progression, consistent with those seen in other animal models (52, 53). However,
758 there were key differences between fast and slow progression for some parameters. Means for innate
759 immune markers, especially, monocytes, were lower in fast progression than slow progression. Of course,
760 this means that monocytes were higher in slow progression, perhaps offering more defense in these early
761 stages. Red cell distribution width (RDW) and corpuscular hemoglobin concentration mean (CHCM) were
762 higher in animals with fast progression and lower in animals with slow progression. High RDW is
763 associated with deficiencies of iron, B-12, or folate and can be due to macrocytic anemia, underlying
764 chronic inflammation, or infection (54). Studies in middle aged and older adults showed that even
765 modestly elevated RDW was associated with an increased risk of all-cause mortality (55). Elevated RDW
766 was also associated with increased severity of sepsis (56, 57).

767 The anthrax toxins actions make the differences in these factors even more important. LTx is known
768 to cause anemia and has also been shown to suppress erythropoiesis, exacerbating the anemia, and
769 promoting progression (58). Hemoglobin and hematocrit have been shown to drop during clinical
770 inhalation anthrax and was observed here (59). In this study, the lone survivor, animal 23, had higher
771 early white blood cells, notably lymphocytes, but also had exceptionally elevated red blood cell count,
772 hemoglobin, and hematocrit (Fig 6). In contrast to animals with fast progression that had higher CHCM,
773 the survivor also had the lowest CHCM (and MCHC) of all animals. These hematological markers will be
774 further evaluated for their association with progression and outcome in future studies.

775 Two animals with long progression, animal 23 that survived and animal 22 that succumbed at 8.4
776 days, had very similar toxemic profiles, especially for LF. All toxins and bacteremia were well below the
777 phase-2 mean out to 108 to 120 hours, but eventually diverged with toxins increasing in the non-survivor
778 and declining to non-detectable in the survivor (Fig 5B and S4 Fig). There were some differences in these
779 animals that might have contributed to the final outcome. The LD_{50} spore dose was higher in the survivor
780 but when adjusted for weight was higher in the non-survivor (Table 1). Also, at 132 hours, the RBC count
781 was within normal limits (5.0 to 7.6 e^6/µL, Battelle report) at 5.24 e^6/µL in the survivor but was below
782 normal in the non-survivor (3.85 e^6/µL). The higher beneficial hematological features described above for
783 the survivor may have provided a better level of resilience against toxins and defense against the
784 organism for recovery.

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785 *Pathological factors associated with disease progression.* Assessment of the degree of microscopic
786 bacteria and other pathology at terminal-stage provides an assessment of the burden and impact of
787 anthrax resulting from the varying toxemic progressions. The general findings were consistent with those
788 described for cynomolgus macaques previously, with primary involvement in the spleen, bronchial lymph
789 nodes, and lungs (37). Overall, animals with longer survival times and thus longer toxin exposure had a
790 higher incidence and/or severity of pathologic findings but slightly lower severity of intralesional bacteria.

791 While both fast and slow progression groups had similar incidences of bacteria in the spleen, the
792 greatest bacterial reservoir organ in this study, the mean severity of splenic bacteria was higher in
793 animals with fast progression than slow progression. Splenic lymphoid necrosis was also seen in twice as
794 many fast progression animals, and with twice the average severity, as compared to those with slow
795 progression. Thus, both bacterial colonization and necrosis in the spleen were early to develop in the
796 course of rapidly progressing anthrax. Conversely, microscopic lesions in other organs were often similar
797 among all animals or had higher incidence and/or severity among slow progression animals. The latter
798 category included bronchial lymph node lymphocyte necrosis and hemorrhage, suggesting that longer
799 toxin exposure may allow for lesion development.

800 Although there were more fast progression animals with visible bacteria in the meninges than slow
801 progression animals, they had little to no associated brain pathology. Studies have indicated that ETx
802 plays a role in disrupting the blood brain barrier (BBB) function and LTx in promoting microbial BBB
803 penetration (60). The higher early toxemia in fast progression may allow BBB penetration and
804 penetration of bacilli, but death is rapid with little associated pathology. In animals with slow
805 progression, the lower end-of-phase-1 and phase-2 toxemia may not facilitate penetration of the BBB
806 quite as readily, but when bacteria did gain access to the brain, the longer low-level toxin exposure in
807 phase-2 may have contributed to gross and microscopic fibrin accumulation, inflammation, and
808 hemorrhage. These differences suggest the rapid sepsis of fast progression doesn't allow sufficient time
809 for a tissue response in the brain, whereas the longer course in slow progression allows for longer toxin
810 exposure resulting in meningitis and death. Notably, meningitis was only seen in animals surviving at
811 least 105 hours post-challenge. More may be learned from later studies in cynomolgus macaques, which
812 have included the analysis of toxin levels in terminal organ tissues to help understand differences in the
813 organ pathologies observed.

814 *Symptom onset biomarkers*. LF, LTx, and EF were positive in all 23 animals at or before symptom
815 onset, with LF the most abundant and EF the least abundant of these (Table 3). Although median PA
816 levels at symptom onset were higher than LF, four animals were negative for PA. However, LTx (PA-LF)
817 was positive (LOD of 0.0075 ng/mL) in these, indicating PA is present but below the PA detection limit
818 (1.87 ng/mL) (Table 2). These four negative PA animals also had the earliest time of symptom onset of

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819 about 30 hours. Six hours later, LF increased 5- to 10-fold, leading to a conversion of PA positivity in two
820 animals at low ng/mL levels, but not enough for all to be above the LOD for PA. The important conclusion
821 is that we have defined the range of levels of multiple toxins at symptom onset in this high dose exposure
822 model. These may help guide development of point of care diagnostics.

823 *Pre-symptom onset detection*. Early diagnosis and treatment will improve the odds of surviving
824 anthrax. With the analytical methods used in this study, LF was the best biomarker for pre-symptom
825 onset detection. It was detected earliest and at higher levels than the next earliest detected biomarker,
826 EF. In this study, LF and EF were detected before bacteremia and before symptom onset in every animal
827 (Fig 1 and Table 3). We previously measured LF in human cutaneous anthrax in the absence of sepsis,
828 and even after antibiotic treatment (61). In these cases, we presume that the LF detected is secreted
829 locally from the site of infection, the cutaneous lesion, and is detected in circulation. Early detection of LF
830 during inhalation anthrax, before bacteria are detectable in the blood, may originate from early events,
831 such as germinating spores within macrophages (62).

832 *Toxemia in the absence of bacteremia*. As shown at pre-symptom onset, anthrax toxins are often
833 present in the serum in the absence of detectable bacteremia. In this study, LF and EF were detected
834 before bacteremia in all of the cynomolgus macaques. In a previous study with 5 rhesus macaques, LF
835 also tested positive before bacteremia, and it continued to test positive throughout the course of
836 infection (46). In that rhesus macaque study, bacteremia went from positive - to negative (during phase
837 2) in some animals - back to positive, while LF was positive at all time points. As described above, LF was
838 detected in serum of patients with cutaneous anthrax; after up to 7 days of antimicrobial treatment, and
839 often when swabs of the cutaneous lesion were negative for tests detecting the bacterium (61).
840 Furthermore, LF was detected in serum and pleural fluid in two inhalation anthrax patients up to 11 days
841 after initiation of antibiotic treatment when bacteria would no longer be detectable, and 5 days after
842 antitoxin treatment. LF was also detected in an anthrax-like illness in a welder, caused by infection with
843 *Bacillus tropicus* that produced anthrax toxins (in review). In this case, LF was detected in serum or
844 plasma 11 days after antibiotics and in pleural fluid collected 38 days after antibiotics and two doses of
845 antitoxin.

846 *Stage dependent toxemia and the phase-2 threshold*. We identified toxemia associated with specific
847 stages (Phase-1, -2, and -3). In the earliest stages of infection, detection was limited to LF and EF. The
848 detection of other toxins and bacteremia came at later stages of phase-1 or early phase-2. The analytic
849 sensitivity of the catalytic toxin methods allowed LTx and ETx to be detected before total-PA, though at
850 lower levels than the total toxins, LF and EF. Both LTx and ETx comprise a fraction of the available PA63
851 and signal the point when active toxin complexes begin to accumulate. The appearance of LTx at 30.5
852 hours closely coincides with the earliest detection of bacteremia at 30.0 hours (including bacteremia that

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853 was below the LOQ and reported as a low '+') (Table 3). The appearance of ETx at 33.4 hours coincides
854 with the detection of low level but quantifiable bacteremia at 34.2 hours. This suggests that toxin
855 complexes (LTx and ETx) are detectable in blood at the same time as bacteremia. PA was detected later,
856 due in part to the higher detection limit, but also possibly due to the rate of cellular uptake of PA during
857 early stages, resulting in less PA in circulation. Though first detection of total-PA was latest, once positive,
858 it was the most abundant toxin; approximately 2.4-fold more abundant than LF at first positive PA. PA83
859 was only detected sporadically and was most consistently positive at high levels of total PA. This indicates
860 that PA83 is rapidly hydrolyzed and activated to PA63, early and at all stages, in all cynomolgus
861 macaques, as also seen in rhesus macaques (45). Therefore, PA83 detection by MS in these studies may
862 occur only when it is being secreted in high levels and has not yet had the time to be processed to PA63.
863 As such, any measures with PA83 are not useful in predicting anthrax stages.

