Suppression of Acute *Ixodes scapularis*–Induced *Borrelia burgdorferi* Infection using Tumor Necrosis Factor-α, Interleukin-2, and Interferon-γ

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Down-regulation of mammalian cytokine production has been demonstrated during tick feeding. To examine the hypothesis that reconstitution of cytokines during tick feeding could facilitate immune containment of *Borrelia burgdorferi*, the following experiments were done. C3H/HeJ mice were given cytokines for 10 days after *Ixodes scapularis* attachment. At day 21, ear biopsies were analyzed for *B. burgdorferi*. Polymerase chain reaction analysis indicated a protection rate of 95% in mice receiving tumor necrosis factor (TNF)-α. Mice that received interleukin (IL)-2 or interferon (IFN)-γ had infection rates of 30%–45% compared with 83% for untreated controls. No correlation was noted between neutralizing antibody, reactivity by Western blot, and subsequent protection. Culture of *B. burgdorferi* in cytokine-conditioned media indicated that TNF-α, IFN-γ, and IL-2 were not cytotoxic for *B. burgdorferi*. These data suggest that cytokine-induced protection from *B. burgdorferi* infection was immune-mediated and that cellular immunity may be associated with protection from *I. scapularis*–induced infection.

Lyme disease, originally described as a clinical entity in 1977, is now the most common human vectorborne disease in the United States [1]. Ninety percent of human cases occur in the northeastern coastal states, the Midwest, and parts of northern California [2, 3]. These areas represent regions of the country associated with the distribution of *Ixodes scapularis* and *Ixodes pacificus,* the principal vectors for the causative agent, *Borrelia burgdorferi* [2, 3]. Although antimicrobial therapy is effective in most cases of early infection, manifestations of chronic disease (arthritis and peripheral neuropathy) develop in ~10% of human patients who remain untreated [1]. The recent rise in incidence and morbidity of this zoonosis has created an immediate need for an effective prevention strategy and vaccine.

In rodent models, protective immune responses to *B. burgdorferi* have been associated with neutralizing antibody responses directed toward the outer surface proteins (Osps) A, B, C, E, and F [4–11]. Thus, recent vaccine strategies to prevent infection in humans have been directed toward the production and use of recombinant Osps [8]. This strategy, however, may be fraught with technical difficulties that could limit its usefulness in outbred species. The primary problem is heterogeneity and genetic diversity among *B. burgdorferi* isolates from the United States, Europe, and Japan [12–16]. *B. burgdorferi* can be subdivided into at least 6 subgroups according to their distinct Osp genotypes [17]. Second, experimental results indicate that there may be no cross-protection induced by individual genotypes of OspA [18] and that antigenic modulation of spirochetal populations may occur in the presence of serum neutralizing antibody [19]. Third, the pathogenesis of Lyme disease is only partly characterized in humans, and it is possible that the humoral immune response to one or more Osps, which occur late in infection and are associated with clinical disease, may initiate or potentiate subsequent immunopathology [20–22]. Finally, the immune response varies widely with both the route and site of inoculation of *B. burgdorferi* [23–25]. Therefore, alternative approaches aimed at preventing or limiting *B. burgdorferi* infection may be warranted.

A more universal aspect of this disease entity involves the events associated with the feeding of *I. scapularis* and the resulting modulation of the host immune response, which greatly influence both the infectivity and dissemination of the spirochete [26, 27]. Systemic dissemination of the tick-inoculated spirochete is delayed in the host for 7–10 days [28]. Nevertheless, early host immune effector mechanisms are ineffective in clearing this organism, due in part to the down-regulation of spirochete Osps such as OspA during nymphal attachment and feeding [29] or perhaps due to the effects tick saliva may have on the generation of an initial host immune response [26].

Tick saliva impairs a range of proinflammatory responses, including macrophage activation [27], secretion of the macrophage-associated cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-α [30], inhibition of neutrophil function [31], suppression of T helper–associated cytokines IL-2 and interferon (IFN)-γ [32], and profound down-regulation of complement activity [33]. In vitro, down-regulating effects on mononuclear cells were induced with <1 μL of saliva; in vivo, these effects were systemic, lasting throughout the period of tick infestation and 3–4 days after engorgement [27]. The relative potency of this effect becomes apparent when one considers

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that ~500 μL of saliva is excreted into the host by adult *Ixodes* ticks over a 96-h feeding [34]. The cumulative effect of this process would be the down-regulation of an initial inflammatory response to spirochete inoculation, specifically in terms of IFN-γ regulation of antigen presentation, NK cell and cytotoxic T cell responses, and immunoglobulin production. Moreover, both IFN-γ and TNF-α have been shown to up-regulate macrophage antimicrobial activity via the induction of intracellular nitric oxide synthesis [35], a crucial factor in the intracellular killing of several macrophagetropic pathogens [36]. Considering that the macrophage is a cellular target of *B. burgdorferi* infection [37], down-regulation of any of these proinflammatory factors would play a critical role in the initial dissemination of spirochete infection.

In evolutionary terms, these events may stabilize an antagonistic tick-host interaction such that the tick can prevent the full expression of host immunity at the feeding site, ensure its survival during a prolonged period of feeding, and inadvertently facilitate the transmission of a pathogen such as *B. burgdorferi* [38]. Moreover, this phenomenon may be common to other arthropodborne diseases. Immunomodulation of host responses has been demonstrated with the insect vectors *Lutzomyia longipalpis* and *Simulium vittatum* [39, 40], potentiating the transmission of human protozoal and filarial diseases, respectively.

The observation that cellular responses are depressed during the earliest phases of *B. burgdorferi* infection [41] and Th1 responses are affected to a greater extent than Th2 responses led us to the hypothesis that administration of Th1 or macrophage-derived cytokines might reverse tick-induced immunosuppression and facilitate an effective immune response to control initial infection and dissemination of *I. scapularis*–transmitted *B. burgdorferi.* This therapeutic approach would not only facilitate the study of specific effector cell populations needed to contain early arthropodborne infection but could have significant implications for the prevention of other *Ixodes*-borne diseases, namely, *Babesia microti* infection and human ehrlichiosis [42, 43]. We examined our hypothesis using tick spirochete transmission in a sensitive mouse model of *B. burgdorferi* infection.

Materials and Methods

Mice. Five to 7 virus antibody–free C3H/HeJ mice (6–7 weeks old; Jackson Laboratory, Bar Harbor, ME) were placed in individual cages with food and water available ad libitum. At the end of the studies, the mice were euthanatized by methoxyflurane anesthesia followed by cervical dislocation.

