

1 **Title: Immunological responses to the relapsing fever spirochete *Borrelia turicatae* in**
2 **infected Rhesus macaques: implications for pathogenesis and diagnosis.**

3

4 **Running title: Immune responses by non-human primates to *Borrelia turicatae***

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29 **Abstract**

30 The global public health impact of relapsing fever (RF) spirochetosis is significant, as the
31 pathogens exist on five of seven continents. The hallmark sign of infection is episodic fever and
32 the greatest threat is to the unborn. With the goal of better understanding the specificity of B cell
33 responses and the role of immune responses in pathogenicity, we infected Rhesus macaques with
34 *Borrelia turicatae* (a new world RF spirochete species) by tick bite and monitored the immune
35 responses generated in response to the pathogen. Specifically, we evaluated inflammatory
36 mediator induction by the pathogen, host antibody responses to specific antigens, and peripheral
37 lymphocyte population dynamics. Our results indicate that *B. turicatae* elicits from peripheral
38 blood cells key inflammatory response mediators (IL-1 β and TNF- α) which are associated with
39 pre-term abortion. Moreover, a global decline in peripheral B cell populations was observed in
40 all animals at 14 days post-infection. Serological responses were also evaluated to assess the
41 antigenicity of three surface proteins, BipA, BrpA and Bta112. Interestingly, a distinction was
42 observed between antibodies generated in non-human primates (NHPs) and mice. Our results
43 provide support for the nonhuman primate model not only in studies of prenatal pathogenesis,
44 but for diagnostic and vaccine antigen identification and testing.

45

46 **Introduction**

47 Relapsing Fever (RF) spirochetosis is a neglected global disease. In parts of Africa, RF
48 spirochetosis is a common bacterial infection [1], and the disease is a significant cause of
49 hospital admissions and child mortality [2-6]. The causative agents are *Borrelia* species that are
50 transmitted by the human body louse, or ixodid and argasid ticks (1-4). The manifestation of
51 disease in humans includes recurrent febrile episodes, rigors, vomiting, severe headache,
52 neurological symptoms, muscle and joint aches and tachycardia (1). Antibiotic treatment may
53 result in the Jarisch-Herxheimer reaction, which is caused by a cytokine release leading to shock
54 (5) and even death (6, 7). Mortality of tick-borne RF spirochetosis is 4-10% and is associated
55 with the burden of spirochetes in the blood (8). RF borreliosis is particularly devastating on fetal
56 and neonatal health (9, 10). For example, in Tanzania a perinatal mortality rate of 436/1000 was
57 reported for *Borrelia duttonii* (11). The disease also has a severe impact in developing countries
58 because of the nonspecific, malaria-like clinical manifestation of the disease. Importantly, with
59 the geographic distribution of RF spirochetes largely overlapping with malaria (12) and studies
60 indicating an often misdiagnosis (13, 14), the true morbidity of RF is underappreciated.

61 The reduction in spirochete levels and eventual clearance has been shown in animal
62 models to be a direct result of the antibody response, especially IgM and IgG3 isotypes (15, 16).
63 The clearance by lymphocytic response was established by Newman and Johnson (17), who
64 showed not only the importance of the B cell response, but that of a T-independent B cell
65 response. Subsequent studies have demonstrated neutralization (18) and a directly bactericidal
66 (19) role of serum IgM in controlling relapsing fever spirochetemia. The contribution of B cell
67 subsets to RF pathogen control has been further delineated in mice (16, 20, 21).

68 Rodent models of RF have contributed immensely to the understanding of infectivity,
69 host-pathogen interactions and immune responses to infection [31-36]. For example,
70 transmission studies in *Borrelia turicatae* demonstrated that RF spirochetes enter the host within
71 seconds of tick bite (22), indicating the importance of preventing early mammalian infection.
72 Moreover, vaccination of mice with the *Borrelia hermsii* variable tick protein (Vtp) has guided
73 vaccine strategies. Vtp is produced in the salivary glands of *Ornithodoros hermsi* and
74 subsequently down-regulated once the pathogens are detectable in murine blood (23).
75 Vaccination studies with Vtp indicated that RF spirochete surface proteins produced in the tick
76 salivary glands could be ideal immunological targets to prevent the establishment of infection
77 (24).

78 Mice are natural reservoir hosts and may have limitations as models for testing
79 intervention and therapeutic strategies. Thermoregulation in mice varies, and they are a limited
80 model to further understand the Jarish-Herxheimer reaction. Mammals have evolved unique
81 thermoregulatory mechanisms in defense against pathogens, with rodents typically remaining
82 afebrile or decreasing body temperatures in response to bacterial challenge and endotoxin
83 administration (25-29). Therefore, mice may not be ideal for the evaluation of vaccine
84 candidates and therapeutics that prevent the clinical sign of fever, which is a hallmark feature of
85 RF.

86 Non-human primates (NHP) infected with RF spirochetes accurately mimic human
87 disease. A 1938 report published by Dr. Edward Francis showed that NHPs infected with *B.*
88 *turicatae* by tick bite exhibited morbidity and mortality commonly observed with human disease
89 (30). We have also demonstrated human-like illness with this model. Four rhesus macaques
90 were infected with *B. turicatae* by tick transmission, and radio telemetry was used to quantify the

91 intricacies of infection (31). Multiple febrile episodes, high spirochete densities in blood, and
92 disruption of cardiac function were observed.

93 In this current report, we further characterized the immune responses of NHPs that were
94 infected with *B. turicatae* by tick bite (31). We originally hypothesized that *B. turicatae* would
95 induce a TH2 type immune response, with concomitant induction of B cell proliferation and
96 antibody production. Rather, we found that in peripheral blood cells, *B. turicatae* induced TH1
97 type cytokines (IL-1 β and TNF- α) and significant declines in B cell populations were observed
98 soon after infection. Changes in peripheral blood lymphocyte subsets, immune mediator
99 production by stimulated PBMCs, and antibody responses reflect a distinct response to RF
100 *Borrelia* in NHPs. We evaluated antibody responses to a known conserved surface protein, the
101 *Borrelia* immunogenic protein A (BipA) (33, 34), and two newly identified surface proteins,
102 Bta112 and the *Borrelia* repeat protein A (BrpA). Bta112 and BrpA are up-regulated in the tick
103 and were evaluated to determine their antigenicity once *B. turicatae* enters the mammalian host.
104 Our results demonstrate differences in the host antibody specificity between mice and NHPs
105 infected with *B. turicatae*, and further indicate the significance of macaques as a model that most
106 accurately represents human RF borreliosis.

107

108 **Results**

109 **Co-culture of macaque PBMCs with *B. turicatae* elicits inflammatory response mediators.**

110 Given the high numbers of RF spirochetes that are observed in the blood during febrile episodes,
111 we sought to measure immune mediators produced by PBMCs in response to stimulation with
112 borreliae. In the analysis of 23 cytokines produced in response to stimulation with *B. turicatae*,
113 both commonalities and differences with *B. burgdorferi* (the Lyme disease causing agent) were

114 observed. While both borreliar pathogens elicited TNF- α , IL-10, G-CSF and IL-12/23p40, *B.*
115 *turicatae* induced a statistically significant higher level of IL-1 β and soluble CD40 ligand
116 (sCD40L) compared to *B. burgdorferi* (Figure 1A and 1D, respectively). We tested stimulation
117 of PBMCs derived from a naïve, uninfected monkey (JD03) in addition to PBMCs derived from
118 infected animals. To preserve the viability of the spirochetes and retain soluble factors, the
119 stimulations were performed with spirochetes in their own growth media (shown as Bt media and
120 Bb media). However, components of the media also had a moderate stimulatory effect for some
121 cytokines/chemokines. Figure 1A shows IL-1 β responses of naïve macaque PBMCs stimulated
122 with borreliae, indicating a significant induction of this inflammatory cytokine specifically by *B.*
123 *turicatae*. For IL-1 β , significance differences were observed at the 12-hour time point when
124 comparing *B. turicatae* (Bt) to BSK (p=0.0231) and *B. burgdorferi* (Bb) to BSK (p=0.001). At
125 24 hours, significant differences in these two groups were observed as well (Bt vs. BSK,
126 p<0.0001; Bb vs. BSK, p=0.0047). In Figure 1B, the effect on TNF- α production indicates that
127 both *Borrelia* species induce production of this inflammatory cytokine by PBMCs. At 12 hours,
128 significance was observed when comparing Bt vs. BSK (p=0.0007), Bb vs. BSK (p<0.0001). No
129 difference was observed between Bt media and BSK, yet the quantity of TNF- α induced by Bt
130 over that of Bt media was significant (p=0.0013), indicating that soluble factors do not drive the
131 induction of TNF- α by *B. turicatae*. At 24 hours, each of these differences remained significant
132 (Bt vs. BSK, p=0.0002; Bb vs. BSK, p=0.0003; Bt media vs. Bt). Figure 1 also shows the
133 specific differences in the induction of G-CSF (C), sCD40 (D) and IL-12/23 (E) from naïve
134 PBMCs stimulated with borreliae. Significant changes in G-CSF production by PBMCs
135 stimulated with *B. turicatae* were only observed at the 24 hour time point. Here, stimulation
136 with Bt vs. BSK was significant (p=0.0005), as was stimulation with Bb vs. BSK (p=0.0002).

