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Role of NK-Like CD8⁺ T Cells during Asymptomatic *Borrelia burgdorferi* Infection

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**ABSTRACT** Lyme disease (LD) due to *Borrelia burgdorferi* is the most prevalent vector-borne disease in the United States. There is a poor understanding of how immunity contributes to bacterial control, pathology, or both during LD. Dogs in an area of endemicity were screened for *B. burgdorferi* and *Anaplasma* exposure and stratified according to seropositivity, presence of LD symptoms, and doxycycline treatment. Significantly elevated serum interleukin-21 (IL-21) and increased circulating CD3⁺ CD94⁺ lymphocytes with an NK-like CD8⁺ T cell phenotype were predominant in asymptomatic dogs exposed to *B. burgdorferi*. Both CD94⁺ T cells and CD3⁺ CD94⁺ lymphocytes, corresponding to NK cells, from symptomatic dogs expressed gamma interferon (IFN-γ) at a 3-fold-higher frequency upon stimulation with *B. burgdorferi* than the same subset among endemic controls. Surface expression of activating receptor Nkp46 was reduced on CD94⁺ T cells from LD, compared to cells after doxycycline treatment. A higher frequency of Nkp46-expressing CD94⁺ T cells correlated with significantly increased peripheral blood mononuclear cell (PBMC) cytotoxic activity via calcein release assay. PBMCs from dogs with symptomatic LD showed significantly reduced killing ability compared with endemic control PBMCs. An elevated NK-like CD8⁺ T cell response was associated with protection against development of clinical LD, while excess IFN-γ was associated with clinical disease.

**KEYWORDS** *Borrelia burgdorferi*, Lyme disease, NK-like cells, NK-like CD8⁺ T cells, NK cells, tick-borne, zoonotic

Lyme disease (LD) is the most common vector-borne disease in the United States, with an estimated ~476,000 annual cases (1, 2). The range and prevalence of the causative spirochete, *Borrelia burgdorferi*, and its vector, *Ixodes* sp. ticks, have been steadily increasing since LD became nationally notifiable in 1991 (3). Following infection, Lyme arthritis occurs in ~60% of untreated patients (4). Most cases are treatable with antibiotics; however, a subset of patients experience antibiotic-refractory joint inflammation, with a suspected immune-based etiology (4). Similar to humans, dogs are incidental hosts of *B. burgdorferi* infection. Most *B. burgdorferi*-seropositive dogs are asymptomatic (AS); Lyme arthritis is the most common clinical manifestation (5). As people do not seek clinical treatment unless they experience symptoms, dogs provide a unique natural model to identify immune processes associated with subclinical *B. burgdorferi* infection.

Invariant natural killer T (iNKT) cells were identified as critical for controlling experimental LD. Both mouse and human iNKT cells recognize *B. burgdorferi* diacylglycerol glycolipid antigens, leading to proliferation, cytokine production, and granzyme-mediated pathogen killing (6, 7). Mice lacking iNKT cells experience prolonged and more severe arthritis, increased carditis, and reduced spirochete clearance compared to wild-type mice (6, 7). In contrast, human iNKT cells are not critical for LD control, suggesting species differences in the role of iNKT cells in LD pathogenesis. However, the role of canine iNKT cells in LD pathogenesis remains to be determined.

In summary, the study of iNKT cells in LD provides insights into the immune mechanisms underlying LD pathogenesis and highlights the potential for therapeutic strategies targeting iNKT cells to control LD.
type controls (8, 9). Further, CD1d−/− mice, unable to present antigen to iNKT cells, have worsened arthritis and increased B. burgdorferi burden (10). iNKT cells were found elevated in LD patient blood, but only in synovial fluid of antibiotic-responsive patients (11). These studies suggest iNKT cells are protective against human LD, but roles for this cell subset in asymptomatic, controlled, natural infection have not been evaluated.

iNKT cells belong to a burgeoning family of unconventional T cells that bridge innate and adaptive immunity (12). While mice express only CD1d, humans and dogs express additional CD1 molecules—CD1a, CD1b, and CD1c (13–16). In humans, CD1 antigen presentation facilitates expansion of a much more diverse and abundant unconventional T cell compartment compared to that observed in mice (14). CD1d presentation of B. burgdorferi glycolipid antigen to iNKT cells is crucial for protection in experimental murine models; however, the potential contribution of other NKT-like cells to protection from clinical Lyme disease has yet to be explored.

Whether peripheral natural killer (NK) cells increase during LD is not clear (17, 18). Antibiotic-refractory human Lyme arthritis was associated with persistence of NK cells expressing CD16, a marker of antibody-dependent cell cytotoxicity (11). CD56bright NK cells in synovial fluid from antibiotic-refractory Lyme arthritis patients had a non-statistically significant, but larger, proportion of gamma interferon (IFN-γ)-producing cells compared to cells from treatment-responsive patients (11). Although Th helper 1 (Th1)-type immunity may contribute to bacterial control, increased serum IFN-γ in erythema migrans-positive patients correlated with increased symptomatology (19). In humans, a higher ratio of Th1 to Th2 cells in synovial fluid directly correlates with arthritis severity (20). Furthermore, excessive type 1 inflammation is associated with increased risk of developing antibiotic-refractory Lyme arthritis (19, 21). These studies may indicate NK cell subsets promote inflammation and immunopathology.

