INFECTION AND IMMUNITY, Dec. 1997, p. 5334–5337 Vol. 65, No. 12
0019-9567/97/$04.00+0

$CD8^+$ T Cells Are Activated during the Early Th1 and Th2 Immune Responses in a Murine Lyme Disease Model

ZENGJUN DONG, MEG D. EDELSTEIN, AND LISA J. GLICKSTEIN*

Department of Medicine, Division of Rheumatology/Immunology, New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts 02111

Received 30 May 1997/Returned for modification 9 July 1997/Accepted 22 September 1997

T-helper responses following *Borrelia burgdorferi* infection in mice determine susceptibility to Lyme arthritis. The ratio of interleukin 4-positive, $CD4^+$ to gamma interferon (IFN-γ)-positive, $CD4^+$ T cells was significantly greater in infected BALB/cJ mice than in infected C3H/HeJ mice. Increased numbers of IFN-γ-producing cells predicted greater arthritis severity, and $CD8^+$ T cells were the main source of IFN-γ in both strains.

The spirochete *Borrelia burgdorferi*, transmitted to humans by infected ixodes ticks, is the causative agent of Lyme disease and its attendant secondary and tertiary cardiac, neurologic, and rheumatic complications (29). Among inbred strains of mice experimentally infected with *B. burgdorferi*, the severity of arthritis is under host genetic control, with BALB/c mice developing only mild disease but C3H/He mice acquiring severe arthritis (2, 26, 33). Severity is linked to higher spirochete burdens, suggesting that susceptibility to pathology represents, in part, a deficiency in antimicrobial immunity (33).

In a number of murine infectious disease models with various degrees of strain-specific susceptibility, polarized lymphokine responses underlie the discordance in disease outcome (8, 9, 15, 22, 23). A panel of *B. burgdorferi* antigen-specific $CD4^+$ T-cell clones derived from chronic Lyme disease patients displayed exclusively the Th1-like cytokine pattern (35). Human rheumatoid arthritis is also associated with an increased Th1-like response (27, 35). Arthritis-susceptible C3H ($H\text{-}2^k$) mice produce a predominately Th1-type immune response following infection with *B. burgdorferi*, while resistant BALB/c ($H\text{-}2^d$) mice produce the opposite Th2-type response (10, 11, 16). When these Th responses are switched by anticytokine antibody treatment of mice, disease outcome, measured as arthritis severity and burdens of the two spirochete strains, is reversed (10, 16).

Infection of mice with *B. burgdorferi*. The N40 isolate of *B. burgdorferi* was kindly provided by Stephen Barthold (Yale University, New Haven, Conn.). Low-passage *B. burgdorferi* N40 cells (20) were frozen into single-experiment aliquots in Barbour-Stoenner-Kelly (BSK) II with 30% glycerol (Sigma Chemical Co., St. Louis, Mo.). Frozen *B. burgdorferi* cells were thawed rapidly, transferred into 10 volumes of BSK II, and cultured at 32°C overnight prior to enumeration of motile spirochetes by dark-field microscopy. Motility, a minimal estimate of viability, was consistently greater than 75%. Female BALB/cJ and C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) at 5 weeks of age and housed in the barrier containment facility (biocontainment level 3) in our Division of Laboratory Animal Medicine. Mice were inoculated in the right hind foot pad with 5×10^5 motile spirochetes in 50 μl of BSK II or mock infected with BSK II alone as previously described (10, 11, 14, 16). Infection was confirmed at the time of sacrifice by culture of ear punch biopsy tissue and/or peripheral blood (data not shown). Infectivity is controlled by the spirochete genotype (6, 19, 34), as well as the dose and route of inoculation (1, 19). However, susceptibility to arthritis following successful infection of mice is strongly determined by host factors, as infectious *B. burgdorferi* induces severe destructive arthritis in C3H/HeJ mice, whether transmitted by needle inoculation or by feeding ticks (10, 11, 16, 30, 31).

