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Programmed Death 1–Mediated T Cell Exhaustion during Visceral Leishmaniasis Impairs Phagocyte Function

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Control of *Leishmania infantum* infection is dependent upon Th1 CD4+ T cells to promote macrophage intracellular clearance of parasites. Deficient CD4+ T cell effector responses during clinical visceral leishmaniasis (VL) are associated with elevated production of IL-10. In the primary domestic reservoir of VL, dogs, we define occurrence of both CD4+ and CD8+ T cell exhaustion as a significant stepwise loss of Ag-specific proliferation and IFN-γ production, corresponding to increasing VL symptoms. Exhaustion was associated with a 4-fold increase in the population of T cells with surface expression of programmed death 1 (PD-1) between control and symptomatic populations. Importantly, exhausted populations of CD8+ T cells and to a lesser extent CD4+ T cells were present prior to onset of clinical VL. VL-exhausted T cells did not undergo significant apoptosis ex vivo after Ag stimulation. Ab block of PD-1 ligand, B7.H1, promoted return of CD4+ and CD8+ T cell function and dramatically increased reactive oxygen species production in cocultured monocyte-derived phagocytes. As a result, these phagocytes had decreased parasite load. To our knowledge, we demonstrate for the first time that pan-T cell, PD-1–mediated, exhaustion during VL influenced macrophage-reactive oxygen intermediate production. Blockade of the PD-1 pathway improved the ability of phagocytes isolated from dogs presenting with clinical VL to clear intracellular parasites. T cell exhaustion during symptomatic canine leishmaniasis has implications for the response to vaccination and therapeutic strategies for control of *Leishmania infantum* in this important reservoir species. *The Journal of Immunology*, 2013, 191: 000–000.
H1 resulted in recovery of Th1-effector function, recovery of phagocyte superoxide production, and decreased parasite load in peripheral blood–derived monocytes from naturally infected dogs. The research within this study is a novel characterization of pan-T cell exhaustion in a major domestic reservoir for visceral leishmaniasis. T cell exhaustion during asymptomatic and symptomatic canine leishmaniasis will impact the efficacy of vaccination and therapeutic strategies to reduce the incidence of leishmaniasis in this important reservoir species.

Materials and Methods

Sample population and study enrollment

Dogs were enrolled by serology, clinical signs, and quantitative RT-PCR (qRT-PCR) positivity, as previously described (5). Dogs were 2–7 y old and had complete veterinary standard of care. Twenty milliliters of blood was collected from each dog at intervals >2 wk. Procedures were performed with approval from Iowa State University Institutional Animal Care and Use Committee.

Clinical staging of study animals

Study animals were assessed via physical and laboratory examination and L. infantum diagnostics (L. infantum kinetoplast DNA-specific quantitative PCR [qPCR]), immunofluorescent Ab test (IFAT), and K39/22 [DDP; Chembio, Medford, NY] serologic analysis. Infected animals were classified as follows: 1) asymptomatic, no clinical signs, qPCR positive, and IFAT <1:256; 2) symptomatic, two or more signs of leishmaniasis (lymphadenomegaly, palpable liver or spleen, alterations in hepatic or renal enzymes), IFAT ≥1:256, and qPCR positive. All symptomatic animals had blood harvested for a complete blood count and serum chemistry performed by the Clinical Pathologists at Iowa State University. A single asymptomatic animal and no asymptomatic animals evaluated had a leukopenia. This animal had a WBC count slightly below the low normal range (5.77 × 103/ml, normal range = 6–17 × 103/ml). Other clinical pathologic findings such as anemia, azotemia, hypocalcemia, hypoproteinemia, proteinuria, and elevated creatinine were common in the symptomatic group.

