

Murine visceral leishmaniasis: IgM and polyclonal B-cell activation lead to disease exacerbation

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In visceral leishmaniasis, the draining LN (DLN) is the initial site for colonization and establishment of infection after intradermal transmission by the sand fly vector; however, little is known about the developing immune response within this site. Using an intradermal infection model, which allows for parasite visceralization, we have examined the ongoing immune responses in the DLN of BALB/c mice infected with *Leishmania infantum*. Although not unexpected, at early times post-infection there is a marked B-cell expansion in the DLN, which persists throughout infection. However, the characteristics of this response were of interest; as early as day 7 post-infection, polyclonal antibodies (TNP, OVA, chromatin) were observed and the levels appeared comparable to the specific anti-leishmaniasis response. Although B-cell-deficient J_hD BALB/c mice are relatively resistant to infection, neither B-cell-derived IL-10 nor B-cell antigen presentation appear to be primarily responsible for the elevated parasitemia. However, passive transfer and reconstitution of J_hD BALB/c with secretory immunoglobulins, (IgM or IgG; specific or non-specific immune complexes) results in increased susceptibility to *L. infantum* infection. Further, J_hD BALB/c mice transgenetically reconstituted to secrete IgM demonstrated exacerbated disease in comparison to WT BALB/c mice as early as 2 days post-infection. Evidence suggests that complement activation (generation of C5a) and signaling via the C5a receptor (CD88) is related to the disease exacerbation caused by IgM rather than cytokine levels (IL-10 or IFN- γ). Overall these studies indicate that polyclonal B-cell activation, which is known to be associated with human visceral leishmaniasis, is an early and intrinsic characteristic of disease and may represent a target for therapeutic intervention.

Key words: Antibody · C5a · Parasitic protozoan

Introduction

Visceral leishmaniasis (VL) is a potentially fatal human disease caused by the intracellular protozoan parasites *Leishmania donovani* and *L. infantum/L. chagasi*. The immune response to

VL is complex and has been shown to be organ-specific, differing significantly dependent upon the site of infection examined (liver versus spleen) [1, 2]. Although the lymph node is thought to be analogous to the spleen, there are considerable developmental as well as structural and functional differences [3, 4]. Reflective of this is the fact that although both spleens and lymph nodes from fatal human cases of VL exhibit destruction of normal architecture, follicular DC (FDC) and GC are lost in spleens, while continuing to be present in lymph nodes [5]. However, few

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experimental studies to date have examined the lymph node responses that occur as a result of infection and have instead focused on the spleen where, akin to observations in humans, the destruction of FDC and GC is evident [6].

Although these observations in the spleen might appear to preclude a role for B cells in disease, other evidence from both human patients and murine model studies indicate that B-cell activation leads to disease exacerbation. Mouse model studies of VL have demonstrated that C57BL/6 B-cell-deficient mice are relatively resistant to intravenous infection [7]. Further, B-cell dysfunction, as evidenced by hypergammaglobulinemia, and the presence of significant non-specific (polyclonal) and autoimmune antibodies are evident and are hallmarks of human VL [8–14].

Moreover, in the case of cutaneous leishmaniasis, polyclonal B-cell activation has been reported in response to *L. major* infection [15, 16]; however, the antigens recognized were not defined. Further, B-cell functions (antibody and antigen presentation) have been shown to lead to disease exacerbation for infection with *L. major* [17], while IgG production but not B-cell antigen presentation promotes infection/disease in the case of *L. mexicana* complex parasites [18–21]. However, the role of immunoglobulins in leishmaniasis is controversial and may depend on the nature of the antigen presenting cell involved [22, 23]. Therapeutic approaches targeting B cells have been shown to be effective for the treatment of autoimmune diseases [24, 25] through not only the reduction of autoantibody levels but also the modulation of T-cell responses. Consequently, the mechanisms by which B cells potentially contribute to disease in VL are of interest and may represent targets for intervention.

In the intradermal murine VL infection model, it has been noted that parasite levels continuously increase with time in the draining LN (DLN) as well as the spleen, while clearing in the liver and skin [26]. Therefore, the lymph node, as well as the spleen, is a site for parasite persistence. In this study, we have focused on the events in the lymph node. Early in infection in the DLN, there was a dramatic rise of the B-cell population, which persists through chronic infection. Interestingly, the antibody response was polyclonal (specific and non-specific). However, neither B-cell-derived IL-10 nor antigen-presentation was found to be relevant to B-cell mediated disease exacerbation. Secretory IgM as well as IgG (specific and non-specific) were found to contribute to disease susceptibility and appear to act in part through the activation of complement and generation of C5a. These findings extend earlier murine VL studies [7] and indicate that a polyclonal B-cell response is an early and intrinsic feature of VL, which helps to establish and maintain infection in the mammalian host.

Results

Histology of infected DLN

Immunofluorescence staining examining the T- and B-cell areas/distribution within the DLN of BALB/c mice was utilized to gain an understanding of cellular architecture in response to

L. infantum infection. As shown in Fig. 1, the DLN becomes enlarged and a loss of normal architecture is evident as early as 3 days post-infection. Instead of the discrete B- and T-cell zones observed in normal LN, B cells are found in both T- and B-cell areas from day 3 post-infection through the chronic phase of the disease (3 months post-infection). As is expected in an ongoing immune response, GC formation and the appearance of FDC in the B-cell follicle regions are observed by day 6 and maintained throughout infection. Overall, these observations differ from what has been reported in the spleen [2, 6]; however, these results support a role for the lymph node in B-cell activation and responses during VL and potentially the hypergammaglobulinemia that is characteristic of this disease.

Given the increase in B cells observed in the DLN, it was of interest to determine whether this was the result of increased proliferation. At various times post-infection with *L. infantum* promastigotes, WT BALB/c mice were evaluated for cellular proliferation in the DLN using BrdU incorporation (Fig. 2). Both T- and B-cell populations initially expand in response to infection. However, B cells continue to proliferate, whereas the level of T-cell proliferation was reduced to levels found in naïve mice by 30 days post-infection. The reduction in T-cell responsiveness in the DLN is similar to recent observations in the spleen, where the expression of B7-H1 was related to the decreased T-cell responsiveness [27]; however, the mechanisms involved in the DLN remain to be determined. The overall increase in B cells (14-fold at 30 days post-infection) is much more prominent than what is observed for the T-cell populations, which display a more modest 3.5-fold increase. Overall, these increases in lymphocyte populations (Table 1) result in a change in the B- to T-cell ratio in the DLN, from 0.29 in the uninfected LN to 0.86 in infected LN by 14 days post-infection. FACS analyses indicated that the B-cell populations were activated, expressing the early activation marker CD69⁺. Although CD69 expression decreased in later stages of infection, it remained above background. Thus, constant local stimulation and expansion of B cells occurs and represents a continued source of new B cells in the DLN. However, this does not exclude the possibility that selective B-cell recruitment and retention may also contribute to the increased B-cell numbers within the DLN.

The expansion and maintenance of B cells throughout the DLN (T- and B-cell areas), in addition to the increasing parasitemia, allude to aberrant B-cell function and a potential B-cell contribution to parasite persistence. Consequently, we examined the nature of this B-cell response and mechanisms by which these cells might modulate *L. infantum* infection.