Infection and propagation of ticks with B. burgdorferi. Nymphal *I. scapularis,* infected with *B. burgdorferi,* were obtained from a laboratory colony maintained by feeding laboratory-reared larvae on mice 2–6 weeks after infection by nymphal ticks. This strain originated from a population of ~100 field-collected nymphs obtained by drag-cloth sampling at the Fordham University Calder Ecology Center in Armonk, Westchester County, New York. This strain of *B. burgdorferi* was maintained in the laboratory through continuous passage between rats and ticks for >1 year before this study. *B. burgdorferi* infection rates for individual nymphal cohorts were 40%–70%, as determined by antigen-capture ELISA, polymerase chain reaction (PCR), or both [44, 45]. A minimum sample of 20 nymphs from each cohort was subjected to either PCR or antigen-capture ELISA. A separate colony of *I. scapularis* was maintained without *B. burgdorferi* infection, and nymphs from this colony were used as negative experimental controls.

Treatment protocol and study design. C3H/HeJ mice (5–7/group) were randomized into separate treatment groups. TNF-α (50 μg/m^2, every other day), IFN-γ (10^4 U/kg once daily), or IL-2 (10^5 U/kg twice daily), alone or in combination, were administered intraperitoneally at the time of *I. scapularis* placement (7 ticks/mouse) and continued for 10 days. Each mouse was housed in an individual cage, and nymphal ticks were allowed to feed to repletion, collected, and pooled for analysis to determine infection rate and relative infectious challenge of individual mice. Twenty-one days after exposure to *I. scapularis,* mice were anesthetized with methoxyflurane, and an ear biopsy was obtained by sterile surgical technique [46]. Biopsies were cultured in BSK II medium (Sigma, St. Louis) and analyzed weekly by darkfield microscopy or fluorescent antibody assay (or both) for 21 days. All negative cultures were reanalyzed by *B. burgdorferi*–specific PCR. All mice were euthanatized at day 50 following exposure to infected *I. scapularis,* and specific target organs (heart, femorotibial joint, spleen, bladder, and ear) were obtained for analysis by culture and PCR as described earlier.

Two separate studies tested IFN-γ and IL-2 (total of 12 animals/group), and a single study examined TNF-α alone and in combination with IL-2 and IFN-γ (5 animals/group). Any disparity in numbers within these groups was due to either the death of an animal before the treatment regimen was concluded (4 animals) or a lack of infectious challenge, as determined by PCR of replete ticks (1 animal). Percent protection was calculated by dividing the number of animals that were culture- or PCR-positive for *B. burgdorferi* by the total number of animals per group and subtracting this number from 100%. In terms of chronic infection (day 50), animals were considered infected if any target organ was positive for spirochetes by culture or PCR. Negative control ticks were laboratory-reared nymphal *I. scapularis* maintained without *B. burgdorferi* infection.

B. burgdorferi–specific PCR. PCR of individual and pooled *I. scapularis* and material from in vitro culture of specific target organs was done using a nested amplification procedure and primer pairs described by Johnson et al. [45] with slight modification. Briefly, tissue cultures (one in vitro passage) were centrifuged to pellet *B. burgdorferi,* and the resulting pellet was digested with proteinase K. Ten microliters of this digest was placed into the PCR reaction mixture containing 200 μ*M* each dNTP (Promega, Madison, WI), 0.5 μ*M* of each primer pair, 2.5 m*M* $MgCl_2$, and 2.0 U of Tth polymerase (Pharmacia Biotechnology Alameda, CA) in 60 m*M* TRIS-HCl and 15 m*M* $(NH_4)_2SO_4$ (pH 8.5). For the second reaction of the nested PCR, 10 μL of the first reaction was placed into 90 μL of the PCR mix containing the internal primer pair. Templates were initially denatured for 2 min at 94°C, and subsequently 40 cycles were performed at 94°C for 1 min, with annealing at 55°C for 1 min, followed by extension at 72°C for 2

min. After 40 cycles, a final extension at 72°C for 5 min was done. PCR products were analyzed on 1% LE agarose gels stained with ethidium bromide and by Southern blotting technique, hybridizing PCR products were hybridized with a ^{32}P-labeled probe [45] spanning internal nucleotides of the flagellin product.

SDS-PAGE and Western immunoblotting. *B. burgdorferi* was isolated from target organs (spleen and bladder) by in vitro culture in BSK II medium, passaged a single time in vitro before being lysed, and fractionated by 12% SDS-PAGE. Resultant protein profiles were elucidated by silver staining of individual lanes (Bio-Rad Laboratories, Hercules, CA).

For immunoblotting, 10 μg of *B. burgdorferi* lysate (Westchester [WC] isolate, derived from in vitro culture of target organs from infected BALB/c and C3H/HeJ mice infested with ticks obtained during sampling at the Fordham University Calder Ecology Center) was fractionated with a 12% slab gel (Bio-Rad Laboratories) in the presence of SDS. Fractionated proteins were transferred to a nitrocellulose membrane (BA-S NC; Schleicher & Schuell, Keene, NH) that was then blocked for 60 min with 5% nonfat dried milk in 10 m*M* TRIS-buffered saline (TBS blotto, pH 8.0). The membrane was cut into strips and incubated with a 1:500 dilution of mouse serum in blotto overnight at 4°C. The strips were then washed five times in TBS with 0.1% Tween 20 and reacted for 2 h with goat anti-mouse IgG and IgM alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at a 1:2000 dilution in blotto. After being washed, the strips were incubated with BCIP/NBT substrate (Life Technologies GIBCO BRL, Gaithersburg, MD) for 20 min. Subsequent color development was stopped by extensive washing of strips with distilled water.

B. burgdorferi fluorescent antibody assay. *B. burgdorferi* were grown to log phase in modified BSK medium. Spirochetes (10^6) were washed twice in PBS–0.1% azide, labeled with 25 μL of fluorescein isothiocyanate–conjugated rabbit anti-*B. burgdorferi* (Biodesign International, Kennebunk, ME) diluted 1:100 in PBS-azide and incubated for 30 min at 4°C. The spirochetes were then washed three times in PBS–azide and analyzed for fluorescence by UV microscopy.

