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Recovery of antigen-specific T cell responses from dogs infected with *Leishmania (L.) infantum* by use of vaccine associated TLR-agonist adjuvant

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Abstract

Visceral leishmaniasis (VL), caused by infection with the obligate intracellular protozoan parasite *Leishmania infantum*, is a fatal disease of dogs and humans. Protection against VL requires a T helper 1 (Th1) skewed $CD4^+$ T response, but despite this knowledge, there are currently no approved-to-market vaccines for humans and only three veterinary-use vaccines globally. As VL progresses from asymptomatic to symptomatic, *L. infantum*–specific interferon gamma (IFNγ) driven- Th1 responses become dampened and a state of immune exhaustion established. T cell exhaustion and other immunoregulatory processes, starting during asymptomatic disease, are likely to hinder vaccine-induced responses if vaccine is administered to infected, but asymptomatic and seronegative, individuals. In this study we evaluated how immune exhaustion, shown previously by our group to worsen in concert with VL progression, effected the capacity of vaccine candidate antigen/toll-like receptor (TLR) agonist combinations to promote protective

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Conflict of interest statement

The vaccine adjuvants utilized in this study are propriety to the Infectious Disease Research Institute. The authors have no competing interests.

Contributors

RGS designed and performed experiments, analyzed data, wrote and revised the manuscript. TLG performed experiments, analyzed data, wrote and revised the manuscript. KJE designed and performed experiments. AJT performed experiments, wrote and revised the manuscript. MSD, RFH, and SGR supplied reagents and revised the manuscript. CAP designed experiments, analyzed data, and wrote and revised the manuscript.

CD4$^+$ T cell responses during progressive VL. In conjunction with Th1 responses, we also evaluated concomitant stimulation of immune-balanced IL-10 regulatory cytokine production by these vaccine products in progressive VL canine T cells. Vaccine antigen L111f in combination with TLR agonists significantly recovered CD4$^+$ T cell IFN-γ intracellular production in T cells from asymptomatic VL dogs. Vaccine antigen NS with TLR agonists significantly recovered CD4$^+$ T cell production in both endemic control and VL dogs. Combinations of TLR agonists and vaccine antigens overcame *L. infantum* induced cellular exhaustion, allowing robust Th1 CD4$^+$ T cell responses from symptomatic dogs that previously had dampened responses to antigen alone. Antigen-agonist adjuvants can be utilized to promote more robust vaccine responses from infected hosts in endemic areas where vaccination of asymptomatic, *L. infantum*-infected animals is likely.

Keywords

Leishmania (L.) infantum; Toll-like receptor agonist; adjuvants; CD4+ T cells; cytokines

1. Introduction

Leishmaniasis is a zoonotic disease that affects approximately 1.3 million individuals per year leading to 20–30,000 deaths [1, 2]. After malaria, *Leishmania* is the most common cause of parasite-induced mortality [3]. Visceral leishmaniasis (VL) is a severe disease form characterized by lymphadenopathy, splenomegaly and hepatomegaly that can be fatal without chemotherapy. Treatment is complicated by antimonial-resistant *Leishmania* strains [4], high drug toxicity [5], and treatment failure or relapse [6]. In North America and the Mediterranean basin, VL is caused by zoonotic *L. infantum*; both dogs and humans are affected. Vaccination as post-exposure immunoprophylaxis, or immunotherapy, in asymptomatic, infected individuals or animals would be an ideal and efficient method to mitigate the need for toxic, expensive, treatments and potentially minimize treatment challenges. Only two anti-*Leishmania* vaccines have been licensed in any country for use in humans and use to date has been limited. In Uzbekistan, a live parasite vaccine is used for prophylaxis [7]. In Brazil, a now discontinued vaccine, had been used for immune therapy [8]. L110f, L111f and KSAC are vaccine candidates currently in clinical trials and successfully promote a robust IFNγ immune response [9–14]. L111f along with a more recently developed subunit peptide vaccine candidate, NS, were used in this study to evaluate ex vivo responses in VL infected and non-infected dogs. L111f is a chimeric, three *Leishmania* gene fusion (TSA, LeIF and LmSTI1) [15]. NS is a two gene fusion of the *Leishmania* nucleoside hydrolase (NH) and a sterol 24-c-methyltransferase (SMT) genes (Fig. 1).

Dogs have similar VL disease progression to that of human patients from endemic areas, making them an ideal population to study vaccine responses [16, 17]. As VL is zoonotic, control of *L. infantum* infection and disease in dogs is critical for overall VL control. Two veterinary vaccines are currently available in Europe and Brazil, respectively. Vaccination of unowned dogs in endemic areas has been implemented using peptide formulations, DNA vaccination or modified-live parasites, with varying degrees of success [18–22].

While vaccine efficacy has been relatively high (~90%) when immunization has occurred in healthy, completely naïve, subjects, efficacy to prevent disease progression declined in infected, asymptomatic or symptomatic individuals [23]. Lower efficacy of vaccines in endemic settings has been observed in large field trials for malaria and tuberculosis vaccination, possibly due to the inclusion of asymptomatic infected individuals [24–26]. Control of *Leishmania* infection requires strong T helper 1 (Th1) responses marked by a robust $CD4^+$ T cell proliferative response and IFNγ production. However, chronic *Leishmania* infection in murine models promotes regulatory responses involving production of classical $CD25^+$, $FoxP3^+$ regulatory T cells [27], IL-10 producing, regulatory, B cells [28] and immune exhaustion characterized by anti-inflammatory responses and an inability to proliferate in response to *Leishmania*-specific antigens [29]. In progressive VL, our data [29] and others [17] demonstrate that immune exhaustion starts prior to onset of clinical disease. Understanding how to stimulate an exhausted immune system to respond to vaccine antigens is essential for successful vaccination/immunotherapy of VL, and perhaps other infections, in endemic settings.

Our group has previously demonstrated that TLR agonist adjuvants protect against cutaneous leishmaniasis caused by *L. major* by recovering effector T cell responses during active infection in mice [10]. Different clinical presentations of *Leishmania* species lead to different responses to therapy. In these studies, we identify vaccine antigen/adjuvant pairings that mount a protective immune response ex vivo from naïve and *L. infantum*-infected dogs across the VL clinical spectrum.

