RESEARCH ARTICLE

The IL-33/ST2 Axis Is Associated with Human Visceral Leishmaniasis and Suppresses Th1 Responses in the Livers of BALB/c Mice Infected with *Leishmania donovani*

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ABSTRACT **During visceral leishmaniasis, the control of hepatic parasite burden is mainly due to granuloma assembly in a microenvironment consisting of both Th1 and Th2 components. Using enzyme-linked immunosorbent assay (ELISA) dosages, quantitative PCR (qPCR), immunohistochemistry, and flow cytometry, we studied the role of interleukin-33 (IL-33), a recently described cytokine signaling through the ST2 receptor, during visceral leishmaniasis. We showed that a higher level of IL-33 was detected in the serum of patients with visceral leishmaniasis than in that from healthy donors and demonstrated the presence of IL-33$^+$ cells in a liver biopsy specimen from a patient. Similarly, in BALB/c mice experimentally infected with *L. donovani*, a higher level of IL-33 was detected in the serum, as well as the presence of IL-33$^+$ cells and ST2$^+$ cells in the mouse liver. In ST2$^{-/-}$ BALB/c mice, better control of the hepatic parasite burden and reduced hepatomegaly were observed. This was associated with strong induction of Th1 cytokines (gamma interferon [IFN-γ] and IL-12) compared to the level in wild-type (WT) mice and better recruitment of myeloid cells associated with strongly induced chemokines (CCL2 and CXCL2) and receptors (CCR2 and CXCR2). Conversely, BALB/c mice treated twice weekly with recombinant IL-33 showed a dramatically reduced induction of Th1 cytokines and delayed inhibition of monocyte and neutrophil recruitment in the liver, which was associated with reduced KC/CXCL1 and CXCR2 expression. Taken together, our results suggest that IL-33 could be a new deleterious regulator of the hepatic immune response against *Leishmania donovani*, via the repression of the Th1 response and myeloid cell recruitment.**

IMPORTANCE **Visceral leishmaniasis is a life-threatening systemic disease due to the *Leishmania* protozoa *L. infantum* and *L. donovani* and is ranked by the World Health Organization as the second most important protozoan parasitic disease after malaria for its grave morbidity, high mortality, and global distribution. *Leishmania* parasites subvert the host's immune response to propagate to target organs, including the spleen, the bone marrow, and the liver. Control of hepatic parasite burdens depends on a delicate and poorly understood Th1/Th2 immune balance. To better understand this complex immune response, new cytokines are interesting targets for research studies. IL-33 is a newly described cytokine usually associated with Th2 response and involved in different diseases, including infectious diseases and hepatitis. Our results suggest that IL-33 could be a new factor of susceptibility and a potential prognostic marker during visceral leishmaniasis.**

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Interleukin-33 (IL-33) is a recently identified member of the IL-1 cytokine family that can act either as a nuclear binding factor or as a cytokine (1, 2). Once secreted, IL-33 signals through a heterodimer composed of the ST2-specific receptor (also called T1 or IL-1RL1) and the IL-1 receptor accessory protein, which is a member of the IL-1 receptor family (2). Before the discovery of IL-33, ST2 was known as a Th2 cell marker constitutively expressed or induced on several immune cell types, such as Th2 lymphocytes, invariant natural killer T (iNKT) cells, natural killer (NK) cells, cytotoxic T cells, monocytes, macrophages, dendritic cells, polymorphonuclear neutrophils (PMN), mast cells, basophils, and eosinophils (3). Accordingly, IL-33 has a broad range of effects on the immune system—either proinflammatory effects or promotion of Th2 immune response—leading to beneficial or worsening outcomes, depending on the clinical setting (4, 5). IL-33 plays a deleterious role in many autoimmune diseases, such as rheumatoid arthritis (6, 7) and inflammatory bowel disease (8), and induces a predisposition to asthma and other allergic diseases (9, 10). Conversely, IL-33 has a protective role in many diseases associated with or exacerbated by Th1 response, like atherosclerosis (11), and in some cardiovascular or metabolic diseases (12, 13). During infectious diseases, IL-33 may also play opposing roles, depending on the organ involved and the Th1/Th2 immune balance necessary to control the infection. IL-33 was shown to be

