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IL-10 Helps Control Pathogen Load during High-Level Bacteremia

Diana Londoño,* Adriana Marques, † Ronald L. Hornung, ‡ and Diego Cadavid*‡

During relapsing fever borreliosis, a high pathogen load in the blood occurs at times of peak bacteremia. Specific IgM Abs are responsible for spirochetal clearance so in absence of B cells there is persistent high-level bacteremia. Previously, we showed that B cell-deficient mice persistently infected with *Borrelia turicatae* produce high levels of IL-10 and that exogenous IL-10 reduces bacteremia. This suggested that IL-10 helps reduce bacteremia at times of high pathogen load by a B cell-independent mechanism, most likely involving innate immunity. To investigate this possibility, we compared *B. turicatae* infection in RAG2/IL-10−/− and RAG2+/+ mice. The results showed that IL-10 deficiency resulted in significantly higher bacteremia, higher TNF levels, and early mortality. Examination of the spleen and peripheral blood showed markedly increased apoptosis of immune cells in infected RAG2/IL-10−/− mice. Neutralization of TNF reduced apoptosis of leukocytes and splenocytes, increased production of IFN-γ by NK cells, increased phagocytosis in the spleen, decreased spirochtemia, and rescued mice from early death. Our results indicate that at times of high pathogen load, as during peak bacteremia in relapsing fever borreliosis, IL-10 protects innate immune cells from apoptosis via inhibition of TNF resulting in improved pathogen control. The Journal of Immunology, 2008, 181: 2076–2083.

Recently, we observed that bacteremia with serotype 2 of *B. turicatae* (Bt2) was much higher in RAG1−/− mice, which are B and T cell deficient, than in Igh6−/− mice, which are only deficient in B cells (6). Moreover, we found a strong positive correlation between the level of bacteremia and circulating levels of IL-10. However, unlike other infectious models where IL-10 impairs pathogen control (12–15), administration of high doses of exogenous IL-10 significantly lowered the pathogen load (6). To further investigate the possibility that IL-10 could help with pathogen control at times of high bacteremia, we studied Bt2 infection in RAG2−/− mice with or without IL-10 deficiency. Our results show that at times of high bacteremia, IL-10 is fundamental to inhibit TNF and prevent apoptosis of innate immune cells, which causes further loss of pathogen control by preventing phagocytosis.

Materials and Methods

Strains and culture conditions

Bt2 has been previously characterized (9, 16). Spirochetes were cultured in BSK-H medium (Sigma-Aldrich) with 12% rabbit serum. Before infection, *Borrelia* viability was assessed by phase-contrast microscopy and serotype identity was confirmed by Western blot with anti-variable small protein 2 (Vsp2) mAb 5F12 (16, 17).

Mouse infections

Female 4- to 5-wk-old C57BL/10SgSnAi(-KO)RAG2 (RAG2−/−) and RAG2/IL-10−/− double-deficient mice generated by crossing C57BL/10SgSnAi(-KO)IL-10 and C57BL/10SgSnAi(-KO)RAG2 were obtained from Taconic Farms (18). The mice were inoculated i.p. with 10^3 Bt2 spirochetes in 200 μl of PBS or with PBS alone for controls and kept for up to 2 wk. Groups of three to eight mice each were used for all experiments. Mice were maintained under specific pathogen-free conditions. Housing and care was in accordance with the Animal Welfare Act in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. For necropsy, mice were anesthetized with isoflurane before euthanasia, blood was collected by exsanguination with cardiac puncture, and plasma was obtained by blood centrifugation. Intra-cardiac total-body perfusion with 30 ml of PBS was performed to minimize blood contamination of tissues. Spirochetes were counted in necropsy plasma using a Petroff-Hausser chamber. Spleen and liver were removed at necropsy and weighted in an electronic balance.

