

Innate Immune B Cell Activation by Leishmania donovani Exacerbates Disease and Mediates Hypergammaglobulinemia.

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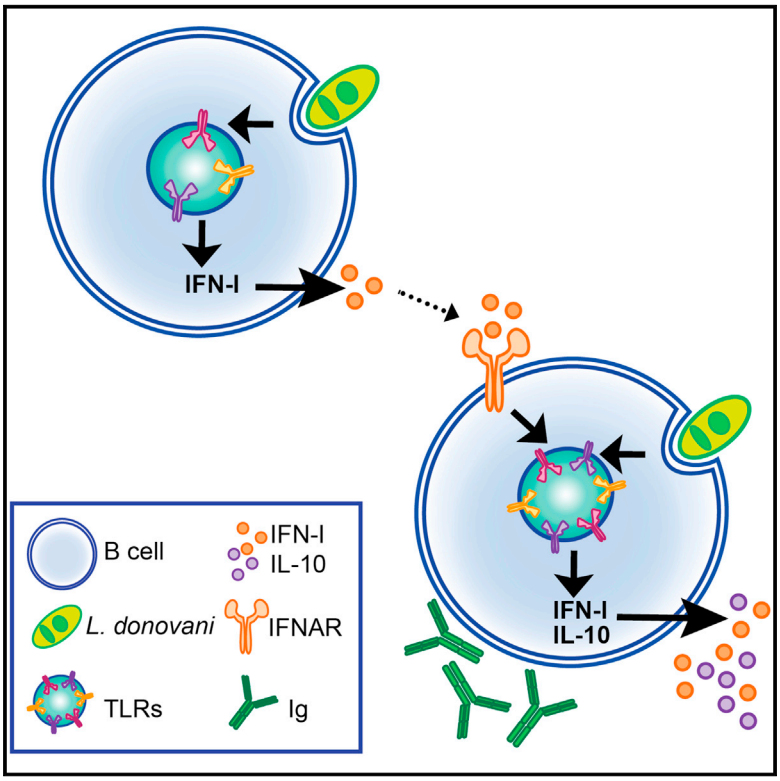
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Article

Cell Reports

Innate Immune B Cell Activation by *Leishmania donovani* Exacerbates Disease and Mediates Hypergammaglobulinemia

Graphical Abstract



Authors

Sasha Silva-Barrios, Mélina Smans, Claudia U. Duerr, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, Simona Stäger

Correspondence

simona.stager@iaf.inrs.ca

In Brief

Silva-Barrios et al. report that the parasite *Leishmania donovani* triggers endosomal TLRs in B cells. This activation induces cytokine and endosomal TLR expression, which are further enhanced by signaling through the type I IFN receptor. Innate B cell activation through endosomal TLRs and IFN-I promotes disease exacerbation and hypergammaglobulinemia.

Highlights

- *Leishmania donovani* triggers endosomal TLRs in B cells
- Innate B cell activation results in IL-10 and type I IFN induction
- IFN-I is involved in a positive regulatory loop enhancing cytokine and TLR expression
- Innate B cell activation and IFN-I govern antibody production during disease

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Innate Immune B Cell Activation by *Leishmania donovani* Exacerbates Disease and Mediates Hypergammaglobulinemia

Sasha Silva-Barrios,[1] Mélina Smans,[1] Claudia U. Duerr,[3] Salman T. Qureshi,[2] Jörg H. Fritz,[3] Albert Descoteaux,[1] and Simona Stäger[1,*]

[1]Institut Armand Frappier and Center for Host-Parasite Interactions, INRS, 531 Boulevard des Prairies, Laval QC H7V 1B7, Canada
[2]Meakins-Christie Laboratories and Department of Medicine, McGill University Health Centre Research Institute, Montreal, QC H4A 3J1, Canada
[3]Department of Microbiology and Immunology and Department of Physiology, Complex Traits Group, McGill University, Montreal, QC H3G 0B1, Canada
*Correspondence: simona.stager@iaf.inrs.ca
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SUMMARY

Participation of B cells in the immune response by various antibody-independent mechanisms has recently been uncovered. B cells producing cytokines have been described for several infections and appear to regulate the adaptive immune response. B cell activation by *Leishmania donovani* results in disease exacerbation. How *Leishmania* activates B cells is still unknown. We show that *L. donovani* amastigotes activate B cells by triggering endosomal TLRs; this activation leads to the induction of various cytokines. Cytokine expression is completely abrogated in B cells from *Ifnar*$^{-/-}$ mice upon exposure to *L. donovani*, suggesting an involvement of IFN-I in a positive feedback loop. IFN-I also appears to enhance the expression of endosomal TLRs following exposure to *L. donovani*. Cell-specific ablation of endosomal TLR signaling in B cells revealed that innate B cell activation by *L. donovani* is responsible for disease exacerbation through IL-10 and IFN-I production and for the promotion of hypergammaglobulinemia.

INTRODUCTION

The main mechanisms leading to antibody production by B cells are largely known. Follicular helper CD4^{+} T cells (TFhs) are thought to play a crucial role in germinal center formation and B cell differentiation into antibody-producing plasma cells (Crotty, 2011); antigen-specific B cell receptor (BCR) recognition is required for this process. Nevertheless, other pathways have been reported to be involved in enhancing antibody production. For instance, Toll-like receptor (TLR) stimulation in B cells can regulate the magnitude of the antibody response and the amount of antigen required for initiating BCR signaling (DeFranco et al., 2012; Freeman et al., 2015). Moreover, *Myd88*$^{-/-}$ B cells were reported to generate decreased antibody responses (Pasare and Medzhitov, 2005; Kasturi et al., 2011).

The role of B cell TLR signaling in antibody production has mainly been studied in models of autoimmune diseases. TLR7 and 9 were shown to contribute importantly to the production of anti-nuclear antibodies in various models of lupus-like disease (Christensen et al., 2006; Han et al., 2015). In contrast, little is known about the role of B cell TLR signaling in infectious diseases. MyD88 appears to be required for preventing lethal dissemination of commensal bacteria during colonic damage caused by dextran sulfate sodium (Kirkland et al., 2012). In this model, MyD88 was essential for the production of immunoglobulin M (IgM) and complement by B cells. TLR9, TLR7, and MyD88 expression in B cells was also shown to be involved in substantially enhancing T cell-dependent germinal center immunoglobulin G (IgG) responses following inoculation with virus-like particles (Hou et al., 2011). During *Salmonella typhimurium* infection, however, MyD88 in B cells was mainly associated with cytokine production and led to disease susceptibility via interleukin-10 (IL-10) induction (Neves et al., 2010). We have also previously reported that B cell-derived IL-10 production following *Leishmania donovani* infection was dependent on MyD88 expression in B cells (Bankoti et al., 2012).

The protozoan parasite *L. donovani* is a causative agent of visceral leishmaniasis (VL), a chronic disease that is characterized, among others, by polyclonal B cell activation and hypergammaglobulinemia. *Leishmania* is an intracellular parasite that preferentially infects macrophages; however, it can also be found in dendritic cells, neutrophils, and fibroblasts (Kaye and Scott, 2011). In a previous study, we have shown that *L. donovani* can also be detected on marginal zone B cells (Bankoti et al., 2012). In the murine model of VL, B cells play a detrimental role (Smelt et al., 2000). IgM, complement, and B cell-derived IL-10 largely contribute to disease susceptibility (Deak et al., 2010; Bankoti et al., 2012). Additionally, IgG immune complexes were shown to exacerbate infection by inducing IL-10 production in macrophages (Miles et al., 2005). IgG is also linked to chronic infection in human VL patients, where high IgG levels are predictive of disease. However, the pathways leading to polyclonal B cell activation and hypergammaglobulinemia in VL are still unknown.

