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Borrelia burgdorferi infection in biglycan knock-out mice

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Short summary

Mice lacking biglycan were infected with bacteria representing the three main genospecies of *Borrelia burgdorferi* sl. Biglycan, an adhesion receptor for the bacteria and a pro-inflammatory signalling molecule, contributes differently to the course of infection depending on the *Borrelia* genospecies.

Foot note page

A conflict of interest statement

The authors have no commercial or other association that might pose a conflict of interest.

A funding statement

The study was financially supported by the Academy of Finland, Jane and Aatos Erkko Foundation, the Special Governmental Fund for University Hospitals (EVO), and by a grant from Turku University Hospital.

Mention of any meeting(s) where the information has previously been presented

Turku Tick Meeting (10/ 2017) in Turku, Finland. Abstract #19.

Gordon Research Seminar & Conference “Biology of Spirochetes 2018” (01/2018) in Ventura,CA, USA. Poster numbers #12 and #27.

NordTick 2018 (04/2018) in Mariehamn, Åland Islands, Finland. Poster number #2.

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Word counts: 196 (abstract) and 3398 (text)

Abstract

Background. *Borrelia burgdorferi* sensu lato spirochetes (*Borrelia*) causing Lyme borreliosis are able to disseminate from the initial entry site to distant organs in the host. Outer surface adhesins are crucial in the bacterial dissemination and adhesion to various tissues. Two well-characterized *Borrelia* adhesins, Decorin binding proteins A and B, have been shown to bind to two host receptors, decorin and biglycan. However, the role of biglycan in *Borrelia* infection has not been characterized in vivo.

Methods. We infected biglycan knock-out (KO) and wildtype (WT) C3H mice with strains representing three *Borrelia* genospecies, *Borrelia burgdorferi* sensu stricto, *Borrelia garinii,* and *Borrelia afzelii*. The infection was monitored by joint swelling measurements, *Borrelia* culture, PCR and serology. The host immune responses were analysed by histological scoring of the inflammation in tissues and by cytokine profiling.

Results. *B. burgdorferi* s.s. and *B. garinii* established long-term infection in both mouse genotypes, while *B. afzelii* failed to disseminate in the KO mice. Further, the *B. burgdorferi* s.s. infected KO mice had persistent inflammation in the joints.

Conclusions. The dissemination and tissue colonization of *Borrelia*, and the inflammatory response of the host differ in a mouse biglycan expression and *Borrelia* genospecies dependent manner.

Key words. Biglycan, Biglycan knock-out mice, Mouse model, *Borrelia burgdorferi* sensu lato, Infection, Adhesion, Immunomodulation

BACKGROUND

Lyme borreliosis (LB) is a tick-borne bacterial infectious disease occurring in Europe, Asia and in North America. The heterogeneous group of *Borrelia burgdorferi* sensu lato (hereafter *Borrelia*) is comprised of about twenty genetically different genospecies, where *Borrelia burgdorferi* sensu stricto (Bbss), *Borrelia afzelii* (Ba) and *Borrelia garinii* (Bg) are the predominant human pathogens causing LB [1, 2]. The clinical manifestations of LB can be observed in the skin, joints, heart and in the peripheral and central nervous system [3]. *Borrelia* is transmitted to humans by infected *Ixodes* ticks [3]. From the initial tick bite site to distant organs, *Borrelia* is thought to disseminate via the cardiovascular, and perhaps also via the lymphatic, system [4, 5]. *Borrelia* has been demonstrated to interact with the endothelium under blood flow by tethering and dragging interactions. These interactions are mediated especially by BBK32 which adheres to fibronectin, an extracellular matrix (ECM) protein, and to glycosaminoglycans (GAG) expressed on the endothelium [6, 7].

In addition to BBK32, *Borrelia* has about 20 described adhesion proteins expressed on its outer surface [8]. Two well-characterized adhesins are the decorin binding proteins (Dbp) A and B mediating adhesion to decorin and biglycan [9-11]. Decorin is an ECM proteoglycan with a single GAG side chain consisting of either dermatan or chondroitin sulfate. Decorin belongs to class I family of small leucine-rich proteoglycans (SLRP) that is expressed in the matrix of collagen-rich connective tissues such as in joints, tendons, skin, and in the connective sheaths of muscles and peripheral nerves [12]. Biglycan is a proteoglycan with two GAG side chains attached to the core protein, and with about 57 % protein sequence homology to decorin [13]. Like decorin, biglycan is expressed ubiquitously in connective tissues. However, biglycan is localized to specialized cell types of connective tissue such as to the

keratinocytes, epidermis of the skin, skeletal myofibers, epithelium in renal tubulars, and to the endothelium of peripheral nerves [12]. Furthermore, biglycan is expressed on endothelial cells lining the blood vessels [11, 14-17]. In addition to providing structural support in the ECM, decorin and biglycan have been described as inflammatory molecules regulating the innate immune response [18-20]. Both proteoglycans can act as danger-associated molecular patterns (DAMPs) on macrophages promoting the release of cytokines and chemokines.

We have characterized the binding properties of DbpAB of three *Borrelia* genospecies (Bbss, Bg and Ba) to biglycan in vitro [11]. Our results showed that the binding activities of DbpA and B to biglycan varied among the genospecies. The DbpA and DbpB of Bg and the DbpB of Bbss were the strongest binders of biglycan under static conditions. Interestingly, the DbpA of Bg bound to biglycan only under flow mimicking the physiological conditions of the shear stress caused by blood. The DbpAB of Ba did not bind to biglycan under static or flow conditions. Based on the fact that DbpAB of *Borrelia* adhere to biglycan expressed in the blood vessel walls, and the pro-inflammatory role of biglycan, we hypothesized that biglycan may have a role in the adhesion of *Borrelia* to endothelial cells, and in the overall host-pathogen interaction during *Borrelia* infection in vivo.

In the present study, we investigated the role of *Borrelia*-biglycan interaction in vivo by infecting biglycan knock-out and wildtype control mice with Bbss, Bg and Ba. We show that the dissemination and tissue colonization of *Borrelia*, and the inflammatory response of the host differ in a mouse biglycan expression and *Borrelia* genospecies dependent manner.

METHODS

Mouse and *Borrelia* strains

Biglycan (*bgn*) knock-out (KO) mice in the C57BL/6 background [21] were backcrossed for seven generations into the wildtype (WT) C3H/HeNHsd background (Envigo, The Netherlands). The genotype of the KO and WT mice was verified with PCR using primers listed in Supplementary Table 1 and biglycan expression was analysed as described in Supplementary Data. Four weeks old female and male KO and WT mice were used for the infection studies. The wildtype *Borrelia* strains Bbss N40, Bg SBK40 and Ba A91 and the culture conditions have been previously described [11, 22].

Experimental infection studies

All experimental infection studies (Figure 1) were approved by the National Animal Experiment Board in Finland (permission ESAVI/6265/04.10.07/2017) and performed in accordance with relevant guidelines and regulations. KO (n=7-8/ experiment) and WT mice (n=7-8/ experiment) were intradermally inoculated with 10^5 Bbss, Bg or Ba. The infections were followed for 28 (experiment I) or 56 days (experiments II to IV). The body weight of all mice was measured before infection and at the end of the study. The development of joint swelling was monitored in a blindfolded manner once a week. Ear biopsy samples were collected at several time-points depending on the experiment. After sacrifice, ear, tibiotarsal joint, heart, bladder and the whole blood were collected.

DNA extraction and qPCR analysis of bacterial load tissue samples

Total DNA of mouse tissues were extracted with High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). The bacterial load in tissues was

analyzed by quantitative PCR as described previously [22, 23]. Data is expressed as bacterial genomes per 100 ng of extracted mouse DNA.

