

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

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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 $V\alpha 14i$ 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. $V\alpha 14i$ 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 $V\alpha 14i$ NKT cells. $V\alpha 14i$ NKT cells in infected wild-type mice expressed surface activation markers and produced IFN γ *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 $V\alpha 14i$ 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 borrelial 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 $V\alpha 14i$ 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 $V\alpha 14$ - $J\alpha 18$ rearrangement (24, 25). These cells have an important regulatory role in innate and acquired immune responses (26). Known as $V\alpha 14i$ NKT cells in the mouse, they recognize autologous and bacterial glycolipids presented by CD1d (24, 25).

Although $V\alpha 14i$ NKT cells are important for the clearance of diverse microbes (25), it has not been shown that recognition of a foreign antigen by the $V\alpha 14i$ TCR is required for pathogen clearance. Our previous data indicated that $V\alpha 14i$ 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 $V\alpha 14i$ 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 $V\alpha 14i$ 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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Table 1. Increased spirochete clearance in wild-type mice

| | Bladder | | Ear | | Heart | | Joint | |
|--------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| | <i>Jα18^{+/+}</i> | <i>Jα18^{-/-}</i> | <i>Jα18^{+/+}</i> | <i>Jα18^{-/-}</i> | <i>Jα18^{+/+}</i> | <i>Jα18^{-/-}</i> | <i>Jα18^{+/+}</i> | <i>Jα18^{-/-}</i> |
| 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. 2D).

***Jα18^{-/-}* 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 *Jα18^{-/-}* 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 variability, 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 *iNKT* 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 *iNKT* cells or not. The odds of clearance to below detectable levels in *Jα18^{-/-}* 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 *iNKT* cells.

***Vα14i* NKT Cell Deficiency Contributes to Elevated Production of Anti-Borrelial Antibodies.** The potential impact of *Vα14i* 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 *Jα18^{-/-}* 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 *Jα18^{-/-}* mice than in *Jα18^{+/+}* animals (Fig. 3A). 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 *Vα14i* NKT cells, also was significantly higher in *Jα18^{-/-}* sera at days 7 and 21 (Fig. 3B).

Infection of Mice Elicits *Vα14i* NKT Cell Activation. To determine whether *Vα14i* NKT cells are activated after *B. burgdorferi* infection, we analyzed *Vα14i* NKT cells in the liver and spleen of infected mice. Flow cytometry with α GalCer-CD1d tetramers was used to identify tetramer positive *Vα14i* 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 *Vα14i* NKT cells isolated from infected mice was observed in both spleen and liver at 7 days after infection (Fig. 4A; Fig. S2A 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 *Vα14i* NKT cells was evidenced by an increase in the percentage of cells staining positive for intracytoplasmic IFN γ when analyzed directly ex vivo without restimulation (Fig. 4B and C; Fig. S2C). Intracellular IL-4 also was increased at this time, although IL-17 was not (Fig. S2D and E, respectively). The combination of IL-4 and IFN γ secretion is suggestive of TCR activation, as opposed to inflammatory activation of *iNKT* cells by IL-12, which tends to induce IFN γ only (31). However, at 14 days after infection signs of activation of *iNKT* cells were greatly diminished, except that intracellular IL-4 remained higher in the spleen only (Fig. S3D).

Despite the robust response of *Vα14i* 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 *Vα14i*-*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

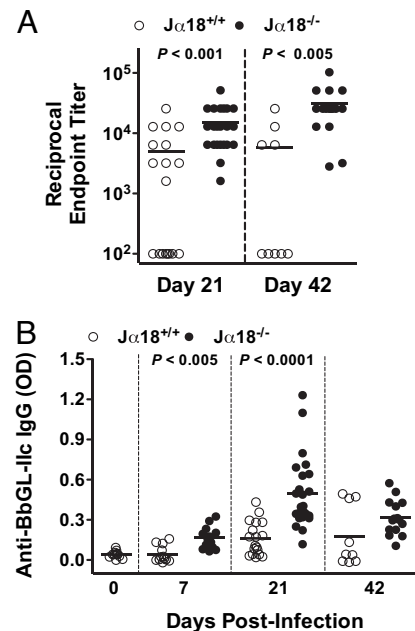


Fig. 3. Increased anti-borrelial Igs in *Vα14i* NKT cell deficient mice. (A) Reciprocal endpoint titers for total anti-borrelial IgG were calculated for serially-diluted sera isolated from individual mice at 21 and 42 days p.i. (B) IgG binding from sera collected at the indicated times p.i. to plates coated with the synthetic BbGL-IIc glycolipid was measured by using a colorimetric ELISA. Combined OD readings are presented for 2 independent experiments.

urated K₂SO₄ 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 Maxisorp) 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-ILc antigen, synthesized as described previously (27). Then, the BbGL-ILc 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-ILc 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 IL-17 staining, cells were cultured for 2 h in the presence of brefeldin A (BD Bioscience) in a CO₂ 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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