1997

Characterization of leukocytes and their effects in Lyme arthritis

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DOI: <https://doi.org/10.31979/etd.p3tw-3rf4>
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**CHARACTERIZATION OF LEUKOCYTES AND THEIR EFFECTS IN
LYME ARTHRITIS**

A Thesis

Presented to

**The Faculty of the Department of Biological Sciences
San Jose State University**

**In Partial Fulfillment
of the Requirements for the Degree
Master of Science**

by

Arlene Diana Bitmansour

December 1997

UMI Number: 1388170

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ABSTRACT

CHARACTERIZATION OF LEUKOCYTES AND THEIR EFFECTS IN LYME ARTHRITIS

by Arlene D. Bitmansour

To investigate the possible involvement of leukocytes in the exacerbation of Lyme arthritis, adoptive transfer experiments were performed using leukocytes isolated from naive C3H/He mouse spleens. The mouse leukocytes were enriched using a nylon wool column and the population was characterized by flow cytometry. Additionally, interferon-gamma and interleukin-4 expression by the brachial, inguinal, and popliteal lymph node lymphocytes of C3H/He mice which had been experimentally challenged with *Borrelia burgdorferi* was investigated using intracellular cytokine staining. Flow cytometry results of nylon wool enriched leukocytes indicated that this pool of leukocytes is comprised of 65% T cells, 5% B cells, 5% neutrophils, and 20% cells of uncharacterized phenotype. These findings revealed that nylon wool enrichment resulted in a mixed population of leukocytes and that this pool of leukocytes does not exacerbate Lyme arthritis. Intracellular cytokine staining revealed that the lymph node lymphocytes predominantly express interferon-gamma.

ACKNOWLEDGMENTS

The author would like to express her sincere gratitude and appreciation to the following San Jose State University technical staff and students that worked on various phases of this thesis project. This project would not have been possible without the dedication and excellent technical support of: Pat Baty (Staff Technician), Andrew Bitmansour, Navdeep Kaur, Linda Goff (Microbiology Technical Staff Manager), Pedram Malek, Jesse Martinez (Animal Care Facility Manager), Carleen Mumaw (Staff Chemist), Reza Riahi, Adam Seddiqui, and A. Mike Tamrazzi.

The author also sincerely thanks Andrew Bitmansour and Dr. Johanna Olweus from Becton Dickinson Immunocytometry Systems (San Jose, CA) for flow cytometry training, providing the flow cytometry facility, expert advice on flow cytometry data analysis for this project, and their generous gift of the following reagents: Thy 1.2, Lyt-2, L3T4, Gr-1, B220, SA APC, SA PerCP, and Ethidium Bromide-Acridine Orange; Dr. John T. Boothby from SJSU (San Jose, CA) for his expert advice and giving me the opportunity to work on this project; Dr. Robert L. Coffman from DNAX Research Institute (Palo Alto, CA) for his generous gift of 11B11 antibody, rIL-4, and expert advice on the T cell aspect of this project; Dr. Ruthann Kibler from SJSU (San Jose, CA) for her expert advice and the use of necessary lab equipment; Dr. Anne O'Garra from DNAX Research Institute (Palo Alto, CA) for providing us with the "Intracellular Cytokine Staining" protocol; Dr. Steven P. Nickell from University of New Mexico (Albuquerque, NM) for helpful discussions and expert advice on *B. burgdorferi*/T cell mouse model; Dr. Louis J. Picker from University of Texas Southwestern Medical Center (Dallas, TX) for expert advice on intracellular cytokine data analysis; and Dr. Irving L. Weissman from Stanford University (Stanford, CA) for his mentoring and support for the last three years which was above and beyond the call of duty for an "outside" committee member. It was through his contacts, suggestions, and challenges that this project was carried out beyond the limitations of SJSU's research facilities. Dr. Weissman's support, expert advice, and time spent on this project is truly appreciated.

DEDICATION

To
the love and grace of God
that has given me strength to endure and overcome all the challenges.

And to
my mother and brother, Elda L. Sargon and Andrew Bitmansour,
whose love, support, and encouragement made this thesis possible.

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GENERAL INTRODUCTION

Lyme disease is a multisystem illness that affects humans as well as other vertebrate hosts. Cases of Lyme disease have been reported in 46 of 48 contiguous United States and in Hawaii (5, 6). The tick species responsible for transmitting *Borrelia burgdorferi* (*Bb*), the causative agent of Lyme disease, belong to the *Ixodes* genus. On the East coast *Ixodes scapularis* and on the West coast *Ixodes pacificus* are the two main tick species associated with transmitting *Bb*. These hard-bodied ticks have four developmental stages which include: egg, larva, nymph, and adult. Each of the three motile stages must have one blood meal so that it can molt into the next stage or lay eggs. In the adult stage only females feed on blood and, after mating with males, convert this blood meal into the next generation of ticks in the form of eggs (10).

The prevalence of *Bb* in nymphs is 20-25% which is half of that found in the adult ticks. However, 90% of Lyme disease cases are due to the *Ixodes* sp. nymphs because host-seeking nymphs appear in late May which happens to coincide with the annual increased incidence of Lyme disease. Nymphs play a bigger role in the epidemiology of Lyme disease due to their smaller size, greater abundance, and the temporal coincidence of their peak feeding activity with human outdoor activity. Adult ticks seek hosts from mid-October until April (10).

Although the immature *Ixodes* ticks parasitize a wide variety of vertebrate hosts, the adult ticks are relatively restricted to the white-tailed deer for their blood meal. However, deer are incapable of infecting ticks with *Bb*. This role is assumed by a variety of small- and medium-sized mammals as well as several bird species. In the Northeast forests, the white-footed mouse, *Peromyscus leucopus*, serves as a reservoir of infection for the immature *Ixodes* ticks. An

individual mouse is capable of infecting nearly 100% of larval ticks during feeding. Larval *Ixodes* ticks that feed on infected hosts acquire *Bb* that are maintained through the larval molt and later transmitted to subsequent hosts during nymphal feeding (10).

Borrelia burgdorferi belongs to the order *Spirochaetales*. The term 'spirochete' encompasses a group of flexuous, thin, gram-negative, chemoheterotrophic, helical-shaped bacteria, which differ morphologically from other prokaryotes by having one or more axial fibrils. Axial fibrils are morphologically similar to prokaryotic flagella comprised of a shaft, its covering sheath, and an insertion apparatus that is differentiated into a terminal knob composed of a proximal hook and insertion disks or bulbous annular swellings (11). The prokaryotic flagellum is attached to the cell by a hook and a basal body, which has a set of rings that attach to the cytoplasmic membrane and a rod that passes through the rings to anchor the flagellum to the cell (1). Prokaryotic flagella propel bacteria from place to place (1), whereas the probable function of the axial fibril and its associated structures is still unclear (11). The order *Spirochaetales* is grouped into five genera which are separated on the basis of morphological and physiological characteristics. *Borrelia*, (one of the five genera) are distinct in size (1 μm by 25 μm), with long and loose coils, and cell poles that are tapered or pointed bearing 15 to 20 axial fibrils (11).

It is estimated that *Ixodes* ticks deliver fewer than 300 *Bb* during their attachment to a host. *Borrelia burgdorferi* travel from the midgut of infected unfed ticks and migrate into the salivary glands prior to transmission to a host. The ticks must be attached for approximately 2 days for infection to be transmitted to the host due to the shift in expression of outer surface proteins (Osp) by the spirochetes. OspA is abundantly expressed on *Bb* in the midgut of

unfed ticks. Within 24 hours of feeding OspA is rapidly lost and OspC is up-regulated on the spirochetes. Once spirochetes have been transmitted to the skin of the host, they reside locally in the dermis for a few days prior to dissemination (2).

The localization of *Bb* in the dermis causes erythema migrans (EM) in 60% to 80% of Lyme disease patients. This EM rash is characteristic of early Lyme disease and was first described in Europe one century before the spirochetal etiology or the tick vector was recognized (21). Erythema migrans develops days to one month after a tick bite and the lesion diameter increases with duration. A central papule which is hyperpigmented is often present at the site of the punctum, giving EM a characteristic target like appearance. As many as 80% of patients with EM have associated systemic complaints such as fatigue (54%), myalgia (44%), arthralgia (44%), headache (42%), fever and/or chills (39%), and stiff neck (35%). Symptoms may occur in the untreated patient prior to the onset of, concomitant with, or after resolution of EM. Cranial nerve palsies and meningitis typically arise earlier and may occur concomitantly with EM. The cardiac features of Lyme disease usually occur within weeks to months post infection and include disruption of the atrioventricular conduction system (first degree, second degree, and complete block of action potentials from the atria into the ventricles), tachyarrhythmias, myopericarditis, and mild cardiac muscle dysfunction with cardiomegaly. Lyme arthritis generally occurs weeks to months after resolution of EM in the untreated patient (21, 23).

Musculoskeletal involvement, particularly arthritis, is a common feature of late Lyme disease. The severity of untreated Lyme arthritis (LA) ranges from subjective joint pain to intermittent attacks or chronic synovitis (painful inflammation of the synovial membrane). Chronic LA is defined as ≥ 1 year of

continuous joint inflammation and primarily involves the knees. Certain class II major histocompatibility genes seem to determine the host immune response to *Bb* that results in chronic LA. The Class II molecules, expressed primarily on B cells, macrophages, dendritic cells, thymic epithelial cells, and in human activated T cells, bind and present antigen to T helper (Th) cells, which then initiate an immune response against these antigens (15, 25). As antigen-presenting structures, the individual differences in MHC molecules expressed by an individual will influence the repertoire of antigens to which the Th cells can respond (15). Genetic variations in the structure of Class II molecules are thought to influence the development of autoimmune responses, either by determining the type and manner of antigen binding or by influencing the composition of the T cell repertoire in the development of self-tolerance during thymic maturation (24). Increased relative risks associated with the HLA-DR4 haplotype and synovial lesion pathology in chronic LA patients are similar to that seen in other types of chronic inflammatory arthritis, including rheumatoid arthritis. Patients with LA usually have high levels of IgG antibody to *Bb* reacting with ≥ 12 *Bb* proteins (25).

