NKT cells prevent chronic joint inflammation after infection with *Borrelia burgdorferi*

Emmanuel Tupin[a,1], Mohammed Rafii-El-Idrissi Benhnia[b,1], Yuki Kinjo[a,1], Rebeca Patsey[c], Christopher J. Lena[a], Matthew C. Haller[c], Melissa J. Caimano[d], Masakazu Imamura[e], Chi-Huey Wong[e], Shane Crotty[b], Justin D. Radolf[d,f], Timothy J. Sellati[c,2], and Mitchell Kronenberg[a,2]

Divisions of [a]Developmental Immunology and [b]Vaccine Discovery, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle Drive, La Jolla, CA 92037; [c]Center for Immunology and Microbial Disease, Albany Medical College, Albany, NY 12208; Departments of [d]Medicine and [f]Genetics and Developmental Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030; and [e]Department of Chemistry and Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037

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***Borrelia burgdorferi* is the etiologic agent of Lyme disease, a multisystem inflammatory disorder that principally targets the skin, joints, heart, and nervous system. The role of T lymphocytes in the development of chronic inflammation resulting from *B. burgdorferi* infection has been controversial. We previously showed that natural killer T (NKT) cells with an invariant (*i*) TCR α chain (*i*NKT cells) recognize glycolipids from *B. burgdorferi*, but did not establish an in vivo role for *i*NKT cells in Lyme disease pathogenesis. Here, we evaluate the importance of *i*NKT cells for host defense against these pathogenic spirochetes by using Vα14*i* NKT cell-deficient ($J\alpha 18^{-/-}$) BALB/c mice. On tick inoculation with *B. burgdorferi*, $J\alpha 18^{-/-}$ mice exhibited more severe and prolonged arthritis as well as a reduced ability to clear spirochetes from infected tissues. Vα14*i* NKT cell deficiency also resulted in increased production of antibodies directed against both *B. burgdorferi* protein antigens and borrelial diacylglycerols; the latter finding demonstrates that anti-glycolipid antibody production does not require cognate help from Vα14*i* NKT cells. Vα14*i* NKT cells in infected wild-type mice expressed surface activation markers and produced IFNγ in vivo after infection, suggesting a participatory role for this unique population in cellular immunity. Our data are consistent with the hypothesis that the antigen-specific activation of Vα14*i* NKT cells is important for the prevention of persistent joint inflammation and spirochete clearance, and they counter the long-standing notion that humoral rather than cellular immunity is sufficient to facilitate Lyme disease resolution.**

cytokines | glycolipids | Lyme disease | spirochetes

Lyme disease, the most common vector-borne illness in the United States, is caused by infection with *Borrelia burgdorferi*, a spirochetal pathogen transmitted to humans and other mammals by *Ixodes scapularis* tick bites (1). The mouse model of borrelial infection has served as an invaluable tool for exploring immunopathogenic mechanisms in Lyme disease (2–4). *B. burgdorferi*-infected severe combined immunodeficient (SCID) mice, which lack functional B and T lymphocytes, exhibit persistent spirochetemia and progressive inflammation of the joints, heart, and liver (5). Adaptive immunity has a critical role in the control and resolution of disease (6–8), as underscored by the persistence of active carditis and the progressively destructive arthritis seen in SCID mice. Disease resolution correlates with the appearance of borreliacidal antibodies that, when passively transferred, protect naive animals against challenge with virulent organisms (6).

However, the role of T cells in disease resolution is somewhat controversial (9–11). There is evidence pointing to the importance of a T_H1/T_H2 balance, because increased IL-12 and T_H1-type cytokines are associated with disease progression in humans and susceptible strains of inbred mice (12–15), whereas cytokines such as IL-10 have a beneficial effect (16–19). In contrast, Bockenstedt *et al*. (20) have shown that $CD4^+$ T_H1 cells were beneficial for the regression of carditis. More recently, Iliopoulou *et al*. (21) reported that C57BL/6 mice deficient for CD28-mediated costimulation develop chronic joint inflammation and have increased titers of anti-OspA antibodies. However, the results from another study (22), relying on adoptive transfer of cells to immune deficient mice, suggested that $CD4^+$ T cells, in the absence of B lymphocytes, exacerbate arthritis and carditis. Last, with regard to the regulation of inflammation and disease resolution, a recent study has shown that T-independent antibodies from marginal zone (MZ) B cells have a major role, because their depletion leads to reduced *B. burgdorferi*-specific IgM and IgG titers, enhanced pathogen burden and more severe arthritis (23).