Herein, a cohort of naturally infected dogs was screened for B. burgdorferi exposure and clinical signs. Due to previous studies indicating a potential protective role for iNKT cells in experimental murine models, we were particularly interested in investigating differences in NKT-like cell types and their critical outputs, inflammatory cytokine production, and cytotoxicity in dogs exposed to B. burgdorferi with and without clinical signs of LD. Our goal was to evaluate cellular responses associated with maintenance or loss of an asymptomatic state and presence of clinical LD. We evaluated inflammatory and cytotoxic functions of circulating lymphocytes expressing CD94, a transmembrane glycoprotein expressed by natural killer family cells (22–24).

RESULTS

Peripheral NK cell frequency not modulated during Lyme disease. We wanted to determine if NK cells were differentially induced in dogs with asymptomatic (AS group) or symptomatic (SY group) Lyme disease. CD94 is a C-type lectin receptor specific for nonclassical major histocompatibility complex (MHC) class I molecules and preferentially expressed by NK cells, NKT cells, and related unconventional T cell subsets (25, 26). CD94 is expressed early in murine NK cell differentiation, and the anti-human CD94 clone HP-3D9 cross-reacts with canine CD94 (27–29). Graves et al. (29) found CD94 surface expression identified cells with an NK/NKT surface phenotype among canine peripheral blood mononuclear cells (PBMCs), and expansion of CD94+ cells by interleukin-2 (IL-2)/IL-15 resulted in a highly cytotoxic, electron-dense granular cell population (29). In our cohort, sorted CD94+ cells also displayed a granular phenotype by light microscopy (see Fig. S1 in the supplemental material). The majority of CD94+ cells were expressed on endemic control (EC group) lymphocytes (data not shown), and similar to humans and mice (22, 30), CD94 expression was observed at both intermediate and high levels on lymphocytes (Fig. S1C).

To determine if CD94+ cells were differentially regulated during Lyme disease, we used flow cytometry to analyze PBMCs from subsets of subjects within a cohort of B. burgdorferi-exposed dogs (see Table S1 in the supplemental material). Lymphocytes were subdivided into CD3-expressing or nonexpressing cells and then positively gated...
by CD94 surface expression (Fig. S1). Circulating CD3-CD94+ lymphocytes, thought to be predominately composed of NK cells, did not differ in frequency between endemic controls and B. burgdorferi-seropositive dogs (Fig. 1A and C). However, in B. burgdorferi-seropositive dogs, the CD3-CD94high subset was no longer dominant (Fig. 1B). This trend is also seen when comparing asymptomatic versus symptomatic Lyme disease. Endemic controls and doxycycline-treated symptomatic LD dogs had a higher ratio of CD3-CD94high cells over CD3-CD94dim cells (Fig. 1D).

Although peripheral NK cell frequency does not appear to be modulated during human LD, the phenotype of these cells differs among patients with antibiotic-responsive versus -refractory Lyme arthritis, suggesting NK cells play an active role in pathology (11). Granzyme B is a serine protease stored in intracellular granules by cytotoxic cells, including NK, NKT, and CD8+ T cells. Upon activation, granules are released onto target cells inducing cell death. NKp46, a conserved activating receptor expressed by mature NK cells (28, 31), clusters at the NK target cell immune synapse, contributing to cytotoxicity (32). Interestingly, the frequencies and geometric mean fluorescence intensities (gMFIs) of granzyme B and NKp46 were similar among CD3-CD94+ lymphocytes independent of B. burgdorferi serostatus (see Fig. S2A to D in the supplemental material).

CD94+ T lymphocytes expanded during asymptomatic Lyme disease. Contrary to the CD3-CD94+ population, the frequencies of CD3+CD94+ T cells were significantly increased among circulating lymphocytes in B. burgdorferi-seropositive dogs compared to seronegative dogs (Fig. 1E). These cells were predominantly CD94high (Fig. 1F to H). When seropositive dogs were divided into asymptomatic or symptomatic LD, the frequency of CD94+ T cells in the peripheral blood of asymptomatic dogs was double the frequency observed among endemic control lymphocytes (P = 0.0041) (Fig. 1G). This
subpopulation remained significantly increased in asymptomatic dogs over endemic controls when dogs were stratified by breed (data not shown).

Based on this, we expected circulating CD94+ T cells from asymptomatic dogs would have a more activated phenotype compared to those from symptomatic dogs. Intracellular granzyme B was found at similar levels in CD94+ T cells independent of *B. burgdorferi* serostatus (Fig. S2E and F). We expected more mature NKp46-expressing CD94+ T cells would be found in *B. burgdorferi*-seropositive compared to endemic controls. Interestingly, among the CD3+ CD94+ subset, there was a nonsignificant decline in the frequency of NKp46+ cells from both asymptomatic and symptomatic *B. burgdorferi*-seropositive dogs (Fig. S2G). When examining NKp46 geometric mean fluorescence intensity (gMFI), asymptomatic and symptomatic *B. burgdorferi*-seropositive dog CD94+ T cells had significantly less NKp46 surface expression compared to doxycycline-treated dogs (Fig. S2H).

**Comparison of CD94dim**- versus **CD94high**-expressing lymphocyte populations. CD94 is differentially expressed during NK cell maturation (22, 30). The CD94+ T cell population from dogs seropositive for *B. burgdorferi* was predominately CD94high (Fig. 1F). Most evidence suggests CD94high NK cells represent a more mature version of CD94dim NK cells (30). To analyze potential differences in these subsets during asymptomatic and symptomatic LD, we compared levels of granzyme B and NKp46 expression between the two CD94+ lymphocyte subpopulations (Fig. 2).