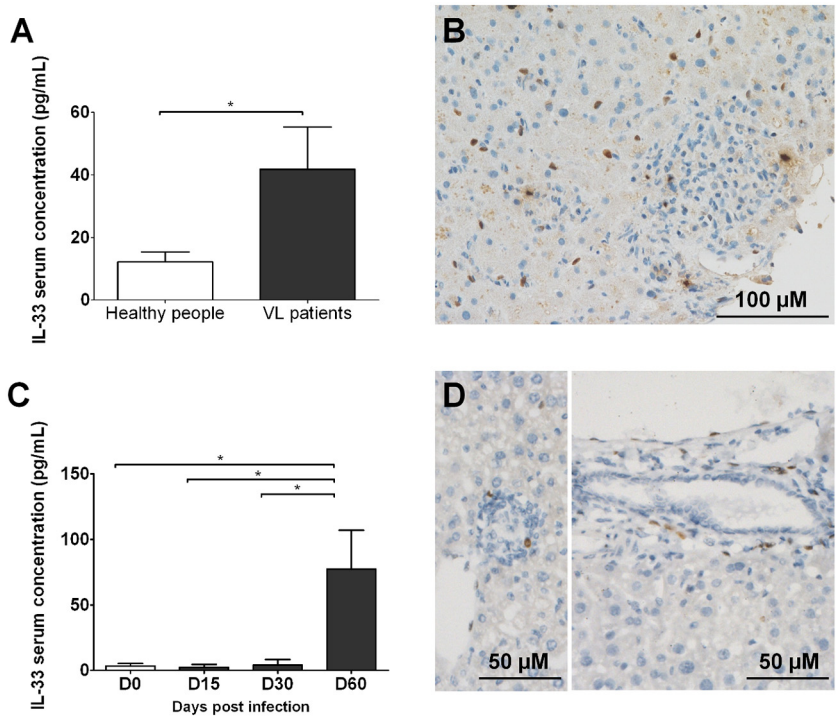

FIG 1 Dosage of serum IL-33 and IL-33 hepatic expression in patients with visceral leishmaniasis and in BALB/c mice infected with *Leishmania donovani*. (A) Quantification of IL-33 by ELISA in the serum of VL patients ($n = 6$) and healthy donors ($n = 21$); the data represent means ± SEM (*, $P < 0.05$). (B) IL-33 expression detected by immunohistochemistry in the liver of a VL patient. The image was acquired at a ×400 magnification. (C) Quantification of IL-33 by ELISA in the serum of BALB/c mice infected with *L. donovani* at 15, 30, or 60 days postinfection (D15, D30, and D60, respectively) and of noninfected mice (D0); the data represent means ± SEM from 4 mice per time point (*, $P < 0.05$). (D) IL-33-expressing cells detected by immunohistochemistry on a liver section from an infected BALB/c mice (D60). Shown is a representative image acquired from 1 mouse out of 7 at a ×400 magnification.

protective during sepsis (14) or keratitis due to *Pseudomonas aeruginosa* (15) and during infections with *Trichuris muris* (16), *Schistosoma mansoni* (17), or *Toxoplasma gondii* (18), whereas it is deleterious during cutaneous leishmaniasis (CL). In *Leishmania major*-infected mice, it was demonstrated that a minor population of ST2$^+$ Th2 cells were specifically enriched in the nonhealing infection site (19) and that blockade of ST2 using an ST2-specific blocking antibody or the fusion protein T1-Fc resulted in the development of less severe disease, with reduced parasite load and a switch in T cell response polarity to a protective Th1 response (20).

During visceral leishmaniasis (VL) due to *L. infantum* and *L. donovani*, the control of hepatic parasite burden is mainly due to a granulomatous inflammatory response, mostly involving Kupffer cells and infiltrating blood monocytes (21). In experimental models of VL, IL-12 plays a pivotal role by initiating a Th1 cell response with the production of gamma interferon (IFN-γ), which activates macrophages, leading to parasite death (22). However, regarding its sustained exposure to many antigens and chemicals, the liver is characterized by a tolerogenic Th2-biased microenvironment, with IL-10 and transforming growth factor β (TGF-β) secretion (23). Thus, a peculiar immune environment involving both Th1 and Th2 cells is usually described during VL and is associated with efficient granuloma assembly and parasite killing (24, 25). Recently described cytokines could be involved in this complex hepatic immune response. Whereas ST2 is clearly associated with the deleterious Th2 response against *L. major* in the skin during cutaneous leishmaniasis (20), no data are available about the roles of IL-33 and ST2 in the liver during VL. Here, we show that IL-33 and ST2 are expressed in the liver during human and experimental murine VL. ST2 deficiency in transgenic BALB/c mice led to a better controlled parasite burden in the liver, which was associated with an early infiltrate of PMN and monocytes and a Th1 polarized immune response. Conversely, injection of recombinant IL-33 in BALB/c mice led to a repressed Th1 response and limited infiltrate of PMN and monocytes.

