Interleukin-35 Enhances Lyme Arthritis in *Borrelia*-Vaccinated and -Infected Mice

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Interleukin-35 (IL-35) has been reported to inhibit the production of interleukin-17 (IL-17) as a means of preventing arthritis and other inflammatory diseases. We previously showed that treatment of *Borrelia*-vaccinated and -infected mice with anti-IL-17 antibody at the time of infection prevented the development of arthritis. The anti-IL-17 antibody-treated mice lacked the extensive tissue damage, such as bone and cartilage erosion, that occurred in the tibiotarsal joints of untreated *Borrelia*-vaccinated and -infected control mice. We hypothesized that IL-35 would reduce the severity of arthritis by suppressing the production of IL-17 in *Borrelia*-vaccinated and -infected mice. Here, we show that administration of recombinant IL-35 (rIL-35) to *Borrelia*-vaccinated and -infected mice augments the development of severe arthritis compared to the results seen with untreated control mice. *Borrelia*-vaccinated and -infected mice treated with rIL-35 had significantly (P < 0.05) greater hind paw swelling and histopathological changes from day 4 through day 10 than non-rIL-35-treated *Borrelia*-vaccinated and -infected mice. In addition, the treatment with IL-35 only slightly decreased the production of IL-17 in *Borrelia*-primed immune cells and did not prevent the development of borreliacidal antibody. Our data do not support a role for IL-35 as a potential therapeutic agent to reduce inflammation in Lyme arthritis.

Lyme borreliosis, caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex, is the most prevalent tick-borne human disease in the world, with approximately 85,500 cases reported annually (18). One of the most frequent pathological manifestations of Lyme borreliosis is arthritis, which develops weeks, months, or years after infection in approximately 60% of infected individuals in North America (38). Despite antimicrobial treatment, 10% of those arthritic patients develop a chronic joint inflammation, leading to permanent cartilage and bone damage (38). Presently, the immune mechanisms responsible for the development of Lyme arthritis are not fully known. Some studies have shown that arthritis following *Borrelia* infection develops in the absence of adaptive immunity (2, 3, 6), while others have shown that T cells are major contributors to pathology (9, 16, 24, 26–28, 39, 40). Recently, considerable evidence has shown that cells and cytokines of the Th17 lineage, such as interleukin-17 (IL-17), may be involved in the development of *Borrelia*-induced arthritis (7, 11, 22, 31–33).

We previously showed that treatment of *Borrelia*-vaccinated and -infected mice with anti-IL-17 antibody at the time of infection prevented the development of arthritis (7). These anti-IL-17 antibody-treated mice lack the extensive tissue damage, such as bone and cartilage erosion, that occurred in the tibiotarsal joints of untreated *Borrelia*-vaccinated and -infected control mice. In addition, treatment of *Borrelia*-vaccinated and -infected mice with antibodies to the IL-17 receptor prevented cartilage and bone destruction. In support of a role for IL-17 in the development of Lyme arthritis, Infante-Duarte et al. (19) showed that outer surface protein A (OspA), a known promoter of Lyme arthritis in experimental animals (13) and in humans (15, 17, 24, 29), primed T cells to produce IL-17. Moreover, Codolo et al. (11) showed that neutrophil-activating protein A of *B. burgdorferi* stimulated the release of IL-17 from synovial T cells of human Lyme arthritis patients. Collectively, these findings provide strong evidence that IL-17 is involved in the pathogenesis of arthritis following infection with *B. burgdorferi*.

Additionally, we previously demonstrated that administration of anti-IL-17 antibody to *Borrelia*-vaccinated and -infected mice led to the production of CD4<sup>+</sup>CD25<sup>+</sup> cells (31) which, upon adoptive transfer, conferred resistance to the development of arthritis (32). A major cytokine produced by CD4<sup>+</sup>CD25<sup>+</sup>Foxp<sub>3</sub> T regulatory (Treg) cells is interleukin-35 (IL-35) (12, 35). IL-35 stimulates expansion of CD4<sup>+</sup>CD25<sup>+</sup> T cells, suppresses the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells, and inhibits the differentiation of Th17 cells in collagen-induced arthritis (CIA) and inflammatory bowel disease models (12, 35). Therefore, IL-35 negatively regulates the proinflammatory cytokine IL-17 (12) and may play a role in the development of *Borrelia*-induced arthritis.