Preparation of antigen and antigen-presenting cells. *B. burgdorferi* soluble antigen was prepared from a 500-ml culture of spirochetes (10^8/ml). After centrifugation for 20 min at 15°C at $12{,}000 \times g$ (10,000 rpm in an SS-34 rotor), the spirochete pellet was resuspended in 5 ml of phosphate-buffered saline (PBS) with Mg_2 (Sigma Chemical Co.) and the suspension was sonicated three times for 2 min each time (15-s intervals) on ice at a 50% duty cycle on a Sonifier 450 (Branson Sonic Power Company, Danbury, Conn.). The sonicate was centrifuged again as described above, and the supernatant of soluble antigens was removed and stored in aliquots at −70°C after filtering through a 0.45-μm-pore-size filter (Millipore, Bedford, Mass.). Irradiated spleen cells (1,700 rads; 10^7 cells/ml) from mock-infected syngeneic mice were pulsed with *B. burgdorferi* soluble antigen (35 μl/ml [final concentration]) for 60 min and washed twice with complete RPMI medium (RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 20 mM HEPES, 1 mM Na pyruvate, 1× nonessential amino acids, 2 mM L-glutamine, and gentamicin [all obtained from BioWhittaker]) before use as antigen-presenting cells.

Antigen-specific recall response in vitro. Draining lymph nodes were harvested from mice 10 to 14 days postinfection, at the onset of measurable arthritis (data not shown). Single-cell suspensions from pooled lymph nodes were washed twice with complete RPMI medium and plated at 5×10^5 cells/well in 96-well plates in the presence or absence of 10^6 antigen-pulsed, irradiated antigen-presenting cells. After 4 to 10 days of in vitro restimulation, cells were activated for the final 5 h of culture with soluble anti-CD3 (1 μg/ml; 145.2C11; PharMingen, San Diego, Calif.) and anti-CD28 (1 μg/ml; 37.51; PharMingen) antibodies in the presence of 3 μM monensin (Sigma Chemical Co.) to retard cytokine export from the Golgi.

Intracellular cytokine analysis by FACS. Intracellular cytokine analysis by fluorescence-activated cell sorter (FACS) was done as previously described (20). Cells were washed in FACS staining buffer I (PBS [BioWhittaker, Walkersville, Md.] with 1% heat-inactivated fetal calf serum [Sigma Chemical Co.] and 0.1% NaN_3 [Sigma Chemical Co.]), and incubated for 5 min

* Corresponding author. Mailing address: New England Medical Center, 750 Washington St., Box 406, Boston, MA 02111. Phone: (617) 636-5789. Fax: (617) 636-4252. E-mail: lisa.glickstein@es.nemc.org.

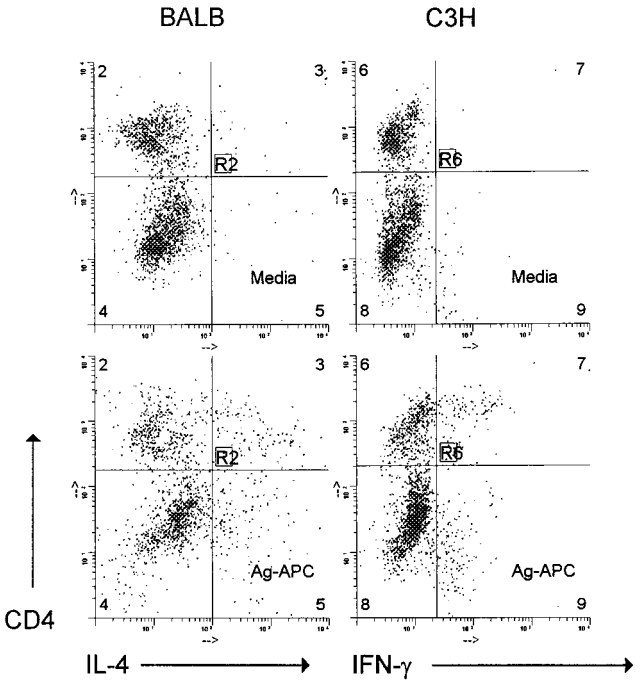

FIG. 1. *B. burgdorferi* antigen-pulsed antigen-presenting cells were required for cytokine production in vitro. (Left) FACS dot plots of CD4 and IL-4 expression in 10-day restimulated cultures of cells from infected BALB/c mice in the presence (Ag-APC) or absence (media) of *B. burgdorferi* antigen-pulsed antigen-presenting cells. (Right) FACS dot plots of CD4 and IFN-γ expression in 10-day cultures of cells from infected C3H mice under the same conditions. The population was gated on forward and side scatter to include only large blast cells.

