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1 **Intraperitoneal treatment with antimicrobial peptide rescues mice from a pulmonary**
2 ***Francisella* infection**

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7 **Abstract**

8 Our long-term goal is to identify new antimicrobial peptides that might be effective
9 against pneumonic *Francisella* infection in mice. Previously, our group searched the peptidome
10 of the American alligator for novel cationic antimicrobial peptides and identified a naturally-
11 occurring C-terminal fragment of apolipoprotein C-1, which we called Apo6. This peptide was
12 found to have antibacterial activity against the ESKAPE pathogens, including those exhibiting
13 multi-drug resistance. In this work, we tested Apo6 and synthetic derivatives for antibacterial
14 activity against *Francisella tularensis* including the virulent strain *F. tularensis* SchuS4.
15 *Francisella* is inherently highly resistant to the cyclic peptide polymyxin antibiotics and beta-
16 lactam antibiotics. We found that our synthetic peptide derivatives (called GATR peptides),
17 designed with increased hydrophobicity and charge, had generally stronger *in vitro* antimicrobial
18 activity against *Francisella* than the parent peptide Apo6. The GATR peptides had a greater
19 effect on the bacterial membrane than the Apo6 peptide and were able to bind *Francisella* LPS,
20 suggesting their mechanism of action against *Francisella*. Cytotoxicity experiments showed low
21 cytotoxicity for most of the GATR peptides, and whole organism toxicity studies in the
22 waxworm allowed us to down-select to two our lead peptides, GATR-3 and GATR-6. These
23 peptides were tested in a murine pulmonary tularemia model. We found that the GATR-3 peptide
24 rescued 50-60% of mice from lethal tularemia infection when administered systemically through

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25 the intraperitoneal route. This peptide is a candidate for further pre-clinical studies for a potential
26 peptide-based approach to tularemia.

27 Keywords: *Francisella tularensis*, tularemia, antimicrobial peptide, *in vivo*, peptide treatment,
28 pneumonic tularemia, MIC, waxworm, cytotoxicity, hemolysis.

29 **Acknowledgements**

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33 discussions.

34 Introduction

35 *Francisella tularensis* is a Gram-negative bacterium that is the causative agent of
36 tularemia. The virulent strains (*F. tularensis tularensis*) can cause disease in humans with
37 inhalation of as few as 10 organisms. In addition, this organism is easily aerosolized and has
38 historically been developed as a bioweapon [1-3]. The United States government has classified
39 *F. tularensis* as a Tier 1 and Category A Select Agent. *F. tularensis* subsp. *tularensis* is found in
40 the United States [4], with localized outbreaks currently occurring across the continent, and is
41 also referred to as the Type A strain. The less virulent Type B strain (*F. tularensis* subsp.
42 *holarctica*) is more commonly found to infect humans in Europe [5]. *F. tularensis* infections
43 (tularemia) are normally treated with fluoroquinolones and aminoglycosides, but are inherently
44 resistant to some antibiotics such as beta-lactams [3, 6] and polymyxins [7, 8]. In addition, drug
45 resistance to conventional antibiotic treatments may be emerging in this species [9, 10], and there
46 is a concern about potentially engineered resistance in the biothreat context. Because of this,
47 there is interest in developing new potential treatments for tularemia [3].

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48 Cationic antimicrobial peptides are small positively-charged peptides, some of which are
49 produced by the innate immune system of vertebrates as well as other organisms [11]. Cryptic
50 cationic antimicrobial peptides are proteolytic fragments of larger proteins; these larger proteins
51 have annotated functions that are not themselves antimicrobial [12, 13]. Cationic antimicrobial
52 peptides can have broad or specific activity against bacteria, viruses, or fungi and can also have
53 host-directed immuno-modulatory functions [11, 14, 15]. Our group has been working to
54 discover novel antimicrobial peptides from reptiles, including the American alligator (*Alligator*
55 *mississippiensis*) and the Komodo dragon (*Varanus komodoensis*) [16-18]. We have discovered a
56 number of antimicrobial peptides with strong antimicrobial activity against a range of bacteria.
57 Previously, we found that peptides representing the C-terminal fragments of an apolipoprotein in
58 *A. mississippiensis* had antimicrobial activity against *Bacillus cereus*, *Escherichia coli*,
59 *Pseudomonas aeruginosa,* and *Staphylococcus aureus*, as well as drug-resistant strains of *E. coli,*
60 *P. aeruginosa, S. aureus,* and *Acinetobacter baumannii* [16, 19].

61 Previous work using canonical and synthetic antimicrobial peptides delivered to the
62 mouse lung as a potential therapy for pneumonic tularemia in mice [20] was not able to
63 demonstrate any significant rescue from infection. A variety of peptides were tested including
64 the human cathelicidin peptide, LL-37, and synthetic fusions of peptides. We sought to identify
65 antimicrobial peptides that might be effective against pneumonic *Francisella* infection in mice.

66 In this work, we tested one of our previously discovered alligator peptides, called Apo6,
67 against *Francisella*. We then created a set of synthetic peptide derivatives of the Apo6 peptide by
68 making amino acid substitutions that increased the hydrophobicity and charge of the peptides in
69 order to increase the potency. These synthetic peptides are named GATR-1 through GATR-7.
70 We examined how these variations change the mechanism of action and the binding to

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71 *Francisella* lipopolysaccharide (LPS). We down-selected the set of peptides based on
72 cytotoxicity, and tested the efficacy of our lead peptides in two infection models: first in the
73 waxworm (*Galleria mellonella*) and then in a murine tularemia pneumonic infection model.
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76 **Materials and Methods**

77 **Bacterial strains.** *Francisella tularensis* subsp. *holarctica* CDC Live Vaccine Strain (NR-646),
78 *F. tularensis* subsp. *tularensis* NIH B38 (NR-50), an attenuated strain, and *F. tularensis* subsp.
79 *tularensis* SchuS4 (NR-10492), the fully virulent strain, were obtained from BEI Resources
80 (Manassas, VA). Bacteria were grown 48-72 h on chocolate II agar (BD 211267) at 37°C with
81 5% CO_2. Prior to the experiments below, bacteria were scraped off the plate and resuspended to
82 0.5 McFarland units in phosphate buffered saline (PBS) or Buffer Q [6.12 mM sodium
83 monohydrogen phosphate heptahydrate; 3.92 mM monosodium phosphate anhydrous; 0.3 g/L
84 tryptic soy broth (BD211825); 1 mg/L cysteine HCl]. A standard curve of bacteria was used to
85 determine the CFU equivalents (0.5 McFarland units = 1×10^7 CFU/ml). Resuspended bacteria
86 were then diluted to the appropriate concentration needed. All work with *F. tularensis* SchuS4
87 was performed in a BSL-3 laboratory following strict safety guidelines.

88 **Peptide synthesis**. Peptides were synthesized by ChinaPeptides, Inc (Shanghai, China) using
89 Fmoc chemistry. Peptide was provided at >95% purity, which was confirmed with RP-HPLC
90 and ESI-MS. Sequences and physico-chemical properties are shown in **Table 1.**

91 **Peptide properties**. Physico-chemical properties including charge, hydrophobic moment and
92 hydrophobicity as well as helical wheels were calculated using Heliquest [21]. In addition, the
93 APD defined total hydrophobic ratio was calculated with the APD3 website [22].

94 **Minimal inhibitory concentration (MIC) determination assay**. MICs were determined
95 according to CLSI guidelines for this organism [23, 24]. Briefly, *Francisella* bacteria were
96 grown on chocolate II agar (BD 221169) for 48-72 h prior to experiments. Minimal inhibitory
97 concentration experiments were performed in Cation-adjusted Mueller Hinton Broth (BD
98 212322, CAMHB) with 2% IsovitaleX (BD 211875) using polypropylene plates [25].

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99 Approximately 3×10^4 bacteria were added to each well, as determined using a McFarland
100 standard curve for *Francisella*. Results were analyzed at 21 and 42 μg/ml using a two-way
101 ANOVA with Sidak's multiple comparisons.

102 **Antimicrobial assays.** The antimicrobial activity of antimicrobial peptides against *F. tularensis*
103 was determined as described previously [26-28]. Briefly, in a 96 well plate, 1×10^5 CFU per well
104 were incubated with various peptide concentrations in Buffer Q for 3 h at 37°C (total volume
105 100 μl). After the incubation, well contents were serially diluted, and 5 μl of each dilution was
106 spotted onto chocolate agar and allowed to dry. Agar plates were incubated overnight at 37 °C
107 and the colonies were counted. The concentration of peptide required to kill 50% of microbial
108 population (EC_{50}) was analyzed by analyzing the percentage of surviving colonies after the
109 overnight incubation as a function of log of peptide concentration. The data was analyzed
110 through GraphPad Prism 6 (GraphPad Software Inc. San Diego, CA, USA). The antimicrobial
111 activity of the derivatives was compared to the activity of LL-37, a human cathelicidin with
112 known antibacterial activity against *Francisella* [27, 29]. The confidence intervals along with the
113 EC_{50} values for each peptide are reported in Table 2. Samples were run in triplicate on three
114 separate occasions.

115 **Membrane depolarization assay**. Membrane potential was measured using a fluorescent assay
116 utilizing $DiSC_3(5)$ dye as previously described with some modification [19, 28]. *F. tularensis*
117 LVS was grown on chocolate II agar (48 h, 37°C, 5% CO_2), and the colonies were suspended in
118 10 mM phosphate buffer to 0.5 McFarland standard. 100 μL of this suspension was added to
119 wells of a black polypropylene 96 well plate. The plate was incubated in a Tecan Infinite F200
120 fluorimeter. A change in the fluorescence was monitored until equilibrium is reached, evidenced
121 by quenching of the fluorescent signal, indicating maximum uptake of the dye by the membrane.