In this study, we examined the role of IL-35 in *Borrelia*-vaccinated and -infected mice. We hypothesized that IL-35 would reduce the severity of arthritis by suppressing the production of IL-17 in *Borrelia*-vaccinated and -infected mice.
However, we show that administration of recombinant IL-35 (rIL-35) to Borrelia-vaccinated and -infected mice augmented the development of severe arthritis compared to the results seen with untreated control mice. In addition, IL-35 only slightly decreased the production of IL-17 in Borrelia-primed immune cells. Our data do not support a role for IL-35 as a potential therapeutic agent to reduce inflammation in Lyme arthritis.

MATERIALS AND METHODS

Mice. Three-week-old inbred male C57BL/6J mice weighing 15 to 20 g were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed at the animal facility located at the University of Wisconsin School of Medicine and Public Health. Mice were maintained under pathogen-free conditions at an ambient temperature of 21°C. Food and acidified water (pH 2.5 to 3.0) were provided ad libitum during daily cycles of 12 h of light and darkness. Experimental protocols were approved by the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health.

Vaccination and preparation. Low-passage-number (<10) virulent B. burgdorferi (formally known as B. burgdorferi strain C-11; isolated from Microtus pennsylvanicus) and B. burgdorferi isolate 297 (isolated from human spinal fluid) (37) were cultured in modified Barbour-Stoener-Kelly (BSK) medium (Gunderson Lutheran Medical Center, La Crosse, WI) until a concentration of 107 spirochetes/ml was reached. Samples of 500 μl were dispensed into 1.5-ml screw-cap tubes (Sarstedt, Newton, NC) containing 500 μl of BSK medium with 10% glycerol (Sigma-Aldrich, St. Louis, MO). The spirochetes were stored at −70°C until used.

Vaccination of mice. A frozen suspension of B. burgdorferi was thawed and heated in a water bath at 56°C for 30 min. These spirochetes were immobile upon examination by dark-field microscopy. In addition, an aliquot of the heat-inactivated suspension of Borrelia failed to grow in fresh BSK medium 7 days after inoculation. The heat-inactivated B. burgdorferi spirochetes were then washed three times by centrifugation at 10,000 × g with ice-cold phosphate-buffered saline (PBS) (pH 7.4) that was sterilized using a 0.2-μm-pore-size filter (Gelman Sciences, Ann Arbor, MI). Subsequently, the washed B. burgdorferi spirochetes were resuspended in PBS and mixed with a volume of 1% sodium hydroxide (Reheis, Berkley Heights, NJ) to yield a concentration of 2 × 108 spirochetes/ml. Mice were then anesthetized with isoflurane in a nose-and-mouth cup and injected subcutaneously in each inguinal region with 0.25 ml of the vaccine by the use of a 23-gauge needle. This whole-cell vaccine is able to consistently induce T cell-driven arthritis (1, 7, 10, 22, 31–33).

Infection of mice. A frozen suspension of B. burgdorferi isolate 297 was thawed and mixed with 4 ml of fresh BSK medium. The spirochetal culture was incubated at 32°C until a concentration of 2 × 107 viable spirochetes/ml was reached. At 21 days after vaccination of mice with heat-inactivated B. burgdorferi, mice were anesthetized with isoflurane in a nose-and-mouth cup and injected subcutaneously in each inguinal region with 0.25 ml of the vaccine by the use of a 23-gauge needle. This whole-cell vaccine is able to consistently induce T cell-driven arthritis (1, 7, 10, 22, 31–33).