with F_c-block (anti-CD16/32; 2.4G2; PharMingen) at 4°C. These cells were then stained conventionally for cell surface markers (Cychrome–anti-CD4 [RM4-5; PharMingen], Cychrome–anti-CD8 [53-6.7; PharMingen], fluorescein isothiocyanate [FITC]–anti-MAC1, biotin-anti-CD45/B220, and streptavidin-phycoerythrin [PharMingen]) and fixed in 4% paraformaldehyde (Sigma Chemical Co.) in PBS for 20 min. FITC–anti-CD11b (MAC1) and biotin–anti-CD45RB/B220 (6B2) antibodies were kindly provided by Henry Wortis of the Tufts University School of Medicine. Fixed cells were washed and permeabilized with FACS staining buffer II (PBS with 1% heat-inactivated fetal calf serum and 0.1% saponin [Sigma Chemical Co.]) and then stained with anticytokine antibodies (phycoerythrin–anti-interleukin 4 [IL-4] [11B11] and FITC–anti-gamma interferon [IFN-γ] [R4 6A2; PharMingen]) in accordance with the protocol obtained from PharMingen. Cells stained in this manner were analyzed on a FACScan using LYSIS II software (Becton Dickinson, San Jose, Calif.). An analysis gate was limited to large, activated blasts.

B. burgdorferi antigen-pulsed antigen-presenting cells elicited accumulation of both $CD4^+$ (32.2 to 79.7%)- and $CD8^+$ (19.3 to 47.4%)-activated T cells (51.5 to 99.6%). No IL-4 or IFN-γ production was observed in the absence of antigen-presenting cells, one indication of specificity of the assay (Fig. 1). Few macrophages ($MAC1^+$) were observed, and B cells, while present in significant numbers, did not produce the cytokines tested (Fig. 2 and data not shown).

Skewing toward a Th1 or Th2 immune response. We questioned whether skewing is present in the early phase of *B. burgdorferi* infection (10 to 14 days postinfection) rather than 5 weeks later, when arthritis is well established (10, 11, 16). $CD4^+$ T cells with a Th1 (IFN-γ-producing) or Th2 (IL-4-