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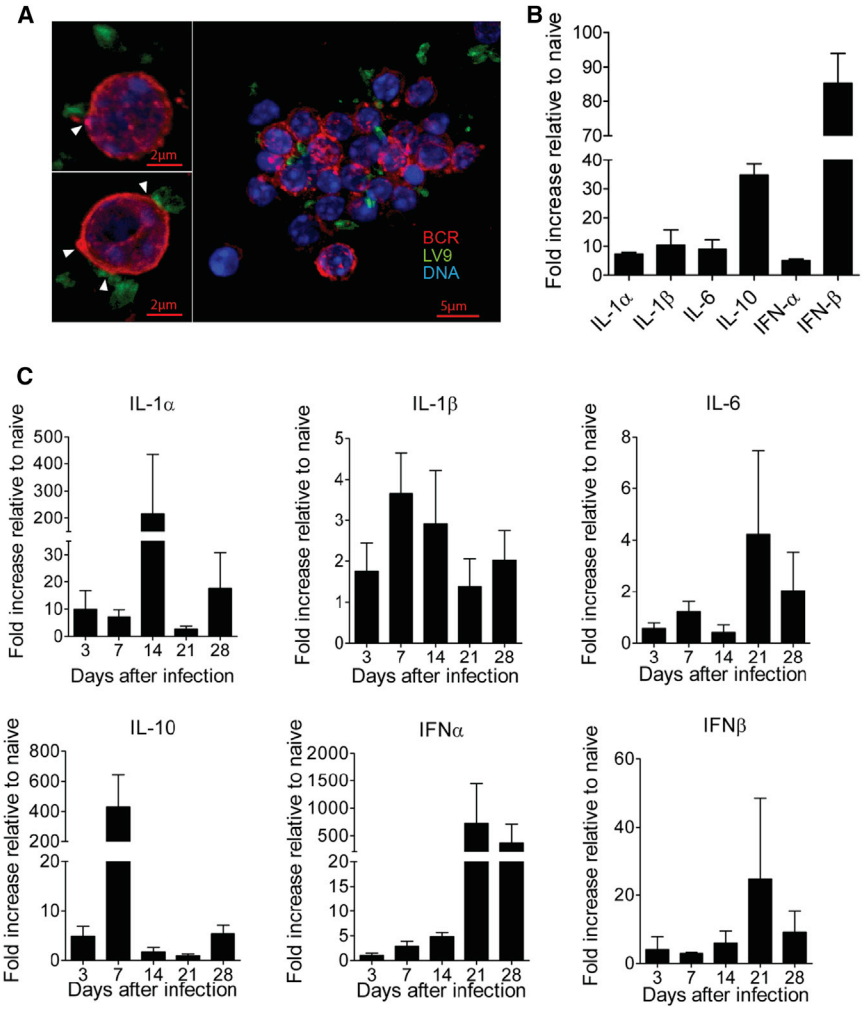


Figure 1. *L. donovani* Promotes Cytokine mRNA Expression in B Cells

(A) Naive splenic B cells were exposed to PKH67-labeled *L. donovani* amastigotes (green) at an MOI of 1:5 for 5 hr. Cells were stained with anti-IgM-AF568 (red) and Hoechst (blue) and visualized by confocal microscopy.

(B and C) Expression analysis by qPCR of cytokine mRNA in naive splenic B cells exposed to *L. donovani* for 8 hr (B) and in splenic B cells purified from infected mice (C). Data represent mean ± SEM from three independent experiments.

In this study, we investigate the mechanisms of B cell activation by *L. donovani*. We show that *L. donovani* amastigotes activate B cells by triggering endosomal TLRs. This activation resulted in the induction of proinflammatory cytokines, IL-10, and type I interferon (IFN) and in the upregulation of endosomal TLR expression. Upregulation of cytokine and endosomal TLR mRNA expression was dependent on the presence of type I IFN receptor (IFNaR) in B cells, suggesting an involvement of type I IFNs in a positive regulatory loop. A functional endosomal TLR pathway was also required to generate non-specific and *Leishmania*-specific antibodies. These results demonstrate that B cell activation by *L. donovani* through endosomal TLRs leads to disease exacerbation through type I IFN production and the promotion of hypergammaglobulinemia.

RESULTS

L. donovani Triggers IL-10 and Type I IFN Expression in Splenic B Cells

We have previously reported that B cells can capture *L. donovani* amastigotes in vivo and in vitro (Bankoti et al., 2012). Upon in vitro exposure to the parasite, B cells form clusters, upregulate the costimulatory molecule CD86 and surface IgM, and eventually die after 48 hr (Bankoti et al., 2012). Confocal microscopy analysis revealed that this interaction does not result in parasite internalization but that *L. donovani* is retained on the surface in IgM-rich pockets (Figure 1A; Movies S1 and S2). B cell activation also resulted in the expression of several cytokines (Figure 1B), with IL-10 and IFN-β being the most highly expressed. Maximal cytokine expression was reached after 8 hr of co-incubation with the parasite (Figure S1). A similar pattern of cytokine expression was also observed in B cells purified from *L. donovani*-infected mice (Figure 1C). Interestingly, IL-10 and IL-1α mRNAs were mainly upregulated during the early stages of infection (days 7–14), whereas type I IFN and IL-6 were prominently expressed during the chronic phase of the disease (days 21–28). We have previously reported that IL-10 secretion is MyD88-dependent (Bankoti et al., 2012). The pathways upstream of MyD88 and of IFN-I induction in B cells following exposure to *L. donovani* are as yet unknown.

L. donovani Activates Endosomal TLRs in B Cells

Because MyD88 is necessary for IL-10 production in B cells (Bankoti et al., 2012), and *L. donovani* is recognized by a number of TLRs (Paun et al., 2011; Flandin et al., 2006; Kropf et al., 2004; Schleicher et al., 2007), we next investigated the role of TLRs in cytokine induction by the parasite. First, we assessed whether TLR agonists could induce a pattern of cytokines in B cells similar to that observed after exposure to *L. donovani*. Interestingly, agonists to TLR3, 7, and 9 triggered the strongest upregulation of mRNA for IL-1α, IL-1β, IL-6, IL-10, and IFN-α/β (Figure 2A) after 8 hr of exposure, suggesting that, although the parasite resides on the cell surface, endosomal TLRs may be triggered by *L. donovani* in B cells. We next examined, by confocal microscopy, the expression of TLR3 (Figure 2B, top) and TLR9 (Figure 2B, bottom) on splenic murine B cells exposed to the parasite. TLR3 and 9 were more intensely present at the