Borrelia culture of tissue samples

All mouse tissue samples were cultured as described previously [22].

Serology

Immunoglobulin G (IgG) antibodies towards *Borrelia* whole cell sonicate (WCS) antigen and towards DbpA and DbpB recombinant proteins were measured with in-house enzyme immunoassays as described previously [22]. Briefly, wells were coated with WCS of Bbss B31 ATCC 35210 (20 µg/ml) or purified recombinant DbpA and DbpB proteins of Bbss, Bg and Ba strain (10 µg/ml). After sample incubation, bound IgG was detected by horseradish peroxidase conjugated IgG antibody (1:8000, Santa Cruz Biotechnology, Santa Cruz, USA). Plasma samples (1:100) were analyzed in duplicates or quadruplicates

Histology and immunohistochemical staining

Findings of inflammation in joints (all experiments) and in hearts (experiments I and II) were evaluated in sagittal tibiotarsal joint and heart sections by an experienced pathologist (MS) blinded to the experimental protocol. The inflammation of the joint was graded from 0 (no inflammation) to 6 (severe inflammation) by scoring the synovial proliferation, and active and chronic inflammation. Myocardial inflammation and fibrosis were graded from 0 (no inflammation or fibrosis) to 8 (severe inflammation with fibrosis). Details of histological and immunohistochemical staining are described in the Supplementary Data.

Statistical analyses

Details of the statistical analyses are described in the Supplementary Data.

RESULTS

Phenotype characterization of biglycan knock-out and wildtype mice

The generation of the KO mice in the *Borrelia* arthritis susceptible C3H background was successful as verified by genotyping, RT-qPCR analysis and immunohistochemistry. The KO mice weighted significantly less than the WT mice at the time of infection at four weeks of age (Figure 2A). There was a statistically significant difference in the biglycan mRNA expression in lungs between the mouse genotypes (P=0.034) (Figure 2B). Biglycan expression was detected in the blood vessel walls in lung tissue of WT mice but not in KO mice by immunohistochemistry (Figure 2C-D).

Bbss is infectious in KO and WT mice

In experiment I, at 7 days post-infection, all Bbss infected KO and WT mice were negative by culture and PCR (Table 1). At day 10, one out of seven KO mice was positive by PCR and two out of eight WT mice were positive by culture. At day 14, five out of seven KO mice and six out of eight WT mice were positive by culture and/or PCR. At 21 and 28 days (experiment I), and at 42 and 56 days (experiment II) post-infection all Bbss infected KO and WT mice were *Borrelia* positive.

The *Borrelia* load in the ear biopsy samples started to rise at 10 and 14 days post-infection in both mouse genotypes (Figure 3A-B). The load peaked at 21 days, after which the load was again lower at days 28, 42 and 56 days post-infection. At 28 days, the highest *Borrelia* load was in the tibiotarsal joints in both mouse genotypes, although all studied tissues were *Borrelia* culture positive. At 56 days post-infection, the *Borrelia* load was statistically significantly higher in the joints of the KO mice

compared to WT mice (*P*=0.037) suggesting that the infection had started to resolve in the WT mice.

In experiments I and II, there were no statistically significant differences in the *Borrelia* load in the ear biopsy samples at 7, 10, 14, 21 and 42 days post-infection between the mouse genotypes (*P*= not determined; *P*=0.285; *P*=0.180; *P*=0.729; *P*=0.729, respectively; Figure 3A-B). At 28 days post-infection, there was a small but statistically significantly higher *Borrelia* load in the bladder of the WT mice compared to KO mice (*P*=0.037). There were no statistically significant differences in the bacterial load in the ear (*P*=0.817), tibiotarsal joint (*P*=0.203) and heart (*P*=0.298) at 28 days, or in the ear (*P*=0.298), heart (*P*=0.418) and bladder (*P*=0.488) at 56 days post-infection between the mouse genotypes.

Bg is also infectious in KO and WT mice

In experiment III, all Bg infected KO and WT mice were negative by culture and PCR at 7, 10 and 14 days post-infection (Table 1), while at 28 and 56 days post-infection, seven out of eight KO mice and all WT mice were positive by culture and/or PCR.

There were no detectable *Borrelia* in the ear biopsy samples at 7, 10 and 14 days post-infection in the KO and WT mice (Figure 3C). At day 28, there was a statistically significantly higher *Borrelia* load in the ear biopsy samples of KO mice compared to WT mice (*P*=0.021), but at day 56, the difference had levelled off. At day 56, there were no statistically significant differences in the *Borrelia* load in the ear (*P*=0.908), tibiotarsal joint (*P*=0.064), heart (*P*=0.563) and bladder (*P*=0.563) between the mouse genotypes, although in the joints of WT mice, there was a trend towards higher bacterial load compared to the joints of the KO mice.

Ba fails to disseminate in KO mice

In experiment IV, all Ba infected KO and WT mice were negative by culture and PCR at 7, 10 and 14 days post-infection (Table 1). At day 28, all KO mice were negative, whereas three out of eight WT mice were positive by culture and/or PCR (*P*=0.200; Fisher's Exact Test; Table 1). Three WT mice had detectable *Borrelia* in the ear biopsy samples, but the difference between KO and WT was not statistically significant (*P*=0.065; Figure 3D). At day 56, all Ba infected KO mice remained negative, while five out of eight WT mice were positive by culture and/or PCR (*P*=0.026; Fisher's Exact Test; Table 1). The WT mice had a statistically significantly higher *Borrelia* load in all post-mortem tissue samples than KO mice (*P*=0.0273 for all tissues). Based on these results, Ba appears to be incapable of disseminating in KO mice and is thus most likely non-infectious in these mice.

All tissue samples of all uninfected control KO and WT mice were negative by *Borrelia* culture and PCR in experiments I to IV (Table 1).

Antibody responses to *Borrelia* infection

In experiments I and II, all infected KO and WT mice had elevated IgG antibodies towards *Borrelia* WCS (*P*=0.908; *P*=0.488, respectively), towards DbpA (*P*=0.420; *P*=0.418, respectively) and DbpB (*P*=0.817; *P*=0.643, respectively) without any significant differences between the mouse genotypes (Figure 4A-C). Similarly, in experiment III, all Bg infected KO and WT mice had elevated antibody levels towards *Borrelia* WCS (*P*=0.643) and DbpB (*P*=0.203) with no significant difference between the genotypes (Figure 4A-C). However, there was a statistically significant difference in the antibody levels towards DbpA (*P*=0.028) between the Bg infected KO and WT mice. In experiment IV, in parallel with the negative infection status of the animals, the KO mice had no antibodies towards *Borrelia* WCS, recombinant DbpA or DbpB (Figure

4A-C). As expected, the Ba infected WT mice had higher antibody levels towards *Borrelia* WCS (*P*=0.036), recombinant DbpA (*P*=0.093) and towards DbpB (*P*=0.027) than the Ba challenged KO mice.

Joint swelling in *Borrelia* infected KO and WT mice

The swelling of the tibiotarsal joints was followed up throughout the experiments by measuring the medio-lateral diameter of the joints once a week (Figure 5A-D). The Bbss infected KO and WT mice developed visible joint swelling, whereas the Bg and Ba infected KO and WT mice had moderate or no joint swelling. The joint swelling difference between the KO and WT mice was calculated by subtracting the joint diameter of the control mice from the infected mice in each genotype. Only in experiment I, there were statistically significant differences in joint swelling between the Bbss infected mouse genotypes at days 14 and 28 (*P*=0.001; *P*=0.018, respectively; Figure 5A). Additional details of the results are described in the Supplementary Data.

