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The Early Dissemination Defect Attributed to Disruption of Decorin-Binding Proteins is Abolished in Chronic Murine Lyme Borreliosis

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1 **The Early Dissemination Defect Attributed to Disruption of Decorin-binding Proteins is**

2 **Abolished in Chronic Murine Lyme Borreliosis**

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23 **Running title:** Dissemination defect of Dbp-deficient *B. burgdorferi*

24 **ABSTRACT**

25   The laboratory mouse model of Lyme disease has revealed that *Borrelia burgdorferi*

26 differentially expresses numerous outer surface proteins that influence different stages of

27 infection (tick-borne transmission, tissue colonization, dissemination, persistence, and tick

28 acquisition). Deletion of two such outer surface proteins, decorin-binding proteins A and B

29 (DbpA/B), has been documented to decrease infectivity, impede early dissemination and,

30 possibly, prevent persistence. In this study, DbpA/B-deficient spirochetes were confirmed to

31 exhibit an early dissemination defect in immunocompetent, but not immunodeficient, mice and

32 the defect was found to resolve with chronicity. Development of disease (arthritis and carditis)

33 was only attenuated in the early stage of DbpA/B-deficient infection in both types of mice.

34 Persistence of the DbpA/B-deficient spirochetes occurred in both immunocompetent and

35 immunodeficient mice in a manner indistinguishable from wild-type spirochetes. Dissemination

36 through the lymphatic system was evaluated as an underlying mechanism for the early

37 dissemination defect. At 12 hours, 3 days, 7 days and 14 days post-inoculation, DbpA/B-

38 deficient spirochetes were significantly less prevalent and in lower numbers in lymph nodes than

39 wild-type spirochetes. However, in immunodeficient mice, deficiency of DbpA/B did not

40 significantly decrease the prevalence or spirochete numbers in lymph nodes. Complementation

41 of DbpA/B restored a wild-type phenotype. Thus, results indicated that deficiency of DbpA/B

42 allows the acquired immune response to restrict early dissemination of spirochetes, which

43 appears to be at least partially mediated through the lymphatic system.

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INTRODUCTION

Borrelia burgdorferi, the etiologic agent of Lyme disease, utilizes a multitude of surface-exposed adhesins to bind to and interact with various components of the extracellular matrix in mammalian hosts. These adhesins include decorin-binding protein (Dbp)A, DbpB, fibronectin-binding protein (Fbp), *Borrelia* glycosaminoglycan-binding protein (Bgp), RevA, *Borrelia* membrane proteins (Bmps), ErpX, and P66. Their respective ligands include decorin, fibronectin, various glycosaminoglycans, laminin and $\alpha_{IIb}\beta_3$ integrin (1, 2, 3, 4, 5, 6, 7). This is by no means a completely inclusive list (8); for example, a yet unidentified borrelial adhesion binds directly to native type I collagen (9) and thus far, ligands for BmpD and members of the OspF family have not been characterized (10). However, the interactions of adhesins and ligands, particularly DbpA/B and decorin, appear to play an important role during all stages of infection.

DbpA and DbpB are encoded in a bicistronic operon (*dbpBA*) on plasmid lp54 of the prototype *B. burgdorferi* B31 strain (11) and were two of the first borrelial adhesins identified (6, 12, 13, 14). These 19-kDa and 20-kDa proteins, respectively, are encoded by and expressed within *B. burgdorferi* sensu stricto strains and also many *B. burgdorferi* sensu lato strains, albeit as heterogeneous homologs (12, 15, 16, 17). Expression is upregulated in the mammalian host after tick-borne infection (18) and DbpA and DbpB are highly antigenic during infection (14, 19, 20). Based on mRNA levels, DbpA and DbpB continue to be expressed throughout chronic infection (12, 14, 18, 19). In comparison to DbpB, DbpA has been established as the more crucial adhesin in the context of pathogenesis, eliciting stronger protective immunity (12, 14) and, on its own, restoring a wild-type phenotype to DbpA/B-deficient mutant *B. burgdorferi* (21, 22).

3

69   In the laboratory mouse model, DbpA and DbpB have been implicated in the

70   establishment of infection, dissemination, tissue colonization, persistence, and tick

71   acquisition/transmission. Disruption of DbpA and DbpB, while nonessential to initial infection

72   (23), will increase the infectious dose (21, 24, 25), decrease total spirochete tissue burdens (25),

73   decrease recovery of spirochetes from tissues distant to the inoculation site (21, 23, 25) and

74   decrease efficiency of tick acquisition/transmission (24). None of the aforementioned studies

75   addressed the influence of DbpA and DbpB disruption on disease development or persistence.

76   The early dissemination defect of DbpA/B-deficient mutants, represented by decreased

77   recovery of spirochetes from tissues distant to the inoculation site (21, 23, 25), seems to be a key

78   to understanding the role of decorin-binding proteins in Lyme borreliosis. With the genetic

79   disruption or absence of these adhesins, spirochetes may be unable to travel by conventional

80   routes or access important microenvironmental niches, and, thus, manifest their altered

81   dissemination phenotype. Although the extracellular matrix (ECM) is important in *B.*

82   *burgdorferi* dissemination, as evidenced by direct dissemination through connective tissue (26,

83   27, 28, 29), spirochetes utilize alternate means to disseminate as well, including bacteremia (19,

84   29, 30, 31). In addition, a relatively unexplored means of dissemination is through lymphatics, as

85   draining lymph nodes are often culture-positive sooner than any other tissues proximal to the

86   inoculation site (20, 25, 32). Few molecular mechanisms that enable the lymphatic route of

87   dissemination have been proposed, but they probably involve the interaction between adhesins

88   and ligands. For example, fibronectin-binding protein, glycosaminoglycans and fibronectin

89   facilitate microvascular interactions observed by intravital microscopy in infected mice (31) and

90   both VlsE and OspC were implicated by phage display for in vivo adherence to vascular

91   endothelium (10), which is likely to include lymphatic vessels as well.

92   The present study concurs with previous studies, in that decorin-binding proteins

93   influence the early stages of infection (dissemination and tissue colonization). These early

94   differences are unique to immunocompetent mice and are abolished in the chronic stage of

95   infection. Results also demonstrate that decorin-binding proteins influence disease severity. We

96   propose that the mechanism of influence pertains to the restricted routes by which spirochetes

97   lacking *dbpBA* are able to disseminate, including lymphatic dissemination.

98

99   **MATERIALS AND METHODS**

100   **Borrelial strains and mutagenesis.** *B. burgdorferi* sensu stricto strain B31-A3, a low-

101   passage infectious clonal isolate of B31-MI, the prototype B31 strain utilized for genome

102   sequencing (33, 34), was utilized as both the wild-type control and the parental strain for genetic

103   manipulation (35). The *dbpBA* operon was disrupted by insertion of *flgBp-aadA* (36) by

104   electroporation of competent B31-A3 as previously described (37) and selection in 50 μg/ml

105   streptomycin, which yielded the B31-Δ*dbpBA* deletion mutant. All *B. burgdorferi* strains were

106   cultivated in liquid modified Barbour-Stoenner-Kelly (BSKII) medium supplemented with 6%

107   normal rabbit serum (38). For isolation of transformants, *B. burgdorferi* was cultured on semi-

108   solid gelatin-free BSKII medium supplemented with 1.7% dissolved agarose plus the appropriate

109   antibiotic (37).

110   The *dbpBA* operon was genetically reconstituted in the B31-Δ*dbpBA* mutant by allelic

111   exchange recombination yielding the B31-*dbpBA*+ complement. The shuttle vector pBSV2G,

112   containing a gentamicin resistance cassette (35) was utilized to create the construct in which the

113   *dbpBA* operon was incorporated. One 1649-bp long fragment of B31 DNA, including the *dbpBA*

114   operon, the promoter region from -266 to -1, and the terminator region after the stop codon from

5

115 1528 to 1649, was amplified by PCR with forward primer P1FBamHI (5′-

116 TCGTGGGATCCCAAGCCAGATTGCATAGC-3′) and reverse primer P7RPstI (5′-

117 TCGTGCTGTGATTATCGGGCGAAGAG-3′). Both pBSV2G and the amplicon were double

118 digested with BamHI and PstI, ligated together and sequenced to ensure the correct orientation of

119 the *dbpBA* operon. The construct was electroporated into B31-Δ*dbpBA* mutants, and successful

120 complements were selected with gentamicin (40 µg/ml). Six complemented mutants were

121 obtained, and confirmed by PCR for the presence of the *dbpBA* operon and gentamicin marker,

122 as well as the absence of the streptomycin marker. Plasmid profiling confirmed that all six

123 complemented mutants contained the plasmids lp25, lp28-1, lp54, cp26 and cp32, which are

124 required for infectivity (39).

125   For construction of suicide vectors and general gene cloning, *Escherichia coli* strain

126 TOP10F′ (Invitrogen, Inc., CA) was utilized and grown in lysogeny broth (LB) broth under

127 aerobic conditions at 37°C. Transformed *E. coli* were cultured in LB medium with 50 µg/ml

128 spectinomycin or 5 µg/ml gentamicin.