RESULTS

Human visceral leishmaniasis is associated with increased serum IL-33 and IL-33 expression in the liver. IL-33 was detected at significantly higher levels in the serum of the 6 VL patients than in the 21 healthy controls (41.8 ±13.5 pg/ml versus 8.6 ±2.2 pg/ml; $P = 0.0105$) (Fig. 1A). All dosages were carried out before onset of treatment.

Immunohistochemical staining of the liver biopsy specimen obtained from a patient with VL revealed a huge parasite burden associated with numerous IL-33-positive cells—either endothelial cells or infiltrating cells and cells surrounding granulomatous foci (Fig. 1B). These observations led us to further investigate the role of IL-33 in murine models.

Infection with *L. donovani* is associated with increasing levels of serum IL-33 and IL-33 expression in the liver. IL-33 levels were measured in the serum of BALB/c mice infected with *L. donovani* at different time points of the disease, and the results revealed a low level of detection in noninfected mice (3.4 ±1.8 pg/ml), as well as at day 15 (D15) and D30 postinfection (2.4 ±2.3 and 4.1 ±4.1 pg/ml, respectively). A significant increase in IL-33 was detected in the serum at day 60, with a mean concentration of 89.7 ±20.1 pg/ml ($P < 0.05$ compared with D0, D15, and D30) (Fig. 1C).

As for humans, immunohistochemical staining of liver biopsy specimens using a goat anti-mouse IL-33 revealed the presence of a specific nuclear staining in cells preferentially located in granulomas and infiltrates surrounding blood vessels at D60 (Fig. 1D), and to a lesser extent at D15 and D30 (data not shown). In addition, as classically described, some endothelial cells were also IL-33$^+$ (2, 26), as confirmed via a costaining of IL-33 and CD31 by immunofluorescence on frozen liver sections at all time points (data not shown).

Infection with *L. donovani* induces the recruitment of ST2$^+$ cells in the livers of BALB/c mice. In order to study the impact of this IL-33 hepatic expression and late systemic secretion, the presence of ST2-expressing cells was first demonstrated by immuno-