Joint inflammation in *Borrelia* infected KO and WT mice

In experiment I, there was a severe active inflammation in the tibiotarsal joints of Bbss infected KO and WT mice with neutrophil infiltration and synovial proliferation resulting in high arthritis score at day 28 (Figure 6A, C, Supplementary Table 2). There was, however, no statistically significant difference between the genotypes (*P*=0.448). In experiment II at 56 days post-infection, the inflammation appeared to be resolving in the joints of the WT mice, since the histological arthritis score was significantly lower at this time point compared to WT mice at day 28 (*P*=0.043). Interestingly, there was a statistically significant difference between the KO and WT mice at day 56 (*P*=0.003). At day 56, the KO mice had chronic inflammation in the tibiotarsal joints with infiltration of lymphocytes, plasma cells and macrophages and synovial proliferation resulting in

the high arthritis scores (Figure 6D). In experiment III, the histological joint inflammation score did not differ statistically significantly between the Bg infected KO and WT mice at day 56 (P=0.067; Figure 6A). At day 56 in the experiment IV, the arthritis scores were at the same level in the control and in the Ba challenged KO mice (P=0.407) and in the control and Ba infected WT mice (P=0.205; Figure 6A). There was no statistically significant difference between the mouse genotypes (P=0.292).

Heart inflammation in Bbss infected KO and WT mice

In experiments I and II, the inflammation of the heart was histologically scored [24] (Figure 6B, Supplementary Table 3). At day 28, there was clear myocardial inflammation resulting in high carditis score in both KO and WT mice without any statistically significant difference between the mouse genotypes (*P*=0.596). At day 56, the carditis score was lower in both mouse genotypes with no statistically significant difference (*P*=0.147).

Immune response of KO and WT mice to *Borrelia* infection

Interestingly, upon infection with Bg in wildtype mice, biglycan expression was upregulated in the aorta (*P*=0.014) and downregulated in the skin (*P*=0.014) compared to control mice (Supplementary Figure 1). Mouse cytokine profiles were also analysed, since biglycan can stimulate especially macrophages to produce various cytokines. However, there were no statistically significant differences between the infected KO and WT mice compared to the control mice in the experiments I to IV (Supplementary Figure 2A-F).

DISCUSSION

The adhesion of *Borrelia* to the host is a complex phenomenon with numerous characterized and many to-be-investigated adhesins on the bacterial surface and

receptors in various host tissues. Also, the role of the DbpAB-adhesins is more multifaceted than assumed at the time they were originally described [25, 26]. We know today that in addition to mediating adhesion to a proteoglycan called decorin, the adhesins carry also biglycan binding activity [11]. This is understandable based on the current knowledge, that the adhesion target in decorin and biglycan is in fact similar, namely the GAG side chain (chondroitin/dermatan sulphate) of the proteoglycans [12]. Therefore, we can conclude that there are actually four different molecules participating in this interaction, two adhesins on the bacterial side, and two receptor molecules with different tissue distribution on the host side.

Previously, the role of DbpAB has been investigated mainly using DbpA and/or DbpB deficient bacteria [22, 27, 28]. The main conclusion of the studies is that both adhesins are needed for full infectivity and pathogenicity of *Borrelia* infection. Importantly, in these studies the investigators have deleted both decorin and biglycan binding activities when using DbpAB knock-out bacteria. There is, however, one study where decorin knock-out mice were used [29]. In this report, it was shown that *Borrelia* colonization of the joints, and arthritis incidence and severity were significantly decreased in the decorin knock-out mice compared to WT mice when an inoculum of 10^4 Bbss was used. However, other tissues of both mouse genotypes were similarly infected based on positive *Borrelia* culture results. On the other hand, when DbpAB knock-out Bbss bacteria were used to infect mice with the inoculum 10^4, none of the mice were infected [30]. Therefore, it seems that the deletion of DbpAB in the bacteria leads to a more dramatic decrease in the infectivity than the deletion of decorin in the mice. This implies the importance of biglycan as an adhesion target for *Borrelia*. However, the specific role of biglycan in *Borrelia* infection in vivo has not been studied before.

In this study, we investigated the *Borrelia*-biglycan interaction by infecting biglycan knock-out mice and wildtype with strains representing the three most prevalent genospecies of *Borrelia*. We have previously shown that the DbpAB adhesins of Bbss, Bg and Ba bind biglycan differently in vitro [11]. Now, our results show that the dissemination and tissue colonization of the *Borrelia* genospecies, and the inflammatory response they induce in the host, are different. Bbss and Bg established long-term infection in the KO and WT mice suggesting that *Borrelia*-biglycan interaction is not a necessity for infection caused by these genospecies. In contrast, Ba failed to disseminate in the KO mice, since no *Borrelia* or antibodies towards *Borrelia* could be detected in any of the Ba infected KO mice. However, Ba demonstrated also lower infectivity in the WT mice (5/8 *Borrelia* culture positive), suggesting that the overall virulence of the Ba strain is lower than the virulence of the Bbss and Bg strains. We know that the DbpA and B of the Ba strain used in this study display poor binding to biglycan and decorin in vitro, while the same adhesins of Bbss and Bg strains are moderate/strong binders [11, 31]. Therefore, it seems that Ba needs the binding activity of both DbpA and B, and both adhesion targets to establish the infection in vivo. However, one limitation of the study is that we collected samples for Borrelia culture and PCR only from the usually analyzed tissues (Salo et al, 2016, Yrjänäinen et al., 2010), but not from the inoculation site in the skin. Therefore, the possibility that Ba survives in the skin of KO mice but is unable to disseminate to other tissues remains to be determined. Taken together, the results suggest that when an appropriate inoculum is combined with a *Borrelia* strain displaying suitable level of virulence, then the importance of *Borrelia*-biglycan interaction becomes apparent.

Another difference was also observed in the KO versus WT mice. The Bbss infected KO mice had persistent inflammation in the joints at 56 days post-infection. At this

time-point, the bacterial load and arthritis score were statistically significantly higher in the joints of KO mice compared to the WT mice. However, the joint samples of both mouse genotypes were *Borrelia* culture positive. These results suggest that Bbss infects KO mice similarly as the WT mice, but biglycan deficiency delays the initiation of inflammation resolution causing chronic inflammation. The faster removal of bacteria in the WT mice compared to biglycan KO mice might be explained by the pro-inflammatory role of biglycan [32]. Upon infection, the soluble form of biglycan is released from the ECM, and it acts as a DAMP stimulating macrophages which in turn start producing cytokines to attract innate immunity cells to the site of inflammation. Tissue release and upregulation of biglycan de novo synthesis might result in faster initiation, and thereby in faster resolution, of inflammation, which is reflected in the lower arthritis score and bacterial load in the Bbss infected WT mice. However, no upregulation of cytokines in the systemic circulation was detected in the Ba, Bbss or Bg infected KO or WT mice. In summary, it seems that biglycan has an immunomodulatory role in the resolution of *Borrelia* infection in the WT mice.

This is the first study on *Borrelia* infection in biglycan knock-out and wildtype mice. The outcome of the infection was dependent on the *Borrelia* genospecies and mouse genotype. The Ba strain was apparently non-infectious or at the very least dissemination defective in the KO mice, while the Bbss and Bg strains were able to establish long-term infection in both mouse genotypes. In addition, there was a remarkable difference in the arthritis score between the Bbss infected KO and WT mice. Consequently, the results suggest that biglycan has the role both as a structural receptor for the DbpAB adhesins and as a pro-inflammatory molecule in the host during *Borrelia* infection.

