Vitamin A Deficiency Exacerbates Murine Lyme Arthritis

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Vitamin A deficiency predisposes the host for a strong inflammatory response, suggesting that it may foster susceptibility to diseases, such as Lyme arthritis, in which activated macrophage and inflammatory cytokine production are pathogenic. Infected mice had a rapid serum retinol decline that correlated with the onset of arthritis. The mice with the least retinol developed acute arthritis earlier and more severely than those with the highest retinol. Earlier and stronger interleukin (IL)-12, interferon-γ (IFN)-γ, and tumor necrosis factor responses were found in *Borrelia burgdorferi*–infected, vitamin A–deficient mice compared with controls. The spirochetes induced IFN-γ secretion from unprimed cells, and retinoid addition in vitro inhibited IFN-γ synthesis. Vitamin A deficiency may exacerbate acute Lyme arthritis by enhancing an acute arthritogenic inflammatory response initiated by spirochete-driven IFN-γ secretion. Conversely, vitamin A may lessen acute Lyme arthritis pathology by blocking IFN-γ and IL-12 synthesis.

The T cell priming microenvironment during vitamin A deficiency includes constitutive interleukin (IL)-12 and interferon (IFN)-γ synthesis, but IL-4 and IL-10 are neither constitutively synthesized nor inducible, rendering the environment favorable for Th1 cell growth [1]. Vitamin A functions in vitro or in vivo to down-regulate IL-12 and IFN-γ synthesis, to enhance Th2 cell expansion or differentiation (or both), and to support antibody responses [1–3]. Together, these observations led us to theorize that transient or chronic vitamin A deficiency might increase susceptibility to proinflammatory cytokine driven diseases such as arthritis.

Arthritis is a frequent and debilitating disease of uncertain etiology. It may result from slow bacterial infections, an autoimmune disease process, or both. The arthritis disease mechanism is incompletely understood, but a chronic inflammatory immune response, perpetuated by persistent bacterial antigen stimulation or by a self antigen that is a molecular mimic of a bacterial antigen, appears to mediate disease pathology. Lyme arthritis is an exceptional model because the causative agent is known to be the tickborne *Borrelia burgdorferi* spirochete [4, 5]. Here we report that vitamin A deficiency does indeed exacerbate Lyme arthritis and we present studies on the acute proinflammatory cytokine responses of vitamin A–replete (A^+) and –deficient (A^-) mice by probing the mechanism for exacerbated arthritic disease.

Materials and Methods

Animals. B10.BR/J breeding mice were obtained from Jackson Laboratory (Bar Harbor, ME) and propagated in our colony. Experiments used weight-matched male and female A^+ and A^- mice produced exactly as described [6, 7]. Experiments began when the mice were 5–6 weeks old, and serum retinol analysis confirmed the vitamin A deficiency [6].

B. burgdorferi and arthritis severity. Spirochetes were grown in BSK-II medium and enumerated as described [8]. Experiments used cloned *B. burgdorferi* strain N40 (provided by S. W. Barthold, Yale University School of Medicine, New Haven, CT) [9]. Mice were injected intradermally with N40 strain spirochetes (10^4) in 0.1 mL of medium or medium only. Spleen, bladder, and ear punch samples were collected 2 weeks after infection, cultured for 2 weeks in BSK-II medium, and examined microscopically to confirm the infection. Animals were considered infected if one or more tissues yielded *B. burgdorferi*–positive cultures. Arthritis was quantitated by measuring changes in the ankle joint and footpads of ether-anesthetized mice [10, 11]; ankle joint measurements indicate the histologic severity of arthritis in mice [11]. Tissue sections were also prepared [9].

Cell cultures. Cells were cultured in HL-1 serum-free medium (Ventrex, Portland, ME) as described [2]. Splenocytes or lymphocytes from the axillary, brachial, and inguinal lymph nodes were pooled from A^+ or A^- mice and stimulated with *B. burgdorferi* spirochetes (equivalent to 5 mg of protein/well) to activate lymphokine secretion. Some cultures included 10 n*M* retinoic acid from a 10 m*M* stock solution of the acid dissolved in dimethyl sulfoxide (DMSO) or DMSO only as a control.