129   **Mice and infections.** Specific-pathogen-free, 3 to 5 week old female C3H/HeN (C3H),

130 C3H.C-*Prkdc^{scid}/*IcrSmnHsd (C3H-*scid*) and IcrTac:ICR-*Prkdc^{scid}* (Swiss-*scid*) mice were

131 acquired from Frederick Cancer Research Center (Frederick, MD), Harlan Sprague Dawley, Inc.

132 (Indianapolis, IN) and Taconic Farms, Inc. (Hudson, NY), respectively. Pregnant outbred

133 Crl:CD1(ICR) mice were acquired from Charles River Laboratories (Hollister, CA). Mice were

134 killed by carbon dioxide narcosis and cardiac exsanguination. Specific isolates of the borrelial

135 mutants, B31-Δ*dbpBA* and B31-*dbpBA*+ were confirmed as infectious to infant ICR mice at all

136 inoculation doses from 10^4 to 10^7 (data not shown). Any individual C3H, C3H-*scid* or Swiss-*scid*

137 mouse, in the experiments included herein, that could not be confirmed as infected (neither PCR-

138   positive nor culture-positive) was excluded from data analysis.

139   **PCR.** DNA was extracted from tissue samples using DNeasy tissue kits, according to the

140   manufacturer's instructions (QIAGEN, Valencia, CA). Samples were analyzed by quantitative

141   PCR (qPCR) using optimized assays for *flaB* and *dbpA*, as previously described (18). Three

142   oligonucleotides, two primers and an internal Taqman probe, for the *flaB* (18) and the *dbpA*

143   genes were used. Primers DbpAB31-247F (5′-GCGAGCTACTACAGTAGCGGAAA-3′) and

144   DbpAB31-444R (5′-TTTCAAGCACTCCTTGAGCTGTA-3′) were created to amplify a 198-bp

145   fragment of *dbpA* DNA. The internal probe DbpAB31-316P (5′- GTGAAACAGGTAGCAAG

146   TATCAGAAAATTCAT -3′) contained 5′ 6-carboxy fluorescein reporter dye and 3′ 6-carboxy-

147   tetramethyl rhodamine quencher dye. Quantification of gene copies was based on absolute

148   standard curves prepared using plasmid standards (18). Target gene copy numbers were

149   expressed as copy number per mg of tissue weight or per µl blood. In addition, DNA extracted

150   from positive cultures and DNA from tissue samples were used to verify *B. burgdorferi*

151   genotypes recovered from infected mice.

152   **Histology.** Tissues were fixed in 10% neutral-buffered formalin, paraffin-embedded,

153   routinely processed and stained with hematoxylin and eosin. Limbs were decalcified prior to

154   processing. Tissue sections were blindly examined and graded for the presence of inflammation.

155   The presence of arthritis in each mouse was determined by examination of knees and tibiotarsi.

156   Sagittal sections through the heart, including sections of great vessels (aorta), were examined for

157   the presence of carditis, as described previously (40, 41). Tibiotarsal arthritis severity was scored

158   on a scale of 0 (no histologic evidence of inflammation) to 3 (severe), as described previously

159   (42).

7

160   **Enzyme-linked immunosorbent assay.** Ninety-six well plates were coated with 1 µg/ml

161   *B. burgdorferi* B31 whole cell lysates in carbonate coating buffer (pH 9.6), as described

162   previously (12). Antibody binding was recognized by a secondary alkaline phosphatase-

163   conjugated goat anti-mouse IgH+L antibody, diluted at 1:5000 (Jackson ImmunoResearch

164   Laboratories Inc., West Grove, PA). Immunoreactivity was revealed using 1 mg/ml phosphate

165   substrate (Sigma-Aldrich, St. Louis, MO) in diethanolamine buffer and optical density values

166   were measured at 405nm on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA), as

167   described previously (41). Individual serum samples were titrated in three-fold dilutions (starting

168   at 1:300). Samples were tested in duplicate, and each assay included uninfected mouse serum as

169   a negative control and 90-day B31-infected mouse serum as a positive control.

170   **Infection, dissemination/colonization, and persistence experiments.** Mice were

171   infected by subdermal inoculation of 10^5 to 10^6 mid-log phase *B. burgdorferi* B31-A3, B31-

172    $\Delta dbpBA$, and/or B31-*dbpBA+* in 0.1 ml BSKII culture medium on the dorsal thoracic midline.

173   Subsets from each group were necropsied at 14, 28, 42, 60 and/or 90 days post-inoculation. Sub-

174   inoculation site and urinary bladder tissues were aseptically collected for culture, as previously

175   described (43). Tissues collected for DNA extraction and qPCR included: skin, sub-inoculation

176   site, heart base, ventricular muscle, quadriceps muscle and left tibiotarsus. Tissues collected for

177   histology included: heart base, left knee and right rear limb. Hearts were bisected along the

178   longitudinal axis to provide samples for both DNA extraction and histology.

179   **Lymphatic dissemination experiment.** Groups of C3H mice were infected by

180   subdermal inoculation of 10^5 mid-log phase *B. burgdorferi* B31-A3, B31-$\Delta dbpBA$, and/or B31-

181   *dbpBA+* in 0.1 ml BSKII culture medium in the skin of the right lateral thigh. Four mice from

182   each group were necropsied at 12 hours, 3 days, 7 days and 14 days post-inoculation. Right and

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183   left inguinal lymph nodes, spleen and urinary bladder were aseptically collected for culture. Both

184   right and left inguinal, popliteal, lumbar, and axial lymph nodes were collected for DNA

185   extraction. Inguinal lymph nodes were bisected to provide samples for both culture and DNA

186   extraction. Extra-lymphatic tissues, including skin at the inoculation site, heart base, and right

187   tibiotarsus, were collected for DNA extraction. To evaluate lymphatic dissemination in the

188   absence of acquired immunity, the experiment was repeated in Swiss-*scid* mice.

189   **Statistics.** Analyses were performed using Fisher's exact test for differences, independent

190   samples *t*-test or two-way analysis of variance, followed by post-hoc pair-wise comparisons

191   (Tukey's HSD test) (PASW Statistics v. 18.0 and Prism v. 5, GraphPad software). Calculated *P*

192   values $\leq$ 0.05 were considered significant.

193

194   **RESULTS**

195   ***Borrelia burgdorferi* deficient in DbpA and DbpB lacks an early dissemination defect**

196   **in immunodeficient mice, but exhibits attenuated disease development.** The dissemination

197   and pathogenic capabilities of the B31-Δ*dbpBA* mutant compared to wild-type B31-A3 was

198   initially evaluated in immunodeficient mice. Groups of 4 C3H-*scid* mice inoculated with 10^6

199   B31-Δ*dbpBA* or B31-A3 were necropsied at 28 days post-inoculation. Sub-inoculation site and

200   urinary bladder from all mice in both B31-Δ*dbpBA* and B31-A3-inoculated groups were culture-

201   positive and there were no statistical differences in tissue spirochete burdens by *flaB* qPCR

202   between groups (data not shown). B31-Δ*dbpBA*-inoculated C3H-*scid* mice developed both

203   arthritis and carditis (Table 1), but the severity of tibiotarsal inflammation was attenuated in the

204   B31-Δ*dbpBA* infection (0.8 mean severity score $\pm$ 0.2 SEM) compared to the wild-type B31-A3

205   infection (2.9 $\pm$ 0.1) (*P* = 0.03). Carditis was milder and in equal prevalence in the B31-Δ*dbpBA*-

9

206 inoculated C3H-*scid* mice compared to mice infected with B31-A3. Therefore, when unrestricted

207 by acquired immunity, B31-Δ*dbpBA* retained the ability to disseminate and colonize distant

208 tissues and was pathogenic, but despite the presence of equal copy numbers of spirochetes in

209 tissue compared to wild type, B31-Δ*dbpBA* elicited less inflammation both hearts and joints.

210   In the above experiment and similar studies by others in immunodeficient mice (24, 25),

211 1 month (28-30 days) post-inoculation was the maximum experiment duration for evaluating

212 infections utilizing DbpA/B-deficient spirochetes. In order to evaluate the capability of B31-

213 Δ*dbpBA* to persist in immunodeficient mice, we extended the duration to 90 days. Groups of 12

214 C3H-*scid* mice were inoculated with 10^6 B31-Δ*dbpBA* or B31-A3 and subsets of 4 mice per

215 group were necropsied at 14 days, 60 days and 90 days post-inoculation. Sub-inoculation sites

216 and urinary bladders from all mice were culture-positive at all intervals and in both groups. Copy

217 numbers of *flaB* DNA in sub-inoculation site, heart base, ventricle, quadriceps muscle and

218 tibiotarsal tissues were not significantly different between B31-Δ*dbpBA* and wild-type B31-A3-

219 inoculated mice at any interval (Fig. 1). The severity of tibiotarsal arthritis and carditis similarly

220 was indistinguishable between B31-Δ*dbpBA* and wild-type B31-A3-inoculated mice at 60 and 90

221 days post-inoculation (Table 1). The qPCR and histology data confirmed that in

222 immunodeficient mice, B31-Δ*dbpBA* spirochetes can disseminate to distant tissues, proliferate

223 therein to an equal degree, incite inflammation and persist in a manner similar to wild-type

224 spirochetes.

