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T-Helper-Cell Cytokines in the Early Evolution of Murine Lyme Arthritis

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Genetic susceptibility to murine Lyme arthritis has been correlated with the dominance of T-helper (Th1)- or Th2-cell-associated cytokines. To determine when commitment of the Th cell phenotype occurs, we examined the kinetics of gamma interferon (IFN-γ) and interleukin-4 (IL-4) production by lymph node T cells of disease-susceptible C3H/HeN and disease-resistant BALB/c mice from days 2 through 30 of infection, a period encompassing the evolution of disease and early regression. BALB/c mice produced more IFN-γ on day 2 of infection than did C3H/HeN mice, whereas IL-4 was first detected on day 14. In contrast, only IFN-γ could be detected in C3H/HeN mice, and the levels steadily increased from day 2 to surpass those seen in BALB/c mice by day 14 of infection. Despite the difference in cytokine profiles, both BALB/c and C3H/HeN mice developed comparable arthritis assessed at 14 days of infection. Arthritis regressed by day 30 in BALB/c mice but persisted in C3H/HeN mice. These studies are the first to demonstrate that the Th2 response to *Borrelia burgdorferi* infection of BALB/c mice is preceded by a Th1 cytokine response. Moreover, the timing of the appearance of IL-4 suggests that its primary effect is not in preventing disease, as suggested by others, but, rather, in hastening the resolution of inflammation. The implications of these findings for the orchestration of host defense against *B. burgdorferi* infection are discussed.

The murine model of Lyme borreliosis, due to infection with the spirochete *Borrelia burgdorferi*, exhibits disease manifestations similar to those seen in human Lyme disease, including arthritis and carditis (6). All inbred laboratory mice are susceptible to infection with *B. burgdorferi*, but the severity of disease varies according to strain, age at the time of infection, and route of inoculation (6, 7). In particular, severe inflammatory arthritis appears 2 to 4 weeks after intradermal inoculation of C3H/HeN mice with *B. burgdorferi* and then subsides, although infection persists (5). In contrast, BALB/c mice infected in a similar fashion develop minimal arthritis (6). Despite the exuberant inflammatory response noted in disease-susceptible C3H/HeN mice, their spirochete burden remains higher than in disease-resistant strains (22). This suggests that the recruitment of inflammatory cells at disease sites is appropriate and is driven by spirochetal antigens.

For a number of infectious microorganisms, polarization of host T helper (Th) cell responses has been implicated as the genetic basis for disease resistance or susceptibility (9, 11, 16, 17, 21). Recent studies of murine Lyme borreliosis have associated the development of a Th1 response in C3H/HeN mice with severe arthritis, as indicated by gamma interferon (IFN-γ) production, whereas the development of a Th2 response in BALB/c mice, with its concomitant interleukin-4 (IL-4) secretion, gives rise to milder disease (13, 15). These reports, however, have examined mice with well-established infection and assessed Th cell responses and the effect of cytokines during the plateau and resolution phases of disease. The direct contribution of T-cell-mediated immunity to host defense remains unclear.

Unlike other infectious pathogens for which T-cell-mediated immunity has been demonstrated, *B. burgdorferi* is not a known intracellular pathogen (as, for example, *Leishmania major*), nor do particular antibody subsets appear necessary for its elimination (such as immunoglobulin E for *Strongyloides*). Innate immune cells, including macrophages and neutrophils, comprise much of the host response to *B. burgdorferi* prior to the development of borreliacidal antibodies. Thus, Th-cell responses that favor the activation of innate immune cells may be an important early determinant of the spirochete burden.

To begin to examine this hypothesis, we have compared the kinetics of Th1 and Th2 cytokine responses as they evolve during the first 30 days of *B. burgdorferi* infection of disease-susceptible C3H/HeN and disease-resistant BALB/c mice. Cytokine levels were correlated with histopathologic assessment of arthritis severity during this period, which encompasses the evolution of arthritis to its peak severity and onset of regression. Our results demonstrate that *B. burgdorferi* infection of C3H/HeN and BALB/c mice induces comparable degrees of arthritis early in infection but that arthritis resolves more rapidly in BALB/c mice. BALB/c mice developed a Th1 cytokine pattern prior to the detection of the Th2 cytokine IL-4, which correlated with the more rapid resolution of arthritis in this mouse strain. Because previous studies have shown that treatment of mice with anti-IL-4 monoclonal antibody (MAb) leads to increased joint swelling during the regression phase of the disease (13), our results support a role for IL-4 in promoting the regression of arthritis, not in preventing its occurrence. The implications of these findings on *B. burgdorferi* host defense are discussed.