Administration of rIL-35. Human rIL-35 (Enzo Life Sciences, Plymouth Meeting, PA) (endotoxin level < 0.1 endotoxin unit [EU]/μg of purified protein), which possesses biological functions similar to those of murine rIL-35 in mice (35), was suspended in PBS to obtain a concentration of 2.5 μg/ml. A 50-μl (0.125-μg) volume of rIL-35 suspended in PBS was injected subcutaneously into each hind paw of 45 Borrelia-vaccinated and -infected mice. That dosage of rIL-35 is larger than one would expect to occur naturally. Two groups of nine mice each were injected with rIL-35 1 h after infection and daily thereafter for 5 days. The third group of nine mice was initially injected with rIL-35 on day 10 after infection and daily thereafter for 5 days. We suspected that rIL-35 would be neutralized by the production of mouse anti-rIL-35 antibody. Therefore, we administered rIL-35 for only 5 days. In addition, two control groups of nine mice each were injected with PBS.

Assessment of swelling. Swelling of the hind paws of mice was measured to determine edematous changes. Hind paws of each mouse were measured every other day for 20 days with a digital caliper (Mitutoyo American Corporation, Aurora, IL) with a sensitivity of 0.01 mm prior to infection with B. burgdorferi isolate 297 or after administration of rIL-35. Mice were anesthetized with isoflurane in a nose-and-mouth cup, and the width and thickness of each hind paw were measured. The caliper values within a group were averaged to obtain the mean caliper value for comparisons of the degree of swelling.

Detection of borreliacidal antibodies. Borrelia-vaccinated mice were euthanized with isoflurane at days 10 and 20 after infection with B. burgdorferi isolate 297. Serum from each mouse within a group was collected, and the serum samples were pooled for detection of borreliacidal antibodies, such as anti-OspA, via flow cytometry (8, 23, 25). Each pooled serum sample was diluted 1:20 with fresh BSK medium and passed through a 0.2-μm-pore-size microcentrifuge filter (Costar, Cambridge, MA). Filtered serum samples were then diluted serially from 1:80 to 1:40,960 with BSK medium in 1.50-ml screw-cap microcentrifuge tubes (Sarstedt, Newton, NC). The serum samples were subjected to heat inactivation at 56°C for 10 min and cooled to 35°C. The inactivated sera were incubated at 35°C for 24 h with 108 viable B. burgdorferi isolate 297 organisms and 10 μl of sterile guinea pig serum (Sigma-Aldrich, St. Louis, MO).

Following incubation, 100 μl of each assay suspension was mixed with 400 μl of PBS and acridine orange (Sigma-Aldrich, St. Louis, MO) (50 μl; 5.4 × 10−9 M) in polyurethane tubes (Becton Dickinson, Franklin Lakes, NJ) (12 mm by 75 mm). A FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used to detect borreliacidal activity. Spirochetes were isolated by gating with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed for 1 to 2 min with the flow rate adjusted to the low setting. When borreliacidal antibodies are present in the serum samples, spirochetes are killed by activated complement that disrupts the outer membrane of the bacteria. The membrane forms blebs, allowing acridine orange to intercalate in nuclear material after passing through the cell membrane. Dead spirochetes show an increase in fluorescence due to the intensity of staining with acridine orange. A shift in mean fluorescence intensity greater than 13% compared to the normal serum control fluorescence is considered to represent a positive result (8, 23, 25). In addition, dark-field microscopy was used to confirm the presence of blebbed, nonviable spirochetes.

Tissue preparation for histological examination. At 10 and 20 days after infection, mice were euthanized with isoflurane and the hind paws of each mouse were amputated at midfemur. The paws were fixed in formalin solution (Sigma-Aldrich, St. Louis, MO). The paws were subsequently placed in decalcifying solution (Lerner Laboratories, Pittsburgh, PA), with fresh decalcifying solution added after 24 h, and stored for 48 h. After decalcification, paws were placed in tissue-embedding cassettes (Fisher Scientific, Hanover Park, IL), embedded in paraffin, and cut into three to five 6-μm-thick sections. Each section of the hind paws was placed onto a glass slide and stained with hematoxylin and cosin. Sections were cryptically coded, and a board-certified pathologist (T. F. Warner) performed fully blinded histopathological examinations of the tissues.

