Fas Ligand Deficiency Impairs Host Inflammatory Response against Infection with the Spirochete *Borrelia burgdorferi*

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Lyme disease represents a complex response to *Borrelia burgdorferi* that involves both bacterial factors as well as host responses. This results in an inflammatory reaction at several sites, including the synovial lining of joints. Synovial tissues of inflamed joints contain cells expressing high levels of Fas and Fas ligand (FasL). Although Fas stimulation is typically associated with cell death, it can also transmit stimulatory signals to certain cell types. Among these are dendritic cells and macrophages, which are abundant in inflamed synovium.

To better assess the role of FasL in the pathogenesis of Lyme arthritis, we evaluated the response to *B. burgdorferi* infection in C3H/HeJ/gld mice that bear a nonfunctional mutation in FasL. Compared to wild-type (C3H)1156-1156 mice, C3H/gld mice had a similar bacterial burden and antibody response 2 weeks and 4 weeks following infection, but they manifested a significantly reduced *Borrelia*-specific cytokine response. In addition, C3H/gld mice developed a greatly reduced incidence and severity of arthritis. The findings document a contribution of FasL to the host inflammatory response to *B. burgdorferi*.

Lyme disease is a multisystem inflammatory disorder and the most prevalent arthropod-borne infection in the United States (25). The agent of Lyme disease, *Borrelia burgdorferi*, is transmitted to mammals via the tick *Ixodes scapularis* (22, 27). The actual pathogenesis of Lyme disease in various organs is only partially understood. Evidence that antibiotic therapy Arrests or reverses manifestations of Lyme disease as well as diminishes antibody titers during the ensuing months establishes an active role of *B. burgdorferi* in this process (22). However, a subgroup of patients with persistent Lyme arthritis is known to be resistant to antibiotic therapy, to contain no detectable *Borrelia* DNA in synovial fluid by PCR, and to have a predominance of HLA-DR4, as in rheumatoid arthritis (26). This suggests that certain chronic features of Lyme disease may manifest an autoimmune basis.

Fas (CD95, Apo-1) is highly expressed by several cells in inflamed synovium, including macrophages, dendritic cells (DC), fibroblasts, and lymphocytes (17, 21). Fas ligand (FasL) is also expressed by some of the same synovial components, including macrophages (21) and γδ T cells (24). Fas recruits FADD (Fas-associated death domain protein) and caspase-8, which form the death-inducing signal complex that promotes apoptosis (8, 9). Although Fas is traditionally viewed as a death receptor that triggers apoptosis, more recent studies have shown that in certain circumstances Fas can also activate the extracellular signal-regulated kinase (ERK) and NF-κB signal pathways (2, 14, 15). In this capacity Fas can stimulate cell growth and/or differentiation in various cell types, including fibroblasts (1), cardiac myocytes (5), certain tumors (20), and T lymphocytes (3).

We recently determined that synovial FasL can stimulate DC to produce interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) and to upregulate CD86 (11). This resulted from the high expression by DC of the Fas inhibitor, c-FLIP, which renders them resistant to Fas-mediated cell death (4, 23). In addition to blocking Fas-mediated cell death, c-FLIP also diverts signals to the mitogen-activated protein kinase ERK, by the association of c-FLIP with Raf1 (15). c-FLIP can also engage the NF-κB pathway through an association with TRAF2 and RIP1 (15, 16). These alternative pathways of Fas signaling were likely responsible for the induction of IL-12 and TNF-α by Fas stimulation (23). Given the prominent role of synovial DC in regulating inflammatory responses in the joints, these collective findings suggested that FasL might contribute to the inflammatory response during the induction of synovitis following infection with *B. burgdorferi*.