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MATERIALS AND METHODS

Mice. Inbred 4- to 5-week-old female BALB/c and C3H/HeN mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and Charles River Laboratories (Raleigh, N.C.), respectively, and housed in the Yale University

School of Medicine animal facility. The mice were given food and water ad libitum and sacrificed by carbon dioxide asphyxiation.

Spirochetes. A clone of *B. burgdorferi* N40 (cN40) with previously verified infectivity and pathogenicity was used in all the experiments (6). Frozen aliquots of low-passage cN40 were thawed and grown to log phase in 8 ml of modified Barbour Stoenner Kelly (BSK II) medium (3) in glass screw-top tubes at 33°C prior to each experiment. Spirochetes were visualized to assess viability and counted by dark-field microscopy before inoculation into mice.

Experimental infection of mice. The mice were infected by syringe inoculation in one or both hind feet with 10^4 cN40 spirochetes in 100 μl of BSK II. At 2, 4, 14 or 30 days after inoculation, the mice were sacrificed and regional draining popliteal lymph nodes were harvested for cytokine analysis. At the 14- and 30-day time points, the infection status was assessed by culturing blood and urinary bladder in 8 ml of BSK II medium at 33°C for 2 weeks. The presence of viable spirochetes was assessed by dark-field microscopy at the end of the culture period. Histopathologic testing was performed on tibiotarsal joints and knees of mice sacrificed on days 14 and 30 of infection. The spirochete burden in regional draining lymph nodes was estimated by quantitative PCR at the 14 day time point.

Cytokine analysis. Popliteal lymph nodes were processed for Th1 and Th2 cytokine analysis from mice infected with *B. burgdorferi* and from control mice mock infected by inoculation of 100 μl of BSK II medium into the hind foot. The popliteal lymph nodes were processed into single-cell suspensions in Click's medium supplemented with 5% fetal calf serum, 0.04 mM β-mercaptoethanol, 200 mM L-glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml (5% Click's medium). Lymph node cells (LNCs) were washed with 5% Click's medium, resuspended in Click's medium with 10% fetal calf serum and supplements (10% Click's medium) at 5×10^6 cells/ml, and aliquoted into 24-well (total volume, 1 ml) or 96-well (total volume, 200 μl) tissue culture plates (Becton Dickson Labware, Lincoln Park, N.J.). LNCs were stimulated with *B. burgdorferi* sonicate at 35 μg/ml (final concentration) or with an equivalent volume of phosphate-buffered saline (15). Supernatants were harvested at 72 h, and IL-4 and IFN-γ were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as specified by the manufacturer (Pharmingen, San Diego, Calif.). Concentrations of IL-4 and IFN-γ were calculated based on standard curves obtained from serial dilutions of recombinant IL-4 and IFN-γ (Biosource, Camarillo, Calif.).

In some experiments, T cells were depleted from LNCs prior to stimulation for cytokine production. Briefly, LNCs were resuspended at 10^7 cells/ml in culture supernatants from the 30 H-12 hybridoma line, which produces anti-Thy 1.2 monoclonal antibody (MAb) (14). After 30 min on ice, the cells were washed in ice-cold 5% Click's medium and resuspended in rabbit serum complement diluted 1:6 (vol/vol) in 5% Click's medium. The cells were incubated at 37°C for 30 min and then washed twice in 5% Click's medium. Unlysed cells were resuspended in 10% Click's medium at 5×10^6 cells/ml, and 200-μl aliquots were stimulated with *B. burgdorferi* sonicate in 96-well plates as described above. The efficiency of T-cell depletion was determined by flow-cytometric analysis of LNCs before and after antibody-mediated cell lysis, with fluorescein isothiocyanate-conjugated rat anti-mouse CD3 antibody (Pharmingen).