Difficulty in assessing the role of T lymphocytes in the response to *B. burgdorferi* could be due to the complexity of mouse T cell subsets. A distinct T lymphocyte subpopulation is the Vα14*i* NKT cells, which are innate-like lymphocytes that coexpress NK receptors, such as NK1.1, and a TCR. The most abundant population of NKT cells in mice expresses an invariant TCR α chain, encoded by a Vα14-Jα18 rearrangement (24, 25). These cells have an important regulatory role in innate and acquired immune responses (26). Known as Vα14*i* NKT cells in the mouse, they recognize autologous and bacterial glycolipids presented by CD1d (24, 25).

Although Vα14*i* NKT cells are important for the clearance of diverse microbes (25), it has not been shown that recognition of a foreign antigen by the Vα14*i* TCR is required for pathogen clearance. Our previous data indicated that Vα14*i* NKT cells recognize galactosyl diacylglycerol antigens from *B. burgdorferi* (27), but did not show a role for these cells in the prevention of inflammation. Here, we show that Vα14*i* NKT cells are important for the prevention of persistent joint inflammation and spirochete clearance, and that specific antibodies are unlikely to mediate these effects. Demonstration that mice deficient for Vα14*i* NKT cells fail to clear *B. burgdorferi*, despite a robust antibody response, challenges the idea that elimination of spirochetes is solely the purview of humoral immunity, and instead suggests an important role for a specialized type of cell-mediated immunity as well.

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[1]E.T., M.R.-E.-I.B., and Y.K. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: sellatt@mail.amc.edu or mitch@liai.org.

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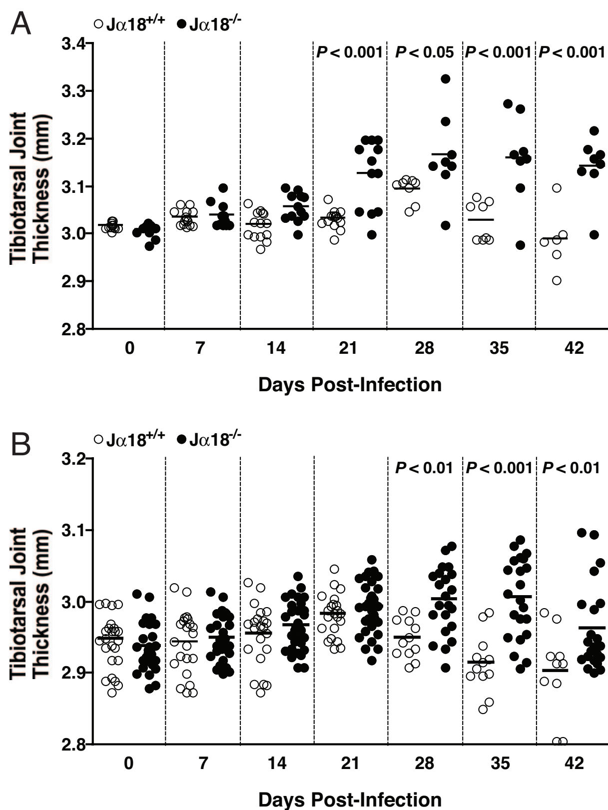

Fig. 1. *B. burgdorferi*-infected tibiotarsal joints from Vα14*i* NKT cell deficient mice exhibit greater and more persistent swelling. $J\alpha 18^{+/+}$ (open circles) and $J\alpha 18^{-/-}$ mice (closed circles) were infected with *B. burgdorferi* and joint thickness was measured by using digital calipers. Each symbol represents the measurement of 1 joint; the 2 hind joints from each mouse were measured and are presented. The horizontal bars indicate mean thickness for each group ($n = 3 - 11$). *A* and *B* show the results from independent experiments.