225   **The early dissemination defect of *dbpBA*-deficient spirochetes in immunocompetent**

226 **mice is abolished in the chronic stage of infection and is rescued by complementation.** To

227 evaluate whether similar spirochete tissue dissemination, persistence and disease development

228 would occur with B31-Δ*dbpBA* infection in immunocompetent mice, groups of 15 C3H mice

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229   were inoculated with 10^5 B31-Δ*dbpBA* or B31-A3. Five mice from each group were necropsied

230   at 14, 28 and 42 days post-inoculation. Fewer culture-positive tissues, and fewer positive mice,

231   were identified in the B31-Δ*dbpBA*-inoculated mice compared to wild type at day 14 and day 28,

232   but by day 42, numbers of culture-positive tissues and numbers of culture-positive mice

233   increased until differences between B31-Δ*dbpBA* and B31-A3 infections were diminished (Table

234   2).

235   Similarly, at day 14, tissue spirochete burdens were undetectable in multiple tissues,

236   including sub-inoculation site, heart base, ventricular muscle, quadriceps muscle and tibiotarsus

237   (all $P = 0.0079$) in B31-Δ*dbpBA*-infected mice compared to wild type (Fig. 2). At day 28,

238   spirochete tissue burdens in heart base ($P = 0.034$) and ventricular muscle ($P = 0.033$) were

239   significantly lower in B31-Δ*dbpBA*-infected mice compared to wild type. However, by day 42

240   post-inoculation, qPCR tissue burdens were equivalent in both groups. No inflammation was

241   observed on day 28 and only minimal carditis (0.1 ± 0.1; 1 out of 4 mice) and mild arthritis (0.4

242   ± 0.2; 2 out of 4 mice) was observed at day 42 in B31-Δ*dbpBA*-inoculated mice (Table 1). By

243   contrast, in the wild type-inoculated mice at day 28, there was statistically significantly greater

244   carditis (1.0 ± 0.0; 5 out of 5 mice; $P < 0.05$) and a mild arthritis ($0.2 + 0.2$; 1 out of 5 mice). At

245   day 42, there was a trend towards slightly more severe and more prevalent disease with mild

246   carditis (0.6 ± 0.2) and mild to moderate arthritis (0.9 ± 0.3) in 4 out of 5 mice. Results

247   demonstrated that B31-Δ*dbpBA* spirochetes retained the capacity to infect, disseminate, and

248   persist in immunocompetent mice, and eventually attain equal levels of tissue burdens and

249   disease, but were delayed and initially only able to induce attenuated disease.

250   The duration of infection in immunocompetent mice was next extended to 90 days post-

251   inoculation in order to fully evaluate the capability of the DbpA/B-deficient mutant to persist.

11

252   The complemented mutant B31-*dbpBA+* was included in the experiment to evaluate whether

253   genetic complementation could rescue the phenotype of the DbpA/B-deficient mutant. Groups of

254   12 C3H mice were inoculated with 10^6 B31-Δ*dbpBA*, B31-*dbpBA+,* or B31-A3. Subsets of 4

255   mice were necropsied at 14 days, 60 days and 90 days post-inoculation. In mice inoculated with

256   B31-Δ*dbpBA*, there were notably fewer culture and/or qPCR-positive mice (1/4) and minimal or

257   no detectable spirochete tissue burdens in B31-Δ*dbpBA*-infected mice at day 14 compared to

258   both wild-type B31 or B31-*dbpBA+*infected mice (Fig. 3). At subsequent intervals (day 60 and

259   90), 3/4 and 4/4 B31-Δ*dbpBA*-inoculated mice were culture and/or qPCR-positive and the level

260   of spirochete tissue burden (Fig. 4) and severity of arthritis and carditis (Table 1) was not

261   significantly different from B31-A3-inoculated mice. All B31-A3 and B31-*dbpBA+*-inoculated

262   mice were positive at 14, 60 and 90 days and tissue spirochete burdens in B31-*dbpBA+*-

263   inoculated mice were either not statistically different or were not significantly less than wild-type

264   B31-A3 (day 14 shown in Fig. 3). Similarly, the severity of arthritis and carditis was not

265   significantly different between B31-A3 and B31-*dbpBA+*-inoculated mice on day 60 and 90

266   (data not shown). The appropriate infecting *B. burgdorferi* genotypes (wild type, mutant,

267   complemented mutant) were confirmed among isolates from each mouse group at necropsy.

268   Thus, DbpA/B-deficient spirochetes, despite their early dissemination defect, were capable of

269   persistence and inducing disease in immunocompetent C3H mice, and complementation of the

270   mutant restored the early dissemination phenotype.

271   **The early dissemination defect is dependent on the presence of an acquired immune**

272   **response.** The *flaB* qPCR data from the above experiments were combined to evaluate

273   spirochete dissemination and colonization kinetics from day 14 to day 90 post-inoculation in

274   immunocompetent C3H mice compared to immunodeficient C3H-*scid* mice (Fig. 4). Heart base

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275   and tibiotarsal results were focused upon because these two tissues are distant from the

276   inoculation site and are often poorly colonized by DbpA/B-deficient spirochetes, due to and

277   representative of the dissemination defect (21, 25). Serology from the above immunocompetent

278   C3H mouse experiments was also combined to evaluate the acquired immune response between

279   DbpA/B-deficient and wild type-inoculated mice. In C3H-*scid* mice, no significant differences

280   were observed in tissue spirochete burdens in heart base (Fig. 4A) or tibiotarsus (Fig. 4B)

281   between the B31-Δ*dbpBA* mutant and wild-type B31-A3. In contrast, B31-Δ*dbpBA* tissue

282   spirochete burdens in C3H mice were markedly lower to absent compared to wild type at early

283   time points (day 14 and day 28), but these differences were abolished by day 42 post-inoculation.

284   Despite a continuous rise in *B. burgdorferi*-specific antibody titer in mice inoculated with both

285   genotypes, differences between the titers in B31-Δ*dbpBA* and wild type infections were not

286   abolished after day 42 and remained statistically significantly greater in the wild type-inoculated

287   mice and in the B31-Δ*dbpBA*-inoculated mice (Fig. 5).

288   ***dbpBA*-deficiency prevents early dissemination though the lymphatic system.**

289   Regional lymph nodes have been reported to become rapidly culture-positive following infection

290   (by needle-inoculation, tick transmission and tissue graft) during infection with wild-type as well

291   as DbpA/B-deficient *B. burgdorferi* (20, 25, 32). One study reported that distant lymph nodes in

292   mice infected with wild-type *B. burgdorferi* became progressively culture-positive over time, in

293   the order of their proximity to the inoculation site (32). The same study concluded that

294   spirochetes were in fact within lymph nodes, rather than in the surrounding connective tissue, by

295   identifying morphologically intact spirochetes in subcapsular sinuses (32). In another study, in

296   mice inoculated with DbpA/B-deficient spirochetes, spirochetes were frequently cultured from

297   lymph nodes at 12 hours and 2 and 3 weeks post-inoculation (25). Based on these observations,

13

298   both wild-type and DbpA/B-deficient spirochetes appeared to be able to enter into, survive

299   within, and potentially migrate through the lymphatic system. This is in contrast to the observed

300   dissemination defect in DbpA/B-deficient spirochetes where heart and joint (tissues that should

301   be accessible by hematogenous or direct routes of dissemination) are less frequently colonized

302   by DbpA/B-deficient spirochetes (21, 25) than by wild-type spirochetes. Based on these

303   observations, we postulated that the lymphatic dissemination route might be utilized by

304   spirochetes lacking DbpA/B more readily than other routes.

305   To investigate this possibility, we determined the prevalence of wild-type B31-A3, B31-

306   Δ*dbpBA* mutant, and B31-*dbpBA+* complemented spirochetes within lymph nodes, both

307   proximal and distal to the inoculation site, and at multiple intervals (0.5, 3, 7 and 14 days) during

308   early infection by culture and qPCR for *flaB* DNA. Any animal that was neither culture nor *flaB*

309   qPCR-positive was considered uninfected and dropped from the data set. Both right and left

310   sides from each pair of lymph nodes (popliteal, inguinal, lumbar, and axillary) were evaluated

311   and if either one or both sides were qPCR or culture-positive, then the pair of lymph nodes was

312   considered positive (Table 3). Initially, we inoculated mice asymmetrically in the right hind limb

313   to evaluate any influence of proximity but the effect of side (right vs. left) was negligible and

314   therefore, each pair of lymph nodes was combined as a unit of evaluation.

