Protective efficacy of mutant strains of *Borrelia burgdorferi* as potential reservoir host-targeted biologics against Lyme disease

Running Title: Reservoir host-targeted biologics against Lyme disease

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Abstract

Lyme disease (LD), caused by *Borrelia burgdorferi* (*Bb*), is the most common vector-borne disease in the United States. Methods to control the transmission of *Bb* to humans via ticks are critical for prevention and elimination of LD. Previously, we showed that deletion of Borrelia host-adaptation Regulator (*badR* mutant) and replacement of 8 conserved residues in Carbon Storage Regulator A of *Borrelia burgdorferi* ($CsrA_{Bb}$, 8S mutant) resulted in upregulation of several lipoproteins due to increased levels of RpoS. Both strains were incapable of colonization in C3H/HeN mice. Clonal isolates lacking linear plasmid 25 derived from strain *Bb*-B31 or *Bb* B31-A3 incapable of infection served as control strains. Infection derived mouse serum and serum from Lyme disease patients exhibited reactivity to lysates and purified borrelial lipoproteins (PBLs) from these mutants. Proteomic analysis of PBLs from the mutants revealed similar and unique antigenic components. Two intradermal vaccinations with the live mutant strains induced anti-borrelial antibody responses and reduced *Bb* load in different tissues compared to unvaccinated C3H/HeN mice following needle challenge with infectious *Bb* strain B31-A3 strain. The *badR* mutant exhibited greater protective efficacy following needle challenge with *Bb* or infected *Ixodes scapularis* (*Is*) nymphs. Transmission of *Bb* to naïve *Is* larvae was reduced from vaccinated mice compared to unvaccinated mice challenged with *Bb* strain B31-A3. PBLs from mutant strains induced protective immunity in C3H/HeN mice against challenge with infected nymphs. Overall, non-infectious mutant strains can serve as a source of antigens for biologics to interfere with the natural life cycle of *Bb*.

Introduction

Lyme disease is the most common vector-borne disease in the US with 89,000 cases reported to Centers for Disease Control and Prevention (CDC, Atlanta) in 2023 and around 476,000 people diagnosed or treated each year reflecting an increase in the incidence of Lyme disease (1, 2). *Borrelia burgdorferi* (*Bb*), the spirochetal agent of Lyme disease is transmitted to vertebrate hosts through the bites of infected *Ixodes scapularis* ticks which are also known to transmit up to 7 pathogens to humans and naïve vertebrate hosts (3). Currently, there are no vaccines available for use in human. Hence, there is a dire need to advance strategies to limit or completely interfere with the transmission of one or more tick-borne pathogens as they cycle through the black-legged tick and a variety of vertebrate hosts (4, 5). One strategy is to reduce the burden of spirochetes in the reservoir hosts notably in the endemic areas, with the expectation that reduced transmission via ticks will gradually reduce the incidence of human Lyme disease (6, 7). Amongst a vast number of mutants strains of *Bb* that have been generated thus far, several are incapable of survival in mammalian hosts or have a highly attenuated phenotype (8-10). However, these mutant strains or their subcellular products provide a rich assortment of candidates that can be tested for efficacy as reservoir host targeted biologics, exploiting their inability to colonize mammalian hosts while capable of inducing protective immune responses.

We have previously shown that the deletion of Borrelia host-adaptation Regulator (BadR) results in de-repression of RpoS and an increase in the levels of expression of several major borrelial lipoproteins that are known to play a critical role in mediating the interactions of the spirochetes with host cell surfaces (11, 12). Levels of Outer surface protein C (OspC), Decorin binding proteins A and B (DbpA/B) and BBK32, among others, were all upregulated in the *badR* mutant. Moreover, replacement of 8 conserved residues with alanines in Carbon Storage Regulator A of *B. burgdorferi* ($CsrA_{Bb}$; 8S mutant) also resulted in increased levels of expression of major lipoproteins of *Bb* (13). Both these mutant strains are incapable of colonization of mouse models of infection and are therefore attenuated for infection likely due to a metabolic defect in the mutant for survival within mammalian hosts or due to innate/adaptive immune mechanisms clearing the spirochetes that hyper-express immunogenic borrelial lipoproteins and other immunologically relevant determinants. However, the increased levels of several RpoS-regulated borrelial lipoproteins in both *badR* and *8S* mutants could be exploited as biologics to enhance host immunity as surface exposed borrelial lipoproteins induce robust antibodies responses in mammalian hosts and are likely to be protective against challenge with infectious strains of *Bb.* In addition to the above mutants, a clonal isolate of *Bb* strain B31 lacking the 25kb linear plasmid

(lp25, ML23) or an lp25-deficient clone from *Bb* strain B31-A3 that are incapable of colonization of mammalian host were also used in the study. The lack of colonization of C3H/HeN or the SCID mice is due to a metabolic insufficiency lacking a key enzyme (*bbe22*, *pncA*, encoding nicotinamidase) critical for metabolism of nicotinamide to generate NAD in lp25 (14-16). The lp25 negative non-infectious borrelial strains are likely to modulate immune responses following multiple administrations via the intradermal route.

While several RpoS-induced lipoproteins were differentially expressed in both *badR* and 8S mutants, the levels of Outer surface protein A (OspA), a major immunogenic lipoprotein, was not altered in these mutants in comparison to the parental wild - type strain under *in vitro* growth conditions (11). Comparable levels of OspA between mutants was expected to induce OspA-specific protective immunity that has been previously shown to be critical for prevention of transmission of *Bb* from infected ticks to vaccinated vertebrate hosts (17-19). Several studies have demonstrated the relevance of the antibody responses directed at borrelial lipoproteins in preventing transmission of *Bb* from ticks. Moreover, a genome-wide proteome analysis of *Bb* (B31) using sera from patients with natural infections revealed approximately 15% of the 1,292 evaluated open reading frames code for immunogenic products (20, 21). The *Bb* genome encodes more than 120 lipoproteins, constituting nearly 8% of open reading frames. Experimental examination of 125 lipoproteins revealed that 86 are secreted to the bacterial surface (20). Consequently, lipoproteins are critical antigenic components of *Bb*, demonstrating efficacy in inducing protective immunity both as individual antigens or as a cocktail of recombinant lipoproteins. Therefore, we hypothesized that these mutant strains incapable of survival in mouse models of Lyme disease, despite hyperexpression of major surface exposed lipoproteins, likely serve as potential biologics to interfere with pathogen survival and transmission from reservoir hosts (7, 17, 22, 23). Moreover, numerous lipoproteins with native composition and distribution/stoichiometry on borrelial outer membranes are expected to induce robust protective immunity (24). Therefore, exploring the protective efficacy of lipoproteins purified from borrelial mutants holds significance, providing valuable insights into the potential use of these strains either as intact spirochetes or utilizing purified lipoproteins derived from these mutants as reservoir host -targeted vaccines.

Mutants strains of *Bb* have been exploited to dissect key features of innate and adaptive immune responses, as well as metabolic fitness to survive during the tick and mammalian phases of infection (25). A mutant lacking the multifunctional outer membrane protein, P66, known to mediate interactions of Lyme spirochetes with integrins was rapidly cleared from the skin of

127 C3H/HeN mice, a phenotype not influenced by skin antimicrobial peptide mCRAMP (26).
128 Moreover, *p66* mutant was unable to colonize neutrophil depleted mice, suggesting a novel role
129 for P66 in the colonization of *Bb*. A borrelial mutant lacking abundant expression of major surface
130 exposed lipoproteins (OspA, OspB and OspC, *ospABC*$^{-}$ mutant) was used to identify non-
131 abundant immunogenic proteins expanding the utility of mutant strains of *Bb* in developing tools
132 or biologics that can be used to induce protective immune responses (27). The overexpression of
133 several surface exposed immunogenic lipoproteins in the *badR* and 8S mutants allows for a rapid
134 and reliable method to partition these lipoproteins from the soluble and membrane proteins using
135 Triton X-114 as the detergent while serving to inactivate viable spirochetes (28, 29). In addition,
136 the partitioned borrelial lipoproteins are likely to be stable vaccine preparations with the caveat
137 that the immunity induced will not reflect that induced by soluble immunogenic proteins. Exploiting
138 mutant strains of *Bb* or their derivatives in conjunction with an expanded array of tools to
139 genetically manipulate *Bb* offers a novel platform to design and deliver antigens against one or
140 more tick-borne pathogens as a multivalent vaccine formulation, notably to prevent pathogen
141 burden in reservoir hosts.

142 In this study, we determined the antigenicity of purified lipoproteins from wildtype *Bb* strain
143 B31-A3, 8S and Δ*badR* using infection derived mouse serum and bar-coded Lyme disease patient
144 serum (blinded with no patient information). We assessed the utility of these mutant strains in
145 providing protection against *Bb* infection in C3H/HeN and in transmission of *Bb* to naïve larvae
146 following intradermal injection of intact spirochetes. Furthermore, the protective efficacy of the
147 lipoproteins purified from these mutant strains following immunization via the intradermal route
148 was determined against tick and needle challenge with infectious *Bb* strain. Correlates of immune
149 response from these studies will aid in formulating purified borrelial lipoproteins for delivery via
150 the oral route and in validation of efficacy of peripheral immune responses induced against *Bb.*
151 These studies are aimed at advancing methods to reduce pathogen burden in reservoir hosts,
152 thereby reducing transmission of *Bb* to humans and naive reservoir hosts via ticks.

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158 **Results**

159 **Extraction and profiling of borrelial lipoproteins**

160 Lipoproteins were extracted from *Bb* strain B31-A3 (wild type parental strain used to generate the
161 mutants), 8S and Δ*badR* using Triton X-114, and the protein profiles were compared between
162 insoluble pellet, aqueous phase (water-soluble proteins), and TX-114 phase (detergent phase).
163 Total protein profile of the partitioned proteins separated on an SDS-PAGE gel and stained with
164 Coomassie blue were distinct among all three strains (Fig. 1A). Immunoblot analysis using a high
165 dilution (1:10,000) of *Bb* strain B31-A3 infection-derived mouse serum from C3H/HeN mice
166 revealed high reactivity with proteins in the detergent phase in all three strains (Fig 1B). Moreover,
167 immunoblot of these partitioned proteins revealed the presence of FlaB (major flagellin of *Bb*)
168 predominantly in the pellet and aqueous phase fractions with no apparent levels of FlaB detected
169 in the detergent phase fraction (Fig 1C). Since much of this study is focused on purified borrelial
170 lipoproteins from *Bb*-B31, 8S and Δ*badR* strains, we compared the detergent phase proteins from
171 these strains and noted apparent differences between them (Fig 2A). It should be pointed out that
172 the infection derived serum had the most reactivity to the detergent phase proteins as a majority
173 of these proteins are surface exposed lipoproteins that are immunogenic. Notably, there are
174 increased levels of an approximately 33kDa protein that is present in the detergent fraction of 8S
175 compared to wild type or Δ*badR* strains (Fig 2A). Based on the size and mass spectrometric
176 analysis, this protein was determined to be Outer surface protein B which has been shown to be
177 a major immunogenic surface exposed lipoprotein (Fig 2B). It should be pointed out that while
178 OspB was apparent in detergent phase extracts of 8S strain, both the WT and Δ*badR* strains
179 expressed OspB as one of the five most abundant lipoproteins (Fig 2C). Three independently
180 purified TX-114 fractions from all three strains had similar protein abundance as the most
181 abundant peptides identified using an unbiased proteomic analysis of the detergent phase
182 proteins from all three strains were derived from major surface exposed lipoproteins of *Bb*
183 validating the detergent extraction procedure (Fig 2C). Notably, there are no significant changes
184 in the serum antibody reactivity between the lipoproteins and the *Bb* lysate, showing that majority
185 of the antigenic proteins are present in the lipoprotein fractions of all three strains. Moreover,
186 immunoblot analysis indicated no major differences between lipoproteins purified from mutants
187 and the B31 strains suggesting that these mutants did exhibit significant differences in major
188 lipoproteins levels which could alter their antigenicity.