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122 The experimental wells were then treated with 100 µl of various concentrations of peptide
123 diluted in 10 mM phosphate buffer. The plate was returned to the spectrofluorometer and
124 readings were taken every min for 15 min (excitation=620 nm; emission=670 nm). Peak RFU at
125 each concentration was used in the analysis. Samples were run in triplicate on two separate
126 occasions. Bacteria without peptide treatment were used as a negative control, and LL-37 was
127 used as positive control. Depolarization results were analyzed using a one-way ANOVA with
128 Dunnett's multiple comparisons.

129 **Ethidium bromide uptake assay.** Pore formation in *F. tularensis* LVS cytoplasmic membrane
130 was assessed using ethidium bromide as described previously with some modification [19, 28].
131 *F. tularensis* LVS was grown on chocolate II agar (48 h, 37°C, 5% CO_2) and colonies scraped
132 into solution. Bacteria were suspended in 10 mM phosphate buffer to 0.5 McFarland standard. In
133 a black polypropylene 96 well plate, 180 µL bacterial culture was then mixed with 10 µM
134 ethidium bromide (final concentration) and incubated with varying concentrations of peptide.
135 The plate was read in a Tecan infinite F200 fluorimeter every 2 min for 20 min at 37°C
136 (excitation=535 nm, emission=590 nm). Data shown is from the 20 min mark. Samples were run
137 in triplicate on three separate occasions. Bacteria without peptide was used as a negative control,
138 and LL-37 was used as positive control. Results were analyzed using a one-way ANOVA with
139 Dunnett's multiple comparisons.

140 **LPS binding**. To examine the potential binding between *F. tularensis* LVS lipopolysaccharide
141 (LPS) and the GATR peptides, an LPS-binding assay using 1,9-dimethylmethyl blue (DMMB)
142 was performed as previously described [30]. LPS from *F. tularensis* subsp. *holarctica* LVS was
143 obtained from BEI Resources (NR-2627). Briefly, 150 µg/ml of LPS was incubated with 10
144 µg/ml of peptide in distilled endotoxin-free water for 1 h. The solution was added to DMMB,

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145 and the absorbance was read at 535 nm on a spectrometer. Samples were run in triplicate on two
146 separate occasions. Results were analyzed using a one-way ANOVA with Dunnett's multiple
147 comparisons.

148 **Hemolysis assay**. The hemolysis assay was performed using washed, defibrinated sheep blood
149 as previously described [31]. Sheep red blood cells (2% RBC) in phosphate buffered saline
150 (PBS) were added to various dilutions of peptide reconstituted in PBS in a sterile U-bottom 96
151 well plate. The plate was incubated for 1 h at 37 °C and then centrifuged at 1000 rpm for 2 min.
152 The supernatant was transferred to a fresh plate and read at 540 nm on a spectrometer. Sheep
153 RBCs (2%) with PBS alone served as the negative control, and 2% RBC in water as the positive
154 control. Experiment was performed twice in triplicate. A representative experiment is shown
155 Results were analyzed using a one-way ANOVA with Dunnett's multiple comparisons.

156 **Cytotoxicity assay.** Cytotoxicity assays were performed using the Vybrant MTT Cell
157 Proliferation Assay Kit (Life Technologies) according to manufacturer's instructions. Assays
158 were performed using human lung epithelial carcinoma line A549 (ATCC CCL-185) and human
159 liver carcinoma line HepG2 (ATCC HB-8065), which were maintained at a low passage in
160 Dulbecco's Minimal Essential Media (Life Technologies 11995073) with 10% heat-inactivated
161 fetal bovine serum and 13 U/ml penicillin-streptomycin. 100 μg/ml of peptide was used for each
162 experimental well, added to the cell growth medium, and incubated for 24 h. Each experiment
163 was performed in triplicate two times. A representative experiment is shown. Results were
164 analyzed using a one-way ANOVA with Dunnett's multiple comparisons.

165 **Peptide toxicity in *Galleria mellonella* larvae.** Larvae were used to assess *in vivo* toxicity of
166 peptides. *G. mellonella* larvae (greater wax moth larvae or "waxworms") were obtained from
167 Vanderhorst Wholesale (Saint Marys, OH, USA). Ten larvae of equal size/weight were randomly

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168 assigned to each group and placed into labeled petri dishes. A 1 ml syringe with a 27G needle
169 was used to inject 10 µl containing 10 µg peptide into each larvae's right proleg. Survival was
170 observed for 48 h. Results from one representative experiment of two total are shown and were
171 analyzed using a Mantel-Cox test.

172 ***G. mellonella* infection and treatment.** Survival assay of wax moth larvae following
173 *Francisella* infection with and without treatment was conducted as previously described [2, 32,
174 33]. *G. mellonella* (wax moth larvae or waxworms) were obtained from Vanderhorst Wholesale
175 (Saint Marys, OH, USA). Ten larvae of equal size/weight were randomly assigned to each group
176 and placed into labeled petri dishes. A 1 ml syringe with a 27G needle was used to inject 10 µl of
177 $1x10^8$ CFU/ml of *F. tularensis* LVS into each larvae's right proleg. After a 60 min incubation to
178 allow the infection to occur, the larvae were then injected with 10 ul of either PBS (no treatment)
179 or 10 ng of the derivatives in the larvae's left proleg. Bacteria treated with 10 µg of levofloxacin
180 was used as a positive control. The experiment was conducted twice; one representative
181 experiment is shown.

182 **Animal model of tularemia infection.** Female BALB/c mice 6-8 weeks of age were obtained
183 from Jackson Laboratories. Animal experiments were approved by and conducted in compliance
184 with regulations of the Institutional Animal Care and Use Committee (Protocol #0328) of
185 George Mason University. All experiments were carried out in accordance with the National
186 Research Council's Guide for the Care and Use of Laboratory Animals (2011) and the Public
187 Health Service Policy on Humane Care and Use of Laboratory Animals (2002). Animals were
188 scored twice daily based on appearance, activity, respiration, and appearance following our
189 protocol. If mice were weighed, weights were taken individually prior to any experimental work
190 each day.

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191 For the inoculum, *F. tularensis* LVS was grown for 2 days on chocolate II agar (37°C,
192 5% CO2). Colonies were scraped and resuspended in sterile PBS to 0.5 McFarland Standard
193 (which is equivalent to ~10^7 CFU/ml for this organism). 36 µl of this suspension was added to 10
194 ml of sterile PBS, and dilution plating was subsequently performed to confirm the inoculation
195 dose. Mice were lightly anesthetized using isoflourane immediately before infection. Each
196 mouse received an intranasal inoculation of 25 µl of this secondary suspension, evenly divided
197 between both nares.

198 Peptide treatments were performed through intraperitoneal (IP) injections. Each injection
199 consisted of 500 µl PBS containing 100 µg peptide or 60 µg levofloxacin. Treatments were
200 performed 3 h, 24 h, and 48 h after infection. In addition, one group (5 mice) received a
201 prophylactic treatment 24 h before infection in the first study, and one group received no
202 treatment. Survival was tracked for 13 days. Survival results were analyzed using a Mantel-Cox
203 test.

204 For organ burden studies, mice were infected and treated as above and sacrificed on Day
205 4. Lungs, livers, and spleens were harvested and homogenized in PBS using DT-20 tubes with an
206 ULTRA-TURRAX Tube Drive (IKA, Wilmington, NC, USA). Homogenate was plated on
207 chocolate II agar and incubated for two days (37 °C, 5% CO2). CFU counts were analyzed using
208 a one-way ANOVA with Dunnett's multiple comparisons.

209 **Statistical analysis.** All statistical analysis was performed in GraphPad Prism 6.0 or 7.0. Tests
210 performed are listed in each methods section and figure legend.

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211 **Results:**

212 **Peptide design and properties:**

213 Apo6 is a naturally occurring (native) peptide identified intact from American alligator
214 blood by *de novo* peptide mass-spectrometry sequencing [16, 17]. It is the C-terminal sequence
215 of alligator apolipoprotein E, and was discovered using our BioProspector process [16].

216 A series of Apo6 derivative peptides, designated GATR-1 through GATR-7 (Table 1),
217 were generated by introducing changes in the original Apo6 sequence, in order to improve the
218 peptide's amphipathicity, hydrophobic face, or net charge, as described below.

219 GATR-1 was produced by replacing the native threonine (T) in position 2 with a
220 phenylalanine (F), and substituting valine (V) at position 13 with phenylalanine (F). These
221 changes increase the hydrophobic moment of the helical peptide from 0.484 μH for Apo6 to
222 0.564 μH as well as raising hydrophobicity from 0.085 H to 0.180 H.

223 GATR-2 was produced by replacing glutamic acid (E) at position 8 with glutamine (Q),
224 glutamic acid (E) at position 15 with glutamine (Q), and aspartic acid (D) at position 19 with
225 asparagine (N). These alterations to the sequence raise the peptides positive charge from +4
226 (Apo6) to +7 and hydrophobicity to 0.130. However, these changes also reduce the hydrophobic
227 moment to 0.441 μH.

228 GATR-3 combines the T2/V13 and E8/E15/D19 amino acid substitutions of GATR-1 and
229 GATR-2. These sequence modifications increase the overall peptide charge to +7, hydrophobic
230 moment to 0.523 μH, and net hydrophobicity to 0.226.