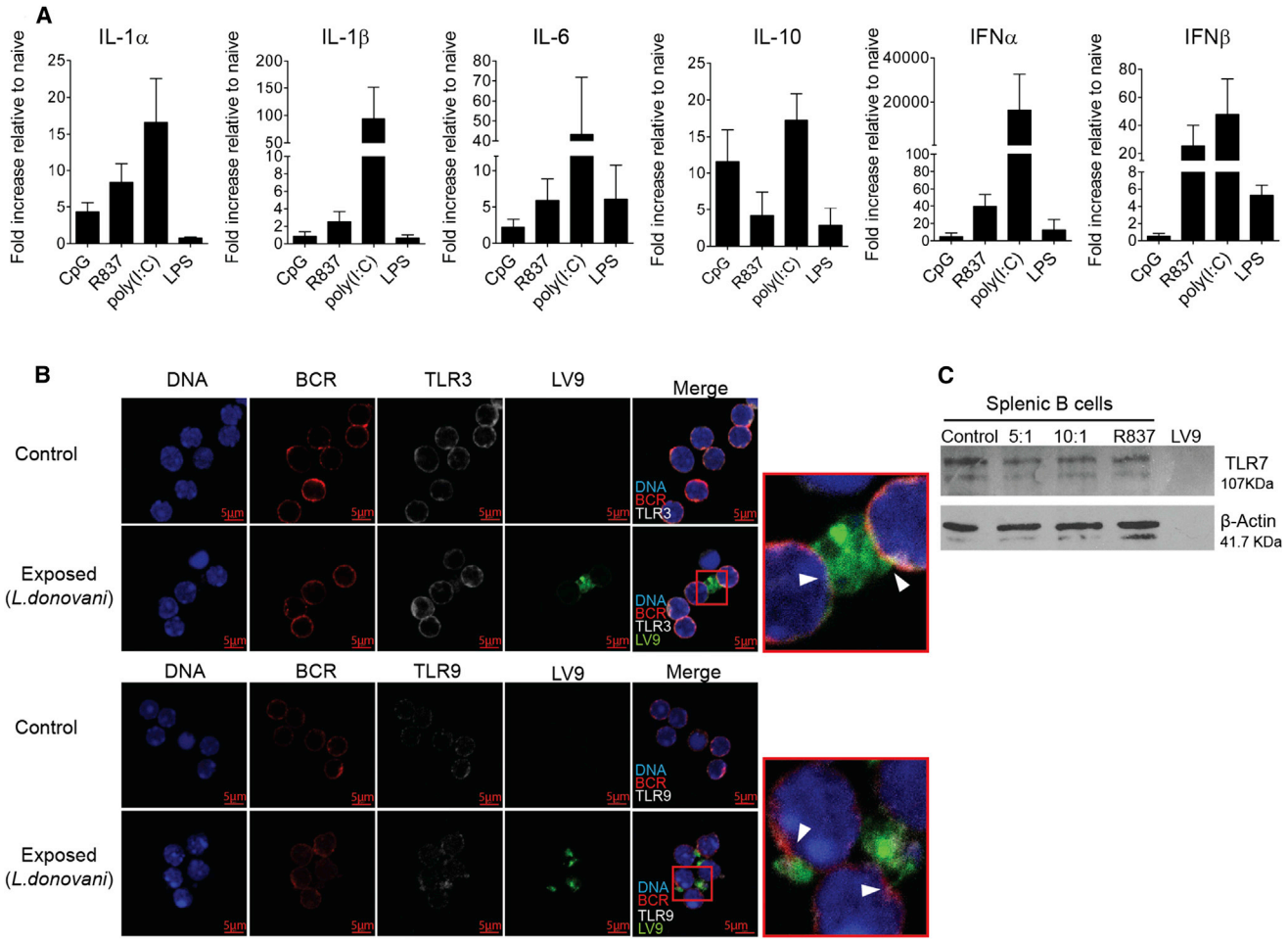


Figure 2. B Cells Express Endosomal TLRs and Respond to TLR Agonist Stimulation

(A) Cytokine mRNA expression by qPCR in splenic B cells activated with TLR agonists for 8 hr.

(B) TLR3 and TLR9 localization by confocal microscopy in B cells exposed or not exposed to *L. donovani* (green) and stained with anti-IgM-AF568 (red), anti-TLR3-Dylight 650 (white), or anti-TLR9-ZenonAF647 (white) and Hoechst (blue).

(C) Immunoblot analysis for TLR7 in B cells exposed to different ratios of parasites or R837-activated and parasites alone (LV9).

Data represent mean ± SEM from three independent experiments.

site of contact with the parasite (Figure 2B), suggesting that endosomal TLRs might be activated by *Leishmania* amastigotes. The presence of TLR7 in murine B cells was determined by western blot (Figure 2C).

B Cell Cytokine Expression following In Vitro Exposure to *L. donovani* Is Dependent on Endosomal TLRs and the IFNAR

To confirm our hypothesis that endosomal TLRs are required for cytokine induction, we exposed splenic B cells purified from *Unc931b*$^{Letr/Letr}$ mice (Lafferty et al., 2014) to the parasite in vitro and assessed cytokine expression. *Unc931b*$^{Letr/Letr}$ mice (Lafferty et al., 2014) have a loss-of-function mutation, known as Letr for "loss of endosomal TLR response," in Unc93b1, which is a chaperone protein for TLR3, TLR7, and TLR9. All cytokine mRNA levels measured failed to be upregulated in *Unc93b1*$^{Letr/Letr}$ B cells exposed to *L. donovani* compared with wild-type (WT) B cells (Figure 3A), suggesting that activation of endosomal TLR pathways was indeed responsible for cytokine expression in B cells. Our results were confirmed using TLR7 and 9 inhibitors (Figure S2). Strikingly, cytokine expression was also abrogated in B cells lacking the IFN-I receptor (IFNAR) (Figure 3A), implying that IFN-α/β may be involved in a positive feedback-regulatory loop. IFN-I signaling was shown to directly regulate TLR7 expression and TLR7 responsiveness by B cells (Doucett et al., 2005; Green et al., 2009; Thibault et al., 2009). Thus, we examined endosomal TLR mRNA expression in B cells purified from C57BL/6 and *Unc931b*$^{Letr/Letr}$ mice following in vitro exposure to *L. donovani*. Activation by the parasites led to the upregulation of endosomal TLR expression in wild-type B cells (Figure 3B). In contrast, endosomal TLR mRNAs failed to be upregulated in the absence of the IFNAR or in *Unc931b*$^{Letr/Letr}$ B cells (Figure 3B). To determine whether endosomal TLR expression was also dependent

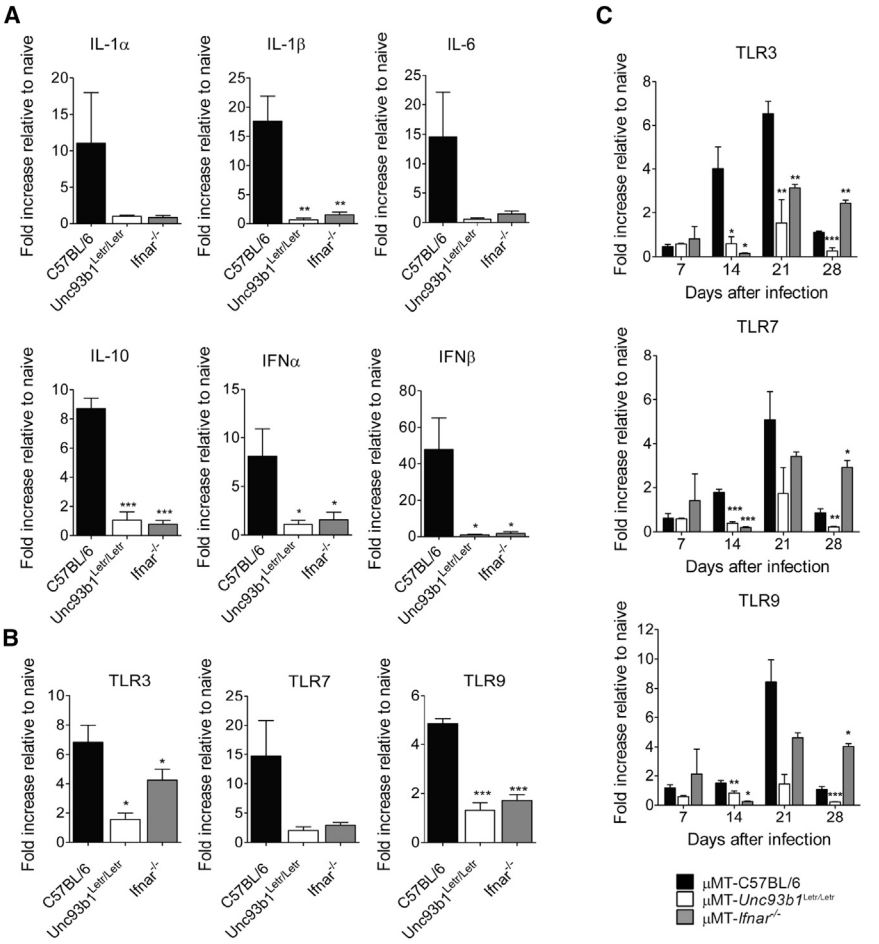


Figure 3. Functional Endosomal TLRs and IFNARs Are Involved in Cytokine and TLR mRNA Expression in B Cells