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190 **Reactivity of serum from Lyme disease patients**

191 Human serum samples with no patient identifiers or clinical information from patients with or
192 without Lyme disease were obtained from the Bay Area Lyme Foundation supported BioBank and
193 tested for reactivity with lipoproteins from parental and mutant strains using an ELISA based
194 method (Fig 3). While several human serum samples showed high titers of IgG specific to
195 lipoproteins from all three strains, there were several samples that had baseline reactivity similar
196 to a normal human serum sample from a local blood bank used as a negative control (normal
197 serum). There were differences in the end-point titers of human serum samples against
198 lipoproteins suggesting differences in either the specificity of the antibodies or potential variations
199 in the levels of borrelial lipoproteins purified from different strains. Four human serum samples
200 tested had titers against lipoproteins from parental wild type strain at levels above base line titers
201 observed with lipoproteins from 8S or Δ*badR* strains (Fig 3). Results showed that all three strains
202 exhibited similar reactivity with the infected human serum samples (Fig 3). Isotypes of the human
203 antibodies indicated a predominance of IgG1 in several samples positive for Lyme disease, while
204 the presence of IgM is suggestive of early stages of infection (Fig 4). Immunoblot analysis of a
205 positive human serum sample (LD585) showed reactivity to lipoproteins of all three borrelial
206 strains (Fig 4B). Although the human serum samples were tested without prior clinical correlates,
207 several serum samples that reacted with both *Bb* lysates or with PBLs correlated with clinical data
208 compared retrospectively (Supplementary Table 1) (30).

209 **Protective efficacy of mutant borrelial strains**. We hypothesized that 8S and *ΔbadR* strains
210 can be exploited to induce protective antibody responses, a key correlate of protection, in
211 C3H/HeN following two immunizations, as both these strains were incapable of survival with
212 murine hosts. As a control, we also used two different clonal isolates lacking lp25, one from *Bb*
213 strain B31- ML23 (14, 15) and the other from *Bb* strain B31-A3 (parental strain for 8S and *ΔbadR*
214 mutants*)*. Two immunizations with $1x10^5$ *Bb*/mouse at day 0 and 14 resulted in increased levels
215 of IgM at day 14 after the second immunization (Fig 5). At day 28 post-second immunization, the
216 levels of total IgG, IgG1, IgG2a and IgG2b were significantly higher compared to that in naïve
217 control mice while the levels of IgG3, IgM and IgA were not significant (Fig 6). Efficacy of protection
218 was determined by challenging mice vaccinated twice with ML23, 8S and *ΔbadR* at day 28 post-
219 second immunization with 5 *I. scapularis* nymphs infected with B31-A3. As shown in Table 1, no
220 spirochetes were detected when different tissues were incubated in BSK-II media from all mice
221 vaccinated with 8S and *ΔbadR* strains while spirochetes were not detected only in 1 out of 3 mice
222 vaccinated with ML23. While one naïve mouse had spirochetes in several tissues tested, there

223 were no spirochetes detected in mice vaccinated with recombinant lipidated OspA via the
224 intradermal route (serving as a positive control for protection). All nymphs used in this experiment
225 were generated from naïve larvae obtained after being fed on a single infected C3H/HeN mouse
226 and hence the total number of mice used in the study were limited depending on the total number
227 of infected nymphs generated from the cohort of fed larvae. This is to ensure a close proximity to
228 number of spirochetes per nymph used to challenge vaccinated mice.

229 Two additional studies were performed using C3H/HeN mice vaccinated twice with viable
230 spirochetes (ML23, 8S and *ΔbadR)* to determine the efficacy of protection and ability to prevent
231 naïve larvae from acquiring *Bb* following challenge of vaccinated mice with $1x10^5$ *Bb* strain B31-
232 A3 spirochetes per mouse. In one experiment (Fig 7A), mice were vaccinated at day 0 and 16.
233 End-point titers of antibodies reacting with total *Bb* lysate needle challenged at day 56 were higher
234 in all vaccinated mice compared to unvaccinated controls (Fig 7B). Following needle challenge at
235 day 56, naïve larvae were allowed to feed on mice to repletion and *Bb* burden was assessed via
236 qPCR. While both unvaccinated mice allowed naïve larvae to acquire *Bb*, only one mouse
237 vaccinated with either 8S or *ΔbadR* had fed larvae with *Bb*. Moreover, it was possible to detect
238 *Bb* specific DNA in the skin, lymph node and joints of vaccinated mice that allowed naïve larvae
239 to acquire *Bb* while skin from one additional mouse vaccinated with *ΔbadR* was also positive for
240 *Bb*. In the second experiment, mice were vaccinated at day 0 and 18 and needle challenged with
241 infectious B31-A3 at day 92 followed by tick feeding at day 120 to determine the levels of
242 protection over a longer duration of time (Fig 7A). There were higher antibody titers in vaccinated
243 animals as expected at day 28 (Fig 7B) while fed larvae from all vaccinated mice were positive
244 for *Bb* by qPCR suggesting the duration of immune response after the second immunization is
245 key to limiting *Bb* acquisition by naïve larvae (Fig 7C). Although larvae fed on *ΔbadR* vaccinated
246 mice had detectable levels of *Bb*, qPCR analysis of skin and joint did not reveal presence of *Bb*
247 specific DNA. However, *Bb* was detected in several tissues from mice vaccinated with ML23 and
248 8S reflecting increased levels of pathogen burden in these mice.

249 Tissue samples from the vaccinated/challenged mice were aseptically isolated following
250 euthanasia at day 92 (Fig 7A) or day 120 (Fig 8A) and processed to facilitate isolation of
251 spirochetes in BSK-II growth medium. Cultures were blind passed after 5 days into fresh BSK-II
252 growth medium to minimize the toxicity associated with the degradation of host tissues and to
253 facilitate growth of spirochetes. As shown in Table 2, 5 out 5 naïve mice challenged with *Bb* B31-
254 A3 had viable spirochetes while only one mouse vaccinated with *ΔbadR* was culture positive for
255 spirochetes. Mice vaccinated with 8S (3/7) and ML23 (4/7) had tissues positive for spirochetes

256 suggesting reduced levels of efficacy in curtailing *Bb* survival although the challenge dose of
257 $1x10^5$ spirochetes of *Bb* strain B31-A3 being relatively high. Moreover, mice with culture positive
258 tissues were the ones that allowed for *Bb* acquisition by naïve larvae and also correlated with
259 high *Bb* load in those tissues by qPCR. These data suggest that vaccination with mutant strains
260 of *Bb* provided select correlates of protection that can be exploited for developing reservoir host
261 targeted biologicals for reducing *Bb* burden.

262 **Protective efficacy of purified lipoproteins from mutant borrelial strains**

263 Since borrelial lipoproteins play a vital role both in the induction of protective immune responses
264 as well as in the pathogenic mechanisms of *Bb*, we hypothesized that purified borrelial
265 lipoproteins (PBLs) from the mutant strains are likely the major determinants influencing the
266 protective capability of intact mutant spirochetes. Correlates of humoral immune response to
267 intradermal inoculation of PBLs in C3H/HeN mice were established using an ELISA based method
268 and levels of IgG, IgG1, IgG2a and IgG2b were found to be significantly elevated compared to
269 that in naïve controls at day 28 dpv, while levels of IgM, IgA and IgG3 were not. Lipoproteins from
270 detergent phase of *Bb* strain B31-A3 were used as the wildtype preparation since both 8S and
271 *ΔbadR* strains were generated using this infectious strain. As shown in Table 3, C3H/HeN mice
272 immunized via intradermal route with PBLs from B31-A3 and 8S were protected following
273 challenge with infected *I. scapularis* nymphs with no viable spirochetes isolated from different
274 infected tissues. The levels of protection conferred by PBL from *ΔbadR* mutant was lower with 2
275 out of six mice positive for viable spirochetes in select tissues. Lipidated OspA was protective with
276 no tissues positive for *Bb* while 5 out of 6 control mice (PBS-treated) had tissues that were culture
277 positive for spirochetes. These data sets are representative of two independent studies with 3
278 mice each to facilitate using *Bb* infected nymphs obtained from a single infected C3H/HeN mice
279 to ensure levels of *Bb* challenge are comparable between each treatment.

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286 **Discussion**

287 There is a dire need to develop products and strategies to reduce the burden of one or
288 more tick-borne pathogens in reservoir hosts in the endemic areas where these diseases are
289 prevalent to prevent their rates of acquisition/transmission via vectors and in the process,
290 eliminate or reduce the incidence of tick-borne diseases in humans (23). A large number of studies
291 have been performed with one or more recombinant proteins from *Bb* that have led to vaccines
292 approved for use in humans (22, 31-33) and dogs (34-36) as well as those targeted for reservoir
293 hosts with varying degrees of protective efficacy (7, 17). Additionally, borrelial antigens as
294 recombinant proteins or those formulated using viral vaccine vectors have been evaluated for
295 efficacy of protection in different experimental models of infection (37-41). However, the rapid
296 expansion of a catalog of mutant strains of *Bb* lacking the ability to colonize different strains of
297 mice or ticks but expressing immunogenic determinants to modulate the vertebrate host immune
298 responses remain to be exploited as biologics capable of interfering with the natural life cycle of
299 *Bb*.