137 For the soluble CD40 ligand, (sCD40l), significant differences were observed only at the 12 hour
138 time point, with stimulation of Bt compared to Bt media demonstrating significance ($p=0.0085$),
139 along with Bt vs. BSK ($p=0.0027$) and Bt vs. BSK ($p=0.0047$). For IL-12/23, significant
140 differences were seen at both 12 and 24 hour time points comparing Bt vs. BSK (12 h: $p=0.0007$;
141 24h: $p=0.0013$) and Bb vs. BSK (12h: $p=0.0033$; 24 h: $p=0.0343$). Figure 2 shows IL-1 β
142 responses among infected monkeys. Stimulation of day 14 p.i. PBMCs with Bt vs. Bt media
143 alone resulted in a significant increase in this inflammatory mediator for all three monkeys that
144 were infected. A two to three fold increase in quantity of IL-1 β produced in response to *B.*
145 *turicatae* compared to media alone indicates the specific effect of the pathogen. Specifically,
146 significant differences were observed at the 12 and 24 h timepoints for JB60 (12 h: $p=0.0070$; 24
147 h: $p=0.0010$), JB23 (12 h: $p=0.0022$; 24 h: $p=0.0005$), and IN57 (12 h: $p=0.0008$; 24 h:
148 $p=0.0005$) when Bt vs. Bt media were compared.

149 Immune regulatory molecules in serum were also quantified. We collected blood
150 droplets between days 0-14, but serum was collected beginning on day 14, as our intent was to
151 evaluate antibody responses. Therefore, we used available sera to test in the 23-plex cytokine
152 magnetic bead panel. The immune mediators that were elevated in serum at various time points
153 included IL-10, sCD40L, IL-8 and MCP-1 (supplemental Figure S2). Both IL-10 and MCP-1
154 were elevated at the earlier time points (14 and 28 days), but declined by 6 weeks post-infection.
155 In contrast, sCD40L and IL-8 appeared to be elevated throughout the infection period in
156 monkeys inoculated with *B. turicatae* (IN57, JB60 and JB23) compared to the animals fed upon
157 by uninfected ticks (JD03).

158 **Characteristic peripheral B cell depletion during acute infection.** T and B-cell phenotypic
159 analyses were performed with PBMCs at days 0, 14, and 70 post-infection time points from all 4

160 NHPs. With respect to the T-cell phenotype, only general CD4 and CD8 T-cell phenotypes were
161 measured in all three time points after infection. Only one animal (IN57) had reduced CD3+
162 populations, detected at day 14 post-infection (Table S1 and Figure 3) and the percentages of all
163 four subsets of CD3 cells (CD4+CD8-, CD4+CD8+, CD4-CD8+ and CD4-CD8-) were reduced.
164 The other 3 animals showed a moderate increase in peripheral T cells at 2 weeks p.i. that
165 declined by day 70.

166 B-cell subsets were distinguished by a panel of markers that included CD5, CD20, CD21,
167 CD138, IgM, IgD, CD27, and CD38. Notable reductions in the percentages of B cells were
168 observed in the serum 14 days after infection, suggesting an infection-induced B cell depletion
169 (Table 1, Figure S1). As shown in Table 1, the B-cell depletion was due to the loss of CD5 (B-1a
170 cells, marker of naïve or immature B-cells (35-37)), CD21 (marker of B cell differentiation and
171 maturation (38-40)), CD86 (activation marker (41, 42)), and CD138 (plasmablasts (37)). In a
172 subsequent staining, we looked at IgM+ B cells, switched memory (CD27+IgD-), non-switched
173 memory (CD27+IgD+), naïve (CD27-IgD+), double negative (CD27-IgD-), and
174 CD27^{high}CD38^{high} plasmablasts. A precipitous and global decline in peripheral B cell populations
175 was observed in all animals at day 14 p.i. (Table 1 and Figure 3). The B cell percentages and
176 different B-cell subsets returned to near pre-infection levels at day 70 post-infection in all
177 animals. The percent drop in total B cell frequency between day 0 and day 14 was significant for
178 all monkeys. Specifically, the CD20+ lymphocytes decreased by 43% for JD03, 84% for IN57,
179 80% for JB60 and 56% for JB23.

180 **Evaluation of Bta112 between strains of *B. turicatae*.** Bta112 was further evaluated as an
181 antigen because computational analyses suggested the protein was exposed on the surface of RF
182 spirochetes. The PROSITE InterPro database identified a predicted lipid attachment site at the

183 N-terminus of the protein (Figure S3). PSIPRED and the Phobius prediction server suggested
184 that the Bta112 was rich with alpha helices and the C-terminus of the protein was soluble and
185 positioned toward the extracellular environment, respectively. Sequence analysis of Bta112
186 between *B. turicatae* 91E135, FCB, TCB1, TCB2, and 99PE-1807, indicated the presence of an
187 intact gene that coded for a protein that was nearly identical in all *B. turicatae* isolates evaluated
188 (Figure S3). Given the presence of Bta112 in multiple *B. turicatae* isolates, we evaluated the
189 protein further.

190 **Expression of recombinant Bta112, temperature-mediated production, and surface**
191 **localization of the native protein.** To evaluate serological responses to *B. turicatae* Bta112, the
192 gene was expressed as a recombinant fusion protein. *bta112* was overexpressed in BL21 Star
193 (DE3) cells (Figure 4A), and rabbit immune serum was generated against the recombinant
194 protein. Since native *bta112* is up-regulated by *B. turicatae* during culture at 22 °C relative to 35
195 °C (43), spirochetes grown at both temperatures were evaluated to assess temperature-mediated
196 protein production. Optical density analysis of immunoblots probed with the rabbit serum
197 sample generated against rBta112 indicated 3.2-fold increase of the protein in *B. turicatae* grown
198 at 22 °C versus 35 °C (Figure 4B, top panel). The rabbit's pre-immunization serum sample was
199 used as a negative control (Figure 4B, middle panel). Moreover, a serum sample generated
200 against *B. turicatae* FlaB was used as a control to indicate similar protein loads were
201 electrophoresed in the immunoblotting assays (Figure 4B, lower panel).

202 Performing proteinase K and immunoblotting assays with *B. turicatae* grown at 35 °C
203 indicated that the Bta112 was surface localized (Figure 4C). Bta112 was degraded following
204 incubation with increasing concentrations (5, 50, and 200 µg per ml) of proteinase K for 15
205 minutes (Figure 4C, upper panel). The relative density of the periplasmic protein FlaB in 5 µg

206 per ml of proteinase K compared to the 0 µg per ml of proteinase K control was 101%. The
207 relative density of FlaB in 50 and 200 µg per ml of proteinase K was 93% and 90% respectively,
208 indicating that the spirochetes' membranes remained intact (Figure 4C, lower panel).
209 Collectively, these results supported that Bta12 was surface localized and the protein's
210 production was elevated at 22 °C. Given these findings, the antigenicity of rBta12 was
211 assessed.