Acknowledgements

We thank Dr. Marian Young (National Institute of Dental and Craniofacial Research, Bethesda, United States) for providing the biglycan knock-out C57BL/6 mouse strain, and the Turku Center for Disease Modeling for generating the biglycan knock-out mice in the C3H background. We thank Sinikka Collanus, Tuula Rantasalo and Meri Rouhiainen (Institute of Biomedicine, University of Turku), Erica Nyman (Turku Center of Disease Modeling, Histology unit) and the Central Animal Laboratory of University of Turku for excellent technical assistance.

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Figure legends

Figure 1. Experimental design of the infection studies. In experiment I, eight biglycan knock-out (KO) and eight wildtype (WT) mice were infected with 10^5 Bbss and followed up for 28 days. Ear biopsy samples were collected at days 7, 10, 14 and 21 post-

infection. In experiment II, eight KO and seven WT mice were infected with 10^5 Bbss and followed up for 56 days. Ear biopsy samples were collected at day 42 post-infection. In experiment III, eight KO and seven WT mice were infected with 10^5 Bg. In experiment IV, eight KO and eight WT mice were infected with 10^5 Ba. In experiments III and IV, ear biopsy samples were collected at days 7, 10, 14 and 28 post-infection, and the infections were followed up for 56 days. The control KO and WT mice were the same in experiments III and IV.

In all experiments, the control KO and WT mice (n=4-5/ experiment) were injected with 100 µl phosphate buffered saline. The body weight of all mice was measured at day 0 and at the end of the study. The joint swelling was measured once a week. The ear biopsy samples were collected for *Borrelia* culture and PCR. At the end of the experiments, mice were sacrificed. Multiple tissue samples were collected for *Borrelia* culture, PCR and histological analysis, and the blood was collected for serology and multiplex cytokine profiling.

Figure 2. Phenotype characterization of the biglycan knock-out C3H mice. Comparison of body weight of KO (n=32-44) and WT mice (n=31-42) (**A**) at the age of four weeks (4w) and twelve weeks (12w). Each symbol represents the body weight of an individual animal. Expression of biglycan mRNA (**B**) in lung tissue of uninfected KO (n=3) and WT mice (n=3) was determined by reverse transcription-qPCR and the results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The results are expressed as the fold change of biglycan relative to GAPDH. Each symbol represents an individual animal. The line indicates the median of each study group. Mann–Whitney U test was used for statistical analysis. P <0.05 are considered as statistically significant. Representative images of immunohistochemically detected biglycan expression in blood vessel walls in lung

476 tissue of Bg infected KO mice without biglycan expression (**C**) and WT mice with high
477 biglycan expression (**D**) in 400x magnification. The arrows indicate the blood vessel
478 walls.

479 **Figure 3.** *Borrelia* load in tissues of KO and WT mice of experiments I-IV (**A**-**D**).
480 Symbols on the left of the dashed vertical line indicate the results of ear biopsy
481 samples, and on the right the results of tissues collected after sacrifice. Each symbol
482 represents individual tissue samples of KO and WT mice analysed by quantitative
483 PCR. The data are expressed as the number of bacterial genome copies per 100 ng
484 of extracted DNA. The short horizontal lines indicate the median of each study group.
485 Mann–Whitney U test was used for statistical analysis. P <0.05 are considered as
486 statistically significant.

487 **Figure 4.** IgG antibody levels in plasma samples of KO and WT mice of experiments
488 I-IV towards *Borrelia* whole cell sonicate (WCS) (**A**), recombinant DbpA (**B**) and DbpB
489 (**C**). Each symbol represents the antibody levels detected in individual KO and WT
490 mice using the in-house enzyme immunoassays. The data are expressed as OD_{492}
491 values. The short horizontal lines indicate the median of each study group. Mann–
492 Whitney U test was used for statistical analysis. P <0.05 are considered as statistically
493 significant.

494 **Figure 5.** Weekly progression of the joint swelling in KO and WT mice of experiments
495 I-IV (**A**-**D**). The data are expressed as the mean lateral diameters of the hind tibiotarsal
496 joints of all mice per study group. Linear mixed models were used for statistical
497 analysis. P <0.05 are considered as statistically significant. The letters connect the
498 study groups with statistically significant differences at indicated time points: a,

499 infected KO and control KO mice; b, infected KO and WT mice; c, infected WT and
500 control WT mice.

501 **Figure 6.** The inflammation score of the tibiotarsal joints (experiments I-IV) and heart
502 (experiments I and II) of KO and WT mice. The arthritis (**A**) and carditis (**B**) severity
503 was assessed by scoring inflammation on the scale 0 (no inflammation or fibrosis) to
504 6 (severe joint inflammation) or 8 (severe heart inflammation with fibrosis). Each
505 symbol represents the inflammation score of individual KO and WT mice. The short
506 horizontal lines indicate the median of each study group. Mann–Whitney U test was
507 used for statistical analysis. P <0.05 are considered as statistically significant.

508 Representative images of joint inflammation in KO (**C, left**) and WT mice (**C, right**) of
509 experiment I show chronic and acute inflammation of synovial membrane with synovial
510 proliferation and thickening, neutrophils present in synovial fluid and periarticular soft
511 tissue swelling and inflammation (**C, insets**). Representative image of joint
512 inflammation in a KO mouse (**D, left**) of experiment II shows synovial hypertrophy,
513 chronic and acute inflammation with infiltration of neutrophils in the synovial fluid and
514 periarticular soft tissues (**D, left inset**). At the same time point, the image of the joint
515 of Bbss infected WT mouse shows only synovial proliferation and chronic inflammatory
516 reaction with lymphocytes and plasma cells (**D, right inset**). Representative images
517 are captured with 50x magnification and the insets with 400x magnification.

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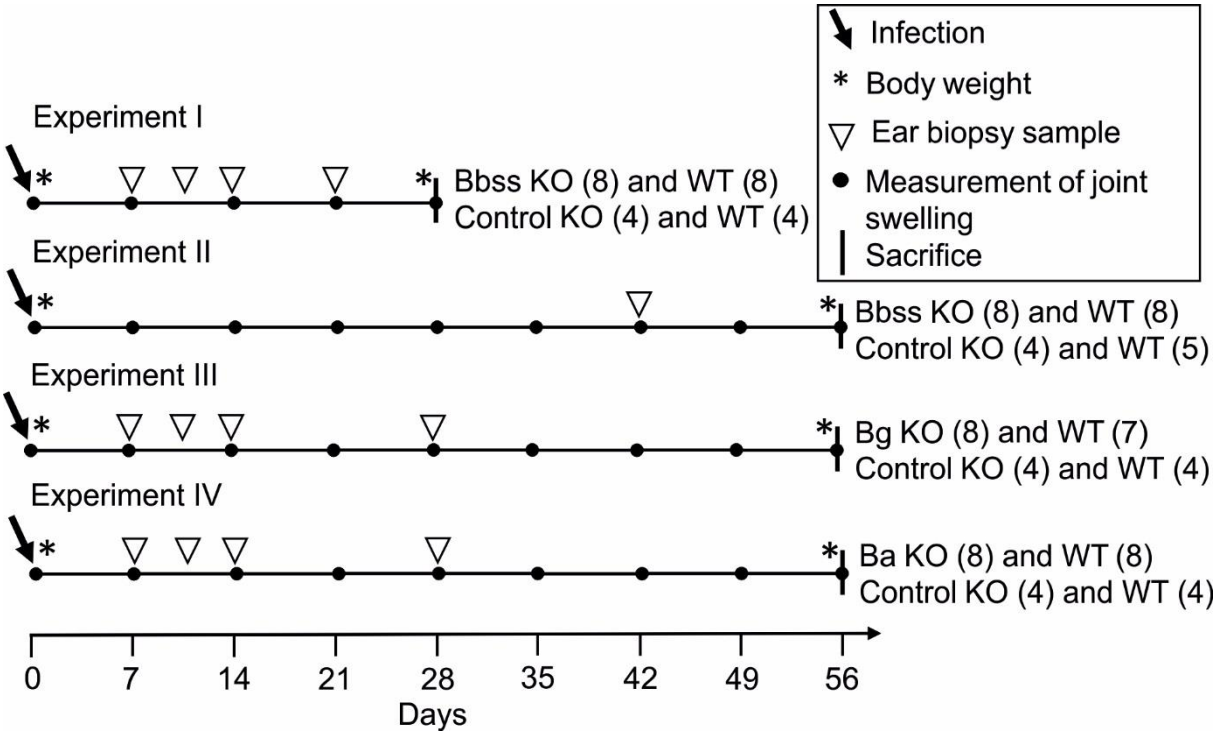