300 In this study, we exploited two mutant strains (8S and *ΔbadR)* of *Bb* that hyper-expressed
301 several major lipoproteins critical for colonization and immunomodulation of mammalian hosts
302 (11, 13). Partitioning of lysates into detergent and aqueous phase proteins using Triton X-114 and
303 immunoblot analysis demonstrated that the detergent phase proteins had significant
304 immunoreactivity with infection derived serum generated against the parental strain of both
305 mutants, *Bb* strain B31-A3 (Fig 1B). A notable difference in the profile of lipoproteins between the
306 mutant and parental wild type strain was increased expression of Outer surface protein B (OspB)
307 in the 8S mutant compared to parental and *ΔbadR* strain while a protein of approximately 17kDa
308 was expressed at a lower level in 8S mutant (Fig 2A, 2B). Additional immunoblot analysis
309 indicated that the 17kDA protein reacted with anti-OspB antibodies in the parental and *ΔbadR*
310 mutant strain (data not shown) suggesting proteolytic processing of OspB in these strains while
311 the full-length protein remained intact in the 8S mutant though the mechanism behind this
312 observation is unclear (42, 43). Moreover, unbiased proteomic analysis of lipoproteins revealed
313 presence of OspB in all three strains was detected as one of the top 5 highly expressed
314 lipoproteins (Fig 2C). While deletion of *badR* resulted in derepression of RpoS leading to an
315 upregulation of several genes encoding major surface exposed *Bb* lipoproteins, the 8S mutant
316 with 8 critical residues in Carbon storage regulator A of *B. burgdorferi* ($csrA_{Bb}$) replaced with
317 alanines resulted in stable expression of $CsrA_{Bb}$ leading to upregulation of RpoS and *rpoS*-
318 regulated lipoproteins resulting in strains that can both be used to harvest immunogenic

319 lipoproteins as biologics to interfere with colonization of *Bb* (13, 44, 45). A well-established
320 procedure using Triton X-114 to partition borrelial lipoproteins provides for simple procedure to
321 extract lipoproteins from these mutant strains while ensuring the inactivation of spirochetes, even
322 though the mutant strains are incapable of survival within mammalian hosts (29). The lack of
323 detection of FlaB, the major flagellin that is not a lipoprotein and is localized in the periplasm of
324 spirochetes, in detergent phase extracts demonstrated the utility of this method to partition
325 lipoproteins from these mutants (Fig 1C). Moreover, unbiased proteomic analysis of peptides from
326 detergent phase extracts of spirochetes identified the top 5 proteins as lipoproteins (Fig 2C). All
327 major lipoproteins purified from these strains have the advantage of being in proportions
328 comparable to their stoichiometry observed in Lyme spirochetes compared to a cocktail of multiple
329 recombinant borrelial lipoproteins to serve as ideal biologics to modulate host responses to
330 interfere with colonization and transmission of *Bb*.

331 Immunogenicity of detergent phase proteins was also determined using infection derived
332 serum from mice and a set of human serum samples from Lyme Biobank with no patient identifiers
333 or patient history of Lyme disease. Comparison of clinical correlates also showed that the specific
334 serum samples positive for interacting with total *Bb* lysates or PBLs were from LD patient (30).
335 As expected, immunoblot analysis with infection derived mouse serum established significant
336 reactivity to the detergent phase extract comprised primarily of lipoproteins providing a correlate
337 of immune response critical for exploiting lipoproteins from *Bb* as source of biologics. Moreover,
338 several samples of human serum were reactive with detergent phase proteins adding to the
339 immunoreactivity of the detergent fraction consistent with prior studies on specificity of Lyme
340 disease patient serum against the major surface-exposed lipoproteins of *Bb* (46). Isotypes of
341 human serum specific to lysates from the parental wildtype strain (B31-A3) indicated 4 human
342 serum samples with higher IgM levels compared to IgG (0526; 0538; 0611) with only two samples
343 (0585 and 0610) having higher levels of IgA above the levels in normal human serum. There were
344 several human serum samples with increased levels of IgG1 reflecting *Bb* specific antibodies
345 likely contributing towards opsonization of the spirochetes and directed at protein antigens (47,
346 48).

347 Immunization of C3H/HeN mice with two doses of 8S and *ΔbadR* with ML23 (incapable of
348 survival in mammalian hosts due to lack of lp25) serving as a control resulted in increased levels
349 of IgM at day 14 after second immunization. While total IgG levels were also significantly higher
350 in vaccinated mice compared to naïve controls, the levels of IgG2a were significantly higher while
351 there were no significant increase observed with other antibody isotypes reacting with total

352 borrelial lysates (Fig 5). However, at day 28 post-second vaccination, there were a significant
353 increase in the levels of total IgG, IgG1, IgG2a and IgG2b in vaccinated animals compared to
354 naïve controls. No significant differences in the levels of IgM, IgG3 and IgA between vaccinated
355 and naïve controls (Fig 6), providing additional correlates of humoral immune response to
356 vaccination with intact mutant spirochetes incapable of survival in murine hosts.

357 Multiple challenge studies were performed where C3H/HeN mice vaccinated twice at day
358 0 and day 14 and challenged at day 28 after the first vaccination. As shown in Table 1, all animals
359 vaccinated with *ΔbadR* or 8S strains, challenged with infected nymphs (5 nymphs per mouse)
360 obtained from a cohort of *I. scapularis* larvae fed on a single mouse infected with *Bb* strain B31-
361 A3, had no colonization of spirochetes in any of the tissues tested similar to mice vaccinated with
362 recombinant lipidated OspA. However, 2 out of 3 mice vaccinated with ML23 had spirochetes in
363 several tissues and one naïve mouse challenged with infected nymphs had spirochetes in several
364 tissues tested. The variation in number of mice used in the study was reflected in the total number
365 of infected nymphs obtained after molting of fed larvae from a single infected mouse to ensure
366 comparable number of spirochetes are delivered to different groups of mice. To overcome the
367 limitations of the small group of mice used in the tick challenge studies, we further performed
368 studies vaccinated twice with ML23, 8S and *ΔbadR* strains as shown in Fig 7 and 8. Levels of
369 antibodies were tested at days 14, 28 and 42 post-first vaccination and were found to be
370 significantly higher in all vaccinated mice compared to naïve controls. Mice were needle
371 challenged with 10^5 *Bb* strain B31-A3 spirochetes to limit variations in the challenge dose at day
372 56 via needle in one experiment and naïve larvae were allowed to feed to repletion at day 84 after
373 challenge. Fed larvae from one out of three mice vaccinated with 8S and *ΔbadR* were positive for
374 *Bb* while larvae from all mice vaccinated with ML23 had no detectable level of *Bb*. Quantitative
375 realtime PCR analysis of tissues from this cohort of mice collected after euthanasia at day 92
376 revealed the presence of *Bb* specific DNA in the corresponding mouse that had fed larvae test
377 positive for pathogen specific DNA. In addition, *Bb* specific DNA was detected in the skin of one
378 mouse vaccinated with 8S or *ΔbadR* while mice vaccinated with ML23 strain had no detectable
379 *Bb* specific DNA in any of the tissues. In the second experiment, a cohort of C3H/HeN mice
380 vaccinated twice with mutant strains of *Bb* were challenged with $1x10^5$ *Bb* strain B31-A3
381 spirochetes at 10 weeks (day 92 from first vaccination) after the second vaccination to determine
382 the duration of protection conferred by the mutant strains. Fed larvae from all mice had detectable
383 *Bb* specific DNA, while the larvae fed on naive control animals had higher *Bb flaB* copies
384 compared to vaccinated animals. Moreover, only the lymph node from one mouse vaccinated with
385 *ΔbadR* had *Bb* specific DNA while several tissues were positive from mice vaccinated with 8S or

386 ML23. These observations are also reflective of the timing of the challenge, the detection of *Bb*-
387 specific DNA from non-viable spirochetal remnants, and the overall challenge dose of $1x10^5$
388 *Bb*/mouse which is considerably higher than what would be acquired from tick-challenge.
389 Nevertheless, the nature of the antibody response in vaccinated animals using the regimen
390 tested, although not sterilizing, resulted in the reduction in *Bb* load, providing insights to the
391 possibility of exploiting these mutant strains or their components for developing biologics to limit
392 *Bb* infection in reservoir hosts.

393 Various tissues isolated from vaccinated/challenged (Fig 7 and 8) mice were propagated
394 in BSK-II growth medium and growth of viable spirochetes were verified using dark field
395 microscopy. As shown in Table 2 (consolidated from experiments described in Fig 7 and 8) only
396 1 in 7 mice vaccinated with *ΔbadR* was positive for growth of spirochetes while 3 out 7 mice
397 vaccinated with 8S were positive. While 4 out of 7 mice vaccinated with ML23 were positive for
398 growth of spirochetes in different tissues, all 5 naive mice had growth (except for spleen where
399 the breakdown of tissues impair recovery of spirochetes) indicating the protective efficacy of
400 mutant strains of *Bb*. It should be pointed out the length of time after the second vaccination
401 impacts the levels of protection, as there was a greater reduction in the levels of fed larvae positive
402 for *Bb flab* as a measure of pathogen burden when the time of challenge was closer to the second
403 vaccination (Fig 7). These studies provide evidence to the utility of these mutant strains or their
404 subcellular components, notably lipoproteins, as potential biologics to induce host responses to
405 reduce the transmission of *Bb* via ticks.

406 Purified borrelial lipoproteins from *Bb* strain B31-A3 (parental wildtype strain), 8S and
407 *ΔbadR* induced significant antibody responses at day 28 post-vaccination with total IgG1, IgG2a
408 and IgG2b being the predominant IgG isotypes against *Bb*. Levels of IgM, IgA and IgG3 were not
409 significantly different between vaccinated and naïve mice. Mice were challenged with infected *Is*
410 nymphs (data from two separate experiments consolidated into a single table) obtained from
411 larvae fed on a single infected C3H/HeN mouse. As noted in Table 3, all mice vaccinated with
412 lipoproteins from B31-A3 and 8S strains did not have any spirochetes in several tissues tested
413 while 2 out 6 mice vaccinated with lipoproteins from *ΔbadR* mutant were positive for spirochetes
414 in select tissues. Five out of 6 control mice (PBS) were positive for infection while all three mice
415 vaccinated with OspA were negative for spirochetes, demonstrating the efficacy of protection of
416 borrelial lipoproteins given as a single intradermal route. These observations are critical to
417 establish correlates of protection for purified borrelial lipoproteins that can be formulated as oral

418 biologics for blocking the transmission of *Bb* between reservoir hosts via ticks, thereby advancing
419 methods to limit the incidence of Lyme disease in humans.

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Materials and Methods

Animals and Ethics Statement

The animal facilities at The University of Texas at San Antonio (UTSA) are part of Laboratory Animal Resources Center (LARC), which is an AAALAC International Accredited Unit. 6-8-week-old female C3H/HeN and BALB/c mice (Charles River Laboratories, Wilmington, MA) were used in this study. All animal experiments were conducted following NIH guidelines for housing and care of laboratory animals and in accordance with protocols approved by the Institutional Animal Care and Use Committee (protocol number MU071) of UTSA. Based on these guidelines, general condition and behavior of the animals were monitored by trained laboratory and LARC staff daily and methods to minimize pain and discomfort were adopted as needed in this study.

Bacterial strains and growth conditions

A low passage infectious clonal isolate of *Borrelia burgdorferi* B31-A3 (49), mutant strains such as Δ*badR* (B31 isolate, *badR* (BB0693) deficient, Str^r) (11), 8S (B31 isolate, $CsrA_{Bb}$ with 8 critical residues replaced with alanine, $Gent^R$) (13) and ML23 (derived from strain *Bb*-B31 lacking linear plasmid 25 or lp25-deficeint strain from *Bb* B31-A3) (14, 15) were propagated at 32°C in liquid Barbour-Stoenner-Kelly (BSK-II, pH 7.6) media supplemented with 6% heat inactivated rabbit serum (Pel-Freez Biologicals, Rogers, AR) with appropriate antibiotics (Sigma-Aldrich, St. Louis, MO) as previously described (11, 13, 44, 50-52). Once the cultures reached a density between 1-$2x10^7$ spirochetes /ml, viable spirochetes were enumerated by dark field microscopy and used for intradermal inoculation of C3H/HeN mice twice at a dose of $1x10^5$ *Bb* per mouse.

