1 Relapsing Fever Borreliosis in IL-10 Deficient Mice

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Conflict of Interest

None of the authors have any conflict of interest to disclose.
ABSTRACT

Relapsing fever (RF) is a spirochetal infection characterized by periods of sickness with fever at time of high bacteremia that alternate with afebrile periods of relative well being during low bacteremia. Patients with epidemic RF who are doing relatively well have extraordinary high levels of IL-10 in the circulation. We investigated the possibility that IL-10 plays an important protective role in this infection using wild type and IL-10 deficient mice inoculated with virulent serotype 2 of the RF spirochete Borrelia turicatae. During peak bacteremia there was increased systemic production of IL-10 that quickly resolved in the postpeak period; in contrast, IL-6 and CXCL13 production increased during the peak but remained elevated during post-peak bacteremia. IL-10 deficiency resulted in lower bacteremia, increased specific-antibody production, higher production of CXCL13 and IL-6, and thrombotic and hemorrhagic complications affecting multiple organs with secondary tissue injury. Our results revealed that production of IL-10 is highly regulated during RF and plays an important protective role in the prevention of hemorrhagic and thrombotic complications at the cost of reduced pathogen control.

Keywords: borrelia, relapsing fever, interleukin 10, CXCL13, TNF, hemorrhage
INTRODUCTION

Relapsing fever (RF) borreliosis is a spirochetal infection caused by different Borrelia species in both endemic and epidemic forms (3). Although RF spirochetes can infect a number of organs they remain predominantly localized in the blood causing recurrent peaks of bacteremia (3, 8). Each peak is rapidly cleared upon development of specific IgM antibodies by B cells (1, 4, 16). The pathogenesis of RF is diverse depending on the species, the serotype, and the host (10, 13, 15). During epidemic RF mortality can reach 70% (32, 37). Patients with epidemic RF have extraordinary high levels of IL-10 in the circulation and still are doing relatively well clinically (17). We recently found similar high production of IL-10 in mice deficient in B and T cells with persistent high level bacteremia with *Borrelia turicatae* (19, 20). Treatment with exogenous IL-10 reduced the clinical manifestations of the infection, systemic production of the B cell chemokine CXCL13, and cerebral microgliosis (20). The absence of IL-10 resulted in rapid death from intracerebral hemorrhage in RAG2 deficient mice infected with virulent serotype 2 of *B. turicatae* (Bt2) (27, 28). The purpose of the current study was to investigate the protective function of IL-10 in RF borreliosis in immunocompetent mice. For this we compared the outcome of acute infection with Bt2 in wild type and congenic IL-10 deficient mice. The results revealed that significant but transient production of IL-10 is a prominent feature of acute RF borreliosis that serves an important protective role from multiorgan hemorrhagic and thrombotic complications.

MATERIALS AND METHODS

*Strains and culture conditions.* Bt2 has been previously characterized (15, 30, 31).

Spirochetes were cultured in BSK-H media (Sigma) with 12% rabbit serum. Prior to infection,
borrelia viability was assessed by motility using phase-contrast microscopy and serotype identity was confirmed by Western blot with anti Vsp2 monoclonal antibody 5F12 (14, 30).

**Mouse infections.** Female 4 to 5-week-old, C57BL/10SgSnAi (WT) and C57BL/10SgSnAi-[KO] IL-10 (IL-10-/-) mice were obtained from Taconic Farms (Germantown, NY). The mice were inoculated intraperitoneally (i.p) with $5 \times 10^4$ Bt2 spirochetes in 200µl of PBS or with PBS alone. Groups of 4-8 mice each were used for all experiments. Mice were euthanized by inhalation with isofluorane followed by cardiac exanguination and extensively perfused with PBS to reduce blood contamination of tissues (11). To confirm infection necropsy plasmas were cultured for 2 weeks and examined for the presence of spirochetes by phase-contrast microscopy. Brain, lungs, spleen, liver, and kidneys were removed at necropsy, fixed in 4% paraformaldehyde overnight at 4°C, paraffin-embedded, and stained by H&E and TUNEL as described (26).