Figure 1. Experimental design of the infection studies. In experiment I, eight biglycan knock-out (KO) and eight wildtype (WT) mice were infected with 10^5 *Borrelia burgdorferi* sensu stricto (Bbss) and were followed for 28 days. Ear biopsy samples were collected at days 7, 10, 14 and 21 post-infection. In experiment II, eight KO and seven WT mice were infected with 10^5 Bbss and were followed 56 days. Ear biopsy samples were collected at day 42 post-infection. In experiment III, eight KO and seven WT mice were infected with 10^5 *Borrelia garinii* (Bg). In experiment IV, eight KO and eight WT mice were infected with 10^5 *Borrelia afzelii* (Ba). In experiments III and IV, ear biopsy samples were collected at days 7, 10, 14 and 28 post-infection, and the infections were followed 56 days. The control KO and WT mice were the same in experiments III and IV.

In all experiments, the control KO and WT mice (n=4-5/ experiment) were injected with 100 µl phosphate buffered saline. The body weight of all mice was measured at day 0 and at the end of the study. The joint swelling was measured once a week. At the end of the infection experiments, mice were sacrificed. Multiple tissue samples were collected for *Borrelia* culturing, PCR analysis and histological analysis and the whole blood was collected for serological analysis and multiplex cytokine profiling.

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541 Table 1. Number of positive *Borrelia* cultures and positive PCR results of total number

542 of tissue samples examined in each experiment.

543 d, days post-infection. [a] Tissues collected after sacrifice. [b] One animal died during the study.

Study group		**Ear biopsy samples (d)**						**At the end of the study [a]**				
		7	10	14	21	28	42	Ear	Bladder	Joint	Heart	Any tissue
Exp I Bbss KO (n=7 [b]-8)	Culture	0/8	0/7	5/7	7/7	-	-	7/7	7/7	5/7	7/7	7/7
	PCR	0/8	1/7	5/7	7/7	-	-	7/7	7/7	7/7	7/7	7/7
Bbss WT (n=8)	Culture	0/8	2/8	6/8	8/8	-	-	6/8	8/8	7/8	8/8	8/8
	PCR	0/8	0/8	3/8	7/8	-	-	7/8	8/8	8/8	8/8	8/8
Exp II Bbss KO (n=8)	Culture	-	-	-	-	-	8/8	8/8	7/8	8/8	8/8	8/8
	PCR	-	-	-	-	-	7/8	8/8	7/8	8/8	8/8	8/8
Bbss WT (n=7)	Culture	-	-	-	-	-	7/7	7/7	7/7	7/7	7/7	7/7
	PCR	-	-	-	-	-	7/7	7/7	7/7	7/7	7/7	7/7
Exp III Bg KO (n=8)	Culture	0/8	0/8	0/8	-	7/8	-	7/8	2/8	1/8	3/8	7/8
	PCR	0/8	0/8	0/8	-	7/8	-	7/8	7/8	7/8	7/8	7/8
Bg WT (n=7)	Culture	0/7	0/7	0/7	-	6/7	-	6/7	0/7	0/7	3/7	6/7
	PCR	0/7	0/7	0/7	-	7/7	-	7/7	7/7	7/7	7/7	7/7
Exp IV Ba KO (n=8)	Culture	0/8	0/8	0/8	-	0/8	-	0/8	0/8	0/8	0/8	0/8
	PCR	0/8	0/8	0/8	-	0/8	-	0/8	0/8	0/8	0/8	0/8
Ba WT (n=8)	Culture	0/8	0/8	0/8	-	3/8	-	4/8	4/8	5/8	4/8	5/8
	PCR	0/8	0/8	0/8	-	3/8	-	4/8	4/8	4/8	4/8	4/8
Exp I-IV Control KO (n=4-12)	Culture	0/8	0/8	0/8	0/4	0/4	0/4	0/12	0/12	0/12	0/12	0/12
	PCR	0/8	0/8	0/8	0/4	0/4	0/4	0/12	0/12	0/12	0/12	0/12
Control WT (n=4-13)	Culture	0/8	0/8	0/8	0/4	0/4	0/5	0/13	0/13	0/13	0/13	0/13
	PCR	0/8	0/8	0/8	0/4	0/4	0/5	0/13	0/13	0/13	0/13	0/13