Histopathologic testing. The joints of hind limbs (knee and tibiotarsal joints) were immersion fixed in neutral-buffered formalin (pH 7.2), demineralized, and then processed and stained with hematoxylin-eosin by routine histologic techniques (6). The tibiotarsal joints were scored for arthritis severity on a scale of 0 (negative) to 3 (severe), based on the degree of leukocytic infiltration and synovial proliferation, by an observer blinded to the experimental protocol (4). Differences in mean values of arthritis severity between BALB/c and C3H/HeN mice were analyzed by Student's two-tailed *t* test.

Quantitative *B. burgdorferi* detection by competitive PCR. DNA was extracted from preweighed mouse lymph node samples by using a modified Qiamp tissue kit protocol (Qiagen, Chatsworth, Calif.) (1). Competitive amplifications of the target DNA were performed with primers targeting a 256-bp region of the flagellin gene of *B. burgdorferi*. These amplification reaction mixtures also contained 100 copies of a 356-bp competitive internal control consisting of a 303-bp segment of *Staphylococcus aureus* plasmid, pub 112, flanked by the same *B. burgdorferi* primer sequences found in the target. The use of digoxigenin-11-dUTP in the reaction mixture allowed the amplified products to be quantitated with the PCR ELISA detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

RESULTS

Th cell-associated cytokines produced during the evolution and early regression of arthritis were assessed in regional draining lymph nodes of mice infected by hind foot inoculation of spirochetes. This inoculation route was chosen so that the T-cell responses in the lymph nodes adjacent to the joints with arthritis could be examined. Previous studies have shown that *B. burgdorferi*-induced IL-4 responses can best be detected when localized to T cells responding near a site of disease (15).

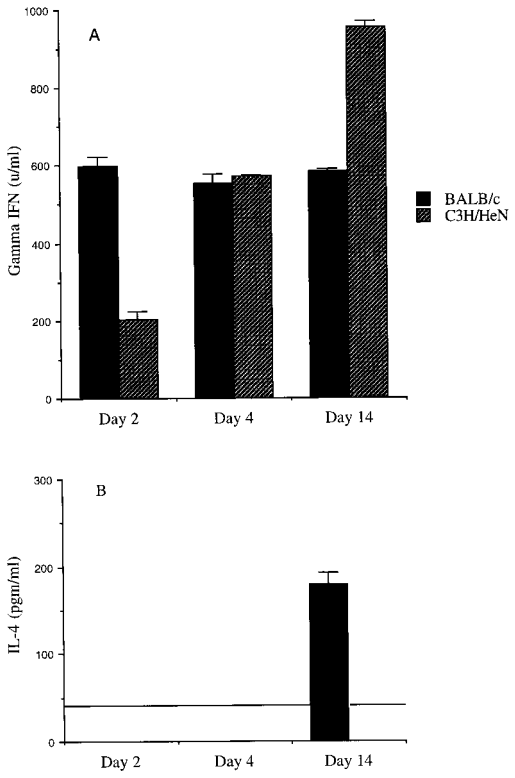

FIG. 1. ELISA measurement of T-cell-associated cytokines in BALB/c and C3H/HeN mice infected with *B. burgdorferi*. Cytokines produced by LNCs after in vitro restimulation were measured by ELISA, and the values expressed as the mean results from triplicate wells ± standard error of the mean (SEM). (A) IFN-γ responses from BALB/c and C3H/HeN LNCs after in vitro restimulation with *B. burgdorferi* sonicate. (B) BALB/c LNC production of IL-4 after in vitro restimulation with *B. burgdorferi* sonicate. No IL-4 responses were detected in C3H/HeN mice. Results are representative of four independent experiments. Although the magnitude of the cytokine responses varied among experiments, the pattern of cytokine secretion by the mouse strains relative to each other was the same. The horizontal line indicates the detection limit (<40 pg/ml) of the IL-4 assay used in this study.