Sample handling and PBMC isolation

Whole blood samples were separated into PBMC, as previously described (5). Whole blood samples collected in heparin-containing tubes were diluted 1:1 with 1× HBSS (Cellgro, Manassas, VA) and 25 mM HEPES. Diluted whole blood was centrifuged at 800 relative centrifugal force (Eppendorf, Hauppauge, NY) for 30 min at room temperature through Ficol-Histopaque 1077 (Sigma-Aldrich, St. Louis, MO), as previously described (5). PBMC were counted with an automated cell counter (Beckman Coulter, Brea, CA). PBMC were washed twice in PBS and suspended in complete tissue culture medium (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 25 mM HEPES buffer). PMBC were counted and adjusted to 4 × 106/ml for further analysis. Cells were then split for evaluation of proliferation and intracellular staining for flow cytometry, or for qRT-PCR, NBT (Sigma-Aldrich), or ELISPOT assays.

Preparation of parasites

L. infantum (LIVT-2) was grown for use as positive control for kinetoplast qRT-PCR or freeze-thaw Ag (f-t.Li.), as previously described (5). L. infantum (LIVT-2) was grown in complete Grace’s medium (incomplete Grace’s supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine). Parasites were harvested, pelleted at 2500 × g for 15 min at 4°C, washed twice with PBS, and suspended in PBS to a concentration of 106 parasites/ml. Ten 1:5 serial dilutions were made, and 50 μL of each dilution was spiked into 150 μL negative canine blood.

Ag stimulation

Cells were stimulated with Con A (5 μg/ml) for 4 d and f-t.Li. (10 μg/ml) for 7 d, as previously described (5). Asymptomatic dog PBMC were stimulated with diestermer virus vaccine (Pfizer, Kalamazoo, MI), a non-Leishmania, specific Ag, positive control (NSC) for 10 d. Twenty-four hours prior to cell harvest, 5-ethyl-5′-deoxyuridine (EdU; Invitrogen, Grand Island, NY) was added at 10 μM, and 10 μg/ml brefeldin A (Sigma-Aldrich) was added 6 h before harvest. Cells were harvested and washed prior to surface and intracellular labeling.

IL-10 and B7.H1 block

Nonadherent PBMC were removed and saved in separate culture. Adherent cell populations were CD3+ CD11b+ by flow cytometry (data not shown). Adherent cells were treated with 10 μg/ml anti-human B7.H1 Ab (clone MH-1; eBioscience, San Diego, CA) or isotype for 4 h and washed, and nonadherent cells were returned to culture for Ag stimulation. This blocked adherent cell B7.H1 specifically. IL-10 block was performed via 10 μg/ml anti-canine IL-10 Ab (R&D Systems, Minneapolis, MN).

PBMC immunolabeling

PBMC were labeled similar to (5), with the following PBMC-labeling panel: canine PBMC labeling panel: EdU-Click-It (Invitrogen), anti-canine CD4/Alexafluor 647 (AbD Serotec, Raleigh, NC), anti-canine CD8/Alexafluor 700 (AbD Serotec, Raleigh, NC), anti-canine IFN-γ/Zenon R-PE (R&D Systems, Minneapolis, MN; Invitrogen, Grand Island, NY), anti-canine IL-10/Zenon allophycocyanin/Alexafluor 750 (R&D Systems; Invitrogen), and anti-human biotinylated PD-1/PE-Cy7 streptavidin (R&D Systems; eBioscience). Cells were blocked with 25 μL canine serum, with mouse and rat nonspecific polyclonal IgG at 20 μg/105 PBMC for 20 min at 4°C. Cells were blocked for 20 min, 4°C, permeabilized with saponin reagent (Invitrogen), and preserved in BD stabilizing fixative (BD Biosciences, San Jose, CA). Samples were analyzed within 48 h. Data acquired via FACScanto (BD Biosciences) with FlowJo analysis (Tree Star, Ashland, OR). Cells were progressively sorted by a live lymphocyte gate, then by CD4 or CD8 positivity for subsequent analysis (Supplemental Fig. I). All gating was based on pooled fluorescence minus one control samples and applied identically across all samples.