CD94high T cells had a higher proportion of granzyme B-positive cells (Fig. 2A). However, CD94dim lymphocytes had more intracellular granzyme B on a per cell basis than their CD94high counterparts (Fig. 2B). Among CD94+ T cells, NKp46 was found to be expressed significantly more often and at a higher level on CD94dim T cells than CD94high T cells in endemic control, asymptomatic, and symptomatic *B. burgdorferi*-seropositive dogs (Fig. 2C and D).

**CD94+ T cell subset has an NK-like CD8+ T cell phenotype.** Within CD3-expressing lymphocyte populations, CD94 has been shown to be expressed by NKT cells, some γδ T cells, and a subset of CD8+ T cells in both mouse and human systems. CD94+ T cells from endemic control dogs, as well as *B. burgdorferi*-seropositive dogs, were predominately CD8– CD4– (mean frequencies: EC, 88.2%; AS, 86.6%; SY, 75.2%) (Fig. 3A). CD8– CD4– double-negative cells made up roughly 10 to 20% of this population, depending on Lyme disease clinical state (mean frequencies: EC, 9.5%; AS, 7%; SY, 21.2%). CD4+ CD8– expressing cells were less than 5% of the CD94+ T cells (mean frequencies: EC, 0.6%; AS, 4.5%; SY, 0.9%).

To establish whether these CD94+ T cells had an antigen binding pattern consistent with iNKT cells, we combined PBMCs with human CD1d tetramers loaded with PBS-57 ligand, an analog of α-galactosylceramide, which binds type 1 CD1d-restricted NKT cells (see Fig. S3 in the supplemental material). CD94+ T cells at 4.7%, 8.1%, and 13.3% were labeled by CD1d/PBS-57 tetramers from endemic control dogs, asymptomatic *B. burgdorferi*-seropositive dogs, and symptomatic *B. burgdorferi*-seropositive dogs, respectively (Fig. 3A). This indicates type 1 CD1d-restricted NKT cells comprise a minor portion of CD94+ T cells in this cohort. The frequency of CD1d/PBS-57 binding cells was significantly higher (1.7-fold, *P* = 0.025) in asymptomatic *B. burgdorferi*-exposed dogs compared to endemic control dogs and highest among cells from symptomatic exposed dogs (2.8-fold, *P* = 0.007) (Fig. 3A). Expansion of type 1 CD1d-restricted NKT cells occurred in dogs in response to *B. burgdorferi* infection, similar to observations from experimental LD (8–11).

Based on these findings, the CD1d restriction of CD94-expressing T cells was of interest. We compared CD1d/PBS-57 binding among CD3+ CD94+ and CD3+ CD94+ lymphocytes from matched subjects (Fig. 3B). We found CD1d/PBS-57 binding cells among both CD94+ and CD94+ T cell subsets. However, the proportion among the CD94+ subset was significantly larger, at double the proportion among CD94+ T cells (8.4% and 3.7%, respectively; *P* < 0.0001). In agreement, sorted CD94+ T cells contained a significantly higher level of iTCRa mRNA than CD94+ T cells compared to CD3+ CD94+ lymphocytes from matched subjects (Fig. 3C) (33). Therefore, type 1 invariant NKT cells are enriched among, but not restricted to, CD94+ T cells.
The majority of CD94$^+$ T cells were CD8$^+$ (Fig. 3A). NK-like CD8$^+$ T cells (sometimes called innate memory CD8$^+$ T cells) are a subset of conventional CD8$^+$ T cells that arise during infection and tumor settings described in mice and humans (34, 35). These cells are most commonly defined as CD3$^+$ CD8$^+$ lymphocytes expressing killer immunoglobulin receptors (CD94/NKG2A, -C, -D, and -E), and distinct from type 1 NKTs by not being CD1d restricted and having a more diverse T cell receptor (TCR) repertoire (34, 36–38). These cells have functional features like those of NK cells, such as cytotoxic activity and rapid IFN-γ response to IL-12/IL-18 stimulation, but display an activated memory-like phenotype (34).

To explore whether the CD94$^+$ CD8$^+$ T cells in our study displayed an NK-like phenotype, we compared CD94$^+$ CD8$^+$ T cells to CD94$^+$ CD8$^+$ T cells in matched subjects (see Fig. S4 in the supplemental

**FIG 2** CD94$^{high}$ T cells have a greater population of granzyme B$^+$ cells and lower NKp46 in *Borrelia* seropositive animals. Shown is a comparison of marker expression between CD94$^{dim}$ (light gray) versus CD94$^{high}$ (dark gray)-expressing CD3$^+$ (left) or CD3$^-$ (right) lymphocytes. (A and C) Percentage of granzyme B$^+$ or NKp46$^+$ of the CD94$^{dim}$ or CD94$^{high}$ parent population. (B and D) Granzyme B or NKp46 gMFI of the CD94$^{dim}$ or CD94$^{high}$ lymphocyte population. EC, endemic control dogs; AS, asymptomatic dogs seropositive for *Borrelia* exposure; SY, symptomatic Lyme; Rx, post-doxycycline treatment. Replicate-matched two-way ANOVA with Sidak’s posttest. The mean and SD are shown (n = 5 to 20). *, P ≤ 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
The CD94$^+$ CD8$^+$ T cells had a significantly higher frequency of intracellular granzyme B cells compared to conventional CD8$^+$ T cells (65.8% versus 51.3%; $P = 0.0001$) (Fig. 3D). Similarly, CD94$^+$ CD8$^+$ T cells had a significantly higher frequency of NKp46$^+$ cells compared to conventional CD8$^+$ T cells (15.3% versus 4.9%; $P = 0.0017$) (Fig. 3E). NKp46 expression was inducible on CD94$^+$ CD8$^+$ T cells, where the portion of NKp46$^+$ cells increased significantly upon exposure to B. burgdorferi spirochetes (medium versus B. burgdorferi, $P = 0.017$), and recombinant canine IL-12 (rIL-12) stimulation also trended to induce NKp46 expression (medium versus rIL-12, $P = 0.057$) (Fig. 3F). In contrast, CD94$^-$ CD8$^+$ T cells from matched subjects did not upregulate surface NKp46 in response to either stimulus. Therefore, CD94$^+$ CD8$^+$ T cells respond to environmental stimuli by increasing expression of an NK cell cytotoxicity receptor and display an NK-like phenotype.