(A and B) Expression analysis by qPCR of cytokine (A) and endosomal (B) TLR mRNA in B cells from C57BL/6, *Unc93b1*$^{Letr/Letr}$, and *Ifnar*$^{-/-}$ mice exposed to *L. donovani*. Data represent mean ± SEM from two independent experiments (n = 6–8). (C) TLR mRNA expression in B cells from infected chimeric mice assessed by qPCR. Data are expressed as fold increase to naive chimeric mice. μMT-C57BL/6 mice were used as comparison group for statistical significance. Data represent mean ± SEM of one of two independent experiments (n = 4–5). *p < 0.05, **p < 0.01, ***p < 0.001.

on IFN-I signaling in vivo, we generated a mixed bone marrow chimera with a targeted ablation of IFNAR in B cells. Chimeric mice were then infected with *L. donovani*, and B cells were purified at various time points after infection. Our in vitro results were confirmed in vivo. IFN-I signaling not only regulated TLR7 expression but was also involved in the upregulation of TLR3 and 9 (Figure 3C).

UNC93b1 Deficiency Abrogates IFN-I and IL-10 mRNA Upregulation in B Cells during *L. donovani* Infection

To determine whether cytokine expression was also lost in vivo in the absence of functional endosomal TLR pathways or the IFNAR, we generated mixed bone marrow chimeras with a specific ablation of UNC93b1 or the IFNAR in B cells. B cell reconstitution levels were evaluated 6 weeks after bone marrow transfer; no significant differences were observed among the three groups (Figure S3). We then purified B cells at various time points after infection from *L. donovani*-infected chimeric mice. With exception of IL-1β and IL-6, most of the cytokine expression was lost in B cells from infected μMT-*Unc93b1*$^{Letr/Letr}$ chimeric mice (Figure 4), indicating that endosomal TLR activation was the main pathway leading to cytokine induction in vivo as well. Similar results were obtained in μMT-*Ifnar*$^{-/-}$ mice, underscoring the important role played by the IFNAR in vivo in enhancing B cell activation by *L. donovani*. Interestingly, B cells from infected μMT-*Unc93b1*$^{Letr/Letr}$ chimeric mice had a similar surface modulation of the costimulatory molecules CD80, CD86, and CD40 (Figure S4) as μMT- C57BL/6 and μMT-*Ifnar*$^{-/-}$ mice, suggesting that endosomal TLR pathways were not the only pathways triggered by *L. donovani* to activate B cells and that induction of cytokine expression was solely dependent on functional endosomal TLR signaling.

Lack of Functional Endosomal TLR Signaling in B Cells Results in Enhanced Th1 Responses

We next sought to determine the biological relevance of endosomal TLR signaling in B cells during *L. donovani* infection. To this end, we infected μMT-*Unc93b1*$^{Letr/Letr}$ and μMT-*Ifnar*$^{-/-}$ chimeric mice and their wild-type controls (μMT-C57BL/6) with *L. donovani* amastigotes and monitored the cellular and humoral immune responses to the parasite. We first monitored the development of protective IFN-γ-producing CD4 T cells in the three groups of mice. μMT-*Unc93b1*$^{Letr/Letr}$ mice displayed a trend toward increased frequencies of IFN-γ$^+$ CD4$^+$ T cells on days 14 and 21 post infection (p.i.) compared with the control group (Figures 5A and 5B). In addition, the mean fluorescence intensity for IFN-γ in the same group of animals was significantly higher on day 21 p.i. (Figure 5C). A characteristic of chronic *L. donovani* infection is the appearance of CD4 T cells co-producing IFN-γ and IL-10. These cells are thought to have immune-suppressive functions and are typically associated with disease progression in humans and mice (Nylén et al., 2007; Stäger et al., 2006). Interestingly, the frequency of IFN-γ$^+$ IL-10$^+$ CD4$^+$ T cells was significantly lower on day 28 p.i. in μMT-*Unc93b1*$^{Letr/Letr}$ mice compared with the control group (Figures 5D and 5E). Although infected μMT-*Ifnar*$^{-/-}$ mice showed stronger Th1 responses and slightly lower frequencies of IFN-γ$^+$IL-10$^+$ CD4$^+$ T cells, the differences were not significant at any time point analyzed.

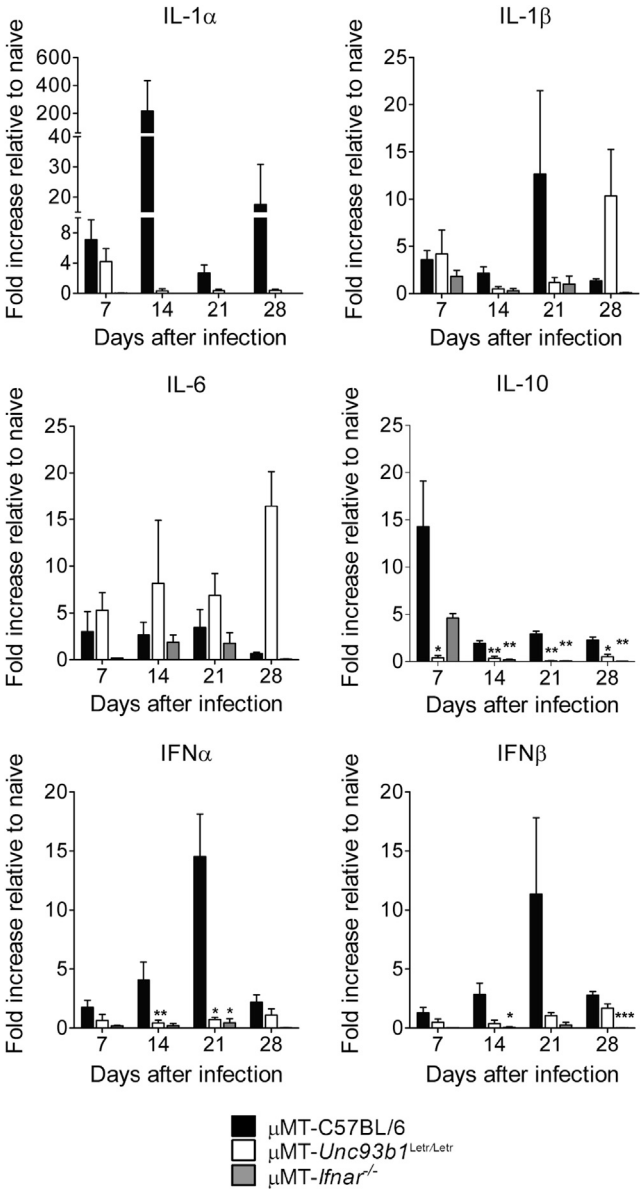


Figure 4. Cytokine mRNA Expression in B Cells during VL Requires Functional Endosomal TLRs and IFNARs

Cytokine mRNA expression in B cells from infected chimeric mice was assessed by qPCR. Data represent mean ± SEM of one of two independent experiments (n = 4–5). *p < 0.05, **p < 0.01, ***p < 0.001.