**Cytokines and chemokines.** Concentrations of TNF, IL-6, IL-10, IL-12 (IL-12p70), GM-CSF, IFN-γ, CCL2, CCL3, CCL5, and CXCL1 were quantified in necropsy plasma in both infected and uninfected control mice with the Luminex 100 Multi-Analyte Profiling System (Luminex Corp, Austin, TX) using BioPlex Manager software (Bio-Rad Laboratories, Hercules, CA) and Lincoplex cytokine assay kits (Linco Research, St. Charles, MO) per the manufacturer’s instructions. We measured CXCL13 by regular ELISA (R&D Systems, Minneapolis, MN).

**DNA extraction and TaqMan PCR.** One hundred microliters of necropsy plasma were centrifuged at 8,500g for 30 min to precipitate spirochetes and the pellets used for DNA extraction with QIAMP DNA Micro kits (Cat#56304) according to the manufacturer’s instructions. The amount of total DNA was quantified with a Nanodrop and determined by the A260/A280 ratio. Quantitative real time PCR was performed on an ABI Prism 7500 Sequence
Detection System (Applied Biosystems) using 100ng of DNA in 50µL of reaction volume with TaqMan Universal master mix reagents (Applied Biosystems) under standard conditions for 40 cycles. The primers and probes for the quantification of spirochetes targeted the borrelia 16S rRNA gene (2, 9, 35). A standard curve using log10 dilutions from DNA extracted from a known number of cultured Bt2 spirochetes in 100ng of uninfected host DNA was used for quantification (35). Analysis was done with the AB Sequence Detection System software version 1.2.3 by extrapolation of the sample’s Ct to the standard curve.

Specific antibody production. Plasma obtained by cardiac exanguination was assayed for the presence of anti Vsp2 IgG and IgM antibodies by ELISA. Microtiter plates were coated with 5µg/ml purified variable surface protein 2 (Vsp2) produced by n-Octyl β-D-glucopyranoside (OGP) non-ionic detergent extraction (14). Plasma dilutions were added to plates for 1h at 37°C and bound murine Ig was detected by addition of AP-conjugated antibody to murine IgG or HRP-conjugated antibody to murine IgM. The relative amounts of plasma anti Vsp2 antibody were determined by comparing the optic density between groups.

Statistical analysis. Results were expressed as median and range or mean and SD. Two sided non-parametric tests were used to determine differences between medians (Mann-Whitney U test) and t-test for differences between means. Differences in percentages were analyzed by Fisher’s exact test. P<0.05 was considered significant. All analysis used GraphPad Prism 4 for windows.

RESULTS

Bacteremia and specific antibody producion in IL-10 deficient mice. First we examined the question of whether IL-10 affects the pathogen load during acute infection. For this, eight
wild type (WT) or IL-10 deficient (IL-10-/-) C57BL/10 mice were inoculated intraperitoneally
with 5x10^4 Bt2 spirochetes and their tail vein blood examined for the presence of spirochetes by
phase-contrast microscopy at various times. No spirochetes were observed in the blood of WT or
IL-10-/- mice in the morning or afternoon of day 3 after inoculation. However, on the morning of
day 4 after inoculation spirochetes were visible in tail vein blood in all 8 WT mice compared
with only 3/8 IL-10-/- mice (p<0.05). This suggested that the kinetic of the first peak of
bacteremia was difference between WT and IL-10-/- mice. To investigate this further we
necropsied half of the animals (N=4 per group) on day 4 and the other half on day 5 (N=4 per
group) and measured the pathogen load in the blood by phase-contrast microscopy with a
Petroff-Hauser chamber; the sensitivity of this method is ≥5x10^4 spirochetes/ml. The results
showed there were significantly more spirochetes in WT than in IL-10-/- mice examined in the
afternoon of day 4: the median (range) was 2x10^6 (5x10^4-4.1x10^6) in WT compared to 2.5x10^4
(0-4x10^5) in IL-10-/- mice (p<0.01, Figure 1A). Mice examined in the morning of day 5 showed
circulating spirochetes by phase-contrast microscopy in 1/4 IL-10-/- and 0/4 WT mice.