864 Phase-2 of slow progression was the most important feature characterized here, since once it is
865 passed, death followed rapidly, irrespective of time. Phase-2 appeared to correspond with the
866 intermediate-progressive clinical stage where the host battles the organism and the effects of
867 intoxication begin to accumulate but have not yet surpassed host defenses. Of all the toxins, LF was the
868 most stable (least variable) during this period, with only one order of magnitude range, from 4.2 to 41.5
869 ng/mL, for all 11 animals and all 58 time-points comprising phase-2. Related toxins, PA and LTx, were also
870 less variable (S3 Fig). The level of stability of LF during phase-2 suggests the possibility that levels are
871 somewhat controlled, either by the host or organism (or both), held below the threshold until host
872 defenses are overcome and disease progresses to the rapidly fatal phase-3. Bacteremia and ETx were the
873 most variable during phase 2. Lower levels seen for ETx may be influenced more by the fluctuations in
874 bacteria and dynamics of intoxication during phase-2.

875 In phase-3, whether fast or slow progression, toxins and bacteremia increase rapidly after passing the
876 phase-2 threshold. So, any toxin levels or bacteremia that are higher than the thresholds suggest there
877 are less than 20 hours of life remaining. The higher the levels, the shorter the interval to death (Fig 5B). In
878 phase-1, lower toxin levels correlate with higher toxin ratios. Conversely, in phase-3 higher toxin levels
879 correlate with lower toxin ratios. This includes all ratios except PA/LF which doesn't change in a
880 consistent manner through the stages of progression. The threshold for LF/LTx is 2.5-5.2 in early stages
881 but declines to 1.7 at the phase-2 threshold and declines further to 1.2 at terminal. It was even as low as
882 1.0 (100% LTx) in some animals with high toxemia (Table 5). This indicates that most of the LF is present
883 as LTx at terminal stage.

884 *Phase-2 threshold and the point of no return*. In 1954, using intradermal *B. anthracis* infection in the
885 guinea pig model, Smith and Keppie described a critical level for bacteremia, a point of no return, above
886 which animals died even after treatment had cleared the organism (11, 63). Animals recovered if treated

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887 when bacteremia was 0.7 to $1.5x10^6$ cfu/mL and died when it was greater than the critical level ($3x10^6$
888 cfu/mL). The threshold for bacteremia in cynomolgus macaques at 1.6E+05 cfu/mL was 20-fold lower
889 than that described in guinea pigs. This is not surprising for different models with different infection
890 routes. Smith and Keppie also described the relationship between the bacterial count in the blood and
891 the time-dependent interval to death. We observed a similar relationship in cynomolgus macaques, a
892 correlation of toxins and bacteremia with survival time. We also determined the phase-2 threshold for
893 entry to phase-3. Medical intervention is usually unsuccessful in the late-fulminant stage-3 (33)
894 (equivalent to phase-3), the phase-2 thresholds could represent the potential point of no return for
895 treatment failures.

896 Conclusions.

897 The assessment of all major anthrax toxins and bacteremia, along with hematology and pathology
898 allowed a comprehensive evaluation of the natural history of inhalation anthrax in the cynomolgus
899 macaque NHP model. We defined a triphasic kinetics of toxemia which could be aligned with the three
900 clinical stages of anthrax. This alignment for fast and slow survival groups indicated that anthrax
901 progression is toxin-dependent rather than time-dependent. Hematological fitness factors may influence
902 the disease course and will be evaluated in other studies. Lethal factor represented the most significant
903 indicator of anthrax progression with a remarkably invariable phase-2, such that a small increase
904 potentially advances infection to phase-3 and a relatively rapid death. Phase-2 thresholds identified,
905 represent a potential point of no return. Ongoing studies will test the critical thresholds for predicting
906 antimicrobial treatment failures. Future studies with combined antimicrobial and antitoxin treatment
907 may advance our understanding of anthrax in humans and when specialized treatments are most
908 efficacious.

909 _____

910 Methods

911 **Reagents.** Materials and reagents were obtained from Sigma-Aldrich (St Charles, MO) except where
912 indicated. LF, PA83, and PA63 were obtained from List Biological Laboratories (Campbell, CA) and EF was
913 from Dr. Stephen Leppla' s laboratory NIH/NIAID as described (23). All other reagents, materials and
914 methods for anthrax toxin measurements were described previously (22, 23, 44, 45, 64).
915 **Safety and quality.** The animal exposure study was conducted in 2014 at Battelle Biomedical
916 Research Center (Columbus, OH) in a BSL3 secured laboratory registered with the Centers for Disease
917 Control and Prevention (CDC) for select agents and inspected by the Department of Defense and U.S.
918 Department of Agriculture. All requirements for select agent laboratories and handling of *B. anthracis*
919 were followed. Battelle performed all aspects of the animal study as well as sample analyses for

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920 quantitative bacteremia and hematology as described in detail below. Samples sent from Battelle to the
921 CDC for analysis of anthrax toxins were filter sterilized and sterility confirmed prior to shipment. Sample
922 analysis at CDC was performed in a BSL2 laboratory and followed all safety requirements for handling
923 biological specimens. All personnel have full training required for safety and follow all general laboratory,
924 chemical and bloodborne pathogens procedures. Chemical hygiene plans are in place. All sample analyses
925 followed high quality procedures using validated methods and conducted in a CLIA-certified laboratory
926 following strict adherence of CLIA regulations. Battelle follows good laboratory practice guidelines as
927 available in their standard operating procedures and facility practices.

928 **Animal study.** The animal exposure study was conducted at Battelle (Columbus, OH), a Public
929 Health Service (PHS) Animal Welfare Assurance approved facility. The study protocol was approved by
930 the Institutional Animal Care and Use Committee (IACUC) (Approved Protocol #3013) as required by PHS
931 policy following HHS, NIH, Office of Laboratory Animal Welfare principles. Work with animals followed all
932 safety and select agent guidelines described above. All aspects of the animal study protocols were
933 designed to minimize stress in the animals. CDC received study samples from Battelle for toxin analysis
934 and was not involved with any component of the animal protocol. Chief of CDC Animal Care confirmed
935 that the use that Battelle IACUC was sufficient.

936 Twenty-three *Macaca fascicularis* cynomolgus macaques (12 males, 11 females) from Covance, Inc.,
937 Alice, TX, weighing 3.1-4.9 kg prior to spore exposure, were confirmed to be healthy, negative for
938 tuberculosis, seronegative for Simian Immunodeficiency Virus, Simian T-Lymphotropic Virus-1, and
939 Macacine herpesvirus 1 and PCR negative for Simian Retrovirus 1 & 2. Animals were quarantined for 5
940 weeks in paired housing, then acclimated to single housing in the biosafety level 3 (BSL3) laboratory one
941 week before exposure.

942 This study was conducted in two challenge groups with the first exposures (Group-1) taking place in
943 November 2013 and the second in February 2014 (Group-2). Group-1 included 10 animals of which 8 (4
944 males, 4 females) were challenged and 2 (1 male, 1 female) were not challenged (naïve controls). In
945 Group-2, 15 CM (8 males, 7 females), which included the 2 controls from Group-1, were challenged.
946 Spore challenge exposures in this model were performed as described previously with a target dose of
947 200 median lethal dose (LD_{50}) *B. anthracis* Ames spores via head-only inhalation exposure, and
948 measurement of actual spore dose per animal by plethysmography (37, 42).

949 Animals were routinely monitored, and clinical signs recorded twice-daily pre-challenge and every
950 6±1 hours commencing at 12 hours post-challenge. The following clinical signs of anthrax were
951 monitored, anorexia, lethargy, respiratory distress, moribundity, abnormal activity such as recumbency,
952 weak, or unresponsive, as well as seizures or any other abnormal signs. Signs of symptoms recorded
953 included normal (N), hunched posture (HP), no stool (NS), soft stool (SS), diarrhea (DI), vomiting (V),

954 lethargic (L), seizures (SE), labored breathing (LB), wheezing (W), moribund (M), unresponsive (UR),
955 prostrate (P). Animals found to be moribund were humanely euthanized. Time to symptom onset was the
956 difference in time hours and minutes from challenge to the hours and minutes of first specific clinical sign
957 described from the 6-hour monitoring during the post-challenge period. NS was not included as a specific
958 sign since it was also described prior to exposure and was not specific.

959 Body temperatures for all animals including group-1 controls were monitored via an implantable
960 programmable temperature transponder (IPTT-300 BMDS, Seaford, DE), one each between the shoulder
961 blades and left hip. Temperatures were recorded daily from day -7 through 0 (before exposure) and every
962 6±1 hours post-challenge. The threshold for a significant increase in body temperature (SIBT) was
963 previously established as the mean pre-challenge (or baseline) temperature plus 2 standard deviations
964 (SD) of the pre-challenge temperature (47).

965 **Sample collection.** Samples collected for Group-1 included whole blood for quantitative bacteremia
966 and plasma for toxins in EDTA tubes at -7 days pre-exposure (-7D) and post-exposure at 18-, 24-, 30-, 36-,
967 48-, 60-, 72-, 84-, and 96-hours and terminal sample when possible. Plasma sample collection was
968 adjusted for Group-2 animals to include a 42-hour collection time and additional samples at 108-, 120-,
969 132-, and 144-hours and end of study (day 14). Whole blood for critical blood counts (CBC) (hematology)
970 was collected in EDTA tubes for Group-1 animals at -7D, 18-, 24-, 30-, 36-, 48-, and 72-hours and for
971 Group-2 at -7D, 18-, 24-, 30-, 36-, 48-, 72-, 84-, 96-, 108-, 120-, and 144-hours post-challenge.