ELISPOT

PBMC were stimulated as previous and incubated on anti-canine IFN-γ or IL-10 capture Ab-coated plates (R&D Systems). A total of 100 μL 0.5 μg/ml biotinylated detection Ab (R&D Systems) was added. Spots were counted as average of duplicate wells over four dilutions, standardized to spots per 106 PBMC.

IL-10 ELISA

 Supernatants from unlabeled PMBC (2 × 105) were collected at indicated time points and stored at −20°C until analysis. IL-10 production was measured via ELISA (R&D Systems) per manufacturer’s recommendations.

NO production

Concentration of nitrite production was assessed via Griess reaction. A total of 50 μL cell culture supernant and Griess reagent (LabChem, Pittsburgh, PA) was mixed and incubated at room temperature, and absorbance was measured at 550 nm via microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentration was determined via sodium nitrite standard curve.

Superoxide production

Production of superoxide was assessed using NBT (Sigma-Aldrich) tablets. NBT tablets were dissolved in 1 mL water, 30 μL added to cells, and incubated for 60–90 min. Coverslips were harvested, fixed, and stained with eosin. Cells were counted as percentage of cells containing formazan precipitate. All evaluations were based on average of 600 cells per experiment, with blinding.

PCR assay

DNA was isolated and RT-PCR was performed, as previously described (12). DNA was isolated using the Qiagen blood DNA isolation kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. DNA quality and quantity were measured using a NanoDrop ND1000 spectrophotometer (Nanodrop, Wilmington, DE). Leishmania small-subunit rRNA-specific fluorescent probe (5′-FAM-[6-FAM]-CCGTTCTGGTGGTGTGCGCGC-3′) and its flankong primers (forward, 5′-AAGCTCTTCTACCCCGACACT-3′; reverse, 5′-CGGACTAAAACCCCTTCCA-3′) (Applied Biosystems, Foster City, CA) previously designed (19) were used. Whole blood DNA samples were assayed via qPCR in duplicates of two dilutions (straight, 1:10) using a Stratagene Mx3005P qPCR system (Agilent, Santa Clara, CA) via a 96-well format and perfeCta qPCR SuperMix, Low ROX master mix (Quanta Biosciences, Gaithersburg, MD). Primers were used at 775 nM and probe at 150 nM, with thinking primers at 95°C for 3 min and 50 cycles of 95°C for 15 s, 60°C for 1 min. Results were analyzed via MxPro QPCR software version 4.01, Microsoft Excel, and GraphPad Prism version 5.04 (GraphPad Software, La Jolla, CA).

Statistical analysis

Statistical analysis was conducted with pairwise Student’s t tests or one-way ANOVA with a Tukey’s posttest via GraphPad Prism version 5.04.
Results

CD4+ T cells from animals with visceral leishmaniasis were functionally exhausted

Alteration in CD4+ effector function during VL is well established, with demonstrated involvement of IL-10, IL-4, TGF-β, and cells able to produce both IL-10 and IFN-γ (2, 3). We previously demonstrated these alterations in L. infantum–infected dogs as they progress to symptomatic VL (5). These findings were suggestive of an exhausted phenotype. Because Leishmania protein GP63 was shown to suppress T cell responses via activation of SHP-1 phosphatase (4), and the exhaustion-associated inhibitory receptor PD-1 functions through increased tyrosine phosphatase activity (7), we hypothesized that T cell exhaustion occurred in naturally occurring VL due to overexpression of PD-1. We obtained PBMC from animals with natural, clinical VL to evaluate whether their CD4+ T cells were functionally exhausted.