2. Materials and Methods

2.1 Animals

A cohort of hunting dogs from the United States, naturally-infected with *L. infantum* and a long history of active VL surveillance was used in these studies [30]. Pen-matched dogs served as endemic controls. The University of Iowa Institutional Animal Care and Use Committee approved animal use, which ensure that the National Institutes of Health guide for the care and use of laboratory animals have been followed. Canine disease progression and VL classification was evaluated as in previous publications (Table 1) [16, 30, 31]. Dogs were examined by veterinarians for signs of leishmaniasis (lymphadenomegaly, palpable liver or spleen, poor hair coat, cachexia, epistaxis, arthrogryposis, alterations in hepatic or renal enzymes on serum chemistry). Dogs with >4 signs of disease were not included in this study. Parasite load was quantified by qPCR and immunofluorescence anti-*Leishmania* antibody testing (IFAT) as performed by the Center for Disease Control and Hygiene [32, 33]. Dogs were stratified into clinical groups: endemic controls (qPCR negative, 0 IFAT titer), asymptomatic (qPCR negative/borderline, >1/16 IFAT titer and ≤ 1/128), and symptomatic (qPCR positive/borderline, ≥1/256 IFAT titer, and ≥ 2 clinical signs) (Table 1).

2.2 Parasite DNA isolation, diagnostic qPCR, and IFAT serology

Parasite DNA isolation and qPCR was performed as previously described [29, 30, 34]. DNA was isolated from canine blood samples with the QIAamp DNA Blood Mini Kit per manufacturer's instruction (QIAGEN, Valencia, CA). Ribosomal primer sequences F (5'-

AAGCCACCCCAGAGGTAAAAA), and R (5'-GACGGGTCTGACCCTTGGTT) (Invitrogen, Life Technologies, Grand Island, NY), and probe: 5' 6FAM-CGGTTCGGTGTGTGGCGCC-MGBNFQ (Applied Biosystems, Life Technologies, Grand Island, NY) were used as previously described [34]. Primers and probe were used at 10nM. Amplification was performed in duplicate using an ABI 7000 qPCR system (Applied Biosystems) with Super Mastermix (Rox) (Quanta Biosciences, Gaithersburg, MD) and cycling protocol as described previously [34]. Results were analyzed by ABI 7000 System SDS Software v1.2.3 (Applied Biosystems). Canine serum samples were stored at −20°C and sent to the Centers for Disease Control and Prevention for IFAT based on *in vitro* promastigote antigen culture as previously described [35, 36].

2.3 Stimulation of whole blood with vaccine antigens and TLR agonists *ex vivo*

Whole blood samples were collected as previously [30] and diluted 1:5 in RPMI. 150 μL of diluted whole blood was plated into 96 well plates. Vaccine antigens L111f [15] and NS [37] were added to wells at a final concentration of 1 μg/mL (Fig. 1). TLR agonists were added at final concentrations as described previously [10]: Glucopyranosyl Lipid Adjuvant (GLA) [TLR4; 1 μg/mL] [38], imiquimod (R837) [TLR7;10 μg/mL] [39], resiquimod (R848) [TLR7/8; 0.1 μg/mL] [39] (IDRI, Seattle, WA). Concanavalin A (ConA) (Sigma Aldrich, St. Louis, MO) [5 μg/mL] was used as a positive control mitogen. Media-only treated cells were used for baseline negative control. ConA treatment groups were cultured for 4 days; all other treatments were cultured for 7 days.

2.5 Flow cytometry

Flow cytometry was performed as previously described [40] and gating strategy demonstrated in Fig S1. Briefly, twenty-four hours prior to cell labeling, EdU (Life Technologies, Madison WI) was added to cells to assess proliferation per manufacturer's directions [29]. Six hours prior to cell labling, 1× Brefeldin A (BD Biosciences, Franklin Lakes, NJ) was added to all cells. Cells were blocked with whole goat serum for 30 minutes, then labeled with anti-CD3 (CA17.2A12), CD4 (YKIX302.9), CD8 (YCATE55.9), (AbD Serotec/Bio-Rad, Hercules, California) IFNγ (142529), and IL-10 (138128) (R&D Systems, Minneapolis, MN) in 1× Perm/Wash Buffer (Life Technologies) per manufacturer's directions. Cells were analyzed via an LSR II flow cytometer (BD Biosciences). 50,000 viable events were acquired and analyzed with FlowJo vX Software (Treestar, Ashland, CA).

2.6 SYBR Green Quantitative PCR and RNA extraction

SYBR green chemistry was utilized for qPCR assay using methods and primers as previously described [40, 41]. Briefly, the $2^{-\Delta\Delta C}{}_{T}$ method was utilized for analysis. RNA was extracted using RNeasy extraction kit following manufacturer's instruction (QIAGEN). RNA was quantified and standardized followed immediately by first strand cDNA synthesis via iScript cDNA Synthesis Kit (Bio-Rad). qPCR reactions were performed in duplicate.

2.7 Statistical Analysis

Differences between experimental group means were assessed using one-way ANOVA with Tukey's post-hoc test via GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). *P* values of ≤ 0.05 were considered statistically significant.

3. Results

3.1 CD4+ T cells from VL symptomatic dogs had reduced responses to vaccine antigens compared to those from asymptomatic dogs

Immune exhaustion, specifically a reduced ability to proliferate after exposure to antigen and reduced production of IFN-γ, was shown to occur in $CD4^+$ and $CD8^+$ T cells from dogs that had progressive VL [29]. $CD4^+$ T cells are largely considered to be the predominant T cell subset for protective anti-*Leishmania* responses[42]; these cells were the primary focus of this analysis. Cells isolated from endemic control dogs, and VL asymptomatic and symptomatic-infected dogs were stimulated with a mitogen and *Leishmania*-specific vaccine antigens to determine if these cells exhibited signs of T cell exhaustion as demonstrated by quantitative PCR (Fig S2). The antigens used were L111f and NS. L111f has been safely administered and well-tolerated in subjects with and without evidence of prior *Leishmania* infection [15, 43, 44] and in combination with MPL-SE vaccine as an adjunct immunotherapy with standard chemotherapy for both cutaneous and mucocutaneous leishmaniasis [45]. NS was recently developed for vaccines targeting VL [37, 46], (Fig 1).

