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Brucella alters the immune response in a *prpA*-dependent manner

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5 **Brucella alters the immune response in a prpA-dependent manner**
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36 **ABSTRACT**

37 Brucellosis, a disease caused by the gram-negative bacterium *Brucella* sp, is a
38 widespread zoonosis that inflicts important animal and human health problems,
39 especially in developing countries. One of the hallmarks of *Brucella* infection is its
40 capacity to establish a chronic infection, characteristic that depends on a wide
41 repertoire of virulence factors among which are immunomodulatory proteins such as
42 PrpA (encoding the proline racemase protein A or hydroxyproline-2-epimerase),
43 involved in the establishment of the chronic phase of the infectious process that we
44 have previously identified and characterized. We report here that, *in vivo*, *B. abortus*
45 *prpA* is responsible for an increment in the B-cell number and in the specific antibody
46 response and that these antibodies promote cell infection. We additionally found that
47 *Brucella* alters the cytokine levels of IFN-γ, IL-10, TGFβ1 and TNFα during the
48 acute phase of the infectious process in a *prpA* dependent manner.

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50 **1. INTRODUCTION**

51 Many microbial pathogens have the ability to establish chronic infections in their
52 hosts and, as such, must be able to overcome the immune response triggered during
53 the infectious process [1]. Although the manipulation and/or modulation of the
54 immune response by pathogens is currently a well-recognized theme in microbial
55 pathogenesis [2, 3] there still are very few examples of how different pathogens
56 (bacterial, virus or eukaryotic) achieve this task. An accepted hypothesis is that
57 pathogens have evolved sophisticated strategies to subvert the immune response
58 tipping the equilibrium between "response" and "non-response" of the immune
59 system. Many pathogens thus, have achieved a balance consistent with the survival of
60 both the microbe and its infected host by fine-tuning the homeostasis of the latter with
61 no major disturbances [4, 5].

62 *Brucella* spp. are Gram-negative facultative intracellular bacteria that cause
63 brucellosis, a worldwide-distributed zoonosis affecting a broad range of mammals
64 including humans. Brucellosis remains a serious problem in many developing
65 countries, causing important economic losses and human health problems. The
66 infection is characterized by an initial acute phase with flu-like symptoms which, if
67 not treated, can become chronic and persist over the life span of the host causing a
68 broad range of disorders, especially osteoarticular complications [6]. The ability of
69 *Brucella* to establish chronic infections in the face of an ongoing immune response,
70 suggests the existence of bacterial virulence factors with immunomodulatory effects.

71 We have previously described a *Brucella abortus* virulence factor (*prpA,* for Proline
72 Racemase Protein A) that i) is secreted during infection, ii) interacts with NMMII-A
73 in macrophages and iii) induces the release of soluble factors responsible for B-cell
74 proliferation *in vitro* [7, 8]. We also showed that *prpA* is required for the

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75 establishment of the chronic phase of infection in mice [8]. This gene has a
76 homologue in *T. cruzi* that also acts as a T-cell independent B lymphocyte mitogen
77 required for virulence [9, 10]. Both genes are hypothesized to act during the acute
78 phase of the infection process, inducing a transient non-responsive state of the
79 immune system that delays or hampers the immune response facilitating chronicity [8,
80 11]. However, if *prpA* acts as a B-cell proliferator *in vivo*, how it alters host immunity
81 has not been elucidated.
82 We report here for the first time that *Brucella* infection induces an increment in B-cell
83 number, as has been described during *T. cruzi's* infection. Moreover, we demonstrate
84 that *prpA* is responsible for this B-cell number increment in infected mice. We also
85 show, *in vivo*, that this virulence factor enhances the production of immunoglobulins
86 directed towards the pathogen and that these antibodies enhance macrophage
87 infection. Finally, we compared the secretion pattern of key inflammatory and anti-
88 inflammatory cytokines in mice during infection with the wild type and the mutant
89 strains and found that they are altered in a *prpA* dependent manner, indicating that this
90 virulence factor also modulates the immune response. Our results show that this gene
91 is clearly involved in the immune modulation process *in vivo* and that alters several
92 aspects of the immune response.
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95 **2. MATERIALS AND METHODS**