In vitro cell culture and ELISA. Inguinal and popliteal lymph nodes were harvested from Borrelia-vaccinated C57BL/6J mice 3 weeks after vaccination with B. burgdorferi. Single-cell suspensions of the lymph node cells were prepared by teasing apart the lymph nodes and passing them through sterile nylon mesh screens (Fisher Scientific, Hanover Park, IL) into cold Dulbecco’s modification of Eagle’s medium (DMEM; Mediatech, Inc., Manassas, VA) containing 10% filter-sterilized calf serum (Sigma-Aldrich, St. Louis, MO) and 0.01% penicillin-streptomycin (HyClone Laboratories, Inc., Logan, UT). A total of 9 × 107 lymph node cells were dispensed into each well of 24-well culture plates. The plates were incubated at 37°C with or without 5 × 105 motile B. burgdorferi isolate 297 organisms and with or without 1 μg of rIL-35 per well. Our results have shown that this amount of rIL-35 was necessary to obtain an effect. At 0, 1, 2, 4, 8, and 14 h of incubation, supernatant from each well was collected after centrifugation of cultures at 10,000 × g for 10 min. IL-17 levels in each supernatant were measured with a mouse IL-17A enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go! kit (eBioscience, San Diego, CA). The changes in optical density at 450 nm and 570 nm were determined for each sample. A standard curve was generated, and data were expressed in picograms of IL-17 per milliliter of serum.

Statistics. Swelling of the hind paws among different groups of Borrelia-vaccinated and -infected mice and concentrations of IL-17 were evaluated by the Fisher least-significant-difference test. The α-level was set at 0.05 before the experiments were initiated. Data are presented with standard errors of the mean.

RESULTS

Effects of rIL-35 on development and progression of arthritis. Forty-five Borrelia-vaccinated C57BL/6J mice were infected with B. burgdorferi isolate 297 21 days after vaccination.
Three groups of nine *Borrelia*-vaccinated and -infected mice each were treated with rIL-35. Two of these groups received rIL-35 1 h after infection and daily thereafter for 5 days, whereas the other group received rIL-35 on days 10 through 15 after infection. Two remaining groups of nine *Borrelia*-vaccinated and -infected mice were injected with PBS. A control group of nine nonvaccinated and noninfected mice was also included. Hind paws of *Borrelia*-vaccinated and -infected mice with or without treatment with rIL-35 were measured for swelling. In addition, mice were killed for histopathological examination at days 10 and 20 after infection. Moreover, sera were collected from all mice before they were killed at days 10 and 20 after infection.

Swelling of the hind paws was detected in *Borrelia*-vaccinated and -infected mice with or without treatment with rIL-35 compared to mice without *Borrelia* vaccination and infection (Fig. 2). The swelling for these two groups peaked on day 10 and rapidly decreased by 20 days after infection. However, *Borrelia*-vaccinated and -infected mice treated with rIL-35 had significantly (*P < 0.05*) greater hind paw swelling from day 4 through day 10 than non-rIL-35-treated *Borrelia*-vaccinated and -infected mice. No significant differences in hind paw swelling were detected between untreated *Borrelia*-vaccinated and -infected mice and *Borrelia*-vaccinated and -infected mice receiving rIL-35 starting on day 10 after infection. However, these mice still had hind paw swelling significantly greater than mice without *Borrelia* vaccination and infection between days 12 and 20 after infection. Nonvaccinated and noninfected mice treated with rIL-35 did not develop arthritis (data not shown). Similar results were obtained when this experiment was repeated twice with 90 *Borrelia*-vaccinated and -infected mice with or without rIL-35 treatment.