Exposure to the vaccine antigen L111f stimulated significant 5.4- and 7.1-fold increases in percent of $CD4^+$ T cells undergoing proliferation from asymptomatic ($p < 0.001$) and symptomatic ($p < 0.01$) dogs, respectively compared to mock-treated cells. L111f exposure induced significant ($p < 0.001$) 8.0- and 8.7-fold increases in percentage of $CD4^+$ T cells with intracellular IFNγ using the same comparisons as proliferation (Fig. 2A). NS stimulated less, yet still significant increase in percent $CD4^+$ T cell proliferation; 3.6-fold ($p < 0.001$) in cells from asymptomatic animals. Similarly there were significant increases in the percentage of $CD4^+$ T cells producing IFNγ of 4.4- ($p < 0.001$) and 4.7-fold ($p < 0.01$) in samples from asymptomatic and symptomatic dogs (Fig. 2B). Similar trends were observed in vaccine antigen responses from $CD8^+$ T cells by clinical group (Fig. S3).

$CD4^+$ T cells from all groups responded significantly ($p < 0.001$) to mitogen (ConA). IFNγ production levels also significantly ($p < 0.001$) increased (Fig. 2C). The percent of $CD4^+$ T cells positive for both IFNγ production and proliferation from VL-symptomatic dogs underwent significant ($p < 0.001$), two-fold, reduction relative to populations of cells from asymptomatic dogs (Fig 2C). This likely indicates a global T cell exhaustion (Fig S2), rather than simply antigen-specific exhaustion, similar to previous observations [29, 30].

3.2 TLR agonist/L111f vaccine antigen modestly increased CD4+ T cell IFNγ responses

Vaccines incorporating multiple TLR agonists significantly stimulated murine $CD4^+$ T cell IFNγ responses during experimental *L. major* infection. We examined if *ex vivo* incubation with *Leishmania* vaccine antigens and TLR agonists could recover effector functions from populations of exhausted symptomatic dog $CD4^+$ T cells. Glucopyranosyl Lipid Adjuvant

(TLR4 agonist) (triangles, Fig. 3A) with L111f modestly (p <0.05) increased $CD4^+$ T cell proliferation 1.6-fold in VL-asymptomatic dogs and IFNγ responses 2.8-fold in $CD4^+$ T cell populations from endemic control dogs, compared to L111f alone in cells from either clinical group (circles, Fig. 3A). Relative to L111f alone, this combination did not enhance recovery of exhausted $CD4^+$ T cell function in VL-symptomatic dogs (Fig. 3A). Addition of imiquimod (TLR7 agonist) or resiquimod (TLR7/8 agonist) did not increase $CD4^+$ T cell proliferation in $CD4^+$ T cells from any clinical group, compared to treatment with L111f alone (Fig. 3B, C). Addition of TLR7 agonist to $CD4^+$ T cells from endemic control dogs compared to cells exposed to L111f alone significantly increased (p <0.01) the percentage of $CD4^+$ T cells producing IFNγ 3.5-fold (Fig. 3B). TLR7/8 agonist addition increased the percentage of endemic control $CD4^+$ T cells producing IFNγ 1.6-fold (Fig. 3C). TLR agonists did not improve $CD8^+$ T cell responses to L111f (Fig. S4). Addition of exogenous TLR agonists to L111f produced significant increases in $CD4^+$ T cell responses from uninfected dogs, likely due to the TLR2 ligating potential of this vaccine antigen, but gave modest or no improvement in L111f-specific responses from cells of VL-asymptomatic or symptomatic dogs.

3.3 TLR agonists with NS significantly increased all $CD4^+$ T cell responses

As NS is a more recently developed vaccine antigen [37, 46], we did not know how NS would perform with other TLR agonists when exposed to canine cells. While NS alone did not induce responses in uninfected dogs, NS with TLR4 agonist stimulated significant (p <0.01) increases in the population of proliferative $CD4^+$ T cells, 4.3-, 1.7-, and 5.8-fold in cells from endemic control, VL-asymptomatic, and symptomatic dogs, respectively (Fig. 4A). Similar significant increases in $CD4^+$ T cell populations were seen for IFNγ production (Fig. 4A; 5.0-, 1.7-, and 2.8-fold). NS with TLR7 or TLR7/8 agonists elicited similar percent population responses across clinical groups, with the most significant alterations in the *L. infantum*-infected dog $CD4^+$ T cell populations producing IFNγ. Symptomatic dog $CD4^+$ T cell percent proliferation increased 4.6-fold and 7.2-fold for TLR7 and TLR7/8 agonists respectively compared to vaccine alone (Fig. 4B and C, left, black triangles). Combination NS and TLR agonist stimulation similarly increased the $CD4^+$ T cell population responses in both asymptomatic and symptomatic dogs. T cells from symptomatic dogs were significantly less responsive to NS alone (Fig. 2), underscoring dramatic improvement of these exhausted T cells from VL symptomatic animals (Fig. 4B and C). $CD8^+$ T cells did not have altered NS vaccine antigen responses after addition of TLR4 agonist in cells from endemic control or symptomatic dogs. $CD8^+$ T cells from VL asymptomatic dogs had decreased populations of proliferative and IFNγ-producing cells after addition of TLR7 or TLR7/8 agonists (Fig. S5), perhaps due to the earlier shift to non-recoverable exhaustion observed in $CD8^+$ T cells vs. $CD4^+$ T cells during progressive VL [17, 29]. In fact, our studies did demonstrate an increase in gene transcription of exhaustion makers LAG3, CLTA4, PD1 and TIM3 as well as secreted IL-27 (Fig S2), which in addition to IL-10 will drive immunoregulatory responses during visceral leishmanaisis [47]. $CD4^+$ T cells from VL-symptomatic dogs were able to recover significant effector function with addition of TLR7 agonist to NS to populations equivalent to those seen from $CD4^+$ T cells from healthy VL-asymptomatic dogs.