Cloning and overexpression of OspA. Genomic DNA from Bb strain B31-A3 was prepared and *ospA* was amplified using primers OspA-F/Xba1- ACGC TCTAGA ATGAAAAAATATTTATTGGGAATAGGTCTA and OspA-R/Sal1 ACGCGTCGACTTATTTTAAAGCGTAATTAATTTCATCAAG. The amplicon was first cloned into pCR2.1 and transformed into E. coli TOP10 cells exploiting the blue/white screening method. The insert was excised using the engineered restriction enzyme sites and cloned into expression plasmid pMAL-2x. Plasmids with the right insert were identified using pMAL2c specific primers flanking the insert and forward and were transformed into *E. coli* RosettaTM (Novagen). OspA fused to Maltose binding protein was induced with IPTG and purified using amylose beads as described previously (53-56). Purified OspA was quantified using BCA and stored at -20˚C in aliquots until used. A similar construct was generated using pET23a vector and OspA fused to 6X-HisTag was purified using NiNTA beads as described previously (50).

474 **Lipoprotein purification from *Bb* strains**

475 Lipoproteins were extracted from the wildtype and mutant strains as previously described (28,
476 57). Briefly, $1x10^9$ *Bb* was solubilized in 1 mL of PBS (Phosphate Buffered Saline, pH7.4)
477 containing 1% Triton® X-114 (TX-114) (Sigma Aldrich) by gentle rocking at 4°C overnight. Pellet
478 containing the TX-114 insoluble material was removed by two centrifugations at 15,000xg at 4°C
479 for 15 mins. The supernatant was transferred to a sterile tube and incubated at 37°C for 15 mins.
480 Then the mixture was centrifuged at 15,000xg for 15 mins at RT. The top aqueous phase was
481 transferred to a new tube and re-extracted one more time with 1% TX-114 as described above.
482 The bottom detergent phase was washed thrice with 1 mL PBS. The final detergent phase proteins
483 were precipitated by adding 10-fold volume of ice-cold acetone, precipitates were collected by
484 centrifugation at 15,000xg at 4°C for 30 mins, acetone was removed by drying, and proteins were
485 resuspended in PBS. Insoluble (Pellet), soluble (aqueous phase) and Lipoprotein (TX-114 phase)
486 fractions were analyzed by SDS-PAGE gel. Purified Borrelial Lipoproteins (PBLs) were quantified
487 using a BCA assay kit (Thermo Scientific) and stored at -20°C until further use.

488 **Proteomic analysis of PBLs**

489 PBLs isolated from infectious B31 strain (WT), 8S and *ΔbadR* mutant strains were submitted in
490 triplicate to UTMB Proteomics facility for Mass Spec analysis to determine the composition of the
491 detergent phase proteins. Each sample mixture was solubilized with 5% SDS, 50 mM triethyl
492 ammonium bicarbonate, pH 7.55 in a final volume of 25 µl. The sample was then centrifuged at
493 17,000 g for 10 min to remove any debris. Proteins were reduced using a solution of 20 mM Tris-
494 2-carboxyethyl phosphine (TCEP, Thermo, Scientific catalog #77,720) and incubated at 65 °C for
495 30 min. The sample was cooled to room temperature and 1 µl of 0.5 M iodoacetamide acid added
496 and allowed to react for 20 mins in the dark. 2.75 µl of 12% phosphoric acid was added to the
497 protein solution. 165 µL of binding buffer (90% Methanol, 100 mM TEAB final; pH 7.1) was then
498 added to the solution. The resulting solution was added to S-Trap spin column (protifi.com) and
499 passed through the column using a bench top centrifuge (30 s spin at 4000×g). The spin column
500 was washed with 400 µL of binding buffer and centrifuged. The binding buffer wash was then
501 repeated two more times. Trypsin was added to the protein mixture in a ratio of 1:25 in 50 mM
502 TEAB, pH 8, and incubated at 37 °C for 4 h. Peptides were eluted with 75 µl of 50% acetonitrile,
503 0.2% formic acid, and then washed again with 75 µl of 80% acetonitrile, 0.2% formic acid. The
504 combined peptide solution was then dried in a speed vac and resuspended in 2% acetonitrile,
505 0.1% formic acid, 97.9% water and placed in an autosampler vial.

506 **NanoLC MS/MS Analysis**

507 Peptide mixtures were analyzed by nanoflow liquid chromatography-tandem mass spectrometry
508 (nanoLC-MS/MS) using a nano-LC chromatography system (UltiMate 3000 RSLCnano, Dionex),
509 coupled on-line to a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San
510 Jose, CA) through a nanospray ion source (Thermo Scientific). A trap and elute method was used.
511 The trap column was a C18 PepMap100 (300 um X 5 mm, 5 um particle size, Thermo Scientific)
512 and the analytical column was an Acclaim PepMap 100 (75 um X 25 cm, Thermo Scientific). After
513 equilibrating the column in 98% solvent A (0.1% formic acid in water) and 2% solvent B (0.1%
514 formic acid in acetonitrile (ACN)), the samples (1 µL in solvent A) were injected onto the trap
515 column and subsequently eluted (300 nL/min) by gradient elution onto the C18 column as follows:
516 isocratic at 2% B, 0-5 min; 5-6 min 2%-4% B, 4% to 32% B, 5-120 min; 32% to 90% B, 120-123
517 min; isocratic at 90% B, 123-126 min; 90% to 2%, 126-129 min; isocratic at 2% B, 129-130 min;
518 2% to 90% B, 130-134 min; isocratic at 90% B, 134-137 min; 90% to 2%, 137-140 min; and
519 isocratic at 2% B, 140-145 min.

520 All LC-MS/MS data were acquired using Xcalibur, version 4.4.16.14 (Thermo Fisher Scientific) in
521 positive ion mode using a top speed data-dependent acquisition (DDA) method with a 3 sec cycle
522 time. The survey scans (m/z 375-1500) were acquired in the Orbitrap at 120,000 resolution (at
523 m/z = 400) in profile mode, with maximum injection mode set to Auto and a normalized AGC target
524 of 100%. The S-lens RF level was set to 60. Isolation was performed in the quadrupole with a 1.6
525 Da isolation window, and CID MS/MS acquisition was performed in centroid mode with detection
526 in the ion trap (Rapid scan rate) with the following settings: CID collision energy = 30%; maximum
527 injection time mode set to Dynamic; normalized AGC target of 20%. Monoisotopic precursor
528 selection (MIPS) and charge state filtering were on, with charge states 2-7 included. Dynamic
529 exclusion was used to remove selected precursor ions, with a +/- 10 ppm mass tolerance, for 30
530 sec after acquisition of one MS/MS spectrum.

531 **Database Searching**

532 Tandem mass spectra were extracted and charge state deconvolved by Proteome Discoverer
533 (Thermo Fisher, version 2.5.0.402). All MS/MS spectra were searched against a concatenated
534 FASTA database which included a Uniprot *Borrelia burgdorferi* database (downloaded 09-16-
535 2022) using Sequest. Searches were performed with a parent ion tolerance of 10 ppm and a
536 fragment ion tolerance of 0.60 Da. Trypsin was specified as the enzyme, allowing for two missed
537 cleavages. A fixed modification of carbamidomethyl (C) and variable modifications of oxidation

538 (M) and deamidation of asparagine and glutamine, were specified in Sequest. Percolator was
539 used to filter the peptide identification results to a false discovery rate of 1%. Label-free
540 quantitation was performed using Minora. Differential expression analysis of sample groups was
541 performed using the Precursor Ions Quantifier node in Proteome Discoverer, with protein
542 abundance ratios calculated using a Pairwise Ratio scheme while hypothesis testing was
543 performed using a t-test of the background population of proteins and peptides. The pathways
544 associated with the identified proteins were determined by KEGG pathway search tool
545 (https://www.genome.jp/kegg/mapper/search.html), against *Borrelia burgdorferi* B31 datasets.

546 **Immunization with lipoproteins or live bacterial strains**

547 For immunization, mutant strains grown in BSK-II media were centrifuged at 4000x g for 20
548 minutes at 4°C and the pellets were washed three times with sterile HBSS at 4000x g for 5 minutes
549 at 4°C and the cells were resuspended in BSK-II medium supplemented with 6% heat inactivated
550 rabbit serum at a final concentration of 10^6 spirochetes/ml as described previously (55, 58-60).
551 Each mouse was injected with 100 µL of spirochetes via intra-dermal injections on Day 0 and Day
552 16. Similarly, purified lipoproteins were injected at a concentration of 100 µg/100 µL via intra-
553 dermal injections on Day 0 and Day 14.

554 **Enzyme Linked Immunosorbent Assay (ELISA)**

555 To determine the peripheral antibody levels, blood was collected from the lateral saphenous vein
556 using Microvette CB 300 capillary tubes (Sarstedt AG & Co.KG), centrifuged at 4000xg for 10
557 minutes and the supernatant were collected and stored at -20°C until further use. ELISA was
558 performed against borrelial lysate or PBLs as required. All the assays were performed using a
559 Costar High binding Assay Plate (Corning). 10 µg of the antigen were resuspended in 10 mL of
560 coating carbonate buffer (0.05M, pH 9.6) and 100 µL of coating antigen were added to each well
561 and incubated overnight at 4°C. After washing three times with Tris Buffered Saline supplemented
562 with Tween 20 (TBST), 100 µL of 1% Bovine Serum Albumin (BSA) (Probumin, Millipore) in TBST
563 was added to each well and incubated for 1 hr at room temperature and washed three times with
564 TBST. Serum samples were diluted in TBST starting from 1:50 up to 1:6400 dilutions and 100 µL
565 of each diluted sample was added. After two hours of incubation at room temperature, the plates
566 were washed five times with TBST and 100 µL of 1:3000 diluted secondary anti-mouse and anti-
567 human IgG antibodies conjugated with Horseradish peroxidase (HRP) was added and incubated
568 for 1 hr. After four washes, 100 µL of OPD substrate solution was added and read at 490 nm using
569 Spark 10M (Tecan) microplate reader. In addition, plates were also developed using anti-mouse

570 isotype specific antibodies conjugated to alkaline phosphatase. Levels of significance were
571 determined using unpaired Student *t*-test using GraphPad Prism 7.0 and indicated in the
572 respective figure legends.

573 **Immunoblot Analysis**

574 Immunoblot analysis was performed to determine the antigenic profile of lipoproteins against
575 mouse and human serum (blinded samples provided by Lyme disease Bio Bank). Equal amounts
576 of purified lipoproteins (5 µg/sample) were separated using 12% polyacrylamide gels and then
577 transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with blocking buffer (10%
578 non-fat dry milk in Tris buffer (pH 7.5) containing 200 mM Tris, 1.38 M NaCl, and 0.1% Tween 20)
579 overnight and then incubated with infected serum collected from mouse or human patients or
580 rFlaB immunized mouse serum at room temperature for 2 hrs in blocking buffer, and treated with
581 horseradish peroxidase (HRP) conjugated Goat anti-mouse or Goat anti-human IgG secondary
582 antibody for 1 hr at room temperature. Immunoblots were developed using an enhanced
583 chemiluminescence system followed by exposure to X-ray film.