212 **Serological responses to *B. turicatae* surface proteins.** Given variations in humoral responses
213 between mammalian species (44, 45), we compared the antigenicity of *B. turicatae* rBta12,
214 rBrpA, and rBipA using serum samples from NHPs and mice that were infected by tick bite.
215 Immunoblotting indicated varying serological responses between NHPs and mice to the
216 recombinant proteins (Figure 5 A-F). All four NHPs produced antibodies that bound to *B.*
217 *turicatae* protein lysates, rBta12, and rBipA, while serological reactivity to rBrpA was only
218 detected in JB60 (Figure 5B). An immunoblot from two mice represented the eight remaining
219 animals (Figure 5E and F). All the mice seroconverted to rBipA, two of eight animals
220 seroconverted to rBta12, while none of the animals seroconverted to rBrpA. Probing
221 immunoblots with a monoclonal antibody for the six histidine epitope was used to as a control
222 for the expected molecular weight of each protein (Figure 5G). These findings suggested
223 varying serological responses to RF spirochete antigens between NHPs and mice.

224 Since rBipA and rBta12 were immunogenic by immunoblotting in all four NHPs, we
225 further evaluated their serological responses over the duration of the study using enzyme-linked
226 immunosorbent assay (ELISA). Assessment of rBipA and rBta12 indicated the temporal
227 persistence of IgG responses to the recombinant proteins (Figure 6 A-D). JB23, JB60, and IN57
228 generated IgG responses for at least 84 days after the animals were infected with *B. turicatae* by

229 tick transmission (Figure 6 A-C). These responses were statistically significant compared to the
230 pre-infection serum samples for each animal to a given recombinant protein. JD03 was a control
231 animal, as described in our previous report (46), and evaluating IgG response at three time points
232 (7, 27, and 43 days) after feeding uninfected ticks indicated that tick saliva did not generate cross
233 reactive antibody responses to rBipA and rBta112 (D). After infecting the animal by tick
234 transmission, IgG responses to rBipA and rBta112 were detected 42 days after feeding (D, day
235 100 of the study). Statistically significant IgG responses were no longer detected to rBta112
236 from animal JD03 84 days after infection. Collectively, these findings indicated temporal
237 persistence of IgG responses to rBipA, while three of four animals generated prolonged IgG
238 responses to rBta112.

239

240 **Discussion**

241 In this study, we identified cytokine profiles associated with pathogenesis and
242 characterized differences in antibody responses of NHPs and mice infected with *B. turicatae*.
243 Evaluating cytokine production from *B. turicatae*-stimulated PBMCs identified mediators
244 involved in disease manifestation. *B. turicatae* induced significant increases in TNF α , IL-1 β ,
245 sCD40, and IL-23 compared to medium controls. The observed TNF α response has been linked
246 to spirochete lipoprotein-induced Jarisch-Herxheimer reactions [14]. Interestingly, *B. turicatae*
247 also induced statistically significant higher levels of IL-1 β compared to cells incubated with
248 *Borrelia burgdorferi*, the Lyme disease pathogen. IL-1 β and TNF α are known to play a primary
249 role in triggering miscarriage and pre-term labor in rhesus macaques (47) and in human patients
250 (48, 49). If significant quantities of RF spirochetes cross the placenta, such a response could be
251 induced *in utero*, and this pathogenic mechanism should be further evaluated. We did not detect

252 elevated levels of these key inflammatory mediators directly in serum of infected monkeys;
253 however, the response in PBMCs was detected between 12-24 hours post-stimulation. We
254 therefore suspect that we missed the height of the inflammatory response with evaluation
255 commencing after 14 days of infection. In mice infected with *B. hermsii*, plasma levels of IFN
256 appear to be elevated at the height of spirochetemia, whereas IL-1 β is detected after clearance of
257 the infection (50). Directly comparable experiments in mice and primates would be of benefit,
258 but consistent detection of these inflammatory mediators in blood cells exposed to RF
259 spirochetes indicates that they are likely important for pathogenesis.

260 Our findings suggest unique characteristics in antibody responses generated to *B.*
261 *turicatae* antigens between mammalian species. BipA is known to be immunogenic in mice (33,
262 34), but previous work screening serum samples from a small cohort of mice naturally infected
263 with *B. turicatae* by tick bite indicated that BrpA was not antigenic (51). The serological
264 responses from the 10 mice that were evaluated in this current study supported previous findings
265 with BrpA. Interestingly, one NHP produced a detectable response against rBrpA. Furthermore,
266 while only two mice seroconverted to rBta112, the protein was antigenic in all four NHPs.
267 While more animals are needed to definitively determine differences in antibody responses
268 between mice and NHPs, these findings suggested that the immune response between the two
269 mammalian species were dissimilar. Future work should evaluate NHPs as a model for antigen
270 discovery and vaccine development.

271 B cells drive the immune effort to control infection with relapsing fever spirochetes, and
272 distinct subsets with roles in immunity have been delineated (20, 52, 53). Mature B cells can be
273 divided into follicular (FO) B cells, present in the lymphoid follicles, marginal zone (MZ) B
274 cells, located in the marginal sinus of the spleen, and B1 cells predominantly found in the mouse

275 peritoneum. These are subdivided into B1a and B1b cells. B1 and MZ B cells are known to
276 engage in the T-cell independent antibody response. In contrast to the Lyme disease (LD)
277 spirochete, which induces an expansion of MZ B cells upon infection, RF spirochetes induce a
278 loss of MZ B cells (54). This may reflect the long-term presence of LD spirochetes in the spleen
279 versus the periodic blood-borne expansion of RF spirochetes and/or the differential responses to
280 antigens. The importance of the B1b cell component of the T-independent response to RF
281 spirochetes was demonstrated by transfer of B1b lymphocytes from convalescent mice to Rag
282 1^{-/-} mice (lacking mature B and T lymphocytes). This subpopulation conferred protection that
283 consisted of a specific IgM response which occurred when mice were challenged 60 days after
284 the reconstitution, indicating that this population alone could confer memory and afford
285 protection (20). Importantly, the identical counterpart of this particular B cell subset has not
286 been identified in humans (55), so it remains to be seen if the same mechanism to control
287 infection occurs in RF patients. Our study shows a precipitous drop in all of the major B cells
288 subsets within the peripheral blood of RF spirochete-infected NHP during the height of
289 bacteremia (2 weeks p.i.). While we did not examine lymph node populations, we surmise that
290 the steep decline in peripheral B cells was met with migration to lymphoid organs. By day 70, no
291 specific B cell subset emerged at an increased frequency over the others. In addition, the specific
292 antibody responses to recombinant proteins were of IgG isotype. Our attempts at screening IgM
293 responses did not produce clear and specific binding to either recombinant proteins or
294 *B.turicatae* lysates. This suggests that B cell responses in primates may rely more on T-
295 dependent IgG subclass responses.

296 RF Borreliosis is a major burden to maternal and fetal health, especially in resource-poor
297 areas and novel intervention strategies are needed. According to the World Health Organization,

298 every year 45% of all deaths in children under 5 years are among newborn infants in their first 28
299 days of life or the neonatal period, and 25% of neonatal deaths result from infections (56). The
300 major threat of RF borreliosis caused by both Old and New World species are pregnancy
301 complications occurring during the perinatal period (~20 weeks after gestation to 1-4 weeks after
302 birth) (57-65). The use of rhesus macaques as an animal model resulted in the identification of a
303 novel antigens (BrpA and Bta112) and confirmed the immunogenicity of BipA. Future studies
304 will determine if these antigens offer potential targets for human vaccination or in diagnosis of
305 RF. We will also expand on the developed NHP model and focus on understanding
306 immunopathology of infected pregnant macaques to identify cytokines in amniotic fluid, which
307 may reveal a mechanism of perinatal effects associated with RF infection.

308

309 **Materials and Methods**

310 **Ethics statement.** Practices in the housing and care of NHP and mice conformed to the
311 regulations and standards of the Public Health Service Policy on Humane Care and Use of
312 Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals. The Tulane
313 National Primate Research Center (TNPRC) and Baylor College of Medicine (BCM) are fully
314 accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care-
315 International. The Institutional Animal Care and Use Committees at the TNPRC and BCM
316 approved all animal-related protocols, including the infection and sample collection from NHPs
317 and mice. All animal procedures were overseen by veterinarians and their staff.