The antibody response to *Bb* in untreated patients develops gradually over the course of months to years with reactivity to an expanding array of proteins including OspA (31 kDa), OspB (34 kDa), OspC (22 kDa), flagellin (41 kDa), P39 (39 kDa lipoprotein), homologs of the GroEL family of heat shock proteins (60-, 66-, 68- kDa proteins), homologs of the DnaK family of heat shock proteins (71- and 73-kDa proteins), and P93 (83 to 100 kDa proteins), a core protein of *Bb*. OspC is recognized by serum of early and late Lyme disease patients whereas OspA/B antibodies are often absent or appear only late in the course of infection (2). This pattern of antibody appearance correlates with the

down-regulation of OspA and up-regulation of OspC during the feeding of the ticks and prior to transmission of *Bb* to the host.

The cellular immune response is not well understood in Lyme disease patients and murine models of Lyme disease. Attempts to detect specific T cell responses to *Bb* or its antigens in mice have been variable, but indicate a relatively weak T cell response during infection. Cellular immune responses in the mouse model of Lyme disease have proven to be similar to those in humans (2). Pathogen-reactive T cell clones, isolated from peripheral blood or synovial fluid of Lyme disease patients, have a Th1-like pattern of cytokine secretion (IFN- γ). The Th1-like pattern of cytokine secretion was found in all of the human CD4⁺ T cell clones even though they were isolated from different patients. Their abundance during a period of active synovium suggests that this T cell subset plays a role in the pathogenesis of reactive arthritis (8, 16, 29). Antigen-specific responses of spleen and lymph node cells from *Bb*-infected C3H/He mice exhibit vigorous clonal expansion characteristic of lymphocytes and Th1-like pattern of cytokine secretion *in vitro* (13, 18).

Transfer of immune cells or serum from infected mice to naive mice has allowed investigation into the relative contribution of the cellular and humoral arms of acquired immunity in host responses to *Bb*. Adoptive transfer of T cell-enriched lymphocytes from infected immunocompetent mice to severe combined immunodeficient (*scid*) mice did not protect against challenge inoculation with *Bb* (2). In contrast passive transfer of immune serum from immunocompetent mice at 18 hours prior or 0 hours relative to *Bb* challenge protected both immunocompetent and *scid* mice from infection. Treatment of mice with immune serum on days 4, 8, and 12 post *Bb* challenge cured mice from infection and the mice did not develop carditis or arthritis. Also, treatment

of *scid* mice with immune serum on days 12 (arthritis onset is on day 12 post *Bb* challenge), 16, 20, 24, and 28 post *Bb* challenge completely resolved arthritis whereas *Bb* challenged *scid* mice treated with normal mouse serum had severe arthritis (2).

Helper T cells regulate virtually every aspect of the antigen-dependent response of B cells, and Th cells are required for the activation, clonal expansion, differentiation, and isotype regulation of B cells which respond to most antigens. The cytokines expressed by the two subsets of Th cells, Th1 and Th2, have well-documented effects on B cells (7). Th1 and Th2 cells can be distinguished by the cytokines they produce (Table 1). Th1 cells produce Interleukin-2 (IL-2), lymphotoxin (TNF- β), and Interferon-gamma (IFN- γ). Th2 cells produce IL-4, IL-5, IL-9, IL-10, or IL-13 (7, 9, 26). Mature (CD4⁺, CD8⁻) Th cells often pass through a "Th0" stage of differentiation in which they secrete both type 1 and type 2 cytokines. These Th0 cells can then be primed to secrete either type 1 or type 2 cytokines by cytokines and/or infectious agents (9). Cytokine responses that resemble Th1 or Th2 responses (but are not necessarily made by CD4⁺ T cells) are referred to as type 1 or type 2 responses respectively (9). Type 1 responses are generally associated with cytotoxic effects {cytotoxic T lymphocyte (CTL), activated macrophages that kill ingested microbes, and complement-fixing antibody}, while type 2 responses are generally associated with non-complement-fixing antibody and allergy related phenomena (eosinophilia, mucosal mastocytosis, and IgE) (9, 20, 27).

Helper T cell cytokine depletion studies indicate that T cells modulate B cell responses. It is known that IFN- γ augments IgG2a whereas IL-4 augments IgG1 production by B cells responding to antigen (7, 20). Since cytokines released by T cells appear to mediate much of their functional activity,

monoclonal antibody (mAb) against IFN- γ and mAb against IL-4 was used to deplete Th1 and Th2 cytokines respectively in C3H/He and BALB/c mice. In these mice, IFN- γ depletion decreased arthritis and *Bb* levels in the joints and augmented production of IgG1 antibody, whereas IL-4 depletion increased arthritis and *Bb* levels in the joints and augmented production of IgG2a antibody (13).

The purpose of this thesis project was to investigate the effect of leukocyte adoptive transfer in LA and to characterize the predominant cytokine pattern in lymph node lymphocytes. Chapter I of this thesis presents *in vivo* adoptive transfer studies using C3H/He mice experimentally challenged with *Bb*. The study described in Chapter II of this thesis is an intracellular cytokine analysis of lymph node lymphocytes from C3H/He mice experimentally challenged with *Bb*.

Chapter I of this thesis presents data from adoptive transfer studies of nylon wool enriched leukocytes (NWEL) and their effects in LA in C3H/He mice. The donor cells were harvested from naive C3H/He mice, nylon wool column enriched, and characterized by flow cytometry. Flow cytometry is a process that allows the simultaneous measurement of multiple physical characteristics of a single cell (3).

C3H/He mice were treated by adoptive transfer of NWEL, whole leukocytes (WL), or medium (RPMI-5 or RPMI). Nylon wool enriched leukocyte-treated mice, WL-treated mice, medium-treated, and control mice were challenged with 1×10^6 *Bb* 24 hours post treatment. Increases in tibiotarsal joint diameter were measured weekly to monitor joint swelling and development of LA, ear punch biopsies were performed on day 14 post *Bb* challenge to confirm disseminated infection, and serum samples were analyzed by ELISA for

the presence of *Bb*-specific IgG to confirm immune response. Results describe the exacerbation of LA with the adoptive transfer of NWEL, the NWEL dose-response curve (10^2 - 10^6 NWEL), the difference in tibiotarsal joint swelling in *Bb*-challenged C3H/He mice receiving NWEL adoptive transfer and WL adoptive transfer, the difference in tibiotarsal joint swelling in C3H/He mice receiving NWEL adoptive transfer, WL adoptive transfer, or adoptive transfer of RPMI-5, and the difference in tibiotarsal joint swelling of *Bb*-challenged C3H/He mice receiving NWEL adoptive transfer (in RPMI-5 or RPMI), adoptive transfer of RPMI-5, or RPMI.

Chapter II of this thesis is a preliminary study performed to assess the type 1/type 2 cytokine response by lymph node lymphocytes in LA. C3H/He mice were challenged with 1×10^6 *Bb*. Increases in tibiotarsal joint diameter were measured on day 0 and day 14 relative to *Bb* challenge to monitor joint swelling and development of LA and serum samples were analyzed by ELISA for the presence of *Bb*-specific IgG to confirm immune response. The brachial, inguinal, and popliteal lymph node cells were harvested from *Bb*-challenged and non-challenged mice on day 14 post *Bb* challenge, pooled separately, and stimulated in culture by PMA/Ionomycin or *Bb*. Post incubation with Brefeldin A, the intracellular cytokine expression of the lymph node cells was detected at the single cell level by flow cytometry.

Table 1. Monoclonal Antibodies for Leukocyte CDs and Cytokines

Antigen	Distribution	Cluster	Clone (mAb)	Cytokine
GR-1	neutrophils		RB6-8C5	
B220	B cells		RA3-6B2	
Thy 1.2	T cells		30-H12	IFN- γ IL-2 IL-4 IL-5 IL-9 IL-10/IL-13 TNF- β
L3T4	helper T cells	CD4	GK1.5	IL-4 IL-5 IL-9 IL-10/IL-13
Lyt-2	cytotoxic T cells	CD8	53-6.7	IFN- γ IL-2 TNF- β
Cytokine	Distribution			Clone (mAb)
IFN- γ	cytotoxic T cells, Th1 cells, NK cells			XMG1.2
IL-4	Th2 cells, NK T cells, basophils, mast cells			11B11

CHAPTER I

**THE EFFECT OF LEUKOCYTE ADOPTIVE TRANSFER ON JOINT
INFLAMMATION IN C3H/He MICE EXPERIMENTALLY
CHALLENGED WITH *Borrelia burgdorferi***

INTRODUCTION

Lyme disease is a multisystem illness caused by the bacterium *Borrelia burgdorferi* (*Bb*). This Gram negative spirochete is transmitted to mammalian hosts by infected *Ixodes sp.* ticks. Lyme disease is the most common vector-borne disease in the United States (9). Most patients develop a distinctive erythema migrans (EM) rash that typically occurs 2 to 32 days after inoculation at the site of the tick bite. The clinical findings in *Bb*-infected patients vary as some patients may be asymptomatic while others develop early Lyme disease symptoms that include fatigue, severe headaches, mild stiff neck, bone and muscle aches, and fever. Early Lyme disease is usually cured by oral antibiotics (21). However, approximately 20 to 40 percent of people infected with *Bb* do not have or do not detect EM. Hematogenous dissemination of *Bb* to secondary sites may occur often resulting in clinical manifestations which include polyarthropathies, mild heart conduction system blockage, and neurological abnormalities. The signs and symptoms of Lyme disease in a typical case can change and are often intermittent. Since the symptoms of Lyme disease may mimic other conditions such as influenza, aseptic meningitis, rheumatoid arthritis, or other conditions, misdiagnosis of *Bb*-infected patients may occur (1). If diagnosed early, some Lyme disease patients respond well to several common antibiotics. Without treatment, Lyme disease in some patients may progress to neurologic disorders, carditis, and arthritis which often resist rigorous anti-microbial therapy. In ~10% of patients with chronic Lyme arthritis (LA), the inflammation persists despite antibiotic therapy (19).