96 **2.1 Bacterial strains and growth conditions**. *Escherichia coli* strains were grown at
97 37°C with aeration in LB broth or Terrific broth. *Brucella* strains were grown at 37°C
98 with aeration in Bacto Tryptic soy broth (Becton Dickinson, Sparks, MD). When
99 necessary, media were supplemented with the appropriated antibiotics: ampicillin at
100 100 µg/ml for *E. coli* and 50 µg/ml for *B. abortus* and gentamicin at 4 µg/ml.
101
102 **2.2 Infection and inoculation of mice.** Infections were carried out as described in
103 [12]. Briefly, female, 60–90 days old BALB/c mice were injected intraperitoneally
104 with 0.2 ml of PBS containing $5x10^4$ CFU of *B. abortus* 2308 or *B. abortus-prpA*
105 mutant. For the PrpA-inoculation experiments, BALB/c mice were injected
106 intraperitoneally with 200 µl of PBS or a sterile solution of PrpA (50 µg/ml) in PBS.
107 At different times after infection or inoculation, animals were sacrificed; the spleens
108 removed, homogenized in RPMI and processed either for direct CFU determination
109 (plating) or fixed and stained for cytometry. All mice were bred in accordance with
110 institutional animal guidelines under specific pathogen-free conditions in the local
111 animal facility (BSL-3, Institute for Research in Biotechnology) of the University of
112 San Martín. Mouse studies were approved by the local regulatory agencies (CICUAE-
113 UNSAM)
114
115 **2.3 Gentamicin protection assays.** J774 A.1 cells were infected as previously
116 described in [13]. Briefly, cells were infected with *Brucella abortus* 2308 with a
117 multiplicity of infection of 20:1 for 1 hr, and Gm and Str (50 and 100 µg/ml) were
118 added to kill non-internalized bacteria. Cells were then washed, lysed with 0,1%
119 Triton X100 and intracellular bacteria were determined by plating dilutions in Difco

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120 Tryptic soy agar. For these infections, wild type bacteria were opsonized for 30 min at
121 37°C with RPMI (control), or sera obtained from 10 days infected mice with either
122 *Brucella abortus* 2308 or *prpA* mutant strains (dilution 1/5000).

123

124 **2.4 Expression of recombinant PrpA.** Recombinant PrpA was produced as
125 previously described [8]. After purification, PrpA was sterilized by filtration through
126 a 0.22μm membrane, and the protein concentration was determined by the Bradford
127 method [14].

128

129 **2.5 Immunoglobulin quantization.** The titer of specific immunoglobulins against
130 *Brucella* was determined by ELISA experiments. Briefly, ELISA Maxisorp plates
131 (Nunc, USA) were sensitized with 0,4 μg/well of a *Brucella abortus* total protein
132 extract overnight and blocked for 2 hrs with 1% BSA in PBS. Serum samples from 10
133 days post-infected mice were serially diluted, and total immunoglobulins, IgG_{2a} and
134 IgM were detected with HRP-secondary antibodies in a colorimetric reaction and read
135 at 450 nm in a MicroPlate Reader Benchmark (BioRad). The closest absorbance value
136 (450 nm) to 0,5 was multiplied by the dilution factor to obtain the titer. Total
137 immunoglobulin concentration was determined by ELISA (Ebiosciences, USA)
138 according to the manufacturer's protocol.

139

140 **2.6 Cytokine analysis.** Spleens from infected female BALB/c mice were
141 homogenized and frozen at -20°C in 2ml PBS, 1% NP40, 2mM PMSF and 1x
142 protease inhibitor cocktail (Sigma Aldrich), thawed, and centrifuged to remove debris.
143 TNFα, IFNγ, TFGβ and IL-10 concentrations were determined by ELISA according
144 to manufacturer´s conditions (eBiosciences).