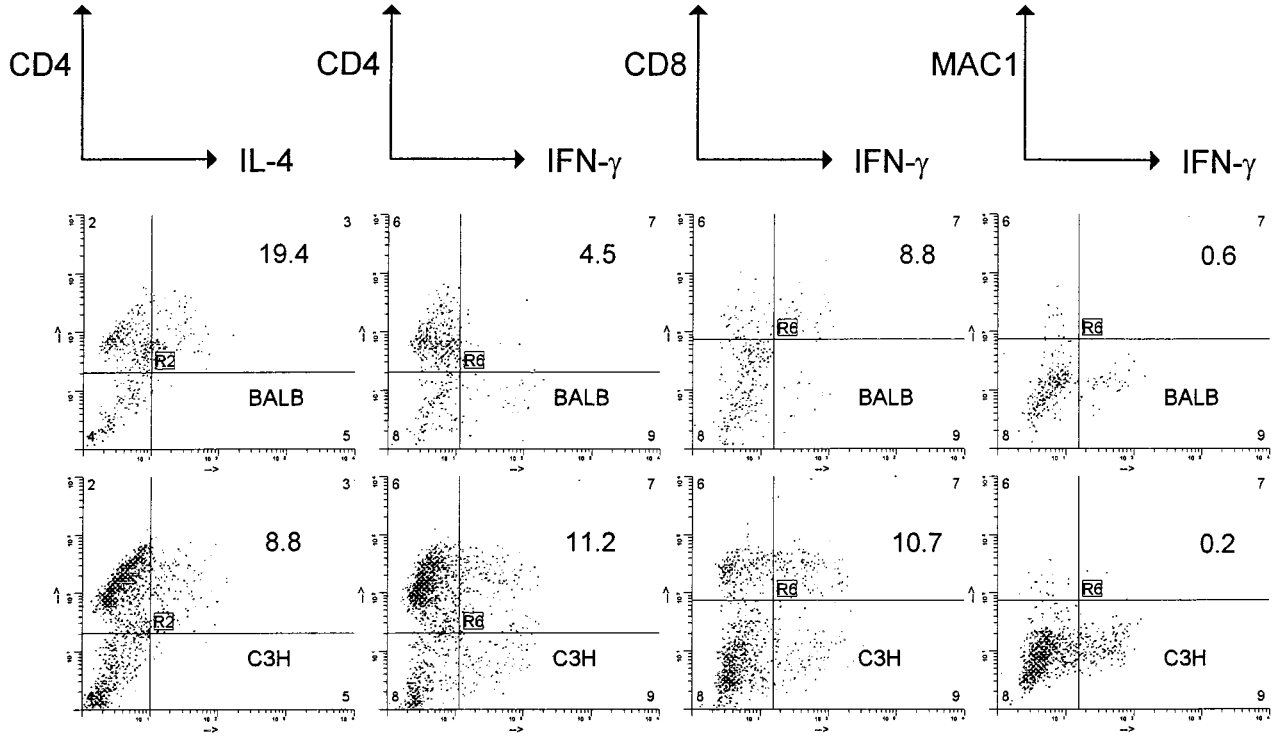

FIG. 2. Representative profiles of large blast cells after tricolor staining. The data shown are from a representative experiment of three after restimulation of lymph node cells from infected BALB/c and C3H mice with *B. burgdorferi* antigen-pulsed antigen-presenting cells in vitro for 10 days. Percentages of double-positive cells after staining with anti-CD4/IL-4/IFN-γ, anti-CD8/IL-4/IFN-γ, or anti-B220/MAC1/IFN-γ antibodies are indicated. The analysis software indicates the center of the quadrant with a small square (labelled R2 or R6).

TABLE 1. Subset distribution after in vitro restimulation of lymph node mononuclear cells with *B. burgdorferi* antigen-pulsed antigen-presenting cells[a]

Time and mouse strain	Mean % ± SD of T cells with phenotype:		
	$CD4^+$ $IL\text{-}4^+$	$CD4^+$ $IFN\text{-}\gamma^+$	$CD8^+$ $IFN\text{-}\gamma^+$
Day 4			
BALB	5.1 ± 3.6	1.6 ± 2.2	7.3 ± 4.0
C3H	6.6 ± 3.9	6.9 ± 7.1	13.1 ± 0.1
Day 6			
BALB	3.5 ± 2.7	0.2 ± 0.3	10.5 ± 8.6
C3H	1.3 ± 1.7	1.0 ± 1.0	7.2 ± 5.4
Day 10			
BALB	21.7 ± 8.0	1.9 ± 0.3	6.0 ± 0.7
C3H	3.4 ± 0.4	7.2 ± 3.4	9.0 ± 4.0

[a] Cells were harvested from cultures on the days indicated and stained for tricolor analysis of CD4, CD8, and cytokine expression by FACS. Percentages of blast cells with the indicated phenotypes are reported as the means of three independent experiments.

producing) phenotype were apparent in both BALB/cJ and C3H/HeJ cultures (Table 1 and Fig. 2). The ratio of percent $IL\text{-}4^+$ $CD4^+$ to percent $IFN\text{-}\gamma^+$ $CD4^+$ T-cell blasts increased with time and was highest in BALB cultures and lowest in C3H cultures after 10 days of in vitro restimulation (Fig. 2 and Table 1). C3H cultures contained the same percentage of $IL\text{-}4^+$ $CD4^+$ T cells as did BALB cultures 4 days after restimulation, suggesting that lack of initial IL-4 production was not the primary reason for the Th1 predominance with time (Table 1). These results are consistent with the finding that CD4 depletion reduced splenic cell in vitro IFN-γ production in C3H mice but not in BALB/c mice (10).