Serum neutralization of B. burgdorferi. Serum neutralizing antibody titers were determined by a colorimetric borreliacidal assay [47]. Hyperimmune canine anti-*B. burgdorferi* serum (neutralization titer of 1/300) was used as positive control for this assay.

Growth of B. burgdorferi in cytokine-conditioned media. *B. burgdorferi* (WC or low-passage B31 isolate, fewer than five in vitro passages) were pelleted by centrifugation at 9000 rpm for 20 min. Spirochetes were then resuspended at a concentration of 5×10^6/mL in BSK II medium and placed in sealed vials (Cryovial; Life Science Products, Denver). After 72 h, spirochetes were washed once with PBS and resuspended in 1 mL of medium for counting by darkfield microscopy using a Petroff-Hausser counting chamber. Only motile spirochetes were counted, and percent growth was calculated by dividing the experimental value by the average control growth value and multiplied by 100. All cytokine combinations and negative controls (media plus spirochetes) were run in triplicate.

Statistical analysis. Significant differences in the mean number of animals protected from *B. burgdorferi* infection by cytokine treatment and differences between groups in mean number of spirochetes grown in cytokine-conditioned media were determined by single-factor analysis of variance test. $P < .05$ was considered a significant difference between groups.

Table 1. Cytokine inhibition of tick-induced *B. burgdorferi* infection in mice as determined by ear biopsy at day 21 after exposure to infected ticks.

Treatment	Ticks*	No. of mice positive for *B. burgdorferi*/ total by: Culture	PCR	% protection†
IFN	2.7	2/12	4/12	66.7
IFN + IL-2	3.8	4/11	5/11	54.6
IL-2	3.9	2/10	3/10	70
TNF + IFN + IL-2‡	3.0	0/17	1/17	95
No treatment	3.0	3/12	10/12	—
Negative controls	2.0	0/12	0/12	—

NOTE. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; PCR, polymerase chain reaction. Percent protection was calculated by dividing no. of animals culture- or PCR-positive for *B. burgdorferi* in the ear by total no. of animals/group and subtracting this no. from 100%.

* Average number of *I. scapularis* fed to repletion/mouse.

† Statistically significant vs. untreated controls (analysis of variance, $P < .05$).

‡ Represents mice treated with TNF, TNF + IL-2, TNF + IFN, and TNF + IL-2 + IFN.

Results

Treatment of acute infection with recombinant cytokines (day 21). Table 1 is a composite of two individual studies that examined the inhibitory effect of recombinant cytokines on *B. burgdorferi* infection, as determined by in vitro culture and PCR analysis of ear punch biopsies harvested 21 days after exposure to infected *I. scapularis*. Exposure to 7 infected *I. scapularis* resulted in an average of 2.7–3.0 ticks/mouse that fed to repletion. No significant difference was noted in the number of infected ticks that fed to repletion on cytokine-treated versus untreated control mice. On the basis of culture and PCR analysis, a significant level of protection from infection was noted in all treatment groups compared with untreated control mice. Only 1 of 17 animals treated with TNF-α, alone or in combination with IL-2 or IFN-γ, was found to be positive by either culture or PCR amplification of *B. burgdorferi* from ear tissue compared with 10 of 12 mice in the untreated control group. Mice receiving IFN-γ, IL-2, or the combination were protected from infection at rates of 55%–70% (table 1). Culture of ear punch biopsies alone was not sufficient to detect *B. burgdorferi* in infected animals. PCR amplification of *B. burgdorferi* demonstrated greater levels of infection in all groups compared with culture alone. This increased sensitivity was most apparent in the untreated control group, where an additional 7 of 12 animals were found to harbor *B. burgdorferi* in biopsy tissue when analyzed by PCR.

Table 2. Cytokine inhibition of chronic *B. burgdorferi* infection as determined by analysis of target organs at day 50 after exposure to infected ticks.

Treatment	Ear	Bl	JT	H	SPL	% protection
IFN	1/12	1/12	0/12	2/12	4/12	50
IFN + IL-2	1/10	1/10	1/10	5/10	2/10	50
IL-2	4/10	4/10	1/10	5/10	4/10	50
TNF + IFN + IL-2*	4/16	7/16	6/16	11/16	4/16	19
No treatment	4/12	6/12	3/12	5/12	4/12	25

NOTE. Bl, bladder; JT, femorotibial joint; H, heart; SPL, spleen; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor. Mice were considered negative for *B. burgdorferi* if culture- and polymerase chain reaction (PCR)–negative in all target organs. Percent protection was calculated by dividing no. of animals culture- or PCR-positive for *B. burgdorferi* in any organ by total no. of animals/group and subtracting this no. from 100%.

* Represents mice treated with TNF, TNF + IL-2, TNF + IFN, and TNF + IL-2 + IFN.

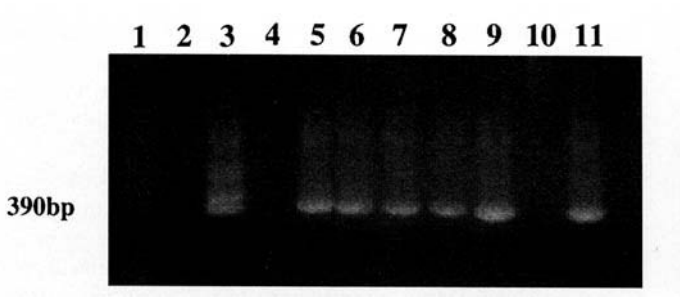

Figure 1. Polymerase chain reaction amplification of *B. burgdorferi* from *I. scapularis*. Lane 1, buffer control; lane 2, *Borrelia hermsii;* lane 3, *B. burgdorferi* (Westchester isolate, <5 in vitro passages); lane 4, uninfected replete ticks; lanes 5–9, pools of replete ticks recovered from individual mice; lane 10, pool of 3 replete ticks recovered from mouse 34; lane 11, 4 replete ticks recovered from mouse 39.

Analysis of chronic infection of target organs (day 50). To enhance the stringency of this bioassay system, mice were euthanatized at day 50 after exposure to infected ticks, and specific target organs (heart, bladder, spleen, ear, and femorotibial joint) were analyzed by culture and PCR. As shown in table 2, the percent protection at day 21 was reduced to 50% in all treatment groups given prophylactic IFN-γ or IL-2. TNF-α alone or in combined cytokine treatment delayed the onset of infection through 21 days, but by day 50 after tick infestation, *B. burgdorferi* had disseminated and localized to selected target organs, namely the bladder, joint, and heart. Those animals treated with IFN-γ, IL-2, or IFN-γ plus IL-2, although showing markedly less infection of target organs than controls, were not statistically different from untreated control animals by day 50 after tick placement. As shown in table 2, the most pronounced infection was localized to the bladder and heart of *I. scapularis*–infected C3H/HeJ mice.