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145 **2.7 Flow cytometry.** Spleens from inoculated or infected mice were homogeneized
146 and depleted of red blood cells using Red Blood Cell lysing buffer (Sigma Aldrich).
147 Total splenocytes numbers were quantified using a Neubauer chamber. 10^6
148 splenocytes were stained for 30 min. with 1 µl of anti-mouse-CD3e-FITC and 1 µl of
149 anti-mouse-CD19-PE (eBiosciences) for T- and B-lymphocytes respectively. Splenic
150 B- and T-cells were quantified using a CyFlow Space (Partec). Percentage of B- and
151 T-cells obtained by flow cytometry were multiplied by the total splenocytes number
152 to obtain the total splenic B- and T-cell number.
153
154 **2.8 Statistical analysis.** The differences between the groups were calculated by using
155 the Student's *t* test for normally distributed variables and nonparametric Mann–
156 Whitney test for non-normally distributed variables. P <0.05 was considered
157 statistically significant.
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162 **3. RESULTS**

163 **3.1 PrpA induces B-cell proliferation *in vivo***

164 We have previously demonstrated that PrpA acts as a T-cell independent B-cell
165 mitogen when mice splenocytes are treated with the purified recombinant protein *in*
166 *vitro* [8]. To determine if this proliferation activity also occurs *in vivo*, we inoculated
167 intraperitoneally 50 μg of recombinant PrpA in PBS and measured at 24 hrs post-
168 inoculation the total number of B-lymphocytes in spleens by flow cytometry (see
169 Materials and Methods). As shown in Figure 1A, injection of PrpA significantly
170 increased (doubled) the total B-cell population in 24 hrs indicating that the effect
171 observed *in vitro* can be reproduced *in vivo*. Mitogenicity due to contaminant *E. coli*
172 LPS has been discarded as heat inactivated PrpA or splenocytes from C3H (LPS-non
173 responding) mice do not proliferate (not shown).
174 To determine if the protein has mitogenic activity in the context of an active infection,
175 we infected mice intraperitoneally with the wild type *B. abortus* and the *prpA* null
176 mutant strains and measured, by flow cytometry at 20 days post-infection, the total
177 number of T- and B-cells in the spleens. Figure 1B shows that the wild type infected
178 mice doubled the B-cell number compared to *prpA* [8] infected or non-infected mice
179 indicating that the gene plays a B-cell mitogenic role during the infectious process.
180 Figure 1C shows the dot plots used to obtain the results shown in panel B.
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187 **3.2 PrpA enhances the specific humoral anti-*Brucella* response**

188 Since one of the normal functions of B-cells is the production of antibodies, we
189 evaluated if the *prpA*-induced increment of B-lymphocytes observed during the
190 infectious process also resulted in an increment in total non-specific immunoglobulin
191 titers. We infected mice intraperitoneally with the wild type and the *prpA* mutant and
192 measured in the serum, at 10 days post-infection, the amount of total
193 immunoglobulins and IgG_{2a} by ELISA. As can be observed in Figures 2A and 2B, no
194 differences in the total amount of these immunoglobulins were produced between the
195 animals infected with the wild type and the *prpA* deficient strain. However, when we
196 measured specific antibody titers directed against the pathogen in the same animals,
197 we observed that wild type infected mice (which previously showed increased splenic
198 B-cell numbers) presented significantly higher titers of specific total
199 immunoglobulins and IgG2a compared to the mutant infected animals (Figure 2C and
200 2D). Altogether these results indicate that *Brucella* alters not only B-cell numbers, but
201 also their function, in a *prpA*-dependent manner.

202 The results shown above could not be attributed to different bacterial loads between
203 wild type and *prpA* null mutant infected mice during acute infection as both strains
204 showed similar bacterial loads at 1 and 3 weeks post infection (Figure 3A).