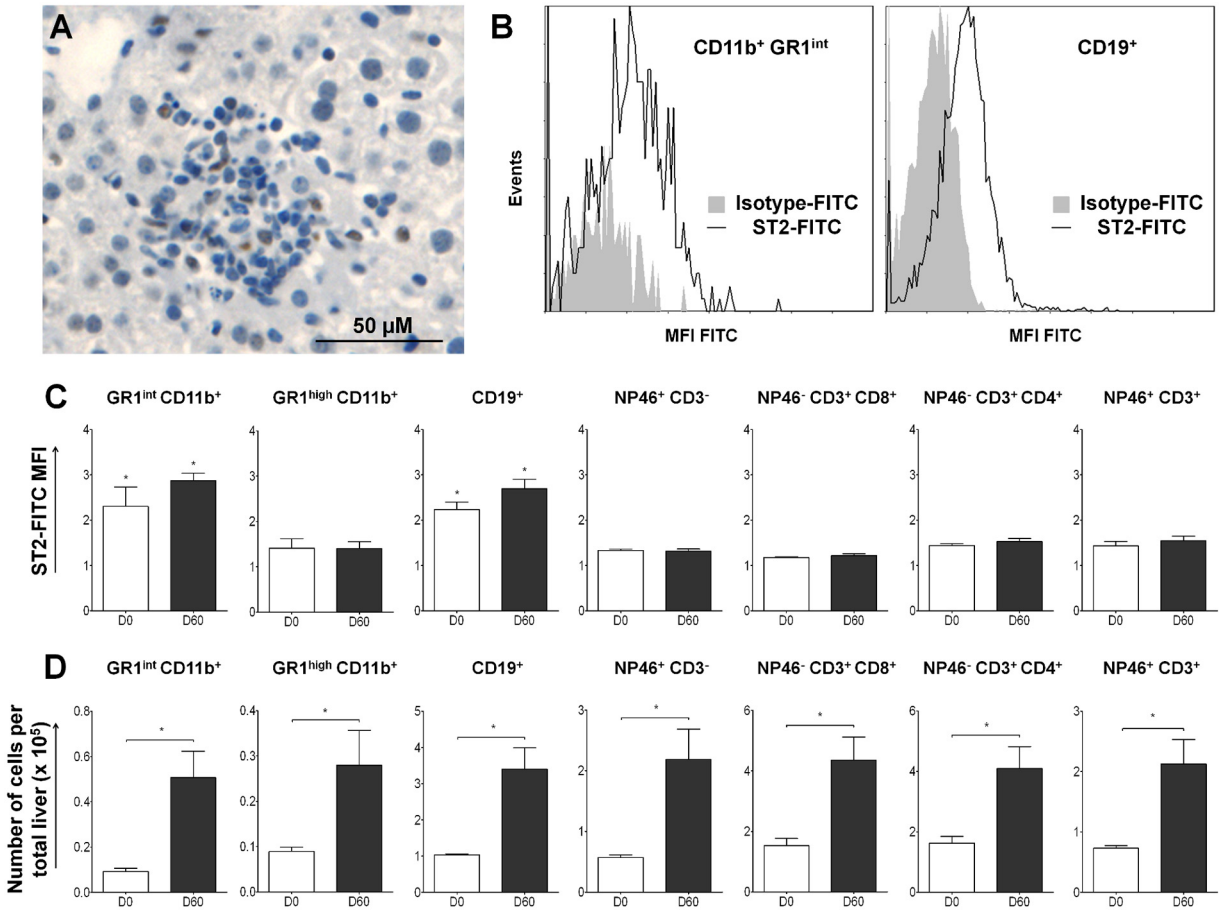

FIG 2 ST2 expression in the liver of BALB/c mice infected with *Leishmania donovani*. (A) Detection of ST2$^+$ cells by immunohistochemistry in liver section of an infected BALB/c mouse on day 60 (D60) postinfection. Shown is a representative image acquired from 1 mouse out of 7 at a ×400 magnification. (B, C, and D) Quantification of ST2$^+$ cell infiltrate in the total livers of BALB/c mice by flow cytometry in noninfected mice (D0) or infected mice at D60. (B) ST2 receptor was detected in GR1^{int} CD11b$^+$ cells and CD19$^+$ cells. The gray curve represents the control isotype, and the black curve represents the specific ST2 staining. This panel is representative of 3 to 4 mice per group. (C) Quantification of ST2 expression on each cell type. ST2 expression was computed as the ratio of ST2/control Ig-FITC mean fluorescence intensities (MFI). The ratio was annotated with * when significantly higher than 1 ($P < 0.05$). (D) Absolute quantification of each cell type in the total liver of mice at D0 and D60. The data represent means ± SEM from 4 mice per time point (*, $P < 0.05$).

histochemistry in the liver at D60 (Fig. 2A). A flow cytometry analysis of the whole liver using the same antibody revealed specific ST2 expression in CD11b$^+$ GR1^{int} cells and in CD19$^+$ cells compared with the isotype control antibody (Fig. 2B). Indeed, the mean fluorescent intensity (MFI) ratios of the anti-ST2 antibody and isotype were 2.2 and 2.3, respectively, and significantly different from 1, whereas other analyzed cell populations showed lower and nonsignificant ratios (Fig. 2C). On day 60 after infection, no significant change was observed in the MFI ratio for any cell type (Fig. 2C). However, a significant infiltrate of all analyzed cell types was observed at D60 compared to noninfected mice, with a 5.5-fold increase of CD11b$^+$ GR1^{int} cell number in the total liver, whereas other cell types were only 2.5- to 3.8-fold increased (Fig. 2D), leading to an enrichment of ST2$^+$ cells after infection.