Because of the limited sensitivity of microscopic examination with the Petroff-Hauser
chamber we wanted to confirm the previous results with a more sensitive method. For this we
used real time PCR amplification of borrelial 16S rRNA. The sensitivity of this assay is about 1
spirochete per ng of DNA. The results revealed that all 8 WT mice and 6/8 of the IL10-/- mice
necropsied on days 4-5 had detectable spirochetes in plasma. In mice examined in the afternoon
of day 4 the median (range) number of spirochetes per 100ng of DNA was higher in WT than in
IL10-/- mice: 4.8x10^3(1.2 x10^3-6.9 x10^5) compared with 1.7x10^3(0.4 x10^1-5.7x10^4) (p=0.05,
Figure 1B). In comparison, the median (range) number of circulating spirochetes was much
lower in the mice examined in the morning of day 5 in both groups: 9.5 x10^2 [3.5 x10^2-9.9 x10^2]
in the WT group and $1 \times 10^2$ [1.1 $\times 10^2$-1.9$\times 10^3$] in IL-10/-/- mice. In WT but not in IL-10/-/- mice
the bacteremia significantly decreased between the afternoon of day 4 and the morning of day 5
(p=0.02 for the WT group and p=0.10 for the IL-10/-/- mice). We concluded that the kinetics and
intensity of bacteremia were different in IL-10/-/- mice.

Since IL-10/-/- mice showed lower bacteremia and Vsp-specific antibodies are responsible
for resolution of peak bacteremia in RF borreliosis next we studied whether IL-10/-/- mice
developed a stronger specific antibody response. To investigate this we compared the amount of
anti Vsp2 antibodies in necropsy plasma by ELISA on days 4-5 after inoculation of Bt2. The
results revealed that IL-10/-/- mice had significantly higher amount of IgM (Figure 1C) and IgG
(Figure 1D) anti Vsp2 antibodies compared to WT mice.

**Cytokine and chemokine response to the infection in IL-10 deficient mice.** Next we
studied the cytokine and chemokine response to the infection in WT and IL-10/-/- mice. Mice
sham-inoculated with PBS were used as controls (N=4 each). For this we measured by ELISA
the levels of various cytokines and chemokines in necropsy plasma from the same mice studied
in the previous section. The cytokine results showed that at times of peak bacteremia in WT
mice there was elevated production of IL-10, about 20 times more than in uninfected controls
(p<0.05, Figure 2A). In contrast, very little IL-10 was detected in the plasma of the 4 infected
WT mice necropsied in the morning of day 5 (Figure 2A). A similar rapid rise and drop was
observed for GM-CSF. The only other cytokine that increased as a result of infection was IL-6.
However, unlike IL-10 and GM-CSF, the circulating levels of IL-6 did not drop in the post-peak
period (Figure 2A). We did not detect any change in the concentrations of TNF, INF-γ or IL-12
in WT mice as a result of the infection. As expected, infected IL-10/-/- mice did not have any
detectable IL-10 in the blood (Figure 2B). The only cytokine that increased with infection in IL-
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10/- mice was IL-6 with marked variability from mouse to mouse (p<0.05, figure 2B). Similar to WT mice, IL-6 did not decrease and in fact was higher in mice examined in the morning of day 5 compared with mice examined the day before. The median (range) levels of IL-6 in infected WT and IL-10/- mice were similar, 60 (2-222) versus 132 (23-644) pg/ml (p=0.27, Figure 2A&B).

The comparison of the chemokine response to the infection revealed that in WT mice there was large production of the B cell chemokine CXCL13 that, unlike IL-10 or GM-CSF but similar to IL-6, remained elevated in the post-peak period (Figure 3A). Small but significant increases in CCL2 and CXCL1 were also observed in WT mice during peak bacteremia, although of smaller magnitude compared with CXCL13 (Figure 3A). CXCL13 was the only chemokine that increased as a result of the infection in IL-10/- deficient mice, with values more than 10 times higher than in uninfected control mice (p<0.05, Figure 3B). Compared with infected WT mice, the median (range) CXCL13 levels in infected IL-10/- mice were also more than 10 times higher: 6.96x10^3 (7 x10^2-8.3x10^3) pg/ml versus 6.1x10^2 (3.3x10^2-9.2x10^2) pg/ml (p<0.001, Figure 3A&B). We concluded that the predominant cytokines and chemokines produced in acute RF borreliosis are IL-10 and CXCL13, respectively. The absence of IL-10 results in much greater production of CXCL13. Unlike CXCL13, the production of IL-10 is transient.