972 **Quantitative bacteremia.** The concentration in colony forming units (CFU) of *B. anthracis*
973 vegetative bacteria per mL of whole blood was determined by serial dilution, plating and colony
974 enumeration as described previously (65). Briefly, 100 µL each whole blood sample was diluted in 900 µL
975 phosphate-buffered saline (PBS), then serially diluted 10-fold in PBS up to 10^7. Then 100 µL of each
976 sample and dilutions were plated in triplicate onto Tryptic Soy Agar, incubated at 37°C for 16-24 h. Mean
977 colony counts from 25-250 cfu/mL, below the quantitative range, were approved for reporting by the
978 Study Director and used here as reported. The quantitative LOD was 100 cfu/mL. Values down to 25
979 cfu/mL when reported were included. A result of '+' indicated that the mean colony counts for a dilution
980 set (2 of 3 plates) was less than 10. For within group quantification, such as at symptom onset, values of
981 '+' were assigned the qualitative bacteremia LOD of 6 cfu/mL (46) and negative samples were assigned a
982 value at ½ the LOD, 3 cfu/mL.

983 **Hematology.** Hematology, CBC (whole blood) measurements were determined at Battelle with the
984 Advia® 120 (Siemens, Deerfield, IL). Measurements included, white blood cell count and differential
985 leukocyte count (absolute and percentage), hemoglobin, hematocrit, red blood cell count, mean
986 corpuscular volume (MCV), mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration

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987 (MCHC), corpuscular hemoglobin concentration mean (CHCM), red cell distribution width (RDW), platelet
988 count, mean platelet volume (MPV), neutrophil:lymphocyte ratio (N/L ratio).

989 **Mass spectrometry (MS) methods for anthrax toxins.** MS methods for anthrax toxins were
990 conducted at CDC. Validated methods for analysis of total-LF and LTx have been described previously (22,
991 44). Activity of EF monomer and ETx (PA_{63}-EF) are congruent and were analyzed for EF activity as
992 described (23). Analysis of total-PA and PA83 for select tryptic peptides in the PA20 and PA63 domains
993 was also performed as described (45). The limits of detection for all the anthrax toxin methods are
994 included in Table 2. Performance characteristics are detailed in the method publications (22, 23, 44, 45).

995 Anthrax toxin MS methods provide high sensitivity, specificity, and accurate measures of all anthrax
996 toxins (22, 45). Magnetic monoclonal antibody bead capture allows selective immunoprecipitation of
997 toxins, (Step 1), followed by endoproteinase activity of LF/LTx, adenylate cyclase activity of EF/ETx and
998 on-bead tryptic digest for PA (Step 2), and detection of LF/LTx and EF/ETx catalytic products and PA
999 tryptic peptides by isotope-dilution MS (Step 3). The detection of catalytic products of LF, LTx, EF and ETx
1000 activity, facilitates signal amplification and enables exquisite analytic sensitivity. LC-MS/MS quantification
1001 of specific PA tryptic peptides allows accurate quantification of both total-PA and PA83, and
1002 determination of the amount of active PA63. These methods were used to measure toxins in cynomolgus
1003 macaques throughout infection. Throughout this report, total LF is referred to as LF and the amount of LF
1004 in complex with PA63 as LTx. Similarly, total EF is EF and in complex as ETx. Total PA is described as PA
1005 and the individual components as PA83 or PA63.

1006 **Pathology.** All cynomolgus macaques, including the survivor to the end of the study, underwent
1007 complete necropsy with recording of gross observations. The brain, kidney liver, lung, bronchial,
1008 mandibular, and mesenteric lymph nodes, spleen, and gross lesions were collected and preserved in 10%
1009 neutral buffered formalin. Preserved tissues were trimmed, paraffin-embedded, sectioned to
1010 approximately 5 microns in thickness, affixed to glass slides, and stained with hematoxylin and eosin for
1011 brightfield microscopic examination by a board-certified veterinary pathologist. Microscopic findings
1012 were graded semi-quantitatively using the following scale: minimal (grade 1) for a minimally detectable
1013 lesion, mild (grade 2) for an easily visualized lesion, moderate (grade 3) for a lesion affecting larger areas
1014 of the tissue and marked (grade 4) for lesions affecting maximum areas of the tissue. Group summary
1015 incidence and average severity calculations were performed for fast and slow progression cohorts. Total
1016 Bacteria scores were calculated for each animal by adding the individual microscopic severity scores for
1017 bacteria in all examined organs. Total Pathology scores were calculated for each animal by adding the
1018 individual microscopic severity scores for all anthrax-related lesions (except for bacteria) in all examined
1019 organs as well as the incidence of any gross lesions in the animal.

1020 **Statistical Analysis and Reporting.** Toxin levels for total LF (LF), LTx, total EF (EF) and ETx were
1021 measured and reported in ng/mL. Total PA (PA) and PA83 were measured in pmol/mL and converted to

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1022 ng/mL. Calculations of total PA, PA63 and PA83 were determined as follows: In pmol/mL total PA minus
1023 PA83 gives PA63 (pmol/mL). PA83 (83 kDa) and PA63 (63 kDa) pmol/mL value multiplied by unit adjusted
1024 83 and 63 ng/pmol, respectively give ng/mL values each which were added to give total PA (PA63+PA83)
1025 in ng/mL. Total PA is referred to as PA and any references to PA63 and PA83 are specified.

1026 All calculations of mean, standard deviation, median and interquartile range (IQR), 95 percent
1027 confidence intervals, and other analyses where reported were performed using standard equations in
1028 Microsoft Excel or JMP®, Version 15, SAS Institute Inc., Cary, NC, 1989-2019. Levels of toxins and
1029 bacteremia at specific time points or stages often varied by multiple logs and were skewed
1030 (nonparametric). Median and interquartile ranges accurately represented the center of data and range.
1031 Mann Whitney Wilcoxon Rank Sum Test (Wilcoxon/Kruskal-Wallis Rank Sum Test in JMP) was used to test
1032 the probability that two populations are equal for toxin levels between animals with fast vs slow
1033 progression (66). P-value (Prob>|Z|) is reported for the normal approximation test for the two groups (Z
1034 test statistic. Alpha level set at 0.05.
1035 For hematological parameters, time post-challenge was limited to 48h because only two animals with
1036 fast progression had later measurements, prohibiting comparisons beyond 48h. This demonstrates early
1037 changes and differences that may contribute to fast and slow progression. Oneway ANOVA was used to
1038 compare differences in means between animals with fast and slow progression at specific time points.
1039 JMP Fit model was used to test the effect of hematology over the time period day -7 pre-challenge, 24-,
1040 36- and 48-hours and for progression (fast vs slow). P-value reported for each term was prob > |t| (t-
1041 ratio) and for overall effect tests was prob > F (F-ratio). Alpha level set at 0.05
1042 **Progression determination and kinetics of toxemia.** Animals fell into two groups defined by the
1043 highest toxin levels and bacteremia reached by 48 hours. Toxemia/bacteremia was higher in 11 animals
1044 that died early (fast progression) and were lower in 11 animals that survived longer (slow progression)
1045 (n=11) and one animal that survived to the end of the study . In four of 23 animals the initial phase-1 rise
1046 proceeded directly to terminal phase-3 (monophasic). The phases were primarily triphasic, consisting of
1047 an initial rise (phase-1), plateau/decline (phase-2), and final rise (phase-3). Of all biomarkers measured,
1048 LF appeared earliest and was the most consistent (least variable) biomarker during the phase-2 plateau.
1049 Therefore, the kinetics of LF was used as the model to estimate the stages of each animal. This estimation
1050 is limited by the collection time points. Bacteremia was used to evaluate the kinetics for missing toxin
1051 measurements at terminal time points.

1052 Phase-1 was defined by the time of first detection of LF during which it rises to a first peak, the end-
1053 of-phase-1, before the plateau or decline. The end-of phase-1 was defined as the time point when
1054 biomarker rise slowed or stopped and consequently begins phase-2. In phase-2 the phase-2 plateau
1055 continued until the final rapid rise to terminal (phase-3). Phase-3 represents the end of the disease

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1056 course at (or near) death/euthanasia. Except for bacteremia, terminal toxin levels can only be estimated
1057 'near' terminal since in each animal, final samples depended on the symptoms and time of euthanasia.
1058 Also, unpredictability and speed of final progression sometimes meant end-stage plasma samples were
1059 not available. A terminal whole blood sample for bacteremia was collected even when a plasma sample
1060 was not.

1061 Note that some animals were missing a sample collection related to a specific stage. Four animals had
1062 no phase-2 (monophasic), of which one animal had no sample for toxemia after 36h (no terminal). Two
1063 animals with long survival had a long collection gap between the sample prior to the terminal time and
1064 terminal samples. Many were missing terminal plasma for toxins. For phase-3 terminal calculations, only
1065 a sample measured ≤12h from the death/euthanasia time point were included.

1066 **Phase-2 and phase-2 threshold.** In slow progression, the low variability of LF over the duration of
1067 phase-2 (< 1-order of magnitude) allowed characterization of this stage as a whole. , Phase-2 was
1068 characterized for all toxins, bacteremia, and toxin ratios and included all measurements for all time
1069 points from the end-of- phase-1 to the end-of- phase-2 from all 11 animals (n=52, 51 for ETx). The
1070 median, upper and lower quartiles, and 95% interval (upper limit) for toxins/bacteremia and 5% interval
1071 (lower limit) for toxin ratios were determined in JMP. The phase-2 upper 95% intervals represent
1072 potential critical toxin thresholds, which when exceeded signal departure from phase-2 and entry to
1073 phase-3. Lower 95% interval applies to toxin ratios.

