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*J Immunol* 2007; 179:8076-8082; doi: 10.4049/jimmunol.179.12.8076

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CD28 Deficiency Exacerbates Joint Inflammation upon Borrelia burgdorferi Infection, Resulting in the Development of Chronic Lyme Arthritis

Bettina P. Iliopoulou,* Joseph Alroy,† and Brigite T. Huber‡*

Lyme disease, caused by the tick-borne spirochete Borrelia burgdorferi (Bb), is a multisystem illness, affecting many organs, such as the heart, the nervous system, and the joints. Months after Bb infection, ~60% of patients experience intermittent arthritic attacks, a condition that in some individuals progresses to chronic joint inflammation. Although mice develop acute arthritis in response to Bb infection, the joint inflammation clears after 2 wk, despite continuous infection, only very rarely presenting with chronic Lyme arthritis. Thus, the lack of an animal system has so far prevented the elucidation of this persistent inflammatory process that occurs in humans. In this study, we report that the majority of Bb-infected CD28−/− mice develop chronic Lyme arthritis. Consistent with observations in chronic Lyme arthritis patients, the infected mutant, but not wild-type mice present recurring monoarticular arthritis over an extended time period, as well as anti-outer surface protein A of Bb serum titers. Furthermore, we demonstrate that anti-outer surface protein A Abs develop in these mice only after establishment of chronic Lyme arthritis. Thus, the Bb-infected CD28−/− mice provide a murine model for studying chronic Lyme arthritis. The Journal of Immunology, 2007, 179: 8076–8082.

Received for publication June 12, 2007. Accepted for publication October 13, 2007.

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‡Abbreviations used in this paper: Bb, Borrelia burgdorferi; OspA, outer surface protein A; Treg, regulatory T cells; wt, wild type; TRLA, treatment-resistant Lyme arthritis; AUC, area under the curve.

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mechanisms, the presence of CD4⁺CD25⁺ regulatory T cells (Treg), is being compromised. Recent studies have reported an essential role of the CD28 costimulatory molecule in the development and maintenance of CD4⁺CD25⁺ Treg cells and have documented a dramatic reduction (80%) of these cells in the CD28⁻/⁻ mouse (18).

When the role of the B7/CD28 costimulatory pathway was addressed in the development of the acute murine Lyme arthritis by CD80/CD86 blockade, no difference in arthritis severity was observed between treated and untreated BALB/c mice (19). In the same study, the authors reported expansion of IFN-γ producing T cells upon Bb infection. These data collectively led to the hypothesis that in the Bb infectious system the CD28 molecule in not required for the induction of an immune response against the pathogen, and that the signal for proper T cell activation could be provided by other costimulatory pathways. In the current study, we demonstrate that even though there is no difference in arthritis development between the Bb-infected CD28⁻/⁻ and the control mice, CD28⁻/⁻ mice develop higher incidence and severity of arthritis at later time points of disease progression, providing a murine model to investigate further the mechanism that regulates chronic Lyme arthritis.

Materials and Methods

Mice

Male CD28⁻/⁻ mice on the C57BL/6J (B6) background were bred at the Tufts University Division of Laboratory Animal Medicine from breeding pairs that were initially obtained from The Jackson Laboratory. Age- and sex-matched wt B6 mice were purchased. All animal experiments were approved by IACUC, the institutional animal review board at Tufts-New England Medical Center. Four- to five-week-old male CD28⁻/⁻ mice and wt mice were infected intradermally in the skin of the tibular area of both hind limbs (1 cm above the knee) with a total dose of 2 × 10⁵ Bb per mouse. This protocol was used for all the infections.

B. burgdorferi

Low passage (passage 2) of the infectious Bb N40 clone D10E9A1-E (gift from Jennifer Coburn) (20, 21) was used for all infections. Bb were cultured in complete Barbour-Stoenner-Kelly medium (Sigma-Aldrich) at 34°C until mid-log phase (5 × 10⁵ Bb/ml) and were counted by darkfield microscopy.