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Abbreviations used in this paper: RF, relapsing fever; Bt2, *B. turicatae* serotype 2; Vsp2, variable small protein 2; TRITC, tetramethylrhodamine isothiocyanate.
The TUNEL assay labels apoptotic and early necrotic cells. For the spleen, 5- to 10-μm frozen axial sections were digested with 20 mg/ml proteinase K (Worthington Biochemical) for 10 min at room temperature. DNA fragments were labeled with biotinylated-conjugated dUTP using TdT (Roche Molecular Biochemicals) for 1 h at 37°C and visualized with HRPlabeled streptavidin with diaminobenzidine as the chromogen. The assay was standardized using sections treated with DNase I as positive control. Negative control sections were incubated without the TdT enzyme. Digital image analysis was used to measure apoptosis in a Petroff-Hausser chamber. Results are shown as the percentage of total cells per 40 field showing TUNEL staining.

Immunofluorescence

Three of 5- to 10-μm frozen axial spleen sections from Bt2-infected RAG2/IL-10−/− mice treated with anti-TNF-Ab necropsied on days 5 (n = 3) and 12 (n = 3) and untreated necropsied on day 5 (n = 3) were mounted on superfrost glass slides (Fisher). Following ice-cold acetone fixation, sections were blocked with a casein solution (Biogenex) and stained with rat mAb anti-mouse F4/80 (MCAP497; Serotec), anti-Vsp2 (16, 17), or anti-mouse IFN-γ FITC-labeled Ab (RM9001; Invitrogen) for 30 min. The secondary reagents were anti-rat IgG FITC- or tetramethylrhodamine isothiocyanate (TRITC)-labeled Ab (TACS TdT kit, catalog no. TA4625; R&D Systems) and counted them by standard light microscopy in 15 consecutive fields at ×40 magnification; all counts were repeated at least three times for consistency. Results were expressed as the percentage of total cells per ×40 field showing FITC or TRITC labeling.

Flow cytometry

One hundred milligrams of spleen from RAG2/IL-10−/− Bt2-infected mice treated or not with anti-TNF Ab were homogenized and splenocytes were
isolated using a cell strainer (Falcon; BD Biosciences). Erythrocytes were lysed with Tris-NH4Cl (Sigma-Aldrich). Cells were recovered by centrifugation and washed once in FACS buffer. Washed splenocytes were incubated for 20 min at 4°C with 10 μg of mouse IgG (Sigma-Aldrich) for FcR blocking, followed by a 20-min incubation with rat anti-NK-1.1 cell PE-conjugated Ab (BD Biosciences). After another wash in FACS buffer, cells were fixed in FACS buffer plus 3.7% paraformaldehyde. For intracellular staining of IFN-γ, cells were permeabilized with 250 μl of Cytofix/CytoPerm solution (BD Biosciences) for 20 min at 4°C and one wash in PermWash solution (Sigma-Aldrich); stimulation of ex vivo IFN-γ production was not done. Cells were then resuspended in 50 μl of PermWash plus 3 μl of anti-IFN-γ FITC Ab (BD Biosciences) and incubated for 30 min at 4°C. After two washes in PermWash, the cells were resuspended in FACS buffer and analyzed immediately using the FACSCalibur flow cytometer with CellQuest analysis software (BD Biosciences). Cells were gated via their forward and size scatter properties, excluding dead cells and debris. For identification of NK-1.1+/IFN-γ+ cells, dot plots quadrants were set so that the NK cells that were stained with the isotype controls were within the 99.5th percentile positive and a total of 10,000 were collected.

Statistical analysis

All statistical analyses were performed using GraphPad PRISM 4 (GraphPad Software). All data are presented as mean ± SD or median (range). The Mann-Whitney or unpaired t test were used to compare data depending on the distribution. The Spearman’s test was used to determine correlations. Results were considered statistically significant if \( p < 0.05 \) and highly statistically significant if \( p < 0.01 \).
increased pathogen load and early mortality in infected RAG2/IL-10−/− mice could be at least partially due to increased production of TNF.