1074 **Time to threshold and time from threshold to terminal.** The time to threshold was calculated
1075 from a linear regression for the two time points between which the threshold fell for all animals with
1076 available points. Animals with phase-2 levels within the typical standard error (≤10%) of the threshold for
1077 LF and followed with lower levels for an extended phase-2, the threshold was determined not to have
1078 been exceeded and was calculated at the later time when the final rise continued. Five animals were
1079 excluded from the threshold calculations. For one animal with fast progression and one with slow
1080 progression the threshold was not exceeded, but later (terminal) samples were missing so the time to
1081 threshold could not be calculated for toxins (Fig 3, animal 1, 19). Animals 20 and 22 with slow progression
1082 had large time-gaps in sample collection (32 and 58 hours) between the last sub-threshold level sample
1083 and final/terminal samples, such that calculation of the time to threshold could not be estimated. The
1084 survivor (animal 23) never exceeded the threshold. Two animals (Fig 3, animal 7, 10) were excluded from
1085 calculations for PA and ETx because they were below the PA, ETx detection limit before the sample that
1086 exceeded the threshold. An n=8 is indicated for the fast group for PA and ETx, versus n=10 for other
1087 toxins. Mean time to threshold was calculated for 8-11 animals with fast progression and 8-9 animals
1088 with slow progression (exact numbers-n in Fig 5). The time from threshold to terminal was determined by
1089 subtracting the calculated time to threshold from the terminal time, estimating how long an animal lived
1090 after reaching the threshold (time in phase-3).

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1262 # Supplemental Material

1263 **In order of their appearance in text:**

1264 **S1 Fig. Survival curve for 23 cynomolgus macaques with inhalation anthrax.** Proportion surviving vs
1265 survival time (hours).

1266 **S2 Fig. Significant increase in body temperature post-challenge.** The threshold for a significant increase
1267 in body temperature (SIBT) is established as the mean pre-challenge (or baseline) temperature plus 2
1268 standard deviations (SD) of the mean pre-challenge temperature. Left hip and right shoulder
1269 transponders recorded temperatures in °F once daily for seven days. The pre-challenge mean, SD and
1270 threshold (mean+2 SD) was determined for both the shoulder and hip transponders for each animal. The
1271 deviation from the pre-challenge threshold was calculated by subtracting the normal threshold
1272 temperature from the post-challenge temperatures. The solid horizontal line represents the thresholds
1273 with zero for the threshold. Deviations from threshold for shoulder and hip graphed for each animal by
1274 number according to survival time. Group-1 controls indicated with -1 (C58003) control corresponding to
1275 group 2 challenged animal number 5 and 0 (C58170) corresponding to group 2 challenged animal number
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1277 **S1 File. All anthrax toxin results in cynomolgus macaques with inhalation anthrax.**

1278 **S2 File. All bacteremia results in cynomolgus macaques with inhalation anthrax.**

1279 **S3 Fig. Kinetics of anthrax biomarkers in cynomolgus macaques with slow progression.** All toxins
1280 (ng/mL) and bacteremia (cfu/mL) over the course of infection in 12 animals with inhalation anthrax and
1281 slow progression, of which 11 were triphasic with phase-2 ≥24 hours and one with phase-3 decline and
1282 survival. One negative value at the end-of-study (EOS) (animal 23 -C58174) was graphed at ½ the limit of
1283 detection for each method as indicated in Table 2. For bacteremia (bact), non-quantifiable results of +

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1284 was assigned a 6 (+ symbols) and negative at 3 cfu/mL (x symbols). The last time point for C59618 at
1285 terminal (Term, 8.4 days) and the survivor which declined to less than the limit of detection at the end-
1286 of-study (EOS, 14-days/336 hours) are not to scale. Black boxes represent the range of levels during the
1287 phase-2 for each biomarker.

1288 **S1 Table. End-of-phase-1 toxins, bacteremia, and toxin ratios.** Individual results at the end-of-phase-
1289 1(P1) are given for animals with high toxemia, low ratios, and early time-to-death (fast progression) and
1290 animals with low toxemia, high ratios, and later time-to-death. Median, upper quartile, and lower
1291 quartile, determined in JMP. P-values comparing fast and slow progression obtained by Mann-Whitney-
1292 Wilcoxon rank sum (non-parametric) test. Levels for survivor shown separately. *Still in phase-1,
1293 excluded. **Less than the limit of detection (<LOD) for PA83 (1.22 ng/mL) given a value at ½ times the
1294 LOD for PA83 (0.61 ng/mL) for calculations.

1295 **S4 Fig. Relationship between anthrax biomarker levels and survival time (days).** Mean $\log_{10}$
1296 concentrations and standard deviations (error bars) for total LF, LTx, total EF, ETx, total PA, and
1297 bacteremia, over the time post-challenge (hours-h) for animals that died/euthanized at 1.9-3.0 days
1298 (n=10), 3.1 to 4.0 days (n=5), 4.1 to 5 days (n=4) and 5.1 to 6 days (n=2), and for one animal at 8.4 days
1299 (animal 22) and survivor to end-of-study (End) (animal 23). Final time points for animal 22 (C59618) at 8.4
1300 days and for 23 (C58174) at end-of-study (14 days) were included together (End). Gray dashed lines
1301 shown for median phase-2 level and phase-2 thresholds (red dotted line).

1302 **S3 File. All hematology results in cynomolgus macaques with inhalation anthrax.**

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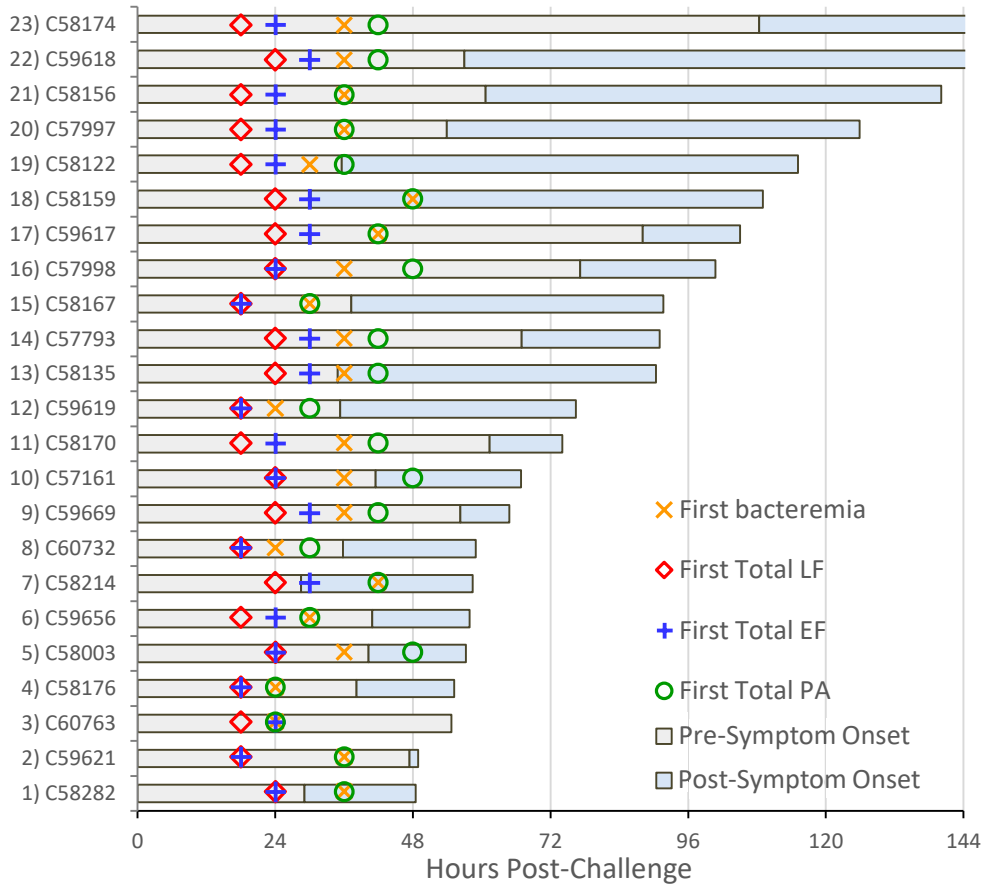
1315 **Figure 1. First detection of anthrax biomarkers.**



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1330 **Figure 2. Kinetics of bacteremia and lethal factor in 23 cynomolgus macaques with variable progression.**