To test more directly the role of FasL in vivo in Lyme arthritis, we examined the infectivity of *B. burgdorferi*, the antibody and cytokine responses, and inflammatory arthritis in C3H/HeJ (wild-type) and C3H/HeJ/FasL mutant (gld) mice. C3H mice develop multisystem infections in response to *B. burgdorferi* with a reproducibly high incidence of polyarthritis within 4 weeks after inoculation (7, 29, 30). As gld mice develop adenopathy and an autoimmune propensity with age (10), only young 4-week-old mice were used prior to the onset of either feature. The findings show that although both groups of mice developed similar burdens of infection and titers of anti-*Borrelia* antibodies, C3H/gld mice manifested a significantly reduced *Borrelia*-specific cytokine response as well as a decreased incidence and severity of arthritis compared to C3H wounds control mice. The results demonstrate a role for Fas/FasL interactions in the host immune response to *B. burgdorferi*.  

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MATERIALS AND METHODS

Mice. Four-week-old wild-type C3H/HeJ (C3H/HeJ+/+) and congenic C3H/HeJ-J/STJf/d6d1J (C3H/gld) male mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolation cages according to The University of Vermont institutional animal care and use guidelines. Experiments were conducted according to Institutional Animal Care and Use Committee-approved protocols.

Infection and evaluation of mice. Low-passage cloned B. burgdorferi strain N40, with proven infectivity and pathogenicity in mice, was used throughout the study. Spirochetes were grown in Barbour-Stoenner-Kelly complex medium (Sigma Chemical Co., St. Louis, MO) at 34°C to mid-log phase and then counted by dark-field microscopy using a Petroff-Hauser bacterial counting chamber. Spirochetes (10⁶) were inoculated subcutaneously at the middle posterior section of the neck. Mice were euthanized after 2 weeks or 4 weeks of infection. Serum was collected and assayed for B. burgdorferi-specific antibody titer and isotype. Tibiotarsal joint thickness was measured by spring-loaded digital calipers (Mitutoyo Corp., Japan). Total genomic DNA was extracted from the ear and then analyzed by SYBR green real-time PCR as previously reported (18). Heart and legs were collected for histology assessment by hematoxylin and cosin staining. Assessment of joint inflammation was made by two readers who were blinded to the code of the slides.

T-cell cytokine response to B. burgdorferi. Splenic CD4+ cells were purified by magnetic bead negative selection using antibodies to CD6 (Tib105), major histocompatibility complex class II (3F12), NK1.1 (RXK3b), and CD11b (BD Pharmingen), San Diego, CA). Splenic CD11c+ dendritic cells were purified by positive selection with biotinylated anti-CD11c beads from miniMACS (Miltenyi Biotec Inc., Auburn, CA). Purified CD4+ cells (10⁴) were plated in 48-well plates with 20 μg/ml Borrelia sonicate and 10⁵ purified CD11c+ dendritic cells in a final volume of 1 ml. Supernatants were collected after 72 h. Quantification of IL-4 and gamma interferon (IFN-γ) using a sandwich enzyme-linked immunosorbent assay (ELISA) was performed as described previously (12). Additional cytokines were also analyzed by cytometric bead array according to the manufacturer’s protocol (BD Pharmingen). Capture beads (50 μl) were added to the assay tube, combined with 50 μl of cytokine standard over a range of 1,250 pg/ml to 20 pg/ml, or with 50 μl of test samples. Phycoerythrin-conjugated detection reagent (50 μl) was added to each tube and incubated for 2 h at room temperature protected from direct light exposure. Wash buffer (1 ml) was added to each tube and centrifuged at 200 × g for 5 min. The supernatant was aspirated, and bead pellets were resuspended in 300 μl of wash buffer. Samples were analyzed by flow cytometry using cytometric bead array software.