231 GATR-4 was produced by adding NP to the N-terminus because N-capping peptides,
232 particularly with a proline residue, has been reported to increase peptide stability and decrease
233 protease susceptibility [34]. Add numbers about charge, HM and H from table.

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234 GATR-5 was produced by combining the GATR-2 alterations with substitutions of
235 phenylalanine (F) at position 10 to tryptophan (W), valine at position 13 to tryptophan (W) and
236 phenylalanine (F) at position 21 to tryptophan (W). These modifications increase the peptide
237 charge from +4 (Apo6) to +7, the hydrophobic moment to 0.566 μH, and hydrophobicity to
238 0.289 H.

239 In GATR-6, the sequence of GATR-5 has been further modified by replacing the lysine
240 (K) residues K11, K12, K14, K16, and K18 with arginine (R) residues. The physicochemical
241 properties of GATR-6 are nearly identical to those of GATR-5 and also GATR-3. Both GATR-5
242 and GATR-6 have a net charge of +7, hydrophobic moments of 0.566 μH and 0.568 μH
243 respectively, and hydrophobicities of 0.289 H and 0.284 H respectively.

244 GATR-7 was produced from the GATR-6 sequence by substituting the lysine (K) at
245 position 1 with arginine (R), the phenylalanine (F) at position 2 to tryptophan (W), the
246 phenylalanine (F) at position 6 to tryptophan (W) and the histidine (H) at position 9 to arginine
247 (R). Due to these substitutions, GATR-7 is predicted to have a net charge of +8, which is higher
248 than that of the other GATR peptides. Additionally, the hydrophobic moment of GATR-7 is
249 0.578 μH and its net hydrophobicity is calculated to be 0.273 H. These values are similar to
250 those calculated for GATR-3, GATR-5 and GATR-6.

251 **GATR peptides are antibacterial against *Francisella tularensis.***

252 Apo6 has been shown to have activity against a broad range of pathogens (*Bacillus*
253 *cereus*, *Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*, as well as drug-
254 resistant strains of *E. coli, P. aeruginosa, S. aureus,* and *Acinetobacter baumannii*) in low salt
255 buffer (EC_{50}) [16, 19]. Apo6 shares a salt-sensitive phenotype with LL-37 [14, 35] and was
256 found to be inactive in Muller-Hinton broth against these bacteria in MIC assays [16, 19]. We

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257 first tested Apo6 and the GATR peptides in MIC assays against *F. tularensis* LVS. Similar to its
258 activity against other bacteria, Apo6 had no observable MIC against *F. tularensis* LVS at the
259 concentrations tested. In addition, GATR-1, GATR-2, GATR-3, and GATR-4 were found to be
260 inactive under these conditions; however, some inhibitory activity was observed when GATR-5,
261 GATR-6, and GATR-7 are tested, with 85% inhibition at 41.5 μg/ml in the case of GATR-7
262 (**Figure 1**). It appears that the antibacterial activity in media increases along with the
263 hydrophobic moment.

264 Next, we tested the antimicrobial activity of Apo6 and its derivatives against *F. tularensis*
265 LVS and *F. tularensis* NIH B38 strain in a low salt buffer, which is an alternate measure of
266 antimicrobial activity reported as EC_{50} values, shown in **Table 2** [16, 19, 26, 27, 34-36]. We
267 performed these experiments using LL-37 as a positive control, which was found to be highly
268 effective against *F. tularensis* LVS (EC_{50}= 0.209 μg/ml), similar to the EC_{50} reported for *F.*
269 *novicida* [27]. As shown in **Table 2**, it was found that the EC_{50} values of the GATR peptides
270 were generally lower than that of Apo6, which has an EC_{50} value of 6.82 μg/ml against *F.*
271 *tularensis* LVS and 16.3 μg/ml and *F. tularensis* NIH B38, with the exception of GATR-4,
272 which had a similar EC_{50} against *F. tularensis* LVS (11.0 μg/ml) but was not effective at
273 concentrations tested against *F. tularensis* NIH B38. Four peptides (GATR-3, GATR-5, GATR-
274 6, and GATR-7) had EC_{50} values lower than 2 μg/ml against both strains of *F. tularensis*, and
275 thus were selected as the most effective peptides against *Francisella.* For comparison, the EC_{50}
276 of levofloxacin for *F. tularensis* LVS is 0.00827 μg/ml (8.27 ng/ml) [36].

277 Apo6, the four most effective GATR variants, and LL-37 were then tested against the
278 highly virulent strain *F. tularensis* SchuS4 in MIC and low salt assays. Results are shown in
279 **Table 3.** Although the parent peptide Apo6 is not effective against *F. tularensis* SchuS4, GATR-

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280 3, GATR-6, and GATR-7 are each moderately effective against this strain, with EC_{50} values
281 around 30 µg/ml. Interestingly, while these 4 peptides are not particularly effective against less
282 virulent strains of *F. tularensis* in MIC assays, GATR-7 displays comparatively strong activity
283 with a MIC of 41.7 µg/ml.

284 **GATR peptides interact with the cytoplasmic membrane of *F. tularensis* LVS**

285 As part of their mechanism of action, antimicrobial peptides can cause bacterial
286 membrane disruption, ranging from slow leakage of cellular contents owing to membrane
287 thinning to formation of large monomeric pores that can lead to cell death [37]. In order to
288 evaluate the interaction between the peptides and the bacterial cytoplasmic membrane, we
289 conducted two fluorescence-based studies. One of the ways in which the structural integrity of
290 cell membrane can be compromised is through disruption of membrane potential [38]. We
291 assessed the depolarization of bacterial membranes using $DiSC_3(5)$, a membrane potential
292 sensitive dye, which intercalates itself in the lipid bilayer resulting in the self-quenching of the
293 dye [38]. If depolarizing compounds are added, the potential decreases, and $DiSC_3(5)$ is released
294 into the solution causing an increase in fluorescence relative to the reduction of membrane
295 potential [31, 37, 38]. **Figure 2A** indicates a concentration-dependent increase in fluorescence
296 when *F. tularensis* LVS was treated with two different concentrations of peptides (10 µg/ml and
297 1 µg/ml). Apo6 and the GATR peptides dissipated the membrane potential in *F. tularensis*
298 LVS at 1 µg/ml, indicating that depolarization of cytoplasmic membrane is a primary mechanism
299 of action of Apo6 and its derivatives. In addition, the derivatives were much more effective in
300 disrupting the membrane potential at 10 µg/ml compared to the parent peptide Apo6 (p
301 values < 0.0001).

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302 Greater disruption can lead to the formation of larger, less transient holes or pores in the
303 bacterial membrane, which will lead to bacterial death. To examine this effect, we conducted a
304 membrane disruption assay using ethidium bromide (EtBr). This larger molecule will pass
305 through a damaged membrane and intercalate with the bacterial DNA resulting in increased
306 fluorescence proportional to the level of membrane disruption. We observed that *F. tularensis*
307 LVS was sensitive to pore-formation by Apo6 and its derivatives (Figure 2B), evidenced by a
308 significant RFU difference between the control and treated bacteria (p-values <0.05). At 10
309 μg/ml, all peptides except GATR-7 demonstrate a significant change in RFU, indicating pore-
310 formation by most of these peptides. Apo6 and GATR-5 also display significant pore formation
311 at a lower concentration of 1 μg/ml. However, GATR-3, GATR-6, and GATR-7 do not show
312 significant pore formation compared to the untreated bacteria at 1 μg/ml. LL-37 was used as
313 positive control in the depolarization and pore formation studies of the peptides [19, 28].

314 **GATR peptides bind *F. tularensis* LVS lipopolysaccharide (LPS).**

315 Lipopolysaccharide (LPS) is a major structural component of the Gram-negative bacterial
316 outer membrane and protects bacteria from antimicrobial compounds [11, 30]. LPS from *E. coli*
317 and other gram-negative bacteria is the endotoxin and activates innate immunity through binding
318 TLR4 receptors [39]. The overall positive charge on cationic antimicrobial peptides assists them
319 to form strong electrostatic interactions with the negatively charged LPS in the membrane of
320 Gram-negative bacteria neutralizing the overall negative charge [40, 41]. The binding of cationic
321 antimicrobial peptides with LPS of Gram-negative bacteria has a major effect on the stability of
322 bacterial membranes. It has been previously demonstrated that several cationic antimicrobial
323 peptides including LL-37, SMAP-29, and CAP18 can bind LPS [42-44]. Some cationic

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324 antimicrobial peptides have been shown to reduce the host immune response to LPS by binding
325 and sequestering it [42].

326 *Francisella* LPS is unusual among gram-negative LPS as it does not induce a strong
327 pyogenic response or activate TLR4 signaling [45-47]. Cationic antimicrobial peptide-LPS
328 binding could lead to greater interaction between the cationic antimicrobial peptides and the
329 *Francisella* bacterial membrane, which might enhance the activity of the peptide against the
330 bacteria. Thus we decided to investigate the ability of Apo6 and GATR peptides to bind purified
331 LPS from *F. tularensis* LVS. In order to analyze the binding between peptides and LPS, we
332 employed a DMMB dye LPS-binding assay. The positively charged dye competes with the
333 positively charged peptide to bind to the negatively charged moieties on the LPS. Upon binding,
334 the dye changes color from blue to purple/pink. As shown in **Figure 3,** we found that although
335 the parent peptide Apo6 does not significantly bind *F. tularensis* LVS LPS, the GATR peptides
336 tested significantly bind this LPS (p value <0.05). GATR peptides with greater charge and
337 hydrophobicity (GATR-6 and GATR-7) bind this LPS in greater amounts than do less charged
338 and hydrophobic peptides (GATR-3 and GATR-5). Thus, LPS binding might contribute to the
339 anti-*Francisella* mechanism of the GATR peptides.