205

206 **3.3 The specific *Brucella* antibody response promotes intracellular replication**

207 The fact that the wild type strain showed higher levels of specific immunoglobulins
208 against *Brucella* in comparison to the *prpA* mutant seemed initially puzzling. Why
209 would a pathogen promote the specific antibody response against itself? Due to the
210 intracellular nature of the infection of *Brucella*, one possible explanation is that these
211 specific antibodies could promote the macrophage uptake. In order to determine if the

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212 *prpA*-dependent increase in the antibody titers directed against *Brucella* has an impact
213 in the infectious process, we performed a gentamicin protection assay with J774 A.1
214 murine macrophage cell line pre-incubating the bacteria 1 hour with serum from
215 uninfected mice or infected with either the wild type or the *prpA* mutant strains. As
216 shown in Figure 3, opsonization of the bacteria with serum from wild type infected
217 mice significantly increased the intracellular bacterial load compared to opsonization
218 with serum from the *prpA* mutant infected or uninfected mice. This result indicates
219 that the increment in the specific antibody titers promoted *in vivo* by PrpA, ultimately
220 promoted the invasion process.
221

222 **3.4 *PrpA* alters the cytokine pattern during *Brucella* infection**

223 Protective immunity against infection by *B. abortus* is directly related to the induction
224 of a pro-inflammatory or type 1-pattern immune response [15-18]. IFNγ and TNFα
225 are two central pro-inflammatory cytokines that promote macrophage activation and
226 elimination of intracellular bacteria [16, 19-23]. To determine if the production of
227 these cytokines were altered *in vivo*, mice were intraperitoneally infected with wild
228 type or the *prpA* mutant strains, and the levels of both cytokines were measured in
229 spleens or serum by ELISA. As can be observed in Figure 4, infection with the mutant
230 resulted in increased levels of INFγ in the serum and in spleens at 7 days post-
231 infection in comparison to the wild type strain (panels A and B). A similar pattern was
232 observed for TNFα, at 7 days post-infection a significant higher concentration of the
233 cytokine was detected in the spleens of mice infected with the mutant strain in
234 comparison with the spleens of mice infected with the wild type strain (Figure 4C).

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235 IL-10 and TGFβ1 are regulatory cytokines that dampen the protective Type-1 or pro-
236 inflammatory immune response of the host. Therefore, their production is exploited
237 by *Brucella* and many other pathogens to promote infection [24-27].
238 We infected mice with the *prpA mutant* or the parental strain of *Brucella* and
239 measured IL-10 and TGFβ1 levels in their spleens. As can be seen in Figure 4E,
240 spleens from animals infected with the *prpA* mutant strain showed higher levels of IL-
241 10 than the ones from mice infected with the wild type strain at 7 days post-infection.
242 In the case of TGFβ1, the *prpA* null mutant showed a statistically lower level of this
243 cytokine in the spleens at 21 days post-infection (Figure 4D). Altogether, these
244 experiments indicate that *prpA* also affects the pattern of pro- and anti-inflammatory
245 cytokines *in vivo*.
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248 **5. DISCUSSION**

249 *Brucella* spp. are pathogens with the capacity to cause chronic infections. This
250 amazing ability to survive in the face of an active immune response highlights the
251 immune modulation capacity of this pathogen.

252 In this report we have further advanced in the molecular characterization of PrpA as a
253 virulence factor of *B. abortus* (PrpA) that induces a transient anergic state of the
254 immune system and participates in the establishment of a chronic infection [8]. PrpA
255 has hydroxyproline epimerase activity [28], induces T-cell independent B-cell
256 proliferation *in vitro* and is homologous to a B-lymphocyte mitogen of *T. cruzi* that is
257 also involved in virulence [9, 28, 29]. Here we report for the first time that *Brucella*
258 infection produces an increment in the B-cell number and specific immunoglobulin
259 titers and demonstrate that both phenomena are produced *in vivo* in a *prpA* dependent
260 manner. Interestingly, these antibodies enhanced the invasion and intracellular
261 survival of the bacteria in macrophages indicating that the humoral response is
262 actually exploited for the infectious process. It has been clearly established that B-
263 lymphocytes play a role in enhancing *Brucella* virulence, since mice lacking B-cells
264 are more resistant to infection [26]. Additionally, it has also been reported that
265 opsonizing antibodies developed against the pathogen or its LPS also promote
266 infection *in vitro* [30-32], strongly suggesting that the specific humoral response is
267 actually detrimental for the control of the infection.