B-cell activation leads to polyclonal antibody production

Hypergammaglobulinemia and non-specific antibody responses are hallmarks of VL normally noted in the chronic stage of the disease [8, 12–14, 28]. Given the level of B-cell activation observed early after intradermal infection, we were interested in the nature and specificity of this B-cell response. To determine

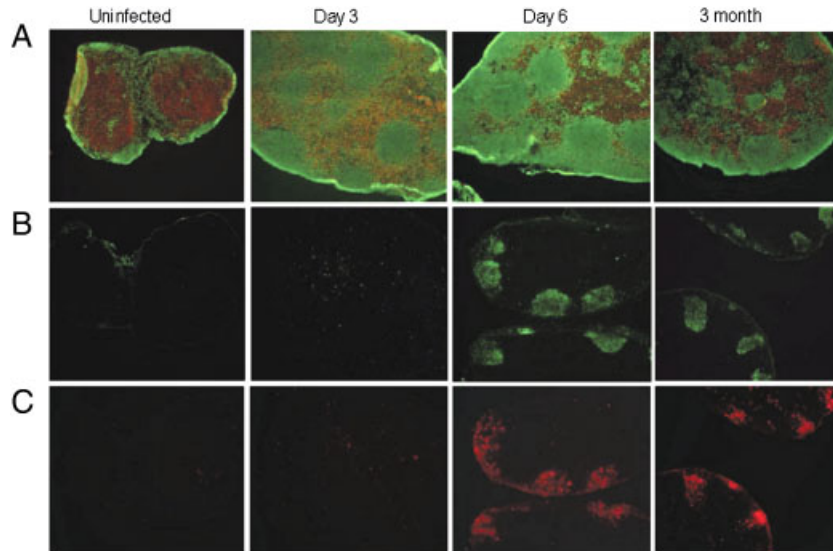


Figure 1. Histology of infected lymph nodes. Immunohistochemical analyses of sections from the DLN of uninfected mice and *L. infantum*-infected mice (days 3 and 6, and at 3 months post-infection). DLN were stained with (A) CD3 (red; T cells) and B220 (green; B cells), (B) peanut agglutinin (GC), or (C) FDC-M1 (follicular DC). There is an early and persistent B-cell expansion and apparent loss of T cells in the DLN. GC and follicular DC (FDC) are evident at day 6 and persist through the chronic stage of the disease (3 months). Data are representative of at least three experiments.

this, BALB/c mice were infected with *L. infantum* and at various times post-infection serum was recovered. Serum antibody levels (IgG and IgM-total) and specificity were assessed by ELISA (Fig. 3). These results indicate, as expected, an increase in antibody production against *Leishmania* antigen (soluble leishmania antigen (SLA)), which is detectable at 7 days after infection and increases with time post-infection. In addition, significant antibody responses were also observed to non-leishmanial antigens (TNP, OVA, chromatin).

To further evaluate the polyclonal responses, ELISPOT analyses for antigen-specific antibody-producing cells (total Ig: IgG+IgM) were performed (Table 2). These data confirmed serological analyses and indicated the fold-increases in the overall antibody responses (leishmanial and non-specific). The polyclonal response, measured by ELISPOT, in the DLN was observed earlier (day 7) than in the spleen (day 14). Overall, the anti-SLA response and the polyclonal antibody responses (OVA and chromatin) appear later in the spleen than the DLN and appears to reflect the relatively lower level of parasites present [26]. Interestingly, the increase of B cells producing anti-OVA and/or anti-TNP-BSA was comparable to or greater than that found for B cells producing anti-*Leishmania* antibodies. These data suggest that overall the polyclonal response (non-specific) was comparable to the anti-parasite response. Therefore, polyclonal B-cell activation is an early, intrinsic, and significant response to infection.

B cells do not contribute to disease susceptibility through IL-10 production or antigen presentation

Given the persistent and polyclonal activation of B cells in response to intradermal infection, the impact of B cells on infection was further examined. Initially, B-cell-deficient BALB/c

J_H D and WT BALB/c mice were infected with *L. infantum*; parasite burdens were determined (DLN, spleens, livers) at 1 and 2 months post-infection. These experiments (data not shown) revealed that BALB/c J_H D mice had reduced parasite numbers at all tissue sites. Overall, these results are similar to those reported for intravenous infection of C57BL/6- μ MT mice [7]; however, the mechanisms by which B cells contribute to increased parasitemia were not established. Consequently, the effects of B-cell antigen presentation, IL-10 production, and secretory immunoglobulin (IgG and IgM) on infection were examined.

One of the mechanisms by which B cells potentially contribute to pathogenesis is through production of immunomodulatory cytokines, such as IL-10. IL-10 has been implicated in disease progression and severity in human patients and in mice [29–31]. Previous studies, using intravenous infection, indicate that mice deficient in IL-10 are able to resolve infection [32]. Further, flow cytometric analyses of intradermally infected mice indicated that in the early DLN response to infection (day 5) B cells produce IL-10 (Fig. 4A), suggesting that early IL-10 production and in particular B-cell-derived IL-10 could be important for infection and parasite survival. Initially, the role of IL-10 in the course of intradermal infection in the lymph node and other tissues was examined using BALB/c mice deficient in IL-10. As seen in Table 3, IL-10-deficient mice have reduced parasite levels; a reduction in parasite levels in the DLN is observed as early as day 3 post-infection. Notably, the effect of IL-10 upon infection was observed at all tissue sites and appears to increase with time post-infection. Therefore, IL-10 appears to contribute to both the early and later phases of disease.

However, to ascertain the direct consequence of B-cell-derived IL-10 on disease, bone marrow chimeras were employed. Chimeric mice were produced using BALB/c J_H D, in which the B cells were derived from either IL-10-deficient or WT BALB/c mice. The J_H D bone marrow recipients, along with BALB/c WT mice, were then

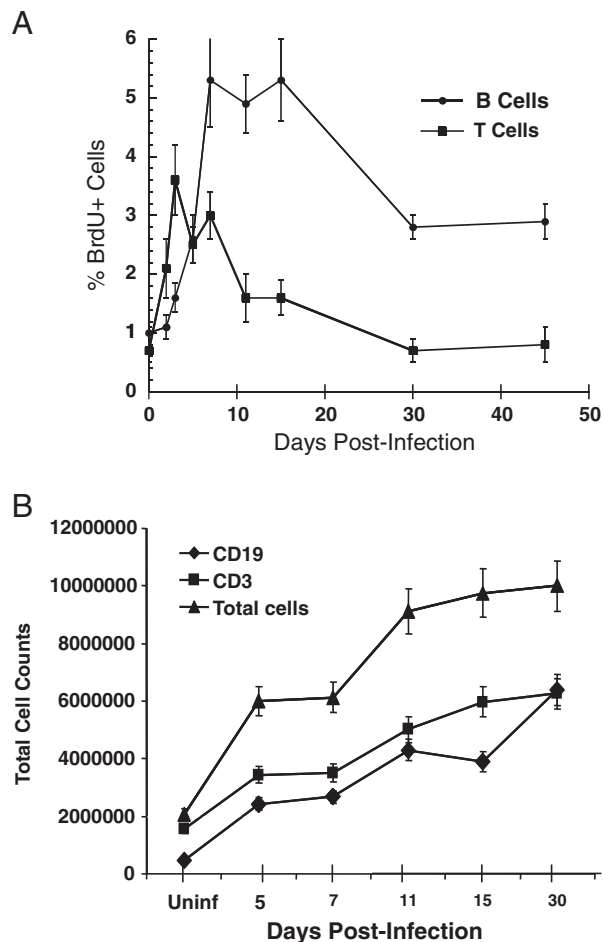


Figure 2. Lymph node responses to *L. infantum* infection. Lymph node T and B lymphocyte responses of BALB/c mice intradermally infected with *L. infantum*. (A) Cellular proliferation was evaluated using BrdU incorporation. BrdU was injected intraperitoneally 4 h prior to obtaining cells at the indicated times for FACS analysis. Results are shown as the percentage of BrdU⁺ T cells (CD3⁺) and the percentage of BrdU⁺ B cells (CD19⁺) cells over time. (B) Increases in cell numbers present in the DLN (total lymph node cells, B cells (CD19⁺), T cells (CD3⁺)). Data show mean \pm SEM ($n = 3\text{--}4/\text{time point}$) and are representative of at least two experiments.