**Disease in IL-10 deficient mice.** Since infection in WT mice resulted in production of elevated amounts of IL-10 during peak bacteremia (Figure 2A), it was possible that IL-10 was helping control disease as previously observed in RAG1 and Igh6 deficient mice (19, 20). To investigate this possibility one of us (DL) had performed masked clinical examinations in WT and IL10/- mice daily after inoculation with Bt2 or PBS for 5 days in the mice previously
discussed. Analyses of the clinical disease severity scores showed that unlike RAG1 or Igh6
deficient mice or scid mice (15, 19, 20), none of the infected WT or IL-10/-/- immunocompetent
mice showed any signs of clinical disease or had lost weight compared with uninfected controls
(not shown). Examination of the spleens removed at necropsy showed splenomegaly of similar
degree in all infected WT and IL-10/-/- mice compared with uninfected controls (not shown).

Next we studied whether IL-10 deficiency resulted in any pathological abnormalities as a result
of the infection. Macroscopic examination of mice necropsied on days 4 or 5 after inoculation
revealed the presence of hemorrhages in several organs in IL-10/-/- mice but not in WT mice or
any of the uninfected controls: All eight infected IL-10/-/- mice had hemorrhage in at least one
organ compared with none of the 8 infected WT mice (Table 1, p<0.01). These hemorrhages
were more common in the kidneys and the liver (62%) than in the brain or the lungs (37%)
(Table 1). Microscopic examination of H&E-stained tissue sections confirmed that hemorrhages
occurred only in infected IL10/-/- mice. The most prominent hemorrhages were found in the
subcapsular area of the kidneys (Figure 4A). In addition to hemorrhage, the livers of IL-10/-/-
mice also showed thrombotic occlusion of multiple vessels surrounded by lobular necrosis
(Figure 4C). Although the livers of infected WT mice did not show hemorrhage or necrosis, they
did show scattered foci of inflammatory cells and hepatocellular degeneration (Figure 4B&D).

We also observed areas with hemorrhage and leukocyte accumulation in and around
microvessels in the lungs, sparse foci of inflammatory cells in the leptomeninges, and small
hemorrhages in the brain parenchyma and cerebellum of infected IL-10/-/- mice (not shown). To
confirm this observation we similarly infected new groups of WT and IL-10/-/- mice with Bt2 or
PBS (N=4 each) and necropsied them 7 or 12 days later. Macroscopic examination at necropsy
confirmed the presence of hemorrhages in most of the IL10/-/- mice (7/8) compared with none of
the WT mice (0/4) (p<0.05). As before, none of the uninfected control mice had any detectable hemorrhage.

To confirm the development of tissue injury in IL-10-/- mice we stained paraffin-embedded liver sections from infected and uninfected WT and IL-10-/- mice with the TUNEL assay, which labels both apoptotic and necrotic cells (21). The results revealed the presence of multiple large areas of TUNEL staining in the liver in the vicinity of thrombosed microvessels only in IL-10-/- deficient mice that corresponded to the necrotic areas previously observed with H&E staining (Figure 4E). Examination of the liver of infected WT mice showed scattered TUNEL positive cells (Figure 4F). We concluded that IL-10 prevented hemorrhagic and thrombotic complications and tissue injury during acute RF borreliosis.

**DISCUSSION**

The first indication that IL-10 plays an important protective role in RF borreliosis came from the clinical study of Cooper et al. that found extraordinarily high levels of IL-10 in the blood of patients with epidemic RF borreliosis who were doing relatively well at the time of admission (17). Further evidence of an important protective role for IL-10 in this infection came from our previous study in RAG1 and Igh6 deficient mice persistently infected with *B. turicatae* that showed a strong negative correlation between circulating levels of IL-10 and clinical disease and improvement of clinical disease by treatment with exogenous IL-10 (19, 20). In a more recent study in RAG2 deficient mice we demonstrated a critical role for IL-10 in survival during acute severe infection with *B. turicatae* and showed that the protective role of IL-10 is mediated in large part by neutralization of TNF (27, 28). In the present study we investigated for the first time the role of IL-10 in acute RF borreliosis in immunocompetent mice. The main findings were the following: (1) Immunocompetent mice produce elevated amounts of IL-10 during acute RF
borreliosis but only during times of high bacteremia. (2) IL-10 deficiency altered the kinetics of bacteremia and lowered the pathogen load in the blood via increased specific-antibody production. (3) The B cell chemokine CXCL13 is the major chemokine produced in response to the infection and significantly increases in the absence of IL-10. (4) In the absence of IL-10, acute RF borreliosis results in multiorgan thrombotic and hemorrhagic complications with secondary tissue injury.