3.4 Percent of CD4+ T cell IL-10 positive population significantly increased after L111f exposure

IL-10, a regulatory cytokine, can dampen harmful effects of unchecked pro-inflammatory responses, but excess IL-10 can suppress leishmanicidal responses leading to progressive disease [48–50]. The percent of $CD4^+$ T cells producing IL-10 significantly increased 3.9-fold in VL-symptomatic dog cells ($p < 0.001$), but only 1.7-fold in asymptomatic dog cells ($p < 0.05$) after stimulation with L111f alone compared to mock-treated cells (Fig. 5A, L111f). This increase in symptomatic dog cells was also a significantly ($p < 0.001$) 2.5-fold larger percent population than observed in endemic control $CD4^+$ T cells following treatment with L111f (Fig. 5A, L111f). Further addition of TLR agonists did not significantly increase the percent population of IL-10 producing $CD4^+$ T cells in endemic control or symptomatic dog cells compared to L111f alone, but there was a significant ($p< 0.05$) 1.7-fold increase in response to L111f and TLR 7/8 in asymptomatic dog $CD4^+$ T cells. The lack of increase in IL-10 observed from symptomatic cells treated with L111f-TLR agonist combinations may be due to the fact that L111f alone causes significant IL-10 production in these cells. IL-10 production to *L. infantum* antigen is not unheralded, as canine $CD4^+$ T cells from symptomatic dogs have previously been shown to respond to freeze-thawed *L. infantum* antigen stimulation with robust IL-10 production [29, 30]. However, when combination treatments were evaluated the percent $CD4^+$ T cell IL-10 population was significantly higher in symptomatic dogs compared to similarly treated endemic controls at 2.1-, 2.9-, and 3.4-fold respectively for TLR4 ($p < 0.01$), TLR7 ($p < 0.001$), and TLR7/8 ($p < 0.001$) agonists (Fig 5B–D, L111f). There was no significant difference between symptomatic and asymptomatic cells treated with combination treatments. This suggests that *L. infantum* infection, regardless of disease progression, has altered the ability of $CD4^+$ T cells to produce IL-10. $CD8^+$ T cells from symptomatic dogs showed similar IL-10 population responses after exposure to L111f compared to mock-treated control $CD8^+$ T cells (Fig S6 A, L111f). IL-10-producing $CD8^+$ T cell populations significantly decreased from symptomatic dogs after exposure to L111f with TLR4 agonist ($p < 0.01$), but in asymptomatic dogs this addition of TLR agonists significantly increased IL-10 production. $CD8^+$ T cell IL-10 populations increased 7.5-fold for TLR4 ($p < 0.001$) and 4.7-fold respectively for both TLR7 ($p < 0.05$) and TLR7/8 ($p < 0.001$) agonists addition (Fig 5B–D, L111f). Concomitantly with the Th1 response, L111f induces a balanced Il-10 regulatory response.

In contrast to L111f, stimulation with NS alone or in combination with TLR agonists had minimal, non-significant effects on $CD4^+$ T cell IL-10 populations from control, asymptomatic, or symptomatic dogs (Fig. 5, NS). $CD8^+$ T cell IL-10 populations were not affected by NS with or without additional exposure to TLR agonists in control or symptomatic dogs. (Fig. S6, NS). $CD8^+$ T cell IL-10 populations from asymptomatic dogs had mild significant decreases ($p<0.05$) of 2.4- and 2.9-fold in response to stimulation with TLR4 and TLR7, respectively, in combination with NS. In parallel with production of robust Th1 responses, NS with TLR agonist exposure did not result in major decreases in IL-10 producing populations, particularly in control and symptomatic dog cells.

4. Discussion

Vaccine-induced responses have been studied in depth under experimental *Leishmania* conditions [48, 51–60], but little is known regarding how these vaccines would serve as a post-exposure prophylaxis or immunotherapy to boost existing responses in already infected animals. This study used dogs, a natural host of *Leishmania infantum*, to demonstrate possible outcomes of vaccination during subclinical and clinical VL, approximating vaccine responses within an endemic population where vaccination could or would often occur after infection. Prophylactic vaccination against *Leishmania* and subsequent protection against experimental infection is enhanced by appropriate formulation with TLR agonists [9, 10]. Our data demonstrate that the inclusion of TLR agonists enhanced IFNγ production and proliferation of $CD4^+$ T cells isolated from VL-infected dogs after stimulation with *Leishmania* vaccine antigens.

The vaccine antigens used in this study are proprietary chimeric fusion proteins currently in (human) clinical trials [9, 13, 37]. Previous studies of L111f demonstrated an ability to stimulate murine $CD4^+$ T cells to produce IFN-γ and TNF-α and when used prophylactically in conjunction with TLR4 agonist MPL-SE, prevented clinical progression of canine VL [14, 55]. Controlling disease in domestic animal reservoirs is important for overall VL control. Provided the close association of human and canine VL, dogs are a commonly targeted vaccine population in *L. infantum* endemic areas. In our U.S. canine cohort NS with TLR-adjuvant supplementation induced greater $CD4^+$ T cell proliferative and IFNγ responses across *L. infantum*-infected groups compared to vaccine L111f. Interestingly, L111f contains a LeIF, a TLR2 agonist glycoprotein, as one of its antigenic components. As TLR2 when paired with TLR6 as a heterodimer can lead to Th2 skewed responses, this TLR agonist effect may dampen antigen-specific responses to produce a less robust $CD4^+$ T cell population responses, as is suggested in this study. Consistent with antigen specific dampening properties, L111f, with or without TLR agonist, significantly increased IL-10 in symptomatic dogs. We hypothesize that this IL-10 increase represents and immune balancing effect, serving to buffer pro-inflammatory Th1 responses induced by the vaccine and/or agonists to prevent immunopathology [61]. IL-10 production has also been demonstrated as being an important prognosticator of disease severity and immunological outcomes [62, 63], nonetheless is an important factor to be observed in response to cellular stimulation. Interestingly, we did not observe elevations in IL-10 responses to NS upon the addition of TLR4, TLR7 or TLR7/8 agonists, suggesting that the TLR2 activating component of L111f may contribute to this response.