268 Cytokines are key effector molecules that orchestrate the immune response of the
269 host. While high levels of anti-inflammatory TGFβ1 and IL-10 have been observed to
270 promote chronic infections [24-27], protective immunity against *B. abortus* is directly
271 related to the induction of pro-inflammatory cytokines [15-18]. Therefore, the strategy
272 of *Brucella* to chronically infect its host seems to be related to its capacity to avoid the

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273 establishment of a protective Type-1 response [19, 33-35]. Consistent with this
274 framework, our results show that *prpA* is associated with the down-regulation of INFγ
275 and TNFα and the up-regulation of TGFβ1 *in vivo*, probably skewing the protective
276 pro-inflammatory towards an anti-inflammatory immune response. This may explain
277 the observation that the *prpA* mutant has significantly affected the capacity to
278 establish a chronic infection [8]. In the case of IL-10, it does not seem to support this
279 Type-1-to-Type-2 hypothesis. However, since IL-10 functions to control an excessive
280 and potentially harmful inflammatory response, the higher levels observed in mice
281 infected with *prpA* null mutant could be a physiological consequence of the increased
282 IFNγ observed in these animals [36].

283 In summary, our data indicate that *Brucella* alters B-cell number and function in a
284 *prpA* dependent fashion. Moreover, we show here that this pathogen exploits B-cell
285 function, specifically antibody production, to its own benefit. Even though infection
286 with the mutant does not show a difference in bacterial load during the acute phase,
287 the fact that the antibodies enhance the macrophagic invasion of the bacteria could
288 indicate that they have a differential distribution in the mouse (i.e. intracellular
289 location) and, thus, are less "visible" to the immune system favoring its persistence.
290 We have also demonstrated that *Brucella* actively alters the cytokine response pattern
291 and that *prpA* is involved in this process indicating that this virulence factor is
292 targeting several arms of the immune response. We have recently shed some light into
293 the molecular mechanism of PrpA. Although this virulence factor is a B-cell mitogen,
294 we have shown that it actually targets $CD11b^{+}F4/80^{+}$ macrophages, where it is
295 translocated during infection [7]. PrpA treated macrophages release soluble factor/s
296 ultimately responsible for B-lymphocyte proliferation. Moreover, we identified
297 NMMHC-IIA as a putative receptor required for PrpA to bind macrophages and

298 exerting its B-cell mitogenic effect. However, the signaling pathways triggered by
299 PrpA in macrophages and the identities of the soluble factors they release still remain
300 elusive. Experiments are in progress to further understand the mechanisms of these
301 pathways for enhancing *Brucella* infectivity.
302

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324 **LEGENDS TO FIGURES**

325 **Figure 1. *PrpA* induces B-cell proliferation *in vivo*. A.** Determination by flow
326 cytometry of B-lymphocyte number in spleens of mice intraperitoneally inoculated
327 with PBS or 50 μg of PrpA at 24 hrs post-inoculation. **B.** Determination by flow
328 cytometry of B and T lymphocyte numbers in spleens of uninfected mice or infected
329 with the wild type 2308 strain or the *prpA* null mutant strain at 21 days post-infection.
330 C. Flow cytometry dot-plots of B and T cell numbers determined in panel B.
331