**Histopathologic confirmation that rIL-35 enhanced the development of arthritis.** The ankle joints of *Borrelia*-vaccinated and -infected mice with (Fig. 3B) or without (Fig. 3A) treatment with rIL-35 exhibited severe inflammation of the tibiotarsal joints, including the synovial space, synovial lining, and perisynovium, 10 days after infection. Many neutrophils were present in the tissues. In addition, erosion of cartilage was observed. However, treatment with rIL-35 enhanced the magnitude of the response in *Borrelia*-vaccinated and -infected mice (Fig. 3B). These mice had a dense infiltration of neutrophils into the synovial space and surrounding tissues. The synovial lining showed extensive proliferation, and the cartilage was eroded along with adjacent bone. Other tissues, particularly those of the perisynovium, exhibited hyperplasia and hypertrophy. The number of neutrophils in the tissue made it difficult to discern individual cells.

**FIG. 1.** Overview of the experimental design by which *Borrelia*-vaccinated and -infected mice, administered rIL-35 or not, were compared. Each of the five groups included nine mice.

**FIG. 2.** Development of hind paw swelling of *Borrelia*-vaccinated and -infected C57BL/6J mice with or without treatment with rIL-35. One group of *Borrelia*-vaccinated and -infected mice received rIL-35 on days 0 to 5 after infection (closed circles). A second group received rIL-35 on days 10 to 15 after infection (open circles). Control groups included untreated *Borrelia*-vaccinated and -infected mice (triangles) and untreated naïve mice (diamonds). Error bars represent the mean hind paw swelling ± standard error. Asterisks denote a significant (*P ≤ 0.05*) difference in swelling among groups of mice. Data are representative of the results of three experiments.
At 20 days after infection, no significant histopathological differences were observed between *Borrelia*-vaccinated and -infected mice receiving no treatment (Fig. 4A) and those receiving rIL-35 from day 0 to day 5 after infection (Fig. 4B). *Borrelia*-vaccinated and -infected mice treated with rIL-35 at day 10 after infection and daily thereafter for 5 days (Fig. 4C) maintained a mild infiltration of neutrophils in the tibiotarsal joint 20 days after infection.

Treatment with rIL-35 failed to alter the development or regression of the borreliacidal antibody response. Pooled sera were obtained from untreated and rIL-35-treated *Borrelia*-vaccinated and -infected mice at day 10 or 20 after infection (Fig. 5). Although sera from rIL-35-treated *Borrelia*-vaccinated and -infected mice had a lower borreliacidal antibody titer (2,560) than sera from untreated controls (5,120), these differences represented a less than 4-fold change. A similar insignificant
A decrease in borreliacidal antibody titer was observed at day 20 after infection. Treatment with rIL-35 failed to significantly alter the development or decline of the borreliacidal antibody response.

**rIL-35 slightly inhibits production of IL-17 by lymph nodes obtained from Borrelia-vaccinated mice.** Inguinal and popliteal lymph node cells were obtained from five to nine Borrelia-vaccinated mice 21 days after vaccination. The lymph node cells were incubated in the presence or absence of 10^6 live B. burgdorferi isolate 297 organisms and with or without rIL-35 treatment. Supernatants from the cultures were obtained at 0, 1, 2, 4, 12, and 24 h after incubation at 32°C. Addition of rIL-35 to cultures of Borrelia-primed cells without Borrelia organisms slightly inhibited the production of IL-17 at all tested intervals (Fig. 6). Statistically significant (P < 0.05) inhibition of IL-17 was achieved at 12 and 24 h in cultures of lymph node cells containing Borrelia organisms and rIL-35. Lastly, no significant differences in IL-17 levels were detected with nonvaccinated and noninfected mice with or without rIL-35 treatment (data not shown).