Vaccination has been previously studied as an immunotherapy for leishmaniasis in Brazil, with conflicting results. These studies demonstrated success following TLR agonist supplementation [64] and contrarily that inclusion of a TLR4 agonist was not successful in halting disease progression [65, 66]. Vaccine dosage, scheduling, or route of infection, could account for these discrepancies. Perhaps more importantly, infected animals progressing to clinical VL have immune exhaustion [29], which our data identify does interfere with vaccine/immunotherapeutic responses. VL symptomatic dog $CD4^+$ T cells had reduced proliferative and IFNγ-production responses to vaccine antigens and mitogen compared to those from asymptomatic dogs (Fig. 2A). While these data are encouraging for use as a post-

exposure prophylactic to prevent *Leishmania* infection progressing to disease in yet asymptomatic hosts, it is imperative to use targeted vaccine formulation able to recover exhausted T cell responses before consideration as a treatment of VL.

5. Conclusion

The foremost challenge in development of VL vaccines is successfully mounting protective immunity in populations with parasite exposure; not naïve hosts. Appropriate pairing of TLR agonists with vaccine antigens may be a way to overcome this challenge as the right pairing(s) were able to ameliorate T cell exhaustion in cells from VL asymptomatic and symptomatic dogs and promote a productive $CD4^+$ T cell response. *In vivo* trials in naturally infected dogs to assess the ability of vaccine constructs with TLR agonist adjuvants may be merited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

VL	visceral leishmaniasis
IFNγ	interferon gamma
Th1	T helper 1
TLR	toll like receptor
IFAT	immunofluorescence anti-*Leishmania* antibody testing
IDRI	Infectious Disease Research Institute
Con A	concanavalin A
TNFα	tumor necrosis factor alpha

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Highlights

- Vaccine-specific T cell responses limited during clinical VL.
- TLR agonists recovered T cell effector functions in cells from *Leishmania*-infected dogs.
- Rational vaccine selection to improve antigen-fatigued responses crucial for endemic areas.

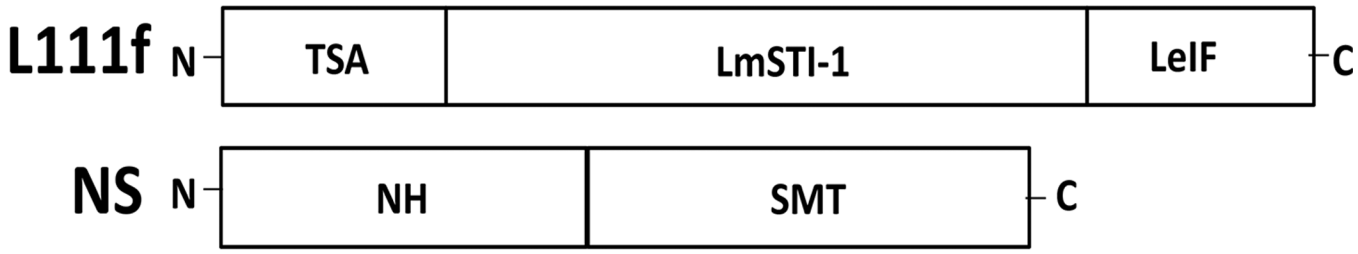

Fig. 1. Construction of anti-*Leishmania* vaccine antigens used for ex vivo stimulation of canine whole blood

L111f is an 111kD fusion protein produced from a fusion of the *L. major* homologue of eukaryotic thiolspecific antioxidant (TSA), the *L. major* stress inducible protein-1 (LmSTI1) and the *L. braziliensis* elongation and initiation factor (LeIF) genes. NS is a 75kD fusion protein produced from the *L. infantum* nucleoside hydrolase (NH) and *L. infantum* sterol 24-c-methyltransferase (SMT) genes.