Lyme disease is a serious illness in North America. The major endemic areas in North America include the Northeast, the upper Midwest, and the Pacific coast. Eleven thousand, six hundred and three cases of Lyme disease

were reported to the Centers for Disease Control in 1995, a 23-fold increase over the 497 cases reported in 1982 (6).

The role of humoral mediated immunity in Lyme disease has been extensively studied (2, 10, 11), but the role of T lymphocytes and their cytokines has not been well characterized. Cytokines are required for the activation, clonal expansion, and differentiation of T cells and B cells which respond to most antigens (7, 13). Helper T cells often pass through a "Th0" stage of differentiation in which they secrete both type 1 and type 2 cytokines. When primed by other cytokines or infectious agents Th0 cells can differentiate into two major subsets, T helper 1 (Th1) and T helper 2 (Th2) which secrete an exclusive set of cytokines. Th1 cells secrete Interleukin-2 (IL-2), Interferon-gamma (IFN- γ), and lymphotoxin (TNF- β), while Th2 cells secrete IL-4, IL-5, IL-9, IL-10, or IL-13 (Table 1). The cellular type 1 and type 2 response is related to humoral immunity as IFN- γ is the cytokine responsible for augmenting IgG2a production and IL-4 is the cytokine responsible for augmenting IgG1 production. Specific cytokine patterns are associated with resolution or exacerbation of both infectious and immune-mediated diseases (12). Recent studies have suggested that Th1 and Th2 cytokines have opposing roles in *Bb*-infected mice (14-16, 22). IFN- γ appears to exacerbate LA whereas IL-4 appears to have an immunoprotective role in that it decreases arthritic joint lesion in *Bb*-infected mice and it decreases the number of spirochetes recovered from the joints of these mice (14-16). These studies were performed in both disease susceptible C3H/He and disease resistant BALB/c mice. The susceptibility of C3H/He mice and the resistance of BALB/c mice appear to be related to the type 1 or type 2 response mounted by these mice respectively.

In vivo T cell depletion studies in mice have demonstrated that T cells

play a critical role in determining the course of *Bb* infection in mice (16). The depletion of CD4⁺ and CD8⁺ T cell subsets via monoclonal antibody (mAb) administration can markedly alter the outcome of *Bb* infection *in vivo*. The depletion of CD8⁺ T cells in *Bb*-infected C3H/He and young BALB/c mice not only reduces the arthritic lesion size, but also reduces spirochete levels in their tissues. Conversely, the depletion of CD4⁺ T cells in *Bb*-infected C3H/He and young BALB/c mice not only increases the arthritic lesion size, but also increases spirochete levels in their tissues. These results suggest that CD4⁺ T cells have an immunoprotective role in *Bb*-infected C3H/He mice, whereas CD8⁺ T cells exacerbate LA in *Bb*-infected C3H/He and BALB/c mice.

Since T cell functional activity is mediated by cytokines, the possible involvement of these cytokines in experimental LA has also been investigated by Keane-Myers and Nickell (15, 19). The selective activation of CD4⁺ T cell subsets with distinct profiles of cytokine production is important in pathogenesis of inflammatory diseases (24). Keane-Myers and Nickell showed that IL-4 and IFN- γ influence the severity of disease in *Bb*-infected mice. The *in vivo* depletion of IL-4 via mAb appeared to promote the number of spirochetes recovered from the joint tissue and increased joint swelling in C3H/He and BALB/c mice. However, the *in vivo* depletion of IFN- γ via mAb appeared to decrease the number of spirochetes recovered from the joint tissue and decreased joint swelling in C3H/He and BALB/c mice. Therefore, these results suggest that IL-4 has an immunoprotective role in *Bb*-infected C3H/He and BALB/c mice, and conversely IFN- γ exacerbates LA in *Bb*-infected C3H/He and BALB/c mice. These two depletion studies imply that IFN- γ and CD8⁺ T cells exacerbate LA whereas IL-4 and CD4⁺ T cells decrease LA in *Bb*-infected mice.

Keane-Myers *et al.* in a recent study also showed that disease-

susceptible C3H/He mice, which normally mount a predominantly type I cytokine response (IFN- γ) during *Bb* infection, become more resistant to infection following treatment with recombinant IL-4 (rIL-4) indicated by reduced joint swelling and fewer spirochetes recovered from their joint tissue. Cytokines may play either an immunoprotective role or exacerbate LA via the T lymphocytes they mediate (14).

Adoptive transfer experiments facilitate the study of various populations of lymphocytes and the cellular interactions required to generate an immune response (17). Lim *et al.* in a recent study obtained popliteal and inguinal lymph node T lymphocytes from inbred normal LSH hamsters and LSH hamsters vaccinated with a whole-cell preparation of formalin-inactivated *Bb sensu stricto* isolate C-1-11 in adjuvant. Adherent B lymphocytes were removed in mAb 14-4-4-s-coated tissue culture plates. Non-adherent, enriched T lymphocytes (5×10^6 immune or normal T lymphocytes in 0.1 ml phosphate buffered saline (PBS)) were injected in each hind paw. Naive recipient hamsters given immune T lymphocytes developed severe destructive arthritis when challenged with *Bb sensu stricto* isolates C-1-11 and 297. By contrast, recipients infused with normal T lymphocytes and challenged with *Bb sensu stricto* isolates C-1-11 and 297 failed to develop severe destructive arthritis (20).

This chapter presents data from adoptive transfer studies using nylon wool enriched leukocytes (NWEL) and their effects in LA in C3H/He mice. The donor cells were harvested from naive C3H/He mice, nylon wool column enriched, and characterized by flow cytometry. Flow cytometry is a process that allows the simultaneous measurement of multiple physical characteristics of a single cell (3).

C3H/He mice were treated by adoptive transfer of NWEL, whole

leukocytes (WL), or medium (RPMI-5 or RPMI). Nylon wool enriched leukocyte-treated mice, WL-treated mice, medium-treated, and control mice were challenged with 1×10^6 *Bb* 24 hours post treatment. Increases in tibiotarsal joint diameter were measured weekly to monitor joint swelling and development of LA, ear punch biopsies were performed on day 14 post *Bb* challenge to confirm disseminated infection, and serum samples were analyzed by ELISA for the presence of *Bb*-specific IgG to confirm immune response. Results describe the exacerbation of LA with the adoptive transfer of NWEL, the NWEL dose-response curve (10^2 - 10^6 NWEL), the difference in tibiotarsal joint swelling in *Bb*-challenged C3H/He mice receiving NWEL adoptive transfer and WL adoptive transfer, the difference in tibiotarsal joint swelling in C3H/He mice receiving NWEL adoptive transfer, WL adoptive transfer, or adoptive transfer of RPMI-5, and the difference in tibiotarsal joint swelling of *Bb*-challenged C3H/He mice receiving NWEL adoptive transfer (in RPMI-5 or RPMI), adoptive transfer of RPMI-5, or RPMI.

MATERIALS AND METHODS

Mice. Four week old C3H/HeJ female mice were obtained from Simonsen Laboratories (Gilroy, CA). The mice were housed three per cage in the Animal Care Facility at San Jose State University.

Preparation of leukocytes for adoptive transfer and flow cytometry. Splenocytes from six naive mice spleens, were pooled and pelleted by centrifugation (200 x *g* for 10 minutes). The pooled splenocytes were washed in 10 ml of RPMI-5 {RPMI 1640 medium with L-glutamine (200 mM), penicillin (10,000 units), and streptomycin (10 mg/ml) (SIGMA Chemical Co., St. Louis, MO) containing 5% heat inactivated (56°C, 1 hour) fetal calf serum (FCS) (HyClone, Logan, UT)}. The red blood cells (RBC) were removed using ammonium-chloride-potassium (ACK) lysing buffer {NH₄Cl (0.15 M), KHCO₃ (1.0 mM), Na₂EDTA (0.1 mM), pH 7.4} and after washing twice with RPMI-5 a viable cell count was performed (see below). A nylon wool column was prepared by packing 1.0 g prewashed, sterile nylon wool (Robbins Scientific, Sunnyvale, CA) into a 12 ml plastic syringe and autoclaving for 30 minutes on dry cycle. The nylon wool column was equilibrated by running 25 to 50 ml of 37°C RPMI-5 through the column. The 37°C warm single cell suspension (WL) was diluted with RPMI-5 to $\leq 1 \times 10^8$ cells in 2 ml and applied to the 12 ml nylon wool column. After a 45 minute incubation at 37°C, RPMI-5 (37°C) was applied to the column and the first 15 ml of the nonadherent NWEL was collected. A viable cell count was performed as before and the cells were injected in a 0.5 ml volume of RPMI-5 by the intraperitoneal (i.p.) route into the mice. A minimum of 6×10^5 cells was saved for flow cytometry.

Leukocyte Viable Cell Count. A 10 μ l aliquot of cells was placed into a 1.5 ml microfuge tube. The cells were diluted by factors of 10 with PBS

containing 2% FCS and stained with a 10 μ l aliquot of Ethidium Bromide (10 μ g/ml) -Acridine Orange (3 μ g/ml) {Molecular Probes, Inc., Eugene, OR}. The diluted and stained cell mixture was loaded onto a standard hemocytometer and counted using a fluorescent microscope equipped with an ultraviolet light source. Cells fluorescing green were counted as viable cells and orange cells were counted as nonviable cells. The cell concentration was calculated as follows:

$$\text{Viable cells/ml} = (\text{Total \# of Viable Cells} / \text{Total \# of Squares Counted}) \times \text{Dilution Factor} \times 10^4$$

Bb strain and mice infections. Low passage (<5) virulent *Bb* N40 (*Bb* N40 isolate provided by Stephen Barthold, Yale University, New Haven, CT) were cultured in Barbour-Stoenner-Kelly (BSK) II medium (SIGMA) at 34°C to a high density. The spirochetes were enumerated using a dark field microscope and a Petroff-Hauser counting chamber. The spirochetes were pelleted and resuspended in sterile PBS to yield 1×10^7 spirochetes/ml. The mice were inoculated with 1×10^6 spirochetes in a total volume of 0.1 ml, by the intradermal (i.d.) route in the mid-back region. *Bb* infection of the mice took place 24 hours after receiving adoptive transfer of NWEL, WL, RPMI-5, or RPMI (RPMI 1640 without 5% FCS).