Results

Mice Lacking Vα14*i* NKT Cells Exhibit More Severe and Prolonged *B. burgdorferi*-Induced Joint Swelling. In this study, we set out to determine whether Vα14*i* NKT cells have a role in host defense against *B. burgdorferi* by using the natural route of tick-mediated infection. We used $J\alpha 18^{-/-}$ mice, which are fully immunocompetent and express CD1d, but lack the $J\alpha$ gene segment required to form the invariant TCR expressed by Vα14*i* NKT cells (28). The impact of this genetic deficiency was evaluated in BALB/c mice, an inbred background known to have greater susceptibility to borrelial infection than C57BL/6 mice (2), reasoning that a nonredundant role for Vα14*i* NKT cells would more likely be uncovered by using a strain that mounts a less protective response to infection.

To assess the role of Vα14*i* NKT cells in arthritis development, the thickness of tibiotarsal joints was measured at weekly intervals postinfection (p.i.), as previously described (29). In 2 independent experiments, shown separately as *A* and *B*, little increase in joint thickness above baseline was observed in either $J\alpha 18^{+/+}$ or $J\alpha 18^{-/-}$ mice for the first 14 (Fig. 1*A*) or 21 days p.i. (Fig. 1*B*). However, the joints of $J\alpha 18^{-/-}$ were significantly larger by days 21 and 28, respectively (Fig. 1), and this increase persisted until day 42. More severe and persistent arthritis also was observed in *B. burgdorferi*-infected BALB/c mice deficient for CD1d (data not shown), and was seen previously in $Cd1d^{-/-}$ mice on the C57BL/6 and 129 strain mixed background (30).

Increased Inflammatory Cell Infiltrate in the Absence of Vα14*i* NKT Cells. A positive correlation between joint size and the intensity and extent of inflammatory cell infiltration in *B. burgdorferi*-infected C3H mice has been reported (29); we observed this same correlation in BALB/c mice. At day 42 p.i., the joints of $J\alpha 18^{-/-}$ mice lacking Vα14*i* NKT cells exhibited a more extensive mixed infiltrate of neutrophils and scattered macrophages, compared with wild-type mice (Fig. 2*A*). Of particular note was the accumulation of inflammatory cells within and around the peritendinous sheaths and the frank tendinitis that was not evident in the $J\alpha 18^{+/+}$ joints. Consistent with these observations, the overall arthritis scores in $J\alpha 18^{-/-}$ mice were significantly greater than those of infected wild-type mice ($P < 0.001$, Fig. 2*C*).

Histological examination of heart tissue revealed mixed infiltrates consisting of macrophages and scattered lymphocytes that were concentrated in the epicardium and atrial myocardium. These infiltrates were present in both groups of mice at day 42 p.i. (Fig. 2*B*). Although quantitative histological evaluation suggested a trend toward more severe carditis in the $J\alpha 18^{-/-}$