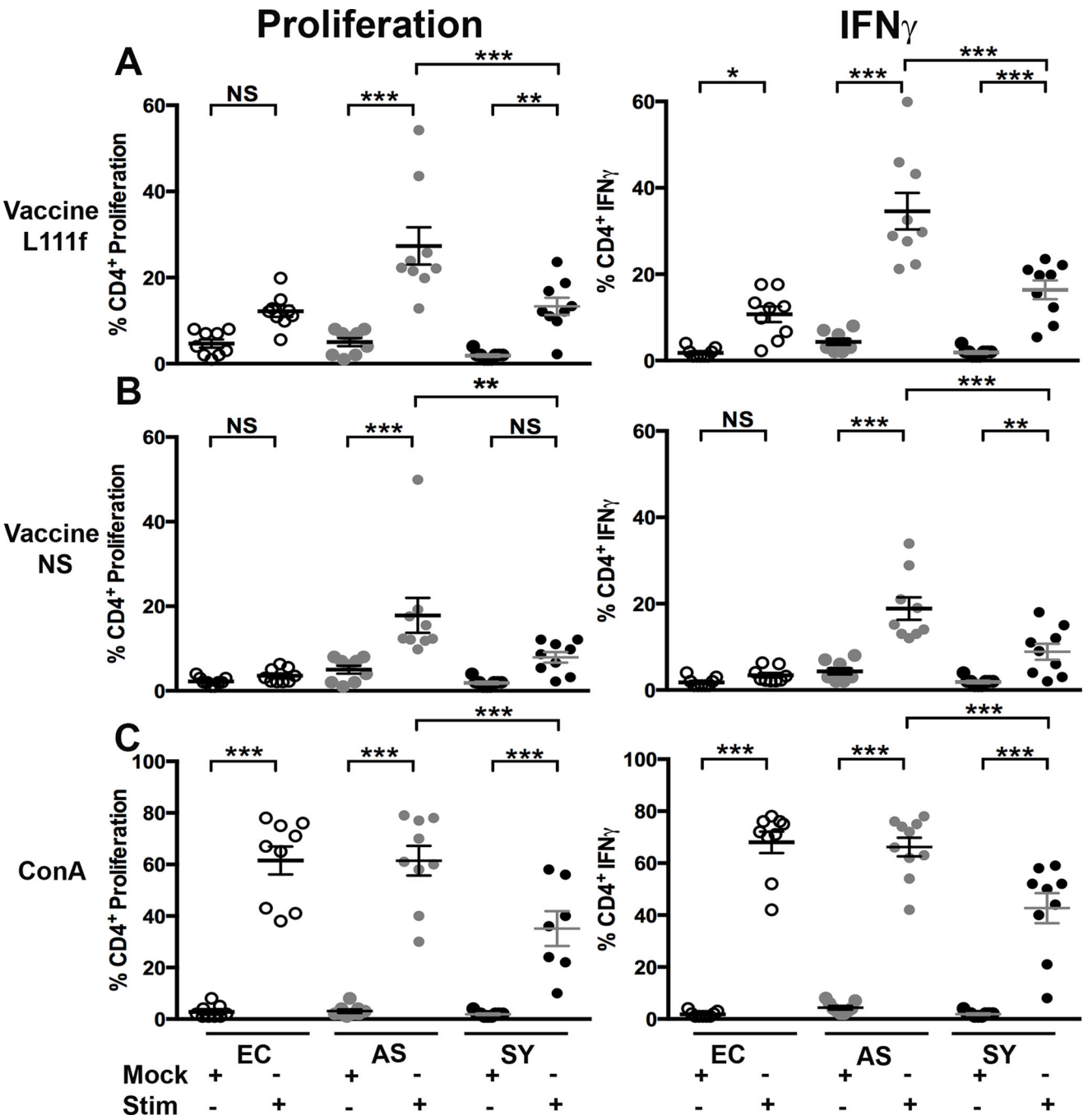

Fig. 2. VL clinical status altered CD4$^+$ T cell response to recombinant *Leishmania* protein vaccine stimulation ex vivo

Whole blood from dogs across the VL clinical spectrum was stimulated ("stim") with 1 μg vaccine L111f (A) or 1 μg vaccine NS (B) or 1 μg ConA (C), or left untreated ("mock"). Each dot represents an individual dog. EC = endemic control (open), AS = asympatomatic, *L. infantum* positive (grey), SY = symptomatic, *L. infantum* positive (black). Error bars are ± SEM. N=7–10 per group. * p<0.05, ** p<0.01, *** p<0.001 via one way-ANOVA.

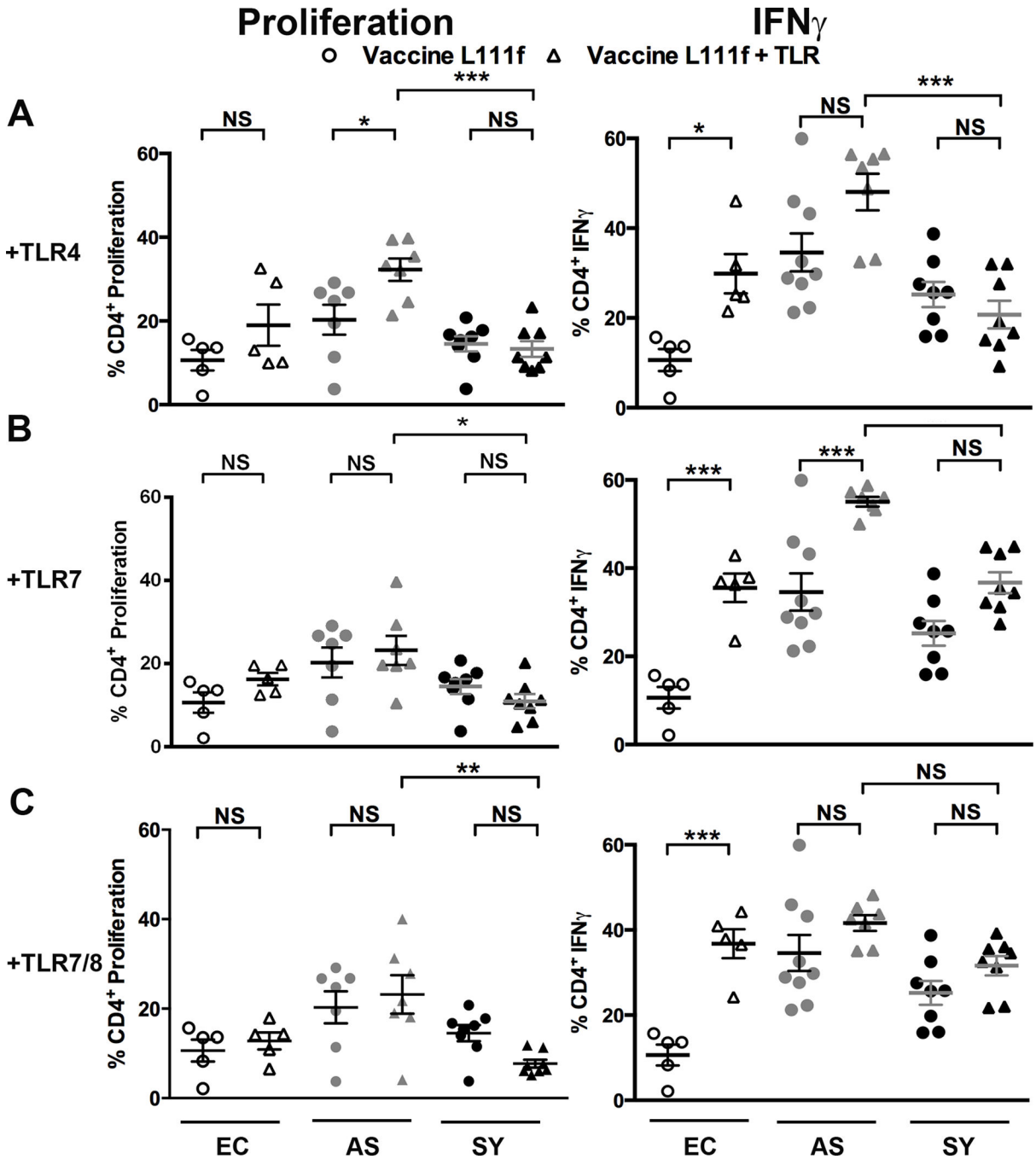

Fig. 3. Vaccine L111f and TLR agonists moderately increased Th1-responses in CD4$^+$ T cells from asymptomatic dogs