**TNF neutralization decreases bacteremia and increases survival**

To investigate the effect of increased TNF in Bt2-infected RAG2/IL-10−/− mice, we treated the mice with neutralizing anti-TNF Ab or PBS as a control beginning 2 h before infection and necropsied them on day 5 (n = 3) or day 12 (n = 6). Mice necropsied on day 12 received a second dose of anti-TNF Ab on day 5. Again, the untreated mice died by day 4–5 and had very high levels of bacteremia at necropsy (Fig. 3A). In contrast, mice treated with anti-TNF Ab did not die and their bacteremia had decreased by ~15-fold when necropsied on day 12 (p < 0.05, Fig. 3A).

**FIGURE 5.** IL-10 deficiency increases apoptosis of innate immune cells. Groups (n = 3 each) of RAG2−/− and RAG2/IL-10−/− mice, untreated or treated with anti-TNF Ab, were infected with Bt2. Results (mean) for uninfected controls are shown by a dotted line. A, The percentage of apoptotic PBMC was determined counting the number of TUNEL-positive nuclei on 15 × 200 phase microscopic fields divided by the total number of cells. The results are presented as mean percentage and the error bars indicate the SD. B, Apoptosis in the spleen was measured using digital image analysis of TUNEL-stained sections. Results are expressed as sum mean density per ×40 microscopic field positive for TUNEL; the error bars indicate SD. Notice the significant increase in apoptosis in PBMC (A) and spleen (B) in infected RAG2/IL-10−/− mice compared with RAG2−/− mice. Also, notice that treatment with anti-TNF Ab significantly reduced apoptosis in both PBMC and spleen.

**FIGURE 6.** TNF neutralization increases IFN-γ production in the spleen by NK cells. A, We measured the concentration of IFN-γ in necropsy plasma in Bt2-infected and uninfected RAG2−/− and RAG2/IL-10−/− on day 5 or 12 after inoculation. The dotted line shows the mean values for uninfected mice. Notice that neutralization of TNF resulted in a significant increase of IFN-γ over time. B and C, Immunofluorescence staining of spleen sections with FITC-labeled anti-IFN-γ Ab in an infected mouse treated with anti-TNF Ab and necropsied on day 12 (B) compared with an untreated infected mouse necropsied on day 5 (C). D, Left panel, The dot plot setting and the central and right panels represent spleen cells from representative RAG2/IL-10−/− deficient mice treated or not with anti-TNF Ab stained for NK-1.1 and IFN-γ and examined by FACS. Note that treatment with anti-TNF Ab increased the percentage of gated cells positive for both NK-1.1 and IFN-γ.
Neutralization of TNF significantly lowered circulating levels of TNF (Fig. 3B). The decrease in bacteremia (Fig. 3A) and circulating levels of TNF (Fig. 3B) of ~20% observed by day 5 in infected RAG2/IL-10−/− mice treated with anti-TNF Ab appeared sufficient to rescue them from early death.

Pathogen control correlates with splenomegaly

The control of pathogen load in the RAG2/IL-10−/− mice treated with anti-TNF Ab could take place in organs of the reticuloendothelial system. To investigate this, we compared the size of the spleen and the liver in Bt2-infected RAG2/IL-10−/− mice treated or not by neutralization of TNF and necropsied on day 5 or 12. RAG2−/− mice necropsied on day 5 were included for comparison. The spleen and the liver were larger in infected RAG2/IL-10−/− mice than in RAG2−/− mice (p < 0.05 for both comparisons, Fig. 4A). Treatment of infected RAG2/IL-10−/− mice with anti-TNF resulted in significantly increased weight of the spleen but not the liver over time (Fig. 4A). There was a highly significant correlation between the increase in the weight of the spleen and the reduction of bacteremia (r = −0.80, p < 0.001) (Fig. 4B).