The hepatic *Leishmania* burden is better controlled in ST2$^{-/-}$ BALB/c mice. To address the role of the ST2$^+$ infiltrating cells, the hepatic immune responses were compared in wild-type (WT) and ST2-deficient mice after infection with *L. donovani*. In WT mice, the parasite burden was significantly higher at D60 (757.0 ± 125.3 *L. donovani* units [LDU]), compared with D15 and D30 (399.4 ±76.86 and 389.5 ± 73.67 LDU, respectively). In ST2$^{-/-}$ mice, the parasite burden was similar to that of WT mice at D15 and D30, but at D60, the liver parasite burden was significantly lower in ST2$^{-/-}$ mice (400.8 ± 69.62 LDU) than in WT mice ($P < 0.05$) (Fig. 3A).

The uncontrolled parasite burden in BALB/c mice at D60 was associated with significant hepatomegaly, a common feature of VL. Indeed, the weight of the liver reached 1.4 ± 0.1 g at D60 and was significantly increased compared with that in noninfected mice (1.1 ± 0.1 g; $P < 0.05$). The basic liver weight of noninfected ST2$^{-/-}$ mice was comparable to that of noninfected WT mice, but at D60 it was significantly lower in ST2$^{-/-}$ mice than that in WT mice (1.0 ± 0.1; $P < 0.05$) (Fig. 3B).

ST2$^{-/-}$ mice infected with *L. donovani* display a Th1 polarized immune response. As the hepatic immune response against *L. donovani* is highly dependent on cytokine induction, quantitative PCR (qPCR) analyses were performed on hepatic lysates to quantify the induction of key Th1 and Th2 cytokines. Whereas IL-12p35 was not significantly induced in WT mice during the course of the disease, strong induction was observed in ST2$^{-/-}$

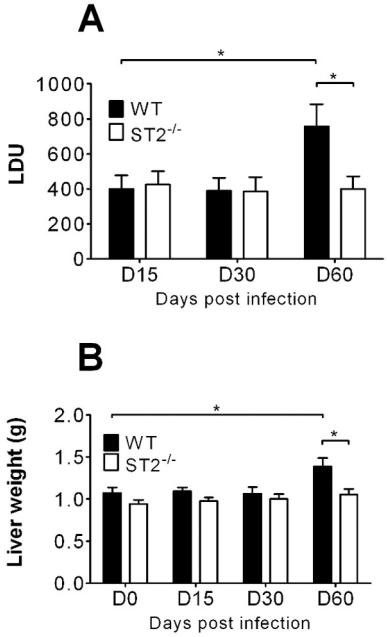

FIG 3 Hepatic parasite burdens and liver weights in BALB/c WT and ST2$^{-/-}$ mice after infection with *Leishmania donovani*. (A) Liver parasite burden was determined on days 15, 30, and 60 postinfection (D15, D30, and D60, respectively) by microscopic counting of Giemsa-stained tissue sections and is expressed as LDU (no. of parasites/1,000 nuclei × liver weight in mg). (B) The liver weight was recorded on days 0, 15, 30, and 60 in WT and ST2$^{-/-}$ mice. Data are expressed as means ± SEM from 7 to 13 mice per mouse strain for each time point; pooled data are from three independent experiments (*, $P < 0.05$).

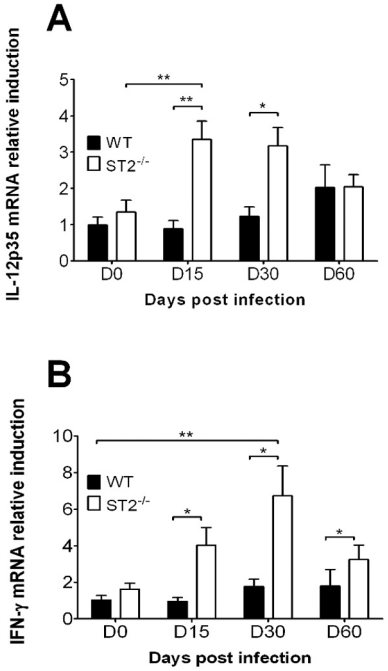

FIG 4 Kinetics of hepatic mRNA induction of IL-12 and IFN-γ in WT and ST2$^{-/-}$ mice infected with *Leishmania donovani*. mRNA induction of IL-12 (A) and IFN-γ (B) was quantified by quantitative PCR in liver extracts at various time points after infection and normalized by comparison to 18S mRNA. Data are the means ± SEM from 7 to 13 mice per mouse strain for each time point; pooled data are from three independent experiments (*, $P < 0.05$; **, $P < 0.01$).