Experimental Mouse Groups. A preliminary study was performed to confirm the exacerbation of LA with the adoptive transfer of NWEL. The three experimental mouse groups designated for this study received adoptive transfer treatment and/or *Bb* challenge, or were non-treated, non-challenged controls (1×10^6 NWEL/ 1×10^6 *Bb*, no treatment/ 1×10^6 *Bb*, and no treatment/no *Bb*).

The NWEL dose-response curve was investigated with seven experimental mouse groups that received adoptive transfer treatment and/or *Bb* challenge, or were non-treated, non-challenged controls (1×10^2 NWEL/ $1 \times$

10^6 *Bb*, 1×10^3 NWEL/ 1×10^6 *Bb*, 1×10^4 NWEL/ 1×10^6 *Bb*, 1×10^5 NWEL/ 1×10^6 *Bb*, 1×10^6 NWEL/ 1×10^6 *Bb*, no treatment/ 1×10^6 *Bb*, and no treatment/no *Bb*).

The difference in tibiotarsal joint swelling of mice receiving NWEL adoptive transfer and WL adoptive transfer was investigated with five experimental mouse groups that received adoptive transfer treatment and/or *Bb* challenge, or were non-treated, non-challenged controls (1×10^2 NWEL/ 1×10^6 *Bb*, 1×10^3 NWEL/ 1×10^6 *Bb*, 6×10^3 WL/ 1×10^6 *Bb*, no treatment/ 1×10^6 *Bb*, and no treatment/no *Bb*).

The difference in tibiotarsal joint swelling of mice receiving NWEL adoptive transfer, WL adoptive transfer, or adoptive transfer of RPMI-5 was investigated with seven experimental mouse groups that received adoptive transfer treatment and/or *Bb* challenge, adoptive transfer treatment and no *Bb* challenge, or were non-treated, non-challenged controls (1×10^3 NWEL/ 1×10^6 *Bb*, 6×10^3 WL/ 1×10^6 *Bb*, RPMI-5/ 1×10^6 *Bb*, 1×10^3 NWEL/no *Bb*, RPMI-5/no *Bb*, no treatment/ 1×10^6 *Bb*, and no treatment/no *Bb*).

Lastly, the difference in tibiotarsal joint swelling of mice receiving NWEL adoptive transfer (in RPMI-5 or RPMI), adoptive transfer of RPMI-5, or RPMI was investigated with six experimental mouse groups that received adoptive transfer treatment and/or *Bb* challenge, or were non-treated, non-challenged controls (1×10^6 NWEL in RPMI-5/ 1×10^6 *Bb*, 1×10^6 NWEL in RPMI/ 1×10^6 *Bb*, RPMI-5/ 1×10^6 *Bb*, RPMI/ 1×10^6 *Bb*, no treatment/ 1×10^6 *Bb*, and no treatment/no *Bb*) {see appendix, Table 2}.

Evaluation of arthritis in the tibiotarsal joints. The tibiotarsal joints in the first study (confirmation of the exacerbation of LA with NWEL adoptive transfer) were assessed visually and swelling was observed and

scored by the same blinded-person on day 7, 10, 14, 17, 21, 24, 28, 31, and 35 post infection in the first study. A 0 to +6 score was recorded for each joint (3 mice/group x 2 joints) and each joint was scored once. The extent of swelling in the groups was determined by subtracting mean joint scores before infection from mean joint scores after infection

Tibiotarsal joints were measured weekly using a spring-loaded microcaliper (Shop Tools Inc., Palo Alto, CA) through the thickest anteroposterior diameter of the ankle for the remaining studies. Joint measurements were taken by the same blinded-person and each joint was measured once. The extent of swelling in the groups was determined by subtracting mean joint diameters before infection from mean joint diameters after infection.

Analysis of systemic Bb infection via ear punch biopsy. On day 14 post *Bb* challenge an ear punch biopsy was performed using a sterile ear punch clip and the tissue was harvested. The ear punch tissue was placed in a tube containing 1 ml of BSK II medium and incubated at 34°C. The presence of spirochetes was ascertained 9 days after incubation in BSK II medium using a dark phase microscope.

Collection of pre-immune and immune serum samples. Three days prior to adoptive transfer treatment pre-immune serum samples were prepared from blood harvested from tail bleeding the mice. A lamp equipped with a 60 volt light bulb was placed directly over the mouse cage to effect an increase in body temperature and vasodilation of the mice. A clean, new razor was used to nick the tail of the mice and blood was collected in 1.5 ml microfuge tubes. The volume of harvested blood varied from 50 μ l to 500 μ l. After allowing the blood to clot for 10 minutes, the tubes were centrifuged (13,000 x

g) and the harvested sera were placed in 1.5 ml cryovials and frozen at -135°C. Immune serum was harvested from mice on day 14 and day 21 of infection as described above. Samples were evaluated for *Bb*-specific reactivity using ELISA.

ELISA for determining relative *Bb*-specific Ab in C3H/He mice. Microtiter plates (96-well) were coated with 100 μ l of *Bb* antigen (20 μ g/ml) in ELISA antigen diluent (carbonate buffer, 0.1 M, pH 9.6), and incubated overnight at 37°C. The plates were washed 3 times with ELISA wash (PBS containing 0.05% Tween 20). Serum harvested from the experimental mice was diluted 1:1000 in ELISA antibody diluent (PBS containing 0.005% Tween 20) and applied to the wells in triplicate in a 50 μ l volume and incubated for 30 minutes at room temperature. After washing 3 times with ELISA wash, 50 μ l of horseradish peroxidase conjugated goat anti-mouse IgG (1:2000) (SIGMA) was applied to all of the wells and incubated for 30 minutes at room temperature. The plates were washed 4 times with ELISA wash and 100 μ l of ELISA substrate {Phosphate-Citrate Buffer Tablet (0.05 M, pH 5.0) with Urea Hydrogen Peroxide + 2, 2'-Azino-bis(2-Ethylbenzthiazoline-6-Sulfonic Acid Tablet (pH 5.0)) (SIGMA) was added to the wells and the plates were incubated for one hour at room temperature. The optical density (OD) was measured using a Bio-Tek EL-310 microtiter plate reader at dual wavelength (A₄₀₅/A₄₅₀ nm). Results are reported as mean ELISA Value % (EV%) where:

$$EV\% = (\text{mouse serum OD} - \text{negative control OD}) / (\text{positive control OD} - \text{negative control OD}) \times 100$$

Sample preparation for flow cytometry analysis. NWEL or WL were placed on ice and distributed in a 96-well V-bottom microtiter plate (6 x 10⁵ cells/well). The cells were incubated with 20 μ l of undiluted normal mouse serum (Caltag, So. San Francisco, CA) for 30 minutes on ice and then stained

using 12 μ l of each fluorochrome-labeled mAb { $\ast$ B220 FITC, $\ast$ Gr-1 PE, $\dagger$ Thy1.2 biotin (b), $\dagger$ Lyt-2 FITC, $\dagger$ L3T4 PE} { $\dagger$ Becton Dickinson Immunocytometry Systems (BDIS) (San Jose, CA), $\ast$ Pharmingen (San Diego, CA)}.

After a 30 minute incubation on ice, the cells were washed two times with PBS containing 2% FCS. Streptavidin Allophycocyanin (SA APC) (BDIS) was added to the wells that received Thy1.2 b and incubated on ice for 30 minutes. The cells were washed two times with PBS containing 2% FCS, resuspended in 200 μ l PBS containing 2% FCS, and subjected to flow cytometry. The data were acquired using a high-sensitive FACScan and CELLQuest software and data analysis was performed using PAINT-A-GATE software (all from BDIS).

RESULTS

Characterization of Whole Leukocytes and Nylon Wool

Enriched Leukocytes used for Adoptive Transfer. Nylon wool enriched leukocytes and WL contained polymorphonuclear cells (neutrophils), large mononuclear cells with large cytoplasm to nucleus ratio (macrophages), as well as lymphocytes (Figure 1). These cytopun slides were prepared using cells from the immunophenotyping tubes. GR-1, B220, and Thy1.2 staining of the WL population yielded 5.38% neutrophils, 57.22% B cells, and 19.05% T cells with 18.34% unclassified cells. GR-1, B220, and Thy1.2 staining of the NWEL population yielded 6.88% neutrophils, 4.99% B cells, and 64.76% T cells with 24.17% unclassified cells (Figure 2). L3T4, Lyt-2, and Thy1.2 staining of the WL population yielded 15.93% L3T4+ T cells, 7.19% Lyt-2+ T cells, and 76.05% unclassified cells. L3T4, Lyt-2, and Thy1.2 staining of the NWEL population yielded 44.44% L3T4+ T cells, 22.67% Lyt-2+ T cells, and 30.71% unclassified cells (Figure 3).

Evaluation of systemic infection. Ear punch biopsies from 81 C3H/He mice were performed on day 14 post experimental *Bb* challenge. Ear punch biopsy cultures from all 60 experimentally *Bb*-challenged mice were positive for *Bb*, whereas, the ear punch biopsy cultures from all 21 non-challenged mice were negative for *Bb* (Figure 4).