584 **Animal injections and tick larvae feeding**

585 For infection studies, *Bb* cultures were verified by PCR for the presence of the virulence plasmids
586 lp25 and lp28-1 on the day of injection. After confirmation, the cultures were centrifuged at 4000x
587 g for 20 minutes at 4°C and the pellets were washed three times with sterile HBSS at 4000x g for
588 5 minutes at 4°C and the cells were resuspended in BSK-II medium supplemented with 6% heat
589 inactivated rabbit serum at a final concentration of 10^6 spirochetes/mL. Each mouse was injected
590 with 100 µL of spirochetes via intradermal injections. After 28 days of needle challenge, animals
591 were moved to the tick facility, hairs were shaved on the dorsal side of the mouse and sterile
592 plastic tubes were glued on to the skin of each mouse using special adhesive material. After
593 overnight drying, 50-100 *Ixodes scapularis* larvae were loaded into the tubes and the tubes were
594 capped to increase the tick movement towards the skin to each mouse. After 3-7 days, fed larvae
595 were collected and stored in a sterile tube covered with perforated parafilm and incubated at 23°C
596 under humid conditions for 6-8 weeks to allow the larvae to molt into nymphs. After collecting the
597 fed larvae, the mice were moved to the regular BSL2 facility for necropsy. All mice infection studies
598 were conducted in a Biosafety Level 2 facility.

599 **Necropsy and quantification of *Bb* burden in tissues**

600 Mice were euthanized by carbon dioxide exposure followed by cardiac puncture, serving as
601 terminal bleed and as a secondary method of euthanasia. Skin, lymph nodes, spleen, heart,
602 bladder and joint tissues were aseptically isolated and inoculated into BSKII medium and scored
603 for growth after a single blind pass at day 5 by examining cultures using dark field microscopy at
604 day 15 post-isolation in primary and blind-pass cultures. Another set of skin, lymph nodes; spleen
605 and joint samples were stored at -80°C for genomic DNA extraction and qPCR analysis.

606 **Genomic DNA extraction and qPCR**

607 Genomic DNA from the skin, spleen and lymph nodes from mice or *Ixodes scapularis* larvae,
608 nymph were extracted using High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN).
609 Briefly, samples were lysed with 200 µL of lysis buffer in a BeadBlaster™ 24 microtube
610 homogenizer using Zirconia beads. Further, 40 µL of proteinase K (20 mg/mL) and 20 µL of
611 Collagenase (1 mg/mL) was addedand incubated overnight at 57°C. Next, DNA was extracted
612 as directed by the manufacturer using the column. *Bb* burden in the samples was determined by
613 a SYBR-based qPCR assay (PowerUp SYBR Master Mix, Applied Biosystems) using *Bb*FlaB
614 gene specific primers and normalized to tick actin or mouse actin and the reaction was performed
615 in a StepOne Plus real time PCR system (Applied Biosystems). Standard curve for all three genes
616 were performed by full length genes cloned into pCR2.1 plasmid and the results are presented
617 as ratio of *flaB* to mouse or tick actin copy number.

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629 **Figure Legends**

630 **Figure 1: Extraction and Immunoreactivity of borrelial Lipoproteins.** *B. burgdorferi* (*Bb*)
631 strains B31-A3 (WT), *ΔbadR* and 8S were propagated at 32˚C, pH7.6 to a density of $1x10^8$ *Bb*/ml
632 cells. The cultures were washed with HBSS and partitioned into aqueous and detergent phase
633 using Triton X-114 were separated using SDS-12.5%PAGE gel. 1A) Total stain of showing the
634 proteins partitioned as the insoluble pellet (Pt), in aqueous (Aq) and Triton X-114 (Tr) phase from
635 all three strains. Immunoreactivity of partitioned proteins with 1B) B31-A3 infected mouse serum
636 and 1C) anti-FlaB mouse serum. Molecular weights in kDA are indicated on the left.

637

638 **Figure 2: Purification and abundance of borrelial lipoproteins from mutant strains of *B.***
639 ***burgdorferi*.** Total lysates of *B. burgdorferi* cultures were partitioned using Triton X-114 and the
640 detergent phase proteins were subjected to proteomic analysis. 2A) Total stain of Triton X-114
641 phase proteins from WT (B31-A3), 8S and *ΔbadR* separated using SDS-12.5% PAGE and stained
642 with Coomassie Brilliant Blue. 2B) Mass spectrophotometric analysis of a detergent phase protein
643 expressed at a higher level in the 8S mutant was identified as OspB. 2C) Relative abundance of
644 OspB and OspA in WT, 8S and *ΔbadR*. 2D) Peptide abundance reflecting the expression of 5
645 predominant lipoproteins in the detergent phase extract of borrelial mutants determined by
646 proteomic analysis.

647

648 **Figure 3: Reactivity of serum from Lyme disease patients with borrelial lipoproteins.**
649 Enzyme linked immuneabsorbant assay (ELISA) was performed with borrelial lipoproteins from
650 B31-A3, *ΔbadR* and 8S mutants coated as antigens on 96 well ELISA plates. Serial two-fold
651 dilution of human serum samples (from 1:50 to 1:6400) from patients with or without Lyme disease
652 (blind, no patient information provided by Lyme BioBank) were tested for reactivity. Plates were
653 developed using anti-human IgG antibodies conjugated with horse raddish peroxidase (HRPO)
654 and o-phenylenediamine dihydrochloride (OPD) substrate solution and read at 490 nm using a
655 microplate reader. Levels of IgG from 15 human serum samples are shown: End point titers of
656 various human serum samples against lipoproteins from *Bb* strain B31, 8S, and *ΔbadR.* Levels
657 of reactivity of normal human serum from the local blood bank served as a negative control.

658

659 **Figure 4: Isotype of human antibodies against *Bb* antigens.** ELISA-based assays were
660 performed using total lysates from *Bb* strain B31-A3, 8S and *ΔbadR* to determine the isotype of
661 the 15 human serum samples from patients with or without Lyme disease as described in Fig 3.
662 4A) Bar graphs showing end point titers of human serum samples determined using HRPO-
663 conjugated secondary antibodies specific to human IgM, IgG, IgA, IgG1 and IgG2. Plates were
664 read at 490 nm using a microplate reader. Levels of reactivity of normal human serum from the
665 local blood bank served as a negative control. 4B) Immunoblot analysis of a sample of human
666 serum that exhibited high titers by ELISA. Proteins from lysates from *Bb* strain B31-A3, 8S and
667 *ΔbadR* were separated on a SDS-12.5% PAGE gel and transferred to PVDF membrane. Blots
668 were developed using HRPO-conjugated anti human IgG and ECL system.

669

670 **Figure 5: Isotype of antibodies induced in C3H/HeN mice at day 14 following vaccination**
671 **with mutant borrelial strains.** Serum from mice vaccinated with ML23, 8S and *ΔbadR* at day 0
672 and 14 were obtained 2 weeks after last vaccination and reactivity/isotype determined using total
673 borrelial lysates from *Bb* strain B31-A3 using ELISA based method. Isotype specific mouse
674 secondary antibodies conjugated to alkaline phosphatase (AP) and PNPP was used as substrate.
675 Absorbance was measured at 405 nm with serum from naïve C3H/HeN mice serving as negative
676 control. Note significant increase in levels of IgM, IgG and IgG2a in vaccinated mice compared to
677 naïve controls. The asterisks indicate levels of significance as follows ***, $P<0.001$, **, $P<0.01$; *,
678 $P<0.05$

679

680 **Figure 6: Isotype of antibodies induced in C3H/HeN mice at day 28 following vaccination**
681 **with mutant borrelial strains.** Serum from mice vaccinated with ML23, 8S and *ΔbadR* at day 0
682 and 14 were obtained 4 weeks after last vaccination and reactivity/isotype determined using total
683 borrelial lysates from *Bb* strain B31-A3 using ELISA based method. Isotype specific mouse
684 secondary antibodies conjugated to alkaline phosphatase (AP) and PNPP was used as substrate.
685 Absorbance was measured at 405 nm with serum from naïve C3H/HeN mice serving as negative
686 control. Note significant increase in levels of IgG, IgG1, IgG2a and IgG2b in vaccinated mice
687 compared to naïve controls. The asterisks indicate levels of significance as follows ****, P< 0001;
688 ***, $P<0.001$, **, $P<0.01$; *, $P<0.05$

689

690 **Figure 7: Levels of protection conferred by live attenuated strains in C3H/HeN mice against**
691 **challenge (4 weeks) with *Bb* strain B31-A3.** 8A) Graphical representation of the timeline of
692 vaccination; needle challenge, larvae feeding and time of euthanasia of C3H/HeN mice. Serum
693 collection timeline is shown at the bottom. 8B) Bar graphs showing the end-point titers of
694 antibodies induced in immunized mice at 28 days post first immunization (dpi) determine using
695 ELISA with *Bb* strain B31-A3 lysate as antigens. 8C) Bar graphs showing *Bb* burden in *I.*
696 *scapularis* larvae fed on challenged mice at 84 days post first immunization. Numbers of borrelial
697 *flaB* copies were normalized against 10^4 total tick actin copies. 8D) Bar graphs showing *Bb* burden
698 in mouse tissues at the time of euthanasia. Numbers of borrelial *flaB* copies were normalized
699 against 10^4 total mouse actin copies in skin, lymph nodes and joints.

700

701 **Figure 8: Levels of protection conferred by live attenuated strains in C3H/HeN mice against**
702 **challenge (10 weeks) with *Bb* strain B31-A3.** 9A) Graphical representation of the timeline of
703 vaccination; needle challenge, larvae feeding and time of euthanasia of C3H/HeN mice. Serum
704 collection time line is shown at the bottom. 9B) Bar graphs showing the end-point titers of
705 antibodies induced in immunized mice at 28 days post first immunization (dpi) determine using
706 ELISA with *Bb* strain B31-A3 lysate as antigen. 9C) Bar graphs showing *Bb* burden in *I. scapularis*
707 larvae fed on challenged mice at 84 days post first immunization. Numbers of borrelial *flaB* copies
708 were normalized against 10^4 total tick actin copies. 9D) Bar graphs showing *Bb* burden in mouse
709 tissues at the time of euthanasia. Numbers of borrelial *flaB* copies were normalized against 10^4
710 total mouse actin copies in skin, lymph nodes and joints.

711

712 **Figure 9: Isotype of antibodies induced in C3H/HeN mice at day 28 following vaccination**
713 **with borrelial lipoproteins.** Serum from mice vaccinated with lipoproteins from *Bb* strain B31-
714 A3 ML23, 8S and *ΔbadR* at day 0 and 14 were obtained 4 weeks after last vaccination and
715 reactivity/isotype determined using total borrelial lysates from *Bb* strain B31-A3 using ELISA
716 based method. Isotype specific mouse secondary antibodies conjugated to alkaline phosphatase
717 (AP) and PNPP was used as substrate. Absorbance was measured at 405 nm with serum from
718 naïve C3H/HeN mice serving as negative control. Note significant increase in levels of IgG, IgG1,
719 IgG2a and IgG2b in vaccinated mice compared to naïve controls. The asterisks indicate levels of
720 significance as follows ****, P< 0001; ***, P<0.001, **, P<0.01; *, P<0.05

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941 Table 1: Efficacy of protection conferred by mutant strains of *Bb* against tick challenge with *Bb*
942 strain B31-A3

# of cultures positive /total #								# of mice infected / total # of mice
	Skin	Lymph node	Spleen	Bladder	Heart	Joint	All sites	
Unvaccinated	1/1	1/1	1/1	0/1	1/1	1/1	5/6	1/1
ML23	1/3	2/3	2/3	2/3	0/3	1/3	6/18	2/3
8S	0/2	0/2	0/2	0/2	0/2	0/2	0/12	0/2
ΔbadR	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
OspA	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

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959 Table 2: Efficacy of protection conferred by mutant strains of *Bb* following needle challenge with
960 *Bb* strain B31-A3*

	# of cultures positive /total #							# of mice infected /total # of mice
	Skin	Lymph node	Spleen	Bladder	Heart	Joint	All Sites	
Unvaccinated	5/5	5/5	1/5	5/5	5/5	5/5	26/30	5/5
ML23	4/7	4/7	3/7	4/7	4/7	4/7	23/42	4/7
8S	3/7	3/7	1/7	3/7	3/7	2/7	15/42	3/7
ΔbadR	1/7	1/7	0/7	1/7	1/7	1/7	5/42	1/7

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962 *Consolidated representation of spirochetal growth in BSKII medium following propagation of
963 tissues isolates from mice in Figures 7 and 8.