Table 1. B-cell activation and expansion in the draining lymph node as a response to infection^{a)}

Days post-infection	B-cell/T-cell ratio	%CD69 ⁺ B cells
Uninfected	0.29	16.3 \pm 2.1
2	0.62	79.5 \pm 8.7**
5	0.76	50.1 \pm 6.6**
14	0.86	24.9 \pm 3.8*
37	1.02	26.2 \pm 7.5 ⁺

^{a)} The ratio of B cells/T cells found in the DLN at various times post-infection with *L. infantum*. An early B-cell activation (CD69⁺) is evident, which decreases but remains above background. The increase in B-cell CD69 expression precedes increasing BrdU incorporation (Fig. 2); the kinetics is consistent with B-cell activation followed by proliferation. Data are representative of two experiments and show mean \pm SE ($n = 3$) per time point. ** $p < 0.001$, * $p = 0.03$, ⁺ $p = 0.09$; Student's *t*-test.

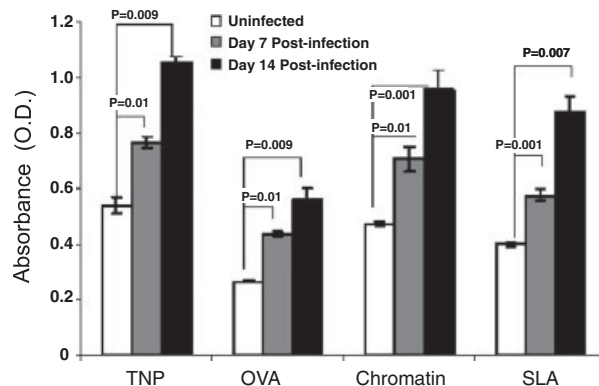


Figure 3. Early serum antibody responses in BALB/c mice infected with *L. infantum* are polyclonal. Sera were collected from mice at days 7 and 14 post-infection and assayed for antibody levels (total Ig (IgG + IgM)) to leishmanial (SLA) and non-leishmanial antigens ((TNP)₁₅-BSA, OVA, chromatin). Statistical significance is indicated and was determined using Student's *t*-test in comparison to uninfected controls for each antigen. Data show mean \pm SEM ($n = 5$ per group) and are representative of two experiments.

intradermally infected with *L. infantum*. Given the more effective control observed in IL-10-deficient mice at 1 month post-infection, parasite levels were determined (DLN, spleens, livers) at 1 month post-infection (Fig. 4B). J_HD mice receiving B cells from either WT or IL-10-deficient mice have parasite burdens that are comparable to WT mice. Overall, these results indicate that although IL-10 can promote parasite survival, B-cell-derived IL-10 is not essential for increased disease susceptibility observed in WT versus B-cell-deficient mice.

Consequently, we examined whether B-cell antigen presentation might be critical to the development of disease. Although controversial, it was possible that B-cell antigen presentation could drive a Th2-like response (IL-10 and IL-4) [17, 33, 34] and thereby potentially could skew ongoing T-cell responses to prevent healing. To examine this possibility, BALB/c WT, J_HD, and mIgM/J_HD mice genetically altered to contain functional B cells (surface IgM but producing no circulating antibody) [35, 36] were infected with *L. infantum*. Parasite burdens (DLN, spleens, livers) at 1 month post-infection (Fig. 4C) clearly indicate that mIgM/J_HD mice were comparable to J_HD mice in their resistance to infection. Therefore, B-cell antigen presentation fails to reconstitute mice to WT susceptibility.

Interestingly, the mIgM/J_HD mice were comparably resistant to infection as the J_HD mice and yet had IL-10 levels higher than the more susceptible WT mice (Table 4). Further, the overall IL-10/IFN- γ ratio did not appear to correlate with the disease susceptibility observed. Therefore, evidence from the mIgM/J_HD mice confirms the bone marrow chimera results indicating that B-cell-derived IL-10 is not critical for disease exacerbation.

Secretory immunoglobulin IgM causes disease exacerbation

Having determined that mice reconstituted for B cells (antigen presentation, IL-10 production) but lacking circulating antibody

Table 2. Early antibody responses in BALB/c mice infected with *L. infantum* are polyclonal^{a)}

	Antigen	ELISPOT lymph node (fold increase)	ELISPOT spleen (fold increase)
Day 7 post-infection	OVA	4.3±0.3*	1.0
	TNP(15)-BSA	27.0±1.6***	1.0
	SLA	4.1±0.6**	1.0
Day 14 post-infection	OVA	13.1±0.6***	8.1±0.2***
	TNP(15)-BSA	9.2±0.8**	4.7±0.21***
	SLA	7.6±0.3***	3.48±0.5*
	Chromatin	4.2±0.04***	4.1±0.2***

a) Elispot analyses of antigen-specific antibody producing cells were determined at days 7 and 14 post-infection. The number of cells secreting antibodies to soluble *Leishmania* antigen (SLA) as well as non-specific antigens (ovalbumin, (TNP)₁₅-BSA, chromatin) were quantified. Data represent the fold-increase in the number of antibody-producing cells over background levels (determined using cells from uninfected mice). Data are representative of two experiments and show mean ± SE (n = 5 mice per group). Background levels of antibody producing cells/10⁶ total cells were: in the spleen: TNP, 1728 ± 72; OVA, 372 ± 8; SLA, 1860 ± 252; chromatin, 222 ± 10; and in the draining lymph node: TNP, 616 ± 40; OVA, 238 ± 38; SLA, 556 ± 28; and chromatin, 44 ± 4. ***p < 0.001, **p = 0.002, *0.002 < p < 0.05; Student's t-test.

exhibit decreased susceptibility to infection with *L. infantum*, the effect of secretory immunoglobulin was examined. Initially, BALB/c (WT) and transgenic mice whose B cells can only express surface and secretory IgM antibody ($\{m+s\}$ IgM/J_HD) [36, 37] were infected with *L. infantum*. As shown in Fig. 5 and in contrast to what was observed for mice expressing only receptor/membrane IgM (mIgM/J_HD mice), the $\{m+s\}$ IgM/J_HD mice exhibited exacerbated infection. Disease exacerbation was evident as early as 2 days post-infection and persisted throughout infection. Parasite levels in the DLN, spleens, and livers of $\{m+s\}$ IgM/J_HD mice were 5- to 25-fold higher than those observed for WT mice.

Although B-cell activation in response to infection is evident in WT BALB/c mice (Fig. 1; Table 1) at early times post-infection, the level of IgM produced in the $\{m+s\}$ IgM/J_HD mice was significantly higher (Fig. 5C) than that in WT mice. Notably, IgM antibodies are commonly observed during active chronic VL [8, 9, 12, 38]. Taken together, these results suggest that secretory IgM can potentially contribute to the establishment and maintenance of infection in VL. This hypothesis was further examined through antibody-transfer experiments.