318 ***B. turicatae* strains used and animal infections by tick bite.** *B. turicatae* strains used in this
319 study were 91E135, Florida canine *Borrelia* (FCB), 99PE-1807, Texas canine *Borrelia* (TCB) 1,
320 and TCB2 (66). Tick transmission studies to were previously reported using a colony of *O.*

321 *turicata* that originated from Kansas (31). Briefly, four male Indian rhesus macaques (JB23,
322 JB60, IN57, and JD03) 2.02-2.85 years of age were used. Animals were sedated with 5-8 mg/kg
323 Telazol by intramuscular injection and ten third stage nymphal ticks infected with *B. turicatae*
324 were fed on each NHP (67). JD03 was initially fed upon by 10 uninfected ticks and monitored
325 for 42 days as a control for tick-specific responses. This animal was subsequently fed upon by
326 infected ticks.

327 Murine infection by tick bite was performed as previously described (34). Eight to 10
328 infected third stage nymphal *O. turicata* were fed to repletion on 10 Institute of Cancer Research
329 (ICR) mice, a Swiss derivative maintained at BCM. Infection was assessed by collecting a drop
330 of blood from the animals and evaluating the specimen by dark field microscopy for the presence
331 of circulating spirochetes. Thirty days after infection by tick bite, the animals were
332 exsanguinated and serum samples were obtained.

333 **Collection and processing of NHP blood.** To evaluate immune responses from NHPs, both
334 whole blood and clotted blood for serum were collected. Animals were anesthetized (Ketamine,
335 0.1 ml/kg, IM) and blood was collected by venipuncture of the femoral vein into either clot tubes
336 or EDTA tubes (whole blood). Blood for serum samples was collected at day 0 (prior to tick
337 feeding), day 28, day 56, day 70 and day 85, as previously described (31). Whole blood for flow
338 cytometry was collected at day 0, day 14 and day 70. Tubes containing clotted blood were
339 centrifuged at 3,000 rpm for 10 minutes to obtain serum samples. Peripheral blood mononuclear
340 cells (PBMCs) were isolated from whole blood using Lymphocyte Separation Medium (MP
341 Biomedicals) (68). The lymphocyte layer was washed once with sterile PBS, then resuspended
342 in PBS/2% FBS and counted. Cells were again pelleted and resuspended in Freeze Medium
343 (Invitrogen) at $\leq 1 \times 10^7$ /ml, then cryopreserved in liquid nitrogen until staining.

344 **Flow cytometry Assay.** Cryopreserved PBMCs were thawed, washed in RPMI-1640 media, ,
345 counted with trypan blue exclusion staining, and adjusted to a concentration of 1×10^7 cells/ml
346 in RPMI-1640 media with 10% FBS. One hundred μ l of cells were used for staining with
347 different concentrations of monoclonal antibodies and incubated for 25 min at room temperature,
348 protected from light, as reported earlier (68-71). The cells were further washed two times with
349 3ml of flow wash buffer (PBS with 0.1% BSA and 7mM sodium azide) and centrifuged at
350 1350rpm for 7 min. Following aspiration of supernatants from cell pellets, the cell pellets were
351 resuspended in 350 μ l of 1% paraformaldehyde buffer (in PBS). For antibodies conjugated with
352 tandem dyes, the cell pellets were dissolved in FACS fixation and stabilization buffer (Becton
353 Dickinson). For T cell phenotyping, CD3-FITC (SP34-2, BD Biosciences), CD8-PerCP (SK1,
354 BD Biosciences), and CD4-APC (L200, BD Biosciences) were used. For B cell phenotyping,
355 anti-CD5-PE-Cy5.5 (CD5-5D7, Invitrogen), anti-CD20-ECD (B9E9, Beckman Coulter), anti-
356 CD21-APC (B-Ly4, BD Biosciences), anti-CD86-PECy5 (FUN-1, BD Biosciences) anti-CD138-
357 FITC (MI15, BD Biosciences), anti-CD27-FITC (M-T271 BD Biosciences), anti-IgM (G20-127,
358 BD Biosciences) and anti-IgD (purified polyclonal, Southern Biotech) antibodies were used.
359 Anti-CD38 antibody (clone OKT10) was obtained from NIH NHP Reagent Resource. Data were
360 acquired within 24 hours of staining using either BD Fortessa instrument (BD Immunocytometry
361 System) or BD Facsverse (BD Biosciences) and FACSDiva software (BD Immunocytometry
362 System). For each sample, 50,000 events were collected by gating either on CD3+ T cells or
363 CD20+ B cells. For B-cell phenotypic analysis, cells were first gated on singlets, followed by
364 lymphocytes, and CD20+ B-cells and CD20- cells. CD20+ B-cells were further gated for
365 CD5/CD21/CD86 and CD138 expression. In cases where enough PBMCs were available, flow

366 cytometry was repeated to give duplicate samples. The gating strategy, along with representative
367 results from a single animal, is shown in supplementary Figure S1.

368 **Cytokine/chemokine array.** A portion of PBMCs derived from whole blood were also used for
369 in vitro stimulation with *B. turicatae*. Cells isolated from blood collected from each animal
370 (which included JD03 following control/uninfected tick feeding) on day 14 post-tick feeding
371 were resuspended in RPMI 1640/10% FBS at 1×10^6 /ml and 0.5 ml was added to each well of a
372 24-well plate. Late log-phase *B. turicatae* was diluted to 1×10^7 spirochetes per ml and 0.5 ml
373 was added to appropriate wells for a 10:1 ratio of spirochetes to cells. Controls included
374 untreated cells, cells incubated with *B. burgdorferi*, and cells incubated with BSK medium
375 (Sigma). To determine the impact of soluble factors produced by the spirochetes, cells were
376 incubated with 0.22 μ m-filtered BSK medium derived from *B. turicatae* and *B. burgdorferi*
377 cultures. Cultures were placed in a 37^o C, 5% CO₂ incubator. Supernatants were collected at 12
378 and 24 hours and stored at -20^o C. Serum samples from days 14, 28 and 41 were also tested by
379 the cytokine/chemokine array. Undiluted samples were analyzed using the MILLIPLEX MAP
380 Non-Human Primate Cytokine Magnetic Bead Panel - Premixed 23 Plex (Millipore) according to
381 the manufacturer's instructions. The bead assay was performed by the Pathogen Detection and
382 Quantification Core at the TNPRC and analyzed on a Bioplex 2000 Suspension Array System
383 (BioRad). Each analyte concentration was calculated by logistic-5PL regression of the standard
384 curve. To determine the statistical significance between the means for two experimental groups,
385 an unpaired, two-tailed Student's t-test was performed using GraphPad Software QuickCalcs.
386 Those differences with $p \leq 0.05$ are reported as significant.

387 **Computational analysis of Bta112.** Initially, *bta112* was identified as a gene up-regulated by
388 *B. turicatae* in the tick and at 22 °C (tick-like growth conditions) compared to spirochetes

389 isolated from infected murine blood and spirochete grown at 35 °C (mammalian-like growth
390 conditions) (43). The protein was evaluated using the Basic Local Alignment Search Tool
391 (BLAST) from NCBI, LipoP1.0, and ScanProsite. The gene sequence of *bta112* was evaluated
392 in *B. turicatae* 91E135, FCB, 99PE-1807, TCB 1, and TCB 2 through ongoing genome
393 sequencing efforts of these isolates.

