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## CONCISE COMMUNICATION

## Tissue Cytokine Responses in Canine Visceral Leishmaniasis

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To elucidate the local tissue cytokine response of dogs infected with *Leishmania chagasi*, cytokine mRNA levels were measured in bone marrow aspirates from 27 naturally infected dogs from Brazil and were compared with those from 5 uninfected control animals. Interferon- $\gamma$  mRNA accumulation was enhanced in infected dogs and was positively correlated with humoral (IgG1) but not with lymphoproliferative responses to *Leishmania* antigen in infected dogs. Increased accumulation of mRNA for interleukin (IL)-4, IL-10, and IL-18 was not observed in infected dogs, and mRNA for these cytokines did not correlate with antibody or proliferative responses. However, infected dogs with detectable IL-4 mRNA had significantly more severe symptoms. IL-13 mRNA was not detectable in either control or infected dogs. These data suggest that clinical symptoms are not due to a deficiency in interferon- $\gamma$  production. However, in contrast to its role in human visceral leishmaniasis, IL-10 may not play a key immunosuppressive role in dogs.

*Leishmania infantum* and *L. chagasi*, which cause human and canine visceral leishmaniasis (VL) in Europe and Latin America, are maintained largely in domestic dog populations. Canine VL is a major veterinary and public health problem in areas where the disease is endemic and has been suggested as a good model for investigating the pathogenesis of human VL. Both diseases are associated with depressed peripheral antigen-specific interferon (IFN)- $\gamma$  production [1, 2], which can be reversed by neutralization of interleukin (IL)-10 in human peripheral blood mononuclear cell (PBMC) cultures [3, 4]. It is surprising that IFN- $\gamma$  mRNA accumulation is observed in the tissues of patients with active VL and that it remains relatively unaltered after curative chemotherapy. In contrast, tissue IL-10 mRNA accumulation decreases after successful chemotherapy, which suggests that IL-10-mediated inhibition of IFN- $\gamma$ -induced macrophage activation, rather than regulation of IFN- $\gamma$  production per se, is central to the pathogenesis of human VL [3–5]. Th2 cytokines, including IL-4 and IL-13, are

variably expressed in human VL, and there is no direct evidence that they inhibit protective responses [3, 6]. Despite accumulating data on human VL, the paucity of comparable data on canine VL has precluded further comparisons.

To address this issue, we measured cytokine (IFN- $\gamma$  and IL-4, IL-10, IL-13, and IL-18) mRNA levels in bone marrow biopsy specimens from dogs with naturally acquired VL in Brazil. These dogs were part of a larger cohort study of *L. chagasi* infection [7, 8]. Here, we provide a comparison of tissue cytokine mRNA accumulation in infected and uninfected dogs and describe the relationships among cytokine mRNA accumulation, anti-*Leishmania* immune responses, and clinical status.

## Materials and Methods

**Study animals.** Details of the study site (Salvaterra, Marajó Island, Pará, Brazil) are described elsewhere [7]. A cohort study was done using 126 initially uninfected dogs in a VL-endemic area; the dogs were sampled approximately every 2 months for  $\leq 823$  days (April 1993 to July 1995). During the study, a proportion of dogs became infected as a result of natural disease transmission; dogs were not experimentally infected. Cytokine mRNA was assessed in 29 of these study dogs (27 infected and 2 uninfected) in May or July 1995, after they were exposed to infection for 208–798 days, as well as in 3 uninfected, unexposed dogs from the city of Belém. Of the 29 exposed dogs, 27 showed evidence of infection. Among 21 of these dogs, infection was confirmed by parasitologic or polymerase chain reaction (PCR) analysis or xenodiagnosis; the other 6 dogs mounted both positive cellular and humoral responses to *Leishmania* antigen. For the purpose of this study, we considered

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all 27 dogs to be infected; comparable results were obtained when analysis was restricted to dogs with confirmed infection. The time of infection was estimated as the earliest time infection was detected by parasitologic, PCR, or serologic analysis [8].

**Clinical examination.** Dogs were scored for 6 symptoms of canine VL: alopecia, dermatitis, chancres, conjunctivitis, onychogryphosis (excessive nail growth), and lymphadenopathy (enlarged popliteal lymph nodes). Each symptom was scored on a semiquantitative scale from 0 (absent) to 3 (severe); scores were added to give an overall clinical score. Dogs were classified as healthy (score, 0–2), oligosymptomatic (score, 3–6), or polysymptomatic (score, 7–18). Severely incapacitated dogs were killed with sodium pentobarbitone.