340 **Toxicity of the GATR peptides**

341 We sought to further down-select the peptides by testing for potential toxicity. To
342 examine whether the GATR peptides may be toxic to mammalian cells (particularly those
343 peptides with higher charge), we performed hemolysis assays, cytotoxicity assays using the MTT
344 assay, and toxicity experiments in *G. mellonella* waxworms. First, hemolysis assays using sheep
345 red blood cells were performed at peptide concentrations of 100 μg/ml for 1 h [19, 31, 34].
346 GATR-5, -6, and -7 showed statistically significant hemolysis, in particular GATR-6 and GATR-

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347 7, each of which had hemolysis levels of greater than 20% of RBCs (**Figure 4A**). Next, we
348 measured cytotoxicity of the GATR peptides by using the MTT assay as a measure of cell
349 viability following peptide treatment [19, 31, 34]. A549 human lung epithelial cells and HepG2
350 liver cells were treated with 100 μg/ml peptide for 24 h. Shown in **Figures 4B** and **4C**, some
351 statistically significant suppression of cell proliferation was seen in A549 cells for GATR-3,
352 GATR-6, and GATR-7; however, no peptides show statistical suppression of cell growth when
353 tested against HepG2 cells.

354 Toxicity assays were also performed in the *G. mellonella* waxworm model. In groups of
355 10, each larvae received 10 μg of peptide, and survival was assessed for 48 h. After this time
356 period, waxworms treated with GATR-3, GATR-6, and GATR-7 were not found to have
357 significant death as measured by larvae survival (**Figure 4D**). However, GATR-5 treated
358 waxworms had only 30% survival, indicating that this peptide could potentially be toxic in an
359 animal model (p=0.0014), and so this peptide will not be carried forward to the *in vivo* testing.

360 **Waxworm *in vivo* infection survival assay**

361 Analysis of the efficacy of antimicrobials utilizing *in vivo* models is conducted to assess
362 the anti-infective potential of the drug in an infected animal. Ideally a mammalian animal model
363 should be employed in order to test the *in vivo* capabilities of antimicrobials; however,
364 alternative models may be appropriate for screening of lead antimicrobial candidates (EC_{50}
365 activity ≤10 μg/ml). *Galleria mellonella,* the greater wax moth, has been proposed as an
366 alternative model that is relatively easy to obtain and has a system of antimicrobial protection
367 similar to that of mammals. These factors make larvae of *G. mellonella* a model of infection for
368 various pathogenic microorganisms [2, 32, 48, 49]. *G. mellonella* has been previously used as an

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369 infection model for *in vivo* effect of antimicrobial peptides and antibiotics against *Francisella*
370 *spp.* infections [2, 48].

371 In the current study, to evaluate the ability of selected antimicrobial peptides to prolong
372 survival of infected *G. mellonella*, larvae were infected with *F. tularensis* LVS and then treated
373 with a single dose of 10 ng of peptides. Shown in **Figure 5,** *G. mellonella* showed statistically
374 significant improved survival when compared to untreated groups ($p<0.05$) when treated with
375 Apo6 and GATR peptides, with GATR-3 having the strongest effect (80% survival, $p=0.0001$).
376 The parent peptide, Apo6, was the next best candidate (60% survival, $p=0.0002$). While GATR-
377 5-treated *G. mellonella* initially demonstrated a strong prolonged survival rate, all of the larvae
378 succumbed to infection by 120 h (0% survival, $p=0.0008$). GATR-6- (30% survival, $p=0.045$)
379 and GATR-7- (30% survival p value= 0.0015) treated waxworms also showed significant
380 prolonged survival.

381 **Murine model *in vivo***

382 After down-selecting our lead peptides through the *G. mellonella* invertebrate model, the
383 3 lead peptides were tested in a murine model of pulmonary tularemia. GATR-3, GATR-6, and
384 GATR-7 were tested. In addition, we tested Apo6, LL-37, and D-LL-37 in this model. LL-37 had
385 stronger activity *in vitro* than any of the GATR peptides. Previously, Flick-Smith et al evaluated
386 the use of LL-37 as a post-exposure intranasal therapy in the treatment of pulmonary tularemia
387 by delivering the peptide directly to the lungs [20]. In that report, LL-37 extended mean time till
388 death but did not increase survival in treated animals. In our study, systemic peptide delivery via
389 the intra-peritoneal (IP) route of treatment appears to have no adverse effect on survival in
390 response to this peptide, i.e. the peptide was not toxic when delivered systemically. Previously,
391 we have found that D-LL-37 has increased activity antimicrobial and protease resistance [35,

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392 36], making it an attractive peptide to use *in vivo*. However, in our study, D-LL-37 had similar
393 activity in this animal model to LL-37 in that it did not extend survival or rescue mice, nor did
394 these peptides lessen signs of disease. Survival data and health scores are shown in **Figure 6A**
395 and **7B**.

396 In the first set of experiments, mice (5/group) were given 1 prophylactic peptide
397 treatment 1 day before infection (Day -1) and 3 treatments after infection at 3, 24, and 48 h. Each
398 intra-peritoneal (IP) injection delivered 5 mg/kg of peptide. Survival is shown in **Figure 6A**. In
399 this model, GATR-3 was found to be the most successful peptide, with 60% survival (p=0.0047)
400 compared to the PBS-injected control, which had a mean time till death (MTD) of 6 days and 0%
401 survival. GATR-6 saved 40% of mice (p=0.0237) compared to the control. GATR-7 saved 20%
402 of mice, but Mantel-Cox tests indicate this was not significant (p=0.0736). When average health
403 scores were examined in **Figure 6B**, GATR-3 delayed the time of disease onset from day 1 to
404 day 3 and lessened severity of clinical signs over the course of infection. GATR-6 and GATR-7
405 did not delay disease onset, but severity of signs were slightly lessened compared to the
406 untreated control. For this set of experiments, mice were weighed daily in the morning. Weights
407 from day 4 are compared in **Figure 6C.** None of the GATR peptide treated mice show a
408 significant difference in weight change compared to the untreated control.

409 In the second set of experiments, the prophylactic pre-treatment was not given and larger
410 groups were used (10 mice/group). Mice received only the 3 treatments after infection at 3, 24,
411 and 48 h. Because LL-37, D-LL-37, and GATR-7 were not found to significantly rescue mice in
412 the first set of studies, they were not used in these experiments. Only GATR-3 and GATR-6
413 were tested. In the survival study shown in **Figure 6D,** GATR-3 once again was the most
414 successful peptide, with 50% survival (p=0.0053) compared to the untreated control, which had

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415 10% survival and a mean time till death of 5.5 days. However, GATR-6 was not found to have
416 significant survival in this study (10% survival, p=0.4347), though mean time till death (MTD)
417 was extended to 6 days. This may indicate the prophylactic treatment was important for the
418 efficacy of GATR-6. When average health scores were compared in **Figure 6E**, differences
419 between peptide-treated and untreated groups were not as apparent as in **Figure 6B**. All groups
420 had signs of disease onset on day 3. Disease signs of GATR-3-treated mice were only slightly
421 lessened compared to the untreated group, while signs of GATR-6-treated mice were slightly
422 more severe than the untreated mice.

423 Because GATR-3 rescued mice in both sets of experiments, an organ burden study was
424 performed with this peptide on day 4 to determine the bacterial burden in the lungs, spleen, and
425 liver (3 mice/group), as shown in **Figure 6F**. Though no significant difference was found
426 between the bacterial burden in the spleens and livers of GATR-3-treated and untreated mice, the
427 bacterial numbers were found to be significantly lower in lungs (p<0.0001). Thus, the survival
428 benefit may have been due to reduced lung burden as opposed to overall bacterial clearance.

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429 **Discussion:**

430 *Francisella* is highly resistant to cationic cyclic peptide antibiotics such as polymyxin B.
431 Indeed, *Francisella* selective growth media contains 100 mg/ml polymyxin B [50]. The
432 resistance to polymyxin B is thought to be due to the special structure of the lipopolysaccharide
433 (LPS) of *Francisella* [46, 51]. Thus, *Francisella* is considered to be resistant to this class of
434 cyclic peptide antibiotics, which are sometimes called cationic antimicrobial peptides by other
435 researchers. However, our previous work has shown that certain cationic antimicrobial peptides
436 can have activity against this organism [27-29, 52, 53]. Experiments to introduce such
437 antimicrobial peptides in the lung by Flick-Smith et al only modestly increased the time-to-death
438 of mice infected with *F. tularensis* LVS [20]. In this work, we sought to develop another
439 antimicrobial peptide that would be effective against *Francisella in vitro* and which would
440 display *in vivo* activity against this infection.

441 Previously, our group identified C-terminal fragments of apolipoprotein C-1 from
442 *Alligator mississippiensis*. These helical fragments, called Apo5 and Apo6, were found to have
443 broad-spectrum activity against a variety of pathogens, including *Pseudomonas aeruginosa,*
444 *Staphylococcus aureus,* and *Acinetobacter baumannii* [16, 19]. In general, these peptides had
445 strong anti-*Francisella* activity in low-salt buffer, with several peptides exhibiting EC_{50} values
446 under 3 µg/ml. When we tested these peptides against *F. tularensis* subspecies, it was found that
447 Apo5 and Apo6 were generally less effective against these subspecies than against other Gram
448 negative bacteria tested, with EC_{50} values ranging from low (~6 µg/ml) against *F. tularensis*
449 LVS to much higher (~16 µg/ml) against *F. tularensis* NIH B38.