**IL-21 is increased in asymptomatic, Borrelia-seropositive dog serum.** IL-21 is a pleotropic cytokine that augments NK and T cell type 1 polarization and promotes NKT cell expansion (39–41). We found significantly elevated serum IL-21 in asymptomatic seropositive dogs over control dogs (Fig. 4A). However, serum IL-21 was not statistically increased in dogs with symptomatic LD. As IL-21 can work with IL-23 to regulate type 17 immune responses, we also measured serum IL-23 levels in dogs at different stages of LD (42). However, IL-23 was not significantly modulated in either seropositive group compared to endemic controls (Fig. 4B).

**B. burgdorferi induces a higher proportion of IFN-$\gamma$-producing CD94$^+$ lymphocytes from symptomatic Lyme disease.** B. burgdorferi infection elicits a mixed adaptive immune in humans, including IFN-$\gamma$, a highly inflammatory cytokine (19, 20, 43). NK, NKT, and CD8$^+$ T cells are strong producers of IFN-$\gamma$. After incubating PBMCs for 24 h with B. burgdorferi spirochetes, we measured CD3$^-$ CD94$^+$ and CD3$^+$ CD94$^+$ lymphocyte intracellular IFN-$\gamma$ by flow cytometry (Fig. 5).
Compared to no stimulation, *B. burgdorferi* induced a significant proportion of CD94+ T cells to become IFN-γ+, with moderate induction within asymptomatic dog PBMCs (1.8-fold over EC, *P* = 0.289) and a significantly greater induction in symptomatic LD PBMCs (2.9-fold over EC, *P* = 0.0037) (Fig. 5B). CD3+ CD94+ lymphocytes also became IFN-γ+ after *B. burgdorferi* stimulation, independent of *B. burgdorferi* serostatus (Fig. 5C). Spirochete-stimulated PBMCs from symptomatic dogs resulted in a 2.7-fold-higher induction of IFN-γ+ CD3− CD94+ lymphocytes over similarly stimulated endemic control PBMCs (SY versus EC, *P* < 0.0001) or asymptomatic dog PBMCs (SY versus AS, *P* = 0.0003), while spirochete-stimulated asymptomatic dog PBMCs resulted...
FIG 6 Decreased cytotoxic activity by symptomatic *Borrelia*-seropositive dog PBMCs against allogeneic target cells. (A) Ratio of released calcein signal in 100:1 E:T-cocultured wells to the signal in 12.5:1 E:T-cocultured wells. One-way ANOVA with Dunnett’s posttest. The mean and SD are shown (n = 5 to 10). (B) Correlation coefficient and (C) P value of Spearman or Pearson test between released calcein signal ratio from panel A and the indicated parameter among CD3+CD94+ or CD3+CD94+ lymphocytes from EC, AS, or SY dogs. Boldface values indicate P ≤ 0.05.

...in a nonsignificant induction of IFN-γ*−* CD3− CD94+ lymphocyte cells over control PBMCs (AS versus EC, P = 0.815). This enhanced induction of IFN-γ-secreting cells might indicate CD94+ cells in symptomatic dogs require a lower threshold of activation.

We evaluated the correlation between surface CD94 expression and intracellular IFN-γ on cells exposed to spirochetes. There was a significant positive correlation between CD94 gMFI and IFN-γ gMFI on CD94+ T cells (Fig. 5D). However, for CD3− CD94+ lymphocytes, CD94 gMFI did not correlate with intracellular IFN-γ (Fig. 5E). NK T cells have been described to recognize *B. burgdorferi* antigens directly or to be indirectly activated by myeloid cell-derived cytokines within the PBMC fraction to induce IFN-γ production.

**Peripheral lymphocytes during symptomatic Lyme disease have decreased cytotoxic activity.** After observing enhanced IFN-γ induction by CD94+ cells from symptomatic LD dogs, we were interested in evaluating whether other critical NK-like functions, particularly cytotoxicity, were enhanced during clinical disease. iNKT cells from mice infected with *B. burgdorferi* were able to directly kill extracellular spirochetes in a granzyme-dependent manner (6). In addition, *B. burgdorferi* is capable of infecting multiple cell types, including synovial cells, endothelial cells, fibroblasts, neurons, and glial cells (44–48). Finally, granzyme A/B-deficient mice have significantly higher joint bacteria burdens, implicating cytotoxic cells contribute to bacterial control (6).