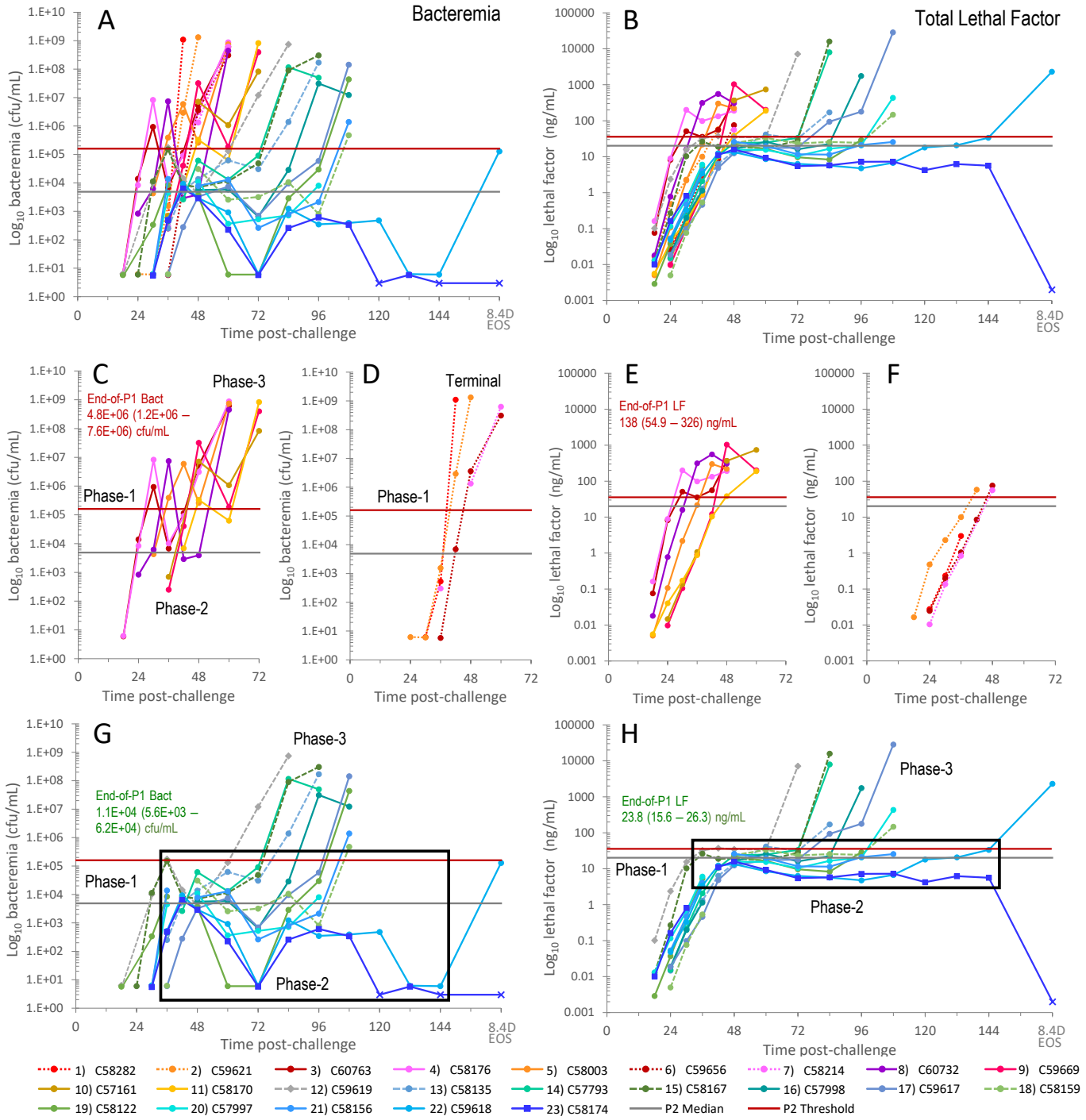

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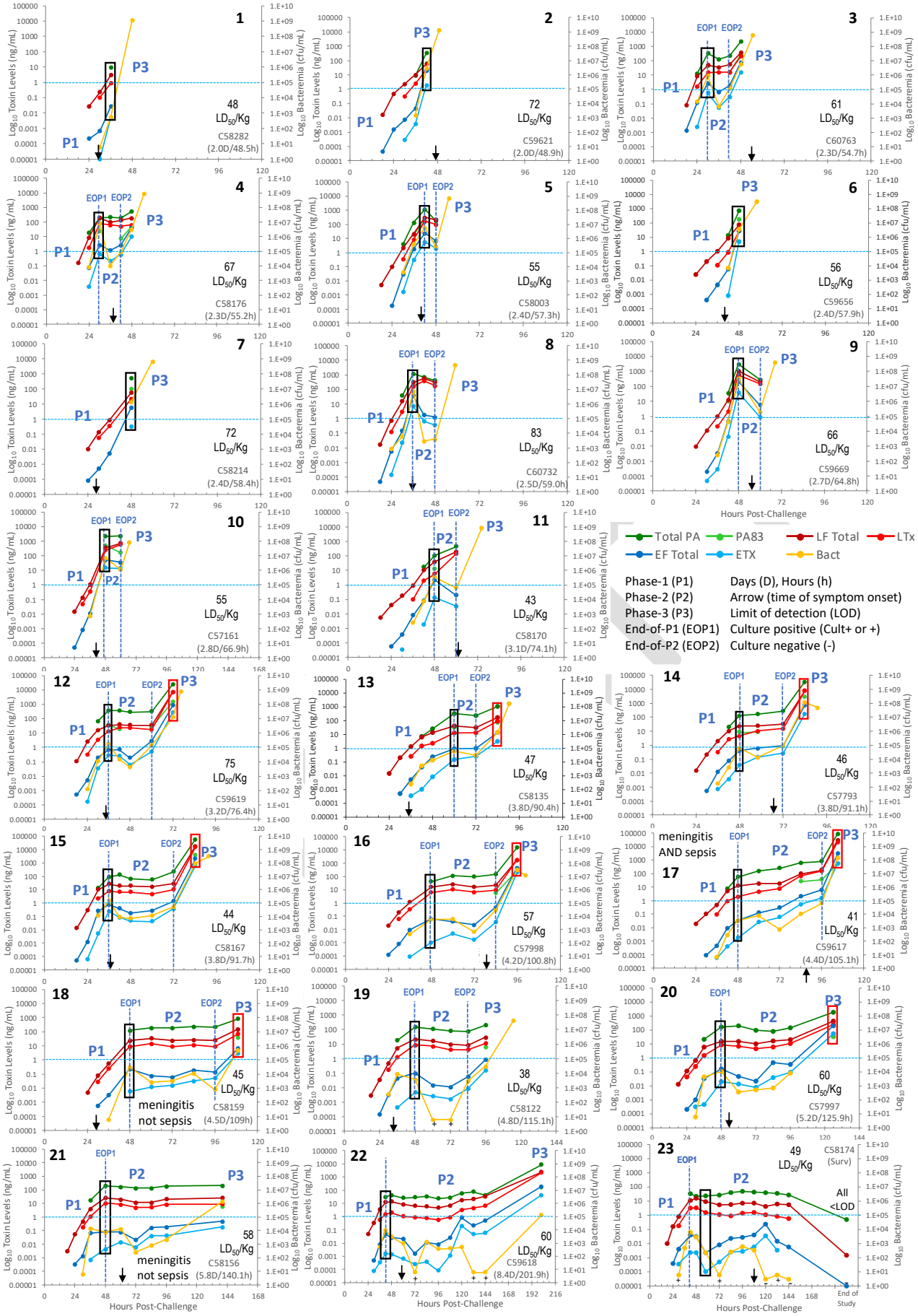
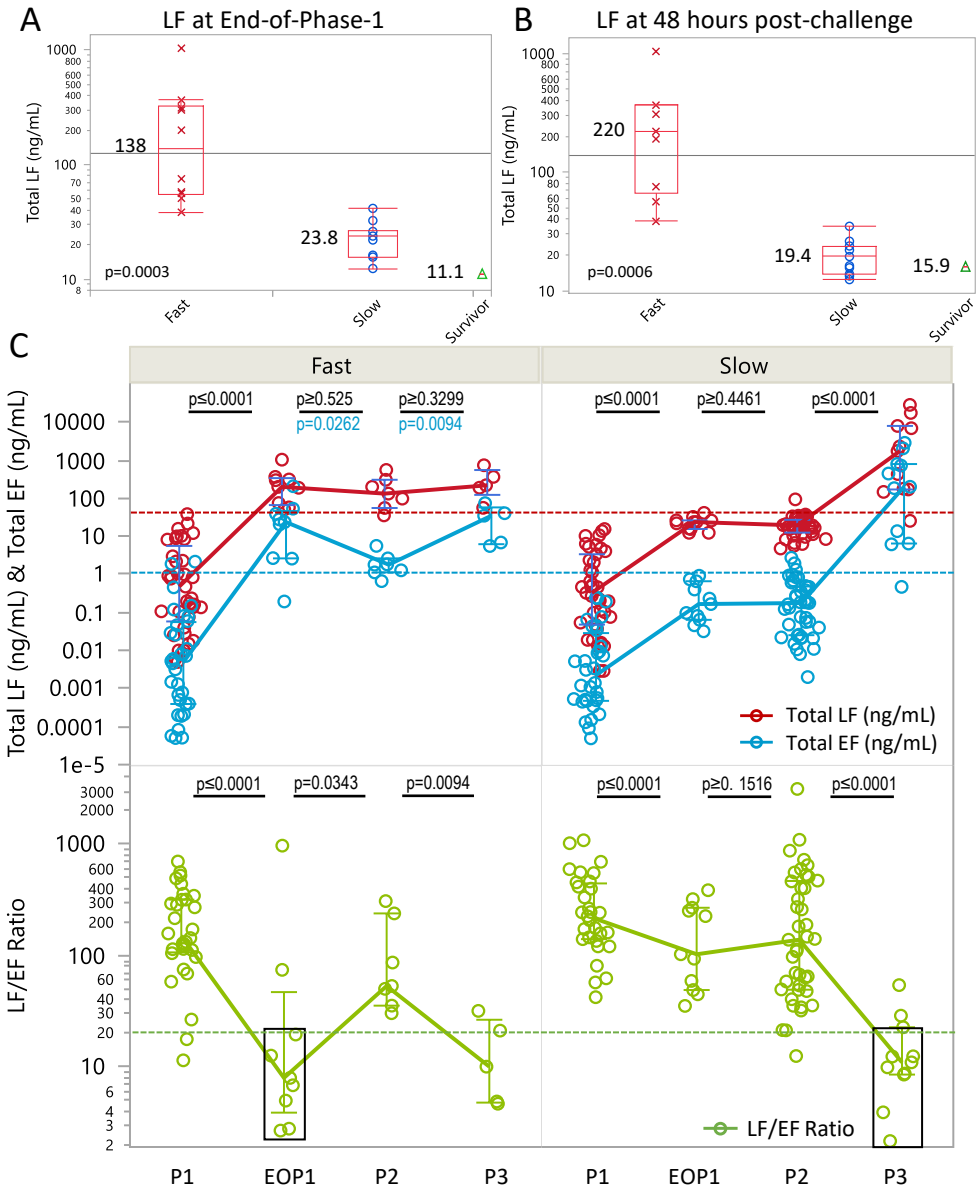
Figure 3. Anthrax toxins and bacteremia during inhalation anthrax in individual cynomolgus macaques.