Whole blood was stimulated with either 1 μg Vaccine L111f (circles) or Vaccine L111f and TLR agonists (triangles). TLR agonists were added at 1μg for Glucopyranosyl Lipid Adjuvant [TLR4] (A), 0.1 μg for imiquimod [TLR7] (B) or 10 μg for resiquimod [TLR7/8] (C). Each dot represent an individual dog. EC = endemic control (open), AS = asympatomatic, *L. infantum* positive (grey), SY = symptomatic, *L. infantum* positive (black). Error bars are ± SEM. N=5–9 per group. * p<0.05, ** p<0.01, *** p<0.001. Statistical analysis was performed via one way-ANOVA.

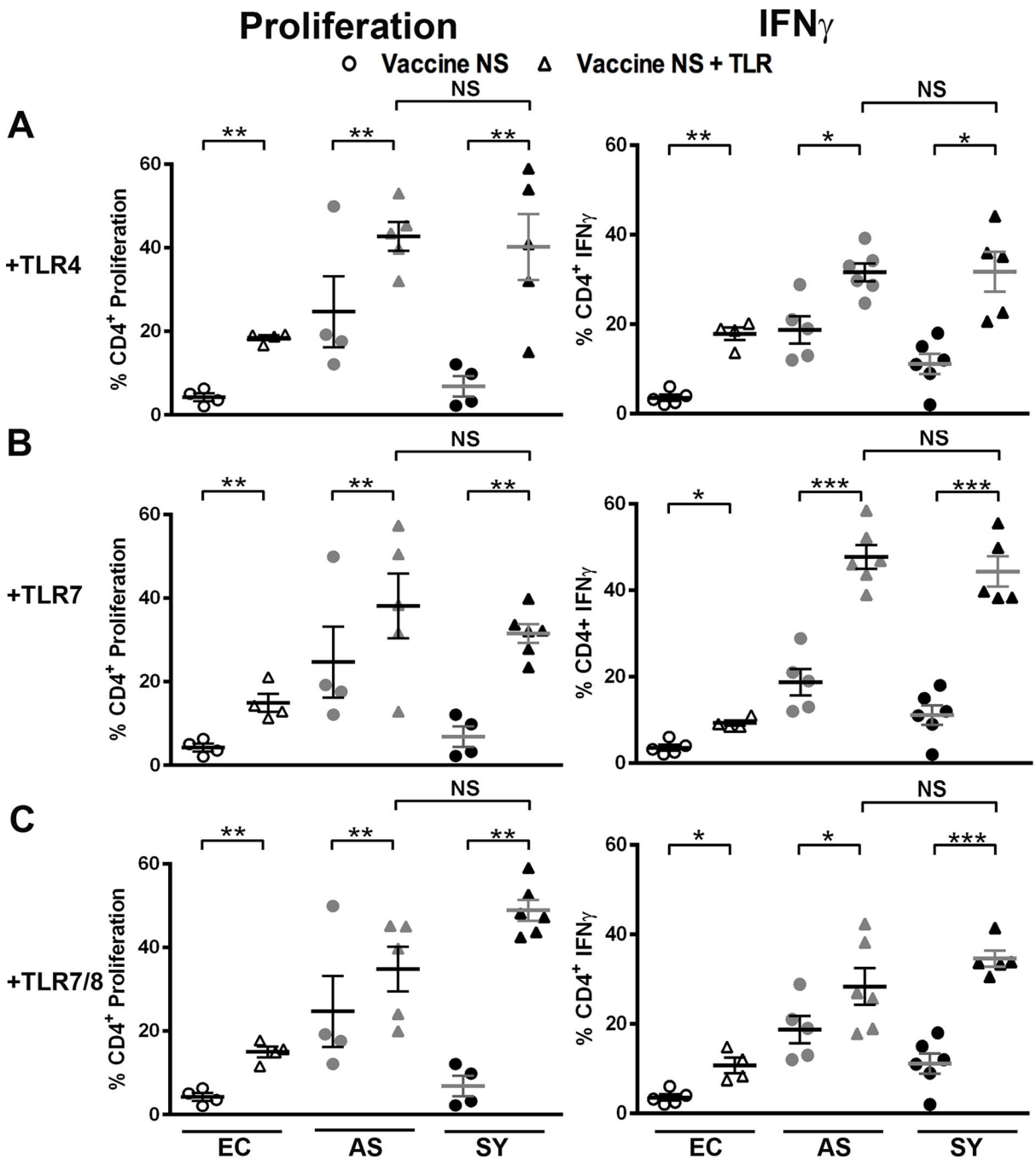

Fig. 4. Vaccine NS and TLR agonists increase Th1-responses in CD4$^+$ T cells from asymptomatic and symptomatic dogs

Whole blood was stimulated with either 1 μg vaccine NS (circles) or vaccine NS and TLR agonists (triangles). TLR agonists were added as in figure 3. Each dot represents an individual dog. EC = endemic control (open), AS = asympatomatic, *L. infantum* positive (grey), SY = symptomatic, *L. infantum* positive (black). Error bars are ± SEM. N=4–6 per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistical analysis was performed via one way-ANOVA.