315   At the earliest time points, qPCR-positive lymph nodes were identified in mice infected

316   with all three *B. burgdorferi* genotypes within hours after inoculation (day 0.5), but the same

317   lymph nodes were universally negative at the following time point (day 3), suggesting drainage

318   of DNA, but not viable spirochetes, from the inoculum. At day 7, the number of positive lymph

319   nodes from B31-Δ*dbpBA*-inoculated mice was significantly lower ($P < 0.0001$) than the number

320   of positive lymph nodes in wild type-inoculated mice. At day 14, the number of positive lymph

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321 nodes from B31-Δ*dbpBA*-inoculated mice was significantly lower (*P* < 0.0001) than from both

322 wild type and complemented mutant infections. Similarly, at day 7 and day 14, spirochete tissue

323 burdens in lymph nodes from B31-Δ*dbpBA*-inoculated mice (10,572 mean copy no. *flaB* DNA

324 per mg tissue $\pm$ 10,536 SEM; 225 $\pm$ 0.0) were lower, though not significantly, than wild type

325 (45,904 $\pm$ 19,596; 38,995 $\pm$ 12,279).

326　　　　Though there was a trend towards greater numbers of PCR-positive tissues in B31-

327 Δ*dbpBA* extra-lymphatic tissues (skin, tibiotarsus and heart base) than in lymph nodes, only on

328 day 3 was the difference significant (*P* = 0.0211). Otherwise, there were significantly fewer

329 PCR-positive extra-lymphatic tissues from B31-Δ*dbpBA*-inoculated mice than in wild type-

330 infected mice at the later time points (day 7 *P* < 0.0001, day 14 *P* = 0.0062) (Table 3). At day 7

331 and day 14, spirochete tissue burdens in extra-lymphatic tissues from B31-Δ*dbpBA*-inoculated

332 mice (25 $\pm$ 4; 54,037 $\pm$ 49,271) were lower, though not significantly, than wild type (7,381,000 $\pm$

333 6,459,000 vs. 103,140 $\pm$ 60,179). Based on culture, viable spirochetes could be recovered from

334 the lymphatic system and extra-lymphatic tissue (urinary bladder) earliest in B31-A3-inoculated

335 mice (day 7), followed by the B31-*dbpBA+*-inoculated mice (day 14) but were not recovered

336 from B31-Δ*dbpBA*-inoculated mice at any interval (Table 3). Therefore, the early dissemination

337 defect of DbpA/B-deficient spirochetes in immunocompetent C3H mice was characterized by

338 minimal presence in lymph nodes, ii) greater presence in extra-lymphatic tissues, and iii) an

339 overall lower spirochete tissue burden in lymph nodes and extra-lymphatic tissues when

340 compared to wild type. These data demonstrate that the lymphatic route is not a dominant means

341 of dissemination/migration utilized by DbpA/B-deficient spirochetes.

342　　　　**Early exclusion of *dbpBA*-deficient spirochetes from the lymphatic system requires**

343 **an acquired immune response.** Results indicated that the early dissemination defect of B31-

15

344 Δ*dbpBA* spirochetes occurs only in C3H, but not C3H-*scid* mice. Therefore, we next sought to

345 determine if an acquired immune response is necessary to exclude B31-Δ*dbpBA* spirochetes from

346 lymphatic dissemination. To investigate this possibility, we intended to repeat the previous

347 experiment in congenic C3H-*scid* mice; however, C3H-*scid* mice became unavailable due to

348 elimination of this mouse strain by the vendor. Therefore, the prevalence and tissue burdens of

349 wild-type, mutant and complemented spirochetes within lymph nodes and extra-lymphatic

350 tissues during the early stage of infection was repeated in equally susceptible Swiss-*scid* mice.

351 　　　　　Culture and PCR-positive lymph nodes were identified in B31-Δ*dbpBA*-inoculated *scid*

352 mice within hours after inoculation (day 0.5) (Table 4). By day 7, the number of positive lymph

353 nodes from B31-Δ*dbpBA*-inoculated *scid* mice was significantly fewer ($P < 0.0001$) than the

354 number of positive lymph nodes in wild type and B31-*dbpBA+*-inoculated *scid* mice. However,

355 by day 14, significant differences between the numbers of positive lymph nodes in wild type,

356 B31-Δ*dbpBA* or B31-*dbpBA+*-inoculated *scid* mice were no longer apparent and spirochete

357 tissue burdens in lymph nodes from B31-Δ*dbpBA*-inoculated *scid* mice ($2,352 \pm 701$) were not

358 significantly different than wild type ($33,497 \pm 11,578$) and B31-*dbpBA+* ($35,938 \pm 10,355$). At

359 this same time point, the number of positive lymph nodes was significantly greater in *scid* mice

360 inoculated with B31-Δ*dbpBA* ($P < 0.0001$) than in similarly inoculated C3H mice. No significant

361 differences were observed between the number of positive lymph nodes and extra-lymphatic

362 tissues in B31-Δ*dbpBA*-inoculated *scid* mice. Viable spirochetes could be recovered from the

363 lymphatic system and extra-lymphatic tissues earliest in B31-A3-inoculated *scid* mice (day 3),

364 followed by the B31-*dbpBA+*-inoculated *scid* mice (day 7) and B31-Δ*dbpBA*-inoculated *scid*

365 mice (day 14) (Table 4). In summary, DbpA/B-deficient spirochetes in immunodeficient Swiss-

366 *scid* mice were not excluded from the lymphatic route of dissemination.

16

367

368 **DISCUSSION**

369   The role of individual borrelial ECM adhesins is a common theme of investigation, given

370 the importance of ECM to the lifecycle and pathogenesis of *B. burgdorferi* (44). Though

371 adhesins may be necessary to a specific stage in borreliosis, no single adhesin has been shown to

372 be absolutely essential. For instance, several studies have independently documented that

373 deletion of *dbpBA* attenuates but does not abolish infectivity of *B. burgdorferi* (21, 23, 24).

374 Similarly, deletion of other adhesins has not been sufficient to alter the course of initial infection.

375 Disruption of Bgp led to an uninterrupted infectious phenotype in immunodeficient mice after 2

376 weeks post-inoculation (45) and deletion of fibronectin-binding protein did not alter infection in

377 immunocompetent mice at 3 weeks (46), although the median infectious dose was increased

378 (47). Deletion of another adhesin, P66, resulted in loss of in vitro spirochetal attachment to the

379 ligand integrin $\alpha_v\beta_3$ (48) and loss of infectivity in both immunocompetent and immunodeficient

380 mice, with retention of the ability to infect ticks and survive in in vivo dialysis membrane

381 chambers (49). Therefore, lack of any single adhesin may not be essential but, as we and others

382 have demonstrated, may influence pathogenicity by altering the course of infection, by changing

383 the ability to disseminate, colonize, cause disease, or persist.

384   While not necessary to establish infection in immunocompetent mice (23), deletion of

385 *dbpBA* was reported to decrease infectivity (21, 24), display a dissemination defect (21, 23, 25)

386 and potentially, alter the ability to persist (25). In this study, we confirmed that DbpA/B-

387 deficient spirochetes manifested an early dissemination defect, but we demonstrated that the

388 defect resolved with chronicity (after day 28 post-inoculation) and that persistence occurred in a

389 manner indistinguishable from wild-type spirochetes. Furthermore, we demonstrated, for the first

17

390 time, that deletion of DbpA/B resulted in early attenuation of disease development and prevented

391 early dissemination and colonization within the lymphatic system. We propose that one

392 mechanism by which the early dissemination defect of DbpA/B-deficient spirochetes occurs is

393 restriction of lymphatic dissemination through which, by comparison, wild-type spirochetes can

394 rapidly migrate.

395 As unlikely as it may seem for an organism dedicated to immune evasion and persistence,

396 there is abundant evidence that *B. burgdorferi* spirochetes actively migrate within the lymphatic

397 system. Lymph nodes are rapidly and consistently culture-positive in both acute and chronic

398 stages of infection (20, 25), become progressively culture-positive in order of proximity to the

399 inoculation site (20), and morphologically intact spirochetes have been identified in subcapsular

400 sinuses of regional lymph nodes (20). Indeed, a recent study found that the direct presence of

401 viable (in contrast to non-viable) spirochetes in lymph nodes deceptively stimulates an atypical

402 immune response that may actually favor survival of spirochetes during early infection (50). In

403 the current study, we provide additional evidence for migration of wild-type spirochetes through

404 the lymphatic system, and demonstrate the diminished ability of DbpA/B-deficient spirochetes to

405 do likewise. Taken together, the lymphatic system appears to be a route of dissemination for *B.*

406 *burgdorferi,* and DbpA and DbpB may be important for that behavior.

407 Based on data presented in this study and by Weening et al. (24), DbpA/B-deficient

408 spirochetes can gain initial and sporadic access to the lymphatic system, but we postulate that the

409 inability to maintain access and migrate therein essentially results in exclusion that coincides

410 with the repeatedly documented early dissemination defect. Involvement of the acquired immune

411 response is strongly implicated as only in immunocompetent mice has the dissemination defect

412 been observed (21, 23, 25) and notably, only in immunocompetent mice have we observed

413   exclusion from the lymphatic system.