394 **Recombinant proteins and rabbit serum generation to recombinant Bta112 (rBta112).**

395 Recombinant BipA (rBipA) and BrpA (rBrpA) were produced as six histidine linked proteins as
396 previously described (34, 51). Recombinant Bta112 was also produced as a six histidine linked
397 recombinant protein using the pEXP1-DEST expression vector (ThermoFisher Scientific,
398 Waltham, MA). The *bta112* gene was amplified by PCR from *B. turicatae* gDNA with
399 Accuprime Pfx (Thermo Fisher Scientific) without its predicted signal sequence (1-69 bp, signal
400 P 3.0 - <http://www.cbs.dtu.dk/services/SignalP-3.0/>). The gene's signal sequence was omitted
401 from amplification using primers SP/1779/70-1461 (5'-CAAACAAGTTTGTACAAAAATTTC
402 AAAAGTCCAAAAGACGCTG-3') and ASP/1779/70-1461 (5'-CGTATGGGTAAAGC
403 TTATTACTACTTGCGGTACTATCTGCTG-3'). The amplicon was cloned by In-fusion (BD
404 Clontech) into pEXP1-HA-DEST, digested with BsrGI and HindIII to create pEXP1-
405 HA::*bta112*, and Top10 *Escherichia coli* were transformed. Plasmid DNA was isolated and
406 submitted for sequencing to ensure that errors were not introduced by PCR. Vector NTI 11.0
407 (ThermoFisher Scientific) was used to assess *bta112* sequence. rBta112 was produced by
408 transforming *E. coli* BL21 Star (DE3) cells (ThermoFisher Scientific) with pEXP1-HA::*bta112*
409 and expression was induced with 0.5 mM IPTG. rBta112 was purified by nickel chelate
410 chromatography.

411 Rabbit anti-rBta112 was produced by Cocalico Biologicals, INC. Pre-immunization
412 serum samples were collected from two rabbits and the animals were immunized
413 intraperitoneally with 50 µg of rBta112 using complete Freund's adjuvant. The animals were
414 immunized three subsequent times in two-week intervals using Freund's incomplete adjuvant.
415 Serum samples were collected and evaluated for specificity to rBta112 and the native protein by
416 immunoblotting.

417 **Surface localization assays, immunoblotting, and densitometry analysis.** To determine the
418 surface localization of Bta112, proteinase K assays and immunoblotting were performed as
419 previously described (51, 72). Moreover, for all immunoblotting assays *B. turicatae* was grown
420 at 35 °C. For proteinase K assays, spirochetes were grown to a density > 5 x 10⁷ cells per ml,
421 pelleted at 1,000 x g for 10 minutes at room temperature, washed in PBS + MgCl₂, pelleted
422 again, and resuspended in PBS + MgCl₂. Spirochetes were incubated with increasing
423 concentrations (5, 50, and 200 µg per ml) of proteinase K (Promega, Madison, WI) for 15
424 minutes at room temperature. PBS + MgCl₂ was used as a vehicle control. Proteinase K was
425 inactivated by boiling samples at 100°C for 10 minutes. SDS-PAGE and immunoblotting were
426 performed as previously described using the Any kD Mini-PROTEAN TGX Stain-free precast
427 gels (BioRad, Hercules, CA) (51). One µg of recombinant protein or 1 x 10⁷ spirochetes were
428 electrophoresed per lane, and the Trans-Blot Cell (BioRad) was used to transfer proteins onto
429 polyvinylidene fluoride membranes. Rabbit, murine, chicken, and NHP serum samples were
430 used to probe immunoblots at a concentration of 1:200, and antibody binding was detected with
431 the appropriate secondary antibody and the ECL Western blotting reagent (VWR, Atlanta, GA).
432 ImageLab (6.0.1) was used to quantify the relative density of FlaB of spirochetes incubated with
433 5, 50 and 200 µg per ml proteinase K to spirochetes that did not undergo proteinase K treatment.

434 **ELISA**

435 Immulon 2HB flat bottom microtiter polystyrene plates (Thermo Fisher, Waltham, MA) were
436 coated with 1 µg/ml of rBipA or r1779 using 1x coating solution (KPL, Gaithersburg, MD). The
437 plates were washed three times with wash buffer (1x PBS and 0.05% Tween20) and blocked
438 with diluent (1x PBS, 5% Horse Serum, 0.05% Tween20, 0.001% Dextran Sulfate) overnight at
439 4 °C. Plates were washed again and probed with the NHP serum samples at a 1:100 dilution in
440 diluent and incubated for one hour at room temperature. Plates were washed again and incubated
441 for one hour at room temperature with peroxidase labeled goat anti-monkey IgG (KPL,
442 Gaithersburg, MD) at a 1:4000 dilution. Plates were washed again and incubated with ABTS
443 Peroxidase Substrate (KPL, Gaithersburg, MD) for 30 minutes and read at 405nm on an Epoch
444 Microplate Spectrophotometer (Biotek, Winooski, VT). Samples were considered statistically
445 significant if their mean optical density was more than three times the SD above the mean of the
446 pre-tick challenge sera ($p \leq 0.003$).

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452 Table 1. Changes in Peripheral B cell Subsets Following Infection with *B. turicatae*.

ANIMAL, DAYS POST INFECTION	CELLS OUT OF TOTAL LYMPHOCYTES										
	CD20+ (%)	CD20+CD5+ (%)	CD20+CD21+ (%)	CD20+CD86+ (%)	CD20+CD138+ (%)	IgM+ (%)	Switch memory (CD27+IgD-)	Non-Switched Memory (CD27+IgD+)	Naïve (CD27-IgD+)	Double negative (CD27-IgD-)	Plasmablast (CD27high CD38high)
JD03u*, d0	26.3 ± 0.71	0.652 ± .032	12.87 ± 0.88	16.43	0.051 ± .008						
JD03u*, d14	30.3 ± 0.85	0.809 ± 0.069	13.87 ± 2.48	14.67	0.113 ± 0.002						
JD03, d0	31.8	0.86	15.20	18.83	0.08	16.75	9.4604	15.8388	0.871	0.61908	0.1059
JD03, d14	18.2 ± 5.09	0.927 ± 0.14	8.71 ± 0.73	8.13	0.233 ± 0.019	16.9332	7.7559	9.3627	7.3233	6.4581	0.11546
JD03, d70	54.7 ± 20.93	2.39 ± 1.78	26.69 ± 8.24	18.87	0.240 ± 0.024	13.4506	6.5836	7.739	4.5562	2.9212	0.10268
IN57, d0	23.9	1.01	12.02	12.45	0.30	54.0015	12.7185	37.947	14.039	4.8094	0.0915
IN57, d14	3.915 ± 1.74	0.225 ± 0.177	1.95 ± 1.17	3.25	0.045 ± 0.006	2.05418	0.43089	0.9214	1.07316	0.28455	0.04293
IN57, d70	58.25 ± 2.05	1.96 ± 0.22	26.76 ± 2.72	28.63	0.342 ± 0.318	42.6855	9.3729	25.8501	16.716	7.761	0.16642
JB60, d0	43.8	1.61	24.66	24.05	0.11						
JB60, d14	8.97 ± 3.02	0.276 ± 0.12	3.99 ± 1.68	8.00	0.026 ± 0.029	4.04336	2.72517	2.58857	1.01767	0.496541	0.2394
JB60, d70	54.4 ± 2.97	2.73 ± 0.23	27.92 ± 1.05	27.67	0.125 ± 0.141	40.3975	18.532	36.2165	1.0283	0.74015	0.47888
JB23, d0	7.3	0.42	3.78	3.96	0.11						
JB23, d14	3.23 ± 0.52	0.132 ± 0.068	1.51 ± 0.41	2.43	0.0158 ± 0.018	1.39282	1.34706	0.94094	0.31746	0.252538	0.30615
JB23, d70	24.05 ± 8.27	0.864 ± 0.35	10.46 ± 1.31	9.50	0.071 ± 0.062	13.7839	15.4583	12.8271	0.7774	0.81627	0.53768

453 *uninfected (fed upon by uninfected ticks)

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665

666 **Figure Legends**

667 **Figure 1. IL-1 β (A), TNF- α (B), G-CSF (C), sCD40 (D) and IL-12/23 (E) responses of naïve**

668 **macaque PBMCs stimulated with *Borreliae*.** PBMCs obtained at day 0 from animal JD03

669 were stimulated with *B. turicatae* (Bt), *B. burgdorferi* (Bb), filtered BSK-H medium derived

670 from *B. turicatae* or *B. burgdorferi* cultures (Btmedia; Bbmedia), uninoculated BSK-H medium

671 (BSK), or left untreated (no trt). Supernatants were collected at 12 and 24 hours, for

672 measurement of inflammatory mediators by a NHP-specific 23-plex cytokine bead assay.