**DISCUSSION**

The complex immunological mechanisms that drive the development and resolution of Lyme arthritis are not fully understood. Recent evidence (7, 22, 31–33) suggests that cells and cytokines of the Th17 lineage may influence the development of Borrelia-induced arthritis. Specifically, we previously showed that IL-17 is a major contributor to inflammation in the Borrelia vaccination and infection model of arthritis (7, 31–33). Treatment of Borrelia-vaccinated and -infected mice with anti-IL-17 antibody at the time of infection prevented the extensive inflammatory changes exhibited in untreated Borrelia-vaccinated and -infected control mice (7, 31–33). Similar preventive results were achieved when Borrelia-vaccinated and -infected mice were treated with antibodies to the IL-17 receptor or treated sequentially with anti-IL-17 antibody followed by antibodies to the IL-17 receptor (7). In addition to preventing arthritis, treatment of Borrelia-vaccinated and -infected mice with anti-IL-17 antibody stimulated an increase in the number of CD4^+ CD25^- effector T cells (31, 32). The production of CD4^+ CD25^- cells was associated with prevention of arthritic development (31, 32) and declined upon cessation of anti-IL-17 antibody administration (31). Importantly, it was shown that these CD4^+ CD25^- cells inhibited the development of arthritis after passive transfer into Borrelia-vaccinated and -infected mice (32), suggesting that these induced cells have a potent immunoregulatory function. In support of these findings, there is a reciprocal developmental relationship between Th17 cells and CD4^+ CD25^- Foxp3^+ regulatory T (Treg) cells (35).

Treg cells produce IL-35, which drives further Treg cell development (12, 35). IL-35 also suppresses the development of CD4^+ CD25^- effector T cells (35), which, as we have previously shown, exacerbate Borrelia-induced arthritis (32). In addition, IL-35 has been shown to attenuate various inflammatory diseases by inhibiting the differentiation of Th17 cells (12, 21, 35). Niedbala et al. (35) inhibited the development of CIA in mice following early-stage IL-35 treatment and showed that the presence of IL-35 decreases the production of IL-17 from CD4^+ T cells. Kochetkova et al. (21) reported similar findings and additionally showed that IL-35 prevented the further progression of established CIA. Moreover, Collison et al. (12) showed that IL-35-secreting Treg cells stimulate recovery...
from established experimental colitis. Collectively, these findings suggest that IL-35 may be a key cytokine in the protection against the T cell-driven arthritis observed in the Borrelia vaccination and infection model of arthritis (1, 7, 10, 22, 31–33) and in humans with Lyme arthritis (11).

However, we have showed that treatment with rIL-35 failed to prevent development of arthritis in Borrelia-vaccinated and -infected mice. In addition, administration of rIL-35 during established inflammation did not resolve arthritis. Instead, treatment with rIL-35 exacerbated the histopathological changes in the tibiotarsal joints compared to those in the ankle joints of untreated control mice. The tibiotarsal joints of rIL-35-treated Borrelia-vaccinated and -infected mice exhibited massive neutrophilic infiltration into the joint spaces and sub-synovial tissues along with erosion of bone and cartilage. Our results suggest that IL-35 may be immunostimulatory immediately after infection of Borrelia-vaccinated mice with B. burgdorferi isolate 297 and that it is not involved in the resolution of the arthritis. Although the Borrelia vaccination and infection model of arthritis exhibits inflammation congruent with the Th17-mediated pathways exhibited in CIA (5, 9, 30, 35) and in synovial cells of human Lyme arthritis patients (11), our findings do not support recent reports of IL-35’s therapeutic potential for alleviation of inflammatory diseases.

We also showed that IL-35 only marginally suppressed the production of IL-17 in vitro. IL-17 levels were decreased by approximately 10% when lymph node cells from Borrelia-vaccinated mice were cultured in vitro with viable B. burgdorferi in the presence of IL-35. Although the decrease in IL-17 levels was statistically significant, the biological relevance is uncertain in light of our histopathological findings. IL-17, even at reduced levels, would likely be constantly present at the local inflammatory site, so long as the spirochetal burden is not reduced. In support of this statement, the presence of viable spirochetes further increased the production of IL-17 by Borrelia-primed lymph node cells. This suggests that the influence of the spirochete burden on propagating inflammation may outweigh the effects of IL-35 in marginally suppressing production of IL-17.