Thus, we used the allogeneic canine MDCK cell line as target cells in an *ex vivo* cytotoxicity assay (Fig. 6). In this assay, target cells released calcein when killed by effector cells. PBMCs from both *B. burgdorferi*-seronegative and -seropositive dogs elicited target cell calcein release. We observed a significantly decreased calcein release in symptomatic *B. burgdorferi*-exposed dog PBMCs compared to endemic control PBMCs (Fig. 6A). Asymptomatic *B. burgdorferi*-exposed dog PBMCs triggered intermediate calcein release that was not statistically different from that of endemic controls. Thus, contrary to IFN-γ production, peripheral lymphocytes from dogs with symptomatic LD had defective cytotoxicity.

We wanted to know which CD94+ cell parameters correlated with target cell calcein release (killing). Endemic control PBMCs had the greatest capacity to elicit calcein release. A higher frequency of CD94+ T cells expressing NKp46 significantly correlated with calcein release in endemic control cells, reinforcing this receptor’s role in cytotoxicity (r = 0.7, P = 0.04). We found no significant association between CD3− CD94+ cell marker expression and released calcein from killed target cells (Fig. 6B and C).

**B. burgdorferi and Anaplasma coinfection.** Complicating LD, *Ixodes* ticks transmit additional medically important pathogens, including *Anaplasma phagocytophilum*, the causative agent of anaplasmosis. Anaplasmosis elicits immunosuppression with leukopenia, leaving subjects susceptible to secondary opportunistic infections (49). A substantial proportion of *B. burgdorferi*-seropositive dogs in our cohort were coexposed to *Anaplasma* (see Table S2 in the supplemental material). We hypothesized *Anaplasma* coexposure would exacerbate LD severity. We evaluated clinical outcome and NKT cell responses in dogs coexposed to *B. burgdorferi* and *Anaplasma* spp.

Upon physical exam, dogs coexposed to both *B. burgdorferi* and *Anaplasma* were no more likely to be LD symptomatic than dogs exposed to only *B. burgdorferi* (P = 0.606,
Fisher’s exact test) (Table S2). Dogs coexposed to B. burgdorferi and Anaplasma did not have increased circulating CD3\(^+\)CD94\(^+\) or CD3\(^-\)CD94\(^+\) lymphocyte frequencies compared to those observed in dogs exposed to B. burgdorferi alone (Fig. 7A and B).

A high proportion of both CD3\(^-\) and CD3\(^+\)CD94\(^+\) lymphocytes from dogs with symptomatic LD produced IFN-\(\gamma\) in response to B. burgdorferi. Neither of these cell subsets from Anaplasma-coexposed dogs had enhanced IFN-\(\gamma\) production compared to B. burgdorferi singly exposed dogs (Fig. 7C and D). The proportion of IFN-\(\gamma\) CD3\(^-\)CD94\(^+\) lymphocytes induced by spirochete stimulation was significantly lower in coexposed dogs compared to that in B. burgdorferi singly exposed dogs (\(P = 0.011\)) (Fig. 7D). Anaplasma coexposure does not exacerbate CD94\(^+\) lymphocyte responses during canine LD and may dampen proinflammatory responses to B. burgdorferi.

**DISCUSSION**

INKT cells have previously been shown to be protective against experimental B. burgdorferi infection and disease severity, but roles for this cell subset in asymptomatic, controlled, natural infection were not evaluated. Within a cohort of naturally infected hunting dogs, asymptomatic B. burgdorferi exposure was associated with increased frequency of CD94\(^+\) T cells compared to seronegative endemic controls (Fig. 1G). We used the human CD1d tetramer and iTCR\(_\alpha\) gene expression to determine if CD94\(^+\) T cells found in dogs corresponded to iNKT cells.

We demonstrate for the first time that there was significantly increased binding of the PBS-S7 ligand-loaded CD1d tetramer in both B. burgdorferi-exposed groups, indicating NKT-like cells are present in this population and importantly increased during clinical Lyme disease (Fig. S3). Previously, Yasuda et al. (33) used a murine \(\alpha\)-GalCer/CD1d dimer and found a present, but minimal, binding population among canine

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**FIG 7** B. burgdorferi and Anaplasma coexposure does not exacerbate CD94\(^+\) lymphocyte frequency. (A and B) Frequency of CD3\(^+\)CD94\(^+\) (A) or CD3\(^-\)CD94\(^+\) (B) lymphocytes among PBMCs. Kruskal-Wallis test with Dunn’s posttest (n = 8 to 24). (C and D) PBMCs from seronegative (n = 10) or seropositive dogs (n = 10 to 20) cultured for 24 h with medium or 1:10 B. burgdorferi spirochetes were stained for intracellular IFN-\(\gamma\) and analyzed by flow cytometry. The frequency of IFN-\(\gamma\)-producing CD3\(^+\)CD94\(^+\) (C) or CD3\(^-\)CD94\(^+\) (D) lymphocytes is shown. Two-way ANOVA (n = 9 to 20). Comparisons within groups by Sidak’s post hoc test: *, \(P \leq 0.05\); **, \(P < 0.01\); and ****, \(P < 0.0001\). Comparisons between groups by Tukey’s post hoc test: #, \(P \leq 0.05\); and ##, \(P < 0.01\). The mean and SD are shown. EC, dogs seronegative by SNAP 4Dx test; Ly, dogs seropositive for B. burgdorferi only; A, dogs seropositive for Anaplasma only; Ly/A, dogs seropositive for B. burgdorferi and Anaplasma.
PBMCs from one healthy lab-bred dog (33). The percentage of CD1d tetramer binding cells among CD3$^+$ cells was higher in our study than that found by Yasuda et al. (33). This could be attributed to our use of the human CD1d tetramers, which have higher homology compared to the murine tetramers. The canine cohort used here is also more antigen experienced, which may increase the circulating iNKT cell compartment, as Yasuda et al. (33) only measured CD1d dimer binding in a dog raised under specific-pathogen-free conditions.