Histopathology

Ankles were harvested and decalcified overnight in Decalcifier I solution (Sur-gipath). Next day, they were rinsed with distilled H₂O for 30 min and then fixed in formalin until they were further processed. Hearts were cut in half and kept in formalin. Fixed ankles and heart tissues were embedded in paraffin, sectioned and stained by H&E. Stained sections were scored histopathologically in a blinded fashion on a scale from 0 to 3, based on the amount of infiltrating cells and morphology, as previously described (22): 0, no inflammation (no infiltrating cells were observed); 1, mild inflammation (one or several small and discrete areas of infiltrate); 2, moderate inflammation (multiple infiltrates without other gross changes); or 3, severe inflammation (heavy contiguous infiltrates in joints, tendons, and muscle).

Bb burden

DNA was extracted from ear punch tissue as previously described (23), with the following modifications. The ear punch tissue was digested for 4 h at 37°C in 125 μl of 1 mg/ml collagenase A (Roche) in Dulbecco’s PBS without Ca²⁺/Mg²⁺ (Cellgro), followed by overnight incubation at 55°C of 0.8 mg/ml Proteinase K (Invitrogen) in 125 μl of 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, and 200 mM NaCl. DNA was first extracted by 250 μl of buffer-saturated phenol (Invitrogen), then by an equal volume of phenol-chloroform-isomyl alcohol 25:24:1 (Invitrogen) and last by an equal volume of chloroform (Fisher Scientific). DNA was then precipitated with 0.3 M NaAc (pH 5.5) in 100% ethanol and resuspended in PCR quality H₂O (Invitrogen) and used as template for determination of Bb burden. The Bb burden is determined by real-time qPCR. Specific primers and 6-carboxyfluorescein-labeled TaqMan MGB (Minor Groove binder) probes for Bb RecA (GenBank accession no. U23457) were designed and used for Bb detection (F-TGGGATAGCTGCTTTTATTGATGCT, R-GACTAAGCCAAAGTTCTGAACATT, P-CCTGTTTATG CAAAGTTCTGAACATT, P-CCTGTTTATG CAAAGTTCTGAACATT, P-CCTGTTTATG CAAAGTTCTGAACATT), whereas primers and VIC-labeled probes specific for murine 18S were purchased (no. 4319413E; Applied Biosystems) and used as loading control. The PCR was conducted using iTaq SuperMix with Rox (Bio-Rad) per the manufacturer instructions. In brief, 100 ng of extracted DNA was used as template in 20 μl of reaction containing primers and probes specific for RecA and 18S (primers at 900 nM each, TaqMan MGB probes at 250 nM each). A Bb standard curve was generated by serial dilutions (1/10) of Bb genomic DNA of know concentration. Based on the calculation that 1 Bb genome is 1.62 × 10⁻⁹ μg, the Bb standard curve spans from 1.5 × 10⁵ to 1.5 Bb in 100 ng of mouse genomic DNA. The qPCR was performed on a 7300 real-time PCR system (Applied Biosystems), using the following PCR cycling parameters (95°C for 2 min, followed by 60 cycles of 95°C for 15 s and 60°C for 1 min). Data were analyzed per manufacturer’s instructions. The amount of template DNA was first normalized by the signal of the 18S housekeeping gene. The normalized RecA signal was then used to determine the Bb burden, based on the standard curve.

Anti-Bb and anti-OspA ELISA

Flat bottom Immulon 2HB plates (Fisher Scientific) were coated overnight with 10 μg/ml Bb lysate or 5 μg/ml recombinant OspA (0.1 M NaH₂PO₄ (pH 9)). Uncoated wells were served as non-Ag control. Coated and uncoated wells were then washed with PBS/0.05% Tween 20 and blocked in 1% milk for 45 min at 37°C. Serum samples were diluted in 1% milk 1/400...
Table I. CD28<sup>−/−</sup> B6 mice develop chronic Lyme arthritis upon infection with Bb<sup>a</sup>

<table>
<thead>
<tr>
<th>Group</th>
<th>Arthritis (arthritic mice/total mice)</th>
<th>Ankle Width (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD28&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>25/28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2 ± 0.14</td>
</tr>
<tr>
<td>B6</td>
<td>7/29</td>
<td>2.9 ± 0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup> CD28<sup>−/−</sup> (n = 28) and wt B6 mice (n = 29) were infected with 2 × 10<sup>4</sup> Bb/mouse. Arthritis was assessed periodically by measuring the ankles using a caliper. The number of mice that develop chronic Lyme arthritis either in one (monooarticular) or both ankles is shown. Data include the pooled results of six independent experiments.