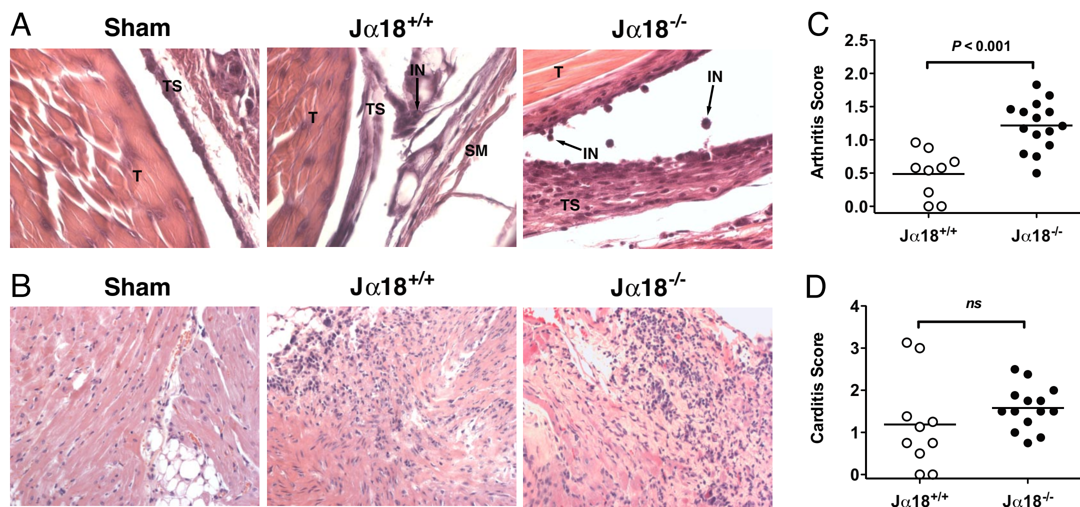

Fig. 2. *B. burgdorferi*-infected tissues from Vα14*i* NKT cell deficient mice exhibit extensive inflammation. (*A* and *B*) Representative sections of joints (400×) (*A*) and hearts (200×) (*B*) from an uninfected (Sham) and *B. burgdorferi*-infected $J\alpha 18^{+/+}$ and $J\alpha 18^{-/-}$ mice at 42 days p.i. T, tendon; TS, tendinous sheath; SM, synovium; IN, mixed inflammatory infiltrates consisting neutrophils and macrophages. Sections of joints (*C*) and hearts (*D*) from mice recovered 42 days p.i. were scored on the basis of histological criteria detailed in *Materials and Methods*. The data shown are combined from 2 independent experiments.

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Table 1. Increased spirochete clearance in wild-type mice

	Bladder		Ear		Heart		Joint	
	Jα*18*$^{+/+}$	*J*α*18*$^{-/-}$	*J*α*18*$^{+/+}$	*J*α*18*$^{-/-}$	*J*α*18*$^{+/+}$	*J*α*18*$^{-/-}$	*J*α*18*$^{+/+}$	*J*α*18*$^{-/-}$
Day 21	6/17	2/21	5/17	2/21	6/17	2/21	7/17*	2/21
Day 42	6/10**	1/15	5/10***	0/15	5/10***	0/15	4/10*	0/15

Clearance is defined when the bacterial burden in individual tissues is below the limit of detection (10 copies of *flaB; 10,000 copies of nidogen*). Results represent combined data from 3 independent experiments. Fisher's exact test, 1 sided: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$

mice, the difference did not reach statistical significance (P = 0.079) (Fig. 2*D*).

***J*α*18*$^{-/-}$ Mice Exhibit a Reduced Ability to Clear Spirochetes.** In some studies (2, 5, 29), severe and prolonged inflammation correlated with decreased clearance of *B. burgdorferi*. Therefore, we compared spirochete burden in the tissues of wild-type and *J*α*18*$^{-/-}$ mice by quantitative (q)PCR, by using the spirochetal *flaB* gene as a target. Considerable numbers of spirochetes were detected in the joints, hearts, ears, and bladders of mice infected for 21 and 42 days, but there was considerable variablity, and the median bacterial burdens in the 2 groups of mice did not differ (data not shown). However, spirochete numbers in wild-type tissues were much more often below the limit of detection, whereas *B. burgdorferi* were almost always found in tissues from mice lacking *i*NKT cells. This dichotomy was clearly evident when the ability to detect spirochetes in tissues from the 2 groups of mice was compared at day 42 (Table 1). When all 148 tissue samples were analyzed together, logistic regression demonstrated that clearance was significantly associated only with whether the animal possessed *i*NKT cells or not. The odds of clearance to below detectable levels in *J*α*18*$^{-/-}$ mice was 0.02 (odds ratio with 95% confidence interval of 0.0 to 0.12) times the odds of clearance in wild-type animals. The reason for the bimodal response in wild-type mice, with clearance observed in some tissues but not others, is not known, but, overall, the results suggest a more active anti-borrelial response in mice with *i*NKT cells.

Vα14*i* NKT Cell Deficiency Contributes to Elevated Production of Anti-Borrelial Antibodies. The potential impact of Vα14*i* NKT cell deficiency on the development of humoral immunity to *B. burgdorferi* also was investigated. As determined by Western blot analysis using a whole cell lysate of *B. burgdorferi* as target, the pattern of borrelial antigen recognition by immune sera from wild-type and *J*α*18*$^{-/-}$ mice was nearly indistinguishable (data not shown). However, although there was some heterogeneity in the wild-type mice, the reciprocal endpoint titer of anti-borrelial IgG antibodies at days 21 and 42 p.i. was significantly higher in the sera of *J*α*18*$^{-/-}$ mice than in *J*α*18*$^{+/+}$ animals (Fig. 3*A*). For wild-type mice, some of the animals had a very low titer of anti-borrelial IgG, and this low titer correlated with spirochete clearance in all of the tissues analyzed [supporting information (SI) Fig. S1]. Similar to the total anti-Borrelia IgG, the level of IgG antibodies with reactivity to the *B. burgdorferi* BbGL-IIc glycolipid antigen, which is recognized by the invariant TCR of Vα14*i* NKT cells, also was significantly higher in *J*α*18*$^{-/-}$ sera at days 7 and 21 (Fig. 3*B*).