**Sampling.** Dogs were anesthetized with a mixture of medetomidine hydrochloride and ketamine. A 20-mL blood sample was obtained from the jugular vein. Bone marrow was aspirated from the iliac crest with a 16 × 25-mm Klima needle (Veterinary Instruments) into a 20-mL syringe containing EDTA, and an aliquot was preserved immediately in liquid nitrogen until used for RNA extraction.

**Reverse transcription-PCR analysis.** Total RNA was extracted, using Tri reagent (Sigma), and then was treated with DNase I (Life Technologies) and was reverse-transcribed (random primers, Superscript II; Life Technologies), according to manufacturer's instructions. Diluted cDNA (1.5  $\mu$ L) was amplified with PCR Master Mix (ABgene) and 10 pmol of each primer in a total volume of 25  $\mu$ L. Reaction conditions were 94°C (1 min), 50°C–55°C (1.5 min), and 72°C (2 min); annealing temperatures were 55°C for the housekeeping gene  $\beta$ -actin and IL-18 and 50°C for other cytokines. Negative controls (water) were run in each set of PCRs. Six microliters of each PCR product was transferred to nylon membrane, was probed with a digoxigenin-labeled internal oligonucleotide (Boehringer Mannheim), according to the manufacturer's instructions, and was assessed by probe binding in a micro-beta counter (Wallac).

**Oligonucleotide primers and probes.** Primers and probes were adapted from published sources using canine sequences from GenBank [9–11]. Forward and reverse primers, internal probes, and the number of cycles, respectively, were as follows:  $\beta$ -actin, CTTCTACAACGAGCTGCGCG, TCATGAGGTAGTCGGTCAGG, and CACACGGTGCCCATCTATGA (29 cycles); IFN- $\gamma$ , TTTTGAA-GAAATGGAGAGAGG, AAATTCAAATAGTGCTGGCAGG, and AAAGAGACAATTTGGCTCTG (40 cycles); IL-4, TATTAATGGGTCTCACCTCCCA, TTGATTCATTCATAGAACAG, and AAGTTATGTCCGTGGACAAAG (45 cycles); IL-10, TACTGGGTTGCCAAGCCCT, TTCACAGAGAAGCTCAGTAAAT, and CACAGGGAAGAAATCGGTGA (40 cycles); IL-13, CTGTGGTTCATTGCTCTCACCT, TCACCAACTGGATCACT-TCAA, and CTCAAGGAGCTCATTGAGGAG (45 cycles); and IL-18, ATGGCTGTAACCTAATAGAAGAC, CTAGCTCTT-GTTTTGAACAGTGAAC, and TACTTTGGCAAGCTTGAA-CC (32 cycles).

**Quantification of PCR analysis.** Samples were amplified, blotted, and probed in duplicate in a single 96-well plate for each cytokine. On each plate, a dilution series of positive control cDNA was included. The positive control cDNA consisted of pooled sample cDNA, except for IL-4 and IL-13, in which cases, cDNA was prepared from a 12-h culture of concanavalin A-stimulated PBMC from an uninfected dog. The dilution of each sample cDNA was

adjusted to approximately equalize  $\beta$ -actin cDNA levels. For each cytokine, undiluted positive control cDNA was assigned an arbitrary number of units of mRNA (1000 U), a standard curve of counts per minute versus log (mRNA units) was constructed, and the number of units of mRNA in each sample was estimated. All samples were on the linear range of the standard curve for  $\beta$ -actin, IL-10, and IL-18; samples below the linear range for IFN- $\gamma$  were scored as zero. For IL-4 and IL-13, all samples were below the linear range, and samples were scored as detectable (i.e., counts per minute greater than background) or not detectable.

**Immunologic assays.** Lymphoproliferative responses to sonicated *L. chagasi* antigen were assessed, as described elsewhere [8], and were expressed as a stimulation index (SI [i.e., the ratio of the geometric mean counts per minute of stimulated wells to unstimulated wells]). IgG and IgG subclass responses to *L. chagasi* antigen were measured by ELISA, as described elsewhere [7], using a panel of monoclonal antibodies [12]. Results were expressed as arbitrary units calculated from a reference serum titrated on each plate. Among the 27 infected dogs, lymphoproliferative responses were measured in 26, IgG subclass responses in 26, and both in 25.