We observed a similar pattern when amplifying iTCR$\alpha$ mRNA corresponding to a putative canine invariant T cell receptor gene. iTCR$\alpha$ mRNA was enriched among CD94$^+$ T cells (Fig. 3C). The canine iVa-iJa gene region used to design iTCR$\alpha$ primers has 72% amino acid homology with the human V$\alpha$24-J$\alpha$Q region (33). The iTCR$\alpha$ mRNA fold change observed in CD94$^+$ T cells in our study (−117-fold) was lower than that reported by Yasuda et al. (33) (−271-fold), which we attribute to their amplifying from a purer, flow-sorted $\alpha$-GalCer/CD1d-binding PBMC population.

Based on our phenotyping, CD1d tetramer binding, and iTCR$\alpha$ gene expression results, it is likely that the CD94$^+$ T cell population described in this work contains a mixture of NK-like CD8$^+$ T cells and a small portion of CD1d tetramer binding iNKT cells. This tetramer-ligand combination does not identify group 2 type 2 CD1d-restricted NKT cells or group 1 NKT-like cells, which may also be present within this population (Fig. 8). As observed in experimental and human Lyme disease, we were very interested to see significantly more CD1d/PBS-57 binding cells among PBMCs from dogs with clinical Lyme disease (Fig. 3A). These findings demonstrate the human CD1d/PBS-57 tetramer has utility for further studies of NKT cell responses in dogs during Lyme disease and other relevant pathological settings.

The majority of circulating canine CD94$^+$ T cells were CD8$^+$ (Fig. 3A). In addition to CD94, these CD8$^+$ T cells expressed granzyme B and Nkp46 more often than CD94$^+$ CD8$^+$ T cells. This phenotype is consistent with NK-like CD8$^+$ T cells that have been recently described (35, 37). NK-like CD8$^+$ T cells are described to possess an effector-memory-reexpressing CD45RA (TEMRA) phenotype (37). Similar to conventional NKT cells, NK-like CD8$^+$ T cells are thought to bridge innate immunity and adaptive immunity, allowing them to respond quickly to both IL-12/IL-18 stimulation and TCR-mediated signals with IFN-\(\gamma\) production and cytotoxic activity (34). Due to the high degree of functional overlap between these cell types, we hypothesize NK-like CD8$^+$ T cells

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**FIG 8** Overview of CD94-expressing lymphocytes during canine Lyme disease. The schematic shows the major CD94-expressing lymphocyte subsets and phenotyping markers found on each subset discussed in this article. Tet$^+$/− refers to PBS-57-loaded CD1d tetramer binding. The percentages at the bottom depict the range found of each subset from PBMCs stimulated with *B. burgdorferi* from seropositive dogs with symptoms of Lyme disease as a percentage of the lymphocyte gate (n = 4).

<table>
<thead>
<tr>
<th>Antigen Presenting Cell</th>
<th>CD1d + Bb Glycolipid Ag</th>
<th>CD1(a/b/c) + Bb Glycolipid Ag</th>
<th>CD1d + Bb Peptide Ag?</th>
<th>CD8</th>
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<td>Nkp46</td>
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<td>Nkp46</td>
</tr>
<tr>
<td>NK cells (CD3-CD94+)</td>
<td>Group 2, Type I NKT cells (CD3+CD94+Tet+)</td>
<td>Group 1, NKT-like cells (CD3+CD94+Tet-)</td>
<td>NK-like CD8$^+$ T cells (CD3+CD94+Tet-CD8$^+$)</td>
<td>Conventional CD8$^+$ T cells (CD3+CD94-Tet-CD8$^+$)</td>
</tr>
<tr>
<td>4.0-5.5%</td>
<td>0.2-0.4%</td>
<td>7.4-16.4%</td>
<td>3.4-11.2%</td>
<td>13.9-17.3%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenotyping Markers</th>
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<tbody>
<tr>
<td>CD8</td>
<td></td>
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<tr>
<td>CD4+</td>
<td></td>
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<tr>
<td>CD4+ or DN</td>
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<td>Nkp46</td>
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<tr>
<td>NKp46</td>
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<tr>
<td>Granzyme B</td>
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<td>TCR</td>
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<td>Diverse TCR</td>
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<td>Conventional TCR</td>
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are likely to also be protective during Lyme disease. This would be consistent with their expansion only in asymptomatic *B. burgdorferi*-exposed dogs.

A caveat of using a naturally infected cohort is that the exact time point postexposure of subjects is not known. This could increase the variability among cellular response observations within each clinical group, if sampling occurred at different phases of the immune response. Further, categorization of dogs as asymptomatic in this study was based on serology and lack of clinical signs. However, antibodies against *B. burgdorferi* can persist for many months postexposure, unless antibiotic treatment occurs (50–52). The medical histories of the dogs indicated dogs categorized as asymptomatic had not received treatment for Lyme disease. Since *B. burgdorferi* PCR is unreliable in peripheral blood, we cannot differentiate asymptomatic dogs harboring active spirochetes from those that have cleared a recent infection (53–55).