Evaluation of antibody-mediated immune response. The mean *Bb*-specific EV% for 12 non-challenged mice on day 14 and 21 was -0.62% and 0.36% respectively, and mean *Bb*-specific EV% for 48 experimentally *Bb*-challenged mice on day 14 and day 21 was 34.98% and 59.13% respectively (Figure 4).

Evaluation of Hind Tibiotarsal Joint Swelling In C3H/He Mice.

The exacerbation of LA with the adoptive transfer of NWEL was confirmed in *Bb*-challenged C3H/He. The tibiotarsal joints of the experimental mice were evaluated by subjective visual examination. At 21 days post-challenge, mice treated with 1×10^6 NWEL appeared to have joint swelling scores that were two times greater than non-treated, *Bb*-challenged mice and six times greater than non-treated, non-challenged mice (Figure 5).

The tibiotarsal joints of experimental mice in the following studies were evaluated by a spring-loaded microcaliper. The NWEL dose-response curve was investigated in *Bb*-challenged C3H/He mice and by day 21 post-challenge *Bb*-challenged mice treated with 100, 1000, and 100,000 NWEL had similar increases in the tibiotarsal joint diameter. The mean increase in tibiotarsal joint diameter of *Bb*-challenged mice treated with 1×10^6 NWEL was 1.43 times greater than non-treated, *Bb*-challenged mice and 5 times greater than non-challenged, non-treated mice (Figure 6).

The difference in tibiotarsal joint swelling in *Bb*-challenged C3H/He mice receiving NWEL adoptive transfer and WL adoptive transfer was investigated. By day 21 post-challenge mean increase of tibiotarsal joint diameter of *Bb*-challenged mice treated with 100 NWEL was 1.14 times greater than the mean increase of tibiotarsal joint diameter of non-treated, *Bb*-challenged mice (note that only one mouse was alive on day 21). Average mean increase of tibiotarsal joint diameter of non-treated, *Bb*-challenged mice was 1.56 times greater than the mean increase of tibiotarsal joint diameter of *Bb*-challenged mice treated with 1000 NWEL, 1.75 times greater than the mean increase of tibiotarsal joint diameter of *Bb*-challenged mice treated with 6000 WL, and 7.00 times greater than the mean increase of tibiotarsal joint diameter of non-treated, non-challenged mice (Figure 7).

The difference in tibiotarsal joint swelling was investigated in *Bb*-challenged C3H/He mice receiving NWEL adoptive transfer, WL adoptive transfer, or adoptive transfer of RPMI-5. By day 21 post-challenge mean increase of tibiotarsal joint diameter of *Bb*-challenged mice treated with RPMI-5 was 1.27 times greater than the mean increase of tibiotarsal joint diameter of non-treated, *Bb*-challenged mice. Non-treated, *Bb*-challenged mice had larger joint diameters than *Bb*-challenged mice treated with 6000 WL and *Bb*-challenged mice treated with 1000 NWEL. Non-challenged mice with and without treatment had similar joint diameters which were ~3 times smaller than *Bb*-challenged mice (Figure 8).

The difference in tibiotarsal joint swelling of *Bb*-challenged C3H/He mice receiving NWEL adoptive transfer (in RPMI-5 or RPMI), adoptive transfer of RPMI-5, or RPMI was investigated. By day 21 post-challenge mean increase of tibiotarsal joint diameter of *Bb*-challenged mice treated with 1,000,000 NWEL in RPMI-5 was 1.11 times greater than the mean increase of tibiotarsal joint diameter of *Bb*-challenged mice treated with 1,000,000 NWEL in RPMI and non-treated, *Bb*-challenged mice, 1.25 times greater than the mean increase of tibiotarsal joint diameter of *Bb*-challenged mice treated with RPMI, 1.43 times greater than the mean increase of tibiotarsal joint diameter of *Bb*-challenged mice treated with RPMI-5, 2.50 times greater than the mean increase of tibiotarsal joint diameter of non-treated, non-challenged mice (Figure 9).

Spleen Leukocytes from Naive C3H/He Mice

A.

B.

Figure 1. Cytospun, Wright-Giemsa stained whole leukocytes (A) and nylon wool enriched leukocytes (B). The cells in these preparations were analyzed by flow cytometry (see Figure 2 and 3). Note that neutrophils and large mononuclear cells (macrophages) are present in both cell preparations.

Spleen Leukocytes from Naive C3H/He Mice

Figure 2. Flow cytometric characterization of whole leukocytes and nylon wool enriched leukocytes. Cells were stained with GR-1 PE, B220 FITC, and Thy1.2 b+SA APC. The top two panels are gated on live cells based on forward scatter (20,000 events shown). The two middle panels show GR-1+ and Thy1.2+ cells present in the cell populations of the top panels. The bottom two panels show Thy1.2+ and B220+ cells present in the cell populations of the top two panels. Note that nylon wool enriched cells consist of ~65% T cells, and ~35% of the cell pool consists of neutrophils, B cells, and other unclassified cells.

Spleen Leukocytes from Naive C3H/He Mice

Figure 3. Flow cytometric characterization of whole leukocytes and nylon wool enriched leukocytes. Cells were stained with L3T4 PE, Lyt-2 FITC, and Thy1.2 b+SA APC. The top two panels are gated on live cells based on forward scatter (20,000 events shown). The two middle panels show L3T4+ and Lyt-2+ cells present in the cell populations of the top panels. The bottom two panels show Thy1.2+ cells present in the cell populations of the top two panels. Note that all Thy1.2+ cells are either L3T4+ or Lyt-2+. However, there are L3T4+ cells that are Thy1.2- (bottom panels: red events in the first two decades).

Figure 4. Summary of ELISAs performed to detect Bb-specific IgG in 60 experimental mice.

#1 Day 21 Mean ELISA Value for Bb-challenged mice=59.13% SD 14.66 n=48

#2 Day 21 Mean ELISA Value for Non-challenged mice=0.36% SD 1.9 n=12

(Note the ELISA Values for the 21 mice from one experiment, 12 Bb-challenged and 9 non-challenged, are not included due to the difference in ELISA conditions. However, Bb-specific IgG was detected in the Bb-challenged mice whereas sera from non-challenged mice did not have Bb-specific IgG.)

† (number of mice with ear punch biopsy positive cultures/ total number of mice in group)

Figure 5. Hind tibiotarsal joint swelling in C3H/He mice. The tibiotarsal joints were evaluated visually and a 0 or plus score was assigned to each joint. The extent of swelling in the groups was determined by subtracting mean joint scores before infection from mean joint scores after infection. Each data point represents 6 scores (3 mice/group x 2 joints). *Data points represent 4 scores (2 mice/group x 2 joints) due to the expiration of one mouse in each group.

Figure 6. Hind tibiotarsal joint swelling in C3H/He mice. The extent of swelling in the groups was determined by subtracting mean joint diameters before infection from mean joint diameters after infection. Each data point represents 6 measurements (3 mice/group x 2 joints).

Figure 7. Hind Tibiotarsal Joint Swelling in C3H/He Mice. The extent of swelling in the groups was determined by subtracting mean joint diameters before infection from mean joint diameters after infection. Each data point represents 6 measurements (3 mice/group x 2 joints). * Data point represents 2 measurements (1 mouse/group x 2 joints) due to the expiration of two mice in Group 1.

Figure 8. Hind Tibiotarsal Joint Swelling in C3H/He Mice. The extent of swelling in the groups was determined by subtracting mean joint diameters before infection from mean joint diameters after infection. Each data point represents 6 measurements (3 mice/group x 2 joints).

Figure 9. Hind Tibiotarsal Joint Swelling in C3H/He Mice. The extent of swelling in the groups was determined by subtracting mean joint diameters before infection from mean joint diameters after infection. Each data point represents 6 measurements (3 mice/group x 2 joints).

DISCUSSION

The cellular immune response is not well understood in Lyme disease patients and murine models of Lyme disease. Attempts to detect specific T cell responses to *Bb* or its antigens in mice have been variable, but indicate a relatively weak T cell response during infection. Cellular immune responses in the mouse model of Lyme disease have proven to be similar of those in humans (2). Pathogen-reactive T cell clones, isolated from peripheral blood or synovial fluid of Lyme disease patients, have a Th1-like pattern of cytokine secretion. The Th1-like pattern of cytokine secretion was found in all of the human CD4⁺ T cell clones even though they were isolated from different patients. Their abundance during a period of active synovium suggests that this T cell subset plays a role in the pathogenesis of reactive arthritis (8, 18, 26). Antigen-specific responses of spleen and lymph node cells from *Bb*-infected C3H/He mice exhibit vigorous clonal expansion characteristic of lymphocytes and Th1-like pattern of cytokine secretion *in vitro* (15, 22). Recent studies (14, 15) present evidence that Th2 cells via IL-4 have an immunoprotective role whereas Th1 cells via IFN- γ are exacerbative in LA.

Adoptive transfer experiments performed by Lim *et. al.* showed that inbred LSH hamsters given T lymphocytes obtained from inbred LSH hamsters vaccinated with formalin-inactivated *Bb* developed severe destructive arthritis when challenged with *Bb sensu stricto* isolates C-1-11 and 297, compared to recipients infused with normal T lymphocytes and challenged with *Bb* who failed to develop severe destructive arthritis (20).

Immunophenotyping showed that nylon wool enrichment yields ~65% T cell enrichment and that ~35% of the nylon wool enriched cells are contaminating leukocytes that include B cells, neutrophils, and other

unclassified cells. C3H/He mice challenged with *Bb* and treated with NWEL from naive mice developed more severe experimental LA than non-treated, *Bb*-challenged mice (5). To further investigate the experimental exacerbation of LA with NWEL adoptive transfer treatment confirmatory experiments were performed using the C3H/He mouse model. The hind tibiotarsal joint swelling in *Bb*-challenged C3H/He mice was larger than non-challenged C3H/He mice (Figures 5-9). This increase in tibiotarsal joint diameter was probably due to the development of arthritis caused by disseminated *Bb* infection. Since, ear punch biopsy cultures from all 60 *Bb*-challenged mice were positive for *Bb*, the inoculation of mice with 1×10^6 *Bb* was successful in inducing a systemic disseminated infection. The humoral immune response mounted by the experimental mice were evaluated by ELISA for *Bb*-specific IgG. Mice that were *Bb*-challenged had a high ELISA value (59.13% by day 21 post-challenge) for *Bb*-specific IgG compared to non-challenged mice and these results suggest that an acquired humoral immune response was mounted by the *Bb*-challenged mice.