Infection of Mice Elicits Vα14*i* NKT Cell Activation. To determine whether Vα14*i* NKT cells are activated after *B. burgdorferi* infection, we analyzed Vα14*i* NKT cells in the liver and spleen of infected mice. Flow cytometry with αGalCer-CD1d tetramers was used to identify tetramer positive Vα14*i* NKT cells. The activation state of these cells then was determined by staining for CD25 and CD69 at day 7 p.i. An increased mean fluorescence intensity of CD25 and CD69 staining on Vα14*i* NKT cells isolated from infected mice was observed in both spleen and liver at 7 days after infection (Fig. 4*A*; Fig. S2 *A* and *B*), but was not observed for conventional T cells (data not shown). These results from tick infection of BALB/c mice were consistent with those obtained earlier from either tick or syringe-infected C57BL/6 mice (27). Also, the potential effector function of the activated Vα14*i* NKT cells was evidenced by an increase in the percentage of cells staining positive for intracytoplasmic IFNγ when analyzed directly ex vivo without restimulation (Fig. 4 *B* and *C*; Fig. S2*C*). Intracellular IL-4 also was increased at this time, although IL-17 was not (Fig. S2 *D* and *E*, respectively). The combination of IL-4 and IFNγ secretion is suggestive of TCR activation, as opposed to inflammatory activation of *i*NKT cells by IL-12, which tends to induce IFNγ only (31). However, at 14 days after infection signs of activation of *i*NKT cells were greatly diminished, except that intracellular IL-4 remained higher in the spleen only (Fig. S3*D*).

Despite the robust response of Vα14*i* NKT cells to *B. burgdorferi* in the liver and spleen, we did not observe recruitment of these cells to the joints and hearts by means of nested-PCR using primers specific for the Vα14-Jα18 TCR as previously described (32) (data not shown). Similarly, no consistent differences in *ifn*γ transcript or secreted protein could be observed in tissues of

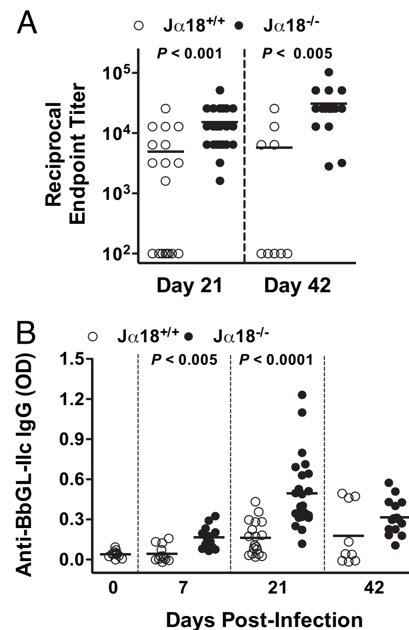

Fig. 3. Increased anti-borrelial Igs in Vα14*i* NKT cell deficient mice. (*A*) Reciprocal endpoint titers for total anti-borrelial IgG were calculated for serially-diluted sera isolated from individual mice at 21 and 42 days p.i. (*B*) IgG binding from sera collected at the indicated times p.i. to plates coated with the synthetic BbGL-IIc glycolipid was measured by using a colorimetric ELISA. Combined OD readings are presented for 2 independent experiments.