This hypothesis is supported by our analysis of borreliacidal antibody production following treatment with rIL-35. The early borreliacidal antibody detected (within ≤10 days) in mice vaccinated with BSK-passed Borrelia is anti-OspA antibody. We showed that rIL-35 reduced (albeit insignificantly) borreliacidal antibody titers, which may account for the early maintenance or elevation of the arthritic stimulus. It is known that an inflammatory environment is required for the differentiation and survival (4) of Th17 cells. It is possible that inefficient early clearance of spirochetes induced by the activity of the borreliacidal antibody results in additional production of Th17-associated cytokines such as IL-6, IL-15, and IL-23. These cytokines have been shown to initiate or propagate Th17 cell-mediated inflammation (4), and they are involved in the development of arthritis in Borrelia-vaccinated and -infected mice (1, 7, 22, 33). In other words, IL-35 may have indirectly augmented inflammation by suppressing the borreliacidal antibody response that is central to removal of the inflammatory stimulus. Therefore, these results suggest that IL-35 may inhibit the activity of potentially protective immune mechanisms in the initial stages of Borrelia-induced arthritis.

FIG. 6. The concentrations (in picograms per milliliter) of IL-17 in the supernatants of cultures of B. bissettii-primed lymph node cells exposed to rIL-35 (light gray and closed bars) or not exposed to rIL-35 (dark gray and open bars) in the presence (dark gray and closed bars) or absence (light gray and open bars) of B. burgdorferi. Error bars represent the mean picogram concentration of IL-17 ± standard error. Asterisks denote a significant ($P \leq 0.05$) difference compared to IL-17 levels in Borrelia-primed lymph node cells without exposure to B. burgdorferi. Data are representative of the results of three experiments.
Our results were unexpected. We hypothesized that administration of rIL-35 would inhibit or resolve the arthritis observed in Borrelia-vaccinated and -infected mice. However, IL-35 increased the severity of inflammation. The lack of a robust borreliacidal antibody response to reduce the spirochete burden is one explanation for the sustained inflammatory environment that would lead to Th17-mediated inflammation in vivo. Another explanation may be that the presence of IL-35 did not cause proliferation of the Treg cells that would have prevented the development of Th17 cells and the production of IL-17. This suggests that other inflammatory mediators are responsible for the increased arthritis severity. Niedbala et al. showed that IL-35 not only reduced production of IL-17 but also increased production of gamma interferon (IFN-γ) (35). This finding supports the idea of the reported antagonistic relationship between Th1 and Th17 cells (14, 36) and may account for the increased inflammation seen in our studies. Although IFN-γ is not required for the development of arthritis in multiple models of Borrelia-induced arthritis (5, 10), its presence likely augments inflammation. Administration of high levels of IL-35 may have induced a strong shift in the type of inflammatory T cell produced. Additional studies to characterize the relationships of different T cell populations in Borrelia-induced arthritis are under way.

Use of the Borrelia vaccination and infection model has been vital for the investigation of roles for other Th17-associated cytokines (1, 7, 22, 31–33) in the development of arthritis following infection with B. burgdorferi. In addition, this model was instrumental in the initial observations that an increase in CD4+ CD25+ T cells with immunoregulatory function correlated to a reduction in IL-17 (31, 32). In contrast, recent findings (34) have shown that a more traditionally used model of Borrelia-induced arthritis, one in which C3H mice are infected with a virulent strain of the Lyme spirochete, does not allow an efficient investigation of IL-17. In support of the statement, it is known that B. burgdorferi organisms (20, 36) or components (19) are capable of stimulating the production of Th17-associated cytokines. More importantly, Codolo et al. (11) showed that synovial cells isolated from humans with Lyme arthritis secrete Th17 cytokines upon interaction with proteins of B. burgdorferi. Thus, the Borrelia vaccination and infection model of arthritis has elucidated pathways of Borrelia-induced arthritis which have not been reported in traditional animal models but which likely reflect events occurring in humans with Lyme arthritis. This is the first documentation of a role of IL-35 and its effect on IL-17 in Borrelia-induced arthritis.