The exacerbation of LA with the adoptive transfer of NWEL was assessed in *Bb*-challenged C3H/He using visual evaluation. A microcaliper was used in the following studies to measure the thickest anteroposterior diameter of the ankle. Although, visual evaluation of the total joint region is more sensitive in that swelling of the total joint region can be observed, spring-loaded microcaliper measurements reflect increases of tibiotarsal joint diameter through the anteroposterior region. Visual evaluation may be more reflective of the swelling state of the total joint region; however, microcaliper measurements are reproducible and can be compared to published studies (5).

The adoptive transfer treatment of mice with of 1×10^6 NWEL appeared

to exacerbate Lyme arthritis (Figure 5 and 6) but subsequent experiments with adoptive transfer control groups show that treated, *Bb*-challenged mice and non-treated, *Bb*-challenged mice had tibiotarsal joint diameter increases that were similar and that the tibiotarsal joint diameter increases were larger than non-treated, non-challenged mice. The mean increase in tibiotarsal joint diameter of non-treated, *Bb*-challenged mice and treated, *Bb*-challenged mice were similar. The increases in tibiotarsal joint diameter were variable within each group of mice and the variability within each group was larger than the differences of tibiotarsal joint diameter between groups.

The NWEL and WL used for adoptive transfer were suspended in RPMI medium containing 5% FCS. Fetal calf serum has many proteins and components that are antigenic and can promote an immune response in mice (4, 23, 25). The variable increases in the tibiotarsal joint diameters of treated, *Bb*-challenged mice may have been the result of the additional immune response to FCS components. Due to the variable increases of tibiotarsal joint diameters of *Bb*-challenged mice the effect of leukocyte adoptive transfer on joint inflammation in C3H/He mice experimentally challenged with *Bb* remains inconclusive.

Therefore, the findings in this adoptive transfer model did not lend support to the possible involvement of NWEL in the exacerbation of experimental LA. However, Lyme disease is the most common vector borne disease in the U.S. and further studies should be performed to establish the mechanism of immunity in Lyme disease until a cure is found.

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CHAPTER II

**DETECTION OF INTRACELLULAR CYTOKINES IN LYMPH NODE
LYMPHOCYTES OF C3H/He MICE EXPERIMENTALLY
CHALLENGED WITH *Borrelia burgdorferi***

INTRODUCTION

The immune response to infectious agents is often characterized by a dominance of either cell-mediated or humoral effector mechanism, which is determined by the cytokine pattern of T helper (Th) cell subsets (9, 16). Th1 cells produce Interleukin-2 (IL-2), lymphotoxin (TNF- β), Interferon-gamma (IFN- γ), and Th2 cells produce IL-4, IL-5, IL-9, and IL-10 or IL-13 (1, 2, 16). Type 1 responses are generally associated with cytotoxic effects (CTL, activated macrophages that kill ingested microbes, and complement-fixing antibody) while type 2 responses are associated with non-complement-fixing antibody and allergy related phenomena (eosinophilia, mucosal mastocytosis, and IgE) (2, 9).

Selective activation of Th cell subsets with distinct profiles of cytokine production is important in the pathogenesis of inflammatory diseases (14). Keane-Myers and Nickell showed that IL-4 and IFN- γ influence the severity of infection in mice experimentally challenged with *Borrelia burgdorferi* (*Bb*). The *in vivo* depletion of IL-4 via monoclonal antibody (mAb) increased the number of spirochetes recovered from the joint tissue and joint swelling in C3H/He mice. However, the *in vivo* depletion of IFN- γ via mAb decreased the number of spirochetes recovered from the joint tissue and joint swelling in C3H/He mice (4).

Disease-susceptible C3H/He mice, which normally mount a predominantly type I cytokine response (IFN- γ) during *Bb* infection, resist disease following treatment with recombinant IL-4 (rIL-4) indicated by reduced joint swelling and fewer spirochetes recovered from their joint tissue. This finding lends more support to the notion that cytokines may play either an

immunoprotective role or exacerbate Lyme disease via the T lymphocytes they mediate (3).

The studies performed by Keane-Myers and Nickell (3, 4) and Matyniak and Reiner (6) to phenotype Th cells used ELISA to evaluate total cytokine levels in cell culture supernatants. Although these studies were important in proposing the dominant Th phenotype in *Bb*-susceptible C3H/He mice and *Bb*-resistant BALB/c mice based on whole leukocyte cytokine secretion in culture, they failed to examine these leukocytes at the single cell level. It is now known that IFN- γ and IL-4 are secreted by a variety of cells such as CD8+, CD4+ (Th1), Natural Killer (NK) cells and CD4+ (Th2), NK T cells (NK cells that express intermediate levels of $\alpha\beta$ T cell receptor), mast cells, and basophils respectively (2, 7, 9, 18). Therefore, to implicate the cells responsible for secreting a particular cytokine, it is more appropriate to analyze cytokine production at the single cell level in concert with other phenotypic markers.

Picker *et al.* have adapted a method to detect the intracellular expression of cytokines in human cells after incubation with Brefeldin A which disrupts intracellular Golgi-mediated transport and allows cytokines to accumulate in the cytoplasm. This method enables detection of multiple cytokines per cell by flow cytometry (12). The intracellular cytokine detection assay by Picker *et al.* was modified by O'Garra and Murphy for murine cells (10, 11).

This chapter presents data from a preliminary study performed to assess the type 1/type 2 cytokine response by lymph node lymphocytes in Lyme arthritis (LA). C3H/He mice were challenged with 1×10^6 *Bb*. Increases in tibiotarsal joint diameter were measured on day 0 and day 14 relative to *Bb* challenge to monitor joint swelling and development of LA and serum samples were analyzed by ELISA for the presence of *Bb*-specific IgG to confirm immune

response. The brachial, inguinal, and popliteal lymph node cells were harvested from *Bb*-challenged and non-challenged mice on day 14 post *Bb* challenge, pooled separately, and stimulated in culture by PMA/Ionomycin or *Bb*. Post incubation with Brefeldin A, the intracellular cytokine expression of the lymph node cells was detected at the single cell level by flow cytometry.

MATERIALS AND METHODS

Mice. Four week old C3H/He female mice were obtained from B & K UNIVERSAL, Inc. (Fremont, CA). The mice were housed two per cage in the Animal Care Facility at San Jose State University.

Bb strain and mice infections. Low passage (<5) virulent *Bb* N40 (*Bb* N40 isolate provided by Stephen Barthold, Yale University, New Haven, CT) were cultured in Barbour-Stoenner-Kelly (BSK) II medium (SIGMA Chemical Co., St. Louis, MO) at 34°C to a high density. The spirochetes were enumerated using a dark field microscope and a Petroff-Hauser counting chamber. The spirochetes were pelleted and resuspended in sterile phosphate buffered saline (PBS) to yield 1×10^7 spirochetes/ml. The mice were inoculated with 1×10^6 spirochetes in a total volume of 0.1 ml, by the i.d. route in the mid-back region.

Evaluation of arthritis in the tibiotarsal joints. Tibiotarsal joints were measured using a spring-loaded microcaliper (Shop Tools Inc., Palo Alto, CA) through the thickest anteroposterior diameter of the ankle. Measurements were taken on day 0, prior to *Bb* challenge, and 14 days post *Bb* challenge by the same blinded-person. Each joint was measured once.

Collection of immune serum samples. Serum samples were harvested from the mice via tail bleeding on day 14 post *Bb* challenge. A lamp equipped with a 60 volt light bulb was placed directly over the mouse cage to effect an increase in body temperature and hence vasodilation of the mice. A clean, new razor was used to nick the tail of the mice and blood was collected in 1.5 ml microfuge tubes. The volume of harvested blood varied from 50 μ l to 500 μ l. After allowing the blood to clot for 10 minutes, the tubes were centrifuged (13,000 $\times g$) and the harvested sera were placed in 1.5 ml cryovials

and frozen at -135°C. The immune serum samples were evaluated for *Bb*-specific reactivity using ELISA.

ELISA for determining relative *Bb*-specific Ab in C3H/He mice. Microtiter plates (96-well) were coated with 100 μ l of *Bb* antigen (20 μ g/ml) in ELISA antigen diluent (carbonate buffer, 0.1 M, pH 9.6), and incubated overnight at 37°C. The plates were washed 3 times with ELISA wash (PBS containing 0.05% Tween 20). Serum harvested from the experimental mice was diluted 1:1000 in ELISA antibody diluent (PBS containing 0.005% Tween 20) and applied to the wells in triplicate in a 50 μ l volume and incubated for 30 minutes at room temperature. After washing 3 times with ELISA wash, 50 μ l of horseradish peroxidase conjugated goat anti-mouse IgG (1:2000) (SIGMA) was applied to all of the wells and incubated for 30 minutes at room temperature. The plates were washed 4 times with ELISA wash and 100 μ l of ELISA substrate {Phosphate-Citrate Buffer Tablet (0.05 M, pH 5.0) with Urea Hydrogen Peroxide + 2, 2'-Azino-bis(2-Ethylbenzthiazoline-6-Sulfonic Acid Tablet (pH 5.0)} (SIGMA) was added to the wells and the plates were incubated for one hour at room temperature. The optical density (OD) was measured using a Bio-Tek EL-310 microtiter plate reader at dual wavelength (A₄₀₅/A₄₅₀ nm). Results are reported as mean ELISA Value % (EV%) where:

$$EV\% = (\text{mouse serum OD} - \text{negative control OD}) / (\text{positive control OD} - \text{negative control OD}) \times 100$$

Removal of Lymph Nodes for Immunophenotyping and Intracellular Cytokine Staining. After cervical dislocation, the brachial, inguinal, and popliteal lymph nodes were removed from each mouse. Lymph nodes from two *Bb*-challenged mice and two non-challenged mice were pooled separately. The pooled lymph nodes were placed on a sterile 70 μ m nylon filter (Falcon, San Jose, CA) and sharp scissors were used to cut the lymph nodes.