Population surface expression of PD-1 on CD4+ T cells was significantly increased in asymptomatic (2-fold) and symptomatic (4-fold) L. infantum–infected animals (Fig. 1), as compared with expression on cells stimulated with a NSC ex vivo (Fig. 1D). The percentage of CD4+ T cells capable of proliferation in response to f-t L.i. Ag was significantly reduced in animals with symptomatic VL, with 5-fold reduction compared with NSC and asymptomatic animals (Fig. 1A, 1E). Intracellular staining revealed a 67% reduction in the percentage of CD4+ cells responding to f-t L.i. stimulation with IFN-γ production compared with either NSC or asymptomatic animals (Fig. 1B, 1F). Consistent with previous studies of leishmaniasis (2) and of T cell exhaustion (20, 21), the percentage of IL-10+ CD4+ T cells in f-t L.i.-stimulated PMBC from symptomatic animals increased almost 6-fold and >2-fold compared with NSC and asymptomatic animals, respectively (Fig. 1C, 1G). CD4+ T cells from symptomatic animals had significantly higher IL-10 compared with asymptomatic animals both with and without stimulation, and significant elevation after Ag stimulation compared with unstimulated PBMC (p < 0.001, data not shown). Within individual gated populations were multiple dual-positive populations, including PD-1+ IFN-γ−, proliferating and IFN-γ+, and even IFN-γ+ IL-10+ populations. These multiple dual-positive populations demonstrate the phenotypic variation possible across a polyclonal, Ag-responsive population of T cells. Alterations in IFN-γ and IL-10 production were similar as measured via ELISPOT (Supplemental Fig. IIA, IIB). Functional exhaustion and symptomatic VL were also associated with a significant increase in L. infantum burden within PBMC (Fig. 1H). The lack of Ag-specific CD4+ effector responses from these cells indicates the presence of CD4+ T cell exhaustion during VL, associated with increased peripheral parasite load.

CD8+ T cells from asymptomatic and symptomatic VL animals exhibit more severe functional exhaustion than CD4+ T cells

CD8+ T cell exhaustion has been characterized during numerous chronic viral infections, protozoal infections, and cutaneous leishmaniasis (8, 9, 12). Proinflammatory cytokines, including IFN-γ, are produced primarily by both CD4+ and CD8+ T cells during chronic VL (22). CD8+ T cell exhaustion has not been previously reported during naturally-occurring VL, and knowledge of CD4+ versus CD8+ T cell exhaustion during VL progression was not previously known. Surface expression of PD-1 was elevated in...
CD8+ T cells from animals with asymptomatic (1.5-fold increase) and symptomatic VL (2-fold increase) as compared with NSC (Fig. 2). VL symptomatic animal CD8+ T cells had dramatic 5-fold reduction in proliferative capacity (Fig. 2A [top row], 2B), and 3-fold reduction in the capacity of CD8+ T cells to produce IFN-γ (Fig. 2A [middle row], 2C) in response to whole f-t L.i. compared either with NSC or asymptomatic animals. There was no significant change in intracellular IL-10 production (Fig. 2A [bottom row], 2D). During asymptomatic infection, evidence of CD8+ exhaustion was more significant than in CD4+ T cells, with a higher number of dogs with significantly increased populations of PD1+ T cells during asymptomatic infection than of CD4+ T cells (Figs. 2E, 1D), with a bimodal population and an increased CD8+ T cell population with poor proliferation (Fig. 2B, 1E). Mean population size of PD-1+ CD8+ versus CD4+ T cells from symptomatic animals was similar (Fig. 1D, 2E). These data indicate the presence of pan-T cell exhaustion during symptomatic VL, and a large percentage (>40%) of asymptomatic animals with phenotypic CD8+ T cell exhaustion and elevated PD-1 (Fig. 2). T cell exhaustion during VL was associated with elevated expression of coinhibitory receptor PD-1, which could be identified prior to onset of symptomatic disease. Exhaustion was associated with clinical progression of VL and could be a predominant contributing factor to the onset of symptomatic disease.