PCR amplification of B. burgdorferi from I. scapularis. To determine that individual mice were indeed challenged with infected *I. scapularis,* DNA was extracted from pools of replete ticks recovered from individual mice, and nested primer pairs were used to sequentially amplify a 390-bp product of the central region of the flagellin gene of *B. burgdorferi.* This assay was specific for *B. burgdorferi*, as no PCR product was generated with cultured *Borrelia hermsii* (figure 1, lane 2). Only 1 animal (figure 1, lane 10) had to be removed from this study because of a lack of evidence of infectious challenge with *I. scapularis.*

Antigenic profile of tissue isolates recovered from C3H/HeJ mice. To determine whether the tick inoculum had been antigenically modified with prophylactic cytokine treatment, *B. burgdorferi* lysates were prepared, fractionated by 12% SDS-PAGE, and silver stained for protein analysis. Spirochete isolates recovered from the bladder of cytokine-treated or untreated mice were antigenically identical to the in vitro–passaged reference strain of the WC isolate (figure 2). As shown, there is a distinct molecular weight shift in OspC that is an identifying characteristic of this isolate compared with low-passage B31. This has been confirmed by immunoblot analysis (data not shown). This SDS-PAGE analysis is representative of isolates obtained from other target organs (spleen, joint, ear, and heart), and no definitive antigenic modulation appeared to occur with any of the prophylactic treatment regimens (data not shown).

Antibody response to B. burgdorferi in mice treated with IFN-γ, IL-2, and TNF-α. Serum reactivity to the WC isolate by C3H/HeJ mice 21 days after exposure to infected *I. scapularis* is shown in figure 3. Regardless of prophylactic treatment or infection status, mice produced antibody to OspC, flagellin, and the 22-kDa protein of *B. burgdorferi.* This immunoblot is representative of all mice examined, and serum from only 1 mouse, treated with IFN-γ alone, reacted with OspA. This

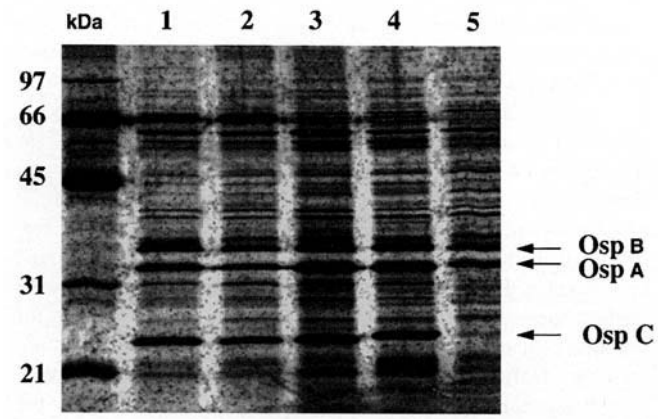

Figure 2. Antigenic profile of Westchester (WC) isolate of *B. burgdorferi* organisms isolated from bladder of infected C3H/Hej mice and cultured for 1 passage in vitro before being lysed, fractionated by 12% SDS-PAGE, and stained with silver. Lane 1, nontreated mouse; lane 2, mouse treated with IL-2; lane 3, WC isolate from BALB/c mouse spleen; lane 4, low-passage B31 strain (<10 passages in vitro); lane 5, high-passage B31 strain (>20 passages in vitro).

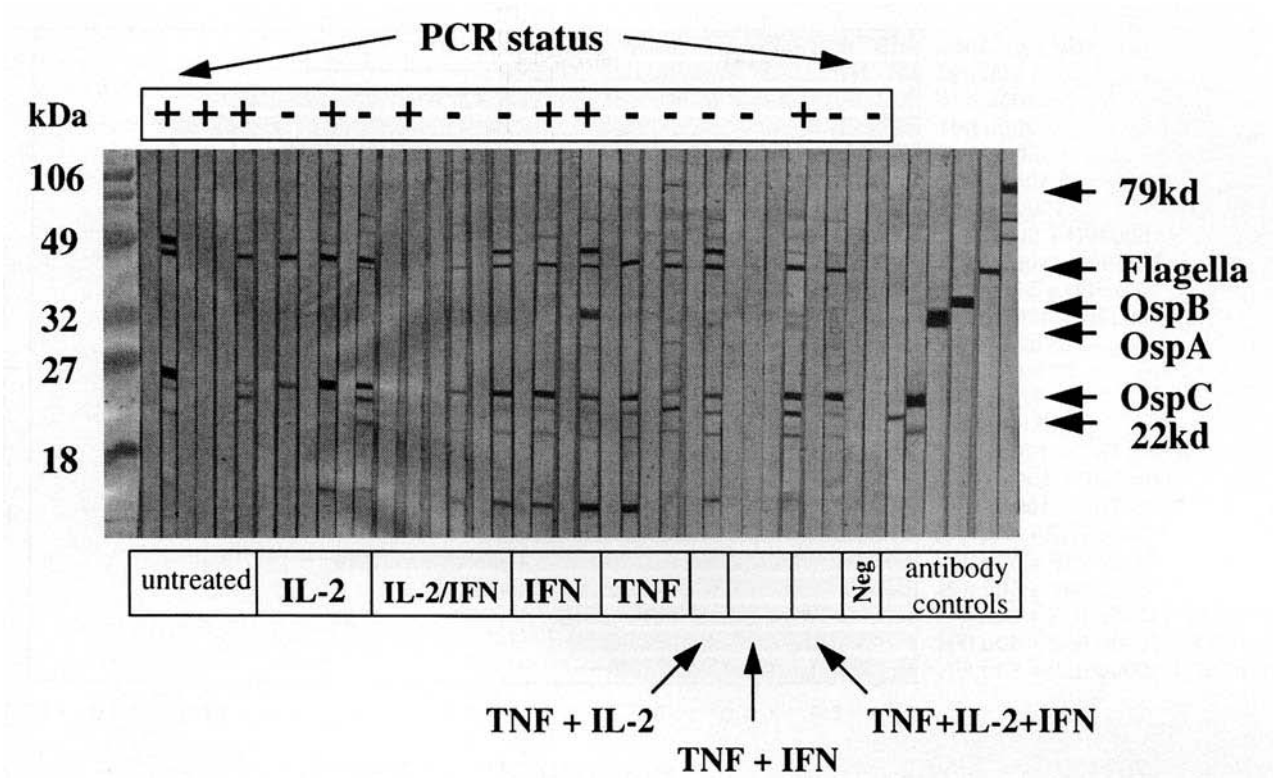

Figure 3. Antibody responses to *B. burgdorferi* in mice 21 days after *I. scapularis* challenge and treatment with IFN-γ, IL-2, and TNF-α. Beginning with lane 2 (lane 1 contains molecular weight markers), every 2 lanes represent individual mouse, with initial lane representing day 0 and second lane day 21 after tick placement. Last 6 lanes contain reference antibodies. Polymerase chain reaction (PCR) status represents infection status at day 21 after tick placement. Neg = challenge with uninfected *I. scapularis.*