B. burgdorferi-specific antibody determination. Ninety-six-well microtiter plates (ICN Biomedical, Aurora, OH) were coated overnight at 4°C with 20 μg/ml Borrelia sonicate in bicarbonate coating buffer, pH 9.6, and blocked with phosphate-buffered saline (PBS) plus 10% fetal calf serum at room temperature for 3 h or at 4°C overnight. After two washes with PBS-0.05% Tween 20, serially diluted sera (from 1:25 to 1:400) were applied and incubated at 37°C for 3 h. Wells were washed three times, and biotinylated anti-immunoglobulin G (IgG), IgG2a, and IgG1 (ZYMED Laboratories, San Francisco, CA) were applied individually and incubated at room temperature for 45 min. After six washes, the plates were incubated with avidin–peroxidase-conjugated anti-mouse antibody at 37°C for 30 min. Plates were washed four times, and 100 μl of substrate (50 μg/ml 3,3′-dicarbophil-P (Sigma) in 1 M sodium acetate, pH 5.6, containing 0.006% H₂O₂) was added. After 20 min at room temperature, plates were read with a Bio-Rad microplate reader at 492 nm.

Detection of Borrelia-specific recA gene by real-time PCR. DNA was extracted from the ears or bladders of mice, or from cultured B. burgdorferi, using the DNeasy method (QIAGEN, Valencia, CA) according to the manufacturer’s recommendations. Briefly, mouse tissue was cut into small pieces and digested with 180 μl of buffer ATL and 20 μl of proteinase K and then incubated at 55°C overnight. Samples were vortexed for 15 s and 200 μl of buffer AL was added, mixed thoroughly, and incubated at 70°C for 10 min. Two hundred microliters of 100% ethanol was added and mixed. The mixture was placed in the DNeasy mini spin column and centrifuged at 8,000 rpm for 3 min. The column was washed with buffer AW2 and centrifuged at 14,000 rpm for 3 min. The column was placed in a 1.5-ml microcentrifuge tube and 200 μl of buffer AE was added to the membrane, and then the tube was centrifuged at 8,000 rpm for 1 min.

Quantitative PCR of B. burgdorferi recA and mouse nidogen genes performed using SYBR green. The oligonucleotide primers used to detect the B. burgdorferi chromosomal recA gene, as previously published (18), were as follows: 5′-CTG GAT CAT TTA TGT TAT TAG ATG AGG CTC TCG-3′ (forward primer) and 5′-GCC AAA GTT CTG CAA CAT TAA CAC CTA AAG-3′ (reverse primer). For standardization of the recA copy number against a reference internal mouse DNA control, the mouse nidogen gene was amplified using the following primers: 5′-GCC ACA GAA TAC CAT CC-3′ (forward primer) and 5′-GGA CAT ACT CTG CTG CCA TC-3′ (reverse primer). PCR was performed on an ABI PRISM 7700. The standard amplification program was as follows: stage 1 (1 cycle of 94°C × 1 min), stage 2 (34 cycles of three steps; 94°C × 1 min, 60°C × 1 min, and 72°C × 1 min), and stage 3 (a three-step process for generating the dissociation curve; 95°C × 1 min, 60°C × 1 min, and 95°C × 1 min). Using this system, a threshold of 10 copies of the recA gene was detectable. The use of different amounts of ear genomic DNA from mice did not interfere with the linearity of the assay.

RESULTS AND DISCUSSION

To monitor the degree of infection following inoculation of mice subcutaneously with B. burgdorferi (strain N40), we used a quantitative PCR-based system using SYBR green that detects the Borrelia chromosomal recA gene (18). This assay could detect as few as 10 copies of the recA gene in DNA from mouse tissues that was normalized to the murine nidogen gene (data not shown). The level of Borrelia burden detectable in ear DNA was considerably higher at 2 weeks than at 4 weeks (Fig. 1A) due to spirochetal migration and is consistent with previous studies (19). At sites more distant from the inoculation area, such as the bladder, levels of Borrelia DNA were higher at 4 weeks (data not shown). However, at both time points there was no difference in spirochetal burden between wild-type and gld mice. There were also no differences in antibody titers to B. burgdorferi between C3H+/+ and C3Hgld mice at either 2 weeks or 4 weeks after infection. As shown in Fig. 1B, titers of total IgG anti-Borrelia antibodies were similar in both strains of mice, as were titers of the IgG2a and IgG1.
pronounced by 4 weeks of infection. CD4+ T cells from mice infected 2 weeks and 4 weeks previously with B. burgdorferi (Bb) were stimulated with purified Borrelia sonicate with 10^6 irradiated syngeneic dendritic cells and harvested after 72 h and analyzed for IFN-γ, IL-4, IL-10, and TNF-α. Supernatants were assayed by ELISA for IFN-γ, IL-4, and IL-10. *, indicates statistically significant differences (P < 0.05) by the Mann-Whitney method. These findings were consistent in three experiments.