* p = 7 × 10<sup>−7</sup> (two-tailed Fisher exact probability test).

for the anti-Bb ELISA or 1/100 for the OspA ELISA. Serially 2-fold dilutions of a known concentration of an OspA-specific mAb (LA-2) were also plated on each plate as a standard curve for the OspA ELISA. Samples were added in duplicates to the ELISA plates and incubated for 45 min at 37°C. Plates were washed and incubated with alkaline phosphatase (AP)-conjugated anti-mouse κ or anti-mouse IgM-AP, IgG3-AP, IgG1-AP, IgG2b-AP, IgG2c-AP (Southern Biotech) for 1 h at 37°C. We assess the levels of IgG2c, because B6 mice lack the gene for IgG2a and express instead the IgG2c isotype. Plates were then developed with the AP substrate, para-nitrophenyl phosphate (Pierce), in 0.2% diethanolamine substrate buffer (Pierce) and were read at 405 nm in a SpectraMax spectrophotometer (Molecular Devices).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. All data were tested for Gaussian distribution, using the Shapiro-Will normality test. Quantitative differences between groups were assessed by a two-tailed Student’s t test (if two groups are compared), and ANOVA (if more than two groups are compared) for normally distributed data and Mann-Whitney U test (if two groups are compared) and Kruskal-Wallis test (if more than two groups are compared) for skewed data. Statistical differences in the proportion of mice with anti-OspA Abs were determined by a two-tailed Fisher’s exact probability test. For outcomes measured multiple times we compared differences between time points using area under the curve (AUC). Significance was declared at a two-sided 0.05 level for all statistical analyses.

**RESULTS**

**CD28<sup>−/−</sup> mice develop recurring chronic arthritis upon Bb infection**

To investigate the role of the CD28 costimulatory molecule in the development of chronic Lyme arthritis upon Bb infection, CD28<sup>−/−</sup> and wt B6 mice were infected with a total dose of 2 × 10<sup>4</sup> Bb per mouse, as described in the Materials and Methods section. Arthritis was assessed in a blinded fashion two to three times a week by measuring the anterior-posterior tibiotarsal joint thickness using a caliper. This allows monitoring the edema formation over the course of the infection. The arthritic manifestations in Bb-infected mice were recorded over a period of 5 mo post infection. As seen in Fig. 1, there was no difference in arthritis development between the CD28<sup>−/−</sup> and the control mice at the early stage of the disease, implying that the ability of the CD28<sup>−/−</sup> mice to mount an innate and adaptive immune response against Bb is not compromised. However, while the majority of the B6 control mice resolved joint inflammation by 3–4 wk post infection, CD28<sup>−/−</sup> mice continued to develop recurring arthritic attacks, similarly to what had been observed in untreated Lyme arthritis patients (Fig. 1). The proportion of CD28<sup>−/−</sup> mice that developed chronic Lyme arthritis over time was statistically significant, as compared with the wt B6 mice (Table I). These episodes of arthritis were brief, lasting ~2 wk, followed by a remission phase.
Interestingly, the arthritic attacks during the later stage of the disease were mostly monoarticular, as seen in a more detailed graphic representation of each ankle (Fig. 2), again consistent with the clinical observations in Lyme patients. Thus, the CD28 deficiency in Bb-infected B6 mice prevents resolution of edema, as measured by joint swelling, leading to a perpetuated joint inflammation that appears to be in most cases occurring and monoarticular.