The results from this study revealed that in immunocompetent mice increased production of IL-10 reduces the host’s ability to control peak bacteremia (Figure 1). This is the opposite of what we observed previously in RAG2 deficient mice persistently infected with *B. turicatae* in which IL-10 deficiency compromised pathogen control via increased leukocyte apoptosis mediated to a great extent by TNF (27, 28). Others have shown that elimination of pathogens from immunocompetent hosts is more effective in the absence of IL-10, including studies with the related Lyme disease spirochete *B. burgdorferi* (22-24). In murine Lyme borreliosis in immunocompetent mice, IL-10 deficiency resulted in a 10-fold decrease in the number of *B. burgdorferi* spirochetes present in ankle tissues (6). It has been hypothesized that IL-10 may suppress the antimicrobial activity of the innate immune system so in its absence there is more-efficient bacterial killing (18). Similar to our results, although IL-10-/- deficiency helped control the pathogen load in the murine model of Lyme borreliosis it also resulted in worsening pathology (6). It is possible that improved pathogen control in IL-10 deficient mice occurs because phagocytosis by the innate immune system improves in the absence of IL-10 (5, 24). However, since specific antibody is primarily responsible for the control and resolution of peak bacteremia in RF borreliosis (1, 4, 11, 29), it is more likely that the lower peak bacteremia in IL10-/- mice in our study was due to stronger and more rapid specific antibody production
(Figure 1C & D). One way this could occur is via increased production of the B cell chemokine CXCL13 in response to infection (Figure 3B). In murine Lyme borreliosis the absence of IL-10 also results in increased antibody production (6).

The cytokine network plays a pivotal role in the orchestration of inflammatory responses to bacterial infection. Unlike RAG1-/- (20) or RAG2-/- mice (27) that showed persistent high level bacteremia and continuous high production of IL-10, in immunocompetent mice this elevation occurred transiently, only at times of peak bacteremia (Figure 2A). It is likely that this transient production of IL-10 attenuated the inflammatory syndrome that would otherwise occur due to the presence of high numbers of bacteria in the blood, and protects against tissue injury. Recently we confirmed that infection with RF spirochetes results in increased production of IL-10 not only systemically but also in tissues (H. Gelderblom and D. Cadavid, unpublished observation). A likely function of this IL-10 surge is suppression of TNF production that can be fatal, as shown in RAG2/IL-10-/- mice infected with Bt2 (27, 28). The patients infected with *B. recurrentis* studied by Cooper et al. that had high levels of IL-10 also had relatively low levels of TNF (17). In infected RAG-/- mice we have shown that IL-10 down regulates both systemic and local production of TNF (19, 20). Although we did not measure TNF production in tissues in this study it is possible that increased local production of TNF in IL-10-/- mice was implicated in the observed microvascular and parenchymal tissue injury. One possible reason for the lack of increased TNF levels in the blood of infected IL-10-/- mice was their lower bacteremia. In RAG1-/- mice there was a strong positive correlation between pathogen load and TNF production systemically and in tissues (20).

Since RF spirochetes remain predominantly localized to the blood it is not surprising that the main complication of IL-10 deficiency was damage to the microcirculation of multiple
organs. However, we were surprised by tissue injury occurred in infected IL-10/- mice despite their lower peak bacteremia (Figures 1 and 4). One alternative explanation is that in IL-10/- mice peak bacteremia may had occurred earlier than day 3 after inoculation when they were first examined by tail-vein puncture or in the night between days 3 and 4; this could be explained by the higher than usual inoculum we used in this study. The alternative explanation is that IL-10 plays a critical role preventing vascular injury even at the lower peak bacteremia observed in IL10/- mice. Vascular injury is a well-known and important complication of infection with most pathogenic spirochetes, notably *Treponema pallidum* (12), RF (10) and Lyme disease (7) borrelias. The mechanism of vascular injury in spirochetal infections is not known. Outer membrane lipoproteins from spirochetes have been shown to activate endothelial cells to produce pro-inflammatory mediators (25, 33, 34, 36, 38). Recently we showed that Bt2 and Vsp2 cause apoptosis of human brain microvascular endothelial cells (Londoño D and Cadavid D, unpublished results) and demonstrated apoptosis in brain endothelial cells from Bt2-infected RAG2/IL-10/- mice (27).