IL-10 deficiency increases apoptosis of immune cells, which is reversed by neutralization of TNF

Increased apoptosis of innate immune cells due to the higher levels of TNF could potentially cause the additional loss of pathogen control in RAG2/IL-10−/− mice. To investigate this, we measured apoptosis by TUNEL in the spleen and PBMC from Bt2-infected RAG2/IL-10−/− and RAG2−/− mice. The results showed that infected RAG2/IL-10−/− mice examined on day 5 had significantly more apoptotic cells in both PBMC (Fig. 5A) and spleen (Fig. 5B) than RAG2−/− mice (p < 0.01 for both comparisons). Uninfected mice had very low apoptosis in spleen and PBMC (Fig. 5). Microscopic examination of TUNEL-stained spleen sections revealed apoptosis of cells with morphological features of macrophages and lymphocytes. Neutralization of TNF significantly reduced apoptosis in both PBMC (Fig. 5A) and spleen (Fig. 5B) in mice examined on day 5 or 12; by day 12, apoptosis in the spleen had decreased to levels similar to uninfected controls. These results indicated that increased apoptosis of macrophages and lymphocytes in RAG2/IL-10−/− mice was mediated by increased TNF.

TNF neutralization increases IFN-γ production by spleen NK cells

There was a significant negative correlation between increased IFN-γ and pathogen load in the blood (r = −0.52, p = 0.02) (Fig. 6A). Because improved pathogen control was strongly associated with splenomegaly (Fig. 4B), we next examined whether treatment with anti-TNF resulted in increased production of IFN-γ in the spleen. Examination of spleen sections by immunohistochemistry with an anti-IFN-γ Ab showed positively stained cells were much more abundant in treated mice examined on day 12 than in untreated mice examined on day 5 (Fig. 6, B and C). Because RAG2−/− mice are T cell deficient, it was likely that NK cells were the source of the IFN-γ in these mice. We investigated this by FACS analysis of splenocytes. The results showed a significantly higher percentage of NK cells producing IFN-γ in spleen cells from anti-TNF treated when compared with untreated RAG2/IL-10−/− mice: the mean (SD) percentage of cells stained for both NK1.1 and IFN-γ was 22.27 (2.6) in treated mice compared with 10.94 (0.6) in untreated mice (p = 0.04). Fig. 6D shows a representative example. We concluded that neutralization of TNF restored production of IFN-γ by NK cells in Bt2-infected RAG2/IL-10−/− mice.

FIGURE 7. TNF neutralization increases phagocytosis of B. turicatae in the spleen. A and B. Immunofluorescence staining for macrophage activation marker F4/80 (green) in spleen sections from a mouse treated with anti-TNF Ab and necropsied on day 12 (A) or untreated and necropsied on day 5 (B) (×100 magnification). C and D. Immunofluorescence microscopic staining for Vsp2 shows Bt2 spirochetes (green) in the spleen capsule from an anti-TNF-treated mouse necropsied on day 12 (C) and in the spleen parenchyma from an untreated mouse necropsied on day 5 (D) (×400 magnification). E and F. Double immunofluorescence microscopy for Vsp2 (green) and F4/80 (red) in the spleen of a mouse treated by TNF neutralization and necropsied on day 12 (E) or untreated and necropsied on day 5 (F) (×400 magnification). Note the yellow color indicating colocalization of Vsp2 and F4/80 in activated macrophages. Inset in E shows a spirochete (arrow) next to an activated macrophage (+) that also stains with Vsp2; G and H. Negative control sections from treated (G) and untreated (H) mice incubated with concentration and isotype-matched primary and secondary Abs.