Cytokine analysis. For transcript measurements, total cellular RNA was reverse-transcribed using an oligo-dT primer, amplified, and quantitated by competitive polymerase chain reaction (PCR) [1, 12]. Decreasing concentrations of competitive mimic DNA were added to a series of identical cDNA samples. Each mimic DNA included the target DNA primer sequences flanking a DNA fragment (600 bp of v-*erbB*) whose length and sequence differed from those of the target [12]. The mimic served to imitate the target cDNA with respect to primer binding and amplification efficiency. The mixtures were amplified under predetermined optimal conditions, and the products were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

The target and mimic PCR bands were identified by size, and the mimic cDNA concentration that yielded a mimic band with fluorescence intensity matching the target band intensity was determined and used to calculate target cDNA copy number in the test cDNA sample. The analyses were repeated until the appropriate

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Animal experimentation guidelines established by the University of Wisconsin-Madison and in accordance with federal guidelines were followed.

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dilution for bands of matching intensity was determined. A negative control PCR without added cDNA or mimic was always included. The glyceraldehyde 3-phosphate dehydrogenase (G3PDH), tumor necrosis factor (TNF)-α, and IFN-γ primers and mimic DNA were from Clontech Laboratories (Palo Alto, CA). The IL-12 p40 subunit primers and mimic DNA were from J. Mansfield (University of Wisconsin-Madison). Values reported are cytokine cDNA copies per 1000 G3PDH cDNA copies.

A two-site ELISA measured IFN-γ as we reported previously [13, 14]. Polyclonal rabbit antiserum to murine IFN-γ was the capture antibody [2], and biotinylated R4-6A2 monoclonal antibody to mouse IFN-γ [15] was the detecting antibody. Color was developed with streptavidin-β-galactosidase and *p*-nitrophenyl-β-D-galactoside. TNF-α and -β were measured by their cytotoxic effects on murine L929 fibroblast cells (from D. Paulnock, University of Wisconsin-Madison) using the crystal violet dye exclusion assay as described [16]. These cells are killed by TNF-α and -β [16]. Recombinant TNF-α (from J. Mansfield) was the standard; the detection limit was 0.2 pg/mL.

Statistical analyses. Each experiment included groups of 5–15 B10.BR mice, and experiments were repeated as indicated in the figure legends. The normally distributed group variances were compared using Student's *t* test; $P < .05$ was considered significant.

Results

Lyme arthritis in A^- and A^+ mice. Vitamin A deficiency worsened acute Lyme arthritis. *B. burgdorferi*–infected A^- mice developed arthritis earlier and more severely than the infected A^+ mice (figure 1). At 4.5 weeks after infection, the ankles and footpads of A^+ mice and the ankles of A^- mice were not significantly larger then before infection, suggesting that the paws had returned to normal. Histologic examinations confirmed the presence of infiltrating lymphocytes in the inflamed but not control joints (not shown). The spirochete cultures derived from the A^+ and A^- mice did not show any differences in the incubation time required for a positive culture (3–6 days), the spirochete density as a function of time, or the proportion of infected mice (100% of those injected). These findings suggest that the spirochete density in vivo was probably not significantly different between the A^+ and A^- mice. Although we did not measure spirochete load in the joints, we think it is unlikely that the more severe acute Lyme arthritis in A^- mice was due to differences in spirochete load.