B Cell Endosomal TLR and IFN-I Are Involved in Hypergammaglobulinemia Induction during *L. donovani* Infection

TLR signaling in B cells is known to potentiate antibody production in autoimmune diseases (Han et al., 2015). Because *L. donovani* induces a very strong antibody response that is detrimental to infection (Deak et al., 2010; Miles et al., 2005), we assessed whether ablation of TLR signaling in B cells would affect the humoral response to the parasite. First, we measured total IgG levels in the sera of infected mice over the course of infection, and we noticed an extreme reduction in serum IgG in μMT-*Unc93b1*$^{Letr/Letr}$ and μMT-*Ifnar*$^{-/-}$ mice compared with the control group (Figure 6A). Serum IgM levels were also significantly reduced, especially on day 14 p.i. Interestingly, both chimeric mouse groups failed to generate an IgM response following infection (Figure 6B). We also found a significant reduction in *Leishmania*-specific IgG antibody levels in μMT-*Unc93b1*$^{Letr/Letr}$ and μMT-*Ifnar*$^{-/-}$ mice compared with the control group (Figure 6C). Collectively, our results indicate that endosomal TLR signaling in B cells strongly enhances antibody production during *L. donovani* infection and may thus contribute to hypergammaglobulinemia. The mechanism of induction partially requires signaling through the IFNAR because μMT-*Ifnar*$^{-/-}$ mice showed an intermediate phenotype with respect to the humoral response (Figures 6A–6C).

To exclude a possible intrinsic B cell defect in our knockout mice, we next immunized *Unc93b1*$^{Letr/Letr}$, *Ifnar*$^{-/-}$, and WT control mice with ovalbumin using saponin as an adjuvant. Immunized *Unc93b1*$^{Letr/Letr}$ produced a lower amount of ovalbumin-specific IgG after the first immunization compared with WT mice. However, when mice were boosted, no significant differences were observed between *Unc93b1*$^{Letr/Letr}$ and WT mice (Figure 6D). *Ifnar*$^{-/-}$ showed a similar response to immunization as WT mice (Figure 6E). This suggests that UNC93b1- and IFNAR- deficient B cells are capable of producing antigen-specific antibodies following an immunization that does not require TLR activation.

B Cell Endosomal TLR Activation by *L. donovani* Exacerbates Infection

Finally, we examined the contribution of B cell endosomal TLR signaling on the parasite burden. *L. donovani* typically establishes chronic infection in the spleen, whereas the parasite is eliminated in the liver. As expected, no major differences in hepatic parasite burden were observed between the three groups of chimeric mice (Figure 7A), suggesting that endosomal TLR signaling in B cells does not substantially contribute to disease resolution or exacerbation in the liver. In contrast, μMT-*Unc93b1*$^{Letr/Letr}$ mice were remarkably resistant to *L. donovani* infection in the spleen, where we observed an 84% reduction in the splenic parasite burden (Figure 7B). These results demonstrate the detrimental role of innate immune B cell activation during visceral leishmaniasis.

DISCUSSION

This study identifies endosomal TLRs as a major pathway of B cell activation by *L. donovani*. Endosomal TLR activation in B cells induced cytokine expression, promoted antibody production, and led to disease exacerbation. The IFNAR was required to enhance this effect.

With exception of IL-10 (Bankoti et al., 2012; Deak et al., 2010; Ronet et al., 2010), the role of B cell-derived cytokines in leishmaniasis has not yet been investigated. We have previously reported that B cell-derived IL10 is MyD88-dependent (Bankoti et al., 2012). In this study, we extend our observation to demonstrate that not only IL-10 but also IL-1, IL-6, and IFN-I are downstream of endosomal TLR signaling following exposure to

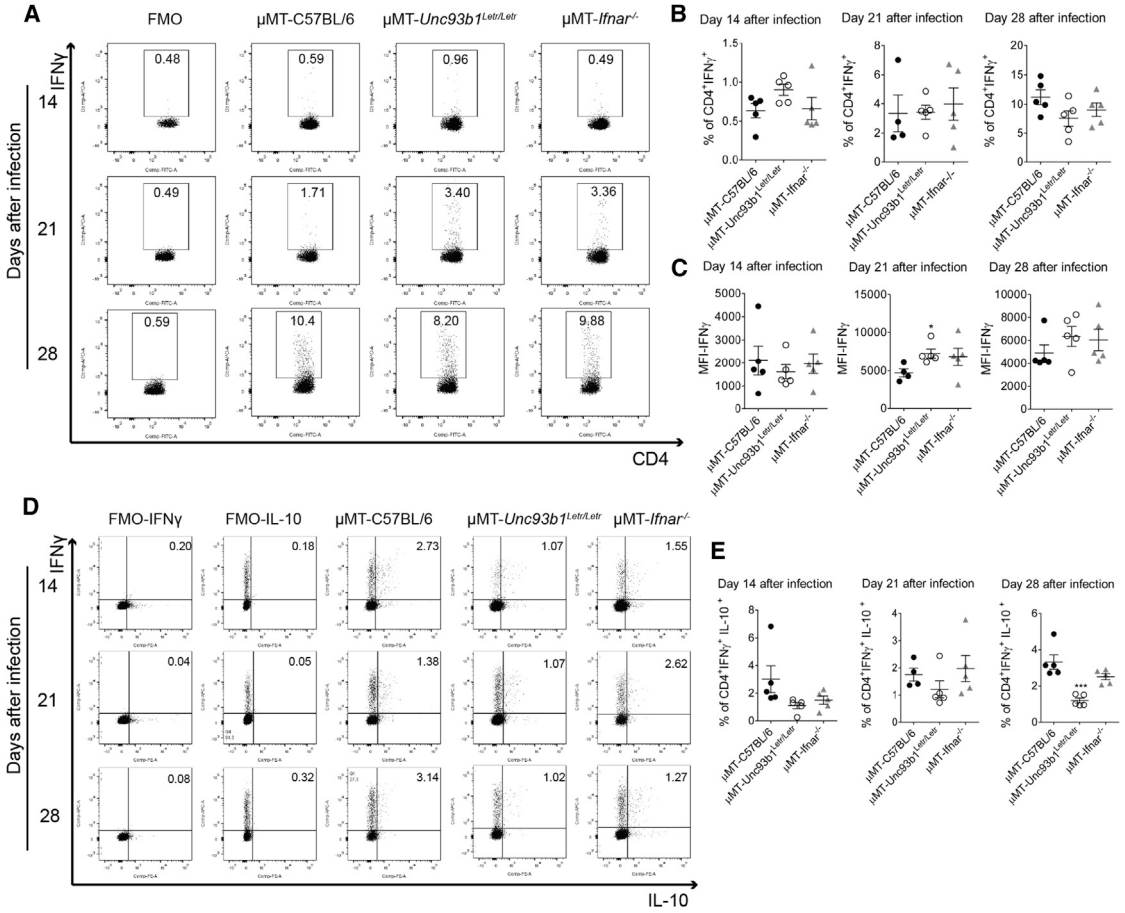


Figure 5. Enhanced Th1 Responses and Lower Frequencies of IFN-γ$^+$ IL-10$^+$ CD4$^+$ T cells in µMT-*Unc93b1*$^{Letr/Letr}$ chimeric mice

Splenocytes from infected µMT-C57BL/6, µMT-*Unc93b1*$^{Letr/Letr}$, and µMT-*Ifnar*$^{-/-}$ mice were stained for CD3, CD4, IFN-γ, and IL-10 after restimulation.