A sterile plunger from a 3 ml plastic syringe (Becton Dickinson, San Jose CA) was used to manipulate the lymph nodes and extract the cells. The cells were washed through the 70 μ m nylon filter with PBS containing 2% fetal calf serum (FCS) (SIGMA). The cells were washed once with PBS containing 2% FCS, counted, and resuspended to yield 1×10^6 cells/ml in RPMI-5 {RPMI 1640 medium containing L-glutamine (200 mM), penicillin (10,000 units), streptomycin (10 mg/ml), and 5% FCS (heat inactivated at 56°C, 1 hour) (SIGMA)}.

Culture, Activation, and Fixing of Cells for Intracellular Cytokine Staining Intracellular cytokine staining was performed by the method of O'Garra and Murphy (10). Briefly, cells were distributed in triplicate wells, with each well containing 2×10^5 cells/200 μ l in a 96-well U-bottom microtiter plate. Mitogen {Phorbol 12-myristate 13-acetate (PMA)+Ionomycin mix (PMA (Sigma) 1μ g/ 1×10^6 cells, Ionomycin (Calbiochem, La Jolla, CA) 10μ g/ 1×10^6 cells)} or antigen {live *Bb* (1×10^7 *Bb*/ 1×10^6 cells)} was added to triplicate wells. The culture plate was incubated in a 37°C, 5% CO₂, humidified incubator.

Mitogen stimulated cells and the mitogen control cells received Brefeldin A (Epicentre Technologies, Madison, WI) (10μ g/ 1×10^6 cells) after 2 hours. The mitogen stimulated cells and the mitogen control cells were harvested at the end of 4 hours in culture. The cells were washed in ice cold PBS containing 2% FCS and resuspended in PBS containing 2% FCS to yield 2×10^6 cells/ml. An equal volume of 4% formaldehyde was added to the cells to yield a 2% formaldehyde final concentration. After 20 minutes at room temperature, the cells were washed in ice cold PBS containing 2% FCS and resuspended at 1×10^6 cells/ml in PBS/BSA/Az buffer {PBS containing 1% bovine serum albumin

1% (BSA) and azide (0.5 M)}. The cells were stored at 4°C in the dark (≤ 48 hours for maximum intensity) until subjected to flow cytometry. By this time the viable cells have been fixed by formaldehyde to retain the same forward and orthogonal scatter properties for gated data acquisition post staining (described below). Antigen stimulated cells and antigen control cells received Brefeldin A ($10 \mu\text{g}/1 \times 10^6$ cells) after 14 hours. The antigen stimulated cells and the control cells were harvested at the end of 16 hours in culture. Washing and fixing of cells was as described above.

Determination of Intracellular IL-4 and IFN- γ . Approximately 2×10^5 formaldehyde fixed cells were added to each well of a 96-well U-bottom plate. Cells were permeabilized with permeabilization buffer {PBS containing 1% BSA, azide (0.5 M), and 0.5% Saponin (SIGMA)} for 10 minutes at room temperature. Next, $25 \mu\text{l}$ permeabilization buffer containing IL-4 specific mAb 11B11 PE ($5 \mu\text{g}/\text{ml}$) (Pharmingen, San Diego, CA) and $30 \mu\text{l}$ permeabilization buffer containing IFN- γ specific mAb XMG1.2 FITC ($2 \mu\text{g}/\text{ml}$) (Pharmingen) was added to the cells. After 30 minute incubation at room temperature the cells were washed once in permeabilization buffer and twice in PBS/BSA/Az buffer. The cells were resuspended in $150 \mu\text{l}$ PBS/BSA/Az buffer and stored in the dark at 4°C until subjected to flow cytometry. The data were acquired using FACSCalibur and CELLQuest software and data analysis was performed using PAINT-A-GATE software (all from BDIS).

Sample preparation for Immunophenotyping. Lymph node cells were placed on ice and distributed in a 96-well V-bottom microtiter plate (6×10^5 cells per well). The cells were incubated with $20 \mu\text{l}$ of undiluted normal mouse serum (Caltag, So. San Francisco, CA) for 30 minutes on ice and then stained using $12 \mu\text{l}$ of each fluorochrome-labeled mAb {Thy1.2 FITC, L3T4 PE,

Lyt-2 biotin (b) (Becton Dickinson Immunocytometry Systems (BDIS) (San Jose, CA).

After a 30 minute incubation on ice, the cells were washed two times with PBS containing 2% FCS. Streptavidin Peridinine Chlorophyll Protein (SA PerCP) (BDIS) was added to the wells that received Lyt-2 b and incubated on ice for 30 minutes. The cells were washed two times with PBS containing 2% FCS, resuspended in 200 μ l PBS containing 2% FCS, and subjected to flow cytometry. The data were acquired using FACSCalibur and CELLQuest software and data analysis was performed using PAINT-A-GATE software (all from BDIS).

RESULTS

Evaluation of antibody-mediated Immune response. Sera from non-challenged mice remained negative for *Bb*-specific IgG (mean EV%= 8.42), and sera from *Bb*-challenged mice had high levels of *Bb*-specific IgG (mean EV%= 66.04) at day 14 post *Bb* challenge (Table 1A).

Evaluation of Hind Tibiotarsal Joint Swelling in C3H/He Mice. *Bb*-challenged mice had a mean increase in tibiotarsal joint diameter that was 3 times greater than the mean increase in tibiotarsal joint diameter of non-challenged mice. Increases of tibiotarsal joint diameter were variable within each group of mice (Table 1B).

Immunophenotyping of Lymph Node Cells. Immunophenotyping of lymph node cells from two *Bb*-challenged mice yielded 44.22% L3T4+ T cells, 20.01% Lyt-2+ T cells, and 33.81% unclassified cells. L3T4, Lyt-2, and Thy1.2 staining of lymph node cells from two non-challenged mice yielded 54.65% L3T4+ T cells, 33.68% Lyt-2+ T cells, and 9.92% unclassified cells (Figure 1).

Characterization of Th1 and Th2 Phenotype via Intracellular Cytokine Staining. The brachial, inguinal, and popliteal lymph node cells from two infected and two non-infected mice were pooled separately. These cells were stimulated with mitogen {PMA+Ionomycin (4 hours)} or antigen {*Bb* (16 hours)} separately and each culture had timed control cultures (4 and 16 hours) (Figures 2 and 3). The overall expression of IL-4 and IFN- γ was higher in the lymph node cells harvested from *Bb*-challenged mice. Nonspecific stimulation with mitogen increased the number of IL-4 (2.84% and 1.86%) and IFN- γ (7.70% and 6.58%) secreting cells in the lymph node cell cultures of both *Bb*-challenged and non-challenged mice when compared to the 4 hour control

cultures. The increase in the IFN- γ secreting cell population was larger in the lymph node cell cultures of both *Bb*-challenged and non-challenged mice compared to the IL-4-secreting cell population (Figure 2).

In vitro stimulation of lymph node cells from *Bb*-challenged and non-challenged mice with *Bb* antigen resulted in an increase in cytokine producing cells by *Bb*-specific lymphocyte clones in the 16 hour cultures. There appears to be a baseline expression of IL-4 and IFN- γ . The proportion of IL-4 producing cells in the 16 hour control culture (*Bb*-challenged and non-challenged) and the 16 hour *Bb* culture (non-challenged) is similar, with an average 1.14% between the three samples. Also, the proportion of IFN- γ producing cells in the 16 hour control culture (*Bb*-challenged and non-challenged) and the 16 hour *Bb* culture (non-challenged) is similar, with an average 1.49% between the three samples. The proportion of IL-4 producing cells (1.68%) in the 16 hour *Bb* culture (*Bb*-challenged) and the proportion IFN- γ producing cells (3.28%) in the 16 hour *Bb* culture (*Bb*-challenged) was higher than the baseline average (Figure 3). Although an increase in the proportion of IL-4 producing cells in the 16 hour *Bb* culture of lymph node cells from *Bb*-challenged mice is apparent, the larger proportion of IFN- γ producing cells in the 16 hour *Bb* culture of lymph node cells from *Bb*-challenged mice depicts a predominantly type 1 response (13).

Mouse Groups	Day 14
A. <i>Bb</i>-specific IgG	EV ($\pm$ SD)
<i>Bb</i> -challenged	66.04% ($\pm$ 0.006)
non-challenged	8.42% ($\pm$ 0.004)
B. Mean increase in joints diameter	mm ($\pm$ SD)
<i>Bb</i> -challenged	0.6 ($\pm$ 0.3)
non-challenged	0.2 ($\pm$ 0.2)

Table 1. Mean ELISA values (A) and mean increase in tibiotarsal joint diameter (B) of *Bb*-challenged and non-challenged C3H/He mice.

Figure 1. Flow cytometric demonstration of combined lymph node cells. Cells were stained with L3T4 PE, Lyt-2 b+SA PerCP, and Thy1.2 FITC. The top two panels are gated on viable cells based on forward scatter (20,000 events shown). The two middle panels show L3T4+ and Lyt-2+ cells present in the cell populations of the top panels. The bottom two panels show Thy1.2+ cells present in the cell populations of the top two panels. Note that all Thy1.2+ cells are either L3T4+ or Lyt-2+. However, there are L3T4+ cells that are Thy1.2- (bottom panels: red events in the first two decades).