**CD28−/− mice develop increased arthritis severity**

To assess cellular infiltration in the inflamed joints, mice were sacrificed 5 mo post infection, during the third recurring arthritic episode. The tibiotarsal joints were processed for histology; slides were stained with H&E and were scored blindly for arthritis severity on a scale of 0 (no inflammation) to 3 (severe cellular infiltration). As shown in Fig. 3A, histological analysis showed increased arthritis severity and joint degeneration, a feature of chronically inflamed joints, in CD28−/− infected mice compared with the control group (p = 0.0003). Since in most cases the observed arthritis was monoarticular, we investigated whether the edema formation measured by the caliper correlated with cellular infiltration in each joint. As seen in Fig. 3B, there was a direct correlation between arthritis severity and ankle thickness (Spearman correlation coefficient = 0.76; p = 0.013).

**CD28−/− mice control Bb burden similarly to wt mice**

Increased arthritis in the Bb-infected CD28−/− mice may reflect the inability of these mice to control the spirochetes. To address this possibility, we compared the systemic Bb burden in these mice and wt controls. DNA was isolated from the ear punches of the infected mice during the chronic phase of Lyme arthritis and tested by real-time qPCR for the level of the Bb-specific gene, RecA. As can be seen in Fig. 4, there was no difference in RecA between the CD28−/− and the control group, indicating that the chronic immunopathology observed in the CD28−/− mice is not due to the inability of these mice to kill Bb.

**CD28−/− mice develop anti-OspA Abs that correlate with the establishment of chronic Lyme arthritis**

To delineate the humoral response in the Bb-infected CD28−/− mice, we bled the mice periodically and assessed the anti-Bb serum levels. Since Lyme arthritis patients develop anti-OspA IgG titers late in the course of the disease (24, 25), we measured the anti-OspA Ab levels in these mice as well. As shown in Fig. 5A, by day 200 post infection the CD28−/− B6 mice developed lower anti-Bb Ab levels compared with the control group (p = 0.001). Surprisingly, however, the humoral response to OspA did not follow the same pattern. Although B6 control mice had no detectable levels of anti-OspA Abs, CD28−/− mice developed substantial anti-OspA

![FIGURE 5. CD28−/− mice develop lower anti-Bb, but higher anti-OspA Ab levels, upon infection with Bb. Total anti-Bb (A) and total anti-OspA (B) Ab response in CD28−/− and wt mice. Mice were infected with 2 × 10^4 Bb/mouse and were bled during an intermittent arthritic attack, on day 200 post infection. The sera were tested by ELISA for anti-Bb and anti-OspA Ab titers. The asterisks indicate a significant difference between the two groups, *, p = 0.001; Student’s two-tailed, unpaired equal variance t test, ***, p = 10^-4; two-tailed Fisher exact probability test. Isotypes of anti-Bb Abs in CD28−/− mice (C) and wt mice (D). Isotype of anti-OspA Abs in CD28−/− mice (E). The difference among the anti-OspA isotypes IgM, IgG3, IgG2c, and IgG2b in CD28−/− mice was not statistically significant (p = 0.34; Kruskal-Wallis). The horizontal lines represent the mean (A, C, and D), and median (B and E), of the mice that developed anti-Bb and anti-OspA Abs. Data include the pooled results of four independent experiments.](http://www.jimmunol.org/Downloadedfrom)
IgM and IgG3 isotypes. We also observed that the CD28 decrease in IgG1, IgG2a, and IgG2b isotypes and an increase in anti-OspA Ab titers during the late stage of the disease, which coincides with the development of chronic Lyme arthritis. Data include the pooled results of four independent experiments.

Ab titers during the late stage of the disease, which coincides with the prolonged episodes of arthritis (Fig. 5B). To further investigate the humoral response in these mice, we determined the isotype of the anti-OspA Abs. As shown in Fig. 5, the pattern of the various OspA-specific isotypes was the same between the CD28−/− mice and control mice, with an overall decrease in IgG1, IgG2a, and IgG2b isotypes and an increase in IgM and IgG3 isotypes. We also observed that the CD28−/− mice developed anti-OspA Ab mainly of the IgM, IgG3, and IgG2b isotype (Fig. 5E).