LNCs from C3H/HeN mice restimulated in vitro with *B. burgdorferi* sonicate produced steadily increasing amounts of IFN-γ from days 2 through 14 of infection (Fig. 1A). In contrast, LNCs from similarly infected BALB/c mice produced higher levels of IFN-γ on day 2 of infection than did C3H/HeN LNCs and maintained this response through day 14 (Fig. 1A). On infection day 30, IFN-γ production by LNCs from both strains of mice had fallen, although the levels remained slightly higher in the C3H/HeN mice (see Tables 1 and 2).

The Th2-associated cytokine IL-4 could not be detected at any time point in stimulated LNCs from C3H/HeN mice, even though the level of IFN-γ, a potentially negative regulator of IL-4 production, had fallen by day 30. In contrast, IL-4 was detected in BALB/c mice at the 14- and 30-day time points (Fig. 1B; see Tables 1 and 2). The absence of detectable IFN-γ and IL-4 in LNCs from mock-infected mice confirmed that cytokine responses were specific to *B. burgdorferi* infection (data not shown). In addition, no difference was noted by flow cytometry in the proportion of B- and T-cell populations in LNCs from C3H/HeN and BALB/c mice (data not shown), indicating that the relative number of T cells in each well was the same.

To confirm that the cytokines detected were produced by T cells, the responses of LNCs depleted of T cells in vitro were

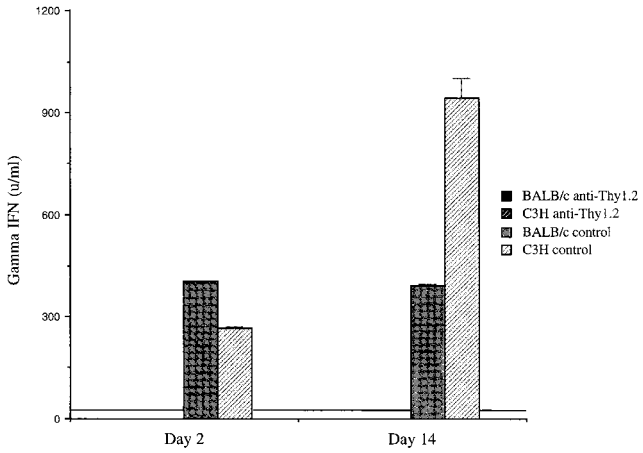

FIG. 2. Depletion of T cells eliminates the IFN-γ response of LNCs to in vitro stimulation with *B. burgdorferi* sonicate. IFN-γ produced by LNCs after in vitro restimulation with *B. burgdorferi* was measured by ELISA before and after depletion of T cells with anti-Thy1.2 MAb. The numerical values are the mean results from duplicate wells ± SEM. Results are representative of two independent experiments. The horizontal line indicates the detection limit (<30 U/ml) of the IFN-γ assay used in this study.

compared to those of bulk LNCs. Flow-cytometric analysis revealed that at least 70% of the T cells in lymph nodes had been depleted in each experiment (data not shown). T-cell depletion of LNCs obtained 2 and 14 days after infection eliminated the IFN-γ response in both strains of mice (Fig. 2).

To correlate arthritis severity with cytokine profiles, the histopathology of the tibiotarsal joints and knees of C3H/HeN and BALB/c mice was examined on days 14 and 30 of infection. Surprisingly, despite the different cytokine profiles at these time points, C3H/HeN and BALB/c mice had similar scores for arthritis severity 14 days after inoculation, the time when disease manifestations were most apparent (Table 1). Arthritis was consistently most severe in the limb ipsilateral to the inoculation site. On day 30 of infection, arthritis in the C3H/HeN mice was still active, whereas disease in BALB/c mice had subsided considerably (Table 2). The appearance of IL-4 consistently preceded the resolution of arthritis in BALB/c mice, whereas no association with arthritis severity could be inferred from IFN-γ levels. Spirochetes were detected in C3H/HeN LNCs on day 14 of infection, whereas no spirochetal DNA could be amplified from LNCs of BALB/c mice at that time point (data not shown), suggesting a trend toward a lower spirochete burden in BALB/c mice at this site.