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977 Table 3: Efficacy of protection conferred by lipoproteins from mutant strains of *Bb* against tick
978 challenge with *Bb* strain B31-A3*

	# of cultures positive /total #							Total # of infected mice/total # of mice
Lipoproteins	Skin	Lymph node	Spleen	Bladder	Heart	Joint	All sites	
Unvaccinated	5/6	5/6	4/6	5/6	4/6	5/6	28/36	5/6
B31-A3	0/6	0/6	0/6	0/6	0/6	0/6	0/36	0/6
8S	0/6	0/6	0/6	0/6	0/6	0/6	0/36	0/6
ΔbadR	0/6	2/6	1/6	2/6	1/6	1/6	7/36	2/6
rOspA	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

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980 * Combined data from two independent studies with 3 mice per treatment/challenge

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995 Supplementary Table 1: Clinical samples and the corresponding ground truth data from the
996 Lyme Disease biobank used in this study.

Sample ID	*Days of EM Prior to Enrollment*	*First-tier tests*						*Second-tier tests*						*Two-tier Positive on 1st Draw*
		ELISA		*IMMUNO BLOT*				*ELISA*		*IMMUNO BLOT*				
		Whole Cell Lysate	*C-6 Peptide*	*IgM*	*IgM Bands*	*IgG*	*IgG Bands*	*Whole Cell Lysate*	*C-6 Peptide*	*IgM*	*IgM Bands*	*IgG*	*IgG Bands*	
LD515	NA	NEG	NEG	NEG	None	NEG	p93, p41	NA	NA	NA	NA	NA	NA	NO
LD526	NA	NEG	NEG	NEG	p41	NEG	None	NA	NA	NA	NA	NA	NA	NO
LD538	NA	NEG	NEG	NEG	p41	NEG	p58, p41	NA	NA	NA	NA	NA	NA	NO
LD585	**10**	**POS**	**POS**	**POS**	**p41, p39, p23**	**NEG**	**p41, p39, p23**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**YES**
LD610	NA	NEG	NEG	NEG	p41	NEG	None	NA	NA	NA	NA	NA	NA	NO
LD611	NA	NEG	NEG	NEG	p41	NEG	p66	NA	NA	NA	NA	NA	NA	NO
LD640	**21**	**POS**	**POS**	**NEG**	**None**	**POS**	**p93, p66, p45, p41, p39, p28**	**POS**	**POS**	**NEG**	**p41**	**POS**	**p93, p66, p45, p41, p39**	**YES**
LD663	**2**	**EQV**	**POS**	**POS**	**p41, p23**	**NEG**	**p41**	**POS**	**POS**	**POS**	**p41, p23**	**NEG**	**p41, p23**	**YES**
LD664	NA	NEG	NEG	NEG	None	NEG	p93	NA	NA	NA	NA	NA	NA	NO
LD673	**14**	**POS**	**POS**	**POS**	**p41, p23**	**POS**	**p66, p58, p41, p39, p23, p18**	**POS**	**POS**	**POS**	**p41, p23**	**NEG**	**p41, p23, p18**	**YES**
LD674	NA	NEG	NEG	NEG	p41	NEG	p58, p41	NA	NA	NA	NA	NA	NA	NO
LD677	**1**	**POS**	**POS**	**NEG**	**p41**	**POS**	**p93, p66, p45, p41, p39, p28, p18**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**YES**

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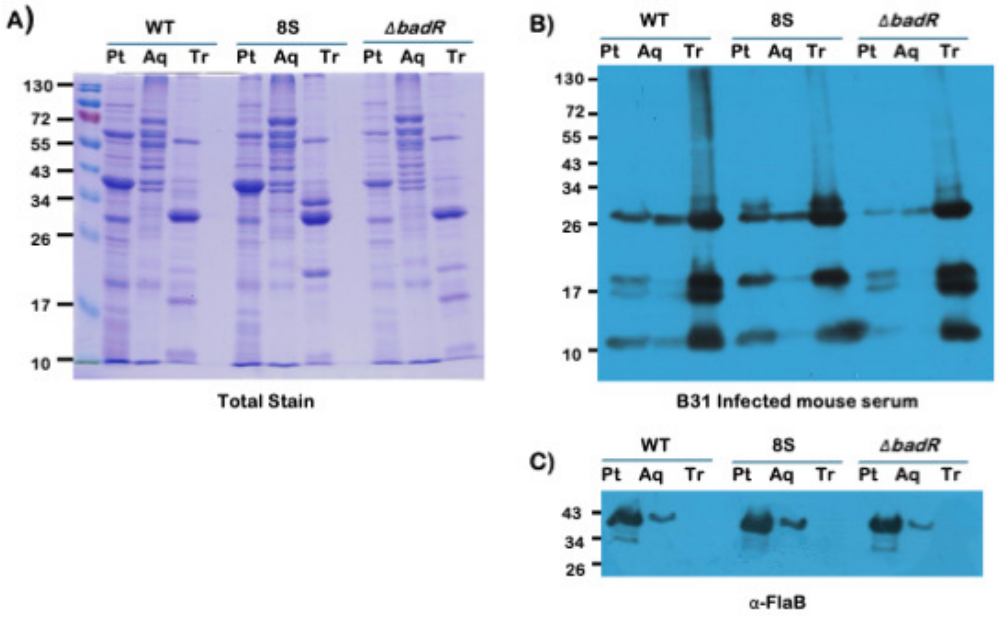
Fig 1


A)
WT
8S
ΔbadR
Pt Aq Tr Pt Aq Tr Pt Aq Tr
130
72
55
43
34
26
17
10
Total Stain
B)
WT
8S
ΔbadR
Pt Aq Tr Pt Aq Tr Pt Aq Tr
130
72
55
43
34
26
17
10
B31 Infected mouse serum
C)
WT
8S
ΔbadR
Pt Aq Tr Pt Aq Tr Pt Aq Tr
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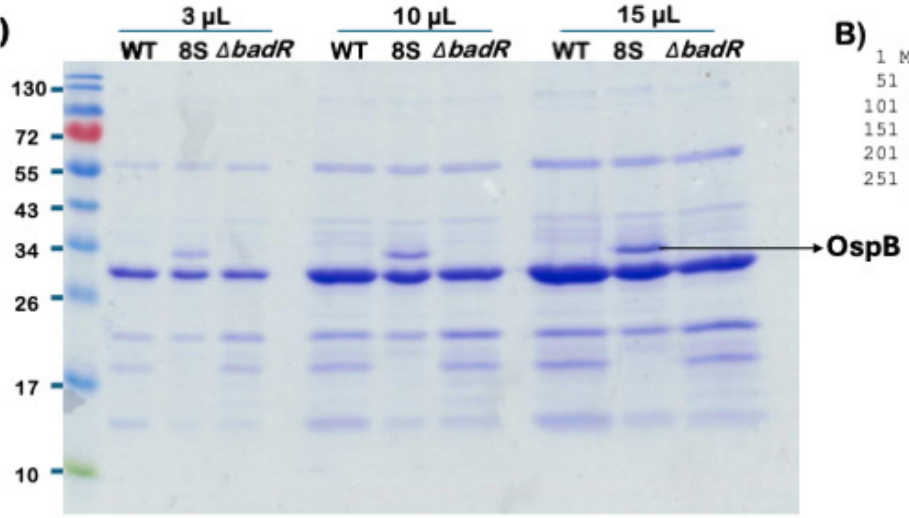
α-FlaB

Fig 2

A)



B)

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  1 MRLLIGFALA LALIGCAQKG AESIGSQKEN DLNLEDSSKK SHQNAKQDLP
 51 AVTEDSVSLF NGNKIFVSKE KNSSGKYDLR ATIDQVELKG TSDKNNGSGT
101 LEGSKPDKSK VKLTVSADLN TVTLEAFDAS NQKISSKVTK KQGSITEETL
151 KANKLDSKKL TRSNGTTLEY SQITDADNAT KAVETLKNSI KLEGSLVVGK
201 TTVEIKEGTV TLKREIEKDG KVKVFLNDTA GSNKKTGKWE DSTSTLTISA
251 DSKKTKDLVF LTDGTITVQQ YNTAGTSLEG SASEIKNLSE LKNALK
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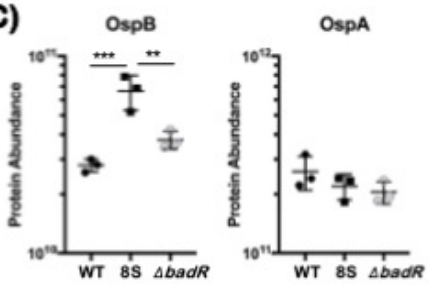
C)



D)

Locus	Protein	MW [kDa]	Abundance								
			WT_1	WT_2	WT_3	8S_1	8S_2	8S_3	*ΔbadR_1*	*ΔbadR_2*	*ΔbadR_3*
BB_A15	OspA	29.4	2.22E+11	2.41E+11	3.17E+11	1.83E+11	2.34E+11	2.41E+11	2.35E+11	1.95E+11	1.85E+11
BB_B19	OspC	22.3	2.93E+10	4.20E+10	5.50E+10	4.88E+10	3.26E+10	4.17E+10	5.63E+10	7.19E+10	7.85E+10
BB_A16	OspB	31.7	2.81E+10	2.58E+10	3.00E+10	5.20E+10	6.86E+10	7.86E+10	4.20E+10	3.46E+10	3.63E+10
BB_J09	OspD	28.4	3.92E+10	1.97E+10	2.79E+10	1.43E+10	1.49E+10	1.81E+10	3.53E+10	2.20E+10	2.44E+10
BB_A62	Lp6.6	7.8	1.31E+10	1.13E+10	1.54E+10	6.25E+09	6.39E+09	7.82E+09	1.39E+10	8.72E+09	1.02E+10