(A) Representative fluorescence-activated cell sorting (FACS) plots for IFN-γ$^+$ CD4$^+$T cells.

(B and C) Percentage of IFN-γ$^+$ (B) and mean fluorescence intensity (C) of IFN-γ in CD4$^+$T cells. Splenocytes were restimulated with PMA/ionomycin for 4 hr and stained for CD3, CD4, IFN-γ, and IL-10.

(D) Representative FACS plots for IFN-γ$^+$ IL-10$^+$ CD4$^+$T cells.

(E) Percentage of IFN-γ$^+$ IL-10$^+$ CD4$^+$ T cells. Data represent mean ± SEM of one of two independent experiments (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001.

L. donovani amastigotes. B cell activation through endosomal TLRs was reported so far only for autoimmune diseases (Avalos et al., 2014; Han et al., 2015) or in a context of vaccination (Hou et al., 2011). Lipopolysaccharides (LPS) and CpG DNA also appear to enhance signaling through the BCR in vitro (Freeman et al., 2015). In contrast, MyD88 expression on B cells was shown to suppress protective immunity against *Salmonella* (Neves et al., 2010) through IL-10 production. In this model, the pathways upstream of MyD88 were not investigated. Nevertheless, the requirement for MyD88 may suggests a role for TLRs in B cell activation and IL-10 induction. TLR signals are also required for the development of regulatory B10 cells (Yanaba et al., 2009).

A large body of literature has now demonstrated that cytokines produced by B cells contribute to the regulation of the adaptive immune response and can inhibit dendritic cells (DC) and macrophage functions. In our infection model, B cell-derived cytokines probably do not play a major role in regulating T cell responses. Indeed, we only observed mild differences in T cell responses between µMT-C57BL/6, µMT-*Unc93b1*$^{Letr/Letr}$, and µMT-*Ifnar*$^{-/-}$, suggesting that B cell-derived cytokines are mainly involved in regulating innate myeloid cell functions. An interesting observation, however, was the induction of IFN-I expression by *L. donovani*. Our results indicate that IFN-α/β may act through autocrine signaling to enhance B cell activation. Indeed, in the absence of a functional IFNAR, cytokine expression was abrogated in B cells exposed in vitro to *L. donovani* or purified from infected mice. IFN-Is are known to exert pleiotropic effects on B cells (Ivashkiv and Donlin, 2014), including enhanced IL-10 production through TLR7/8 in B cells (Liu et al., 2014), induction of marginal zone B cells (MZB) follicular shuttling (Li et al., 2015), stimulation of B cell-activating factor (BAFF) production in monocytes (Gomez et al., 2015), and enhancement of antibody responses by promotion of isotype switching (Le Bon et al., 2001).

In VL, we demonstrate that IFN-I signaling is also involved in the upregulation of TLR3, 7, and 9 in B cells. A positive feedback

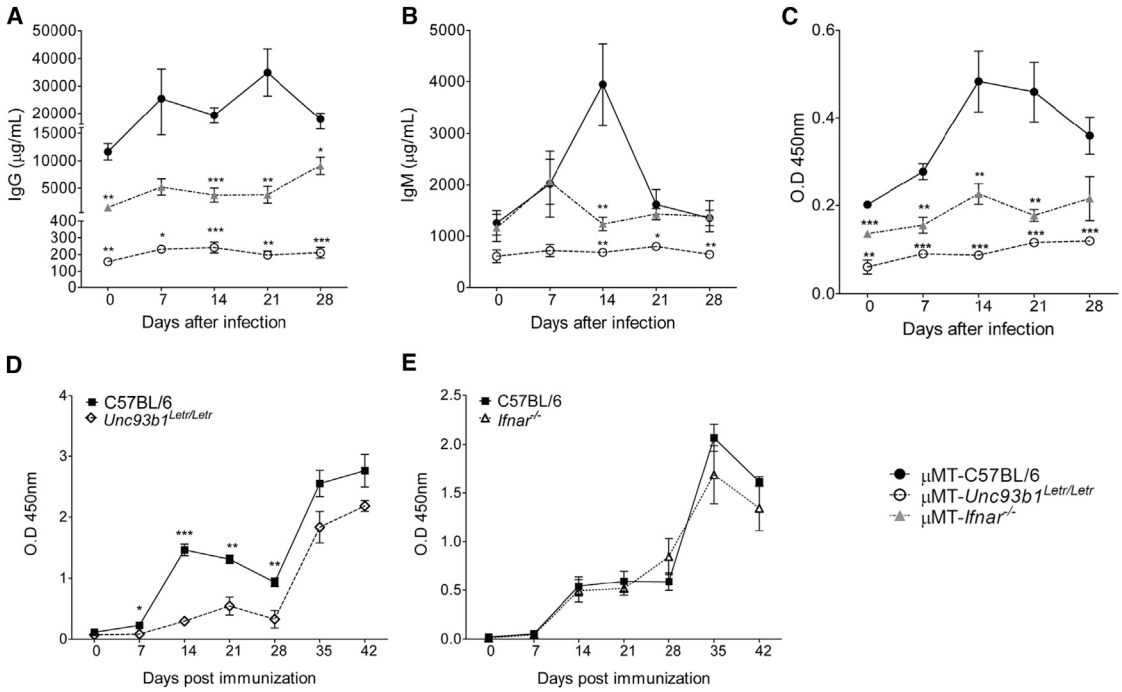


Figure 6. Endosomal TLRs and IFNARs in B Cells Promote Hypergammaglobulinemia

(A–C) Total levels of IgG (A), IgM (B), and *Leishmania*-specific IgG (C) in the sera of infected μMT-C57BL/6, μMT-*Unc93b1*$^{Letr/Letr}$, and μMT-*Ifnar*$^{-/-}$ mice over the course of infection. Data represent mean ± SEM of one of two independent experiments (n = 4–5). O.D., optical density.

(D and E) C57BL/6, *Ifnar*$^{-/-}$, and *Unc93b1*$^{Letr/Letr}$ mice were immunized with ovalbumin and saponin. The graphs show the level of ovalbumin-specific IgG in the sera of immunized mice at various time points after immunization. Data represent mean ± SEM of one of two experiments (n = 5–6). *p < 0.05, **p < 0.01, ***p < 0.001.

loop involving IFN-β and TLR7 has also been proposed by Green et al. (2009). In our model, the interplay between IFN-I and endosomal TLRs has been extended to the regulation of the humoral response. The fact that treatment with IFN-I can lead to lupus-like symptoms (Gota and Calabrese, 2003) suggests that this positive loop may apply to other diseases. A possible role for IFN-I in enhancing antibody production has already been ascribed to various models of viral infections (Lund et al., 2004, Heer et al., 2007) and seem to be mainly dependent on IFNAR expression in B cells (Marzo et al., 2005). However, a direct role for B cell TLRs in regulating the humoral response was only demonstrated in autoimmune diseases. For instance, TLR7 and 9 in B cells have been shown to be involved in enhancing autoantibody production in systemic Lupus Erythematosus (SLE) (Christensen et al., 2006, Han et al., 2015). Moreover, in patients suffering from Wiskott-Aldrich syndrome, increased TLR signaling in B cells promotes the enrichment of self-reactive transitional B cells (Kolhatkar et al., 2015). Our study demonstrates that a parasite can directly activate endosomal TLRs in B cells and that this activation results in enhanced non-specific and *Leishmania*-specific antibody production.