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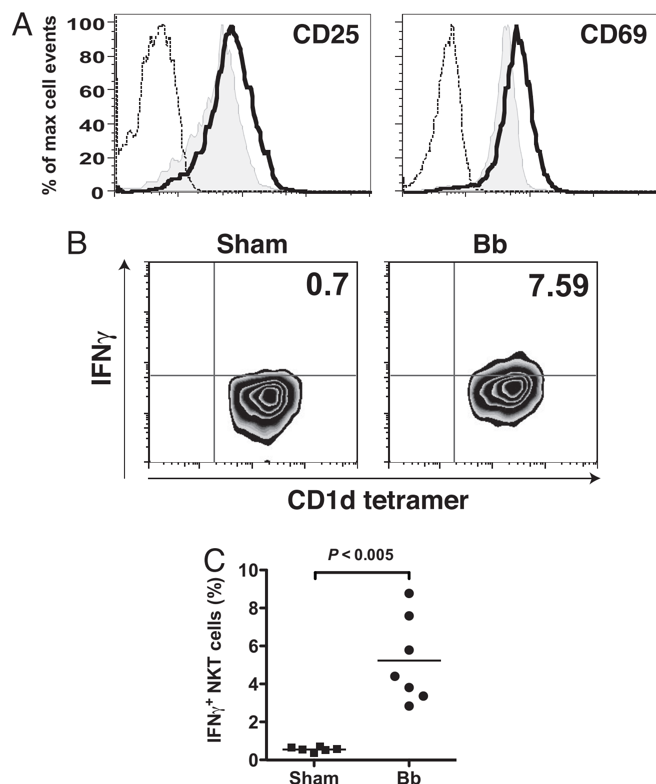

Fig. 4. Vα14*i* NKT cells from *B. burgdorferi*-infected mice at day 7 are activated. (*A*) Expression of activation markers CD25 and CD69 on Vα14*i* NKT cells gated on αGalCer-CD1d tetramer$^+$ CD19$^-$ liver mononuclear cells recovered from *B. burgdorferi*-infected wild-type mice 7 days p.i. Data are from a single mouse representative of 6 uninfected (gray histograms) and 7 infected mice (solid lines); similar data were obtained in 2 experiments. The dashed line is the isotype control. (*B*) Staining for intracellular IFNγ from representative samples is depicted. (*C*) The percentage of IFNγ^+ Vα14*i* NKT cells for individual sham treated (n = 6) or *B. burgdorferi* (Bb) infected (n = 7) mice is shown. IFNγ^+ Vα14*i* NKT cells were detected directly ex vivo without restimulation ($P < 0.005$).

infected wild-type and $J\alpha 18^{-/-}$ mice (data not shown). Collectively, these results imply a role for Vα14*i* NKT cells in modulating Lyme disease pathogenesis early in the infection process and perhaps in sites where immune responses are primed as opposed to the site of inflammation.

Discussion

In this study, we set out to determine whether Vα14*i* NKT cells contribute to host defense against *B. burgdorferi*. $Cd1d^{-/-}$ C57BL/6 mice were previously reported to have an increased bacterial burden and joint inflammation after syringe infection with *B. burgdorferi* (30). However, T cells with more diverse TCRs also recognize CD1d; also, CD1d has been reported to have a signaling function that may be independent of T lymphocyte activity (33, 34). In fact, in several experimental systems (35–37), analysis of $Cd1d^{-/-}$ and $J\alpha 18^{-/-}$ mice gave discordant results, suggesting either a role for CD1d-reactive T cells with more diverse TCRs or another function for CD1d besides antigen presentation. Consistent with this additional function for CD1d, MZ B cells from $Cd1d^{-/-}$ mice have reductions in Borrelia-specific IgM and IgG production in response to infection with the related spirochete, *Borrelia hermsii* (38). This IgM production is believed to reflect a T-independent B cell response; and, therefore, the influence of CD1d in this case implicates a CD1d function separate from antigen presentation to T cells. Consequently, we used $J\alpha 18^{-/-}$ mice to address more precisely the role of Vα14*i* NKT cells in vivo. $J\alpha 18^{-/-}$ BALB/c mice exhibited a markedly altered immune response to tick-transmitted *B. burgdorferi*, characterized by increased and persistent joint inflammation, some impairment of bacterial clearance, and elevated Borrelia-specific antibody titers. Increased titers of IgG antibody directed toward both total spirochetal antigens, and the abundant diacylglycerol glycolipid antigen BbGL-IIc, were likely fueled by the persistence of spirochetes in infected tissues. Such anti-borrelial antibodies, including those with affinity for glycerol glycolipids, also are found in Lyme disease patients (39, 40). Our data do not rule out a role for IgM antibodies in clearance, and in fact, such antibodies could contribute to the rapid spirochete clearance seen in some wild-type mice. In addition to serving as a target for the antibody response, BbGL-IIc also is an antigen recognized by the invariant TCR expressed by Vα14*i* NKT cells (27). IgG antibodies very likely are T cell-dependent, but these data indicate that cognate help provided to glycolipid-reactive B cells by Vα14*i* NKT cells is not the main mechanism driving the synthesis of anti-glycolipid antibodies during infection. A provocative corollary is that Vα14 *i*NKT cells may instead have an indispensable role in cell-mediated immunity to *B. burgdorferi*.