**T cell exhaustion in CD4+ T cells from symptomatic VL animals reversed by B7.H1 block**

An increased percentage of PD-1–expressing CD4+ T cells was progressively associated with CD4+ T exhaustion during symptomatic VL. Previous studies utilizing B7.H1 Ab block demonstrated recovery of CD8+ T cell effector function (20, 23). Our previous studies demonstrated an increased percentage of IL-10–producing CD4+ T cells and increased IL-10 production during symptomatic VL. Others have postulated that IL-10 may induce CD4+ T cell suppression during chronic VL (2, 24). Based on this, our hypothesis was that IL-10/IL-10R or PD-1/PDL-1 (B7.H1)
signaling was necessary for CD4+ T cell exhaustion in PBMC from symptomatic VL animals. Block of B7.H1 resulted in significant recovery in CD4+ T cells able to proliferate to f-t L.i. Ag both in asymptomatic and symptomatic infection-derived PBMC (Fig. 3A, 3B). IL-10 block trended toward increased proliferation-capable CD4+ T cells in PBMC from both asymptomatic and symptomatic animals but was not statistically significant (Fig. 3). B7.H1 block resulted in significant recovery of a population of CD4+ IFN-γ+ T cells compared with isotype or IL-10 Ab-treated PBMC from asymptomatic and symptomatic animals (Fig. 3A, 3C). The number of IFN-γ–producing PBMC was also significantly increased after B7.H1 blockage compared with isotype or with IL-10 treatment as measured via ELISPOT (Supplemental Fig. IIIA). Recovery of a population of IFN-γ–producing cells after anti–IL-10 Ab treatment trended toward significance but was not significantly altered compared with isotype-treated PBMC (Fig. 3A, 3C). IL-10 production as measured by ELISA was significantly reduced after B7.H1 block as compared with IL-10 production from PBMC treated with isotype Ab (Fig. 3D). The population of IL-10+ PBMC quantified via ELISPOT increased after blockage of B7.H1 and stimulation with f-t. L.i., perhaps suggesting transformation of IL-10–expressing cells into IFN-γ coexpression, similar to previous findings (2) (Supplemental Fig. IIIB).

Blockage of B7.H1 was consistently effective in increasing the Ag-responsive population of CD4+ T cells from both asymptomatic and symptomatic VL animals, significantly increasing the percentage of cells able to proliferate and produce IFN-γ after B7.H1 treatment. PD-1 is therefore necessary for CD4+ T cell exhaustion during VL, and blocking the PD-1/B7.H1 interaction recovered a population of functional Th1 CD4+ T cells.

**Blockage of B7.H1/PD-1 recovers proliferation in exhausted CD8+ T cells but not IFN-γ production**

Exhaustion of CD8+ T cells from peripheral blood in both asymptomatic and symptomatic animals was associated with a significant elevation of PD-1 surface expression (Fig. 2). Blockage of the B7.H1/PD-1 interaction, but not IL-10, significantly recovered both proliferation and IFN-γ production within the CD4+ T cell population in response to stimulation with f-t L.i. (Fig. 3). To evaluate the role of PD-1 and IL-10 in suppressing CD8+ T cell function, we blocked B7.H1 and IL-10 prior to stimulation with f-t L.i. and evaluated CD8+ T cell function (Fig. 4). Consistent with B7.H1 blockage in the CD4+ T cell population, B7.H1/PD-1 blockage on adherent PBMC significantly increased CD8+ T cell proliferative capacity in response to f-t L.i. in both asymptomatic and symptomatic animals (Fig. 4A, 4B). As opposed to the CD4+ T cell population, blockage of IL-10 also significantly recovered CD8+ T cell proliferation after f-t L.i. stimulation in asymptomatic animals (Fig. 4A, 4B). However, within the CD8+ T cell population, blockage of B7.H1 did not significantly recover IFN-γ production in either asymptomatic or symptomatic dogs (Fig. 4B). In symptomatic dogs, blockage of IL-10 significantly recovered IFN-γ production, although recovery was minimal compared with positive control (Fig. 4B). This indicates that in the CD8+ T cell population of asymptomatic and symptomatic dogs with VL, IFN-γ production is largely nonresponsive to blockage of B7.H1/PD-1.