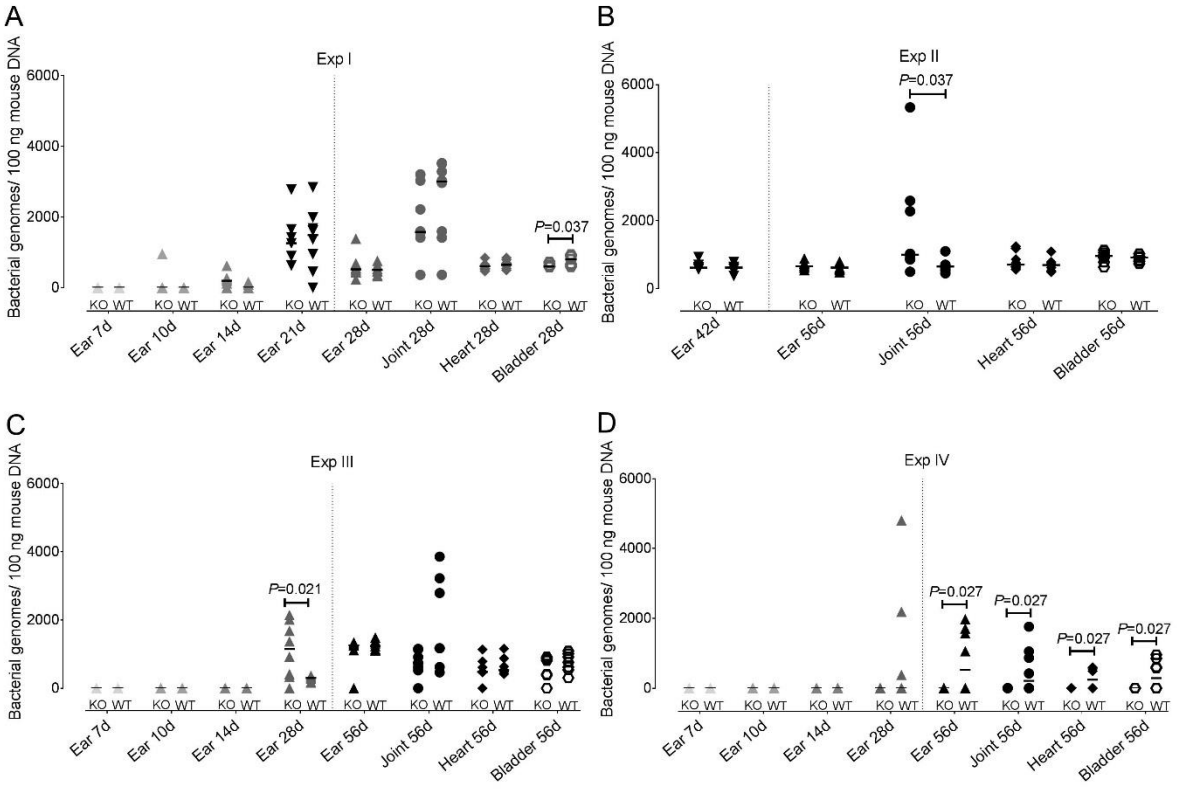
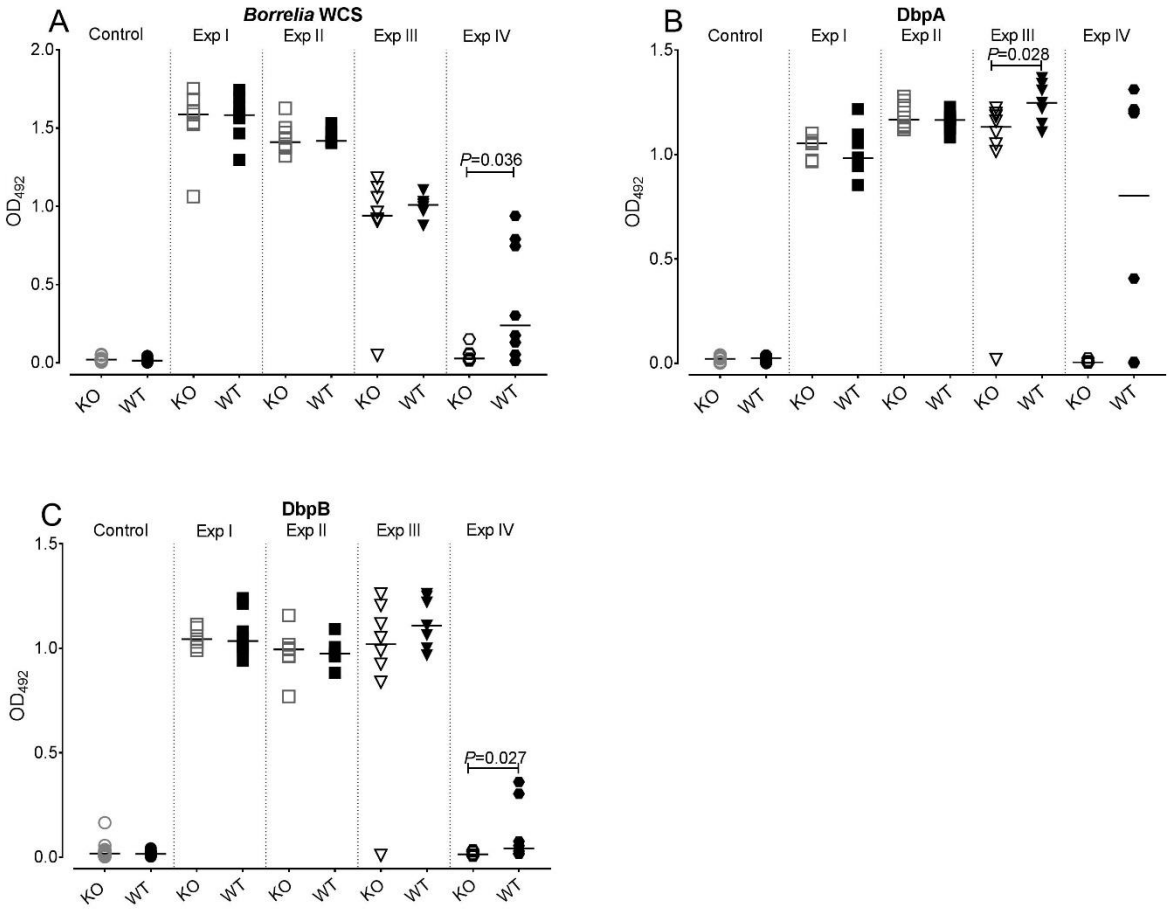


Figure 2. *Borrelia* load in tissues from biglycan knock-out (KO) and wildtype (WT) mice infected with *Borrelia burgdorferi* sensu stricto for 28 days (**A**) or 56 days (**B**), with *Borrelia garinii* (**C**) or with *Borrelia afzelii* (**D**). Data on the left of the vertical line indicate ear biopsy samples and on the right side indicate tissues collected after sacrifice. Data points indicate individual tissue samples of KO and WT mice analysed by quantitative PCR. A 102 base pair fragment of the *ospA* gene was amplified from mouse tissue samples collected at indicated days (d) post-infection. The data were expressed as the number of bacterial genome copies per 100 ng of extracted mouse DNA. The line indicates the median of each study group. Mann-Whitney U -test was used for statistical analysis. $P<0.05$ are statistically significant.



558

559 **Figure 3.** IgG antibody levels in plasma samples of biglycan knock-out (KO) and wildtype (WT) mice
560 infected with *Borrelia burgdorferi* sensu stricto for 28 days (ExpI) or 56 days (ExpII), with *Borrelia garinii*
561 (ExpIII) or with *Borrelia afzelii* (ExpIV) and of control KO and WT mice (Control) towards the *Borrelia*
562 whole cell sonicate (WCS) (**A**), recombinant DbpA protein (**B**) and recombinant DbpB protein (**C**). The
563 antibody levels were detected with in-house enzyme immunoassays. In the aligned dot blots, the data
564 were expressed as OD_{492} values. Each symbol represents an individual animal. The line indicates the
565 median of each study group. Mann-Whitney U -test was used for statistical analysis. $P<0.05$ are
566 statistically significant.

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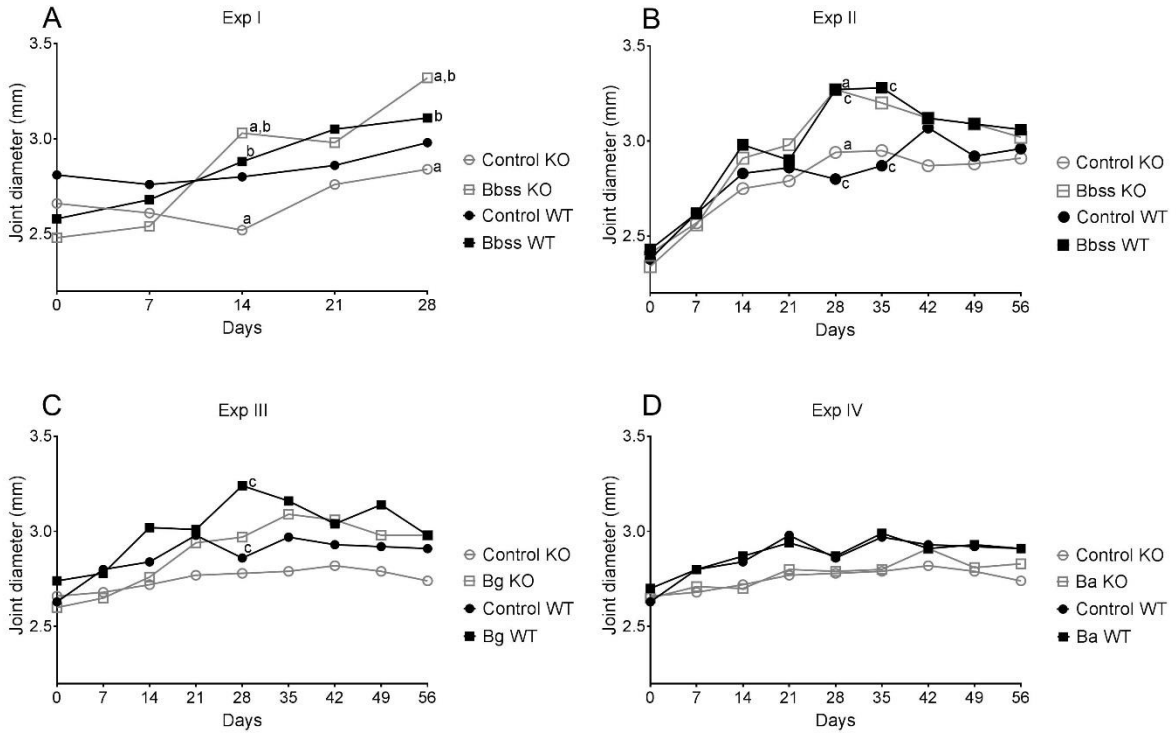