414   The importance of the acquired immune response, B cell and antibody-mediated

415   immunity in particular, to disease resolution and spirochete reduction in the host is well

416   established (41, 51, 52, 53). How this clears or prevents access of DbpA/B-deficient spirochetes

417   to lymphatics is perplexing because these genetically manipulated spirochetes lack one of the

418   more immunogenic antigens, DbpA (12, 14). Without a vulnerable target, one might expect

419   DbpA/B-deficient spirochetes to escape immune pressure; however, based on our observations,

420   this is incorrect. We showed that the acquired immune response to DbpA/B-deficient spirochetes

421   (by *B. burgdorferi*-specific serum titer) remains significantly lower than the wild-type immune

422   response to wild type (Fig. 5) despite equilibration of tissue spirochete burdens to a wild-type

423   level (Fig. 4C and 4D). This reduced immune response remains capable of excluding DbpA/B-

424   deficient spirochetes from the lymphatics, at least within the early stages of infection.

425   Several mechanisms that would prevent lymphatic dissemination of DbpA/B-deficient

426   spirochetes in immunocompetent mice are possible: i) DbpA/B-deficient spirochetes have

427   increased vulnerability to antibody clearance within lymphatics, ii) DbpA/B-deficient spirochetes

428   have increased vulnerability to non-antibody-mediated clearance within lymphatics, or iii)

429   lymphatics become inaccessible to DbpA/B-deficient spirochetes after the initial establishment

430   of infection. Our observations are more consistent with the first two possibilities since

431   involvement of the acquired immune response is implicated. If DbpA/B-deficient spirochetes are

432   more vulnerable to antibody clearance, then increased exposure to IgM could account for the

433   greater susceptibility. IgM dominates the anti-borrelial immune response (50) and though it may

434   be too large and unwieldy to penetrate collagenous tissues, it is present in blood and lymph (54).

435   The caveat remains that evidence exists to refute the hypothesis that steric hindrance alone

436   prevents the antibody response from targeting spirochetes embedded in collagen (55, 56). As for

437   non-antibody-mediated clearance, recent investigations into invariant natural killer T (iNKT)

438   cells are reminders that there are alternate immune mechanisms to consider (57, 58). For

439   instance, disruption of the phagocyte (macrophage or Kupffer cell)-iNKT cell interaction results

440   in diminished IFN-γ production, decreased phagocytic clearance, and increased bacterial loads

441   (57) and dissemination (58).

442   Similarly, the exact mechanism by which the DbpB/A-deficient spirochetes maintain the

443   capability to incite inflammation despite the absence of a strongly immunogenic antigen is

444   speculative at best. Only during the earlier stage of infection (day 28) was there a statistically

445   significant difference in severity of arthritis (in C3H*scid* mice) or carditis (in C3H mice) between

446   B31-Δ*dbpBA* and wild type-inoculated mice. However, in C3H mice, there was a slight

447   attenuation in disease severity in B31-Δ*dbpBA* extending to day 60. Relative tissue spirochete

448   burdens are not sufficient to explain the difference in disease severity since attenuation of disease

449   in B31-Δ*dbpBA*-inoculated mice extends past the point (day 42) of equilibration between

450   genotypes (Fig. 4C and 4D). Rapidity of dissemination to and colonization of a site of

451   predilection for inflammation (heart base or tibiotarsus) may be an alternate possible explanation

452   for the initially attenuated inflammation associated with B31-Δ*dbpBA* spirochetes. For example,

453   in the earlier time points (<14 days), histologically evident inflammation often lags behind the

454   wave of directly disseminating wild-type spirochetes in immunodeficient C3H*scid* mice (D. M.

455   Imai, unpublished).

456   In summary, we demonstrated and confirmed that disruption of *dbpBA* results in an early

457   dissemination defect that is dependent on the presence of acquired immunity, resolves with

458   chronicity of infection, and appears to reflect restricted migration through the lymphatic system.

20

459   We confirmed that deficiency in *dbpBA* does not diminish the ability to infect, to cause disease

460   or to persist. The counterintuitive dispensability of DbpA and DbpB, immunodominant (19, 12,

461   20) but potentially protective (19, 12, 59) outer surface proteins that afford the ability to

462   disseminate in the face of acquired immunity, is only one indication of the complexity of the

463   borrelial pathogen-host relationship.

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471   **REFERENCES**

472   **1.**   **Brissette CA, Verma A, Bowman A, Cooley AE, Stevenson B.** 2009. The *Borrelia*

473   *burgdorferi* outer-surface protein ErpX binds mammalian laminin. Microbiology.

474   **155**:863-872.

475   **2.**   **Brissette CA, Bykowski T, Cooley AE, Bowman A, Stevenson B.** 2009. *Borrelia*

476   *burgdorferi* RevA antigen binds host fibronectin. Infect. Immun. **77**:2802-2812.

477   **3.**   **Coburn J, Chege W, Magoun L, Bodary SC, Leong JM.** 1999. Characterization of the

478   candidate *Borrelia burgdorferi* β_3-chain integrin ligand identified using a phage display

479   library. Mol. Microbiol. **34**:926-940.

480   **4.**   **Fischer JR, Parveen N, Magoun L, Leong JM.** 2003. Decorin-binding proteins A and

481   B confer distinct mammalian cell type-specific attachment by *Borrelia burgdorferi*, the

482   Lyme disease spirochete. Proc. Natl. Acad. Sci. **100**:7307-7312.

483   doi:10.1073/pnas.1231043100.

484   **5.   Fischer JR, LeBlanc KT, Leong JM.** 2006. Fibronectin binding protein BBK32 of the

485   Lyme disease spirochete promotes bacteria attachment to glycosaminoglycans. Infect.

486   Immun. **74**:435-441.

487   **6.   Guo BP, Norris SJ, Rosenberg LC, Höök M.** 1995. Adherence of *Borrelia burgdorferi*

488   to the proteoglycan decorin. Infect. Immun. **63**:3467-3472.

489   **7.   Parveen N, Leong JM.** 2000. Identification of a candidate glycosaminoglycan-binding

490   adhesin of the Lyme disease spirochete *Borrelia burgdorferi*. Mol. Microbiol. **35**:1220-

491   1234.

492   **8.   Antonara S, Ristow L, Coburn J.** 2011. Adhesion mechanisms of *Borrelia burgdorferi*.

493   Adv. Exp. Med. Biol. **715**:35-49.

494   **9.   Zambrano MC, Beklemisheva AA, Bryksin AV, Newman SA, Cabello FC.** 2004.

495   *Borrelia burgdorferi* binds to, invades and colonizes native type I collagen lattices.

496   Infect. Immun. **72**:3138-3146.

497   **10.   Antonara S, Chafel RM, LaFrance M, Coburn J.** 2007. *Borrelia burgdorferi* adhesins

498   identified using *in vivo* phage display. Mol. Microbiol. **66**:262-276.

499   **11.   Hagman KE, Lahdenne P, Popova TG, Porcella SF, Akins DR, Radolf JD, Norgard**

500   **MV.** 1998. Decorin-binding protein of *Borrelia burgdorferi* is encoded within a two-gene

501   operon and is protective in the murine model of Lyme borreliosis. Infect.

502   **12.   Feng S, Hodzic E, Stevenson B, Barthold SW.** 1998. Humoral immunity to *Borrelia*

503   *burgdorferi* N40 decorin binding proteins during infection in laboratory mice. Infect.

504   Immun. **66**:2827-2835.

505   **13.   Guo BP, Brown EL, Dorward DW, Rosenberg LC, Höök M.** 1998. Decorin-binding

506   adhesins from *Borrelia burgdorferi.* Mol. Microbiol. **30**:711-723.

507   Immun. **66**:2674-2683.

508   **14.   Hanson MS, Cassatt DR, Guo BP, Patel NK, McCarthy MP, Dorward DW, Höök**

509   **M.** 1998. Active and passive immunity against *Borrelia burgdorferi* decorin binding

510   protein A (DbpA) protects against infection. Infect. Immun. **66**:2143-2153.

511   **15.   Benoit VM, Fischer JR, Lin Y, Parveen N, Leong JM.** 2011. Allelic variation of the

512   Lyme disease spirochete adhesin DbpA influences spirochetal binding to decorin,

513   dermatan sulfate and mammalian cells. Infect. Immun. **79**:3501-3509.

514   **16.   Roberts WC, Mullikin BA, Lathigra R, Hanson MS.** 1998. Molecular analysis of

515   sequence heterogeneity among genes encoding decorin binding proteins A and B of

516   *Borrelia burgdorferi* sensu lato. Infect. Immun. **66**:5275-5285.

517   **17.   Salo J, Loimaranta V, Lahdenne P, Viljanen MK, Hytönen J.** 2011. Decorin binding

518   by DbpA and B of *Borrelia garinii, Borrelia afzelii* and *Borrelia burgdorferi* sensu

519   stricto. J. Infect. Dis. **204**:65-73.

520   **18.   Hodzic E, Feng S, Freet KJ, Barthold SW.** 2003. *Borrelia burgdorferi* population

521   dynamics and prototype gene expression during infection of immunocompetent and

522   immunodeficient mice. Infect. Immun. **71**:5042-5055.