Here, we show that not only do Vα14*i* NKT cells increase expression of activation markers transiently after infection, significantly preceding joint inflammation, but they also contain higher amounts of intracellular IFNγ and IL-4 when analyzed ex vivo, suggestive of TCR activation. The inability to detect Vα14*i* NKT cells in sites of inflammation hints at a role in modulating Lyme disease pathogenesis through priming immunity at distal locations. Collectively, these results suggest that Vα14*i* NKT cells are a critical element of the early response to *B. burgdorferi*, at least 2 weeks before inflammation is apparent, although it remains to be determined whether this early response results primarily from the ability of these cells to stimulate innate or adaptive immunity. Regardless, the importance of Vα14*i* NKT cells, and the increased anti-borrelial Igs observed in the absence of these cells, certainly challenges the notion that humoral immunity is sufficient for resolution of Lyme borreliosis, although it may be necessary. Also, it should be noted that, although there was a quantitative increase in anti-borrelial antibodies in the absence of Vα14*i* NKT cells, we cannot exclude the possibility that there was some positive, qualitative influence on humoral immunity exerted by these T cells.

In conclusion, we have shown that Vα14*i* NKT cells are activated in vivo after *B. burgdorferi* infection and they contribute to host defense by facilitating spirochetal clearance, thereby limiting the severity and duration of Lyme arthritis. These data provide the strongest evidence linking antigen recognition by the invariant TCR to the clearance of a pathogen. For unknown reasons, the frequency of *i*NKT cells in the peripheral blood of humans varies greatly, from almost undetectable up to several percentage (25, 41). Therefore, it is possible that a low frequency of *i*NKT cells in some individuals is a risk factor that may increase an individual's susceptibility to Lyme disease.

Materials and Methods

Mice. Six to eight week-old BALB/c mice (Jackson Laboratory) and $J\alpha 18^{-/-}$ BALB/c mice, provided by Dr. Masaru Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan), were housed under specific pathogen-free conditions at the La Jolla Institute of Allergy and Immunology. All experimental procedures were approved by the institutional animal care committee.

Generation of *B. burgdorferi*-Infected *I. scapularis* Nymphs and Tick-Mediated Transmission. Three to five week-old C3H/HeJ mice (Jackson Laboratories) were syringe-inoculated intradermally with 10^4 *B. burgdorferi* strain 297; ≈400 pathogen-free *I. scapularis* larvae (Oklahoma State University, Stillwater, OK) were allowed to feed on *B. burgdorferi*-infected mice and were collected over water after repletion. Fed larvae were stored over a supersat-

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urated K_2SO_4 solution in an environmental incubator maintained at 22 °C with a 16 h:8 h light:dark photoperiod until they molted to the nymphal stage. To infect mice by means of tick-transmission, naive animals were each infested with 4 *I. scapularis* nymphs confined within a capsule placed on the back. Each capsule consisted of the screw-cap portion of a 1.5-mL polypropylene conical tube secured to closely clipped fur by a mixture (wt/wt) of 4 parts rosin gum (Sigma-Aldrich) and 1 part beeswax (Fisher Scientific). After tick-mediated infection with *B. burgdorferi*, various tissues isolated from the mice were bisected with one-half used for histopathological evaluation, and the remaining half snap-frozen in liquid nitrogen and stored at −80 °C for subsequent genomic DNA extraction.