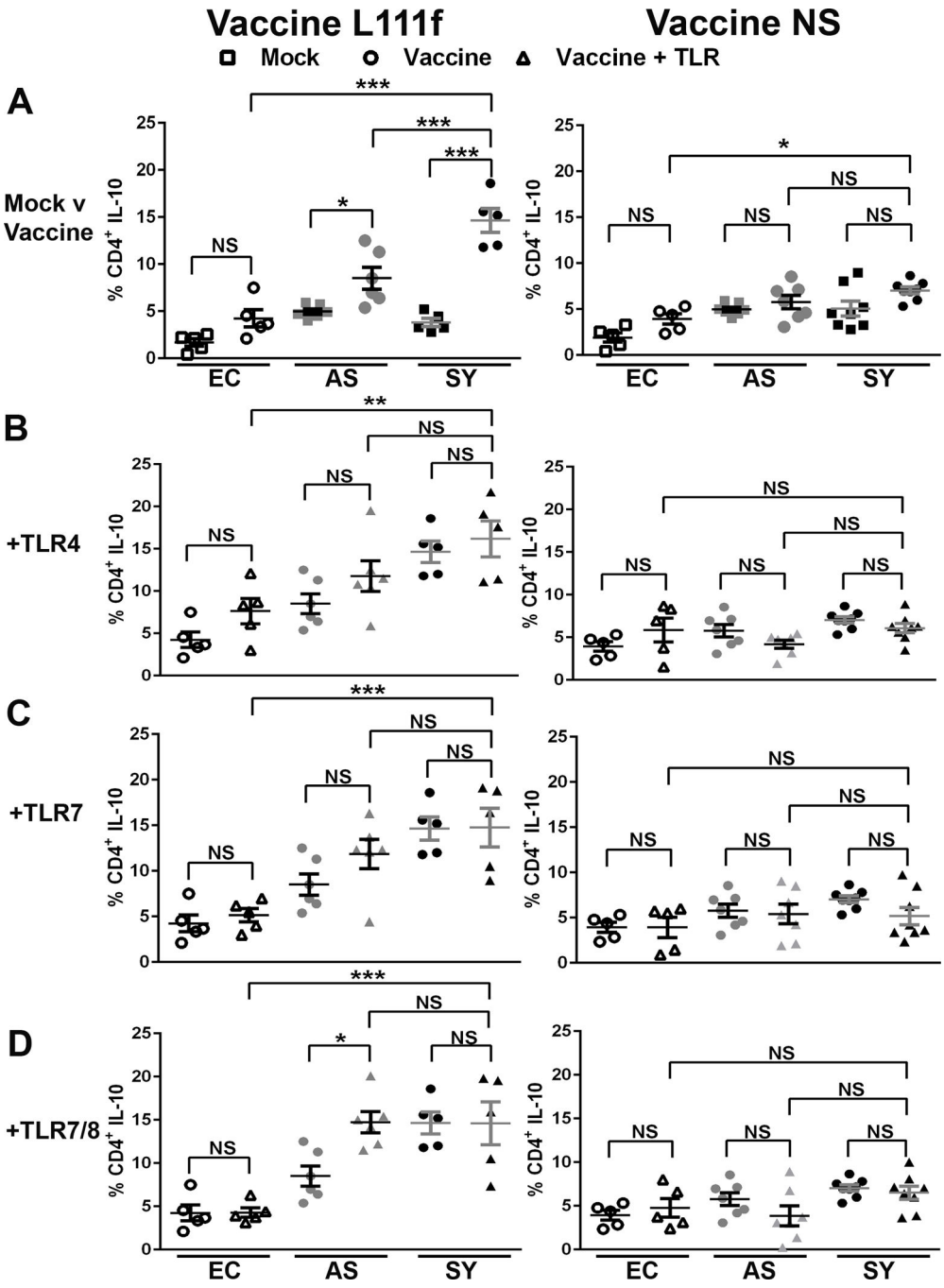

Fig. 5. Vaccine L111f increased IL-10 secretion from CD4$^+$ T cells of symptomatic dogs

Whole blood was stimulated (A) 1 μg vaccine (circles), or left untreated "mock" (squares) for 7 days. Vaccine, L111f or NS, used for stimulation is indicated in each column. In B to D whole blood was stimulated with either 1 μg vaccine L111f or NS alone (circles) or vaccine with TLR agonists (triangles). TLR agonists were added at 1 μg for TLR4 (B), 0.1 μg for TLR7 (C) or 10 μg for TLR7/8 (D). Each dot represents an individual dog. EC = endemic control (open), AS = asympatomatic, *L. infantum* positive (grey),SY = symptomatic, *L.*

infantum positive (black). Error bars are ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistical analysis was performed via one way-ANOVA.

Table 1

Clinical groups by diagnostic status; qPCR and serology[1]

Sex	Group (Infected)	Group (Clinical)	7/2013 IFAT[2]	7/2013 qPCR[2]	12/2013 IFAT[3]	12/2013 qPCR[3]
F	Infected	Symptomatic	512	Positive	256	Positive
F	Infected	Symptomatic	512	Positive	512	Borderline
F	Infected	Symptomatic	512	Positive	256	Positive
M	Infected	Symptomatic	128	Positive	512	Positive
F	Infected	Symptomatic	128	Negative	256	Borderline
F	Infected	Symptomatic	256	Negative	256	Positive
F	Infected	Symptomatic	256	Negative	512	Borderline
M	Infected	Symptomatic	0	Positive	512	Positive
M	Infected	Asymptomatic	128	Negative	32	Negative
F	Infected	Asymptomatic	64	Negative	16	Negative
M	Infected	Asymptomatic	128	Negative	64	Negative
M	Infected	Asymptomatic	0	Negative	128	Negative
M	Infected	Asymptomatic	32	Negative	16	Negative
F	Infected	Asymptomatic	16	Negative	0	Borderline
F	Infected	Asymptomatic	0	Negative	32	Borderline
M	EC[4]	Negative	0	Negative	0	Negative
F	EC	Negative	0	Negative	0	Negative
F	EC	Negative	0	Negative	0	Negative
F	EC	Negative	0	Negative	0	Negative
F	EC	Negative	0	Negative	0	Negative

[1] Serology by IFAT performed by the Centers for Disease Control, Atlanta, GA.

[2] status 6 months prior to blood collection.

[3] status at time of blood collection for vaccine antigen exposure.

[4] EC- endemic control, pen-matched, dogs