The *B. burgdorferi* N40 infection dramatically decreased serum retinol levels in both A^- and A^+ mice (figure 2). At the time of infection, A^- mice had serum retinol levels that were just 61% of those in the A^+ controls and well below normal for a mouse. After infection, retinol levels dropped ~57% in A^+ mice and ~39% in A^- mice. Nevertheless, A^+ mice had significantly more retinol ($P < .005$) 2–4 weeks after infection than did A^- mice. The retinol level in A^+ mice recovered ($P < .005$) during the experiment but not to the level in uninfected A^+ mice. These data indicate that *B. burgdorferi* infection can dramatically lower retinol levels, leading to transient or lasting vitamin A deficiency, depending on access to dietary vitamin A or stores of retinyl palmitate in the liver.

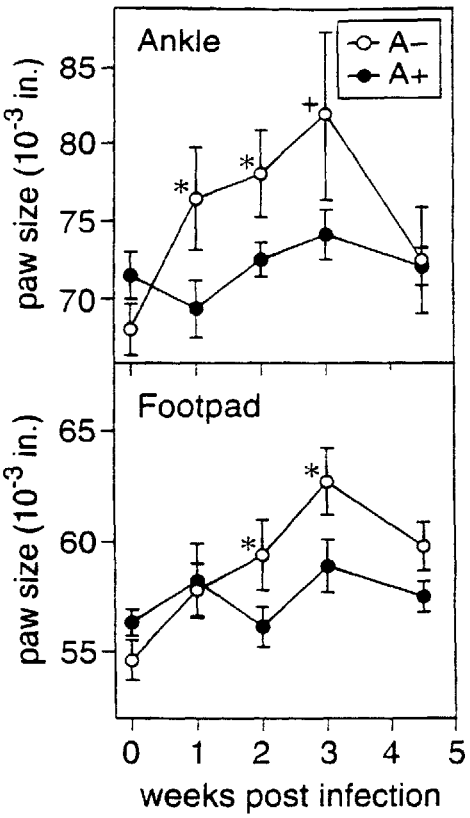

Figure 1. Paw swelling in *Borrelia*-infected vitamin A deficient (A^-) and -replete (A^+) mice. Mice (5 weeks old) were infected intradermally on their backs with 10^4 N40 strain spirochetes. Uninfected paw measurements are from adult 8- to 10-week-old A^+ and A^- mice. Paws of anesthetized animals (9–15/group, 2 paws/mouse) were measured by engineer's caliper; in. = inches. Values are mean ± SD of 18–30 paws from 1 of 3 experiments. Significantly different: * $P < .001$, + $P < .005$.

IFN-γ and IL-12. We analyzed lymph node cells derived from the nodes draining the intradermal injection site for proinflammatory cytokine transcripts. Uninfected animals served as controls. IL-12 is a potent stimulus for T cell and NK cell IFN-γ secretion [17], and IFN-γ has been implicated as arthritogenic [18–20]. The draining lymph nodes were significantly enlarged compared with those of uninfected control mice, indicating an immune reaction to the pathogen. The transcript measurements were made in freshly explanted lymph node cells without restimulation of the cells in vitro.

Consistent with our previous report [1], cells from uninfected A^- mice transcribed IFN-γ and IL-12 constitutively (figure 3) and in larger amounts than did cells from A^+ control mice. Control cells transcribed few IFN-γ and no IL-12 transcripts (figure 3). IL-12 and IFN-γ transcripts in A^- mice peaked at 1 week after infection and decreased thereafter (figure 3). At 1 week after infection, A cells had ~4-fold more IFN-γ transcripts than did A^+ cells. Thus, the IFN-γ and IL-12 tran-