1339 **Figure 4. Stage-dependent biomarker levels.**



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1349 **Figure 5. Relationship of toxemia and time.**

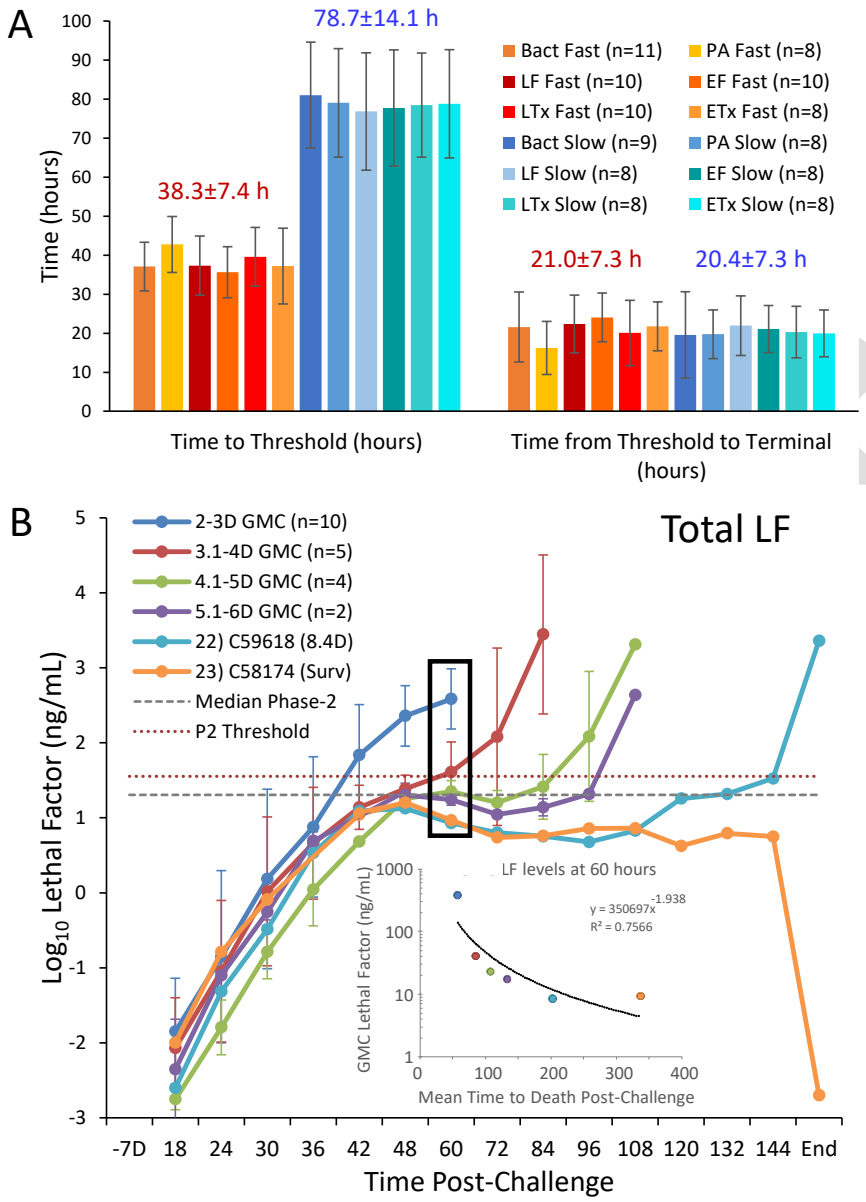

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Figure 6. Early hematology during inhalation anthrax.

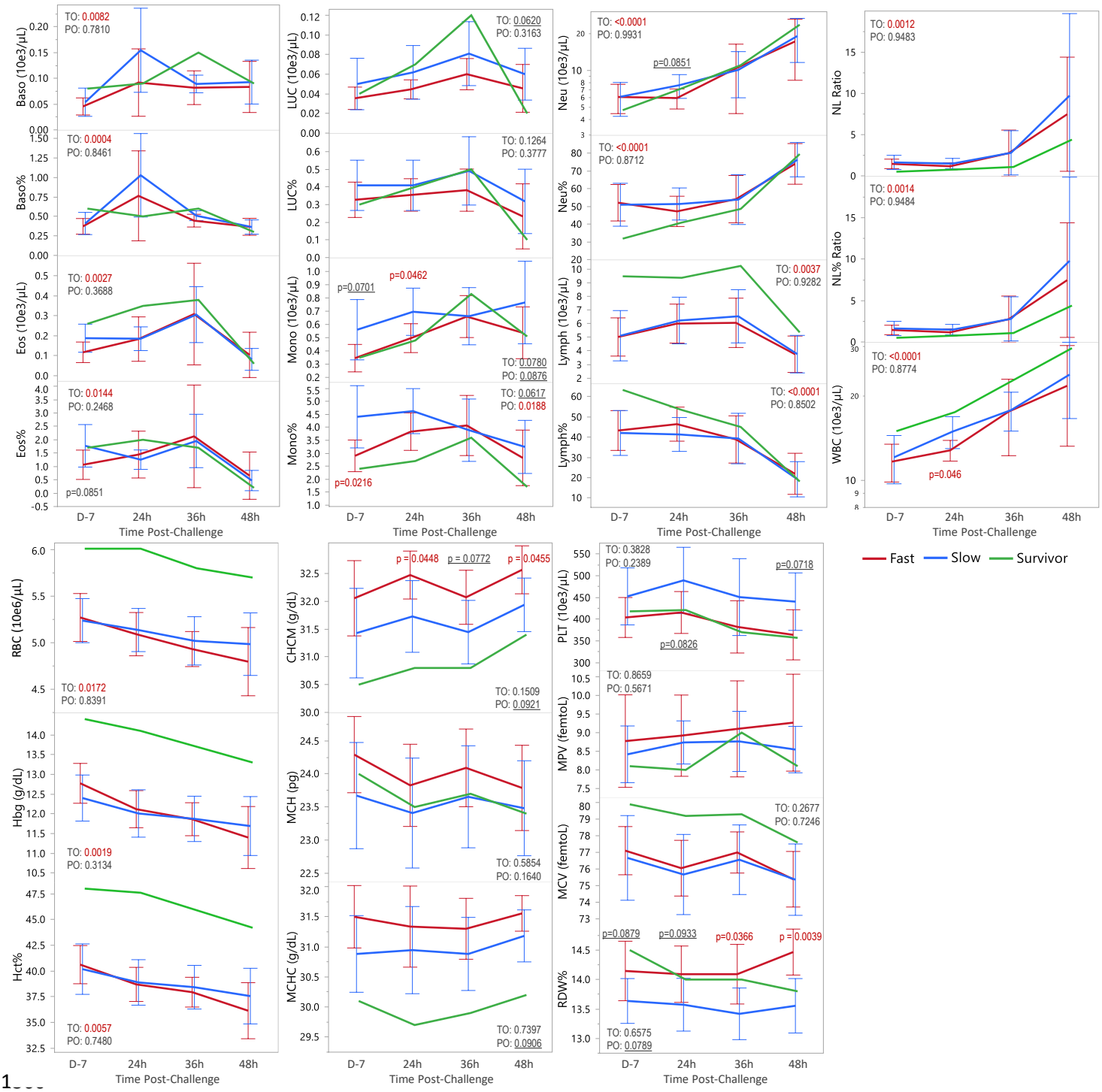
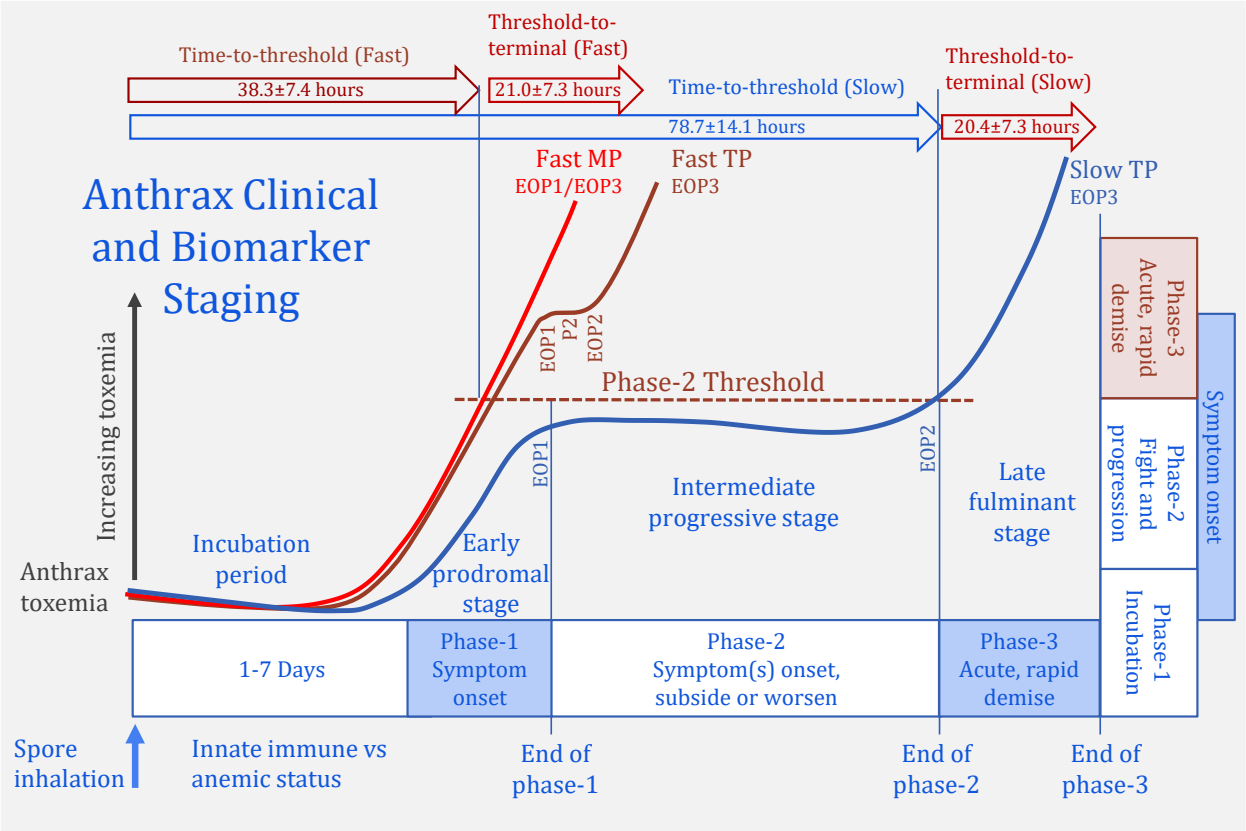
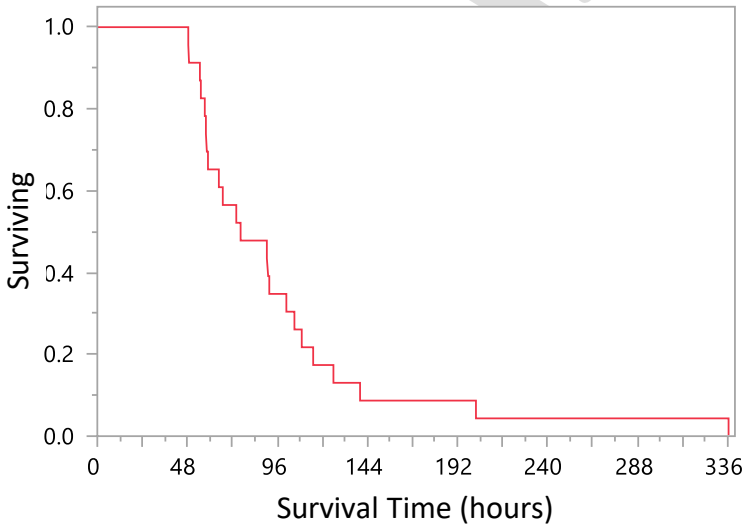