332 **Figure 2. *PrpA* induces specific anti-Brucella immunoglobulin production *in vivo*.**
333 Determination by ELISA of **A**, total or **B**, IgG2a immunoglobulins in uninfected mice
334 or infected with the wild type 2308 strain or the *prpA* null mutant strain at 10 days
335 post-infection. Determination of **C**, total specific or **D**, IgG2a specific anti-*Brucella*
336 immunoglobulins in uninfected mice or infected with the wild type 2308 strain or the
337 *prpA* null mutant strain at 10 days post-infection.
338

339 **Figure 3. The specific antibody response promoted by *prpA* enhances bacterial**
340 **invasion of macrophages. A.** Bacterial load in the spleens of mice infected with the
341 wild type 2308 and the *prpA* null mutant strains during the acute phase of the
342 infectious process does not vary. **B.** Quantification by the gentamicin protection assay
343 of intracellular bacteria 1 hr post-infection with *Brucella abortus* 2308 pre-incubated
344 for 30 min with RPMI or sera from mice infected with either the wild type or the *prpA*
345 null mutant strain at 10 days post-infection. *P<0.05
346

347 **Figure 4. PrpA alters the levels of pro- and anti-inflammatory cytokines *in vivo*.**
348 ELISA quantitation of IFNγ in serum **(A)** and IFNγ **(B)**, TNFα **(C)**, IL-10 **(D)** in

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349 spleens of mice infected with the wild type and the *prpA* null mutant strains at 7 days

350 post-infection. Concentration of TGFβ1 **(E)** in spleens from mice 21 days post-

351 infection.

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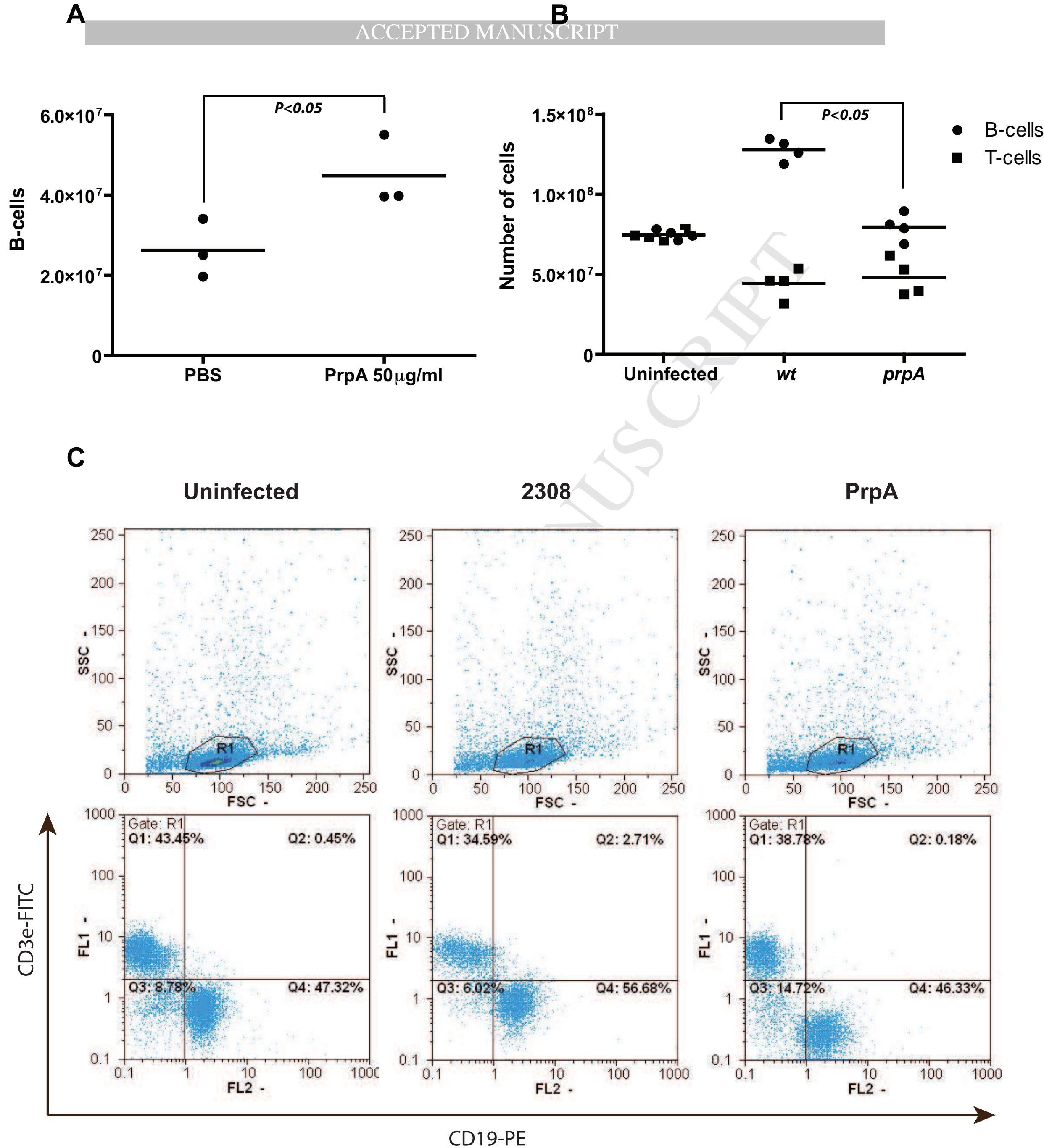