523   **19.   Cassatt DR, Patel NK, Ulbrandt ND, Hanson MS.** 1998. DbpA, but not OspA, is

524   expressed by *Borrelia burgdorferi* during spirochetemia and is a target for protective

525   antibodies. Infect. Immun. **66**:5379-5387.

526   **20.   Tunev SS, Hastey CJ, Hodzic E, Feng S, Barthold SW, Baumgarth N.** 2011.

527   Lymphadenopathy during Lyme borreliosis is caused by spirochete migration-induced

528   specific B cell activation. PLoS Pathog. **7**:e1002066. doi:10.1371/journal.ppat.1002066.

529   **21.**   **Shi Y, Xu Q, McShan K, Liang FT.** 2008. Both decorin-binding proteins A and B are

530   critical for the overall virulence of *Borrelia burgdorferi*. Infect. Immun. **76**:1239-1246.

531   **22.**   **Shi Y, Xu Q, Seemanaplli SV, McShan K, Liang FT.** 2008. Common and unique

532   contributions of decorin-binding proteins A and B to the overall virulence of *Borrelia*

533   *burgdorferi*. Plos ONE. **3**: e3340. doi:10.1371/journal.pone.0003340.

534   **23.**   **Shi Y, Xu Q, Seemanaplli SV, McShan K, Liang FT.** 2006. The *dbpBA* locus of

535   *Borrelia burgdorferi* is not essential for infection of mice. Infect. Immun. **74**:6509-6512.

536   **24.**   **Blevins JS, Hagman KE, Norgard MV.** 2008. Assessment of decorin-binding protein A

537   to the infectivity of *Borrelia burgdorferi* in the murine models of needle and tick

538   infection. BMC Microbiol. **8**:82.

539   **25.**   **Weening EH, Parveen N, Trzeciakowski JP, Leong JM, Höök M, Skare JT.** 2008.

540   *Borrelia burgdorferi* lacking DbpBA exhibits an early survival defect during

541   experimental infection. Infect. Immun. **76**:5694-5705.

542   **26.**   **Motameni AT, Bates TC, Juncadella IJ, Petty C, Hedrick MN, Anguita J.** 2005.

543   Distinct bacterial dissemination and disease outcome in mice subcutaneously infected

544   with *Borrelia burgdorferi* in the midline of the back and the footpad. FEMS Immunol.

545   Med. Microbiol. **45**:279-284.

546   **27.**   **Shih CM, Pollack RJ, Telford SR, Spielman A.** 1992. Delayed dissemination of Lyme

547   disease spirochetes from the site of deposition in the skin of mice. J. Infect. Dis. **4**:827-

548   831.

549   **28.**   **Shih CM, Telford SR, Pollack RJ, Spielman A.** 1993. Rapid dissemination by the

550   agent of Lyme disease in hosts that permit fulminating infection. Infect. Immun. **61**:2396-

551   2399.

552   **29.   Wormser GP.** 2006. Hematogenous dissemination in early Lyme disease. Wien. Klin.

553   Wochenschr. **118**:634-637.

554   **30.   Barthold SW, Persing DH, Armstrong AL, Peeples RA.** 1991. Kinetics of *Borrelia*

555   *burgdorferi* dissemination and evolution of disease after intradermal inoculation of

556   mice. Am. J. Pathol. **139**:263-273.

557   **31.   Norman UM, Moriarty TJ, Dresser AR, Millen B, Kubes P, Chaconas G.** 2008.

558   Molecular mechanisms involved in vascular interactions of the Lyme disease pathogen in

559   a living host. PLoS Pathog. **4**:e1000169. doi:10.1371/journal.ppat.1000169.

560   **32.   Straubinger RK, Straubinger AF, Härter L, Jacobson RH, Chang Y, Summers BA,**

561   **Erb HN, Appel MJG.** 1997. *Borrelia burgdorferi* migrates into joint capsules and

562   causes up-regulation of interleukin-8 in synovial membranes of dogs experimentally

563   infected with ticks. Infect. Immun. **65**:1273-1285.

564   **33.   Casjens S, Palmer N, van Vugt R, Huang WM, Stevenson B, Rosa P,**

565   **Lathigra R, Sutton G, Peterson J, Dodson RJ, Haft D, Hickey E, Gwinn M, White**

566   **O, Fraser CM.** 2000. A bacterial genome in flux: the twelve linear and nine circular

567   extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia*

568   *burgdorferi.* Mol. Microbiol. **35**:490-516.

569   **34.   Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O,**

570   **Ketchum KA, Dodson R, Hickey EK, Gwinn M, Dougherty B, Tomb J, Fleischmann**

571   **RD, Richardson D, Peterson J, Kerlavage AR, Quakenbush J, Salzberg S, Hanson**

572   **M, van Vugt R, Palmer N, Adams MD, Gocayne J, Weidman J, Utterback T,**

573   **Watthey L, McDonald L, Artiach P, Bowman C, Garland S, Fujii C, Cotton MD,**

574   **Horst K, Roberts K, Hatch B, Smith HO, Venter JC.** 1997. Genomic sequence of a

575   Lyme disease spirochaete, *Borrelia burgdorferi*. Nature. **390**:580-586.

576 **35.**   **Elias AF, Stewart PE, Grimm D, Caimano MJ, Eggers CH, Tilly K, Bono JL, Akins**

577   **DR, Radolf JD, Schwan TG, Rosa P.** 2002. Clonal polymorphism of *Borrelia*

578   *burgdorferi* strain B31 MI: implications for mutagenesis in an infectious strain

579   background. Infect. Immun. **70:**2139-2150.

580 **36.**   **Frank KL, Bundle SF, Kresge ME, Eggers CH, Samuels DS.** 2003. *aadA* confers

581   streptomycin-resistance in *Borrelia burgdorferi*. J. Bacteriol. **185:**6723-6727.

582 **37.**   **Samuels DS.** 1995. Electrotransformation of the spirochete *Borrelia burgdorferi*, p. 253-

583   259. *In* Nickoloff JA (ed), Electroporation Protocols for Microorganisms, vol. 47.

584   Humana Press, Totowa, New Jersey.

585 **38.**   **Barbour AG.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol.

586   Med. **57**:521-525.

587 **39.**   **Casjens SR, Mongodin EF, Qui WG, Luft BJ, Schutzer SE, Gilcrease EB, Huang**

588   **WM, Vujadinovic M, Aron JK, Vargas LC, Freeman S, Radune D, Weidman JF,**

589   **Dimitrov GI, Khouri HM, Sosa JE, Halpin RA, Dunn JJ, Fraser CM.** 2012. Genome

590   stability of Lyme disease spirochetes: comparative genomics of *Borrelia burgdorferi*

591   plasmids. PLoS ONE. **7**:e33280. doi:10.1371/journal.pone.0033280.

592 **40.**   **Armstrong AL, Barthold SW, Persing DH, Beck DS.** 1992. Carditis in Lyme disease

593   susceptible and resistant strains of laboratory mice infected with *Borrelia burgdorferi*.

594   Am. J. Trop. Med. Hyg. **47**:249-258.

595 **41.**   **Barthold SW, Hodzic E, Tunev S, Feng S.** 2006 Antibody-mediated disease remission

596   in the mouse model of Lyme borreliosis. Infect. Immun. **74**:4817-4825.

597 **42. Barthold SW.** 1991. Infectivity of *Borrelia burgdorferi* relative to route of inoculation

598   and genotype in laboratory mice. J Infect. Dis. **163**:419-420.

599 **43. Barthold SW, de Souza MS, Janotka JL, Smith AL, Persing DH.** 1993. Chronic

600   Lyme borreliosis in the laboratory mouse. Am. J. Pathol. **143**:959-971.

601 **44. Cabello FC, Godfrey HP, Newman SA.** Hidden in plain sight: *Borrelia burgdorferi* and

602   the extracellular matrix. Trends Microbiol. **15**:350-354.

603 **45. Parveen N, Cornell KA, Bono JL, Chamberland C, Rosa P, Leong JM.** 2006. Bgp, a

604   secreted glycosaminoglycan-binding protein of *Borrelia burgdorferi* strain N40, displays

605   nucleosidase activity and is not essential for infection of immunodeficient mice. Infect.

606   Immun. **74**: 3016-3020.

607 **46. Li X, Liu X, Beck DS, Kantor FS, Fikrig E.** 2006. *Borrelia burgdorferi* lacking

608   BBK32, a fibronectin-binding protein, retains full pathogenicity. Infect. Immun. **74**:3305-

609   3313.

610 **47. Seshu J, Esteve-Gassent MD, Labandeira-Rey M, Kim JH, Trzeciakowski JP, Höök**

611   **M, Skare JT.** 2006. Inactivation of the fibronectin-binding adhesion gene *bbk32*

612   significantly attenuates the infectivity potential of *Borrelia burgdorferi*. Mol. Microbiol.

613   **59**:1591-1601.

614 **48. Coburn J, Cugini C.** 2003. Targeted mutation of the outer membrane protein P66

615   disrupts attachment of the Lyme disease agent, *Borrelia burgdorferi*, to integrin $\alpha_v\beta_3$.