TNF neutralization increases phagocytosis in the spleen

The above experiments showed that neutralization of TNF in infected RAG2/IL-10−/− mice improved pathogen control, decreased immune cell apoptosis, increased production of IFN-γ by NK cells, and increased splenomegaly. It was likely that the increased production of IFN-γ resulted in increased phagocytosis in
the spleen, which would explain the improved control of bacteremia. We examined this possibility by measuring phagocytosis in the spleen by immunofluorescence microscopy. For this, we stained spleen sections from Bt2-infected RAG2−/− and RAG2/IL-10−/− mice with mAb against the variable major protein of Bt2 (Vsp2) (9, 16) and against F4/80, a known marker of macrophage activation (10). F4/80 immunostaining showed that neutralization of TNF greatly increased the number of activated macrophages in the spleen compared with untreated mice (Fig. 7, A and B). Vsp2 immunostaining in mice treated with anti-TNF showed intact spirochetes mostly in the capsule (Fig. 7, C–E); the majority of the Vsp2 signal from the spleen parenchyma corresponded to cells with morphology suggestive of macrophages (Fig. 7E). In contrast, frequent intact spirochetes were observed in untreated mice throughout the spleen parenchyma (Fig. 7D). Double immunostaining for F4/80 and Vsp2 showed colocalization of Vsp2 within activated macrophages only in treated mice (Fig. 7, E and F). No staining was observed with negative control Abs (Fig. 7, G and H). We concluded that treatment of RAG2/IL-10−/− mice by TNF neutralization increased phagocytosis of Bt2 by macrophages in the spleen.

Discussion

Our findings provide novel insights into the role of IL-10 and TNF in the control of the pathogen load at times of high bacteremia as in B cell-deficient mice infected with the RF spirochete B. turicatae. Although several studies have reported on the critical role of Ab-mediated immunity in RF borreliosis (3–5, 21), much less is known about the role of innate immunity in this disease. However, an innate effector mechanism has been shown to partially control RF bacteremia (5, 21). Our results indicate that IL-10 deficiency compromised the ability of the innate immune system to control the pathogen load in RAG2−/− mice. In the absence of IL-10, infection resulted in higher production of TNF, markedly increased apoptosis of splenocytes and PBMC, impairment of phagocytosis, higher bacteremia, and early mortality. Neutralization of TNF decreased TNF levels, reduced apoptosis of immune cells, improved production of IFN-γ by NK cells, enhanced phagocytosis in the spleen, lowered the pathogen load, and rescued the mice from early death.

Proinflammatory cytokines, in particular TNF, play a key role in amplification of the inflammatory response in the course of sepsis. Although a proinflammatory response can be beneficial for bacterial clearance, too much activation can lead to cell injury and even shock. Anti-inflammatory mediators, especially IL-10, are essential to counterbalance the proinflammatory response. In sepsis, the concentration of IL-10 is often indicative of the magnitude of the inflammatory stress, being the highest in patients with the most severe disease (23–28). Moreover, the strong correlation between TNF and IL-10 concentration in septic patients demonstrates an autoregulatory feedback loop between these two mediators (23).

Studies in endotoxemic mice have shown that IL-10 determines the amount of LPS that can be tolerated without death (29, 30) and that this effect occurs by suppressing the production of TNF (30).

Although we expected that IL-10 deficiency could increase disease, its effect in the control of pathogen load was most surprising. In most situations, IL-10 has a suppressive effect on both macrophages and dendritic cells (12, 22) and the majority of animal models have shown that IL-10 inhibits antimicrobial response while protecting from immunopathology (12–15). IL-10 deficiency increases spirochetal clearance but leads to more severe arthritis compared with wild-type mice during infection with the related spirochete Borrelia burgdorferi (12, 22). Similarly, TNF inhibition is usually associated with increased susceptibility to infection (14, 31–33). The majority of murine models with IL-10 deficiency have reported a positive correlation between improved pathogen control and increased production of TNF and IFN-γ (14, 34–36).