Figure 1

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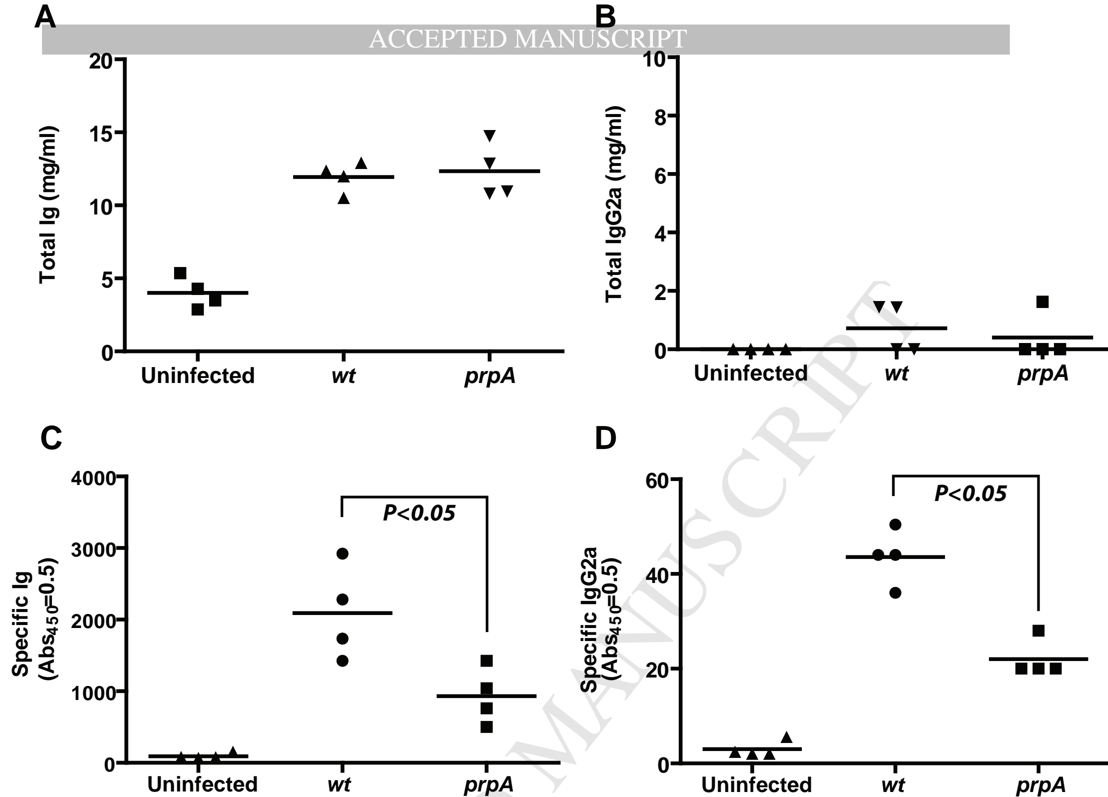