mice at D15 and D60 ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 4A). Similarly, IFN-γ was not significantly induced in infected WT mice compared to noninfected mice, but a significant induction was observed in ST2$^{-/-}$ mice at early and late time points ($P < 0.05$) (Fig. 4B). On the contrary, Th2 cytokines, such as IL-4, IL-10, and IL-13, were not induced or were weakly induced, and there were no significant differences between infected and noninfected mice (data not shown).

Monocytes and PMN are recruited early in the liver of ST2$^{-/-}$ BALB/c mice in response to *L. donovani* infection. As monocytes and PMN are important cell types recruited early in the liver for an efficient immune response against *L. donovani*, some key chemokines involved in their attraction, CCL2 and CXCL2, were quantified by qPCR. A significant elevation in CCL2 mRNA was observed at D15 ($P < 0.05$) and D30 ($P < 0.01$) in the ST2$^{-/-}$ samples compared to those from WT mice (Fig. 5A). A significant rise in CXCL2 expression was also observed in ST2$^{-/-}$ mice at D30 ($P < 0.001$), with significantly stronger induction than in WT mice ($P < 0.05$) (Fig. 5B).

To quantify the recruitment of CCL2- and CXCL2-responding cells, the induction of CCR2 and CXCR2 receptors was analyzed by qPCR in hepatic lysates. CCR2 was not perceptibly induced in WT mice, whereas a significantly higher induction was observed in ST2$^{-/-}$ mice at D15 ($P < 0.05$) and D30 ($P < 0.01$) (Fig. 5C). Similarly, a significant induction of CXCR2 was observed at D15 in ST2$^{-/-}$ mice compared to that in WT mice ($P < 0.001$) (Fig. 5D).

To address the role of CCL2 and CXCL2 to recruit myeloid cells in the liver during the course of infection, the expression of myeloperoxidase (MPO) was quantified by qPCR in the liver. The expression of this enzyme, mostly expressed in PMN, was higher in ST2$^{-/-}$ mice at all time points after infection ($P < 0.05$ at D30) (Fig. 5E). To confirm the stronger myeloid cell recruitment in ST2$^{-/-}$ mice, an immunohistochemical staining using an anti-MPO antibody was performed on liver sections. The number of MPO$^+$ cells observed on tissue sections was expressed in terms of the tissue surface (mm^2). A striking infiltrate of MPO$^+$ cells was observed at D15 and D30 ($P < 0.05$) in ST2$^{-/-}$ mice (Fig. 5F to H). In particular, an early infiltrate was observed at D15, where the number of MPO$^+$ cells was 2-fold higher in ST2$^{-/-}$ mice compared to WT mice ($P < 0.05$) (Fig. 5F), but no significant difference was observed between WT and ST2$^{-/-}$ mice at later time points (Fig. 5F).

Recombinant IL-33 treatment limits the Th1 immune response in infected BALB/c mice. In order to consolidate the data obtained with ST2-deficient mice and address more specifically the role of free IL-33 in the liver, BALB/c WT mice were infected with *L. donovani* and treated intraperitoneally with recombinant IL-33 (rIL-33) twice a week until sacrifice on day 15, 30, or 60.

A dramatic reduction of hepatic IFN-γ and IL-12p35 induction was observed in rIL-33-treated mice compared to nontreated (NT) mice ($P < 0.001$ and $P < 0.05$ on day 60, respectively) (Fig. 6A and B). Again, no differences were observed in the induction levels of the Th2 cytokines IL-4, IL-10, and IL-13 at all time