In addition, as seen in Fig. 6, A and B, the CD28−/− mice mounted anti-OspA Abs only after the establishment of chronic arthritis, implying that perpetuated inflammation is a prerequisite for the development of an anti-OspA humoral immune response. Indeed, anti-OspA Ab titers were detectable only 200 days post Bb infection in the majority of CD28−/− mice (Fig. 6A), although recurring arthritic episodes presented before this date (Fig. 6B). Collectively, these results indicate that the humoral immune response to OspA upon Bb infection is unique for the CD28−/− system and is dependent on the establishment of chronic Lyme arthritis.

**FIGURE 6.** The development of anti-OspA Abs in the CD28−/− mice requires the establishment of chronic Lyme arthritis. The anti-OspA Ab titers (A) as well as the ankle width (B) were monitored over the course of 200 days, in Bb-infected CD28−/− mice (n = 18). The horizontal line in B represents the ankle width of mock-infected CD28−/− mice. Absolute numbers represent mice with anti-OspA Ab/total mice and ankle width/total mice (B). Even though CD28−/− mice develop arthritis early on, anti-OspA Abs are detected after a prolonged period of chronic Lyme arthritis. Data include the pooled results of four independent experiments.

**FIGURE 7.** Chronic Lyme arthritis in CD28−/− mice resolves upon antibiotic treatment

Upon establishment of chronic Lyme arthritis, CD28−/− mice were treated with antibiotic, and the joint inflammation was followed over a period of 2 mo. As shown in Fig. 7, arthritis did not persist after antibiotic treatment, as assessed by monitoring edema formation using a caliper. In contrast, untreated Bb-infected CD28−/− mice never resolved joint inflammation spontaneously, and a representative experiment is shown on Fig. 1. In addition, no Bb DNA was detectable in the ear punch of the CD28−/− mice, after antibiotic treatment (data not shown). The results were confirmed by histological analysis of the joints, performed upon termination of the experiment. No cellular infiltration was observed in the joints of the antibiotic-treated CD28−/− mice (data not shown).

**Discussion**

One of the most prominent clinical manifestations of Lyme disease is the development of chronic Lyme arthritis. Months after Bb infection, ~60% of untreated Lyme patients experience intermittent arthritic attacks that may last for years. Affected individuals have persistent Bb infection, as measured by spirochetal DNA PCR in their joints (5, 26). However, the immune mechanisms that govern this inflammatory process have been elusive thus far, mainly because of the lack of an animal system for chronic Lyme arthritis. Mice develop acute arthritis in response to Bb infection; their joint inflammation peaks within the first few weeks post infection and then spontaneously resolves, even though Bb establishes life-long persistent infection. It is likely, therefore, that immunoregulatory mechanisms control excessive immune activation in mice, allowing perseverance of the spirochetes. According to this model, interference with immunoregulation would lead to...
chronic arthritis in mice, similar to what is seen in the majority of Bb-infected humans. To test this working hypothesis, we analyzed arthritis in CD28⁻/⁻ mice after infection with Bb, because these mutant mice have a substantial reduction in the number of Treg cells (18). No difference was observed in the development of edema formation during the acute phase of the disease in these mice compared with wt mice. These data indicate that even in the absence of the CD28 T cell costimulatory molecule an immune response against Bb is taking place, implying that other molecules provide proper costimulation. This result is consistent with a previous report that taking place, implying that other molecules provide proper co-stimulation. In our system, the acute inflammatory response was not affected, because no differences in the ankle width were observed between wt B6 mice. However, the B6 control mice cleared joint inflammation, we observed that CD28⁻/⁻ B6 mice manifested intermittent chronic arthritic attacks that were mainly monoarticular, similar to Lyme arthritis presented in humans.

A similar clinical observation was made in Bb-infected IL-10⁻/⁻ mice, where immunoregulatory mechanisms are abrogated. IL-10 is an anti-inflammatory cytokine, induced by Bb lipoproteins, that negatively regulates the immune response against Bb by inhibiting the production of proinflammatory cytokines by macrophages (28). IL-10⁻/⁻ mice on an arthritis-resistant B6 background showed increased acute arthritis upon Bb infection (8) (29).