616   Proc. Natl. Acad. Sci. **100**:7301-7306. doi:10.1073/pnas.1131117100.

617 **49. Ristow LC, Miller HE, Padmore LJ, Chettri R, Salzman N, Caimano MJ, Rosa PA,**

618   **Coburn J.** 2012. The β_3-integrin ligand of *Borrelia burgdorferi* is critical for infection of

619   mice but not ticks. Mol. Microbiol. **85**:1105-1118.

27

620 **50. Hastey CJ, Elsner RA, Barthold SW, Baumgarth N.** 2012. Delays and diversions

621   mark the development of B cell responses to *Borrelia burgdorferi* infection. J. Immunol.

622   **188**:5612-5622.

623 **51. Barthold SW, deSouza M, Feng S.** 1996. Serum-mediated resolution of Lyme arthritis

624   in mice. Lab. Invest. **74**:57-67.

625 **52. McKisic MD, Redmond WL, Barthold SW.** 2000. T cell-mediated pathology in murine

626   Lyme borreliosis. J. Immunol. **164**:6096-6099.

627 **53. Schaible UE, Wallich R, Kramer MD, Nerz G, Stehle T, Museteanu C, Simon MM.**

628   1994. Protection against *Borrelia burgdorferi* infection in SCID mice is conferred by

629   presensitized spleen cells and partially by B- but not T cells alone. Intern. Immun. **6**:671-

630   681.

631 **54. Murphy K, Travers P, Walport M.** 2008. The humoral immune response. P. 400-401.

632   *In* Janeway's Immunobiology, 7th ed. Garland Science, New York, NY.

633 **55. Liang FT, Brown EL, Wang T, Iozzo RV, Fikrig E.** 2004. Protective niche for

634   *Borrelia burgdorferi* to evade humoral immunity. Am. J. Pathol. **165**:977-985.

635 **56. Strother KO, Hodzic E, Barthold SW, de Silva AM.** 2007. Infection of mice with

636   Lyme disease spirochetes constituitively producing outer surface protein A and B. Infect.

637   Immun. **75**:2786-94.

638 **57. Hawley K, Navasa N, Olson CM Jr, Bates TC, Garg R, Hedrick MN, Conze D,**

639   **Rincon M, Anguita J.** 2012. Macrophage p38 mitogen-activated protein kinase activity

640   regulates invariant natural killer T-cell responses during *Borrelia burgdorferi* infection. J.

641   Infect. Dis. **206**:283-91.

642 **58. Lee WY, Moriarty TJ, Wong CH, Zhou H, Strieter RM, van Rooijen N, Chaconas**

643   **G, Kubes P.** 2010. An intravascular immune response to *Borrelia burgdorferi* involves

644   Kupffer cells and iNKT cells. Nat. Immunol. **11**:295-302.

645   **59.**   **Xu Q, McShan K, Liang FT.** 2008. Essential protective role attributed to the surface

646   lipoproteins of *Borrelia burgdorferi* against innate defences. Mol. Microbiol. **69**:15-29.

647 **Table 1.** The inflammation associated with B31-Δ*dbpBA B. burgdorferi* infection is not significantly different from inflammation

648 associated with wild-type *B. burgdorferi* infection after day 28 post-inoculation, in either immunodeficient or immunocompetent mice.

649 More severe inflammation does not absolutely correspond with a significantly greater spirochete tissue burden.

Mouse strain	Isolate	Day	Tibiotarsus			Heartbase		
			No. spirochetes[a]	Prevalence[b]	Arthritis severity[c]	No. spirochetes	Prevalence	Carditis severity
T/B cell-deficient	Δ*dbpBA*	28	2.39E+04	8/9	$0.8 \pm 0.2^{d,e}$	4.10E+04	6/9	0.4 ± 0.1
		42	ND	ND	ND	ND	ND	ND
		60	5.55E+03	4/4	2.8 ± 0.3^{f}	9.90E+05	4/4	1.0^{g}
		90	3.19E+04	4/4	3.0^{h}	1.37E+06	4/4	1.0^{i}
	wild type	28	3.81E+04	8/8	2.9 ± 0.1^{d}	3.61E+04	8/8	0.8 ± 0.1
		42	ND	ND	ND	ND	ND	ND
		60	5.68E+04	4/4	3.0	2.06E+06	4/4	1.0
		90	3.00E+01	4/4	3.0	7.10E+06	4/4	1.0
Immuno-competent	Δ*dbpBA*	28	5.69E+02	0/4	0.0^{e}	2.28E+03	0/4	0.0^{j}
		42	6.17E+04	2/4	0.4 ± 0.2	1.79E+03	1/4	0.1 ± 0.1
		60	ND	3/4	0.4 ± 0.1^{f}	9.51E+02	1/4	0.1 ± 0.1^{g}
		90	1.45E+02	3/4	0.8 ± 0.3^{h}	4.33E+03	1/4	0.1 ± 0.1^{i}
	wild type	28	2.59E+04	1/5	0.2 ± 0.2	3.92E+04	5/5	1.0^{j}

30

42	1.08E+05	4/5	0.9 ± 0.3	6.43E+03	3/4	0.6 ± 0.2
60	ND	4/4	1.1 ± 0.3	2.27E+03	1/4	0.1 ± 0.1
90	3.45E+02	4/4	0.8 ± 0.1	2.30E+03	1/4	0.1 ± 0.1

650

651　　[a] No. of spirochetes in respective tissues represented as mean copy no. *flaB* per mg tissue.

652　　[b] No. of mice/Total no. of mice.

653　　[c] Mean severity + SEM

654　　[d,h] Differences in arthritis severity are statistically significantly different (all *P* values < 0.05) but differences in spirochete tissue

655　　burdens are not statistically significant.

656　　[e] Arthritis severity is significantly different ($P < 0.05$) and corresponds with significantly greater tissue spirochete burden ($P = 0.007$).

657　　[f] Arthritis severity is significantly different ($P < 0.05$).

658　　[h] Carditis severity is significantly different ($P < 0.05$) and corresponds with significantly greater tissue spirochete burden ($P = 0.0005$).

659　　[i] Carditis severity is significantly different ($P < 0.05$) and corresponds with significantly greater tissue spirochete burden ($P = 0.002$).

660　　[j] Carditis severity is significantly different ($P < 0.05$) and corresponds with significantly greater tissue spirochete burden ($P = 0.003$).

31

661 **Table 2:** Viable, cultivable spirochetes lacking *dbpBA* are recovered from tissue in increasing

662 frequency over time in immunocompetent C3H mice.

Isolate	Day	No. positive cultures / total no.		No. positive mice/total no.
		Sub-inoc site	Bladder	
Δ*dbpBA*	14	2/5	0/5	2/5
	28	4/5	0/5	4/5
	42	4/5	4/5*	4/5
wild type	14	5/5	2/4	5/5
	28	5/5	5/5	5/5
	42	5/5	1/5	5/5

663 * In 3 of the 4 positive cultures, spirochetes were observed only rarely.

664

665 **Table 3:** Dbp-deficiency prevents the recovery of spirochetes from the lymphatic system in the

666 early stage of infection in immunocompetent laboratory mice. Complementation of *dbpBA*

667 recovers the wild-type phenotype.

Isolate	Day	*flaB* PCR (culture)*				
		Popliteal^	Inguinal	Lumbar	Axillary	ExtraLN†
wild type	0.5	2/4	3/4 (0/4)	4/4	1/4	2/12 (0/4)
	3	1/3	0/3 (0/3)	0/3	0/3	2/9 (0/4)
	7[a,x]	4/4	4/4 (4/4)	4/4	4/4	12/12 (4/4)
	14[b,y]	4/4	4/4 (4/4)	4/4	4/4	12/12 (4/4)
Δ*dbpBA*	0.5	4/4	2/4 (0/4)	1/4	1/4	6/12 (0/4)
	3	0/3	0/3 (0/3)	0/3	0/3	4/9 (0/4)
	7[a,x]	2/4	1/4 (0/4)	0/4	2/4	3/12 (0/4)
	14[b,c,y]	1/3	0/3 (0/3)	0/3	0/3	4/9 (0/4)
dbpBA+	0.5	1/4	2/4 (0/4)	1/4	0/4	4/12 (0/4)
	3	0/2	0/2 (0/2)	0/2	0/2	2/6 (0/4)
	7	1/1	1/1 (0/1)	1/1	1/1	2/3 (0/4)
	14[c]	3/4	3/4 (3/4)	3/4	3/4	9/12 (1/4)

668 * No. pos/total

669 ^ Includes both right and left-sided nodes.

32

670 † Extralymphatic tissues collected for PCR included skin, heart base, and tibiotarsus. ExtraLN

671 tissues collected for culture included spleen and urinary bladder.

672 [a,b] Prevalence of *flaB* DNA in lymph nodes from Δ*dbpBA* infected mice is significantly lower (P

673 < 0.0001 by Fisher's exact test) than in wild type infected mice.