Figure 2. Flow cytometric analysis of collective lymph node cells harvested from Bb-challenged and non-challenged C3H/He mice on day 14. Harvested lymph node cells were cultured in RPMI-5 for 4 hours (control) or PMA (1 ug/ml)-Ionomycin (10 ug/ml) for 4 hours in a humidified incubator, 37°C, 5% CO₂. Brefeldin A (10 ug/ml) was added to the cells for the last 2 hours of culture. Lymph node cells were harvested post 4 hour culture, washed, permeabilized, and stained with IL-4 PE and IFN- γ FITC. The panels are gated on viable cells based on forward scatter (14,000 events shown).

Figure 3. Flow cytometric analysis of collective lymph node cells harvested from Bb-challenged and non-challenged C3H/He mice on day 14. Harvested lymph node cells were cultured in RPMI-5 for 16 hours (control) or 1×10^7 Bb/ 1×10^6 cells for 16 hours in a humidified incubator, 37°C, 5% CO₂. Brefeldin A (10 ug/ml) was added to the cells for the last 2 hours of culture. Lymph node cells were harvested post 16 hour culture, washed, permeabilized, and stained with IL-4 PE and IFN-γ FITC. The panels are gated on viable cells based on forward scatter (14,000 events shown).

DISCUSSION

T cell-associated cytokines IL-4 and IFN- γ influence the severity of disease in *Bb*-infected mice (3-6). Infection of C3H/He and BALB/c mice with *Bb* has proven to be a useful model in illustrating a genetic basis for susceptibility to Lyme disease (6). Differences appear to correspond to the Th cell response (Th1 vs Th2) mounted against the invading pathogen (6, 8, 15, 17, 19). BALB/c mice tend to mount a Th2 response and are *Bb* resistant, whereas, C3H/He mice are *Bb* susceptible and tend to mount a Th1 response (3-6). Most studies have analyzed 72 hour culture supernatants of bulk lymphoid populations by ELISA (3-6). Several different leukocyte populations are known to secrete the same cytokine. Therefore, to implicate the cells responsible for secreting a particular cytokine, it is more appropriate to analyze cytokine production at the single cell level in concert with other phenotypic markers.

Since the C3H/He mice challenged with *Bb* in this experiment mounted a *Bb*-specific IgG response and developed swollen joints compared to non-challenged mice it can be concluded that disseminated infection in the *Bb*-challenged mice had resulted in disease. Immunophenotyping results revealed that the majority of lymph node lymphocytes consist of T cells. *In vitro* stimulation of lymph node lymphocytes from non-challenged mice with *Bb* antigen and 16 hour control cultures from *Bb*-challenged and non-challenged mice results depict a baseline expression of IL-4 (mean 1.14%) and IFN- γ (mean 1.49%). The amount of IL-4 producing cells (1.68%) in the 16 hour *Bb* culture (*Bb*-challenged) and the amount IFN- γ producing cells (3.28%) in the 16 hour *Bb* culture (*Bb*-challenged) was higher than the baseline average. The

larger amount of IFN- γ producing cells in the 16 hour *Bb* culture of lymph node cells from *Bb*-challenged mice depicts a predominantly type 1 response.

These preliminary results confirm other published studies (3, 4, 6, 19) in that C3H/He mice apparently mount a predominantly type 1 response when challenged with *Bb*, and that this response is not immunoprotective in that Lyme arthritis is manifested two weeks post *Bb* challenge. Although IL-4 and IFN- γ secreting cells were detected at the single cell level, attempts to determine the identity of these cells failed. The collective lymph node cells were surface stained with the APC conjugate of L3T4. Preliminary data, however, indicate that most of the IL-4 and IFN- γ secreting cells were L3T4 negative (data not shown).

There is mounting evidence that IFN- γ is the exacerbative cytokine and although IFN- γ is a type 1 cytokine, Th1 cells have not been conclusively implicated as being the sole producers of IFN- γ in LA. Other effector cells may determine the pathogenesis of LA with type-1 like responses such as CD8⁺ and NK cells. Since the phenotype of IFN- γ secreting cells in LA has not yet been characterized, further studies are required to examine the effector cell(s) and IFN- γ secretor(s) in LA.

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GENERAL CONCLUSIONS

Recent studies have shown the involvement of T cells and their cytokines in experimental LA models (12-14, 18). Adoptive transfer of immune enriched T lymphocytes into naive recipient LSH hamsters resulted in severe destructive arthritis when challenged with *Bb*. Recipients infused with normal T lymphocytes challenged with *Bb* failed to develop severe destructive arthritis (17).

Previous studies indicated that adoptive transfer of NWEL obtained from naive C3H/He mice exacerbate LA (4). However, data presented here with adoptive transfer control groups show that adoptive transfer treated, *Bb*-challenged mice and non-treated, *Bb*-challenged mice had tibiotarsal joint diameter increases that were similar but larger than non-treated, non-challenged mice. The mean increase of tibiotarsal joint diameter of adoptive transfer treated, *Bb*-challenged mice and non-treated, *Bb*-challenged mice were similar. The increases of tibiotarsal joint diameter were variable within each group of mice and the variability within each group was larger than the differences of tibiotarsal joint diameter between groups. The findings in this adoptive transfer model did not lend support to the possible involvement of NWEL in the exacerbation of experimental LA.

Infection of C3H/He and BALB/c mice with *Bb* has proven to be a useful model in illustrating a genetic basis for susceptibility to Lyme disease (18). Differential susceptibilities in several infectious disease animal models appear to correspond to the type of Th cell response (Th1 vs Th2) mounted against the invading pathogen (18, 19, 22, 28, 29). BALB/c mice tend to mount a Th2 response and are *Bb* resistant, whereas, C3H/He mice are *Bb* susceptible and tend to mount a Th1 response (12-14, 18).

Most *in vitro* cytokine studies to date have analyzed 72 hour culture supernatants of bulk lymphoid populations by ELISA (12-14, 18). Several different leukocyte populations are known to secrete the same cytokine. Therefore, it is more appropriate to analyze cytokine production at the single cell level in concert with other phenotypic markers to implicate the cells responsible for secreting a particular cytokine. The results of this preliminary study confirm other published studies (12, 13, 18, 29) where C3H/He mice apparently mount a predominantly type 1 response when challenged with *Bb* which is not immunoprotective in that Lyme arthritis is manifested two weeks post *Bb* challenge. Mounting evidence suggests that IFN- γ exacerbates LA. Although IFN- γ is a type 1 cytokine, Th1 cells have not been conclusively implicated as the sole producers of IFN- γ in LA. Other effector cells may be involved in the pathogenesis of LA.

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APPENDIX

<u>Table 1. Fluorochromes Used with FACScan Analysis</u>			
<u>Fluorochromes</u>	<u>Excitation (nm)</u>	<u>Emmission (nm)</u>	<u>Laser</u>
Fluorescein Isothiocyanate (FITC)	488	515	Blue
Phycoerythrin (PE)	532	585	Green
Allophycocyanin (APC)	633	647	Red
Peridinine Chlorophyll Protein (PerCP)	488	677	Blue

Table 2. C3H/He Mouse Experimental Groups

Mouse groups Adoptive Transfer Treatment, <i>Bb</i> challenge	
EXPERIMENT 1: Confirmation of Exacerbation LA With Adoptive Transfer of NWEL	
Group 1	1×10^6 NWEL, 1×10^6 <i>Bb</i>
Group 2	no treatment, 1×10^6 <i>Bb</i>
Group 3	no treatment, no <i>Bb</i>
EXPERIMENT 2: Determination of NWEL Dose-Response Curve in C3H/He Mice Experimentally Challenged with <i>Bb</i>	
Group 1	1×10^2 NWEL, 1×10^6 <i>Bb</i>
Group 2	1×10^3 NWEL, 1×10^6 <i>Bb</i>
Group 3	1×10^4 NWEL, 1×10^6 <i>Bb</i>
Group 4	1×10^5 NWEL, 1×10^6 <i>Bb</i>
Group 5	1×10^6 NWEL, 1×10^6 <i>Bb</i>
Group 6	no treatment, 1×10^6 <i>Bb</i>
Group 7	no treatment, no <i>Bb</i>
EXPERIMENT 3: Determination of the Difference in Tibiotarsal Joint Swelling in <i>Bb</i> Challenged C3H/He Mice Receiving NWEL or WL Adoptive Transfer	
Group 1	1×10^2 NWEL, 1×10^6 <i>Bb</i>
Group 2	1×10^3 NWEL, 1×10^6 <i>Bb</i>
Group 3	6×10^3 WL, 1×10^6 <i>Bb</i>
Group 4	no treatment, 1×10^6 <i>Bb</i>
Group 5	no treatment, no <i>Bb</i>
EXPERIMENT 4: Determination of the Difference in Tibiotarsal Joint Swelling in <i>Bb</i> Challenged C3H/He Mice Receiving NWEL, WL, or RPMI-5 Adoptive Transfer	
Group 1	1×10^3 NWEL, 1×10^6 <i>Bb</i>
Group 2	6×10^3 WL, 1×10^6 <i>Bb</i>
Group 3	RPMI-5, 1×10^6 <i>Bb</i>
Group 4	1×10^3 NWEL, no <i>Bb</i>
Group 5	RPMI-5, no <i>Bb</i>
Group 6	no treatment, 1×10^6 <i>Bb</i>
Group 7	no treatment, no <i>Bb</i>
EXPERIMENT 5: Determination of the Difference in Tibiotarsal Joint Swelling in <i>Bb</i> Challenged C3H/He Mice Receiving NWEL in RPMI-5 or RPMI, RPMI-5, or RPMI Adoptive Transfer	
Group 1	1×10^6 NWEL in RPMI-5, 1×10^6 <i>Bb</i>
Group 2	1×10^6 NWEL in RPMI, 1×10^6 <i>Bb</i>
Group 3	RPMI-5, 1×10^6 <i>Bb</i>
Group 4	RPMI, 1×10^6 <i>Bb</i>
Group 5	no treatment, 1×10^6 <i>Bb</i>
Group 6	no treatment, no <i>Bb</i>