In our system, the acute inflammatory response was not affected, because no differences in the ankle width were observed between the CD28⁻/⁻ and the control group during the early phase of the disease. Likewise, when CD28⁻/⁻ mice were infected with Plasmodium chabaudi parasites, the acute response was similar to that of wt B6 mice. However, the P. chabaudi infected CD28⁻/⁻ mice developed chronic parasitemia, compared with the control mice which cleared the parasites (30).

The prevalence of the observed recurring monoarticular arthritic episodes in the Bb-infected CD28⁻/⁻ mice is even more significant, because these mutants are on the B6 background, which is relatively resistant to Bb-induced arthritis. The intermittent inflammatory reactions and remission phases might reflect an immune response against different Bb variants. Bb has the ability to modulate the expression of its highly immunogenic outer surface proteins (31). It is possible that in an environment where immunoregulatory mechanisms are compromised, expression of Bb variants might flare up the host immune response.

Upon the investigation of the humoral response to Bb Ags, CD28⁻/⁻ mice showed decreased titters of anti-Bb Abs compared with the control mice. This outcome was anticipated in light of the observation that the basal IgG levels are reduced in the CD28⁻/⁻ mice (15). However, despite the overall lower humoral response to Bb lysate, the mutant mice, but not the control mice, mounted a significant Ab response against OspA. Furthermore, the anti-OspA Abs in the CD28⁻/⁻ mice developed only after the establishment of chronic Lyme arthritis. This is particularly interesting, because OspA, mainly expressed in the midgut of Bb-infected ticks, gets down-regulated upon feeding of the infected tick on a mammalian host (32). Thus, it is not surprising that anti-OspA Abs are barely detectable in mice during acute arthritis, nor in patients during early Lyme arthritis (24, 33, 34). Nevertheless, patients with chronic Lyme arthritis are seropositive for OspA Abs, and this humoral response parallels the severity and duration of arthritis (24, 25). Collectively these studies suggest that OspA expression gets up-regulated in the mammalian host during a chronic inflammatory reaction, a hypothesis that we were able to confirm in vivo (35). Our results fit with this model and provide additional evidence for the notion that perpetual inflammation is a prerequisite for the development of an anti-OspA humoral immune response.

The majority of chronic Lyme arthritis patients clear arthritis upon antibiotic treatment. In contrast, 10% of these patients continue to show inflammation of their joints, a condition termed treatment-resistant Lyme arthritis (TRLA) (36). When Bb-infected CD28⁻/⁻ mice were treated with ceftriaxone, an antibiotic that has been reported to effectively eradicate Bb (37, 38), all animals cleared arthritis, with no signs of recurring joint inflammation for at least 2½ months post antibiotic therapy. This finding, however, was not surprising, since it is well established that the risk of developing TRLA is highest in individuals with rheumatoid arthritis-associated HLA-DR alleles, mainly of HLA-DR4 haplotype (39). In these TRLA patients the magnitude of the anti-OspA immune response correlates with the severity of Lyme arthritis, even in the absence of active Bb infection (4, 25). This observation led to the hypothesis that an autoimmune response is induced in these individuals. Indeed, we have recently shown that the presence of a HLA-DR4 transgene into CD28⁻/⁻ mice allows the development of TRLA (manuscript submitted for publication).

In conclusion, we have generated a mouse model for chronic Lyme arthritis. This murine system addresses the role of immunoregulation in the development of chronic Lyme arthritis and provides a tool for the dissection of the immunological manifestation that perpetuates chronic joint inflammation due to Bb infection.

Acknowledgments

We are grateful to Dr. Jenifer Coburn for the gift of R. burgdorferi and to Dr. Jeffrey Bluestone for guiding us to the CD28⁻/⁻ system. We thank members of the Huber laboratory for critical reading of the manuscript and Lin Miao and Francesca Chang for expert technical help.

Disclosures

The authors have no financial conflict of interest.

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