Fig 3

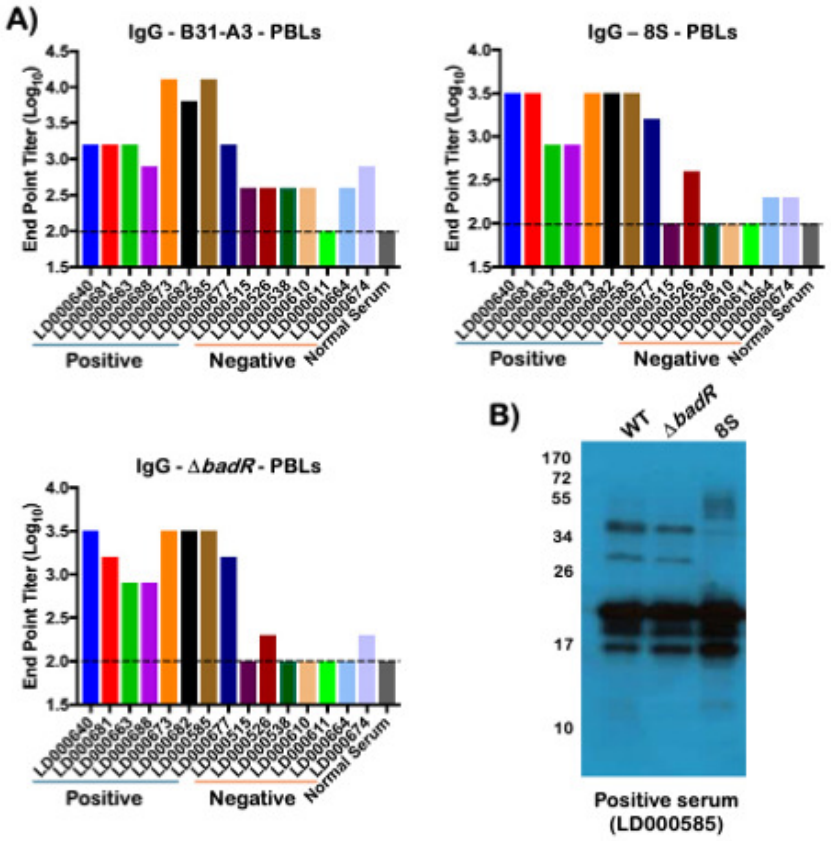

A)
IgG - B31-A3 - PBLs
IgG – 8S - PBLs
IgG - ΔbadR - PBLs
End Point Titer (Log10)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
LD000640
LD000681
LD000683
LD000688
LD000673
LD000682
LD000585
LD000677
LD000515
LD000526
LD000538
LD000610
LD000611
LD000664
LD000674
Normal Serum
Positive
Negative
B)
WT
ΔbadR
8S
170
72
55
34
26
17
10
Positive serum
(LD000585)

Fig 4

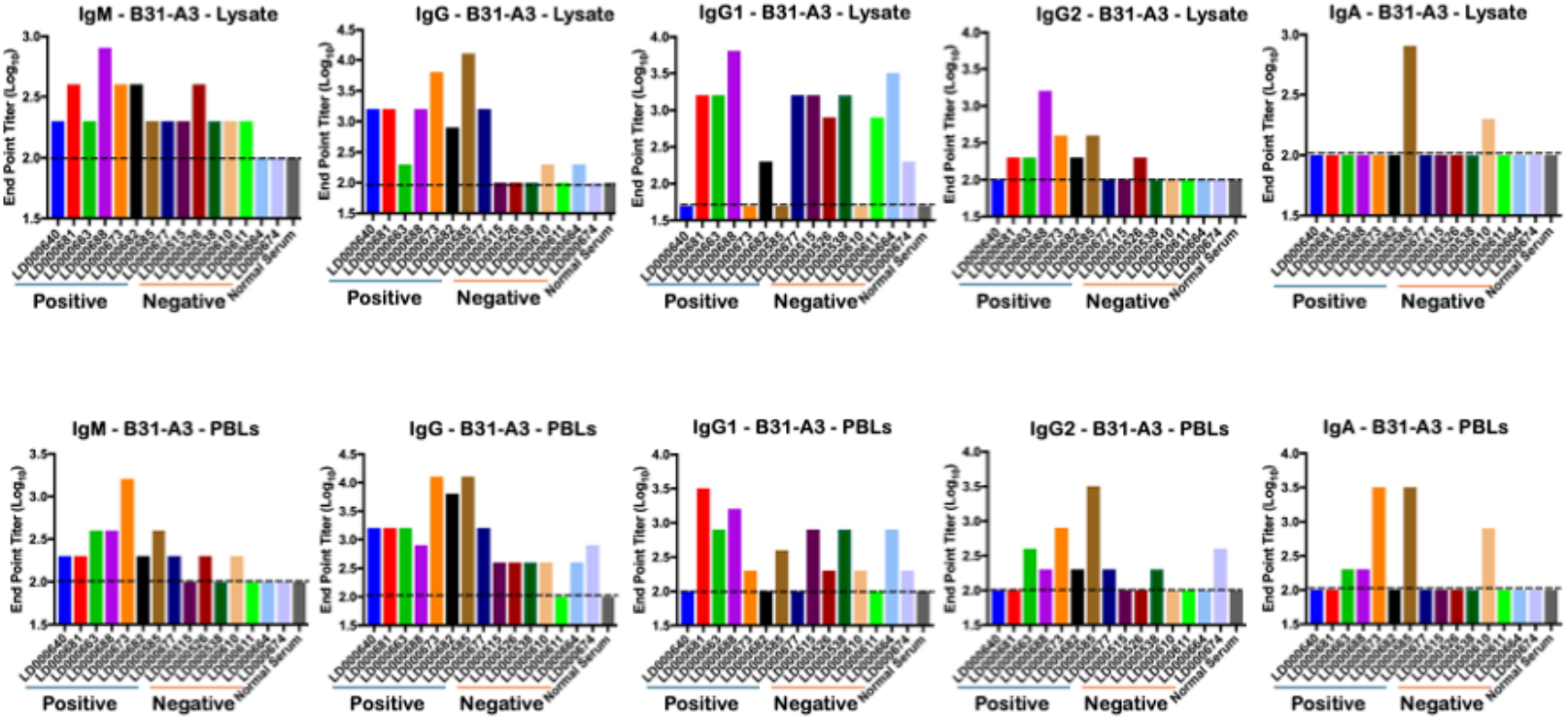
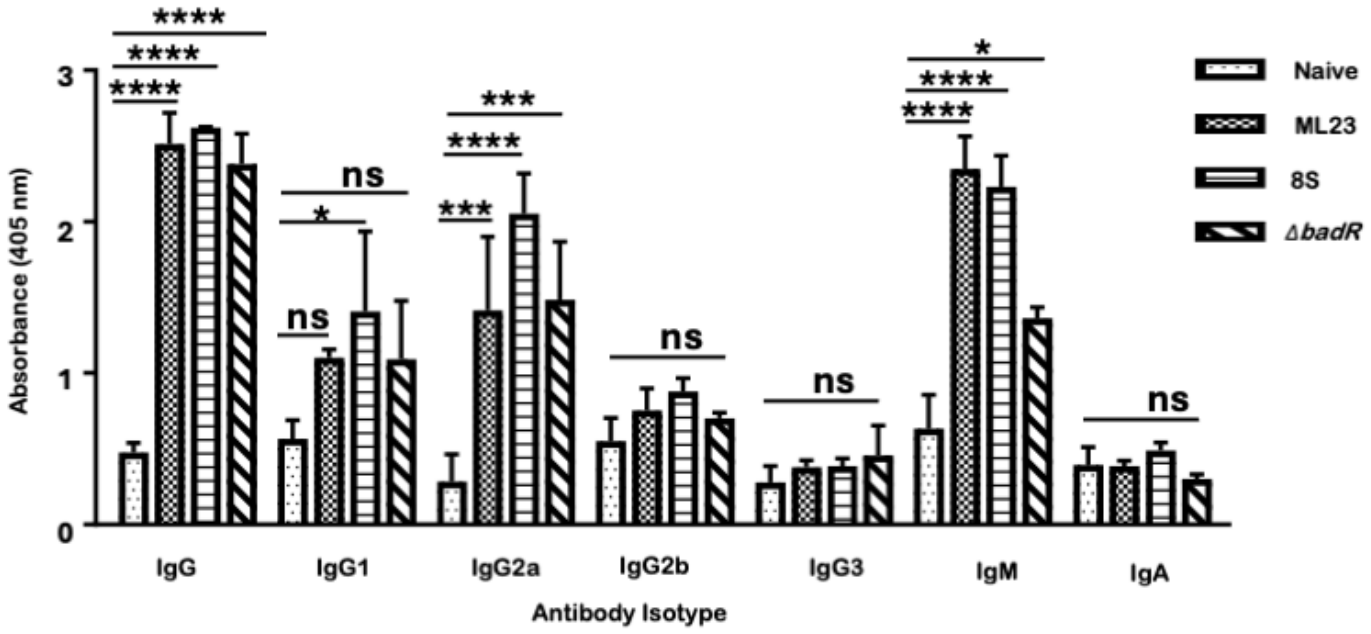

IgM - B31-A3 - Lysate
IgG - B31-A3 - Lysate
IgG1 - B31-A3 - Lysate
IgG2 - B31-A3 - Lysate
IgA - B31-A3 - Lysate
IgM - B31-A3 - PBLs
IgG - B31-A3 - PBLs
IgG1 - B31-A3 - PBLs
IgG2 - B31-A3 - PBLs
IgA - B31-A3 - PBLs
End Point Titer (Log10)
Positive
Negative
Normal Serum

Fig 5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ns
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Naive
ML23
8S
ΔbadR
Absorbance (405 nm)
3
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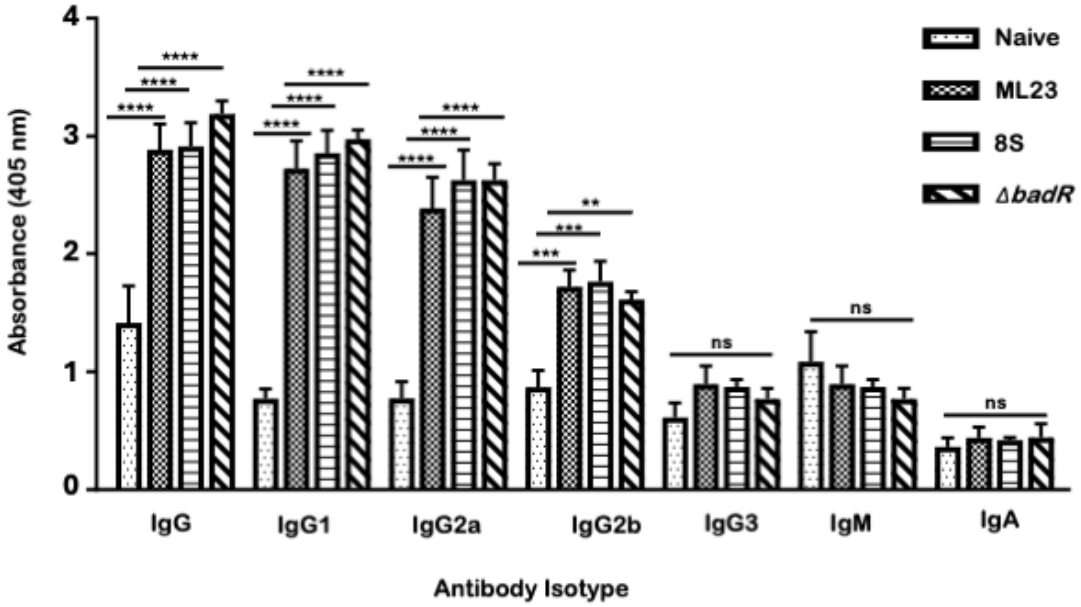
0
IgG
IgG1
IgG2a
IgG2b
IgG3
IgM
IgA
Antibody Isotype