mouse was PCR-positive for *B. burgdorferi.* Serum reactivity, as demonstrated by Western blot, was not demonstrably different by day 50 after tick exposure (data not shown). Moreover, none of the mice, regardless of infection status or treatment regimen, produced significant neutralizing antibody titers (data not shown), as measured by the in vitro borreliacidal assay.

Growth of B. burgdorferi in cytokine-conditioned media. To determine whether cytokines, used alone or in combination, would be directly cytotoxic to *B. burgdorferi,* spirochetes (WC isolate or B31) were cultured for 72 h in cytokine-conditioned media and counted under darkfield microscopy. As seen in figure 4, TNF-α, IL-2, or IFN-γ, alone at concentrations 10- to 100-fold higher than that delivered in vivo, were not directly toxic to *B. burgdorferi* in vitro. In fact, 100 or 200 U/mL IFN-γ, 500 U/mL IL-2, combinations of IFN-γ plus IL-2, or 25–100 ng/mL TNF-α used alone significantly enhanced the proliferation of spirochetes in vitro compared with those cultured in medium alone (figure 4). Only the triple combination of TNF-α plus IL-2 plus IFN-γ resulted in decreased growth relative to controls. This was not, however, a statistically significant decrease in growth over 72 h.

Discussion

The studies reported here indicate that Th1–derived or macrophage-associated cytokines, used during the earliest period of *I. scapularis* inoculation of spirochetes, significantly inhibited *B. burgdorferi* infection. Using a C3H/HeJ mouse model of natural infection [48], TNF-α alone or in combination treatment inhibited *B. burgdorferi* infection for 21 days after tick placement in 95% of challenged mice. Fifty-five to 70% of mice administered Th1-derived cytokines, IFN-γ or IL-2, resisted acute infection over this same time period compared with untreated controls, in which the vector-induced infection rate was 83.3%. By day 50 after tick infestation (chronic infection), infection of IFN-γ– and IL-2–treated mice was reduced by 50% compared with untreated animals, in which the infection rate of specific target organs was 75%. Although protection against chronic infection was maintained in 50% of the animals challenged with *I. scapularis,* this protection was not statistically significant compared with that of untreated controls. Furthermore, mice given TNF-α alone or in combination could not maintain control of *B. burgdorferi* after the initial 21-day period, and resultant chronic infection of target organs occurred at a rate similar to that in untreated control mice.

To our knowledge, this is the first report of the suppression of natural, vector-induced *B. burgdorferi* infection in vivo using prophylactic cytokine therapy. Although inhibition of *I. scapularis* infectivity was not maintained at a significant level throughout the entire 50-day period, significant inhibition of acute infection was maintained for at least 21 days. The failure