Hypergammaglobulinemia, which commonly leads to antibody-mediated immunopathology, is a hallmark of VL. The mechanisms leading to hypergammaglobulinemia in VL are poorly understood. A recent study conducted in human VL patients revealed that serum levels of the B cell-activating factor BAFF were elevated during active disease (Goto et al., 2014). However, a clear positive correlation between BAFF and IgG levels in VL patients was not found. Hence, the authors suggested that other factors may act synergistically with BAFF to promote hypergammaglobulinemia. During chronic lymphocytic choriomeningitis virus (LCMV), hypergammaglobulinemia and autoantibody production arise when B cells process viral antigen and present it to TFhs (Hunziker et al., 2003). Interestingly, viral antigen processing did not require BCR, complement receptors, or Fc receptors. We also observed with *Leishmania* that induction of cytokine expression was not dependent on a specific BCR (data not shown), suggesting that perhaps *Leishmania* antigens are also internalized by a non-specific pinocytosis of either parasite exosomes or surface molecules cleaved by B cell proteases. The role of TFhs in VL has recently been clarified in a study in macaques (Rodrigues et al., 2014). TFhs appear to expand during acute infection, paralleling the differentiation of activated memory B cells and the production of parasite-specific IgG. In contrast, the authors observed a contraction of germinal centers during chronic infection, confirming results reported previously in experimental VL (Smelt et al., 1997). This contraction affected the production of parasite-specific IgG, whereas hypergammaglobulinemia persisted. Hence, it appears that TFhs may play a limited role in the maintenance of hypergammaglobulinemia during VL. Nevertheless, it is possible that TLR-activated B cells may induce abortive TFh responses while enhancing the production of non-specific antibodies. Further investigations are warranted to understand the interaction between these two cell populations.

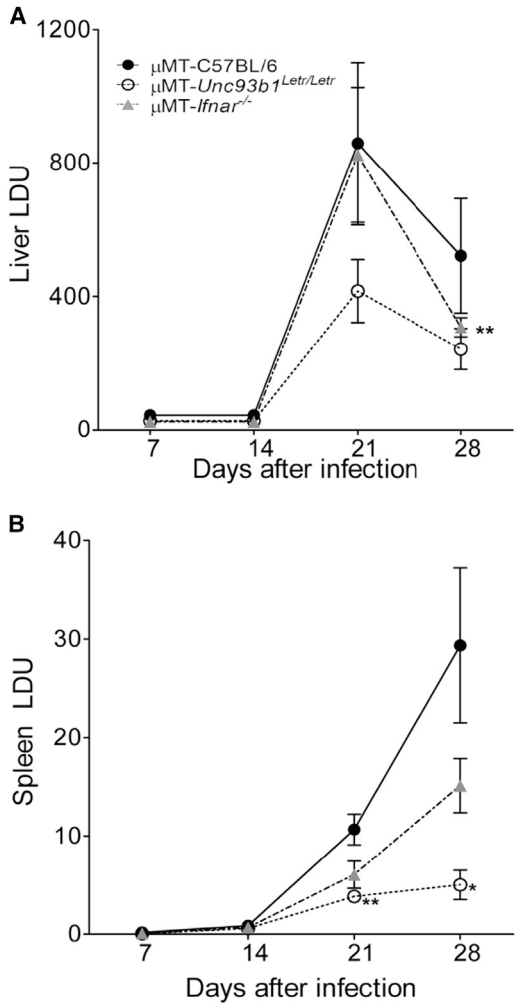


Figure 7. Endosomal TLRs in B Cells Contributes to VL Exacerbation
(A and B) Liver (A) and splenic (B) parasite burden in chimeric mice over the course of infection, represented as LDUs. Data represent mean ± SEM of one of two independent experiments (n = 4–5). *p < 0.05,**p < 0.01.

In conclusion, we discovered that a parasite can directly activate endosomal TLRs in B cells. We propose that this activation is largely responsible for promoting hypergammaglobulinemia in an IFNAR-dependent manner. Hypergammaglobulinemia and elevated levels of IFN-I are characteristics of several chronic infections and autoimmune diseases and are often associated with immunosuppression and/or increased pathology. Thus, innate activation of B cells may be an unappreciated mechanism that underlies the development of hypergammaglobulinemia and may therefore contribute to immunopathology in other chronic infections.

EXPERIMENTAL PROCEDURES

Mice and Parasites

C57BL/6 and μMT mice were purchased from The Jackson Laboratory. *Unc93b1*$^{Letr/Letr}$ (Lafferty et al., 2014) and *Ifnar*$^{-/-}$ (Müller et al., 1994) mice were bred at the animal facility of McGill University. All mice were housed at the Institut National de la Recherche Scientifique (INRS) animal facility under specific pathogen-free conditions and used at 6–12 weeks of age. Experiments involving mice were carried out under protocols approved by the Animal Care and Use Committee of the INRS, Institut Armand-Frappier. These protocols respect procedures on good animal practice provided by the Canadian Council on Animal Care.

L. donovani (strain LV9) was maintained by serial passage in B6.129S7-Rag1^{tm1Mom} mice, and amastigotes were isolated from the spleens of infected animals. Mice were infected by injecting 2 × 10^7 amastigotes intravenously via the lateral tail vein (Stäger et al., 2003). Splenic parasite burdens were determined by examining methanol-fixed, Giemsa-stained tissue impression smears. Data are presented as number of parasites per spleen or as Leishman Donovan Units (LDUs) (Bankoti et al., 2012).

B Cell In Vitro Studies

Naive splenic B cells were purified using the B cell isolation kit (Miltenyi Biotech) according to the manufacturer's protocol. Purified B cells (purity 90%–92%) were then incubated either alone, with different ratios of parasites, or with TLR agonists: 3 μg/ml CpG (InvivoGen), 5 μg/ml R837 (InvivoGen), 3 μg/ml poly (I:C) (Amersham), and 10 μg/ml LPS (Sigma). B cells were also incubated with 1 μg/ml ODN2088 (Miltenyi) and a TLR7 and 9 inhibitor (Stunz et al., 2002). Cells were cultured in supplemented DMEM (Gibco, Invitrogen). For cytokine mRNA expression analysis, confocal microscopy, and immunoblots, cells were incubated for 8 or 24 hr at 37°C.

Quantitative Real-Time PCR

RNA from isolated B cells from in vitro or in vivo experiments was extracted using the RNeasy mini kit (QIAGEN) as described by the manufacturer. Reverse transcription was carried out using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. Real-time PCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The IL-1α, IL-1β, IL-6, IL-10, IFN-α, IFN-β, and HPRT genes were amplified using primers whose sequences are described below. All PCRs were carried out with the Stratagene mx3005p real-time PCR system. Data were normalized to HPRT and expressed as fold increase to naive controls.

Primers

The following primers were used to determine the relative gene expression using qPCR: *Hprt*, 5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′ and 5′-GAT TCA ACC TTG CGC TCA TCT TAG GC-3′; *Il1a*, 5′-CAA ACT GAT GAA GCT CGT CA-3′ and 5′-TCT CCT TGA GCG CTC ACG AA-3′; *Il1b*, 5′-AAC CTG CTG GTG TGT GAC GTT C-3′ and 5′-CAG CAC GAG GCT TTT TTG TTG T-3′; *Il6*, 5′-ACA ACC ACG GCC TTC CCT ACT T-3′ and 5′-CAC GAT TTC CCA GAG AAC ATG TG-3′; *Il10*, 5′-AGG GTT ACT TGG GTT GCC AA-3′ and 5′-CAC AGG GGA GAA ATC GAT GA-3′; *Ifna*, 5′-CAT CTG CTG CTT GGG ATG GAT-3′ and 5′-TTC CTG GGT CAG AGG AGG TTC-3′; *Ifnb*, 5′-TCA GAA TGA GTG GTG GTT GC-3′ and 5′-GCA CTT TCA AAT GCA GTA GAT TCA-3′; *Tlr3*, 5′-AAG ACA GAG ACT GGG TCT GGG-3′ and 5′-TGA AAC TTC GTC CGC AGG AA-3′; *Tlr7*, 5′-GGC ATT CCC ACT AAC ACC AC-3′ and 5′-TTG GAC CCC AGT AGA ACA GG-3′; and *Tlr9*, 5′-CTA GAT GCT AAC AGC CTC GCC-3′ and 5′-GTC CTC GCC ACT TCC ACT G-3′.