450 We designed the GATR series of peptides with the aim of improving upon the promising
451 antimicrobial performance demonstrated by these peptides. Because there was no discernable

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452 difference in activity between Apo5 and Apo6 against the *F. tularensis* subspecies, we chose to
453 focus on derivatives of the shorter Apo6. Little is known regarding the antimicrobial mechanisms
454 and the associated specific interactions employed by these peptides, hence the GATR peptide
455 variants (GATR-1 to -7) were designed based on incremental minor changes to the peptide
456 sequences. Individually, these modifications were anticipated to minimally impact the peptide
457 structural properties and preserve amino acid side-chain groups present in the parent peptide that
458 may participate in critical interactions with bacterial targets such as the membrane or LPS. By
459 substituting amino acids to increase peptide hydrophobicity and overall positive charge, we were
460 able to create peptides with stronger *in vitro* and *in vivo* activity. All of the peptides, except
461 GATR-4, exhibited superior performance over the parent peptide Apo6 against *F. tularensis*
462 LVS under EC_{50} conditions. All the GATR variants, except GATR-1 and GATR-2, also
463 demonstrated superior performance against *F. tularensis* NIH B38, the *F. tularensis* type strain,
464 compared to parent peptide Apo6. The more substituted peptides, GATR-5, -6, and -7, began to
465 show activity in cation-adjusted Mueller Hinton Broth, though a MIC could not be determined
466 based upon the concentrations tested. The most efficacious peptides were also found to have
467 stronger activity against *F. tularensis* SchuS4 compared to Apo6. Most notably, GATR-7 had a
468 determinable MIC against *F. tularensis* SchuS4 at concentrations tested.

469 Our previous studies showed that the Apo6 peptide affected bacteria by disrupting the
470 bacterial membrane, primarily through depolarization [19]. To examine if this was also the case
471 with the synthetic peptides with *F. tularensis*, we examined membrane binding and disruption.
472 $DiSC_3(5)$ measures depolarization and transient holes in a previously hyperpolarized membrane.
473 It was found that as hydrophobicity and cationicity increase, so does depolarization activity.
474 However, this is not the case when the ethidium bromide uptake assay was performed, which

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475 measures larger pores or disruption that allow the passage of ethidium bromide into the cell.
476 While Apo6 shows significant membrane disruption at both 10 and 1 μg/ml tested, none of the
477 other peptides show significant depolarization at 1 μg/ml. In general, the GATR peptides have a
478 similar ΔRFU to Apo6 at 10 μg/ml. It is not clear why this occurs based on physico-chemical
479 properties. The charge and hydrophobicity of Apo6 is generally much lower than that of the
480 GATR peptides, but considering the greater antimicrobial efficacy of the GATR peptides, it
481 appears that the pore-forming activity is less important to its antibacterial mechanism than the
482 depolarization activity, which may suggest an intracellular target.
483 Some antimicrobial peptides, such as LL-37, bind to bacterial LPS [14]. In addition,
484 some apolipoproteins have been shown to bind LPS [54, 55]. Our prior experiments had shown
485 that Apo6 did not bind *E. coli* LPS (data not shown), and we found, similarly, that Apo6 does not
486 significantly bind *F. tularensis* LVS LPS. The synthetic GATR peptides, however, were found to
487 bind greater amounts of *F. tularensis* LVS LPS as hydrophobicity and cationicity increased,
488 leveling off with GATR-6 and GATR-7. It is unclear if increasing LPS binding leads to
489 increased depolarization, but it is possible that increased attraction between peptide and LPS
490 allows higher-binding peptides to better associate with the membrane. It seems that there is no
491 correlation, either positive or negative, between LPS binding and pore formation in the
492 membrane.
493 After dropping one peptide (GATR-5) for toxicity issues, the 3 best performing peptides,
494 GATR-3, GATR-6, and GATR-7, were tested for activity *in vivo*. Though GATR-7 had the best
495 performance in MIC assays, initial studies in *G. mellonella* waxworms did not clearly show this
496 peptide to be the best performer. Instead, GATR-3 and the parent peptide Apo6 saved more
497 larvae from a lethal *F. tularensis* infection, though GATR-6 and GATR-7 also significantly

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498 rescued the waxworms. Because the *in vitro* and *in vivo* data together did not point to a clear
499 front-runner peptide, we tested the three top-performing peptides (GATR-3, GATR-6, GATR-7)
500 in a murine model infected with pulmonary tularemia. In initial studies, we tested Apo6 *in vivo*
501 because of its strong performance in *G. mellonella* studies. However, this peptide was not
502 effective in murine studies (data not shown). In a set of further experiments, we tested GATR-3,
503 GATR-6, and GATR-7, as well as LL-37 and its D-enantiomer D-LL-37. We have previously
504 shown that LL-37 is a highly effective peptide against *Francisella in vitro* [27, 29, 34-36]. LL-37
505 peptide has been previously tested in a murine pulmonary tularemia model [20]. Flick-Smith et
506 al previously reported that when LVS-infected mice are treated with LL-37 via the intranasal
507 route, the peptide significantly extended mean time to death, but did not ultimately rescue any
508 mice. In our experiments, we treated at a higher concentration than Flick-Smith et al and also by
509 a different route (via intraperitoneal injection), and similarly found that LL-37 had no effect on
510 survival of infected mice. We also tested D-LL-37 *in vivo* because we have shown in *in vitro* that
511 this chiral enantiomer is equally or more effective than the native peptide [35, 36]. In addition, it
512 has the advantage of protease resistance, which should allow it to circulate in the body longer.
513 When D-LL-37 was tested in this model, this peptide was also ineffective at rescuing infected
514 mice or even prolonging mean time till death. Thus, LL-37 is not effective against a pulmonary-
515 based tularemia infection when given systemically, in agreement with previous reports [20].
516 When the GATR peptides were tested in this model with a prophylactic treatment, it was
517 found that both GATR-3 and GATR-6 peptide treatments significantly rescued mice infected
518 with *F. tularensis* LVS. GATR-7 did not, though this peptide had the strongest activity in MIC
519 assays, which are considered the gold standard for activity [19, 24]. In a second set of
520 experiments, GATR-3 and GATR-6 were tested in larger groups without the prophylactic

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521 treatment. GATR-3 maintained its efficacy without the prophylactic treatment, while GATR-6
522 did not. This indicates that the pre-infection administration at Day -1 may have been important
523 for the activity of GATR-6, which makes this peptide less favorable as a candidate to take
524 forward for further testing.

525 Other groups have used peptides to treat a variety of bacterial infections in animal models
526 with varying levels of success. Silva et al treated mice infected with *E. coli* and *S. aureus* with
527 peptides derived the marine tunicate *Styela clava* and found that a single dose of 10 mg/kg
528 yielded survival rates of 80-90% [56]. In another study, it was found that a single 80-200 mg/kg
529 dose boosted survival of rainbow trout infected with *Yersinia ruckeri* from 20% to 70% [57].
530 Additionally, mice infected with *Bacillus anthracis* spores were treated with a single dose of 1
531 mg/kg synthetic protease-resistant peptides, and survival was boosted to 20-30% [58]. Thus, 3-4
532 doses of 5 mg/kg GATR-3 yielding 50-60% survival in infected mice compares favorably with
533 the results of other trials. The dosage of GATR-3 in this study is also comparable to levofloxacin
534 (5 mg/kg vs. 3 mg/kg), though GATR-3 did not rescue all mice in the treated cohort. Larger
535 doses of GATR-3 may increase efficacy of the peptide; however, we must first study safety of
536 these larger doses in mice. Based on the treatment used in the study, a preliminary dosage for
537 human infection can be inferred using guidelines put forth by the FDA [59]. The dosage of 5
538 mg/kg in mice would convert to approximately 0.4 mg/kg in humans with a dose of 24 mg
539 presuming a 60 kg human.

540 *F. tularensis* disseminates from the lung to the liver and spleen during infection [20].
541 GATR-3 was tested in an organ burden study to examine whether bacterial burden reduction was
542 the cause for *in vivo* activity. Although *Francisella* burden was reduced in the lungs of GATR-3-
543 treated mice, it was not significantly reduced in the liver or spleen. Further studies are needed to

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544 determine whether GATR-3 clears the infection directly as its mode of action *in vivo* or whether
545 it activates the host immune response to promote survival. Due to its strong and consistent
546 performance, GATR-3 has strong potential as an anti-tularemia peptide and will be the subject of
547 further pre-clinical development to determine its pharmacokinetic and pharmacodynamics
548 properties.
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687 **Tables and Figures:**

688 **Table 1. Sequences and physico-chemical properties of GATR antimicrobial peptides.**
689 Physico-chemical properties and helical wheels were calculated using Heliquest [21] and APD3
690 [22].