Reconstitution of J_HD mice with immunoglobulin (IgG or IgM) restores WT susceptibility to infection

To further examine the role of immunoglobulins in disease exacerbation, J_HD mice were passively reconstituted with serum from chronically infected WT or $\{m+s\}$ IgM/J_HD mice or mice immunized with amastigote membrane preparations or normal mouse serum (controls). The transfer of immune serum to the J_HD mice was found to restore susceptibility (parasitemia) to WT levels. Passive transfer of normal mouse serum into J_HD mice, however, failed to modulate disease resistance. Consequently, antibodies present during infection contributed to disease susceptibility in VL. These results (data not shown) were similar to those previously observed for cutaneous leishmaniasis (caused by *L. major*, *L. mexicana*, and *L. amazonensis*) [18–22] where antibody

contributes to disease progression. However, these results differ from those previously reported for murine VL [7] and also studies where the opsonized *L. major* organisms were found to stimulate DC function (IL-12 and antigen presentation) leading to parasite containment [23]. Therefore, to further validate that disease exacerbation is a direct result of circulating immunoglobulins (IgM and/or IgG) in murine VL, and to examine whether non-specific as well as specific antibodies are capable of promoting disease, passive antibody-transfer experiments were performed.

Purified immunoglobulins (from either OVA-immunized BALB/c mice (IgG), or naïve or chronically infected WT (IgG) or $\{m+s\}$ IgM/J_HD BALB/c mice (IgM)) were employed to reconstitute J_HD mice. The transfer of purified antibody from naïve (normal) mouse serum (IgG from WT or IgM from $\{m+s\}$ IgM/J_HD, respectively) did not lead to increases in parasite levels (Fig. 6). In contrast, the transfer of purified immunoglobulins from chronically infected WT (IgG) or $\{m+s\}$ IgM/J_HD mice (IgM) led to significantly increased parasite levels in the DLN, spleen, and liver (Fig. 6). Further, the transfer of purified anti-OVA IgG to J_HD mice, which were then given OVA antigen at the time of infection, led to increased parasite levels in the spleen and liver. These results indicate that non-leishmanial antigen-antibody complexes could also impact on disease progression and are of interest, given the early polyclonal activation observed. Although the transfer of IgG appeared somewhat more effective in enhancing parasitemia (in the LN, spleen, and liver) than IgM, this may be due, in part, to the known differences in half-life of these two immunoglobulins (6–8 days *versus* 2 days, respectively) [39]. However, overall these results clearly indicate that antigen-antibody complexes (IgG or IgM; specific and non-specific) can contribute to overall disease pathogenesis in murine VL.

Blocking the action of C5a reduces parasitemia in BALB/c (WT) mice

Although IgG has been shown to promote *Leishmania* infection through ligation to macrophage FcR [18, 19, 21, 22] and

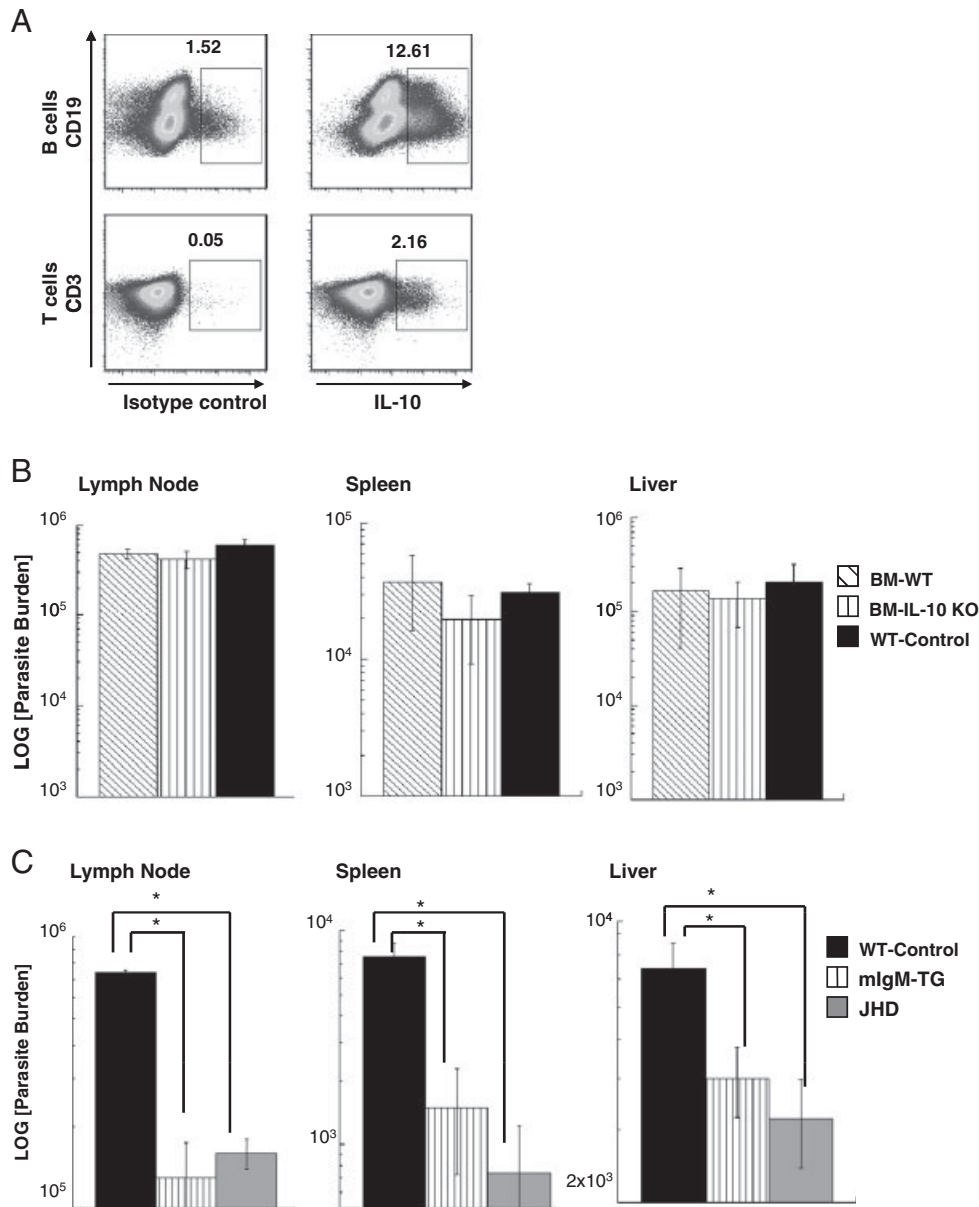


Figure 4. B-cell-derived IL-10 is insufficient to restore susceptibility. (A) FACS analyses of the DLN cells from WT BALB/c mice ($CD3^+$ T cells or $CD19^+$ B cells) at 5 days post-infection with *L. infantum*. B cells and T cells contribute to IL-10 production early in infection. (B) Bone marrow chimeric J_{HD} mice were employed to evaluate the effect of B-cell-derived IL-10 on disease progression. Shown are the parasite burdens 1 month after infection with *L. infantum* (DLN, spleen, liver) of J_{HD} mice reconstituted with B cells not producing IL-10 (20% IL-10-deficient+80% J_{HD} bone marrow cells) or with IL-10-producing B cells (20% WT+80% J_{HD} bone marrow cells). WT BALB/c mice were employed as controls. Data show mean \pm SE ($n = 5$ mice per group). No statistical differences were found between various groups of mice ($p > 0.2$). (C) Parasite burdens (LN, spleen, liver) and immune responses (Table 4) of BALB/c WT, J_{HD} , and mice transgenically altered to have functional B cells but no circulating antibody (mIgM/ J_{HD} transgenic mice) were compared at 1 month post-infection. Data are representative of three experiments and show mean \pm SE ($n = 3$ mice per group). * $p = 0.01$, Student's *t*-test.