Production of the majority of IFN-γ by $CD8^+$ T cells following *B. burgdorferi* infection. IFN-γ production was observed in cultures from infected BALB/cJ mice, but almost exclusively in the $CD8^+$ T-cell subset (Table 1 and Fig. 2). Cytokine production by $CD4^+$ T cells was augmented significantly by $CD8^+$ T cells in C3H mice as well (Table 1 and Fig. 2). IFN-γ production and arthritis decreases dramatically in both strains following anti-CD8 antibody depletion (10). *B. burgdorferi*-specific $CD8^+$ T-cell lines have been generated from peripheral blood and synovial fluid of patients with Lyme arthritis (5). Although T-cell activation is not required for Lyme disease in mice, these results, taken together, suggest a role for $CD8^+$ T cells in disease pathogenesis (11, 24).

It is known that in the absence of antibiotic treatment, people can remain infected with *B. burgdorferi* spirochetes for years, with bouts of arthritis alternating with asymptomatic periods (29). It is not known how or if the spirochetes evade immune system detection during this long course of disease. The $CD8^+$ T-cell recall response to *B. burgdorferi* antigen that we have identified suggests that one or more *B. burgdorferi* proteins enter into host cytosol and the class I presentation pathway. Secretion mechanisms allow proteins from cytoplasmic and noncytoplasmic bacteria access to host cytosol (7, 18). These mechanisms include specific transport of proteins by bacteria adherent to mammalian cells or transport of proteins via a bacterium-derived channel, as in the case of the bacterial toxins (7). Such antigen transfer has not been previously described for *B. burgdorferi* and may be of interest in understanding both the arthritogenic immune response and the natural life cycle of the bacteria in the infected human host.

In Lyme disease of immunocompetent mice, T cells are an important component of the immune defense against infection (11). However, unlike the protective role of $CD8^+$ T cells and IFN-γ in *Chlamydia trachomatis* (28), $CD8^+$ T cells have a negative influence on the immune response to *B. burgdorferi*, as their presence increases both arthritis and spirochete burdens (11). This resembles the role of $CD8^+$ T cells and IFN-γ in autoimmune syndromes (12). For example, IFN-γ exacerbated multiple sclerosis in human patients (21). Myelin-specific $CD8^+$ T cells were cloned from such patients and may play a role in the disease process (32). $CD8^+$ T cells can play both regulatory and pathogenic roles in some autoimmune diseases (17). In the murine diabetes model, oral tolerance induced $CD8^+$ suppressor T cells but also a population of $CD8^+$ effectors which exacerbated the disease (4, 13). Lyme arthritis in response to heat-inactivated or frozen-thawed spirochetes was not described, although these stimuli induce protective immunity in the recipient animals (3, 25). $CD8^+$ T cells and the Th1 immune response are likely to be arthritogenic through multiple synergistic mechanisms. First, the Th1 immune response does not control spirochete burdens as well as a Th2 response, and these spirochetes in the joints would lead to increased recruitment of neutrophils and more severe arthritis. In addition, IFN-γ from $CD4^+$ or $CD8^+$ T cells could increase inflammation and arthritis by inducing macrophages and other phagocytic cells to produce inflammatory mediators. Finally, $CD8^+$ T cells could be arthritogenic as cytotoxic effector cells directed against antigen-presenting cells, altering the immune response, or against synovial cell targets, directly damaging the joint.

This work was supported by grants from the Harold G. and Leila Y. Mathers Foundation (L.J.G.) and the National Institutes of Health (training grant AR07570 [Z.D.]).

We thank Allen Steere for support and discussions, Deepak Kaul for technical assistance, and Dawn Gross for advice on the FACS staining protocol.