This study confirms at the experimental level an important protective role for IL-10 in acute RF borreliosis in immunocompetent mice via prevention of hemorrhagic and thrombotic complications. Since injury occurred in IL-10/- mice despite lower bacteremia, this indicates that the improved pathogen control conferred by the absence of IL-10 is ultimately not advantageous to the host because of the resultant vascular injury that took place. These findings add to the body of evidence on the important and complex protective role that IL-10 plays in bacterial infections.
FIGURE LEGENDS

Figure 1. A Bacteremia and specific antibody production in WT and IL-10-/- mice infected with Borrelia turicatae. Blood was harvested by cardiac puncture from groups of 4 mice each infected for 4 days. Spirochetes were counted by phase contrast microscopy. Results are presented as spirochetes/ml plasma (log scale) in box plots. The bacteremia was significantly higher in WT mice (shaped bars) than IL-10-/- mice (open). B Spirochetal load per 100 ng of plasma DNA using TaqMan PCR. For this total DNA was extracted from 100μl of plasma pellets and measured for Borrelia spp. 16S rRNA gene. Results for WT (shaped boxes) and IL-10-/- (open boxes) mice are presented as box plots. C&D Detection of Vsp2-specific IgM (C) and IgG (D). Plasma was collected from the indicated WT and IL-10-/- on days 4 and 5 after inoculation of Bt2. Vsp2-specific antibody levels were determined by ELISA using Vsp2 extracted from cultured Bt2 as antigen. Data represents the fold OD (optical density) difference and bars indicate the mean (SD) scores. Uninfected controls are shown by a dotted line.

Figure 2. Cytokine response to the infection in wild type and IL-10 deficient mice. We measured the concentrations of cytokines in wild type (WT, panel A) and IL-10-/- (panel B) mice in necropsy plasma by ELISA on days 4 (N=4 each) or 5 (N=4 each) after inoculation of serotype 2 of B. turicatae (Bt2) or PBS as a control (N=4). Results are presented as mean (SD) pg/ml by whether mice were necropsied during peak (day 4) or post-peak (day 5) bacteremia.

Figure 3. Chemokine response to the infection in wild type (WT) and IL-10 deficient mice. We measured the concentrations of several chemokines in WT (panel A) and IL-10-/- (panel B) mice in necropsy plasma by ELISA after inoculation of serotype 2 of B. turicatae (Bt2) or PBS as a
control. Results are presented as mean (SD) pg/ml and by whether mice were necropsied during periods of high (day 4) or low (day 5) bacteremia.

**Figure 4.** Effect of IL-10 deficiency on the pathology of acute RF borreliosis. Representative histological H&E-stains (A to D) and TUNEL staining (E&F) from kidney and liver of IL-10-/- (A&C and E&F) and wild type (B&D) mice necropsied 4-5 days after inoculation of serotype 2 of *B. turicatae*. The kidneys in IL-10-/- mice show large sub-capsular hemorrhages (*) that were not seen in infected WT mice (Figure 4B). The liver in IL-10-/- mice showed necrotic areas (*) next to thrombosed microvessels and a higher influx of inflammatory cell in vessels (arrow on Figure 4D) not seen in WT mice. TUNEL shows large areas of positive staining in necrotic areas in the IL-10-/- mice (Figure 4E) and scattered TUNEL positive cells in the WT mice (Figure 4F) (20X magnification).
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a Hemorrhages were studied macroscopically at necropsy 4-5 days after intraperitoneal inoculation of 5x10^4 B. turicatae spirochetes or PBS as a control.

b Number of mice affected/total of mice examined.
References