subsequent production of IL-10 (upon TLR activation), the contribution of IgM to disease exacerbation was unexpected. Further, the early effect observed (2 days post-infection), lack of a correlation of parasite burdens with IL-10 levels (Table 3; data not shown) as well as the ability of non-specific immune complexes to cause disease exacerbation suggested an alternate mechanism might be involved. Given the early time course of disease exacerbation in {m+s}IgM/ J_{HD} mice and the effect of

IgM as well as IgG, the role of complement was considered. Consistent with the activation of complement, as a consequence of *L. infantum* infection, there is a heightened cellular (macrophages, neutrophils, DC) response in the DLN. Moreover, cells from the DLN of infected mice expressed reduced levels of C5a receptor (C5aR, CD88) (data not shown), suggesting downregulation and activation through this receptor during infection. As C5a is known to be involved in the

Table 3. IL-10 deficiency leads to lower parasite numbers and disease amelioration^{a)}

		DLN	Spleen	Liver
3 days post-infection	BALB/c-WT	$1.4 \pm 0.3 \times 10^4$	$1.0 \pm 0.9 \times 10^1$	$2.6 \pm 2.0 \times 10^2$
	IL-10 ^{-/-} BALB/c	$5.7 \pm 0.2 \times 10^{3*}$	$1.0 \pm 0.9 \times 10^1$	$1.2 \pm 0.4 \times 10^2$
15 days post-infection	BALB/c-WT	$6.9 \pm 0.6 \times 10^4$	$6.2 \pm 0.2 \times 10^4$	$3.1 \pm 0.9 \times 10^5$
	IL-10 ^{-/-} BALB/c	$3.4 \pm 0.01 \times 10^{4****}$	$4.6 \pm 1.1 \times 10^{3****}$	$1.1 \pm 0.2 \times 10^{4**}$
1 month post-infection	BALB/c-WT	$2.7 \pm 0.1 \times 10^5$	$7.3 \pm 0.2 \times 10^4$	$8.8 \pm 0.6 \times 10^4$
	IL-10 ^{-/-} BALB/c	$2.6 \pm 0.01 \times 10^{4****}$	$4.5 \pm 0.2 \times 10^{3****}$	$3.2 \pm 0.5 \times 10^{3****}$

^{a)} The effects of IL-10 upon the course of infection in the LN, spleen and liver of BALB/c mice intradermally infected with *L. infantum*. BALB/c IL-10-deficient and WT mice were infected with *L. infantum* and parasite burdens determined. Data are representative of three experiments and show mean \pm SE ($n = 3-4$ mice/group). * $p = 0.01$, ** $p = 0.005$, *** $p = 0.0001$, **** $p < 10^{-6}$; Student's *t*-test. The parasite levels at day 3 in the liver and spleen were not statistically different between the BALB/c WT and IL-10-deficient mice

Table 4. Cytokine responses in the draining lymph node of B cell deficient and transgenetically reconstituted BALB/c mice^{a)}

Time post-Infection	Mouse	IL-10 (pg/mL)	IFN- γ (pg/mL)	Ratio:IL-10/IFN- γ
6 Days	BALB/c-WT	$187 \pm 5^{***}$	$683 \pm 41^{***}$	0.27
	mIgM-J _H D-BALB/c	$505 \pm 17^{***, \ddagger\ddagger}$	$1672 \pm 87^{***, \ddagger}$	0.30
	J _H D-BALB/c	0.0 ± 4.0	183 ± 23	0.00
16 Days	BALB/c-WT	$30 \pm 10^{***}$	$1842 \pm 88^{***}$	0.016
	mIgM-J _H D-BALB/c	$381 \pm 50^{***, \ddagger}$	$6132 \pm 221^{***, \ddagger\ddagger}$	0.060
	J _H D-BALB/c	0 ± 1.7	79 ± 16	0.00
2 Months	BALB/c-WT	10 ± 18^{ns}	$339 \pm 32^{***}$	0.029
	mIgM-J _H D-BALB/c	$5 \pm 8^{**}, \ddagger$	$78 \pm 16^*, ns$	0.064
	J _H D-BALB/c	56 ± 10	268 ± 85	0.21

^{a)} Cells from the DLN of uninfected BALB/c and *L. infantum*-infected mice at 6, 16 days and 2 months post-infection were isolated and stimulated with SLA. Levels of IL-10 and IFN- γ were determined. No correlation of susceptibility to infection with IL-10 or IFN- γ levels or IL-10/IFN- γ is evident. Data show mean \pm SE ($n = 3$ mice/group) for each time post-infection. Shown are the *p* values for comparisons of J_HD (to either WT BALB/c or IgM-Tg): * $p < 0.02$, ** $p < 0.002$, *** $p < 0.0002$; comparisons of WT BALB/c to IgM-Tg: \ddagger $p < 0.02$, $\ddagger\ddagger$ $p < 0.002$, $\ddagger\ddagger\ddagger$ $p < 0.0002$, *ns* = not significant; Student's *t*-test.

chemotaxis and recruitment of both monocytic, dendritic, and neutrophil cell populations [40, 41] critical to leishmaniasis [42–45], we examined the involvement of C5aR on infection.

To evaluate the role of complement/C5a on infection, the effect of treatment with a C5aR antagonist on infection of WT BALB/c mice was examined. Mice were treated with the C5aR antagonist (Ac-Phe-[Orn-Pro-dCha-Trp-Arg]), and parasite burdens in these mice were evaluated and compared to those receiving a control peptide (Ac-Phe-[Orn-Pro-dCha-Ala-D-Arg]) and to control (untreated) mice. As shown in Fig. 7, blocking the C5aR reduces the level of infection in the liver, and DLN as early as 6 days post-infection. Comparative analyses of the ongoing immune response indicated that treatment with C5aR antagonist does not lead to the preferential development of a Th1-like response. Comparable levels of IFN- γ and IL-10 were found in the treated mice. The levels of IFN- γ found in response to SLA for the control infection (PBS), control peptide, and C5aR antagonist groups were 61.5, 50.9, and 67.7 ng/mL, respectively; the respective levels of IL-10 were 1.8, 2.25, and 2.38 ng/mL. These results concerning the cytokines levels differ from reports of C5aR-deficient mice [46], where heightened IFN- γ responses were found to limit cutaneous infection with *L. major*; however,

this may reflect the short duration of treatment as well as possibly differences in the mouse genetic background.

Overall, these results suggest that immunoglobulins can contribute to disease severity through the activation of complement, production of C5a, and consequent signaling *via* its receptor.