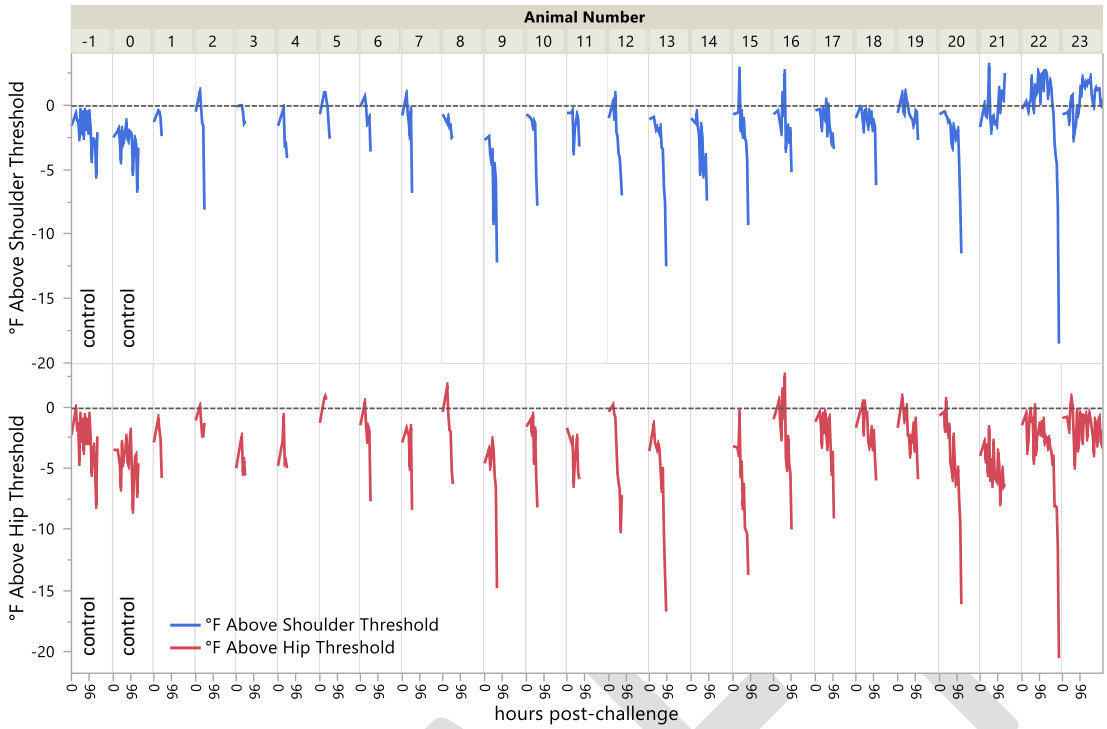
Figure 7. Proposed relationship of toxemia-defined progression with clinical staging.



S1 Fig. Analysis of survival time in 23 cynomolgus macaques with inhalation anthrax.



S2 Fig. Significant increase in body temperature post-challenge.



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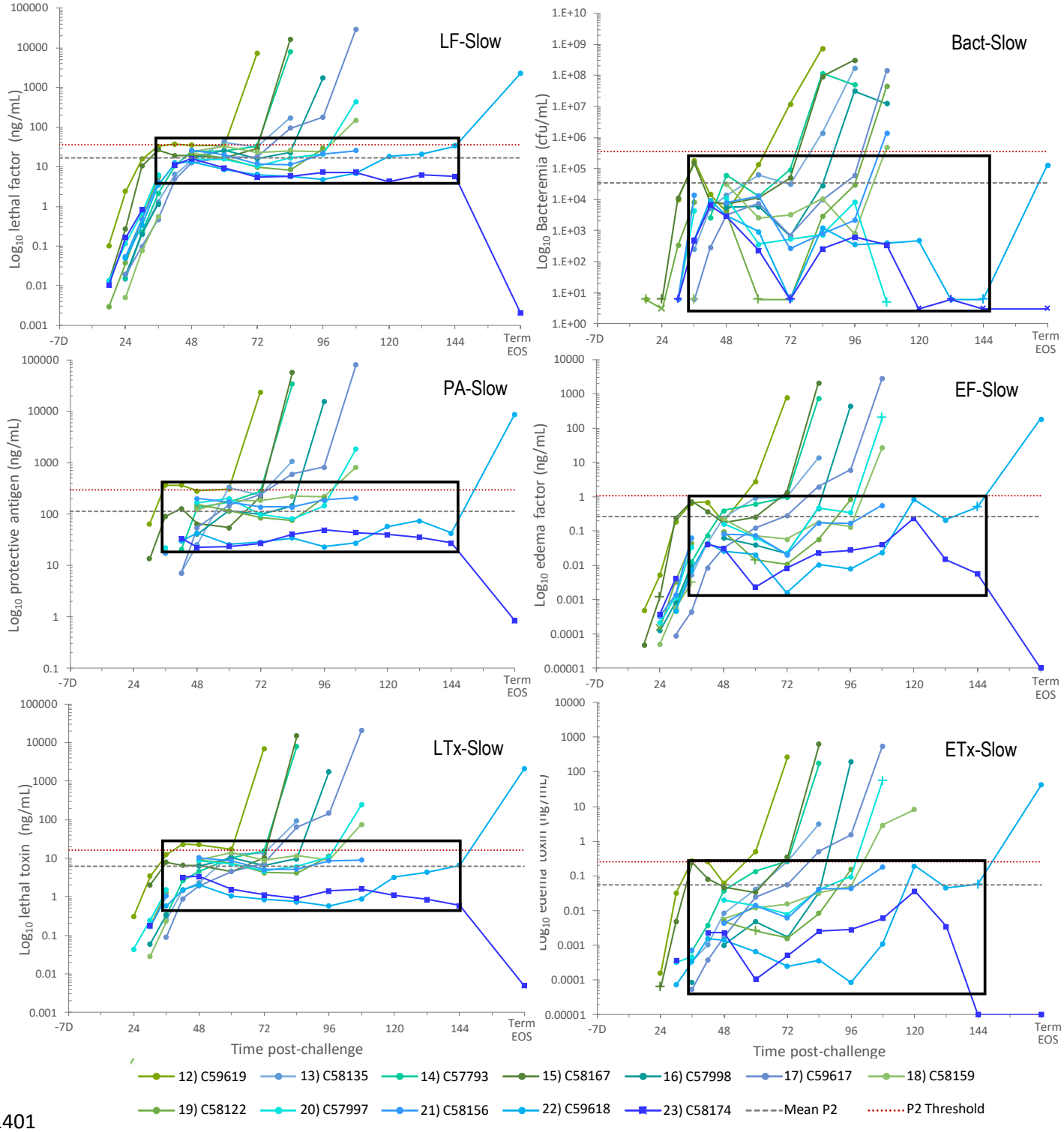
1400 **S3 Fig. Kinetics of anthrax biomarkers in cynomolgus macaques with slow progression.**