Confocal Microscopy

L. donovani parasites were stained with PKH67 (Sigma) following the manufacturer's instructions and incubated with purified naive splenic B cells (B cell isolation kit, Miltenyi). 2 × 10^6 B cells were incubated either alone or with PKH67-*L. donovani* amastigotes (at a multiplicity of infection [MOI] of 5:1 or 10:1). 8 or 24 hr post-incubation, cells were fixed with 2% paraformaldehyde for 15 min at 4°C. Permeabilization and unspecific staining were blocked using 0.1% Triton X-100, 1% bovine serum albumin, 5% goat serum, and 5% horse serum in PBS for 30 min at 4°C. For immunostaining, cells were labeled with coupled antibodies against BCR (goat anti-mouse IgM-AF568, Life Technologies), TLR3 (anti-TLR3 antibody (40C1285.6)-DyLight 650, Novus), and TLR9 (anti-TLR9 monoclonal antibody, Abcam) coupled to IgG1 Zenon-Alexa Fluor-647, Life Technologies). NucBlue (Life Technologies) was used to visualize cell nuclei, following the manufacturer's instructions. Labeled cells were washed in PBS and resuspended in 100 μl of PBS and then transferred

onto a polylysine-coated coverslip. Coverslips were subsequently mounted with mounting medium (Fluoromount G, Southern Biotechnology Associates). Analysis of protein localization was performed using a Zeiss LSM780 system equipped with a 30-mW, 405-nm diode laser; 25-mW, 458/488/514 argon multiline laser; 20-mW DPSS 561-nm; laser; and 5-mW HeNe 633-nm laser mounted on a Zeiss Axio Observer Z1 and operated with Zen 2011 software (Zeiss). We used a Plan-Apochromat 63× oil differential interference contrast (DIC) 1.4 numerical aperture (NA) objective for our observations, and images were acquired via sequential acquisition.

Immmunoblot

Total cell protein extracts from 3×10^6 purified B cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with protease inhibitors (Complete mini, Roche). Equal amounts of protein (15 μg) were fractionated by 8% SDS-PAGE, and transferred to a nitrocellulose membrane (Hybond, Amersham). Rabbit anti-mouse polyclonal antibody against TLR7 (Imgenex) was used for the immunoblot assay. Blots were stripped (stripping buffer, Thermo Fisher), and equal loading was confirmed with a monoclonal antibody against β-actin (Abcam).

Generation of Mixed Bone Marrow Chimeras

Chimeras were generated by reconstituting lethally irradiated C57BL/6 mice with 2×10^7 bone marrow cells from μMT, *Unc93b1*$^{Letr/Letr}$, *Ifnar*$^{-/-}$, or C57BL/6 mice as described previously (Bankoti et al., 2012). The bone marrow was mixed at a ratio of 4:1 for the following combinations: 80% μMT with 20% *Unc93b1*$^{Letr/Letr}$, 80% μMT with 20% *Ifnar*$^{-/-}$, and 80% μMT with 20% C57BL/6. The reconstitution level was evaluated 6 weeks after bone marrow transfer (Figure S3). Chimeric mice were infected 7–8 weeks after reconstitution with 2×10^7 *L. donovani*-LV9 amastigotes intravenously via the lateral tail vein.

Flow Cytometry

Endogenous CD4 T cell responses in infected chimera were analyzed as described previously (Nothelfer et al., 2015). Briefly, splenocytes were either restimulated with bone marrow-derived dendritic cells (BMDCs) pulsed with fixed parasites or phorbol 12-myristate 13-acetate (PMA)/ionomycin in the presence of Brefeldin A (BD Biosciences). Cells were then stained with anti-CD4-fluorescein isothiocyanate (FITC), anti-CD8 Pacific Blue, anti-IFN-γ-allophycocyanin, and anti-IL-10-phycoerythrin (BD Biosciences). 350,000 cells were acquired on a BD LSRFortessa cell analyzer (Becton Dickinson), and analysis was performed using FlowJo software (Tree Star).

The expression of costimulatory molecules by B cells from infected chimeric mice was assessed using the following antibodies: FITC-conjugated anti-major histocompatibility complex class II (MHCII) (BD Biosciences), eFluor 450-conjugated anti-CD19 (eBioscience), PE-conjugated anti-CD40 (eBioscience), PE-conjugated anti-CD80 (eBioscience), and PE-conjugated anti-CD86 (eBioscience). Cells were acquired with a BD LSRFortessa cell analyzer (Becton Dickinson), and analysis was performed using FlowJo software (Tree Star).

Antibody Production

Sera from infected mice were analyzed by ELISA. Total IgM and IgG levels were measured using the Mouse IgM ELISA Ready-SET-Go! kit and Mouse IgG ELISA Ready-SET-Go! kit (eBioscience) following manufacturer's recommendation. *Leishmania*-specific IgG was measured using the following protocol. Nunc Maxisorp 96-well plates (eBioscience) were coated for 2 hr at 37°C and then overnight at 4°C with 8 μg of soluble *Leishmania* antigens (SLAs) in coating buffer (15 mM Na_2CO_3 and 35 mM NaH_2CO_3 [pH 9.6]). Plates were washed three times with 0.1% Tween 20 in PBS (PBS-T) and further blocked with 0.1% Tween 20 and 0.5% gelatin for 2 hr at room temperature. After washing, sera diluted in PBS (1:400) were added and incubated for 2 hr at 37°C. Wells were then washed five times with PBS-T, followed by the addition of enhanced chemiluminescence (ECL) anti-mouse IgG and horseradish peroxidase-linked species-specific whole antibody (GE Healthcare, Amersham) diluted 1:12,000 in PBS and were incubated for 1 hr at 37°C. Substrate solution (3, tetramethylbenzidine (TMB) liquid substrate, Sigma) was then added. The reaction was developed at room temperature for 15 min and stopped with 1 M H_2PO_4. Absorbance was measured at 450 nm in a microplate reader (model 680, Bio-Rad).

Ovalbumin Immunization

C57BL/6, *Unc93b1*$^{Letr/Letr}$, and *Ifnar*$^{-/-}$ mice were immunized with 400 μg ovalbumin (Ova) (Sigma) and 0.05% saponin (Sigma) in sterile PBS at the base of the tail. Mice were boosted 28 days after the first immunization with the same amount of protein and adjuvant. Blood was collected weekly by the lateral saphenous vein; serum was separated by centrifugation and stored at −80°C. Ova-specific IgG were measured following the protocol described by Garulli et al. (2008). A serial dilution of the sera was performed; the data represent the results of dilution at 1:200. The reaction was developed at room temperature for 5 min and stopped with 1 M H_2PO_4. Absorbance was measured at 450 nm in a microplate reader (model 680, Bio-Rad).

Statistical Analysis

Statistical analysis was performed using Student's t test. Differences were considered to be statistically significant when $p < 0.05$. All experiments were conducted independently at least twice.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.028.

AUTHOR CONTRIBUTIONS

S.S.-B. and S.S. conceived the project, designed the experimental approach, performed experiments, interpreted data, and wrote the manuscript. A.D. provided key expertise and interpreted data. M.S. and C.U.D. performed experiments and analyzed data. S.T.Q. and J.H.F. provided key expertise and key materials.

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