Ag stimulation does not induce further apoptosis in exhausted T cells

Previous research has demonstrated a stepwise manner in which CD4+ and CD8+ T cell populations progressively exhaust, ending with clonal deletion of exhausted cells (10, 12). Conversely, Shin

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**FIGURE 3.** PD-1/B7.H1 interaction necessary for CD4+ T cell exhaustion during symptomatic VL. Adherent PBMC treated with B7.H1 blocking Ab or IL-10 blocking Ab. PBMC stimulated as previous. (A) CD4+ cellular proliferation (top row) and CD4+ T cell IFN-γ (bottom row), compared with PD-1 (x-axis) in PBMC from negative control (left), asymptomatic VL (middle), and symptomatic VL (right) dogs, with isotype control (left), anti-IL-10 Ab (center), or anti-B7.H1 Ab treatment (right). (B) CD4+ cellular proliferation, n = 15. (C) CD4+ PBMC IFN-γ intracellular production, n = 21. (D) Production of IL-10 via ELISA, n = 11. **p < 0.01, via one way ANOVA with Tukey’s posttest.
et al. (13) demonstrated persistence of exhausted T cells in vivo, maintained through basal levels of proliferation and Ag stimulation. To evaluate whether there was maintenance of exhausted T cells during symptomatic VL, we evaluated PBMC for annexin V and propidium iodide positivity (Fig. 5). Annexin V positivity was higher within the CD8+ T cell population than within the CD4+ T cell population from both symptomatic and asymptomatic animals across all experimental treatments (Fig. 5). However, annexin V positivity was higher in CD4+ and CD8+ T cells from asymptomatic animals than in T cells from symptomatic animals (Fig. 5). Propidium iodide positivity indicative of cell death was minimal in all groups (data not shown). After IL-10 or B7.H1 block, CD4+ and CD8+ T cell apoptosis was still significantly lower within the more phenotypically exhausted symptomatic T cell populations as compared with cells from asymptomatic animals (Fig. 5C, 5D). There were no significant differences in annexin V positivity between experimental treatments (Fig. 5C, 5D). Blocking of B7.H1 in CD4+ T cells from symptomatic VL animals resulted in a trend toward increased annexin V positivity, corresponding with more pronounced proliferation recovery. Three VL symptomatic dogs had increased PBMC proliferation and increased annexin V positivity, whereas two dogs, animals with lowest proliferative and IFN-γ responses to B7.H1 block (Fig. 3B, 3C), had no alteration in PBMC annexin V positivity.

B7.H1 blockage improves the production of superoxide and parasite clearance in phagocytic monocytes

Previous reports demonstrated the importance of ROI and RNI in reducing intracellular Leishmania burden (1, 17). Phagocyte production of ROI and RNI required IFN-γ production by T cells (18). We demonstrated that blocking PD-1/B7.H1 signaling recovered a population of CD4+ T cells with IFN-γ production. We sought to determine how functional Th1 recovery altered phagocyte function, as phagocytes are both the target of L. infantum infection and necessary for L. infantum removal. After PD-1/B7.H1 block, L.i. Ag-stimulated monocyte-derived phagocytes from asymptomatic and symptomatic dogs produced significantly more superoxide compared with all other treatments (Fig. 6A, Supplemental Figure IID). NO production, as measured by Griess reaction performed on cell supernatants from identical PBMC cultures, had no significant differences between any experimental groups (Supplemental Fig. IID). Dogs, like humans, have limited inducible NO synthase activity. There is continuing debate as to the importance of RNI in the killing of intracellular pathogens in species other than mice, including humans and dogs (18, 25).