In contrast to the similar levels of Borrelia antibodies, however, differences in cytokine responses to B. burgdorferi became pronounced by 4 weeks of infection. CD4+ T cells from spleens of infected or PBS-injected control mice were stimulated with B. burgdorferi in the presence of syngeneic irradiated dendritic cells. Supernatants were harvested after 72 h and analyzed for production of IFN-γ and IL-4 by ELISA. As shown in Fig. 2A, the levels of these cytokines after 2 weeks of infection tended to be higher in C3H+/+ CD4+ T cells, although this was not statistically significant. However, by 4 weeks postinfection, C3H+/+ CD4+ T cells did indeed produce significantly more IFN-γ and IL-4 than did C3Hgld CD4+ T cells (Fig. 2A). The cytokine production was due to Borrelia infection as there was no significant cytokine response of CD4+ T cells from uninfected mice. These findings were observed in three separate experiments. The ELISA findings were also confirmed and extended using cytokine bead array analysis. This revealed a statistically significant decrease in IFN-γ, TNF-α, and IL-6, but not IL-10, for C3Hgld CD4+ T cells (Fig. 2B). These differences were not due to an inherent unresponsiveness of C3Hgld CD4+ cells, as the cytokine response to CD3/CD28 stimulation was equivalent to C3H+/+ CD4+ T cells (data not shown).

Joint inflammation in response to infection with B. burgdorferi was measured by ankle thickness and histological analysis of joints. As shown in Fig. 3, there was a progressively greater increase in ankle swelling from C3H+/+ mice compared with C3Hgld mice between 2 weeks and 4 weeks following infection. Both time points revealed a statistically significant difference. Consistent with these findings, histological analysis of knees revealed a greater incidence and severity of arthritis in C3H+/+ mice as reflected in the degree of fibroblast proliferation over and into joint surface cartilage, the presence of macrophages and lymphocytes, and the destruction of meniscal tissue (Table 1). These findings were statistically significant for both incidence and severity of arthritis.

The current findings are consistent with a model in which the presence of FasL influences the nature of the immune response to B. burgdorferi by promoting an increased cytokine response as well as an augmented inflammatory synovitis. Because apoptosis of immune cells is reduced in FasL mutant gld mice, it might have been anticipated that C3Hgld mice would have a decreased response to infection. However, the opposite was observed. This could be explained by a decreased immune response to the infection. The current findings are consistent with a model in which the presence of FasL influences the nature of the immune response to B. burgdorferi by promoting an increased cytokine response as well as an augmented inflammatory synovitis. Because apoptosis of immune cells is reduced in FasL mutant gld mice, it might have been anticipated that C3Hgld mice would have a decreased response to infection. However, the opposite was observed. This could be explained by a decreased immune response to the infection.