TABLE 1. Comparison of arthritis severity and cytokine levels in BALB/c and C3H/HeN mice on day 14 of infection

Mouse strain	Severity of[a]:		Production of[b]:	
	Contralateral arthritis	Ipsilateral arthritis	IFN-γ (U/ml)	IL-4 (pgm/ml)
BALB/c	0.2 ± 0.6	1.5 ± 0.8	263 ± 156[c]	296 ± 3
C3H/HeN	0.1 ± 0.2	1.1 ± 0.4	912 ± 43	<40[d]

[a] Arthritis was scored in severity on a scale of 0 to 3 (4), and values represent the mean ± standard deviation of scores averaged from 10 mice/strain. The difference in arthritis scores between BALB/c and C3H/HeN mice was not statistically significant ($P > 0.05$, two-tailed t test).

[b] Cytokines produced by pooled LNCs after in vitro restimulation were measured by ELISA, and the numerical values shown are the mean results from triplicate wells ± SEM.

[c] The large SEM reflects one outlying triplicate value.

[d] Values below 40 pgm/ml fall below the lower limits of IL-4 detection in this assay.

TABLE 2. Comparison of arthritis severity and cytokine levels in BALB/c and C3H/HeN mice on day 30 of infection

Mouse strain	Severity of arthritis[a]	Production of[b]:	
		IFN-γ (U/ml)	IL-4 (pgm/ml)
BALB/c	0.8 ± 0.4	44 ± 11.6	310 ± 65
C3H/HeN	1.7 ± 0.7	225 ± 88.1	<40[c]

[a] Values for arthritis are the mean ± standard deviation of averaged scores (on a scale of 0 to 3) of both tibiotarsal joints from mice inoculated with *B. burgdorferi* in both hind feet. Five mice were analyzed per strain. The difference in arthritis severity between BALB/c and C3H/HeN mice was statistically significant ($P < 0.05$, two-tailed t test).

[b] Cytokines produced by LNCs after *in vitro* stimulation were measured by ELISA in individual mice. The numbers represent the mean of values (averaged from duplicate wells) from individual mice ± SEM.

[c] The lower detection limit for IL-4 is 40 pgm/ml.

DISCUSSION

Experimental infection of inbred laboratory mice with *B. burgdorferi* results in arthritis, whose severity appears to be based on genetically determined host immune responses which control the spirochete burden and the inflammatory response (5, 6). The host immune response to microbial pathogens in the earliest stages of infection is a critical determinant of disease resistance and susceptibility. These responses are dedicated to containment of the pathogen and subsequent control of inflammatory cell-associated tissue damage. The cytokine pattern elicited by pathogens has a profound influence on the developing specific immune response (9, 11, 16, 17, 21). In murine Lyme borreliosis, polarized Th1 and Th2 cytokine responses have been documented in disease-susceptible and disease-resistant strains of mice, respectively, and neutralization of those cytokines results in a change in disease phenotype (13, 15). Humans with chronic Lyme disease also appear to have skewing of the T-cell repertoire toward the production of Th1-like cytokines and comparative diminution of IL-4 responses (18, 23, 24). This has led to the widely held assumption that the Th2 cytokine IL-4 plays a protective role whereas the presence of IFN-γ is detrimental to the host. Our examination of the kinetics of the Th1 and Th2 cytokine response in murine Lyme borreliosis and its relationship to arthritis severity provides a different view of the role of these cytokines in the disease process.

In in vitro restimulation assays, we demonstrated that LNCs from *B. burgdorferi*-infected BALB/c and C3H/HeN mice produce different patterns of the Th-cell-associated cytokines IFN-γ and IL-4 over time. Surprisingly, in four independent experiments, IFN-γ levels were consistently higher in BALB/c mice than in C3H/HeN mice at the earliest stage of infection analyzed (day 2). These findings suggest that a greater number of IFN-γ-producing T cells were primed in the regional draining node of BALB/c mice than of C3H/HeN by day 2 of infection, even though the initial spirochete inoculation dose was the same. One possible consequence of the initial peak in IFN-γ production in BALB/c mice is a more effective control of spirochetes through macrophage activation, thereby reducing the antigen load. Because extremes of antigen concentration (high or low) appear to favor Th2-cell development (10), a lower local antigen concentration could induce the later differentiation of primed precursor Th0/Th1 cells into IL-4-producing Th2 cells. In fact, by day 14 of infection, IL-4 can be detected in LNC restimulation assays from BALB/c but not

C3H/HeN mice, and PCR analysis of spirochetal DNA in lymph node tissues indicated a lower spirochete burden in BALB/c than C3H/HeN mice.