Blocking PD-1/B7.H1 increased superoxide production in adherent monocytes from symptomatic dogs chronically infected with L. infantum (Fig. 6A). Regaining an ability in these cells to produce parasite-lethal reactive oxygen species provides a link

![FIGURE 4. PD-1/B7.H1 interaction necessary for suppression of proliferation but not IFN-γ production during symptomatic VL-associated CD8+ T cell exhaustion. Adherent PBMC treated with B7.H1 blocking Ab or IL-10 blocking Ab. PBMC stimulated as previous. (A) CD8+ cellular proliferation (top row) and CD8+ T cell IFN-γ (bottom row), compared with PD-1 (x-axis) in PBMC from negative control (left), asymptomatic VL (middle), and symptomatic VL (right) dogs, with isotype control (left), anti–IL-10 Ab (center), or anti-B7.H1 Ab treatment (right). (B) CD8+ cellular proliferation, n = 13. (C) CD8+ PBMC IFN-γ intracellular production, n = 22. *p < 0.05, **p < 0.01, via one way ANOVA with Tukey’s posttest.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)

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between restoration of CD4+ Th function and improved monocyte-derived phagocyte function. We next wanted to identify whether this increase in ROI-producing phagocytes led to reduced intra-PBMC parasites ex vivo from VL asymptomatic and symptomatic dogs. We quantified PBMC parasite load after PD-1/B7.H1 treatment via RT-PCR. After B7.H1 block, parasite-stimulated PBMC from symptomatic animals had significantly fewer *L. infantum* parasites than f-t *L.i.*-stimulated PBMC or isotype Ab-treated PBMC using paired PBMC from the same dogs (Fig. 6B). Average reduction in *L. infantum* after B7.H1 blockage was 0.58 log or 67.36%. Although not significant, IL-10 block reduced parasite load in the four dogs with highest parasitemia ($p = 0.16$) (Fig. 6B, IL-10).

**Discussion**

Effective vaccination and immunotherapeutic strategies for VL have remained elusive (26). Host and parasite factors regulating the balance of an asymptomatic state versus symptomatic clinical disease are incompletely understood for VL. A better understanding is integral for addressing VL immunity (1). Dogs with leishmaniasis have a similar pathogenesis to human VL. In addition,
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FIGURE 6. B7.H1 block increased phagocyte superoxide production and decreased parasite load. (A) NBT assay performed on adherent PBMC from asymptomatic and symptomatic VL dogs after isotype, B7.H1, or IL-10 Ab treatment and stimulation with f-t Li. (B) L. infantum–specific quantitative RT-PCR performed on PBMC DNA. Data presented as number of genomic copies of L. infantum10⁵ PBMC from symptomatic dogs, after f-t Li. stimulation (Li) compared with stimulation and addition of isotype, (left, Iso), anti–IL-10 (middle, IL-10), or anti-B7.H1 Ab (right, B7.H1). (A) n = 13 and (B) n = 7. *p < 0.05, **p < 0.01, via one-way ANOVA with Tukey’s postest on averaged (A) or log-transformed (B) data.

canine leishmaniasis is a primary target for public health interventions in multiple endemic areas, including culling, vaccination, and/or therapy (5, 27).

Using naturally-occurring canine VL, we identified decreased PBMC production of IFN-γ, decreased CD4⁺ T cell proliferation to Ag stimulation, and increased IL-10 production during clinical disease (5), an exhausted phenotype. CD8⁺ T cell exhaustion has been identified in numerous infections, including disseminated cutaneous leishmaniasis (9, 10, 12, 28). CD8⁺ exhaustion is characterized by absence of Ag-specific responses, including proliferation, CD8⁺ cytotoxicity, and IFN-γ production (29). Knowledge of CD4⁺ T cell exhaustion is less extensive (10). In this study, to our knowledge, we demonstrate for the first time concurrent CD4⁺ and CD8⁺ T cell exhaustion during naturally-occurring canine VL, mediated by PD-1/B7.H1. We demonstrate CD8⁺ T cell exhaustion, partially mediated by PD-1, which occurs prior to the onset of symptomatic disease, and prior to the onset of functional CD4⁺ T cell exhaustion. It is logical, from an evolutionary standpoint, that CD8⁺ T cells are more sensitive than CD4⁺ to tolerogenic stimuli, including prolonged Ag stimulation during chronic infection. This would protect infected cells from being the target of cytotoxic responses, and significantly dampen proinflammatory stimuli that induce host pathology (30).