**Statistical analysis.** Levels of IL-10, IL-18,  $\beta$ -actin mRNA, and antibodies and cellular responses were approximately log-normally distributed and were analyzed after log-transformation. IL-4 mRNA was analyzed as a binary variable by logistic regression. IFN- $\gamma$  mRNA levels were not normally distributed after transformation and were analyzed by nonparametric statistics. To control for the relative amount of mRNA in each sample, the  $\beta$ -actin mRNA level was included as a covariate in parametric analyses, and IFN- $\gamma$  mRNA levels were divided by the  $\beta$ -actin mRNA level before analysis. The clinical score was analyzed as a Poisson variable, with overdispersion accounted for by adjusting the scale parameter to equal the Pearson's  $\chi^2$  divided by the residual degrees of freedom. Stata 6.0 (Stata) was used for analyses.

## Results

**Cytokine mRNA levels in infected and uninfected dogs.** All 5 uninfected dogs had detectable IL-10 and IL-18 mRNA in their bone marrow. However, IFN- $\gamma$  and IL-13 mRNAs were undetectable, and IL-4 was detected in only 1 of 5 dogs (table 1). In infected dogs, there was a significant increase in bone marrow IFN- $\gamma$  mRNA ( $P = .015$ , Mann-Whitney  $U$  test). However, there was no increase in the accumulation of mRNA for IL-10 ( $P = .62$ ) or IL-18 ( $P = .52$ ) or in the proportion of dogs with detectable IL-4 ( $P = .35$ ). No infected dog produced detectable levels of IL-13 mRNA. Cytokine mRNA accumulation in infected dogs did not vary significantly with estimated time since infection. Of the 27 infected dogs, 25 were seropositive at the time of sampling, and 2 had serorecovered. Of 26 infected dogs, 23 had a positive cellular response ( $SI > 3$ ).

**Relationships between clinical status and immune responses in infected dogs.** There was a significant positive relationship between clinical score and detectable IL-4 mRNA in infected dogs ( $\chi^2 = 3.98$ ;  $P = .046$ ). There was a similar relationship between clinical score and anti-*Leishmania* IgG levels ( $\chi^2 = 10.71$ ;  $P = .0011$ ), but there was no relationship with cellular

**Table 1.** Cytokine mRNA levels (arbitrary units) in uninfected and *Leishmania chagasi*-infected dogs.

Variable	Uninfected (n = 5)	Asymptomatic (n = 14)	Oligosymptomatic (n = 7)	Polysymptomatic (n = 6)
Interleukin-18	39 (14–104)	43 (30–62)	34 (15–74)	50 (27–92)
Interleukin-10	28 (11–72)	50 (28–89)	37 (16–86)	63 (24–170)
Interferon- $\gamma$	0 (0–0)	5 (0–13)	8 (0–33)	11 (0–21)
Interleukin-4	0.20 (0.01–0.72)	0.29 (0.08–0.58)	0.43 (0.10–0.82)	0.67 (0.22–0.96)
$\beta$ -Actin	30 (23–40)	56 (41–77)	35 (22–57)	50 (31–80)
Days after infection	0	397 (289–505)	382 (208–557)	459 (363–554)
IgG ( $\times 10^{-3}$ )	1.5 (0.5–4.5)	11 (5–25)	141 (66–302)	185 (65–528)
Stimulation index	1.9 (0.59–6.3)	9.5 (5.5–17)	12 (4.2–32)	5.1 (1.7–15)

NOTE. Data are geometric mean (95% confidence interval [CI]), except for the following: interferon- $\gamma$ , which is shown as median (interquartile range); interleukin-4, which is shown as proportion of dogs producing detectable interleukin-4 (95% CI); and days after infection, which are shown as arithmetic mean (95% CI). Infected dogs were grouped according to their clinical score.

response or other cytokines. When the relationship between clinical score and immune responses was tested by multiple regression, there was a highly significant positive relationship between clinical score and both IgG and IL-4 mRNA, and there was a negative relationship with both cellular responsiveness and IL-18 mRNA (table 2).