Figure 2

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A

7 days
21 days
Log(CFU/spleen)
7
6
5
4
3
2308
PrpA
2308
PrpA

B

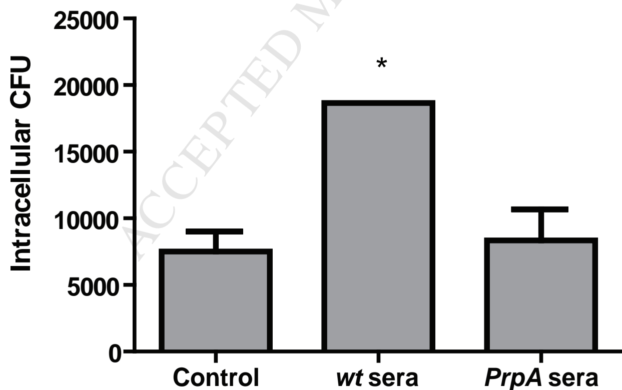

Figure 3

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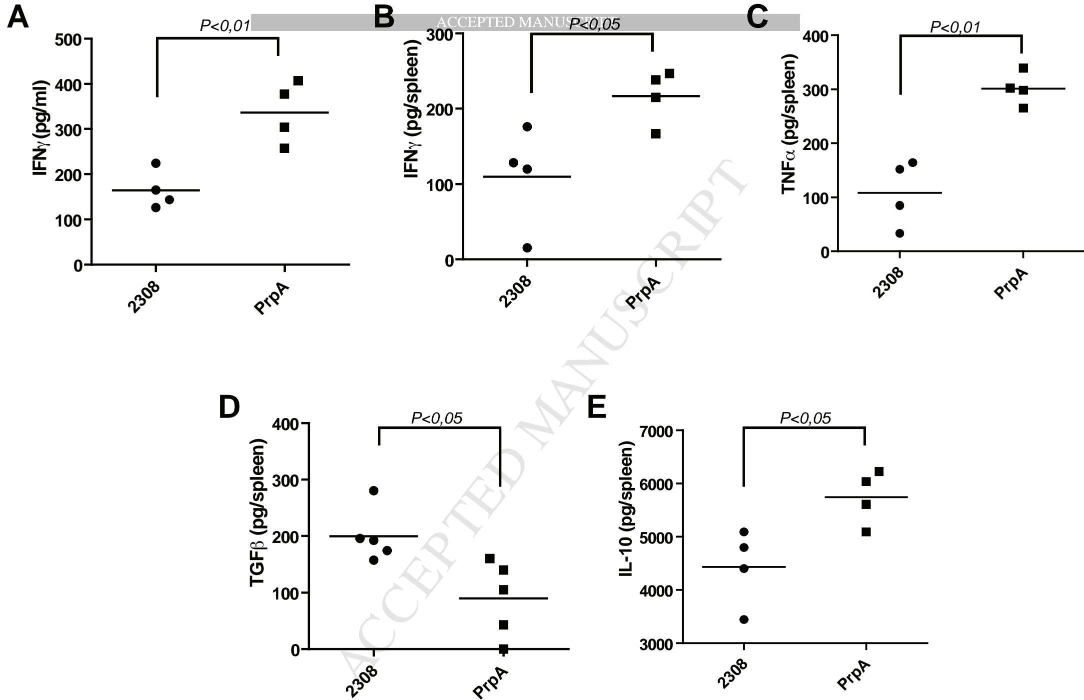

Figure 4