Discussion

Although immunoglobulins can provide protection against infectious organisms, antibodies are also known to contribute to the disease pathology associated with infection (viral, bacterial, and parasitic [47–51]) as well as autoimmune diseases [52–54]. Hypergammaglobulinemia and polyclonal B-cell responses are hallmarks of VL [8–14]. Elevated levels of total antibody (IgG; IgM) have been shown to correlate with disease pathology of human [8, 55, 56] and canine VL [28, 57]. Notably, VL patients display detectable antibody titers against antigens unrelated to the parasite such as haptens (DNP, FITC), protein antigens (KLH), and autoantigens (*e.g.* DNA, IgG, and smooth muscle), in addition to anti-leishmanial antibodies [8–14]. However, while abnormal B-cell

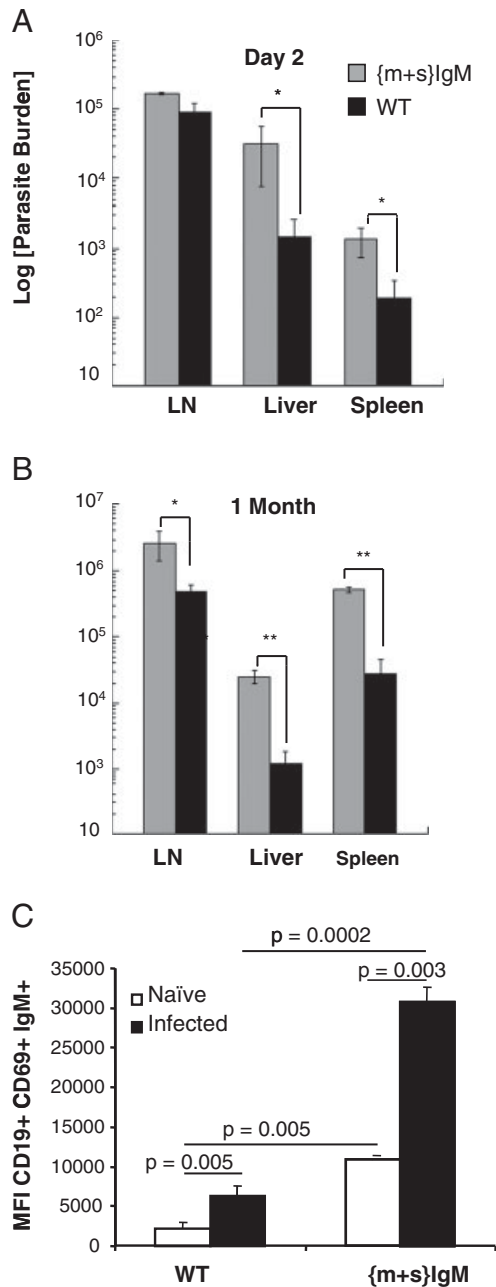


Figure 5. Secretory IgM causes disease exacerbation. Parasite burdens in mice transgenically altered to produce membrane and secretory IgM ($\{m+s\}$ IgM/ $J_{H}D$ mice) were compared to that of WT mice by limiting dilution analysis at (A) 2 days and (B) 1 month after infection with *Leishmania infantum*. Parasite burdens are shown for the DLN, spleen, and liver tissue sites. Data are representative of at least two experiments and are similar to what was found at other times post-infection (days 6, 10, 14, and 2 months). (Minimum of $n = 3$ mice per group). $*0.04 \geq p \geq 0.02$; $**0.002 \geq p \geq 0.001$; Student's *t*-test. (C) In response to infection, B-cell activation and IgM production in $\{m+s\}$ IgM/ $J_{H}D$ mice is elevated in comparison to WT mice. Data show summary of FACS analyses examining IgM⁺ (intracellular and extracellular), CD69⁺, CD19⁺ cells at 5 days post-infection. Data are representative of two experiments and show mean \pm SE ($n = 3$).

responses are evident in VL, the mechanisms by which B cells may contribute to disease in VL are not fully understood.

In the murine intradermal model for VL, a pronounced and early B-cell response is observed in the DLN, which is sustained in response to infection. Although a B-cell response to infection is not unusual, this B-cell response, as found in human disease, was notably polyclonal in nature, with the production of immunoglobulins against non-specific as well as leishmanial antigens. The early onset of the polyclonal B-cell response suggests an intrinsic response to infection and prompted an investigation into the mechanisms by which B cells might contribute to disease.

BALB/c mice lacking B cells ($J_{H}D$) intradermally infected with *L. infantum* were found, in fact, to have significantly reduced parasitemia (all tissue sites – liver, spleen, lymph node). Potentially several B-cell-mediated mechanisms (APC function, cytokine (IL-10), and/or immunoglobulin production) could lead to the increased disease severity. Although IL-10 contributes to susceptibility and parasitemia in murine VL, B-cell-derived IL-10 appeared to have little, if any, effect on parasite levels at the various tissue sites. Studies with bone marrow chimera mice indicated that B cells deficient in IL-10 were comparable to WT B cells in reconstituting susceptibility of $J_{H}D$ mice. Further BALB/c mice that were transgenetically reconstituted [35, 36] for surface IgM expression (and also antigen presentation; IL-10 production) displayed comparable levels of resistance to infection as $J_{H}D$ mice. Notably, similar results were obtained for mIgM transgenic mice infected with the New World species, *L. pifanoi* or *L. amazonensis* [18, 19]. In these studies, infected mIgM transgenic mice developed comparable cytokine responses to infected WT mice (IL-10, IL-4, no IFN- γ), yet had significantly lower parasite burdens. However, our observations do differ from recent observations for *L. major*, where the APC function of B cells can contribute to disease; nonetheless, this contribution varied and was dependent upon the infecting strain [17, 22]. Consequently, the mechanisms of B-cell modulation of infection may vary and be dependent upon the species/strain of *Leishmania*. Overall, our results indicate that neither B-cell antigen presentation nor B-cell-derived IL-10 is the primary B-cell mechanism leading to increased parasitemia and disease in murine VL.

In contrast, B-cell-derived immunoglobulins (IgM and IgG) were found to play a critical role in disease progression in VL. Passive antibody transfer experiments indicate that IgG, IgM, and non-specific antigen–antibody complexes restored disease susceptibility to $J_{H}D$ mice. These observations differ with previous studies of murine VL using a high dose intravenous infection model of VL and mice that lack mature B cells (μ MT; C57BL/6), where the transfer of serum from chronically infected WT mice failed to restore susceptibility to infection for the μ MT mice [7]. It is unclear whether the differences in *Leishmania* species (*L. donovani* versus *L. infantum*), infection model, and/or the use of serum versus purified antibodies contribute to these different observations.

Interestingly, disease exacerbation (in comparison to WT BALB/c mice) was evident for ($\{m+s\}$ IgM/ $J_{H}D$) BALB/c