570 **Figure 4.** The weekly progression of the joint swelling in biglycan knock-out (KO) and wildtype (WT)
571 infected with *Borrelia burgdorferi* sensu stricto (Bbss) for 28 days (**A**) or 56 days (**B**), with *Borrelia garinii*
572 (Bg) (**C**) or with *Borrelia afzelii* (Ba) (**D**) and of the corresponding control KO and WT mice (Control).
573 The data were expressed as the mean lateral diameters of the hind tibiotarsal joints of all mice per study
574 group. Linear mixed models were used for statistical analysis. *P*<0.05 are statistically significant. The
575 letters connect the study groups with statistically significant differences at indicated time points. a,
576 statistically significant difference between infected KO and control KO mice; b, statistically significant
577 difference between joint swelling of KO and WT mice; c, statistically significant difference between
578 infected WT and control WT mice.

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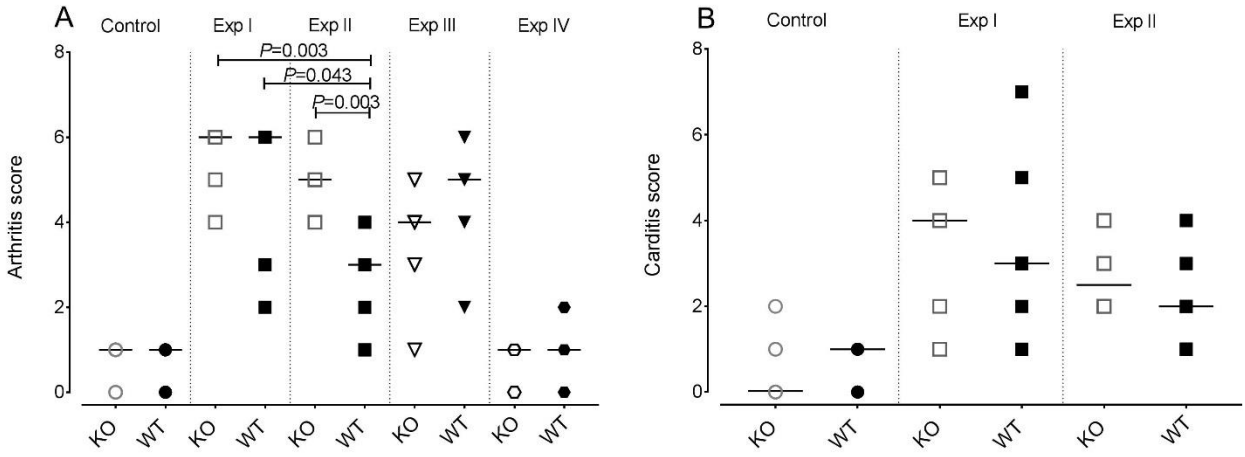

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584 **Figure 5.** The inflammation score of the tibiotarsal joints and heart of biglycan knock-out (KO) and
585 wildtype (WT) mice infected with *Borrelia burgdorferi* sensu stricto for 28 days (Exp I) or 56 days (Exp
586 II), with *Borrelia garinii* (Exp III) or with *Borrelia afzelii* (Exp IV) and of the control KO and WT mice
587 (Control). The arthritis (**A**) and carditis score (**B**) were assessed by scoring inflammation on the scale 0
588 (no inflammation) to 6 (severe inflammation). In the aligned dot blots, each symbol represents an
589 individual animal. The line indicates the median of each study group. Mann-Whitney U -test was used
590 for statistical analysis. $P<0.05$ are statistical significant.

591

Supplementary data

Supplementary Methods

Mouse strains

Biglycan (*bgn*) knock-out (KO) mice in the C57BL/6 background [1] were backcrossed for seven generations into the wildtype (WT) C3H/HeNHsd background (Envigo, The Netherlands). The genotype of the KO and WT mice was verified with PCR using primers listed in Supplementary Table 1 [1]. The biglycan allele is in the X-chromosome. Therefore, male knock-out mice are *bgn* $^{-/0}$ and female mice are *bgn* $^{-/-}$. For clarification, KO refers to male and female biglycan knock-out mice. Four weeks old female and male KO and WT mice were used for the infection studies. Female wildtype C3H/HeNHsd mice (Envigo, The Netherlands) were used for biglycan expression studies.

Borrelia strains

The wildtype *Borrelia* strains (*Borrelia burgdorferi* sensu stricto N40 (Bbss), *Borrelia garinii* SBK40 (Bg) and *Borrelia afzelii* A91 (Ba)) and the culture conditions have been previously described [2]. For inoculations, the spirochetes were enumerated with Neubauer counting chamber and were diluted to 10^6/ml in phosphate buffered saline (PBS).

RNA extraction and reverse transcriptase-qPCR analysis of biglycan expression

Lung tissue samples of uninfected biglycan KO and WT and multiple tissues from control and Bg infected WT mice (Envigo) were collected and stored in RNA*later* (Qiagen, Hilden, Germany) at -20 °C. The total RNA was extracted by RNeasy Mini kit (Qiagen) and reverse transcribed to cDNA by QuantiTect Rerverse Transcription Kit (Qiagen) according to manufacturer's protocols. The LightCycler 480 SYBR Green I

Master mix (Roche Diagnostics, Mannheim, Germany) and LightCycler 480 II equipment (Roche Diagnostics) were used to detect the biglycan gene expression and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with primers listed in Supplementary Table 1. The biglycan mRNA abundance was determined from the quantification cycle (Cq) for biglycan and normalized against the Cq for GAPDH using the $2^{-\Delta\Delta Cq}$ method [3]. Data are expressed as fold changes.

DNA extraction and qPCR analysis of bacterial load tissue samples

Total DNA of mouse tissues was extracted by High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's protocols. Quantitative PCR (qPCR) was performed using LightCycler 480 Probes master kit (Roche Diagnostics) and LightCycler 480 II equipment (Roche Diagnostics). A 102 base pair fragment of the *ospA* gene was amplified [4]. Samples of control mice were analysed once, whereas samples of infected mice were run in triplicates or quadruplicates. A sample was accepted as positive when the sample was positive in at least two separate runs. All analyses included a negative control sample. The load of *Borrelia* in tissue samples was calculated using a standard curve ranging from 10^2 to 10^6 copies of *Borrelia* genome. The data are expressed as the number of bacterial genomes copies per 100 ng of mouse DNA.