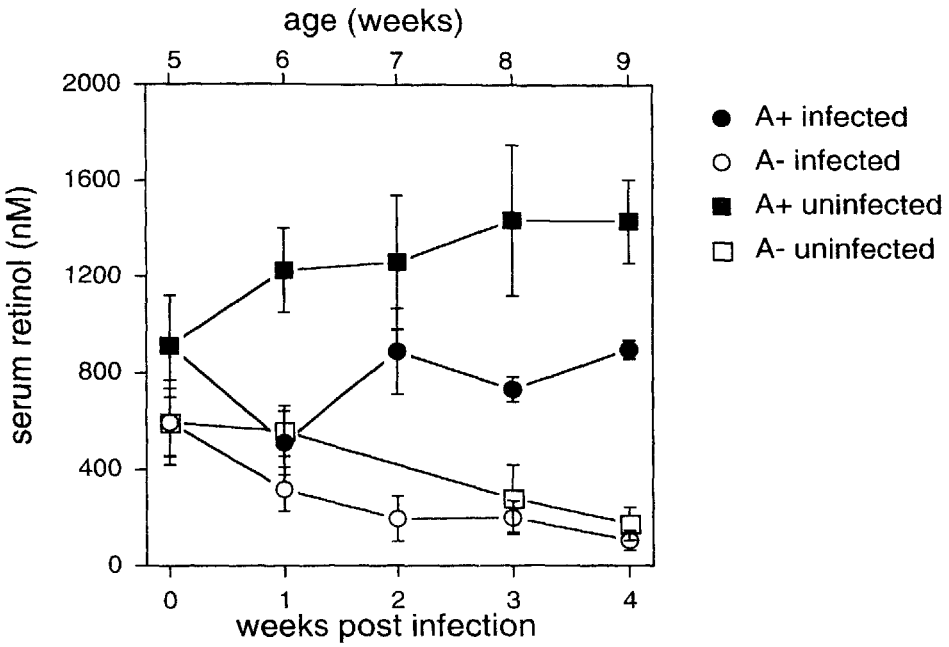

Figure 2. Serum retinol levels in uninfected vitamin A–replete (A^+) and –deficient (A^-) mice and *B. burgdorferi*–infected A^+ and A^- mice. Animals were age-matched. Values are mean ± SD of ≥5 samples from 1 of 3 experiments.

script prevalence immediately preceded the appearance of acute arthritis symptoms in A^- mice.

We detected no cytokines from freshly explanted lymphocytes without antigen restimulation, as we reported previously for *T. spiralis*–infected mice [2, 14]. Therefore, we collected lymph node cells from mice infected 1–3 weeks earlier with *B. burgdorferi*, restimulated the cells in vitro for 24 h with spirochetes [21], and measured cytokines in the supernatants. Consistent with the transcript analysis, cells from A^- mice produced more IFN-γ (59.6 ± 5.1 ng/mL) protein than did A^+ control cells (35.2 ± 6.6 ng/mL) at 1 week after infection. This high IFN-γ secretion by cells from A^- mice is consistent with the increased susceptibility of these mice to acute Lyme arthritis.

TNF-α. TNF-α is reportedly arthritogenic in rheumatoid arthritis [18, 20]. For TNF-α, transcripts correlate imperfectly with protein secretion, due to posttranscriptional controls [22]. We therefore bioassayed the TNF-α/β proteins generated from lymphocytes of *B. burgdorferi*–infected A^+ and A^- mice. The restimulated A^- (14 pg/mL) but not A^+ (0 pg/mL) cells produced TNF. The bioassay probably detected TNF-β protein, since few TNF-α transcripts were detected in the A^- cells (data not shown).

Nonimmune IFN-γ secretion. *B. burgdorferi* organisms are reportedly B cell mitogens [11, 23]. To analyze whether the spirochete-stimulated cytokine release was due to antigen-specific or nonspecific stimulation, we measured cytokine secretion from unprimed A^+ lymphocytes cultured with *B. burgdorferi* for 24 h. The spirochetes stimulated IFN-γ secretion from unprimed lymph node cells (3.9 ± 0.4 ng/mL; mean ± SD for triplicate cultures) and splenocytes (6.4 ± 0.3 ng/mL; one representative experiment of two); unstimulated cells produced no IFN-γ. Addition of retinoic acid (10 n*M*), the active vitamin A metabolite, inhibited IFN-γ synthesis to <1.0 ng/mL, consistent with our previous reports [2, 3, 13]. Spirochetes did not stimulate TNF synthesis (data not shown). We suggest that the ability of *B. burgdorferi* to stimulate high levels of IFN-γ secretion from unprimed cells may be a critically important virulence factor in Lyme arthritis.