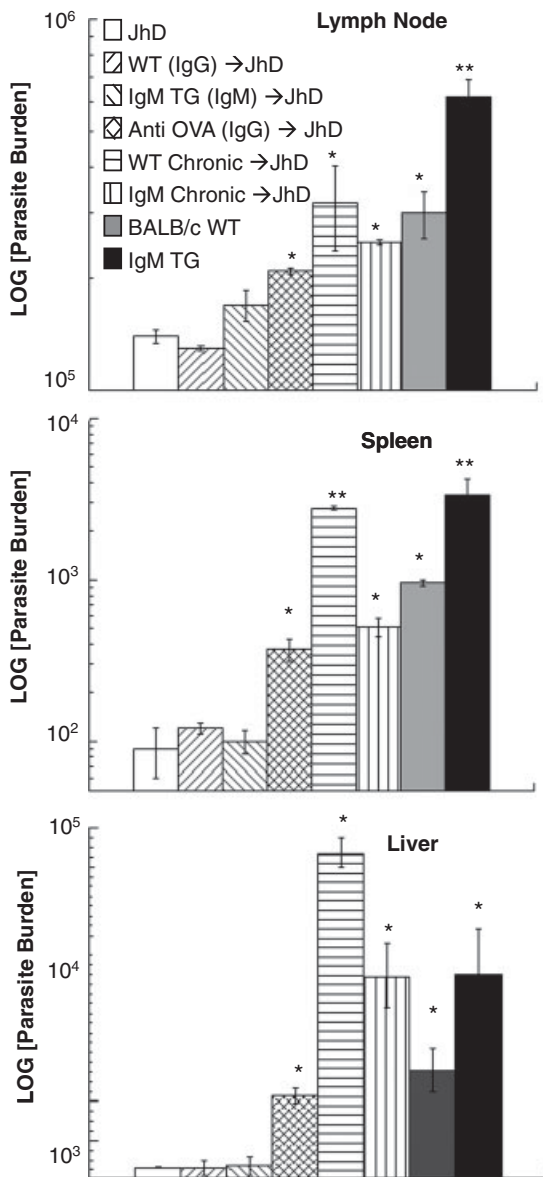


Figure 6. Reconstitution of $J_{H/D}$ mice with IgG or IgM antibodies restores WT susceptibility to infection. To assess the role of antibody or antigen-antibody complexes, isolated IgG or IgM from naive or chronically *L. infantum*-infected WT or $\{m+s\}$ IgM/ $J_{H/D}$ Tg mice was employed. Additionally, IgG was isolated from BALB/c mice immunized with OVA. Mice were given 600 μ g of purified antibody intraperitoneally, 1 day pre-infection, and, subsequently, every 3 days for IgM, and every 7 days for IgG. OVA antigen was injected together at the time of infection in mice receiving anti-OVA antibody to create nonspecific antigen-antibody complexes. Parasite burdens were determined 3 wk post-infection. Data show mean \pm SE ($n=3-5$ mice per group). * $0.05 \geq p \geq 0.01$, ** $p < 0.002$ in comparison to $J_{H/D}$ mice; Student's *t*-test.

mice, which are genetically reconstituted to secrete only IgM, indicating for the first time that IgM can facilitate parasite survival. Further, IgM antibody from infected $\{m+s\}$ IgM/ $J_{H/D}$ BALB/c mice transferred into $J_{H/D}$ BALB/c mice exacerbated infection. Importantly, disease exacerbation was evident as early as 2 days post-infection, and it was sustained throughout infection. The early effect of IgM as well as the observation

that the cytokine responses of $\{m+s\}$ IgM/ $J_{H/D}$ mice appeared to be comparable to WT mice suggested that alternate mechanisms contributed to the observed enhanced susceptibility to infection.

Immunoglobulins may act through the activation of complement. Although complement activation can lead to the lysis of *Leishmania* parasites, it is well established that infective metacyclic promastigotes as well as intracellular amastigotes are resistant to complement-mediated lysis [58]. Further, leishmanial promastigotes subvert the complement system by utilizing CR3 for macrophage entry and to downregulate host oxidative burst and IL-12 responses, essential for parasite containment [59, 60]. The results herein for IgM differ from vaccine studies [61] where IgM from B-1 B cells was found to enhance protection induced to repetitive antigen HASPB1. However, the situation in naive animals may differ from vaccinated mice. A selective expansion of B-1 B cells in the DLN during infection was not observed in the DLN of infected mice [62]; consequently, the level of antibody from B-1 B cells may be obscured in the context of the ongoing response to infection.

In addition to cell lysis, the activation of complement results in the generation of cellular mediators (C3a, C5a) that can lead to cellular recruitment and the modulation of the host immune response [40, 63]. Notably, as a consequence of *L. infantum* infection, there is a heightened cellular response in the lymph node; in addition to the increases in B cells, increases are observed in the numbers of macrophages, neutrophils, and DC [62]. Further, a reduced level of expression of CD88 on lymph node cells of *L. infantum* infected mice was observed, suggesting an ongoing generation, binding, and uptake of C5a during infection. Significantly, treatment with a C5aR antagonist causes a reduction in parasitemia in WT BALB/c mice in the lymph node and liver. These effects were noted early in infection (day 6). Notably, treatment with the C5aR antagonist peptide did not appear to lead to changes in the overall cytokine responses (IL-10; IFN- γ). These results are consistent with the response to infection of IgM transgenetically reconstituted mice, where cytokine responses do not correlate with disease. However, the effect of C5aR antagonist treatment on the mouse immune response appears to differ from those reported for C5aR^{-/-} C57BL/6 mice infected with *L. major*. These mice have been shown to upregulate IL-12 expression, promoting a heightened Th1 response and parasite control [46]. Nonetheless, the lack of an effect on the ongoing immune response by the C5aR antagonist peptide in comparison to the immunodeficient mice may reflect pharmacokinetics and/or short duration treatment with the C5aR antagonist peptide. Although the mechanisms responsible for disease modulation by the C5aR antagonist peptide remain to be determined, it is possible that changes in cellular recruitment may contribute. Additionally, C5a is known to upregulate CR3 expression on macrophages [64]; thus, increased infection of host macrophages and parasite survival [59] through C5a modulation of CR3 could contribute to disease.

Overall, observations suggest that B-cell activation and the production of IgM as well as IgG contribute to disease patho-

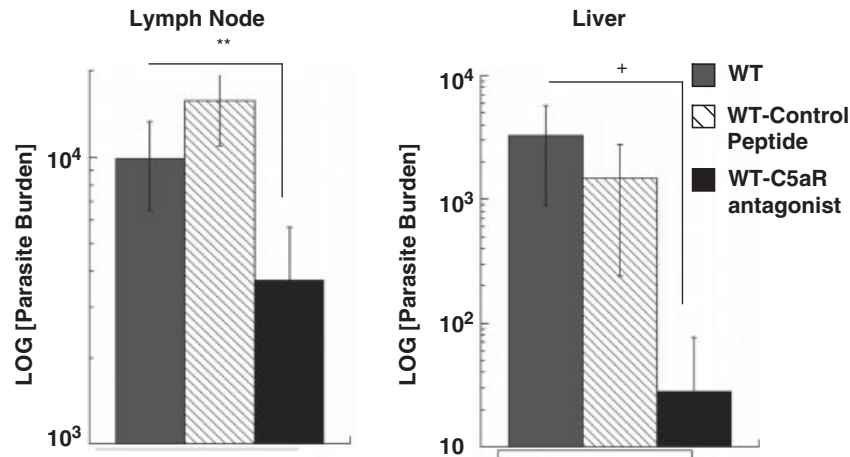


Figure 7. C5aR antagonist peptide treatment ameliorates disease in WT BALB/c and $\{m+s\}$ IgM/ $J_{H}D$ Tg mice. BALB/c mice were treated with a C5aR antagonist C5aRa (Ac-Phe-[Orn-Pro-dCha-Trp-Arg]) or control peptide (Ac-Phe-[Orn-Pro-dCha-Ala-D-Arg]) on days -1 and 0 pre-infection and days 2 and 4 post-infection intradermally with 5×10^5 *L. infantum* promastigotes. Parasite burdens were evaluated at day 6 post-infection in the DLN and liver. Parasite numbers were too low in the spleen at this early time point for evaluation. *p*-values are for mice treated with C5aR antagonist in comparison to control mice. Data are representative of three independent experiments and show mean \pm SE ($n = 3-5$). ** $p = 0.005$, + $p = 0.06$; Student's *t*-test.

genesis in murine VL. These findings in light of the polyclonal B-cell responses observed for human VL are noteworthy and reveal that the apparently early and continuous B-cell expansion/activation is not only an intrinsic feature of the initial parasite–host interaction but is important for overall disease severity. The fact that there is a lack of a requirement for antibody specificity (parasite) in pathogenesis would suggest that antibody-antigen complexes, in general, are detrimental to the host. IgM responses are known to persist throughout the course of human VL [8, 55]. Therefore, B-cell-derived immunoglobulins (IgM and IgG), immune complexes, and consequent complement activation, with the generation of C5a, potentially may contribute to disease and parasite persistence throughout infection. Overall, these results indicate a new mechanism of disease pathogenesis in VL and suggest that intervention in the action of C5a could potentially be considered for immunotherapeutic approach to alleviate disease.