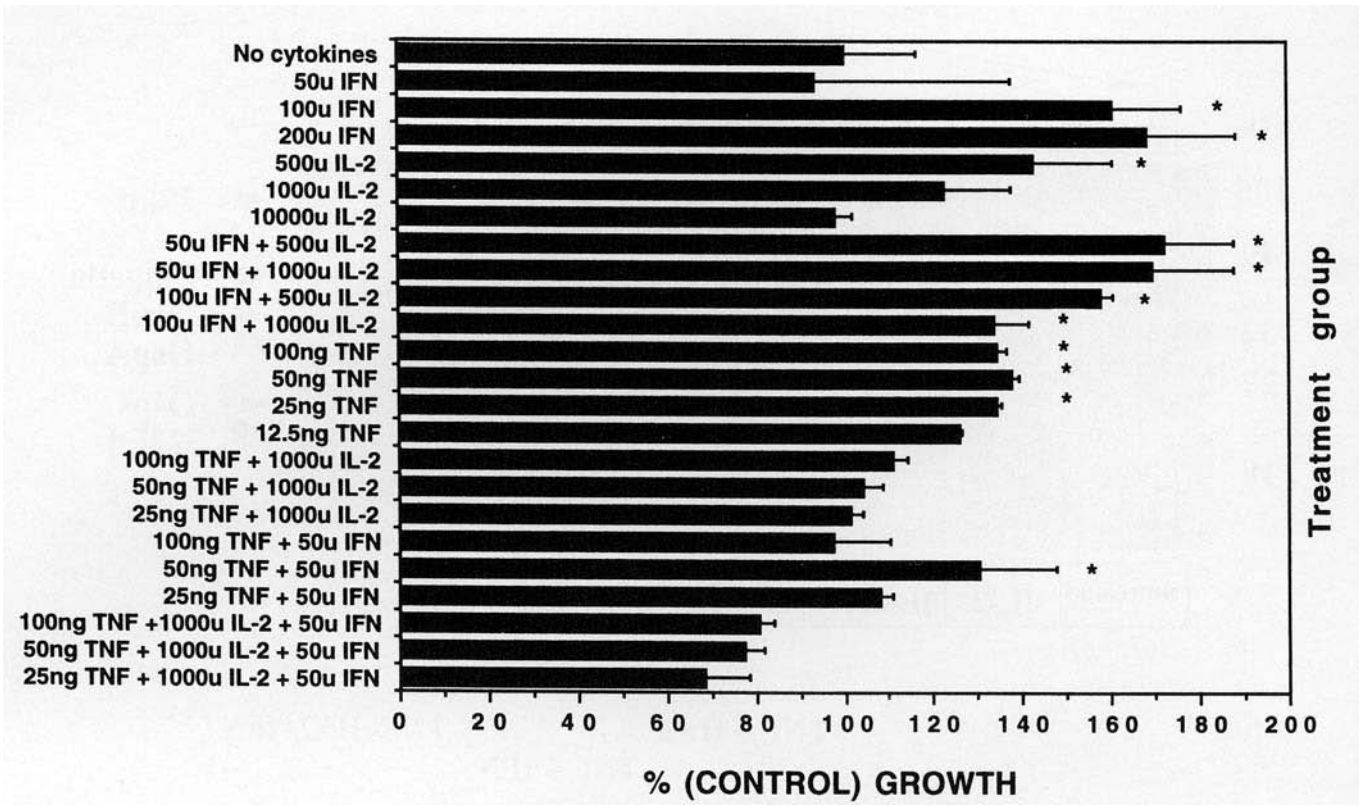

Figure 4. Growth of *B. burgdorferi* in cytokine-conditioned media. Bars indicate average of 3 cultures; SE bars represent SD from mean. u IFN (IFN-γ) and u IL-2 are in U/mL. * Statistical significance ($P < .05$, analysis of variance) relative to control growth in unconditioned media.

to control chronic infection in >50% of animals challenged with *I. scapularis* was most likely the result of several factors. These would include a lack of optimization of specific treatment regimens and enhanced stringency involved in our experimental approach, which involved a highly sensitive C3H/HeJ mouse model combined with a specific PCR detection method to amplify *B. burgdorferi* in target organs. Since there can be no control over the absolute level of infectious inoculum utilizing a natural vector for challenge studies, the results obtained from these experiments should be contrasted against those of the vast majority of the studies examining prophylactic immunization regimens, which were only 14–21 days long, did not use the natural vector, and did not analyze a panel of specific target organs by both culture and PCR [5, 7–10, 49].

Our in vitro studies, which demonstrated significantly enhanced growth of *B. burgdorferi* in cytokine-conditioned media, indicated that inhibition of spirochete infection in vivo was most likely an immune-mediated event and not the result of direct killing of spirochetes by recombinant cytokines. In fact, recombinant cytokines enhanced the growth of spirochetes in vitro, a phenomenon that correlated with several cytokine combinations that inhibited infectivity in vivo. This phenomenon of cytokine enhancement of microbial growth is not unique to *B. burgdorferi*. Specific receptors for host-derived cytokines have been described for protozoa [50], as IL-2 has been shown to enhance the in vitro growth and in vivo pathogenicity of *Leishmania amazonensis* [51]. Likewise, IFN-γ has been shown to play a role in enhancing both the growth and pathogenicity of *Trypanosoma brucei* in vivo [52]. Whether there are specific receptors on *B. burgdorferi* for Th1 or macrophage-derived cytokines or a specific role for cytokine growth enhancement to facilitate dissemination of this organism in vivo remains to be elucidated.

The likelihood of cellular immune-mediated containment is significant in this mouse model, as previous studies have demonstrated an inability of C3H/HeJ mice to respond adequately to *B. burgdorferi* infection [48]. Several perturbations in cytokine responsiveness to infection have been noted in C3H/HeJ mice, which are exquisitely sensitive to infection and disease compared with BALB/c mice, which become infected with *B. burgdorferi* but do not develop clinical symptoms. Ramachandra et al. [30] demonstrated an inability of C3H/HeJ macrophages to produce TNF-α when stimulated by *B. burgdorferi* in vitro. Moreover, relative to responses of BALB/c mice, Il-2 and IL-4 levels in C3H/HeJ mice did not increase with infection, although both strains of mice developed similar humoral responses to *B. burgdorferi* during chronic infection [32]. This correlated with earlier work by Souza et al. [41], which described impairment in lymphocyte proliferation and a decline in both IL-2 and IL-4 production in disease-susceptible strains

of mice versus resistant BALB/c mice. Ma et al. [53] demonstrated the in vitro macrophage stimulatory properties of both OspA and OspB and the relevance of TNF-α in mediating both the production of IFN-γ from NK cells and ultimately the production of nitric oxide by stimulated macrophages, a critical component of antimicrobial activity in vivo.

Taken together, these data suggest that cell-mediated immune responsiveness may be impaired in the earliest phases of spirochete infection and dissemination in susceptible animals, and thus may be a significant factor in control of early *B. burgdorferi* infection. As noted in previous studies, specific immune unresponsiveness waned over several weeks, and cytokine production returned to normal with increasing chronicity of infection [32]. In fact, experimental data suggest that Th1 cytokine expression in chronic infection may be associated with arthritis in susceptible C3H/HeJ mice, while high levels of IL-4 production was noted in regional lymph nodes of disease-resistant BALB/c mice 4–5 weeks after needle inoculation of spirochetes [54]. Thus, early control over Th1/Th2 cytokine regulation would be paramount in regulating not only early infection but subsequent immunopathology in the susceptible host.

Immune impairment during acute infection may be further compounded in the earliest phases of vector inoculation of spirochetes by the immune regulatory properties associated with *I. scapularis* feeding and saliva deposition within the host [55]. Tick saliva has been shown both in vitro and in vivo to have profound down-regulatory effects on TNF-α and IL-1 production by macrophages, IL-2 and IFN-γ release by T cells, and induction of a general state of immune nonresponsiveness by blocking specific modulators of the initial inflammatory response during tick attachment and feeding [55].

In terms of the data presented here, early reconstitution of both Th1 and macrophage-derived cytokines enabled disease-susceptible mice to circumvent both tick- and spirochete-induced immunosuppression and to mount an early and effective immune response sufficient to contain *Ixodes*-induced spirochete infection. Antigenic modulation by cytokine treatment and humoral immunity, in terms of nonspecific reactivity measured by immunoblot and a lack of neutralizing antibody activity, did not appear to be a significant factor in the protection of cytokine-treated C3H/HeJ mice. Because TNF-α, IL-2, and IFN-γ would be expected to up-regulate macrophage cytotoxicity, enhance class I and II antigen presentation, and reinforce NK and T cell cytotoxicity, it should follow that a state of cellular unresponsiveness would be reversed and cellular immunity would then become established in cytokine-treated mice. Although the direct role of cellular immunity has never been established for containment of early *B. burgdorferi* infection in vivo, experimental evidence suggests that cellular protective mechanisms are grossly and directly down-regulated in these animals and therefore should play a positive role in containment of infection.