Discussion

We found evidence that vitamin A deficiency worsens acute Lyme arthritis and, in addition, that this outcome is due at least partially to unregulated IL-12 and IFN-γ synthesis. The IL-12 and IFN-γ transcripts were constitutive in A^- mice before infection, and both transcripts increased and peaked immediately before arthritis symptoms appeared in the tibiotarsal joints. Retinoic acid, a vitamin A metabolite, is a known inhibitor of IL-12 [1] and IFN-γ transcription (this report and [1–3, 13]), suggesting a mechanism to explain the correlation between higher levels of retinol and milder arthritis. Apparently, vitamin A deficiency exacerbates an antigen-nonspecific proinflammatory cascade, leading to acute Lyme arthritis.

B. burgdorferi infection lowers serum retinol levels by an unknown mechanism. Many infectious pathogens induce transient vitamin A deficiency in well-nourished subjects [24–28]. For example, human immunodeficiency virus type 1–infected persons are vitamin A deficient compared with uninfected persons, and vitamin A deficiency correlates strongly with increased mortality [24]. Tuberculosis [25], measles [26], leprosy [27], and influenza [28] also induce transient vitamin A deficiency.

There is good evidence that nonspecific immune responses are pathogenic in Lyme arthritis. The *scid* mice lack T and B cells and do not make antigen-specific responses, but they de-

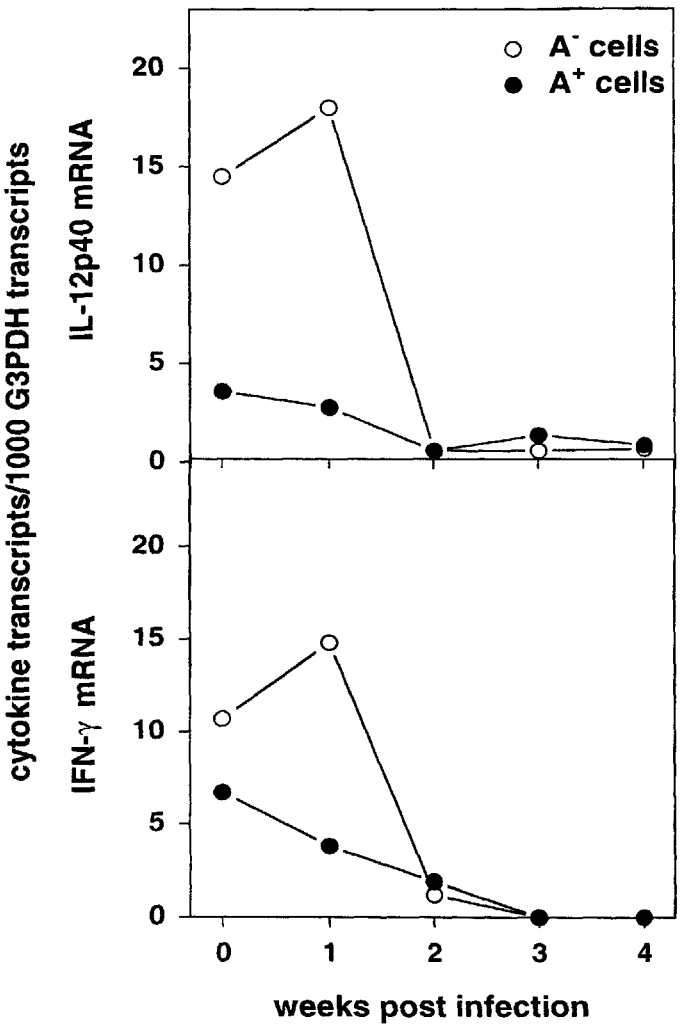

Figure 3. Interferon (IFN)-γ and interleukin (IL)-12 transcripts from lymph nodes of vitamin A–replete (A^+) and –deficient (A^-) mice were analyzed by quantitative competitive polymerase chain reaction. Infection was as described in figure 1. Values are reported as cytokine cDNA copies/1000 cDNA copies of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) from 1 of 2 experiments.