Name	Sequence	Molecular Weight	Charge	Hydro-phobic Moment (μH)	Hydro-phobicity (H)	Defined Total Hydrophobic Ratio
Apo5 [36]	FSTKTRNWFSEHFKKVKEKLKDTFA	3103.57	+4	0.436	0.155	32%
Apo6 [19]	KTRNWFSEHFKKVKEKLKDTFA	2768.21	+4	0.484	0.085	31%
GATR-1	K**F**RNWFSEHFKK**F**KEKLKDTFA	2862.31	+4	0.564	0.180	36%
GATR-2	KTRNWFS**Q**HFKKVK**Q**KLK**N**TFA	2765.34	+7	0.441	0.130	31%
GATR-3	K**F**RNWFS**Q**HFKKFK**Q**KLK**N**TFA	2859.35	+7	0.523	0.226	36%
GATR-4	NPKTRNWFSEHFKKVKEKLKDTFA	2973.41	+4	0.405	0.082	29%
GATR-5	K**F**RNWFS**Q**H**W**KK**W**K**Q**KLK**N**T**W**A	2976.46	+7	0.566	0.289	36%
GATR-6	K**F**RNWFS**Q**H**WRR**W**RQR**L**RN**T**W**A	3116.55	+7	0.568	0.284	36%
GATR-7	**RW**RNWWS**QRWRRWRQR**L**RN**T**W**A	3241.69	+8	0.578	0.273	36%
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKD FLRNLVPRTES	4493.33	+6	0.521	0.201	37%

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692 **Table 2. Antibacterial activity of GATR peptides against *F. tularensis* LVS and NIH B38.**
693 EC_{50} values of Apo6 and its derivatives were determined in Buffer Q against *F. tularensis* LVS
694 *and F. tularensis* NIH B38 (the Type strain). For statistical comparison, the 95% confidence
695 intervals ($p < 0.05$) are listed. The values are also expressed as µM for direct comparison.

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	F. tularensis LVS		***F. tularensis* NIH B38**	
Peptide name	**EC_{50} (95% CI) [µg/ml]**	**EC_{50} [µM]**	**EC_{50} (95% CI) [µg/ml]**	**EC_{50} [µM]**
Apo6	6.8 (5.9-7.8)	2.5	16 (8.6-31)	5.89
GATR-1	0.76 (0.54-1.1)	0.26	16 (9.5-27)	5.62
GATR-2	2.4 (1.5- 3.7)	0.86	11 (7.7-15)	3.9
GATR-3	0.53 (0.42-0.66)	0.19	0.80 (0.56-1.1)	0.28
GATR-4	11.0 (7.5-16)	3.7	Not active	Not active
GATR-5	0.76 (0.56- 1.0)	0.26	1.9 (1.6-2.3)	0.65
GATR-6	0.89 (0.60-1.3)	0.29	0.16 (0.06-0.43)	0.051
GATR-7	1.0 (0.81-1.3)	0.32	1.7 (1.4-2.1)	0.53
LL-37	0.21 (0.14-0.31)	0.047	0.13 (0.076-0.21)	0.028

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708 **Table 3. Antibacterial activity of selected GATR peptides against *F. tularensis tularensis***
709 ***(Ftt)* SchuS4.** The more active GATR derivatives were tested against *Ftt* SchuS4 in CAMHB
710 with 2% IsovitaleX and in Buffer Q. For statistical comparison, the 95% confidence intervals (p
711 < 0.05) are listed. The EC50 values are also expressed as µM for direct comparison.

Peptide Name	**MIC [µg/ml]**	**EC_{50} (95% CI) [µg/ml]**	**EC_{50} [µM]**
Apo6	Not tested	Not active	Not active
GATR-3	>83.3	28.6 (13.3 to 61.6)	10.0
GATR-6	>83.3	32.3 (15.8 to 66.2)	10.4
GATR-7	41.7	24.2 (wide)	7.47
LL-37	Not tested	0.562 (0.195 to 1.61)	0.125

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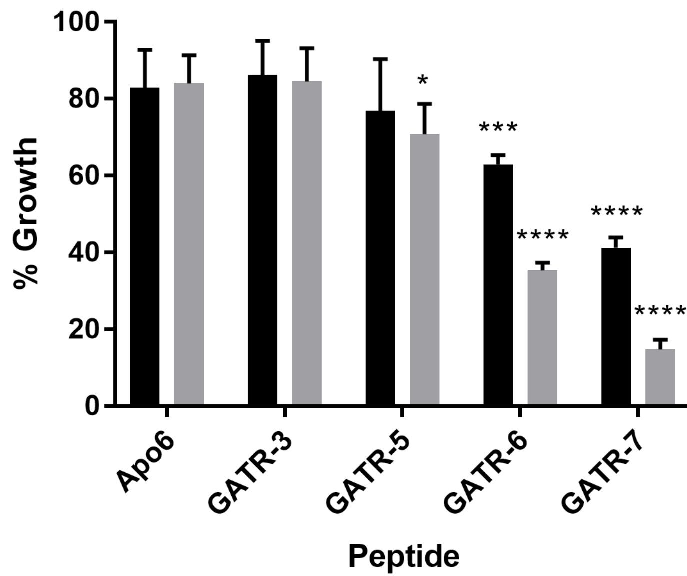
714 **Figure 1. GATR peptides have improved antibacterial activity in broth against *F.*
715 *tularensis* LVS.** MIC assays were performed in Cation-adjusted Mueller Hinton Broth with 2%
716 IsoVitaleX (black=21 µg/ml; gray=42 µg/ml) on *F. tularensis* LVS with 5 replicates per
717 experiment. Experiment was performed twice. Results were analyzed using a 2way ANOVA
718 with Sidak's multiple comparison against Apo6. Error bars indicate standard deviation. (*
719 p<0.05; *** p<0.001; ****p<0.0001).



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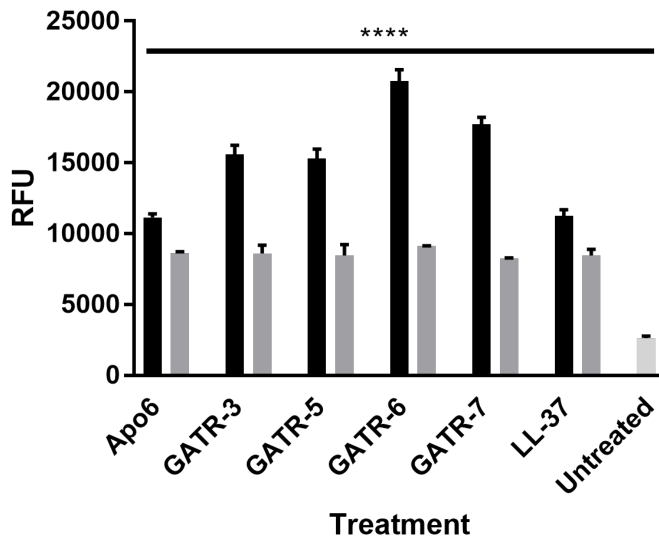
722 **Figure 2. GATR peptides disrupt the bacterial membrane of *F. tularensis* LVS**. A.
723 Membrane depolarization was measured using DiSC3(5) in 10 mM phosphate buffer with at
724 least 2 replicates per experiment (black=10 µg/ml; gray=1 µg/ml). Experiment was performed 3
725 times. B. Pore formation or greater membrane perturbation was measured using ethidium
726 bromide in 10 mM phosphate buffer with 3 replicates per experiment (black=10 µg/ml; gray=1
727 µg/ml). Experiment was performed 3 times. Results were analyzed using a 1way ANOVA with
728 Dunnet's multiple comparisons. Error bars indicate experimental variation. (** p<0.01; ****
729 p<0.0001)

730 A.



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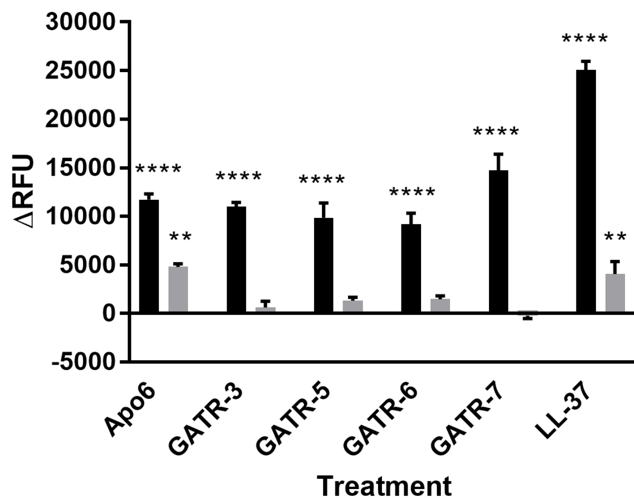
732 B.



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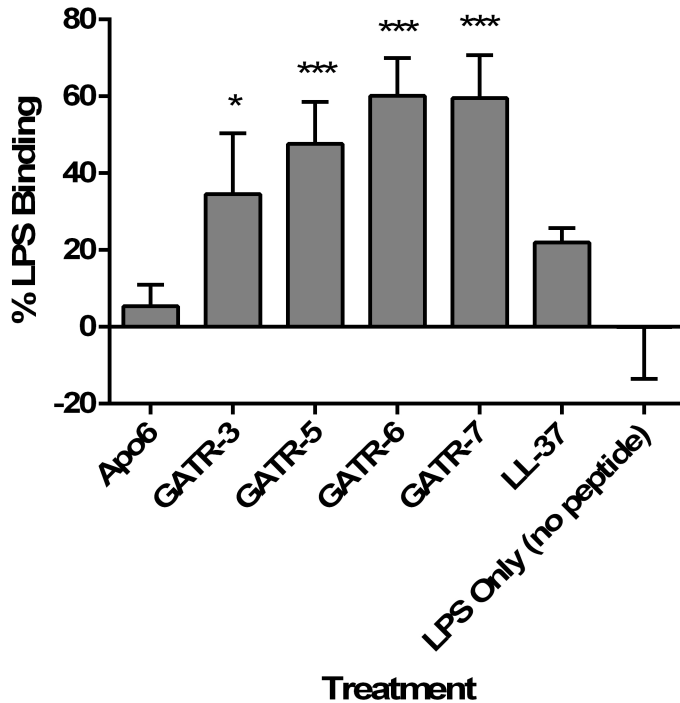
735 **Figure 3. GATR peptides bind *F. tularensis* LVS LPS.** 150 μg/ml of LPS was incubated with
736 10 μg/ml of peptide in distilled endotoxin-free water for 1 h and then added to DMMB. The
737 experiment was performed twice with 3 replicates per experiment. Results were analyzed using a
738 1way ANOVA with Dunnett's multiple comparisons tests. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)
739 Error bars indicate standard deviation.