In conclusion, the studies presented here indicate that delivery of appropriate cytokines during the earliest phases of tick infestation and spirochete inoculation inhibit the establishment of acute *B. burgdorferi* infection in a highly susceptible mouse strain. These data suggest that this is accomplished without the benefit of a specific humoral immune response and that cytokines that enhance initial containment of spirochetes do so without directly killing *B. burgdorferi.* In light of the fact that the efficacy of a vaccine program using spirochete Osps is presently unknown in humans, it may be prudent to consider alternative vaccine strategies. To that end we are currently using this therapeutic model to establish the role of specific cellular compartments in the containment of *Ixodes*-induced *B. burgdorferi* in mice. Establishment of the role of specific, early cellular immunity should have ramifications not only for successful vaccine development in *I. scapularis*–induced infections but, given the ubiquitous nature of host immune suppression induced by hematophagous vectors [38–40, 55], should have broad application in the prevention and treatment of other arthropodborne diseases.

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References

1. Centers for Disease Control and Prevention. MMWR Morb Mortal Wkly Rep **1995**;44:459–62.
2. Barbour AG, Hayes SF. Biology of *Borrelia* species. Microbiol Rev **1986**;50:381–400.
3. Brown RN, Lane RS. Lyme disease in California: a novel enzootic transmission cycle of *Borrelia burgdorferi.* Science **1992**;256:1439–42.
4. Schaible UE, Kramer MD, Eichmann K, Modolell M, Museteanu C, Simon MM. Monoclonal antibodies specific for the outer surface protein A (OspA) of *Borrelia burgdorferi* prevent Lyme borreliosis in severe combined immunodeficiency (scid) mice. Proc Natl Acad Sci **1990**;87:3768–72.
5. Fikrig E, Barthold SW, Kantor FS, Flavell RA. Long-term protection of mice from Lyme disease by vaccination with OspA. Infect Immun **1992**;60:773–7.
6. Fikrig E, Telford SR, Barthold SW, Kantor FS, Spielman A, Flavell RA. Elimination of *Borrelia burgdorferi* from vector ticks feeding on OspA-immunized mice. Proc Natl Acad Sci **1992**;89:5418–21.
7. Preac-Mursic V, Wilske B, Patsouris E, et al. Active immunization with pC protein of *Borrelia burgdorferi* protects gerbils against *B. burgdorferi* infection. Infection **1992**;20:342–9.
8. Stover K, Bansal GP, Hanson MS, et al. Protective immunity elicited by recombinant bacille Calmette-Guérin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine. J Exp Med **1993**;178:197–209.
9. Norton-Hughes CA, Engstrom SM, Coleman LA, Kodner CB, Johnson RC. Protective immunity is induced by a *Borrelia burgdorferi* mutant that lacks OspA and OspB. Infect Immun **1993**;61:5115–22.