### Table 1. Decreased arthritis in B. burgdorferi-infected gld mice

<table>
<thead>
<tr>
<th>Age and type</th>
<th>No. of mice</th>
<th>No. of arthritic joints/no. of joints examined</th>
<th>Avg arthritis severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>7</td>
<td>11/24</td>
<td>1.6 ± 0.81</td>
</tr>
<tr>
<td>gld</td>
<td>7</td>
<td>4/22</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>7</td>
<td>14/19</td>
<td>1.7 ± 0.66</td>
</tr>
<tr>
<td>gld</td>
<td>7</td>
<td>4/24</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Incidence of arthritis was determined in knee and tibiotarsal joints of infected mice, and the number of affected joints was reported as a fraction of the total number of joints examined histologically by hematoxylin and eosin staining. Arthritis severity was based on the degree of leukocyte infiltration and synovocyte proliferation over, and invasion into, articular cartilage, using a scale of 0 to 3. Average arthritis severity represents the mean score of affected joints ± standard deviations. The results are pooled from two independent observers who were blinded to the genotype of the mice. The differences in incidence of arthritis were statistically significant at both time points (day 14, P = 0.046; day 28, P = 0.025) (chi-square analysis) as was severity (day 14, P = 0.040; day 28, P = 0.045) (Mann-Whitney test).
manifest a greater inflammatory response. In addition, as C3Hgld mice age, they accumulate memory T cells that are high producers of many cytokines, particularly IFN-\(\gamma\) (10). For these reasons, only young 4-week-old mice were used in the current studies. Nonetheless, the cytokine profile of C3Hgld CD4\(^+\) T cells in response to B. burgdorferi was considerably diminished compared to wild-type CD4\(^+\) T cells, particularly at 4 weeks. This paralleled a decreased incidence and severity of arthritis in C3Hgld mice. Hence, while the absence of FasL signaling by T cells may not influence the burden of Borrelia, it greatly alters the extent to which the immune response provokes a secondary inflammatory synovitis.

The decreased cytokine response of CD4\(^+\) T cells from Borrelia-infected C3Hgld mice might result from either a decreased frequency of Borrelia-reactive T cells or a decreased responsiveness on a per cell basis. However, cytokine responses of CD4\(^+\) T cells from infected wild-type versus gld mice were very similar following anti-CD3/CD28 activation. This would favor a decreased frequency of Borrelia-reactive CD4\(^+\) T cells in gld mice. In either event, the findings indicate that in the absence of FasL there is a lack of optimal activation of at least the CD4\(^+\) T-cell cytokine response to B. burgdorferi.

Although there is no known signaling abnormality in T cells from gld mice, other studies using wild-type T cells have demonstrated a possible positive retrograde signaling via FasL to costimulate T-cell proliferation (28). In addition, we (11) and others (23) have demonstrated that dendritic cells are strongly activated via FasL signaling to produce IL-12, TNF-\(\alpha\), and IL-1\(\beta\) as well as to upregulate expression of surface CD40, CD80, CD86, and major histocompatibility complex class II. A likely explanation for the FasL-mediated activation, rather than cell death, of dendritic cells is that they express high levels of the Fas inhibitor, c-FLIP, a caspase-8 homolog that can also divert signals toward the mitogen-activated protein kinase ERK, as well as augment NF-kB activation (4, 15). Fas-mediated activation of dendritic cells could provide a strong stimulus toward proinflammatory cytokine production, which is observed in the synovial fluid of Lyme arthritis patients (32).

The contribution of Fas/FasL interactions in murine Lyme disease could also occur in other cell types in addition to dendritic cells. Fibroblasts are resistant to Fas-mediated cell death and proliferate faster in the presence of Fas stimulation (1). This could promote the expansion of synovial fibroblasts and macrophages in inflamed joints, a hallmark of rheumatoid arthritis and chronic Lyme arthritis (6, 21). In this regard, mice deficient for Fas also manifest diminished collagen-induced arthritis, and Fas-deficient macrophages have less induction of NF-kB and cytokine response following stimulation with the TLR4 agonist lipopolysaccharide (17). In addition, macrophages, like dendritic cells, are moderately resistant to Fas-induced death (21) and secrete large amounts of chemokines in response to Fas ligation, which was critical for tumor eradication in a murine model (13). Macrophage activation and recruitment are also central to the inflammatory features of murine borreliosis, and we observed substantially fewer macrophages in the synovium of Borrelia-infected gld mice. Fas signaling engages recruitment of FADD, caspase-8, and c-FLIP, and deficiency in any of these molecules is embryonically lethal (31, 33). Hence, Fas may function at many levels in cell responses during development of Borrelia infection as well as during infection to regulate inflammatory responses.

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