674 [c] Prevalence of *flaB* DNA in lymph nodes from Δ*dbpBA* infected mice is significantly lower ($P <$

675 0.0001) than in *dbpBA+* (complemented mutant) infected mice.

676 [x] The number of PCR-positive extralymphatic tissues from Δ*dbpBA* infected mice are

677 significantly fewer ($P < 0.0001$) than in wild type infected mice.

678 y The number of PCR-positive extralymphatic tissues from Δ*dbpBA* infected mice are

679 significantly fewer ($P = 0.0062$) than in wild type infected mice.

680

681 **Table 4:** Dbp-deficiency decreases but does not prevent spirochetes from utilizing the lymphatic

682 system in the early stage of infection in immunodeficient laboratory mice.

Isolate	Day	*flaB* PCR (culture)				
		Popliteal^	Inguinal	Lumbar	Axillary	ExtraLN†
wild type	0.5	0/4	0/4 (0/4)	0/4	1/4	5/12 (0/4)
	3	2/4	1/4 (1/4)	0/4	1/4	5/12 (0/4)
	7[a,x]	4/4	4/4 (4/4)	4/4	4/4	12/12 (3/4)
	14[c]	4/4	4/4 (4/4)	4/4	4/4	12/12 (4/4)
Δ*dbpBA*	0.5	4/4	0/4 (4/4)	1/4	4/4	7/12 (0/4)
	3	na	na	na	na	na
	7[a,b,x]	0/4	0/4 (0/4)	2/4	0/4	5/12 (0/4)
	14[c]	3/3	3/3 (3/3)	3/3	3/3	9/9 (3/3)
dbpBA+	0.5	1/4	0/4 (0/4)	0/4	0/4	4/12 (0/4)
	3	3/4	1/4 (0/4)	0/4	0/4	3/12 (0/4)
	7[b,x]	4/4	3/4 (4/4)	4/4	4/4	12/12 (0/4)
	14[c]	4/4	4/4 (4/4)	4/4	4/4	12/12 (4/4)

683 * No. pos/Total

33

684   ^ Includes both right and left-sided nodes.

685   † Extralymphatic tissues collected for PCR included skin, heart base, and tibiotarsus. ExtraLN

686   tissues collected for culture included spleen and urinary bladder.

687   [a] Prevalence of *flaB* DNA in lymph nodes from Δ*dbpBA* infected mice is significantly lower ($P <$

688   0.0001 by Fisher's exact test) than in wild type infected mice.

689   [b] Prevalence of *flaB* DNA in lymph nodes from Δ*dbpBA* infected mice is significantly lower ($P <$

690   0.0001 by Fisher's exact test) than in *dbpBA+* infected mice.

691   [c] All lymph nodes from wild type, Δ*dbpBA*, and *dbpBA+*(complemented mutant) infected mice

692   are positive for *flaB* DNA and therefore, could not be analyzed by Fisher's exact test.

693   [x] The number of PCR-positive extralymphatic tissues from Δ*dbpBA* infected mice is significantly

694   fewer (all $P = 0.0046$) than in wild type and *dbpBA+* infected mice.

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707 **FIGURE LEGENDS.**

708

709 **FIG. 1.** DbpA/B are not essential for dissemination, colonization or persistence in

710 immunodeficient mice. *B. burgdorferi flaB* DNA per mg tissue weight (mean $\pm$ SEM) in sub-

711 inoculation site (A), heart base (B), ventricle (C), tibiotarsus (D) and quadriceps muscle (E) from

712 C3H-*scid* mice inoculated with B31-Δ*dbpBA* (white bars) compared to wild-type B31-A3 (black

713 bars) at 14 days, 60 days and 90 days post-inoculation. No significant differences observed.

714

715 **FIG. 2.** Early defects in dissemination and colonization, attributed to the disruption of DbpA/B,

716 are not observed in the chronic stages of Lyme borreliosis in immunocompetent mice. *B.*

717 *burgdorferi flaB* DNA per mg tissue weight (mean $\pm$ SEM) in tissues from C3H mice inoculated

718 with B31-Δ*dbpBA* (white bars) compared to wild-type B31-A3 (black bars) at 14 days, 28 days,

719 and 42 days post-inoculation (*, all $P \leq 0.034$).

720

721 **FIG. 3.** Complementation of the *dbpBA*-deficient mutant restores a wild-type phenotype. *B.*

722 *burgdorferi flaB* DNA per mg tissue weight (mean $\pm$ SEM) in tissues from C3H mice inoculated

723 with B31-Δ*dbpBA* (white bars) compared to the complemented mutant B31-d*bpBA*+ (gray bars)

724 and wild-type B31-A3 (black bars) (*, $P \leq 0.03$).

725

726 **FIG. 4.** The early dissemination defect is dependent on an acquired immune response. *B.*

727 *burgdorferi flaB* DNA per mg tissue weight (mean $\pm$ SEM) in heart base (A) and tibiotarsus (B)

728 from C3H-*scid* mice and heart base (C) and tibiotarsus (D) from C3H mice at days 14, 28, 42,

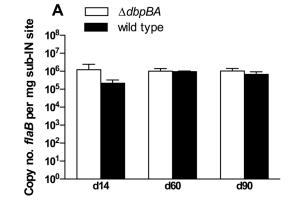729 60, and 90 post-inoculation. Mice were inoculated with B31-Δ*dbpBA* (white circles) or wild-type

35

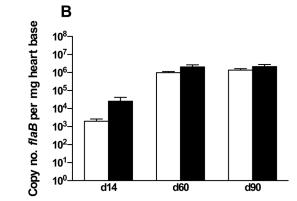730   B31-A3 (black circles). Each data point represents 4 to 9 mice from 2 separate experiments (*, P
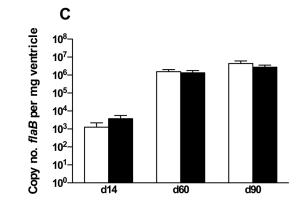
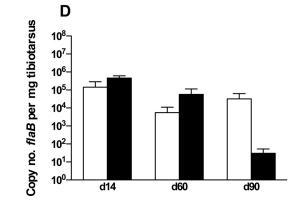731 $\leq$ 0.035).

732

733   **FIG. 5.** *Borrelia burgdorferi*-specific antibody titers steadily rise over time, regardless of

734   borrelial genotype, but remain significantly greater in mice inoculated with wild-type spirochetes

735   compared to mice inoculated with DbpA/B-deficient spirochetes. Mice were inoculated with

736   B31-Δ*dbpBA* (white circles) or wild-type B31-A3 (black circles). Each data point represent mean

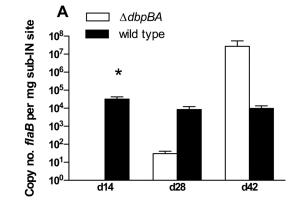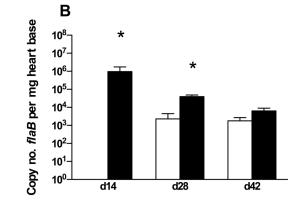737   reciprocal dilutions $\pm$ SEM of 4 to 5 mice from 2 separate experiments (*, P = 0.006, P = 0.05, P
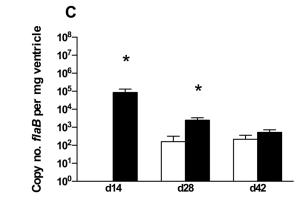
738   < 0.001, respectively).

739

B

C

D

Y-axis: **Copy no. *flaB* per mg tibiotarsus**, with values 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8

X-axis: d14, d60, d90

E

Copy no. *flaB* per mg quadriceps

10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0

d14   d60   d90

B

C

D

E

Y-axis: Copy no. *flaB* per mg quadriceps

10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0

*

d14   d28   d42

A

Copy no. flaB per mg heartbase

10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0

○— $\Delta dbpBA$
●— wild type

d14   d28   d60   d90

T/B cell-deficient

B

T/B cell-deficient

C

Y-axis: Copy no. *flaB* per mg heart base

Y-axis values: 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7

X-axis: d14, d28, d42, d60, d90

Immunocompetent

D

10^7
10^6
10^5
10^4
10^3
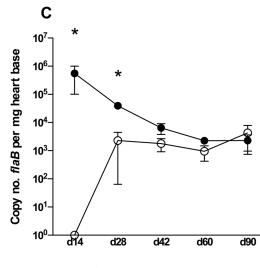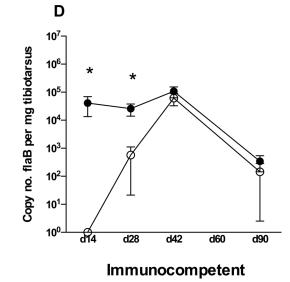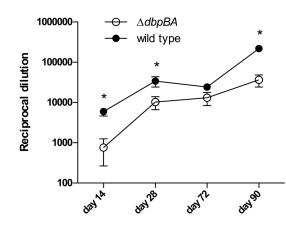10^2
10^1
10^0

Copy no. flaB per mg tibiotarsus

d14   d28   d42   d60   d90

Immunocompetent

Anti-*B. burgdorferi* serum titer