673 **Figure 2. IL-1 β production by the PBMCs of *B. turicatae*-infected macaques.** Cells isolated

674 from blood on day 14 post-tick feeding were incubated with *B. turicatae* at a 10:1 ratio of

675 spirochetes to cells (+Bt), untreated (no trt), incubated with BSK-H medium (+BSK), or

676 incubated with filtered mBSK medium derived from *B. turicatae* cultures (+Btmedia).

677 Supernatants were collected at 12 and 24 hours, for measurement of inflammatory mediators by

678 a NHP-specific 23-plex cytokine bead assay.

679 **Figure 3. Frequency of B and T cells in the peripheral blood after infection with *B.***

680 ***turicatae*.** PBMCs were subjected to flow cytometry to detect the relative percentages of B cells

681 (CD20+) and CD4+/CD8+ T cell subsets. Each staining experiment was performed twice and

682 the standard deviation is indicated with error bars.

683 **Figure 4. Expression of *bta112* as a recombinant protein, temperature mediated protein**

684 **production and surface localization of Bta112.** Bta112 was produced in *E. coli* and purified

685 (A). *E. coli* samples were taken prior to induction (Tp0), three hours after induction (Tp3), and

686 the purified protein. Immunoblots using rabbit serum samples generated against rBta112

687 indicated the protein's increased production at 22 °C compared to 35 °C (B, upper panel).

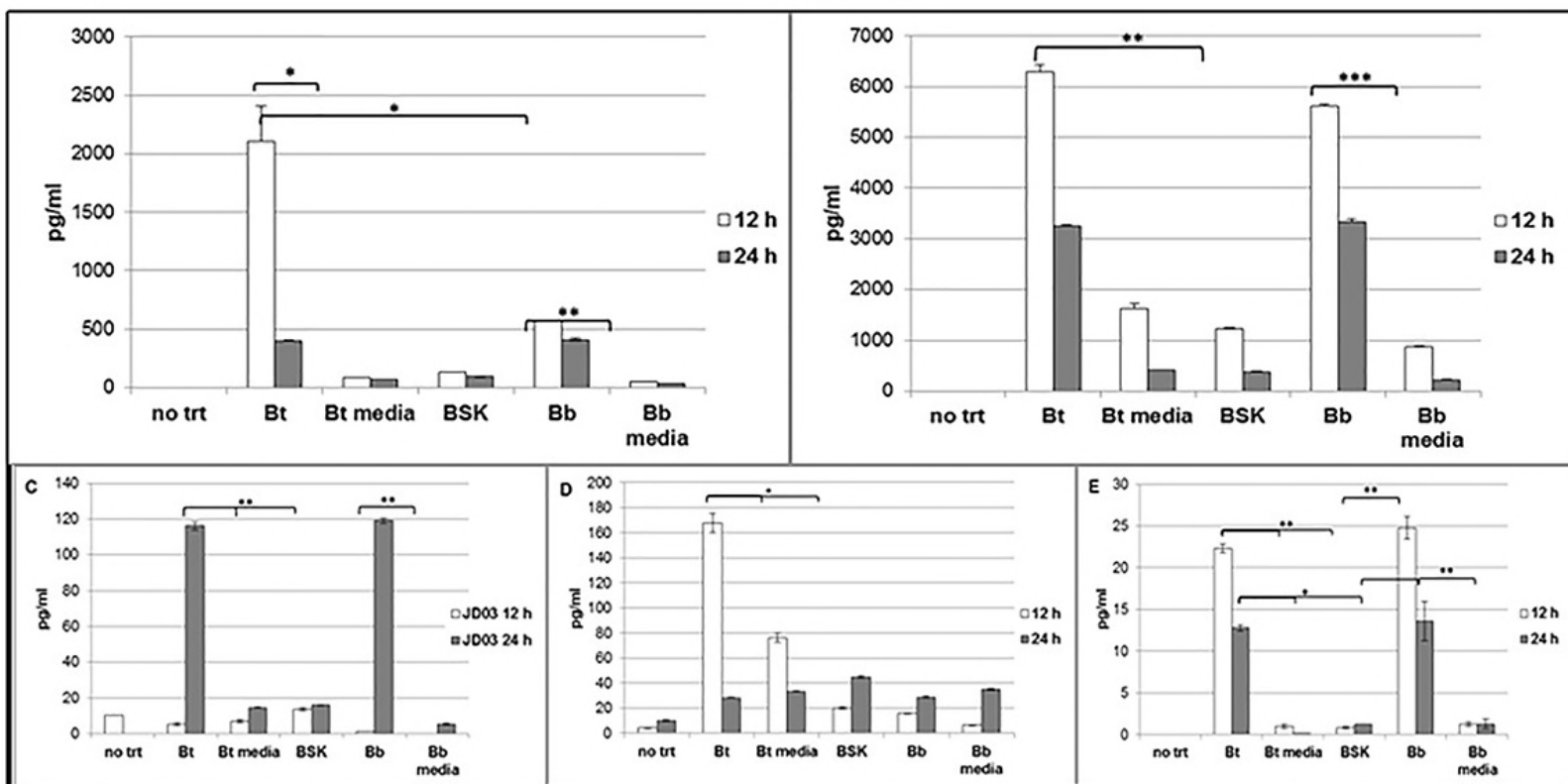
688 Preimmune serum samples (B, middle panel) and serum samples generated to the flagellin (FlaB)

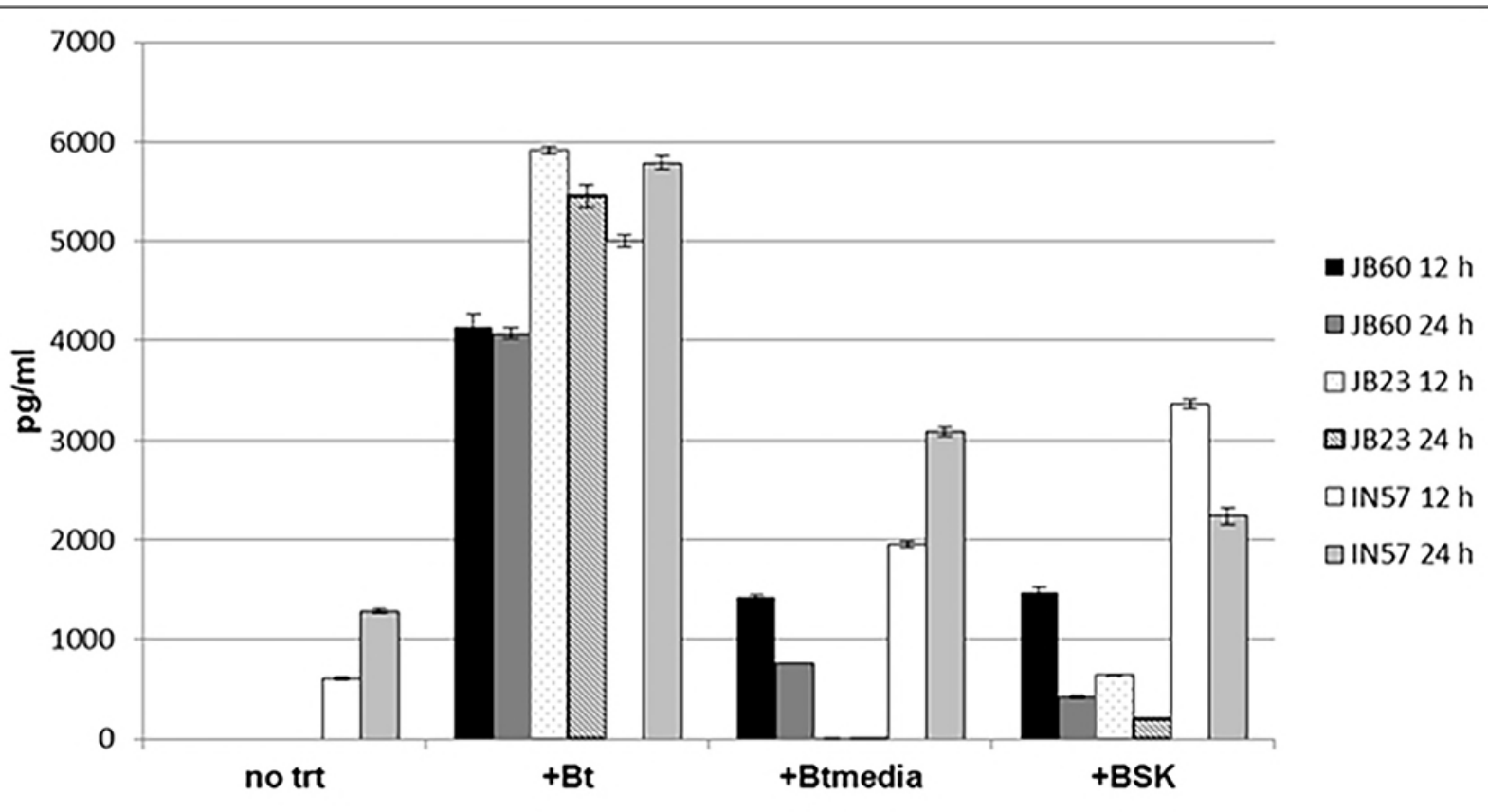
689 protein (B, lower panel) are shown. Immunoblotting was also performed to evaluate the surface
690 localization of Bta112. Proteinase K (PK) was used at concentrations of 5 to 200 µg per ml (C).
691 Membranes were probed with anti-Bta112 serum samples (C, upper panel), and anti-FlaB (C,
692 lower panel) as a control to indicate equal protein loads in the gels (73).. Molecular weight
693 markers (MWM) are show on the left of gel (A), and molecular masses are indicated on the left
694 of each immunoblot.