***Borrelia* culture of tissue samples**

All tissue samples of the KO and WT mice were cultured as described previously [5].

Blood collection and serology

The blood of control and infected mice was collected into heparinized tubes and was centrifuged at 6000 x g for 10 minutes at room temperature. The resulting plasma was stored at -20 °C. Immunoglobulin G (IgG) antibodies towards *Borrelia* whole cell

sonicate (WCS) antigen and towards DbpA and DbpB recombinant proteins were measured with in-house enzyme immunoassays as described previously [5]. Briefly, wells were coated with WCS of Bbss B31 ATCC 35210 (20 µg/ml) or purified recombinant DbpA and DbpB proteins of Bbss, Bg and Ba strain (10 µg/ml). Bound IgG was detect by horseradish peroxidase conjugated IgG antibody (1:8000, Santa Cruz Biotechnology, Santa Cruz, USA). Plasma samples (1:100) were analyzed in duplicates or quadruplicates.

Tissue samples, histological analysis and immunohistochemical staining of biglycan

For histologic examination and immunohistochemistry the tibiotarsal joint, heart and lung tissue samples of infected biglycan KO and WT mice were fixed in 10 % phosphate buffered formaldehyde, dehydrated in a graded ethanol series and embedded in paraffin. The tibiotarsal joint samples were also decalcified in EDTA. In all cases, 5 µm thick serial paraffin sections were cut. Tibiotarsal joints and hearts were stained with haematoxylin and eosin as well as with Weigert Van Gieson staining for light microscopy using routine histology techniques. Findings of inflammation in joints (all experiments) and in hearts (experiments I and II) were evaluated in sagittal tibiotarsal joint and heart sections by an experienced pathologist blinded to the experimental protocol. The inflammation of the joint was graded from 0 (no inflammation) to 6 (severe inflammation) paying attention to synovial proliferation, and active and chronic inflammation. Myocardial inflammation and fibrosis were graded from 0 (no inflammation or fibrosis) to 8 (severe inflammation with fibrosis).

For biglycan immunohistochemical staining, lung tissue samples were digested with 0.0045 U/ml chondroitinase ABC (Sigma-Aldrich) and stained with a rabbit IgG polyclonal antibody LF-159 (1:500; Kerafast, Boston, USA). Immunodetection was

665 performed using the avidin-biotin complex method (Sigma-Aldrich), diaminobenzidine
666 was used as the chromogen, and finally the sections were counterstained with
667 hematoxylin and eosin.

668 **Multiplex cytokine profiling of KO and WT mice**

669 Plasma cytokine levels were measured by using Luminex 200 equipment (Luminex,
670 Austin, USA) and a customized MILLIPLEX MAP Mouse Cytokine/Chemokine
671 MCYTOMAG-70K (Merck Millipore, Billerica, USA) according to manufacturer's
672 protocol in one well/sample. The results were calculated based on a standard curve
673 of each analyte using the xPONENT 3.1.software (Luminex). The analytes measured
674 included interleukin 1 beta (IL-1B), the C-C motif chemokine ligand (CCL) 2, CCL3,
675 CCL5, tumor necrosis factor (TNF) and the C-X-C motif chemokine ligand (CXCL) 2.

676 **Statistical analyses**

677 Categorical variables were characterized using frequencies and differences between
678 the groups were tested using Fisher's exact test. For continuous variables means or
679 medians were used to describe the data and the Mann–Whitney U test or the Kruskal–
680 Wallis test was used for the analyses to compare the infected KO and WT mice. Data
681 are presented in the figures as individual data points and medians of each study group
682 are indicated as horizontal lines. The progression of joint swelling was analyzed using
683 linear mixed models. The final model included group, week, sex and group*week –
684 interaction. Also, the mouse housing cage was used in a model to take account the
685 dependencies between several measurements, because it was not possible to identify
686 each mouse. If the interaction of group and time was statistically significant, the
687 pairwise comparisons between control mice and infected mice in KO and WT groups
688 were performed in each time-point. Also, the differences between control mice and
689 infected mice were compared between KO and WT groups in each time-point. P-

values of those comparisons were corrected using Sidak's method for multiple comparisons. P-values below 0.05 were considered as statistically significant. Statistical analyses were performed using SAS System for Windows (Version 9.4, SAS Institute Inc., Cary, USA).

Supplementary Results

Phenotype characterization of biglycan knock-out and wildtype mice

The KO mice in the C3H background were born with no apparent deficiencies. However, the KO mice weighted significantly less than the WT mice at the time of infection at four weeks of age (Supplementary Figure 1A). There was a statistically significant difference in the biglycan mRNA expression in lungs between the mouse genotypes (*P*=0.034) (Supplementary Figure 1B). Biglycan expression was detected in the blood vessel walls in lung tissue of WT mice but not in KO mice by immunohistochemistry (Supplementary Figure 1C-D).

Antibody responses to *Borrelia* infection

In experiments I and II, all infected KO and WT mice had elevated IgG antibodies towards *Borrelia* WCS (*P*=0.908; *P*=0.488, respectively), towards DbpA (*P*=0.420; *P*=0.418, respectively) and DbpB (*P*=0.817; *P*=0.643, respectively) without any significant differences between the mouse genotypes (Supplementary Figure 2A-C). Similarly, in experiment III, all Bg infected KO and WT mice had elevated antibody levels towards *Borrelia* WCS (*P*=0.643) and DbpB (*P*=0.203) with no significant difference between the genotypes (Supplementary Figure 2A-C). However, there was a statistically significant difference in the antibody levels towards DbpA (*P*=0.028) between the Bg infected KO and WT mice. In experiment IV, in parallel with the negative infection status of the animals, the KO mice had no antibodies towards

Borrelia WCS, recombinant DbpA or DbpB (Supplementary Figure 2A-C). As expected, the Ba infected WT mice had higher antibody levels towards *Borrelia* WCS (*P*=0.036), recombinant DbpA (*P*=0.093) and towards DbpB (*P*=0.027) than the Ba challenged KO mice.

Joint swelling in *Borrelia* infected mice

In experiments I and II, the analysis of the joint swelling of the Bbss infected KO and WT mice in the interaction of time and study group was statistically significant (*P* <0.001; *P* <0.001, respectively; Figure 3A-B). The joint swelling of infected KO mice was statistically significantly increased compared to control KO mice at day 14 (*P* <0.001) in experiment I and at day 28 in experiments I and II (*P* <0.001; *P*=0.014, respectively; Figure 3A-B). The joint swelling in infected WT mice was significantly different from control WT mice at days 28 and 35 in experiment II (*P* <0.001; *P* <0.001, respectively; Figure 3B).

In experiment III, the joint swelling in the Bg infected KO and WT mice was similar but less prominent than in experiments I and II (Figure 3C). The analysis of the joint swelling in the interaction of time and study group was statistically significant (*P* <0.001). The joint swelling of infected WT mice was significantly increased compared to control WT mice at 28 days post-infection (*P*=0.006). However, there were no statistically significant differences in joint swelling between the mouse genotypes.

In experiment IV, in line with the negative Ba infection status of the KO mice, there were no apparent joint swelling (Figure 3D). Also, the WT mice had no significant joint swelling, even though five out of eight WT mice were *Borrelia* culture positive. The differences in the joint swelling in the control and Ba infected KO and WT mice were not statistically significant in the different time-points (group*time interaction *P*=0.255).

739 However, the joint diameter increased statistically significantly during the experiment
740 in all study groups (P <0.001) due to growth of the mice, but there were no significant
741 differences in joint swelling between the study groups (P=0.167).

742 **References**

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