In mice, IFN-γ produced during *B. burgdorferi* infection does not contribute to disease resistance (56). In human Lyme disease, the serum IFN-γ concentration is significantly elevated in active erythema migrans patients, correlates with an increased number of symptoms, and may promote chronic inflammation (19, 56–58). Although neither circulating CD3−CD94+ nor CD3+CD94+ lymphocyte frequencies were modulated among symptomatic dog PBMCs in this report, a significantly augmented fraction of these cells produced IFN-γ when exposed to *B. burgdorferi*, indicating differential activation of these cells (Fig. 5B and C). NK cell priming by various cytokines, including IFN-α/β, IL-2, IL-12, and IL-18, has been described, where NK cells cultured with these cytokines secreted significantly increased inflammatory cytokines, including IFN-γ (59, 60). IL-2 and IL-18 have been shown to be elevated in acute Lyme disease patient sera: thus, increased sensitivity of canine CD94+ lymphocytes to *B. burgdorferi* during symptomatic Lyme disease may be due to cytokine priming (61).

Interestingly, we found IL-21 was significantly increased in asymptomatic, but not symptomatic, *B. burgdorferi*-seropositive dog serum. Activated CD4+ T cells, T follicular helper cells, and NKT cells are most commonly observed sources of IL-21 (62, 63). IL-21 can synergize with IL-2, IL-7, and IL-15 to decrease apoptosis and enhance proliferation of NKT cells and CD8+ T cells (39–41, 62, 64–66). Increased serum IL-21 may contribute to the expanded CD94+ T cell population in dogs with asymptomatic Lyme disease; however, this association needs to be strengthened with correlation analyses between serum IL-21 concentration and CD94+ cell frequencies among a larger sample of subjects.

How IL-21 exerts regulation is complex, often dependent on dose, differentiation state of targets, and cytokine milieu (64, 65). In peripheral human NK cells and CD8+ T cells, IL-21 reduced expression and activity of the activating receptor NKG2D, while upregulating other activating receptors, such as 2B4 and NKp30 on NK cells and CD28 on CD8+ T cells (67). Thus, IL-21 may alter the activation profile of NK-like CD8+ T cells in *Borrelia*-exposed dogs to tune their response.

Despite exacerbated IFN-γ induction by symptomatic dog CD94+ lymphocytes in response to *B. burgdorferi*, we observed decreased cytotoxicity by PBMCs from symptomatic dogs compared to seronegative dog PBMCs. One previous study found PBMCs from active Lyme disease patients also showed a defect in cytotoxicity (68). This study found that addition of *B. burgdorferi* spirochetes, or *B. burgdorferi* culture supernatant, within cytotoxicity assay cultures significantly reduced the ability of healthy control PBMCs to exert cytotoxic activity. Thus, the potential presence of *B. burgdorferi* spirochetes or secreted factors in actively infected dogs may affect their cytotoxic ability.

NK cell exhaustion has also been described in settings of chronic antigen exposure and inflammation, with both decreased NKp46 expression and impairment of NK cell cytolytic function characteristic of exhausted cells (69). In our assay, there was a significant positive association between calcine release and NKp46 expression on endemic control dog CD94+ T cells (70–72). However, this correlation was lost among *B. burgdorferi*-seropositive dog cells. In Fig. 1D, CD94+ T cells from both asymptomatic and symptomatic dogs expressed less NKp46 compared to posttreatment. However,
cytotoxicity was only significantly reduced in the symptomatic group. IL-21 can augment IFN-γ production in some settings and is a potent enhancer of cytotoxic activity (40, 41, 66, 73). IL-21-expanded murine NKTs display increased intracellular granzyme B, cytotoxicity in vitro, and tumor control in vivo (39, 41, 66). As IL-21 is known to enhance cytotoxicity, we hypothesized increased serum IL-21 in asymptomatic dogs may buffer the cytotoxic capability of NK, NKT, and NK-like CD8+ T cells in these dogs. Further experiments using recombinant IL-21 during in vitro cytotoxicity assays would be needed to confirm this interaction.

In regions where B. burgdorferi is endemic, Ixodes scapularis also transmits Anaplasma phagocytophilum and coexposure is common (74–76). Although a subset of dogs was seropositive to both pathogens, our analyses did not reveal a negative synergistic effect of coexposure on CD94+ lymphocyte subsets (Fig. 7). This agrees with physical exam findings; dogs coexposed to B. burgdorferi and Anaplasma were no more likely to be symptomatic than dogs exposed to only B. burgdorferi (Table S2). Although the IDEXX 4Dx SNAP Plus test detects both A. phagocytophilum and Anaplasma platys, based on geography and B. burgdorferi coexposure, A. phagocytophilum is the most likely coinfecting species. Therefore, our data do not support an exacerbatory effect of A. phagocytophilum exposure on Lyme disease in this hunting dog cohort.

Together, we found expansion of an NK-like CD8+ T subset with reduced propensity to express IFN-γ and elevated serum IL-21 characterized dogs with asymptomatic Lyme disease. These cells were cytotoxic without excessive inflammation, leading to maintenance of a subclinical infection. CD94+ lymphocytes prone toward IFN-γ production with reduced cytotoxic activity characterized dogs with symptomatic disease. These biomarkers may aid diagnosis of Lyme disease in environments with transmission of multiple tick-borne pathogens. Regulatory therapies directed at limiting NK and NKT-like cell IFN-γ expression may improve the clinical course.