Fig 6


Absorbance (405 nm)
4
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Naive
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ns
IgG
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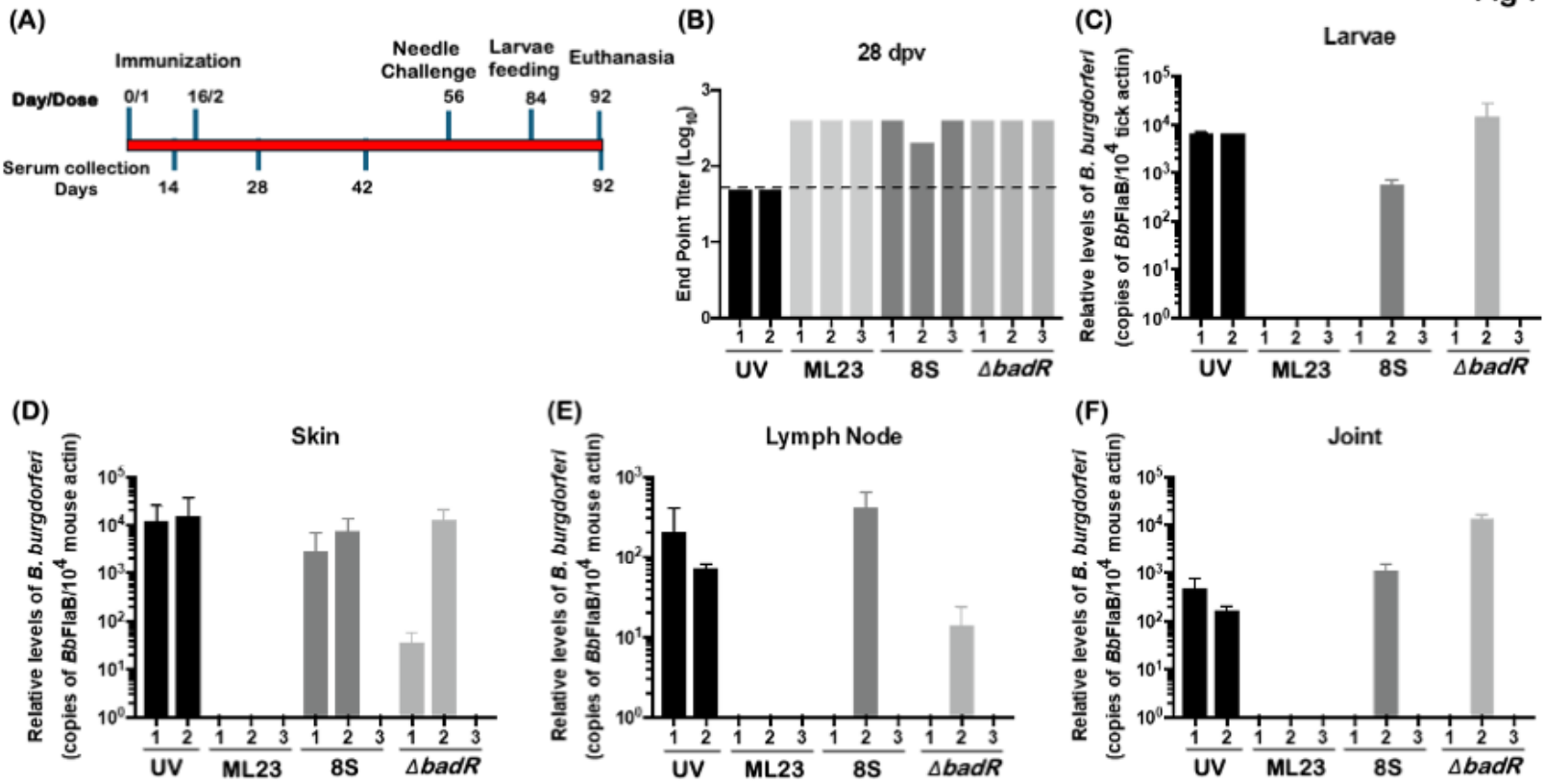
IgG3
IgM
IgA
Antibody Isotype

Fig 7


(A)
Immunization
Needle Challenge
Larvae feeding
Euthanasia
Day/Dose
0/1
16/2
56
84
92
Serum collection Days
14
28
42
92
(B)
28 dpv
End Point Titer (Log10)
UV
ML23
8S
ΔbadR
(C)
Larvae
Relative levels of B. burgdorferi (copies of BbFlaB/10^4 tick actin)
(D)
Skin
Relative levels of B. burgdorferi (copies of BbFlaB/10^4 mouse actin)
(E)
Lymph Node
(F)
Joint

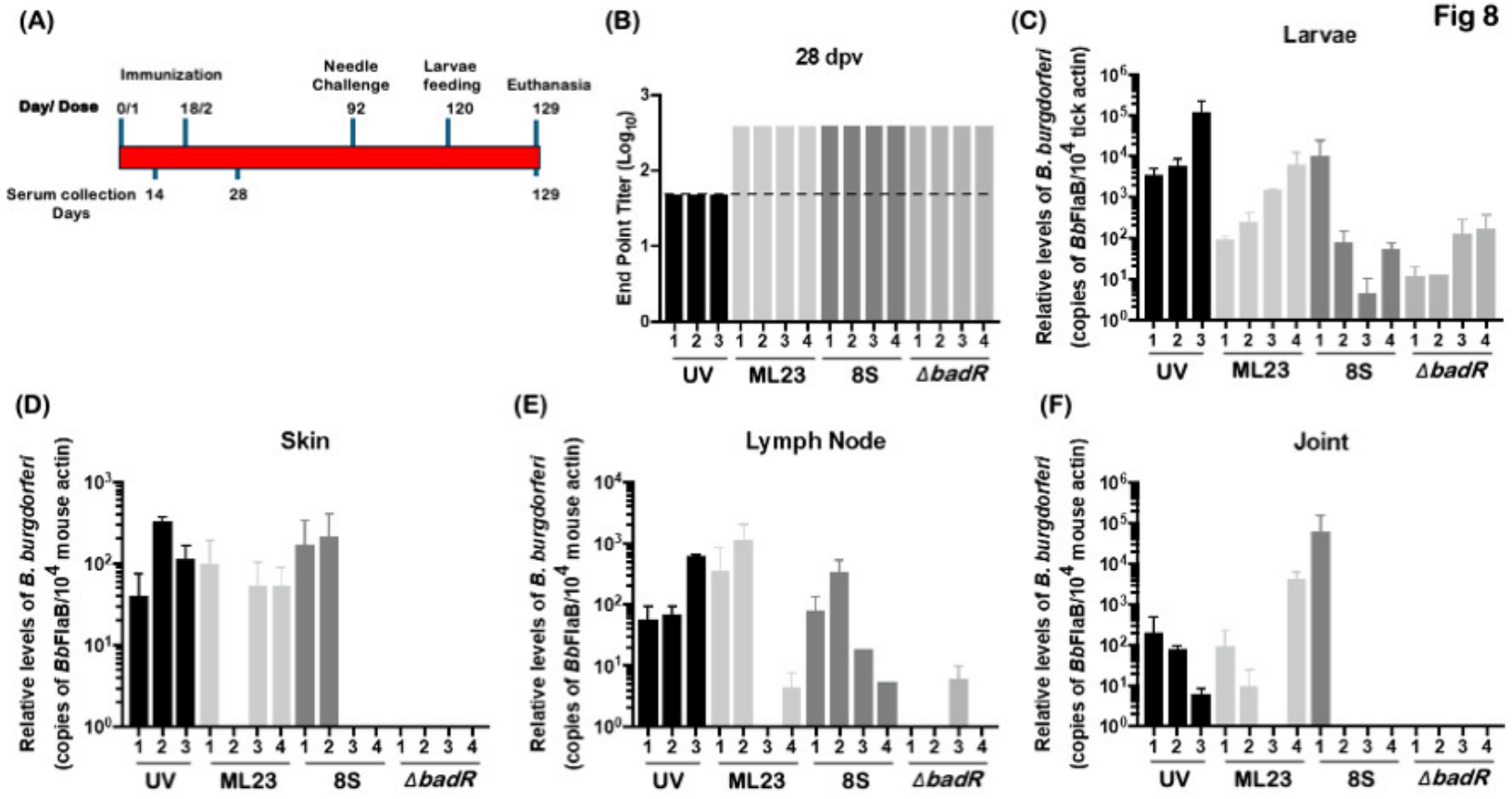
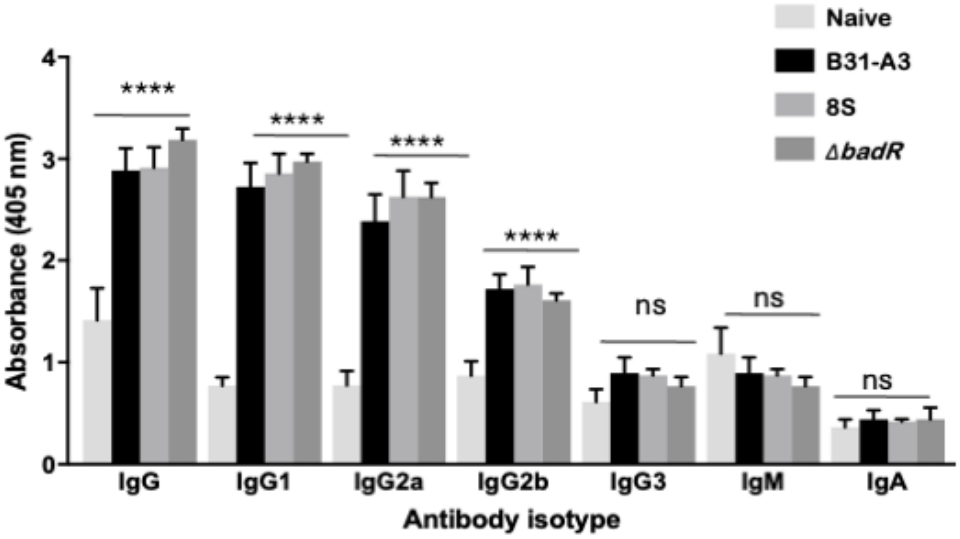


Fig 8
(A)
Immunization
Needle Challenge
Larvae feeding
Euthanasia
Day/ Dose
0/1
18/2
92
120
129
Serum collection Days
14
28
129
(B)
28 dpv
End Point Titer (Log10)
(C)
Larvae
Relative levels of B. burgdorferi (copies of BbFlaB/10^4 tick actin)
(D)
Skin
Relative levels of B. burgdorferi (copies of BbFlaB/10^4 mouse actin)
(E)
Lymph Node
Relative levels of B. burgdorferi (copies of BbFlaB/10^4 mouse actin)
(F)
Joint
Relative levels of B. burgdorferi (copies of BbFlaB/10^4 mouse actin)
UV
ML23
8S
ΔbadR

Fig 9


Naive
B31-A3
8S
ΔbadR
Absorbance (405 nm)
0
1
2
3
4
****
****
****
****
ns
ns
ns
IgG
IgG1
IgG2a
IgG2b
IgG3
IgM
IgA
Antibody isotype