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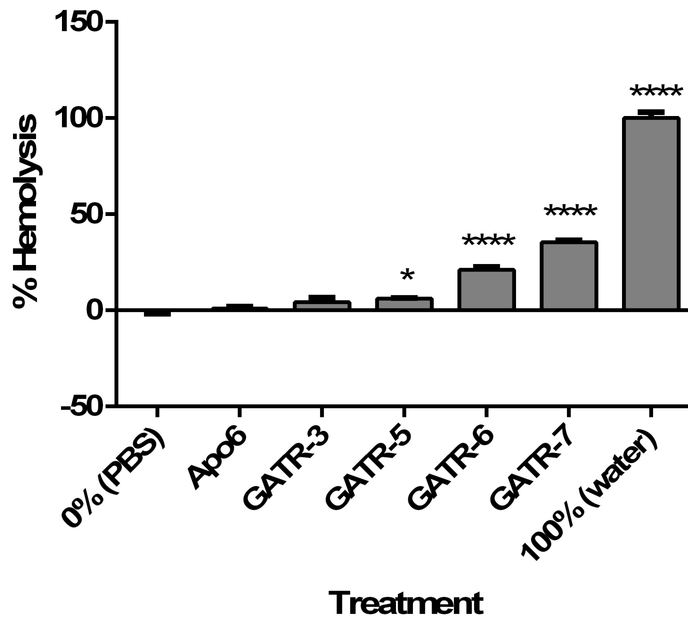
743 **Figure 4. Toxicity of GATR peptides. A.** Hemolysis assay using 2% sheep red blood cells.
744 Peptides are reconstituted in sterile PBS. For 0% hemolysis, RBCs are exposed to PBS. For
745 100% hemolysis, RBCs are exposed to sterile water. Experiment was performed twice with 6
746 replicates per experiment. Results were analyzed using a 1way ANOVA with Dunnett's multiple
747 comparisons. **B.** MTT cell proliferation assays using A549 human lung epithelial cells and **C.**
748 HepG2 human hepatocytes with 24 h exposure to 100 µg/ml peptide. Experiments were
749 performed twice each with 3 replicates per experiment. Results were analyzed using a 1way
750 ANOVA with Dunnett's multiple comparisons. (* $p<0.05$; ** $p<0.01$; **** $p<0.0001$) **D.**
751 Toxicity in *G. mellonella* larvae was measured by injecting each worm with 10 µg of peptide (10
752 larvae/group). Survival was measured for 48 h.

753 **A.**



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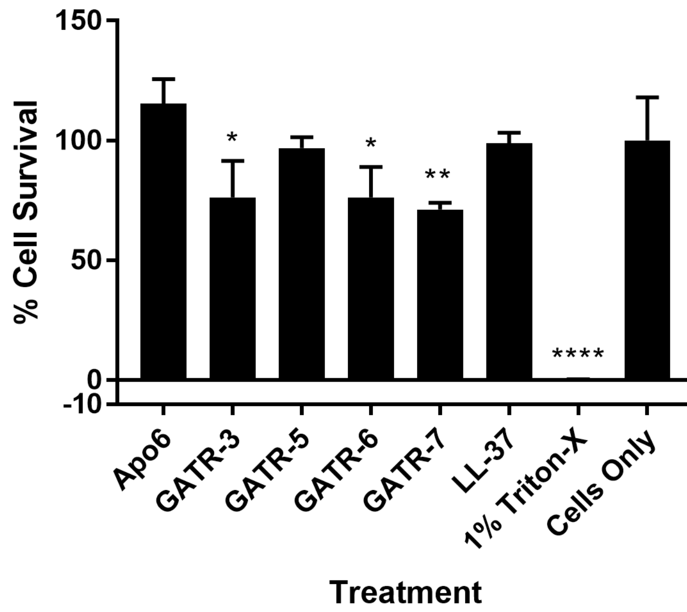
757 **B.**



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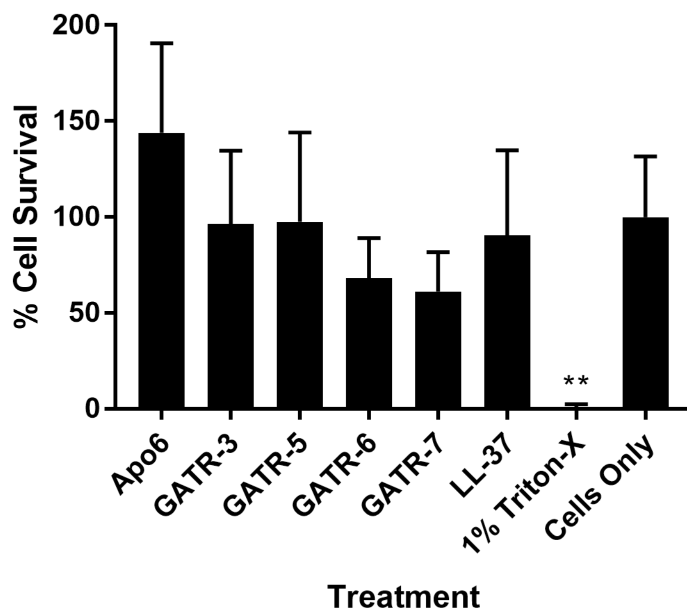
761 C.



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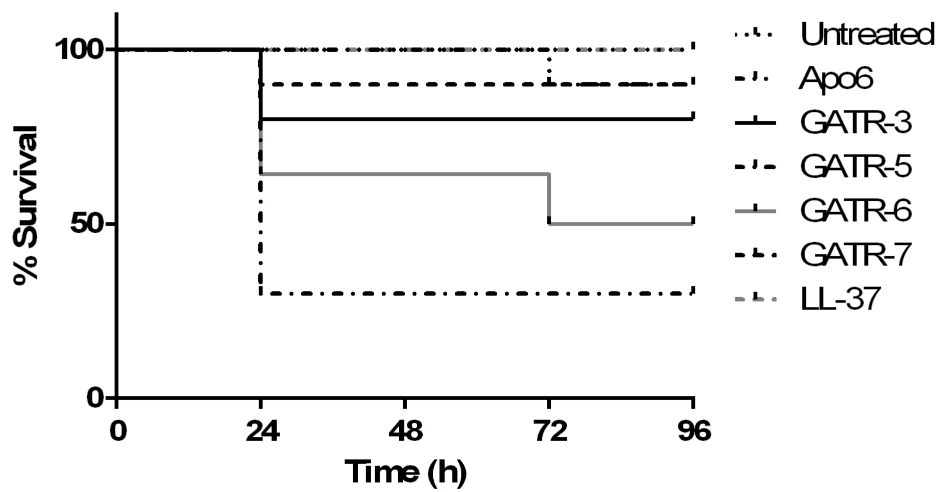
764 D.



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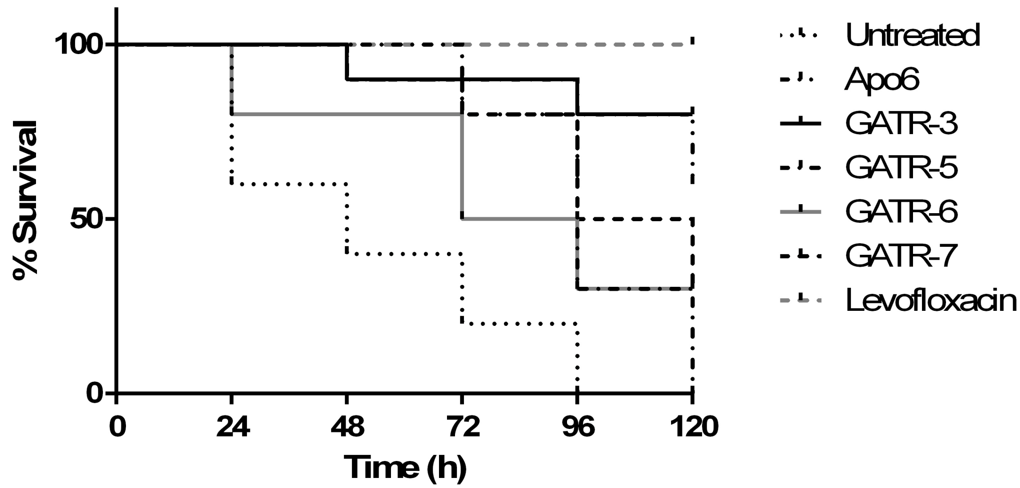
767 **Figure 5. *G. mellonella* survival following GATR peptide treatment.** *G. mellonella* larvae
768 were infected with *F. tularensis* LVS and treated with a single injection of 10 ng peptide or 10
769 μg levofloxacin (10 larvae/group). Survival was monitored for 120 h after infection.