velop an inflammatory reaction that does not eradicate the spirochete and leads to chronic Lyme arthritis (reviewed in [29]). The *scid* mice have NK cells that can produce IFN-γ and macrophages that can become activated in response to IFN-γ. The activated macrophages secrete IL-12, TNF-α, IL-1β, and IL-6, all of which can be stimulated by *B. burgdorferi* spirochetes in vitro [11, 30] and three of which (TNF-α, IL-1β, and IL-6) have arthritogenic function [18–20]. It is likely that NK cells and macrophages, respectively, produced the constitutive IFN-γ and IL-12 that we observed. Our data are therefore consistent with a pathologic role for nonspecific immune responses in acute Lyme arthritis.

TNF-α has been reported to be arthritogenic in rheumatoid arthritis [18, 20], but our experiments suggest that it may not be arthritogenic in Lyme arthritis. We observed little if any TNF-α mRNA in cells from A^- animals when they clearly had severe acute arthritis. In contrast, TNF-β may be arthritogenic in Lyme disease. Our bioassay detected a significant amount of TNF in the samples obtained from A^- mice immediately preceding their most severe acute arthritis symptoms. Since little TNF-α mRNA was present at this time (data not shown), it seems likely that the samples contained TNF-β. The appearance and disappearance of this TNF correlated with the appearance and disappearance of IFN-γ protein. Thus, the possible arthritogenic or protective functions (or both) of TNF in Lyme arthritis remain to be defined by further experimentation.

In summary, in this study, vitamin A deficiency was both a consequence and an arthritis susceptibility factor in *B. burgdorferi* infection. In our model, the *B. burgdorferi* spirochetes began the arthritogenic acute-phase reaction by directly activating macrophages to produce inflammatory cytokines and by either directly or indirectly stimulating IFN-γ secretion. The infection also decreased serum vitamin A levels. Our data suggest that transient vitamin A deficiency leads to constitutive transcription of the IL-12 and IFN-γ genes [1], predisposing an animal to produce a strong inflammatory reaction. Thus, when the *B. burgdorferi* spirochetes interact with lymphoid cells of a vitamin A–deficient host, the outcome is a very high rate of IL-12 and IFN-γ synthesis and severe acute arthritis. This example illustrates the circular relationship among infection, vitamin A deficiency, and immune system dysfunction.

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References

1. Cantorna MT, Nashold FE, Hayes CE. Vitamin A deficiency results in a priming environment conducive for Th1 cell development. Eur J Immunol **1995**;25:1673–9.
2. Cantorna MT, Nashold FE, Hayes CE. In vitamin A deficiency multiple mechanisms establish a regulatory T helper cell imbalance with excess Th1 and insufficient Th2 function. J Immunol **1994**;152:1515–22.
3. Chun TY, Carman JA, Hayes CE. Retinoid repletion of vitamin A–deficient mice restores IgG responses. J Nutr **1992**;122:1062–9.
4. Barbour AG, Fish D. The biological and social phenomenon of Lyme disease. Science **1993**;260:1610–6.
5. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease—a tick-borne spirochetosis? Science **1982**;216:1317–9.
6. Smith SM, Levy NS, Hayes CE. Impaired immunity in vitamin A–deficient mice. J Nutr **1987**;117:857–65.
7. Smith SM, Hayes CE. Contrasting impairments in IgM and IgG responses in vitamin A–deficient mice. Proc Natl Acad Sci USA **1987**;84:5878–82.
8. Barbour AG. Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med **1984**;57:521–5.
9. Barthold SW, de Souza MS, Janotka JC, Smith AL, Persing DH. Chronic Lyme borreliosis in the laboratory mouse. Am J Pathol **1993**;143:959–71.