Despite the dramatic difference in IFN-γ levels between BALB/c and C3H/HeN mice on day 14 and the presence of IL-4 in the former strain, arthritis severity assessed by histopathologic testing was not significantly different at that time point. These results differ from those reported in previous studies examining Th-cell-associated cytokines in murine Lyme borreliosis (13, 15). Those studies relied on caliper measurement of the tibiotarsal joint diameter to assess arthritis severity and on analysis of cytokine levels at later times (after 4 weeks) of infection, during the plateau and resolution phases of arthritis. Our studies are the first to examine the cytokine environment during the evolution of disease as assessed by histopathologic testing.

In the murine model of Lyme borreliosis, strain-specific disease resistance and susceptibility has been defined by histopathologic examination of joints of mice infected by intradermal inoculation (22). However, our results show that 14 days after hind foot inoculation, disease-resistant BALB/c mice can develop arthritis comparable in severity to that in disease-susceptible C3H/HeN mice. The discrepancy in the severity of arthritis between hind foot and intradermal inoculation may be related to the route of spirochete dissemination and accessibility of anatomical sites in which spirochetes may preferentially replicate and cause disease. We have found that mice infected by intradermal inoculation into the shoulder region more readily develop carditis than do mice inoculated via the hind foot route (6a). Other investigators have noted that viable spirochetes are easier to detect in the somatic quadrant near the site of the tick bite than at more distant sites and have raised the possibility that dissemination of *B. burgdorferi* occurs more readily by migration through tissues than by blood-borne spread (20).

The arthritis-modulating activity of IL-4 has been shown by depletion of IL-4 with MAb and by providing large amounts of this cytokine exogenously during the course of *B. burgdorferi* infection of mice (12, 13, 15). The mechanism by which this cytokine exerts its effects on joint inflammation is unclear. The inhibitory action of IL-4 on proinflammatory cytokine secretion by Th1 cells and macrophages (2) could lead to a decrease in joint inflammation independent of direct effects on the pathogen burden. Indeed, depletion of IL-12 (a cytokine promoting Th1 cell differentiation) with MAb early in infection resulted in a modest attenuation of arthritis severity in C3H/HeN mice, but at the expense of enhancing the spirochete burden (1). In a separate study, we have demonstrated that blockade of the B7-2/CD28 T-cell costimulatory pathway during the first 14 days of infection markedly reduces IL-4 production but does not enhance arthritis severity assessed at 14 days (19a). In our experiments, IL-4 was first detected in BALB/c mice 14 days after inoculation, the time of peak arthritis, and persisted at least through day 30 of infection, coincident with arthritis resolution. Taken together, these results suggest that the arthritis-modulating effect of IL-4 does not occur immediately, and they support an indirect mechanism such as inhibition of inflammatory cytokine secretion by other cell types. Although IL-4 depletion studies have demonstrated a role for this cytokine in reducing established *B. burgdorferi* inflammation in mice, other Th2-associated cytokines may also be important. It is noteworthy that in studies of *B. burgdorferi*-specific T cells from humans with chronic Lyme disease, IL-10 but not IL-4 suppressed the secretion of proinflammatory cytokines in in vitro culture systems (23).

In summary, our analysis of T-cell cytokine secretion during the development of murine Lyme arthritis suggests that the evolution of a Th2-type immune response evolves from a Th1 response in disease-resistant mice. The timing of the appearance of the Th2 cytokine correlates with accelerated regression of inflammatory changes. We postulate that it is a deficiency in activation of the innate immune response early in infection that leads to more severe disease in disease-susceptible mice, rather than the presence of IFN-γ, which may be an entirely appropriate specific immune response in the setting of higher spirochete loads. Studies addressing these issues are in progress.

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