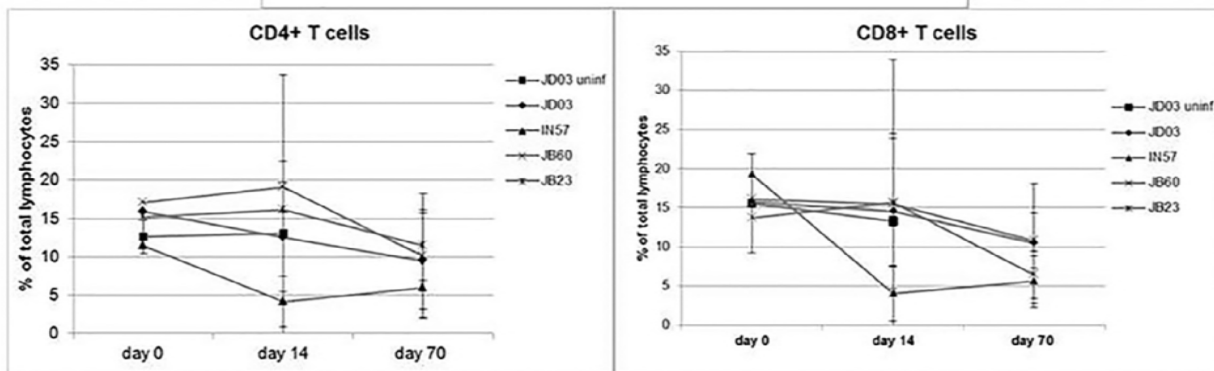
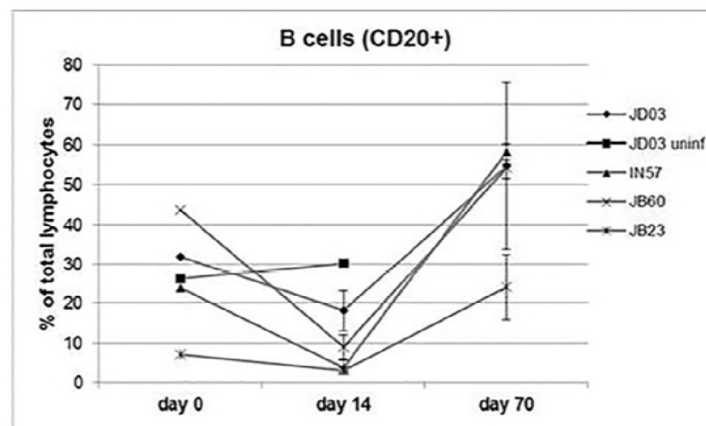
695 **Figure 5. Immunoblot analysis using serum samples from NHPs and mice to rBta112,**
696 **rBrpA, and rBipA.** Immunoblots of JB23 (A), JB60 (B), IN57 (C), and JD03 (D) are shown.
697 Membranes were probed with preinfection serum samples (left blot) and serum samples collected
698 at days 84 (JB23, JB60, and IN57) and 100 (JD03). Immunoblots from two mice (E and F),
699 which represent the remaining 10 animals are shown. Membranes were also probed with an anti-
700 6 histidine monoclonal antibody (G), and indicate the molecular weight of each recombinant
701 protein. An asterisk is next to each recombinant protein that was antigenic. Molecular weights
702 are indicated at the left of each immunoblot.

703 **Figure 6. Evaluation of temporal serological responses to rBipA and rBta112 by ELISA.**
704 Serum samples were evaluated prior to infection and at days 28, 44, 56, 70, and 84 for animals
705 JB23, JB60, and IN57 (A-C). Serum samples from JD03 were collected prior to and seven, 27,
706 and 43 days after feeding the animal with uninfected (D, uninfected ticks). The animal was then
707 infected with *B. turicatae* by tick bite and serum samples were collected at days 58, 72, 100, and
708 142 of the study (D, infected ticks). Pre-infection serum samples from each animal were used to
709 establish a statistically significant threshold ($p \leq 0.003$) for rBipA (dashed line) and rBta112
710 (dotted line).

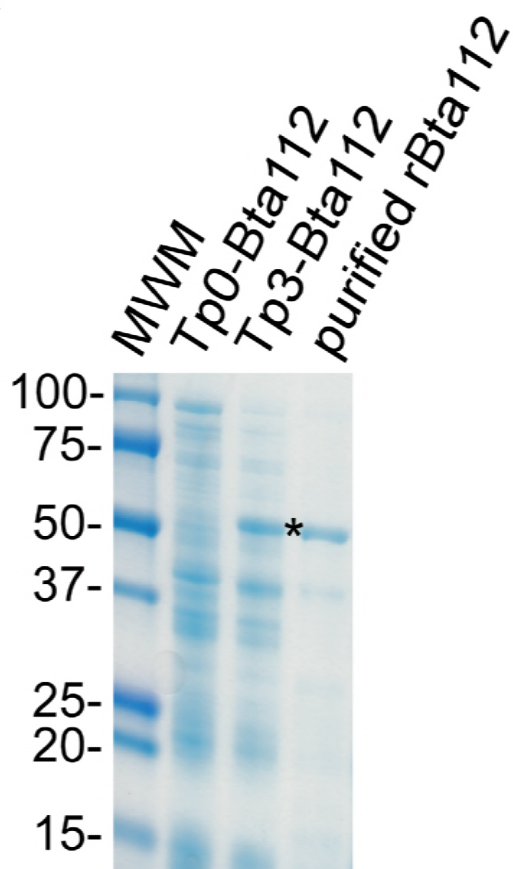
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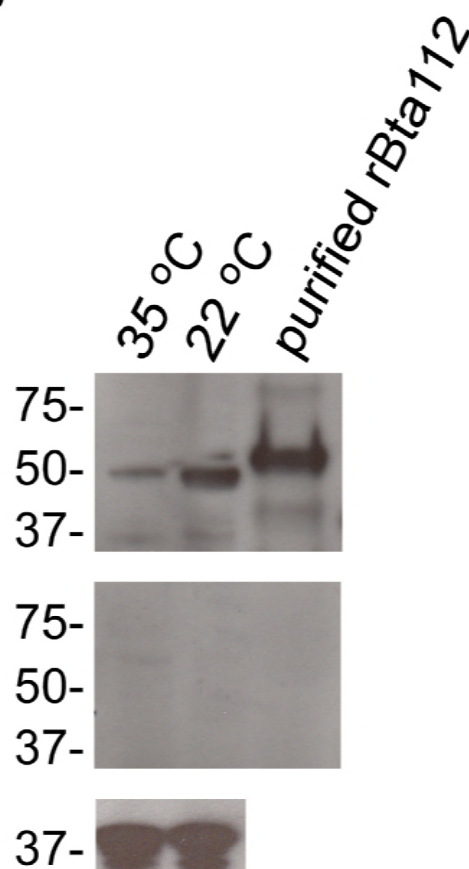