REFERENCES

1. **Barthold, S. W.** 1991. Infectivity of *Borrelia burgdorferi* relative to route of inoculation and genotype in laboratory mice. J. Infect. Dis. **163:**419–420.
2. **Barthold, S. W., D. S. Beck, G. M. Hansen, G. A. Terwilliger, and K. D. Moody.** 1990. Lyme borreliosis in selected strains and ages of laboratory mice. J. Infect. Dis. **162:**133–138.
3. **Barthold, S. W., E. Fikrig, L. K. Bockenstedt, and D. H. Persing.** 1995. Circumvention of outer surface protein A immunity by host-adapted *Borrelia burgdorferi*. Infect. Immun. **63:**2255–2261.
4. **Blanas, E., F. R. Carbone, J. Allison, J. F. A. P. Miller, and W. R. Heath.** 1996. Induction of autoimmune diabetes by oral administration of autoantigen. Science **274:**1707–1709.
5. **Busch, D. H., C. Jassoy, U. Brinckmann, H. Girschick, and H. I. Huppertz.** 1996. Detection of *Borrelia burgdorferi*-specific $CD8^+$ cytotoxic T cells in patients with Lyme arthritis. J. Immunol. **157:**3534–3541.
6. **Coburn, J., S. W. Barthold, and J. M. Leong.** 1994. Diverse Lyme disease spirochetes bind integrin $\alpha_{IIb}\beta_3$ on human platelets. Infect. Immun. **62:**5559–5567.
7. **Finlay, B. B., and P. Cossart.** 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science **276:**718–725.
8. **Flores Villanueva, P. O., T. S. Harris, D. E. Ricklan, and M. J. Stadecker.** 1994. Macrophages from schistosomal egg granulomas induce unresponsiveness in specific cloned Th-1 lymphocytes in vitro and down-regulate schistosomal granulomatous disease in vivo. J. Immunol. **152:**1847–1855.
9. **Heinzel, F. P., M. D. Sadick, S. S. Mutha, and R. M. Locksley.** 1991. Production of interferon-γ, interleukin 2, interleukin 4, and interleukin 10 by $CD4^+$ lymphocytes in vivo during healing and progressive murine leishmaniasis. Proc. Natl. Acad. Sci. USA **88:**7011–7015.
10. **Keane-Myers, A., and S. P. Nickell.** 1995. Role of IL-4 and IFN-γ in modulation of immunity to *Borrelia burgdorferi* in mice. J. Immunol. **155:**2020–2028.
11. **Keane-Myers, A., and S. P. Nickell.** 1995. T cell subset-dependent modulation of immunity to *Borrelia burgdorferi* in mice. J. Immunol. **154:**1770–1776.
12. **Liblau, R. S., S. M. Singer, and H. O. McDevitt.** 1995. Th1 and Th2 $CD4^+$ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol. Today **16:**34–38.
13. **Lider, O., L. M. B. Santos, C. S. Y. Lee, P. J. Higgins, and H. L. Weiner.**