A major issue with our approach to investigation of the mechanisms of Lyme arthritis has been the use of Borrelia-vaccinated and -infected mice. The mouse has emerged as the commonly accepted animal with which to investigate Lyme arthritis. More specifically, strains within the “arthritis-susceptible” C3H genotype (2) have served as better indicators of arthritic mechanisms than less-susceptible strains. Young C3H mice infected, via needle injection or tick bite, with particularly pathogenic Borrelia bacteria display infiltration of neutrophils and other leukocytes into the joint space, as well as inflammation of connective tissue and synovial tissue, within days of infection. Synovial hyperplasia, fibrin deposition, and increased cellular infiltration are observed 2 weeks after infection. Arthritis peaks 3 weeks after infection and gradually declines (2). However, humans do not develop Lyme arthritis in the days following a tick bite. Humans develop Lyme arthritis several weeks to months after infection (38). During this lag time, components of the immune system, including T cells, are primed to recognize arthrogenic antigens found on B. burgdorferi. These adaptive immune events have been shown to be involved in the pathology of human Lyme arthritis. Synovial fluid of human Lyme arthritis patients contains CD4+ T lymphocytes capable of producing Th1 (16) or Th17 (11) cytokines. Moreover, those arthritic patients produce T cells specific for the putative arthrogenic borrelial antigen OspA and an increase in anti-OspA antibodies that coincides with late-stage arthritis. Collectively, these findings provide support for the idea that cells of adaptive immunity—particularly T cells—are significant contributors to human Lyme arthritis.

We have used a model of arthritis which incorporates the activity of primed and activated T cells (7, 22, 31–34). C57BL/6 mice are vaccinated with heat-killed B. burgdorferi in aluminum hydroxide in order to induce priming of T cells. Three weeks later, mice are infected with a heterologous strain of Borrelia that is capable of driving the primed T cells to induce arthritis while evading the protective antibody response generated by vaccination. In our model, mice develop arthritis regardless of age or gender. This model exhibits an antigen-specific reaction to viable Lyme spirochetes and is dependent on the presence of CD4+ T cells. Infection with homologous spirochetes induces arthritis if infection occurs prior to development of Borrelia-specific antibodies; however, infection with a heterologous strain allows a degree of pathology more suitable for recognition of the effects of immune modulators, such as antibodies and recombinant cytokines. The greatest advantage of using the Borrelia vaccination and infection model is its ability to reflect the effects of activated T cells in the development of arthritis following infection with Borrelia organisms. In support of this statement, humans develop arthritis several months after immune priming with infection with B. burgdorferi. In contrast, infected C3H mice develop arthritis within days of infection (2, 3, 6). In addition, the latter animal models of Lyme arthritis do not highlight the role of T cells in pathology. We do not claim that our model mimics routes of natural infections in humans; indeed, infection via the bite of Borrelia-infected ticks is the only natural route of infection. However, the rationale for the use of the Borrelia vaccination and infection model, as well as its exhibition of antigenic specificity and requirement for T cells, lends significant credibility to its use. In summary, the Borrelia vaccination and infection model is a viable and valuable model by which to investigate the adaptive immune events responsible for Lyme arthritis.

In conclusion, we found that IL-35 does not play a major role in preventing the induction of severe Lyme arthritis. Our results conflict with previous reports that IL-35 is an immunosuppressive agent. Additional studies are needed to more fully characterize the role of IL-35 in Lyme arthritis.

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