Blockade of B7.H1/PD-1 significantly recovered CD4⁺ and CD8⁺ T cell proliferation and CD4⁺ IFN-γ production in response to L. infantum Ag. In addition, this blockade also significantly reduced the presence of IL-10 in cell culture supernatants. This is consistent with the findings and hypothesis suggested by Mou et al. (11) that PD-1 exerts its effects both directly through immunoreceptor tyrosine switch motif–associated SHP phosphatases and indirectly through regulation of IL-10 production from monocytes during Leishmania infection. Based on clinical data in this study, it is likely that concurrent CD4⁺ and CD8⁺ T cell exhaustion preceding the onset of clinical disease contributes to precipitation of symptomatic VL.

CD4⁺ T cells from symptomatic animals had a significant reduction in annexin V positivity, with minimal apoptosis. CD8⁺ populations also had reduced annexin V positivity compared with control, although the CD8⁺ T cell population overall had a greater degree of apoptosis in culture than CD4⁺ T cells. Reductions in apoptosis could be due to previous clonal deletion of responsive Ag-specific T cells. Within both CD4⁺ and CD8⁺ T cell populations, blockade of B7.H1 significantly increased proliferative capacity and in the CD4⁺ population recovered IFN-γ. This makes clonal deletion of all responsive cells unlikely, as an Ag-specific memory T cell population could be recovered. A more likely explanation is the Ag-addiction theory put forth by Wherry and others (10, 13). This would suggest that both CD8⁺ and CD4⁺ exhausted T cell populations contain Ag-experienced cells that do not respond to Ag by undergoing proliferation, but also do not undergo apoptosis after Ag stimulation. Possible mechanisms for this would include Ag-driven survival, perhaps through cytokines such as IL-2 and TCR engagement, or less likely through replicative senescence due to epigenetic modification and telomere shortening during aging and prolonged cellular replication (31–33).

We identified increased APC superoxide production and reduced intracellular parasites ex vivo after PD-1/B7.H1 block, indicating a role for this signaling pathway in hindering phagocyte function as VL progresses. The importance of ROI and RNI production is well documented for effective immunity against Leishmania species (1). To our knowledge, this is the first report linking T cell exhaustion and PD-1/B7.H1 signaling directly to macrophage responses. Due to dramatic alterations in phagocyte, CD4⁺ T cell, and CD8⁺ T cell effector function after ex vivo PD-1/B7.H1 block, this treatment may be a means of inducing increased proinflammatory phagocyte function, and therefore a strong target for VL immunotherapy.

A recent report demonstrates the importance of CD4⁺ T cell exhaustion in the development of CD8⁺ T cell exhaustion (34). In the presence of chronic infection, it is likely that these two processes are extensively intertwined. Previous studies aimed at VL immunotherapy have targeted IFN-γ, blocking IL-10, or using TLR agonists (24, 35). Limited success of these immunotherapeutic approaches suggests there are other, perhaps more significant, mechanisms of immune dysregulation during VL. This study demonstrates concurrent CD4⁺ and CD8⁺ T cell exhaustion, often present prior to the onset of symptomatic VL, was mediated by significantly elevated surface expression of coinhibitory molecule PD-1. This may have significant ramifications for prevention and treatment interventions for dogs as a reservoir of Leishmania infantum, as T cell exhaustion may reduce the efficacy of vaccination or therapeutic strategies.

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