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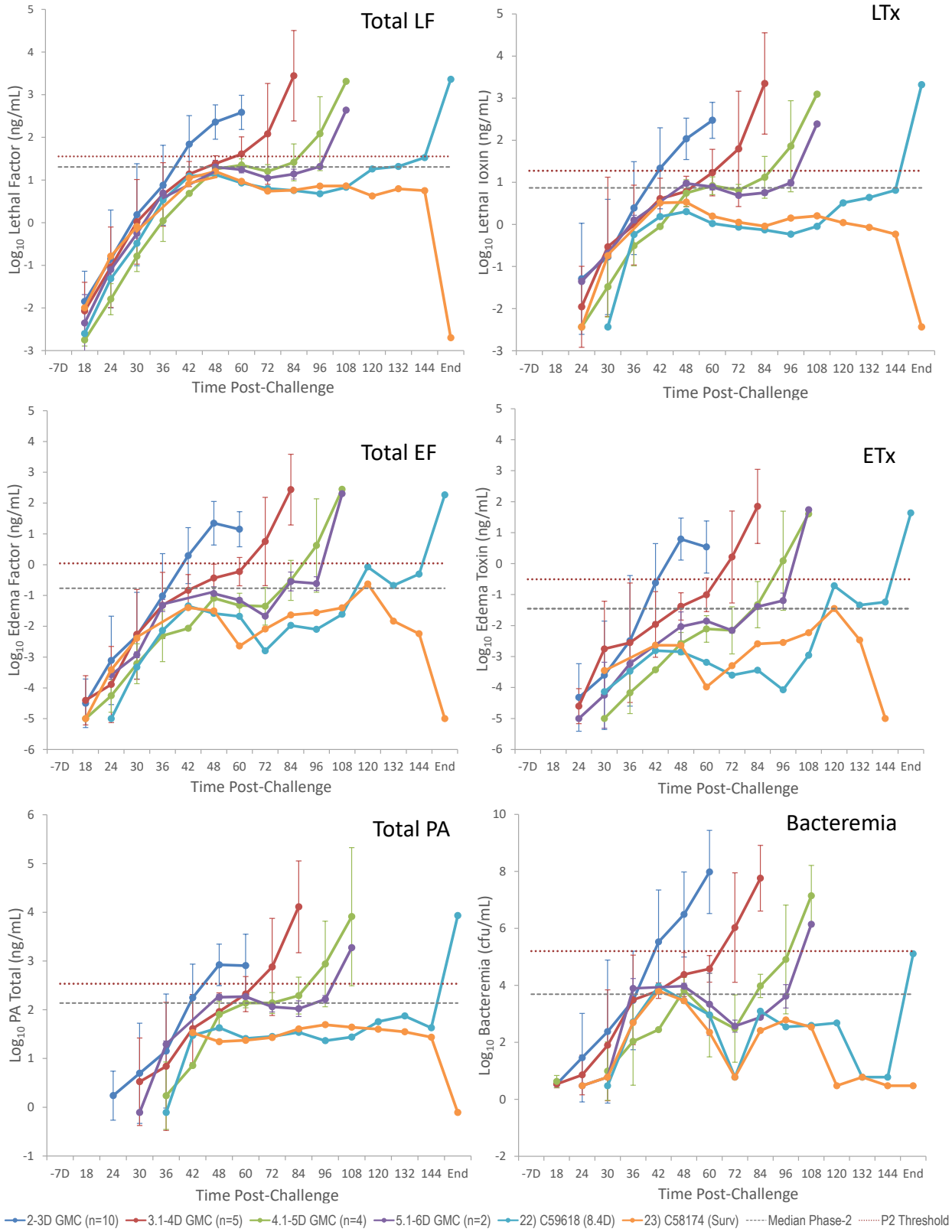
S1 Table. End-of-phase-1 toxins, bacteremia, and toxin ratios.

Animal ID	Time to Death (hours)	End-of-P1 Time Point	Total-PA (ng/mL)	PA83 (ng/mL)	Total LF (ng/mL)	Lethal Toxin (ng/mL)	Total EF (ng/mL)	Edema Toxin (ng/mL)	Bact (cfu/mL)	PA/LF Ratio	LF/LTx Ratio	LF/EF Ratio	EF/ETx Ratio	PA/ETx Ratio	Phase
1) C58282*	48.5	48 h NA (36h shown)	9.11	<LOD	2.99	0.865	0.026	0.0045	5.3E+02	3.0	3.5	115.0	5.8	2024	P1*
2) C59621	48.9	42 h	333	67.4	57.8	23.7	20.7	1.872	2.9E+06	5.8	2.4	2.8	11.1	178	EOP1-P2
3) C60763	54.7	30 h	326	45.2	50.9	15.3	2.6	0.576	9.4E+05	6.4	3.3	19.6	4.5	566	EOP1-P2
4) C58176	55.2	30 h	188	25.6	200	81.7	2.68	0.635	8.3E+06	0.9	2.4	74.6	4.2	296	EOP1-P2
5) C58003	57.3	42 h	1143	163	300	169	23.7	5.64	5.9E+06	3.8	1.8	12.7	4.2	203	EOP1-P2
6) C59656	57.9	48 h	731	182	75.2	27.7	28	4.97	3.6E+06	9.7	2.7	2.7	5.6	147	EOP1-P2
7) C58214	58.4	48 h	509	97.6	56.2	21	5.58	0.307	1.3E+06	9.1	2.7	10.1	18.2	1658	EOP1-P2
8) C60732	59	36 h	1189	176	313	165	39.2	7.1	7.4E+06	3.8	1.9	8.0	5.5	167	EOP1-P2
9) C59669	64.8	48 h	3035	621	1038	573	208	38.9	3.2E+07	2.9	1.8	5.0	5.3	78	EOP1-P2
10) C57161	66.9	48 h	2221	516	366	245	53.4	15	7.2E+06	6.1	1.5	6.9	3.6	148	EOP1-P2
11) C58170	74.1	48 h	102	12.1	38.4	5.53	2.16	0.134	3.3E+05	2.7	6.9	17.8	16.1	761	EOP1-P2
	Median		620	130	138	54.7	22.2	3.4	4.8E+06	4.8	2.4	9.0	5.4	190	
	lower quartile		328	40.3	54.9	19.5	2.7	0.51	1.2E+06	2.9	1.8	4.4	4.2	148	
	upper quartile		1447	266	138	188	42.8	9.1	7.6E+06	7.1	2.9	18.2	12.3	615	
12) C59619	76.4	36 h	368	36.2	32.5	12.5	0.661	0.278	1.8E+05	11.3	2.6	49.2	2.4	1324	EOP1-P2
13) C58135	90.4	60 h	328	33.1	41.5	13	0.922	0.153	6.2E+04	7.9	3.2	45.0	6.0	2144	EOP1-P2
14) C57793	91.1	48 h	133	8.8	23.8	4.55	0.401	0.038	6.1E+04	5.6	5.2	59.4	10.6	3500	EOP1-P2
15) C58167	91.7	36 h	89.7	7.6	26.3	7.94	0.743	0.242	1.5E+05	3.4	3.3	35.4	3.1	371	EOP1-P2
16) C57998	100.8	48 h	41	<LOD**	16.2	6.32	0.064	0.001	5.6E+03	2.5	2.6	253.1	64.0	41000	EOP1-P2
17) C59617	105.1	48 h	54.2	<LOD**	12.4	1.91	0.032	0.002	3.3E+03	4.4	6.5	387.5	16.0	27100	EOP1-P2
18) C58159	109	48 h	121	7.89	23.8	8.76	0.231	0.0057	3.1E+04	5.1	2.7	103.0	40.5	21228	EOP1-P2
19) C58122	115.1	48 h	152	<LOD**	22	9.03	0.097	0.0048	3.6E+03	6.9	2.4	226.8	20.2	31667	EOP1-P2
20) C57997	125.9	48 h	165	11.8	15.6	8.62	0.166	0.020	1.1E+04	10.6	1.8	94.0	8.3	8250	EOP1-P2
21) C58156	140.1	48 h	200	<LOD**	26.1	10.3	0.081	0.0043	7.8E+03	7.7	2.5	322.2	18.8	46512	EOP1-P2
22) C59618	201.9	42 h	29.6	<LOD**	12.4	1.53	0.046	0.0015	9.4E+03	2.4	8.1	269.6	30.7	19733	EOP1-P2
	Median		133	7.6	23.8	8.6	0.17	0.0057	1.1E+04	5.6	2.7	103	16	19733	
	lower quartile		54.2	0.61	15.6	4.6	0.064	0.0020	5.6E+03	3.4	2.5	49.2	6.0	2144	
	upper quartile		200	11.8	26.3	10.3	0.66	0.15	6.2E+04	7.9	5.2	270	30.7	31667	
	Fast-v-Slow p-value		0.0035	0.0003	0.0002	0.0011	0.0001	0.0003	0.0001	0.5495	0.149	0.0004	0.098	0.0004	
23) C58174	Survivor	42 h	33.9	<LOD	11.1	3.25	0.040	0.0023	6.4E+03	3.05	3.42	278	17.4	14739	EOP1-P2

DRAFT

1 **S4 Fig. Relationship between anthrax biomarker levels and survival time (days).**



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