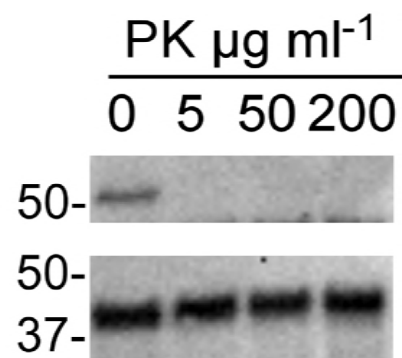
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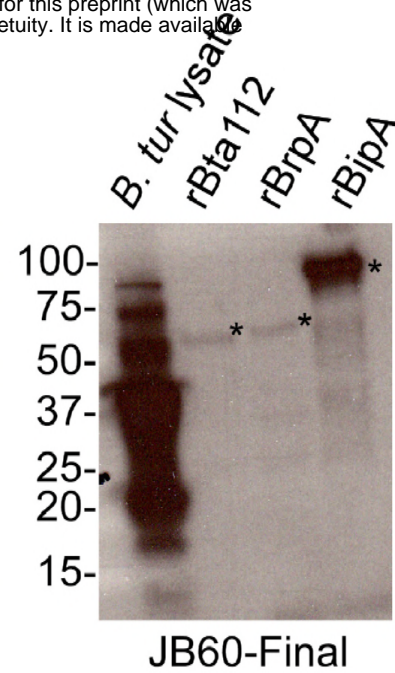
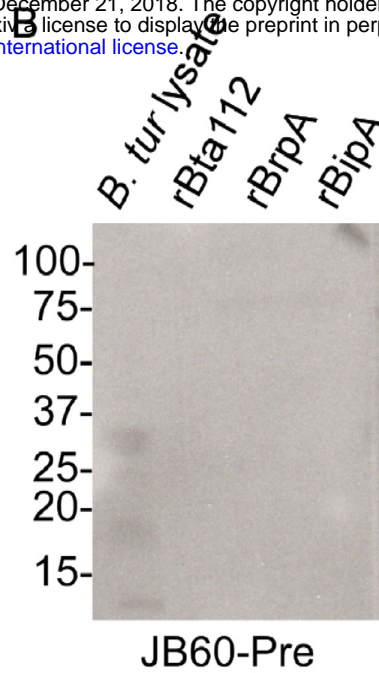
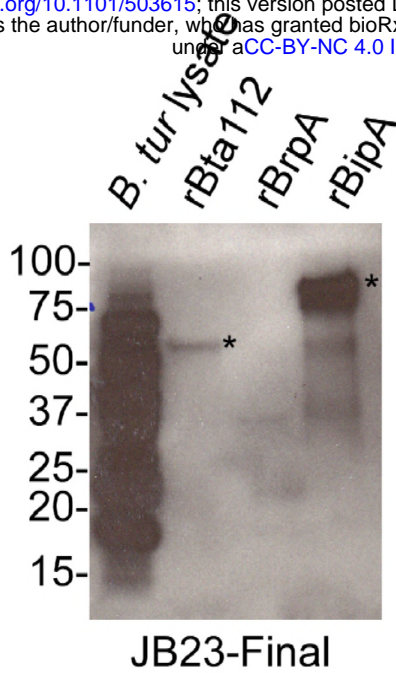
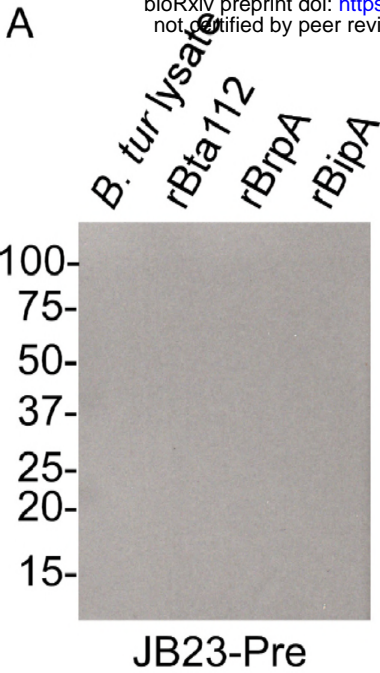
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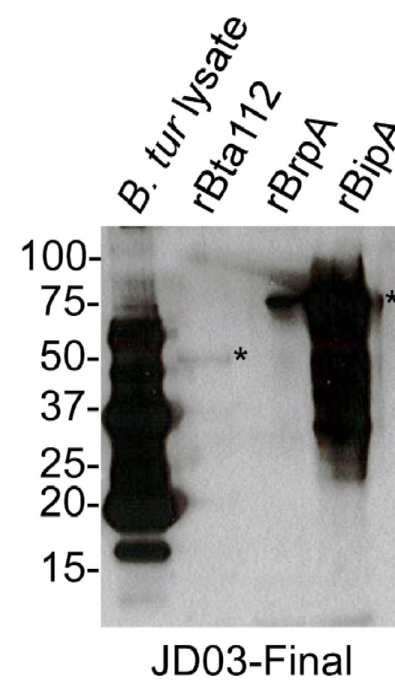
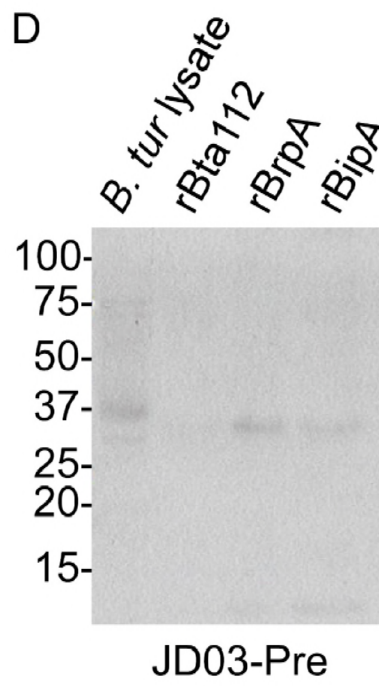
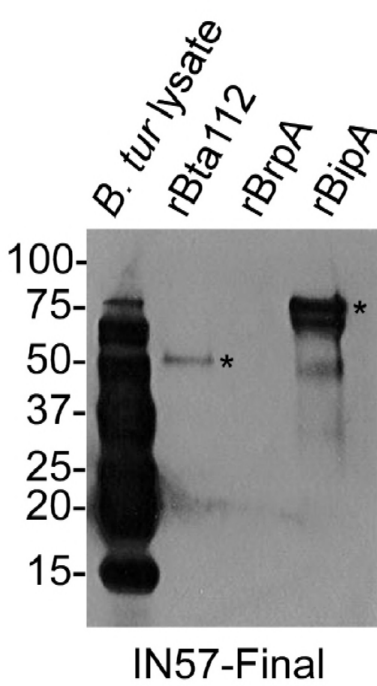
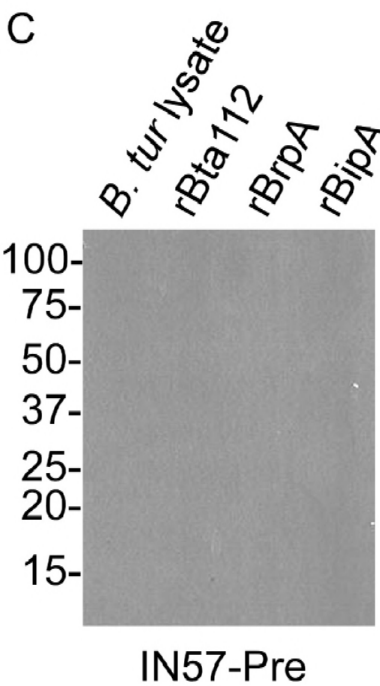
C



A



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E