10. Keane-Myers A, Nickell SP. T cell subset–dependent modulation of immunity to *Borrelia burgdorferi* in mice. J Immunol **1995**;154: 1770–6.
11. Yang L, Ma Y, Schoenfeld R, et al. Evidence for B-lymphocyte mitogen activity in *Borrelia burgdorferi*–infected mice. Infect Immun **1992**;60: 3033–41.
12. Siebert PD, Larrick JW. PCR MIMICS: competitive DNA fragments for use as internal standards in quantitative PCR. Biotechniques **1993**;14: 244–9.
13. Carman JA, Hayes CE. Abnormal regulation of IFN-γ secretion in vitamin A deficiency. J Immunol **1991**;147:1247–52.
14. Carman JA, Pond L, Nashold F, Wassom DL, Hayes CE. Immunity to *Trichinella spiralis* infection in vitamin A–deficient mice. J Exp Med **1992**;175:111–20.
15. Spitalny GL, Havell EA. Monoclonal antibody to murine γ interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. J Exp Med **1984**;159:1560–73.
16. Hogan MM, Vogel SN. Measurement of tumor necrosis factor α and β. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds. Current protocols in immunology. Vol I, section 6.10. Bethesda, MD: John Wiley & Sons, **1994**:1–4.
17. Trinchieri G. Interleukin-12 and its role in the generation of Th1 cells. Immunol Today **1993**;14:333–8.
18. Al-Janadi M, Al-Balla S, Al-Dalaan A, Raziuddin S. Cytokine profile in systemic lupus erythematosus, rheumatoid arthritis, and other rheumatic diseases. J Clin Immunol **1993**;13:58–66.
19. Hahn G, Stuhmuller B, Hain N, Kalden JR, Pfizenmaier K, Burnmester GR. Modulation of monocyte activation in patients with rheumatoid arthritis by leukapheresis therapy. J Clin Invest **1993**;91: 862–70.
20. Williams RO, Feldmann M, Maini RN. Anti–tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci USA **1992**;89:9784–8.
21. Habicht GS. Cytokines in *Borrelia burgdorferi* infection. In: Schutzer SE, ed. Lyme disease. Molecular and immunologic approaches. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, **1992**:149–60.
22. Lindsten T, June CH, Ledbetter JA, Stella G, Thompson CB. Regulation of lymphokine messenger RNA stability by a surface mediated T cell activation pathway. Science **1989**:244;339–43.
23. de Souza MS, Fikrig E, Smith AL, Flavell RA, Barthold SW. Nonspecific proliferative response of murine lymphocytes of *Borrelia burgdorferi* antigens. J Infect Dis **1992**;165:471–9.
24. Semba RD, Graham NMH, Caiaffa WT, Margolick JB, Clement L, Vlahov D. Increased mortality associated with vitamin A deficiency during human immunodeficiency virus type 1 infection. Arch Intern Med **1993**; 153:2149–54.
25. Bresse BB, Watkins E, McCoord AB. The absorption of vitamin A in tuberculosis. JAMA **1942**;119:3–4.
26. Markowitz LE, Nzilambi N, Driskell WJ. Vitamin A levels and mortality among hospitalized measles patients. J Trop Pediatr **1989**;35:109–13.
27. Sher R, Shulman G, Baily P, Politzer WM. Serum trace elements and vitamin A in leprosy subtypes. Am J Clin Nutr **1981**;34:1918–24.
28. Sommer A, Katz J, Tarwotjo I. Increased risk of respiratory disease and diarrhea in children with preexisting mild vitamin A deficiency. Am J Clin Nutr **1986**;40:1090–8.
29. Bartold SW, Sidman CL, Smith AL. Lyme borreliosis in genetically resistant and susceptible mice with severe combined immunodeficiency. Am J Trop Hyg **1992**;47:605–13.
30. Radolf JD, Arndt LL, Akins DR, et al. *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytes/ macrophages. J Immunol **1995**;154:2866–77.