10. Probert WS, LeFebvre RB. Protection of C3H/HeN mice from challenge with *Borrelia burgdorferi* through active immunization with OspA, OspB, or OspC, but not with OspD or the 83-kilodalton antigen. Infect Immun **1994**;62:1920–6.
11. Nguyen PK, Lam TT, Barthold SW, Telford SR, Flavell RA, Fikrig E. Partial destruction of *Borrelia burgdorferi* within ticks that engorged on OspE- or OspF-immunized mice. Infect Immun **1994**;62:2079–84.
12. Dykhuizen, DE, Polin DS, Dunn JJ, et al. *Borrelia burgdorferi* is clonal: implications for taxonomy and vaccine development. Proc Natl Acad Sci **1993**;90:10163–7.
13. Zingg BC, Anderson JF, Johnson RC, LeFebvre RB. Comparative analysis of genetic variability among *Borrelia burgdorferi* isolates from Europe and the United States by restriction enzyme analysis, gene restriction fragment length polymorphism, and pulsed-field gel electrophoresis. J Clin Microbiol **1993**;31:3115–22.
14. Zingg BC, Brown RN, Lane RS, LeFebvre RB. Genetic diversity among *Borrelia burgdorferi* isolates from wood rats and kangaroo rats in California. J Clin Microbiol **1993**;31:3109–14.
15. Schwan TG, Schrumpf ME, Karstens RH, et al. Distribution and molecular analysis of Lyme disease spirochetes, *Borrelia burgdorferi,* isolated from ticks throughout California. J Clin Microbiol **1993**;31:3096–108.
16. Nakao M, Miyamoto K, Fujita H. Characterization of *Borrelia burgdorferi* isolated from *Ixodes persulcatus* and *Ixodes ovatus* ticks in Japan. Am J Trop Med **1992**;47:505–11.
17. Adam T, Gassman GS, Rasiah C, Gobel UB. Phenotypic and genotypic analysis of *Borrelia burgdorferi* isolates from various sources. Infect Immun **1991**;59:2579–85.
18. Schaible UE, Wallich R, Kramer MD, et al. Immune sera to individual *Borrelia burgdorferi* isolates or recombinant OspA thereof protect scid mice against infection with homologous strains but only partially or not at all against those of different ospA/OspB genotype. Vaccine **1993**;11:1049–56.
19. Sadziene A, Rosa PA, Thompson PA, Hogan DM, Barbour AG. Antibody-resistant mutants of *Borrelia burgdorferi:* in vitro selection and characterization. J Exp Med **1992**;176:799–809.
20. Aberer E, Brunner C, Suchanek G, et al. Molecular mimicry and Lyme borreliosis: a shared antigenic determinant between *Borrelia burgdorferi* and human tissue. Ann Neurol **1989**;26:732–7.
21. Sigal LH. Cross-reactivity between *Borrelia burgdorferi* flagellin and a human axonal 64,000 molecular weight protein. J Infect Dis **1993**;167:1372–8.
22. Mensi N, Webb DR, Turck CW, Peltz GA. Characterization of *Borrelia burgdorferi* proteins reactive with antibodies in synovial fluid of a patient with Lyme arthritis. Infect Immun **1990**;58:2404–7.
23. Moody KD, Barthold SW. Relative infectivity of *Borrelia burgdorferi* in Lewis rats by various routes of inoculation. Am J Trop Med **1991**;44:135–9.
24. Roehrig JT, Piesman J, Hunt AR, Keen MG, Happ CM, Johnson BJ. The hamster immune response to tick-transmitted *Borrelia burgdorferi* differs from the response to needle-inoculated cultured organisms. J Immunol **1992**;149:3648–53.
25. deSouza MS, Smith AL, Beck DS, Kim LJ, Hansen GM, Barthold SW. Variant responses of mice to *Borrelia burgdorferi* depending on the site of intradermal inoculation. Infect Immun **1993**;61:4493–7.
26. Ribeiro JM, Makoul GT, Levine J, Robinson DR, Spielman A. Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, *Ixodes dammini.* J Exp Med **1985**;161:332–44.
27. Ramachandra RN, Wikel SK. Modulation of host-immune responses by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production. J Med Entomol **1992**;29:818–26.
28. Shih CM, Pollack RJ, Telford SR, Spielman A. Delayed dissemination of Lyme disease spirochetes from the site of deposition in the skin of mice. J Infect Dis **1992**;166:827–31.
29. Burkot TR, Piesman J, Wirtz RA. Quantitation of the *Borrelia burgdorferi* outer surface protein A in *Ixodes scapularis:* fluctuations during the tick life cycle, doubling times, and loss while feeding. J Infect Dis **1994**;170:883–9.
30. Ramachandra RN, Wikel SK. Effect of *Borrelia burgdorferi* strain 297 on peritoneal macrophages of different strains of mice: elaboration of tumor necrosis factor-α and interleukin-1. Immunol Infect Dis **1993**;3:203–7.
31. Ribeiro JM, Weis JJ, Telford SR. Saliva of the tick *Ixodes dammini* inhibits neutrophil function. Exp Parasitol **1990**;70:382–8.
32. Ramachandra RN, Dalton DA, Wikel SK. Antibody and cytokine response in mice experimentally infected with *Borrelia burgdorferi* strain 297. Immunol Infect Dis **1993**;3:299–08.
33. Ribeiro JM. *Ixodes dammini:* salivary anti-complement activity. Exp Parasitol **1987**;64:347–53.
34. Balashov YS. Bloodsucking ticks (Ixodoidea): vectors of diseases in man and animals. Misc Pub Entomol Soc Am **1972**;8:161.
35. Karupiah G, Xie Q, Buller R, Nathan C, Duarte C, MacMicking JD. Inhibition of viral replication by interferon-γ–induced nitric oxide synthase. Science **1993**;261:1445–8.
36. Green SJ, Nacy CA. L-arginine–derived nitric oxide is an antimicrobial effector molecule. ASM News **1994**;60:83–5.
37. Peterson PK, Clawson CC, Lee DA, Garlich DJ, Quie PG, Johnson RC. Human phagocyte interactions with the Lyme disease spirochete. Infect Immun **1984**;46:608–11.
38. Ribeiro JM. Role of saliva in tick/host interactions. Exp Appl Acarol **1989**;7:15–20.
39. Titus RG, Ribeiro JM. Salivary gland lysates from the sand fly *Luzomyia longipalpis* enhance *Leishmania* infectivity. Science **1988**;239:1306–8.
40. Cross ML, Cupp MS, Cupp EE, Galloway AL, Enriquez FJ. Modulation of murine immunological responses by salivary gland extract of *Simulium vittatum* (Diptera: Simulidae). J Med Entomol **1993**;30:928–35.
41. deSouza MS, Smith AL, Beck DS, Terwilliger GA, Fikrig EF, Barthold SW. Long-term study of cell-mediated responses to *Borrelia burgdorferi* in the laboratory mouse. Infect Immun **1993**;61:1814–22.
42. Magnarelli LA, Andreadis TG, Stafford KC, Holland CJ. Rickettsiae and *Borrelia burgdorferi* in Ixodid ticks. J Clin Microbiol **1991**;29:2798–803.
43. Bakken JS, Dumler JS, Chen SM, Eckman MR, Van Etta LL, Walker DH. Human granulocytic ehrlichiosis in the upper midwest United States. A new species emerging? JAMA **1994**;272:212–8.
44. Burkot TR, Wirtz RA, Luft B, Piesman J. An OspA antigen-capture enzyme-linked immunosorbent assay for detecting North American isolates of *Borrelia burgdorferi* in larval and nymphal *Ixodes dammini.* J Clin Microbiol **1993**;31:272–8.
45. Johnson B, Happ CM, Mayer LW, Piesman J. Detection of *Borrelia burgdorferi* in ticks by species-specific amplification of the flagellin gene. Am J Trop Med Hyg **1992**;47:730–41.
46. Sinsky RJ, Piesman J. Ear punch biopsy method for detection and isolation of *Borrelia burgdorferi* from rodents. J Clin Microbiol **1989**;27:1723–7; erratum **1989**;27:2401.
47. Ma J, Coughlin RT. A simple, colorimetric microtiter assay for borreliacidal activity of antisera. J Microbiol Methods **1993**;17:145–53.
48. Barthold SW, Beck DS, Hanse GM, Terwilliger GA, Moody KD. Lyme borreliosis in selected strains and ages of laboratory mice. J Infect Dis **1990**;162:133–8.
49. Dunne M, Al-Ramadi BK, Barthold SW, Flavell RA, Fikrig E. Oral vaccination with an attenuated *Salmonella typhimurium* strain expressing *Borrelia burgdorferi* OspA prevents murine lyme borreliosis. Infect Immun **1995**;63:1611–4.
50. Hide G, Gray A, Harrison CM, Tait A. Identification of an epidermal growth factor receptor homologue in trypanosomes. Mol Biochem Parasitol **1989**;36:51–60.

51. Mazingue C, Cottrez-Detoeuf F, Louis J, Kweider M, Auriault C, Capron A. In vitro and in vivo effects of interleukin-2 on the protozoan parasite *Leishmania*. Eur J Immunol **1989**;19:487–91.
52. Olsson T, Bakhiet M, Edlund C, Hojeberg B, Van der Meide PH, Kristensson K. Bidirectional activating signals between *Trypanosoma brucei* and $CD8^+$ T cells: a trypanosome-released factor triggers interferon-γ production that stimulates parasite growth. Eur J Immunol **1991**;21:2447–54.
53. Ma Y, Seiler KP, Tai KF, Yang L, Woods M, Weis JJ. Outer surface lipoproteins of *Borrelia burgdorferi* stimulate nitric oxide production by the cytokine-inducible pathway. Infect Immun **1994**;62:3663–71.
54. Matyniak JE, Reiner SL. T helper phenotype and genetic susceptibility in experimental Lyme disease. J Exp Med **1995**;1251–4.
55. Ribeiro JMC. Role of saliva in blood-feeding arthropods. Ann Rev Entomol **1987**;32:463–78.