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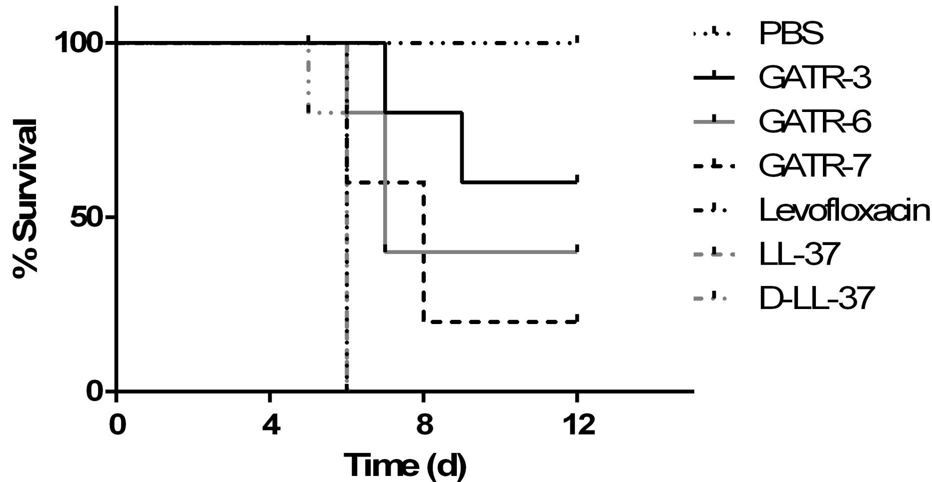
775 **Figure 6. GATR Peptide treatment of *F. tularensis* LVS infected mice.** BALB/c mice were
776 infected with 50 LD50 of *F. tularensis* LVS and treated with peptide 24 h before and 3, 24, and
777 48 h after infection (5 mice/group). A. Survival curves of mice with prophylactic treatment, B.
778 Average health scores over course of study, C. percent initial weight on day 4 after infection, in
779 which results were analyzed using a 1way ANOVA with Tukey's multiple comparisons (****
780 p<0.0001) Next, BALB/c mice were infected with 10 LD50 of *F. tularensis* LVS and treated
781 with peptide 3, 24, and 48 h after infection (5 mice/group). D. Survival curves of mice with post-
782 infection treatment only E. Average health scores of mice during survival study. F. Organ burden
783 study comparing untreated (black bars) and GATR-3-treated (gray bars) organs (3 mice/group).
784 Lungs, liver, and spleen were harvested on day 4 after infection homogenized in PBS, and plated
785 on chocolate agar. Results were analyzed using a 1way ANOVA with Tukey's multiple
786 comparisons. (**** p<0.0001)

787 A.



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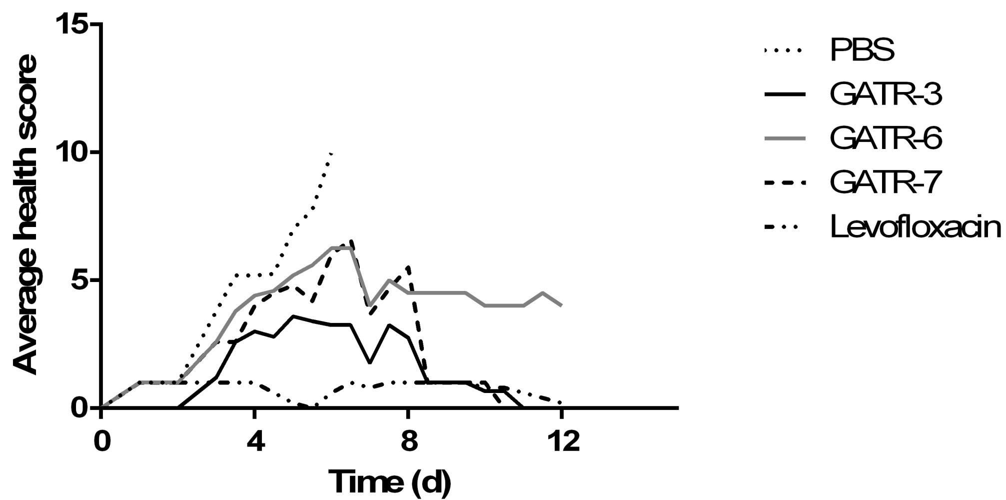
790 B.



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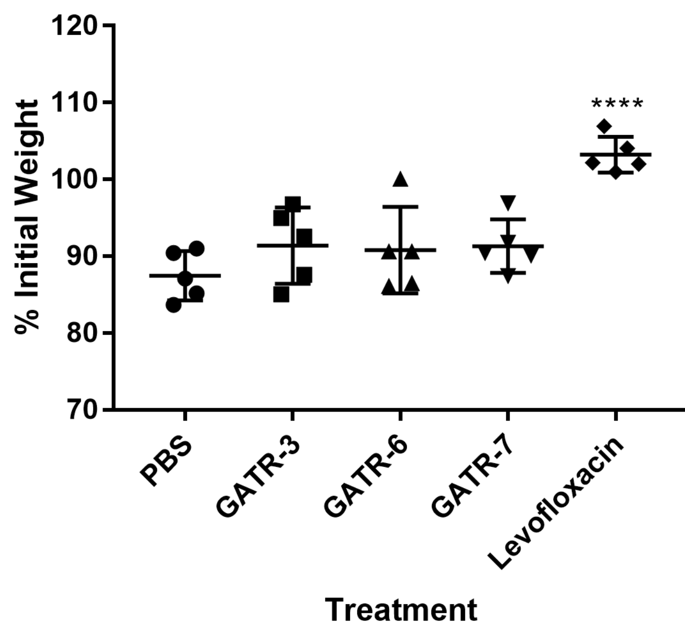
792 C.



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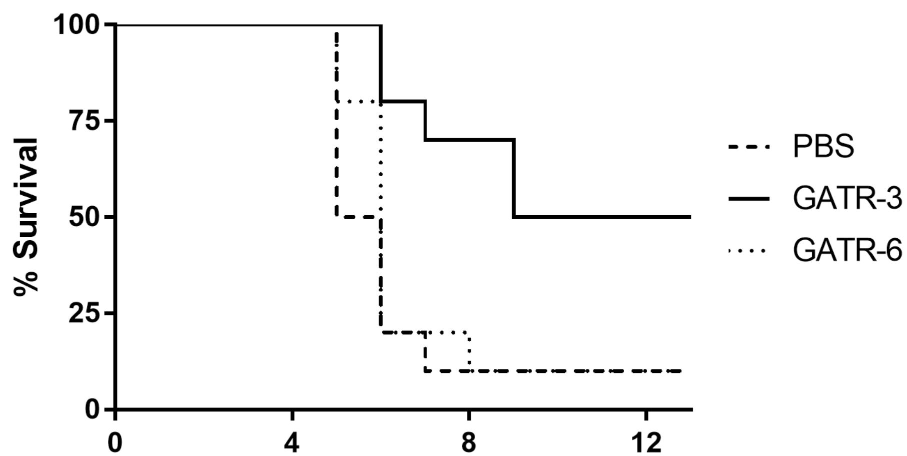
795 D.



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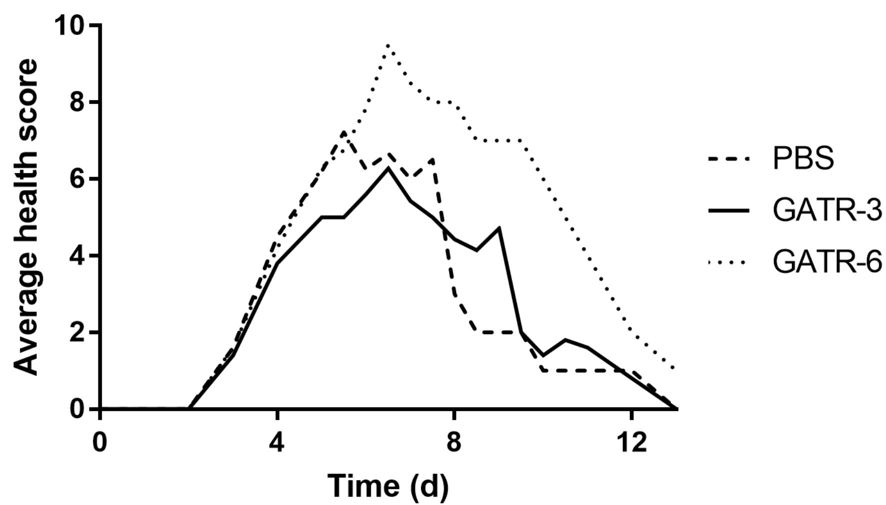
797 E.



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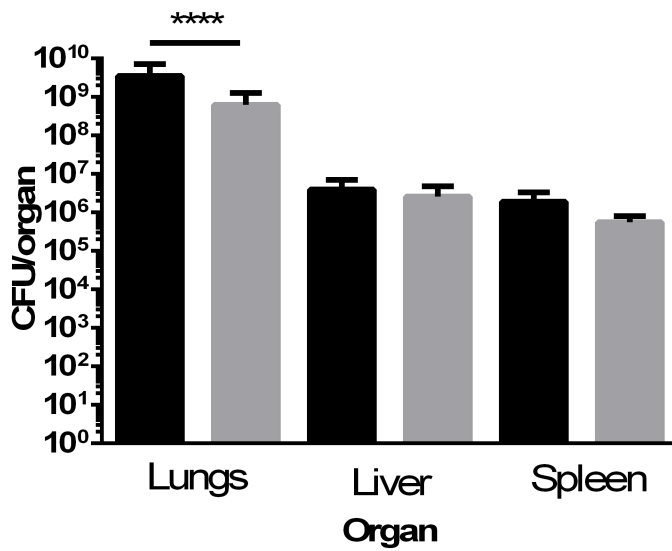
800 F.



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