Materials and methods

Mice

BALB/c mice were purchased from the NCI or from Taconic Farms. Breeding pairs of BALB/c $J_{H}D$ mice, VH186.2- $\{m+s\}$ IgM/ $J_{H}D$ / $J_{H}D$ transgenic mice ($\{m+s\}$ IgM/ $J_{H}D$ Tg; produce membrane and soluble IgM), and VH186.2-mIgM/ $J_{H}D$ / $J_{H}D$ transgenic mice (mIgM/ $J_{H}D$ Tg; functional B cells with surface IgM) [35, 36, 65] were kindly provided by Dr. Shlomchik (Yale University). Breeding pairs of IL-10-deficient BALB/c mice were originally provided by Dr. Coffman (DNAX) through Dr. Kullberg and Dr. Sher (NIH). All experiments using mice were reviewed by

the Yale University Animal Care and Use Committee. All procedures were conducted in accordance with U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training. Yale University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Parasites, infection, and parasite burden analyses

Mice were infected with isolated late stationary-growth-phase promastigotes (9–12 days of culture) of *L. infantum* (MHOM/ES/92/LLM-320; isoenzyme typed MON-1), expressing the P-8 PGLC (an amastigote associated molecule) as previously described [26]. Mice were infected intradermally either in ear pinnae or rear hind feet, using $5-10 \times 10^6$ organisms unless otherwise indicated. Parasite burden analyses for the lymph node, spleen, and liver were performed as previously described using 3–5 mice/group [26]; under these conditions, the cloning efficiency for this strain was $\geq 90\%$.

Immunofluorescence staining of lymph nodes and spleens

Organs were frozen in O.C.T. compound; sections ($7 \mu\text{m}$) were fixed in acetone, blocked with TNB buffer (3% Casein in PBS; NEN Life Science Products) containing 5% normal donkey serum, and then treated with Avidin-Biotin block (Vector Laboratories) and endogenous peroxidase activity was quenched. Using antibodies from BD Biosciences, slides were stained with rat IgG anti-mouse CD45R/B220, hamster IgG1k anti-mouse CD3 ϵ , or rat anti-mouse FDC (Clone FDC-M1), followed by biotin-conjugated donkey

anti-rat IgG or biotin-conjugated anti-hamster antibody. GC were stained with biotin-labeled peanut lectin followed by streptavidin-HRP conjugate. The antigens were detected using tyramide-FITC or tyramide-tetramethylrhodamine (NEN Life Science Products). Slides were mounted with Fluoromount-G (Southern Biotechnology Associates) and analyzed by fluorescence microscopy (Leitz Orthoplan 2).

FACS analyses

DLN were excised and single cell suspensions were prepared and cells were stained for CD3, CD19, or CD69; all antibodies were from BD Biosciences. To determine lymphocyte populations expanding in response to infection, mice were injected with 2 mg BrdU intraperitoneally. Four hours later, DLN were excised and cells were stained with cell surface markers (CD3 or CD19). After washing, cells were fixed and permeabilized with BD Cytofix/Cytoperm Buffers (BD Biosciences). Cells were treated with DNase to expose incorporated BrdU and were stained with FITC-conjugated anti-BrdU. For intracellular detection of IgM, cells were fixed with 2% paraformaldehyde after surface staining, permeabilized with 0.05% saponin and stained with anti-IgM (eBiosciences). For intracellular cytokine staining of IL-10, cells were stimulated with SLA at 25 µg/mL in RPMI medium (supplemented with 10% FBS, 2-mercaptoethanol, glutamine, and antibiotics) for 72 h followed by 6 h of culture with PMA, ionomycin, and monensin and stained using anti-IL-10 or an isotype control (PE-labeled; eBiosciences). Data were acquired by using FACSCalibur and were analyzed by using FlowJo software (Tree Star).

Determination of antibody levels and antibody-producing cells by ELISA and ELISPOT

For ELISA and ELISPOT analyses, plates were initially coated with 10 µg of TNP₁₅-BSA, chicken egg albumin (OVA; Sigma Chemical), chromatin, or soluble *Leishmania* antigen in PBS overnight at 4°C. For ELISA, after overnight antigen incubation, plates were blocked (3% BSA in PBS). Serial dilutions of serum from uninfected and infected mice incubated overnight at 4°C, followed by incubations with biotinylated anti-mouse Ig antibody (BD Biosciences). Plates were then incubated with streptavidin-HRP, followed by development using TMB substrate. Plates were analyzed at 450 nm. Statistical significance was measured using a *t*-test for infected mice compared to uninfected controls.

For ELISPOT analysis, after overnight antigen incubation (as indicated above), plates were blocked (3% BSA in PBS). Single-cell suspensions of lymph node or splenic cells (serial dilutions) were then added to wells and incubated at 37°C. Subsequently, plates were incubated with biotinylated anti-mouse Ig, followed by phosphatase-labeled streptavidin and developed using SigmaFast BCIP/NBT as substrate. Plates were examined micro-

scopically. Statistical significance was measured (*t*-test) for each antigen in comparison to uninfected controls.

Passive antibody transfer experiments

To assess the role of antigen–antibody complexes, isolated IgG or IgM from naïve or chronically *L. infantum*-infected WT or {*m+s*}IgM/J_HD Tg mice or IgG from OVA-immunized BALB/c mice were used. Antibody was isolated by ammonium sulfate precipitation followed (in the case of IgG) by protein G column purification. All antibody preparations were then fractionated over polymyxin B columns. All preparations were endotoxin-free, as assessed using a Limulus amoebocyte lysate assay (Cambrex). SDS-PAGE was used to assess antibody purity. Mice were injected intraperitoneally with 600 µg of purified antibody, 1 day pre-infection, and, subsequently, every 5 days for IgM and every 7 days for IgG. OVA (50 µg) was injected at the time of infection in mice receiving anti-OVA antibody to create OVA-antibody complexes.

Bone marrow chimeras

BALB/c J_HD mice were reconstituted as previously described after irradiation with 350 rads [66], using a mixture of either 20% WT/80% J_HD bone marrow cells or 20% IL-10-deficient bone marrow/80% J_HD bone marrow cells (9×10^6 cells/mouse). Reconstitution of B cells was confirmed by FACS.

C5aR antagonist treatment

To examine the role of complement and specifically C5a on infection, mice were treated with either C5aR antagonist (C5aRa; Ac-Phe-[Orn-Pro-dCha-Trp-Arg]) or control peptide (Ac-Phe-[Orn-Pro-dCha-Ala-D-Arg]), according to described methods [67]. Additionally, a group of untreated mice were used as infection controls. Briefly, mice were injected intraperitoneally with 1 mg/kg body weight of peptide suspended in PBS at days –1 and 0 pre-infection, and, subsequently, every other day post-infection [67, 68]. Mice were infected using 5×10^5 *L. infantum* parasites.

Statistical analysis

A Student's *t*-test was used to determine statistical significance in all experiments.

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J_HD/J_HD and VH186.2-mIgM/J_HD/J_HD transgenic mice, and Drs. Craft, Ruddle and Mamula for helpful discussions. This study was supported by NIH grants AI45044 and AI27811 (to D. Mc. P.) and GM-62134 and AI-068730 (to J. L.) and AI-43603 (M. J. S.); E. D. was supported in part on an NIH Training Grant, T32 AI07404 and a grant from Fort Dodge Animal Health.

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Abbreviations: DLN: draining LN · FDC: follicular DC · SLA: soluble leishmania antigen · VL: visceral leishmaniasis

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