In contrast, the current (Fig. 1B) and previous (6, 10) results by our group in RF borreliosis indicate that during high bacteremia IL-10 plays an opposite role: it helps control the pathogen load and protects from death, and this effect occurs via TNF suppression and prevention of immune cell apoptosis. We propose that in situations where there is a rapid and overwhelming growth of organisms in the blood, as seen in our model, IL-10 is essential to counterbalance TNF production, allowing the host to tolerate the high pathogen load, while waiting for a more effective response to develop, in the case of RF serotype-specific Ab production. Without IL-10, the high pathogen load resulted in increased production of TNF that led to prominent leukocyte apoptosis resulting in a further increase in the pathogen load to lethal levels. Local or systemic production of TNF has been implicated in induction of apoptosis in several types of eukaryotic cells, including leukocytes (14, 34–38). In sepsis caused by Gram-negative bacteria, also characterized by high production of TNF and persistent bacteremia, several studies have evaluated the importance of apoptosis of immune cells as contributing to the high mortality (39–42). Some studies have reported that persistent bacteremia is the results of loss of pathogen clearance due to apoptosis of spleen and/or liver macrophages and lymphocytes (39, 41, 43). Also, there is evidence that patients dying from sepsis have markedly increased lymphocyte apoptosis in the spleen (43). Increase in spleen lymphocyte apoptosis has been shown to reduce survival in experimental animals with sepsis (41, 43, 44). The immune cells most affected by dysregulated apoptotic cell death in sepsis appear to be lymphocytes (41, 43, 44).

Our results suggest that the markedly increased leukocyte apoptosis in blood and lymphoid tissues in Bt2-infected RAG2/IL-10−/− mice caused the lack of IFN-γ production and decreased macrophage phagocytosis. IFN-γ induces multiple microbialidal functions in macrophages and also activates neutrophils; both cell types are important in controlling infections. The related spirochete B. burgdorferi has been shown to elicit IFN-γ production from NK cells (45). Furthermore, TNF has been shown to induce apoptosis of NK and macrophage cells in a dose-dependent manner (46, 47). The lack of production of IFN-γ and apoptosis of macrophages and lymphocytes in our model likely explains the failure of phagocytosis that resulted in further increase in the pathogen load to fatal levels. Consistent with this are the findings that neutralization of TNF reduced apoptosis and restored production of IFN-γ, increased phagocytosis, and lowered the pathogen load. TNF levels in the RAG2/IL-10−/− mice on day 12, 7 days after the last dose of neutralizing Ab was given, were similar to those of the RAG2−/− mice on day 5. Therefore, the excessive levels of TNF that had led to marked apoptosis of immune cells in untreated mice were clearly reduced allowing the protective phagocytic function of the innate immune system to take place. An interesting observation was that pathogen control correlated with increased weight of the spleen but not the liver (Fig. 4). The spleen has been shown to play an important role in pathogen control in severe RF borreliosis (4, 48). Splenectomized mice have a more severe and of longer duration first episode of bacteremia when infected with a strain that causes high-level bacteremia, but not with a strain that reaches only moderate levels (4). Also, splenomegaly temporally correlates inversely with bacteremia and thrombocytopenia, consistent with both being simultaneously cleared by the mononuclear phagocytic system (48).
Differences in the effects of IL-10 deficiency on pathogen control in our model compared with the results from models of B. burgdorferi infection (12, 22) suggest that IL-10 plays different roles depending on particular characteristics of the infection. B. burgdorferi infection is mostly associated with certain target tissues, and its presence in blood is brief and at low numbers, as it is demonstrated by the low pathogen detection by blood PCR and culture (49). Therefore, most of the effect of the immune response to B. burgdorferi is localized to tissue and in presence of a relatively low bacterial load. In contrast, RF Borrelia control infection by protecting innate immune cells from apoptosis critical role in counteracting the inflammatory response and helps specific Ab and/or insufficient production of IL-10 may result in significant morbidity and mortality, which may explain why epidemic RF killed millions before antibiotics were available (2). Our results indicate that, at times of high levels of bacteremia, IL-10 plays a critical role in counteracting the inflammatory response and helps control infection by protecting innate immune cells from apoptosis via down-regulation of TNF.

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