MATERIALS AND METHODS

Lyme disease cohort. Naturally B. burgdorferi-exposed, owned hunting dogs were enrolled after caretakers provided informed consent (76). Subjects included foxhound and English springer spaniel breeds at high risk of exposure due to their hunting activities. All animal work was reviewed and approved by the University of Iowa Institutional Animal Care and Use Committee. A total of 717 dogs from Midwestern and Eastern United States were screened for seropositivity to Borrelia burgdorferi, Anaplasma phagocytophilum, Anaplasma platys, Ehrlichia ewingii, and Ehrlichia canis (SNAP 4Dx Plus test; IDEXX Laboratories, Inc.) (Table S1). All B. burgdorferi-seropositive samples were also tested via C6 quanti- tative enzyme-linked immunosorbent assay (ELISA), and those with a C6 value of >10 U/mL were enrolled (IDEXX Reference Laboratories). Caretakers provided relevant medical history, and veterinarians performed a physical exam for signs of tick-borne illness, including lymphadenopathy, rash or petechiation, erythema, scleral injection, injected or pale mucous membranes, loss of body condition, and more specific signs of Lyme disease, including shifting lameness or other arthropathic without history of musculoskeletal injury or neurological signs. Doxycycline was administered to symptomatic dogs, and a subset of these cases were redrawn approximately 4 weeks posttreatment. Dogs were allocated into groups: endemic control (EC) (negative SNAP 4Dx Plus test), asymptomatic (AS) (B. burgdorferi seropositive with no clinical signs [33.5% of dogs B. burgdorferi seropositive]), symptomatic (SY) (B. burgdorferi seropositive and 2+ clinical signs [18.7% of dogs B. burgdorferi seropositive]), and treated (doxycycline-treated symptomatic dogs) (Table S1). There was no significant association between age or sex and Lyme disease clinical outcome in B. burgdorferi-seropositive dogs. Additionally we included evaluation of responses in Anaplasma coexposed (B. burgdorferi and Anaplasma seropositive [47.8% of dogs Borrelia seropositive]) (Table S2).

Serum cytokine bioplex. IL-21 and IL-23 serum protein concentrations were measured using a custom Milliplex MAP magnetic bead panel assay according to the manufacturer’s instructions (Millipore Sigma). Detection was performed on a Luminex 200 (Bio-Rad) instrument in the University of Iowa Flow Cytometry Core.

Peripheral blood mononuclear cell isolation. Diluted blood was underlaid with Ficoll-Paque PLUS (GE Healthcare) and counted as previously described (77). Peripheral blood mononuclear cells (PBMCs) were resuspended at 5 × 10^6 cells/mL for further analyses in complete tissue culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum [FBS], 2 mM L-glutamine, 100 U/mL penicillin-streptomycin, and 1 × nonessential amino acids). In some experiments, recombinant canine IL-12 (rcl-12) (B&D System) was used to stimulate PBMC cultures at 10 ng/mL.

Immunoblotting for flow cytometry and cell sorting. PBMCs were blocked with 2 mg/mL cells dog gamma globulin (Jackson ImmunoResearch) in 50% FBS/PBS. Mouse anti-canine NKp46 clone 48A generated by J. A. Foltz and D. Lee from Nationwide Children’s Hospital (now commercially available as MABF2109 from Millipore Sigma) was applied at 4 μg/mL, followed by 1:50 anti-mouse IgG conjugated...
with allophycocyanin (APC) (Jackson ImmunoResearch) (31). A 1:25 dilution of anti-canine CD3 conjugated with fluorescein isothiocyanate (FITC) (clone CA17.2A12), 1:100 anti-canine CD8 conjugated with A700 (clone YCATESS.9; Bio-Rad Antibodies), and 1:25 anti-human CD94 conjugated with BV421 (clone HP-3D9; BD Biosciences) were used (29). Intracellular staining used 1:50 anti-human granzyme B conjugated with phycoerythrin (PE) and Texas Red clone GB11 (78-80) or 5 μg/mL anti-canine IFN-γ (MAB781; R&D) conjugated with Zenon R-PE (Thermo Fisher).

Unloaded or PBS-57-loaded human CD1d tetramers fluorescently tagged with APC were obtained from the NIH Tetramer Core Facility and stored at 4°C. For labeling of PBMCs, tetramers were included with other surface staining antibodies at 1:50 dilution for 60 min on ice, protected from light.

“Fluorescence minus one” controls were performed. Cell events were acquired at University of Iowa Flow Cytometry Core on an LSRII flow cytometer (BD Biosciences) and analyzed via Flowjo v10 software.

For flow-assisted cell sorting, PBMCs were stimulated with 10 ng/mL recombinant canine IL-2 (R&D Systems). Anti-human CD94-BV421 alone or in combination with anti-canine CD3-FITC was applied as described above. Cells were sorted using the FACS Aria II or FACS Aria Fusion fluorescence-activated cell sorter (Becton Dickinson) at the University of Iowa Flow Cytometry Core. Sorted PBMCs were cytospun onto SuperFrost slides, fixed briefly in methanol, and stained with Hema-3 solutions I and II. Coverslips were mounted with Permount, and bright-field images were obtained using cellSams Standard software on an Olympus BX51 microscope with a 100× objective and 1.30 aperture under oil immersion.

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REFERENCES


We declare no conflict of interest.