Assessment of Inflammation. Joint inflammation was evaluated (*i*) grossly by digital caliper measurement of tibiotarsal joint thickness, and (*ii*) histologically by examination of decalcified, paraffin-embedded specimens stained with hematoxylin and eosin. Disease severity was assessed on the basis of edema, inflammatory cell infiltration, and thickening of the tendon sheath as previously described (29). The amount of polymorphonuclear and mononuclear cell infiltration was graded in a blinded manner as 0 (none), 1 (light), 2 (moderate), or 3 (heavy). Hearts from the same time points also were evaluated for histopathological alterations as previously described (29).

Quantification of *B. burgdorferi* DNA. Quantification of *B. burgdorferi* DNA was performed by qPCR by using TaqMan Universal PCR Master Mix and the iQ5 real-time PCR Detection System (BIO-RAD Laboratories). Genomic DNA was extracted and qPCR was performed in triplicate by using 40 ng of target DNA, along with *B. burgdorferi*-specific *flaB* primers (200 nM) and probe (320 nM) or primers (400 nM) and probe (320 nM) directed against the single-copy mouse *nidogen* gene and quantification of target DNA was accomplished as described previously (29).

***B. burgdorferi*-Specific Antibodies.** Anti-borrelial antibody endpoint titers were determined by ELISA. Flat-bottomed 96-well microtiter plates (Nunc Maxisorpcopy) were coated overnight at 4 °C with each well containing 0.5 μg of *B. burgdorferi* strain 297 whole cell lysates in PBS. A standard ELISA protocol was followed by using serially-diluted preimmune and immune sera in blocking buffer (PBS containing 0.5% BSA and 0.1% Tween 20) reacted against whole cell lysates of *B. burgdorferi* in 96-well microtiter plates, starting at 1:200 with subsequent 2-fold serial dilutions to 1:102,400. To measure glycolipid-specific antibodies, hexane solvent was evaporated from the synthetic *B. burgdorferi* BbGL-IIc antigen, synthesized as described previously (27). Then, the BbGL-IIc glycolipid was resuspended in blocking buffer, used to coat 96-well microtiter plates and then reacted with a 1/20 dilution of preimmune or immune sera to measure BbGL-IIc reactivity. After washing, plates coated with borrelial whole cell lysates or synthetic antigen were handled similarly for detection by addition of streptavidin-horseradish peroxidase conjugated goat anti-mouse IgG (CALTAG Laboratories) diluted 1:1,000 in blocking buffer. Plates were developed by using *o*-phenylenediamine dihydrochloride, and after reaction quenching by using 1N HCl, the optical density was read on a SpectraMax 250 (Molecular Devices) at 492 nm. The titer for an individual mouse serum sample was determined to be the reciprocal of the highest dilution that had a reading above the cutoff. Cutoff values were determined as the average of the preimmune serum samples plus the SD multiplied by the factor 1.833, based on readings obtained from 14 preimmune sera samples.

In Vivo NKT Cell Response and Flow Cytometry. Liver mononuclear cells and spleen cells were collected from sham (uninfected) and *B. burgdorferi*-infected mice 7 and 14 days after ticks fed to repletion. Activation markers and intracellular cytokine staining of αGalCer-CD1d tetramer positive cells were carried out according to a published protocol (42) with slight modifications; αGalCer-CD1d tetramer positive, CD19 (clone 1D3, BD PharMingen) negative cells were analyzed for activation markers. For intracellular IFNγ, IL-4 or 1L-17 staining, cells were cultured for 2 h in the presence of brefeldin A (BD Bioscience) in a CO_2 incubator before staining. Cells were analyzed and data acquired by using a FACS-Caliber (BD Bioscience) instrument and results were analyzed by using FlowJo software (Treestar).

Statistical Analysis. Tests were performed by using Prism 4.0 (GraphPad) and a 2-tailed Mann–Whitney test, unless otherwise indicated. Joint thickness data were analyzed by 1-way ANOVA with Newman Keuls test. To evaluate bacterial clearance, a logistic regression model was used to test for associations between such clearance in all 4 tissues and 3 factors: (*i*) knockout versus wild-type animals, (*ii*) time of measurement (day 21 versus day 42), and (*iii*) organ system (joint, heart, bladder, and ear). The odds ratio for the association between the dependent variable (clearance) is reported for associations with $P < 0.05$. The logistic models were estimated by using MINITAB Statistical Software. Data for individual tissues were evaluated by using 1-sided Fisher's exact test as shown in Table 1.

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