*Relationships between immune responses in infected dogs.*

There was a significant positive correlation between IFN- $\gamma$  mRNA accumulation and anti-*Leishmania* IgG in infected dogs (Kendall's  $\tau = .400$ ;  $n = 27$ ;  $P = .005$ ). This correlation was significant for IgG1 ( $\tau = .323$ ;  $n = 26$ ;  $P = .027$ ); however, it was weaker and not significant for IgG2 ( $\tau = .222$ ;  $n = 26$ ;  $P = .14$ ), IgG3 ( $\tau = .164$ ;  $n = 26$ ;  $P = .27$ ) and IgG4 ( $\tau = .127$ ;  $n = 26$ ;  $P = .40$ ). There were no other significant correlations between cytokine mRNA levels and either antibody or cellular responses. IL-18 and IL-10 mRNA levels were significantly positively correlated in infected dogs (partial  $r = .593$ ;  $P = .0014$ ). There were no other significant correlations among cytokine mRNA levels.

**Discussion**

Tissue cytokine responses have been measured in human VL and in hamster and murine models [3–5, 13, 14], yet, to our knowledge, a similar approach has not been applied previously to canine VL. Here, in a study of naturally infected dogs, the primary feature identified as distinguishing canine VL from that in man [3–5], mouse [13], and hamster [14] is the absence of high-level accumulation of IL-10 mRNA in infected tissues. The basal levels of IL-10 mRNA in normal bone marrow presumably reflect its regulatory role in hematopoiesis [15], although concurrent infections cannot be ruled out. However, even in dogs with severe disease, IL-10 mRNA accumulation was comparable with that of uninfected control dogs. Furthermore, there was no relationship between IL-10 and any of the immune parameters measured. In contrast, human VL is associated with increased tissue accumulation of IL-10 mRNA and increased levels of serum IL-10 [3–6]. Indeed, a recent report suggests that serum IL-10 is independently regulated com-

pared with other Th2 (IL-4 and IL-13) or Th1 (IFN- $\gamma$ ) cytokines and that IL-10 in the absence of these other cytokines is associated with relapsing disease [6].

In contrast to the IL-10 results, our data show that mRNA accumulation for IFN- $\gamma$  was, as expected, increased as a consequence of infection. Indeed, dogs with asymptomatic and polysymptomatic infections accumulated similar levels of IFN- $\gamma$  mRNA. IFN- $\gamma$  production was strongly associated with antibody production, which suggests a general up-regulation of anti-*Leishmania* immune responses in heavily infected hosts. These data support the notion that, as in humans, mice, and hamsters, levels of tissue IFN- $\gamma$  in dogs may not be the primary indicator of disease status or eventual cure [3–6, 13, 14], but, rather, that the inhibition of macrophage activation may play a key role [16]. We found no evidence of a role for IL-10 in this process, although it is possible that changes in IL-10 message during infection may have been masked by any generalized up-regulation of bone marrow mRNA synthesis. Further studies including other inhibitory cytokines, such as transforming growth factor- $\beta$ , are now required.

The role of IL-4 in VL remains controversial. Serum IL-4 has been detected readily in some clinical studies but has not been detected in others (see discussion in [6]). Although IL-4 has been detected in murine VL, studies in IL-4<sup>-/-</sup> mice surprisingly indicate an essential role for this cytokine in both natural resistance and successful chemotherapy [17]. Here, we found only a very low accumulation of IL-4 mRNA in some, but not all, infected dogs. However, there was a significant positive relationship be-

**Table 2.** Multiple regression analysis of the relationship between clinical score and immune responses in *Leishmania chagasi*-infected dogs.

Variable	Slope (SE)	$\chi^2$	P
Interleukin-18	-1.229 (0.557)	4.87	.027
Interleukin-10	-0.256 (0.274)	0.87	.351
Interferon- $\gamma$	0.033 (0.021)	2.57	.109
Interleukin-4	1.646 (0.524)	9.86	.0017
$\beta$ -Actin	0.596 (0.610)	0.96	.328
IgG	1.046 (0.275)	14.46	.0001
Stimulation index	-1.981 (0.609)	10.57	.0011

tween IL-4 detection and disease severity, which suggests that IL-4 production is associated with pathology.

IL-18, which may play an important role in the priming for IFN- $\gamma$  production by T cells, has not previously been evaluated in VL. Here, we detected IL-18 mRNA accumulation in both control and infected dogs but did not identify any association with infection per se. However, there was some evidence for a protective role of IL-18 in infected dogs: there was a negative relationship between IL-18 and clinical score, although this was evident only when controlling for other immune responses. There also was a positive correlation between IL-18 and IL-10, which may reflect coordinate regulation of these macrophage-derived cytokines.

In summary, this study indicates that some, but not all, of the features of cytokine regulation are shared between the various forms of VL in different hosts. This finding cautions against wholesale extrapolation of immune mechanisms between these different diseases.

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