1989. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. II. Suppression of disease and in vitro immune responses is mediated by antigen-specific $CD8^+$ T lymphocytes. J. Immunol. **142:**748–752.
14. **Lim, L. C. L., D. M. England, B. K. DuChateau, N. J. Glowacki, J. R. Creson, S. D. Lovrich, S. M. Callister, D. A. Jobe, and R. F. Schell.** 1994. Development of destructive arthritis in vaccinated hamsters challenged with *Borrelia burgdorferi*. Infect. Immun. **62:**2825–2833.
15. **Matsuzaki, G., F. Song, and K. Nomoto.** 1996. Suppression of T-helper type-1 immune response against *Listeria monocytogenes* by treatment of mice with goat antibodies to mouse IgD. Immunology **87:**15–20.
16. **Matyniak, J. E., and S. L. Reiner.** 1995. T helper phenotype and genetic susceptibility in experimental Lyme disease. J. Exp. Med. **181:**1251–1254.
17. **McFarland, H. F.** 1996. Complexities in the treatment of autoimmune disease. Science **274:**2037–2038.
18. **Normark, S., and H. Wolf-Watz.** 1997. Common themes amid diversity. Science **276:**659.
19. **Norris, S. J., J. K. Howell, S. A. Garza, M. S. Ferdows, and A. G. Barbour.** 1995. High- and low-infectivity phenotypes of clonal populations of in vitro-cultured *Borrelia burgdorferi*. Infect. Immun. **63:**2206–2212.
20. **Openshaw, P., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra.** 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. J. Exp. Med. **182:**1357–1367.
21. **Panitch, H. S., R. L. Hirsch, J. Schindler, and K. P. Johnson.** 1987. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. Neurology **37:**1097–1102.
22. **Reiner, S. L., S. Zheng, Z.-E. Wang, L. Stowring, and R. M. Locksley.** 1994. Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from $CD4^+$ T cells during initiation of infection. J. Exp. Med. **179:**447–456.
23. **Romani, L., A. Mencacci, E. Cenci, R. Spaccapelo, P. Mosci, P. Puccetti, and F. Bistoni.** 1993. $CD4^+$ subset expression in murine candidiasis: Th responses correlate directly with genetically determined susceptibility or vaccine-induced resistance. J. Immunol. **150:**925–931.
24. **Schaible, U. E., M. D. Kramer, K. Eichmann, M. Modolell, C. Museteanu, and M. M. Simon.** 1990. 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. USA **87:**3768–3772.
25. **Schaible, U. E., M. D. Kramer, C. W. E. Justus, C. Museteanu, and M. M. Simon.** 1989. Demonstration of antigen-specific T cells and histopathological alterations in mice experimentally inoculated with *Borrelia burgdorferi*. Infect. Immun. **57:**41–47.
26. **Schaible, U. E., M. D. Kramer, R. Wallich, T. Tran, and M. M. Simon.** 1991. Experimental *Borrelia burgdorferi* infection in inbred mouse strains: antibody response and association of H-2 genes with resistance and susceptibility to development of arthritis. Eur. J. Immunol. **21:**2397–2405.
27. **Simon, A. K., E. Seipelt, and J. Sieper.** 1994. Divergent T-cell cytokine patterns in inflammatory arthritis. Proc. Natl. Acad. Sci. USA **91:**8562–8566.
28. **Starnbach, M. N., M. J. Bevan, and M. F. Lampe.** 1994. Protective cytotoxic T lymphocytes are induced during murine infection with *Chlamydia trachomatis*. J. Immunol. **153:**5183–5189.
29. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321:**586–596.
30. **Telford, S. R., III, E. Fikrig, S. W. Barthold, L. R. Brunet, A. Spielman, and R. A. Flavell.** 1993. Protection against antigenically variable *Borrelia burgdorferi* conferred by recombinant vaccines. J. Exp. Med. **178:**755–758.
31. **Telford, S. R., III, F. S. Kantor, Y. Lobet, S. W. Barthold, A. Spielman, R. A. Flavell, and E. Fikrig.** 1995. Efficacy of human Lyme disease vaccine formulations in a mouse model. J. Infect. Dis. **171:**1368–1370.
32. **Tsuchida, T., K. C. Parker, R. V. Turner, H. F. McFarland, J. E. Coligan, and W. E. Biddison.** 1994. Autoreactive $CD8^+$ T-cell responses to human myelin protein-derived peptides. Proc. Natl. Acad. Sci. USA **91:**10859–10863.
33. **Yang, L., J. H. Weis, E. Eichwald, C. P. Kolbert, D. H. Persing, and J. J. Weis.** 1994. Heritable susceptibility to severe *Borrelia burgdorferi*-induced arthritis is dominant and is associated with persistence of large numbers of spirochetes in tissues. Infect. Immun. **62:**492–500.
34. **Yang, L., Y. Ma, R. Schoenfeld, M. Griffiths, E. Eichwald, B. Araneo, and J. J. Weiss.** 1992. Evidence for B-lymphocyte mitogen activity in *Borrelia burgdorferi*-infected mice. Infect. Immun. **60:**3033–3041.
35. **Yssel, H., M.-C. Shanafelt, C. Soderberg, P. V. Schneider, J. Anzola, and G. Peltz.** 1991. *Borrelia burgdorferi* activates a T helper type 1-like T cell subset in Lyme arthritis. J. Exp. Med. **174:**593–601.

Editor: R. N. Moore