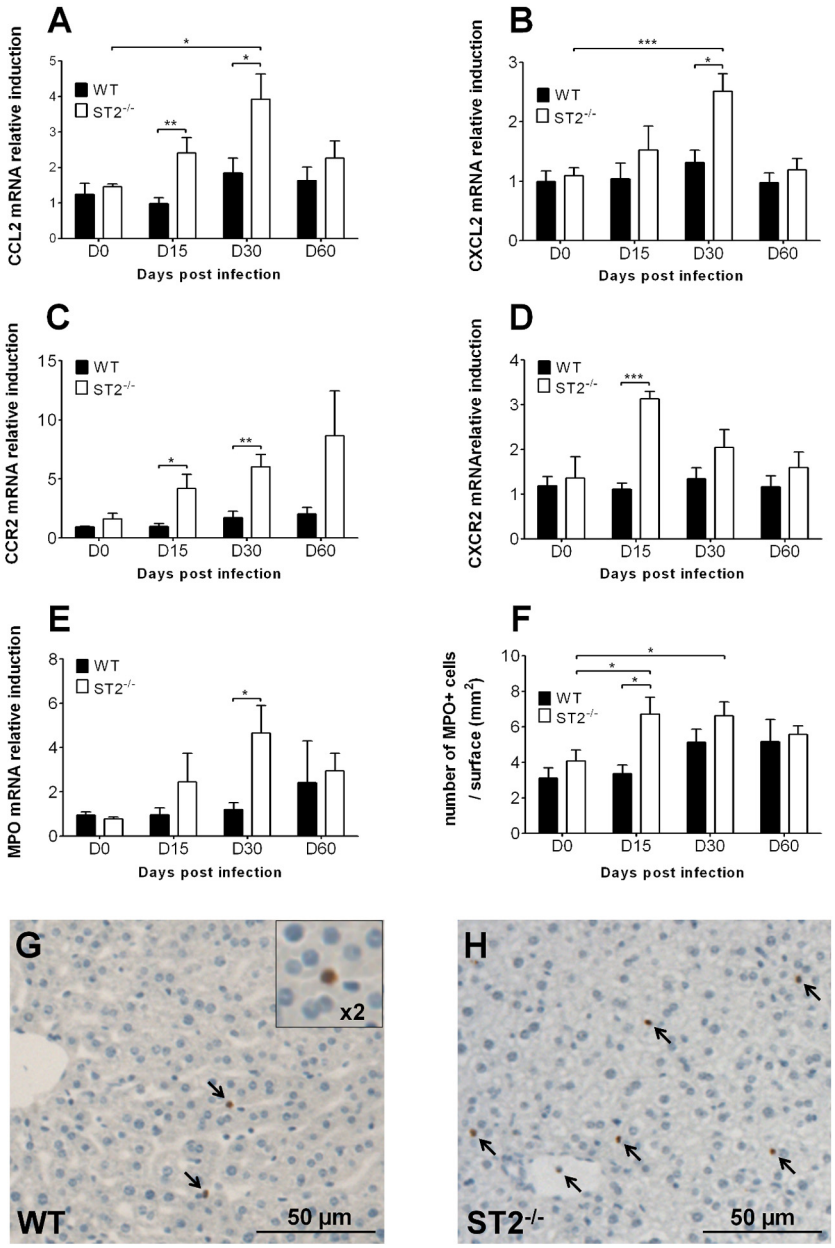

FIG 5 Recruitment of polymorphonuclear neutrophils and monocytes in the livers of WT and ST2$^{-/-}$ mice infected with *Leishmania donovani*. Quantification of mRNA induction of CCL2 (A), CXCL2 (B), and their corresponding receptors, CCR2 (C) and CXCR2 (D), as well as MPO (E) by qPCR at various time points after infection and normalized by comparison to 18S mRNA. Data are expressed as means ± SEM from 7 to 13 mice per background for each time point; pooled data are from three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (F) Quantification of MPO$^+$ cells detected by immunohistochemistry using an anti-MPO antibody after microscopic observation. Data represent the mean number ± SEM of MPO$^+$ cells counted per mm^2 of liver section during the course of the disease in infected WT and ST2$^{-/-}$ mice (7 to 13 mice per mouse background for each time point). Representative fields were observed at 15 days postinfection in WT (G) or ST2$^{-/-}$ mice (H) at ×100 magnification. MPO$^+$ cells are indicated by black arrows.

points (data not shown), suggesting a specific Th1 repression in the livers of BALB/c mice in response to IL-33. Additionally, a mild increase in parasite loads was observed in treated mice compared to NT mice (380 ±27 versus 528 ±83 LDU on day 30, respectively, and 450 ±58 versus 685 ±193 LDU on day 60; *P* not significant) (Fig. 6C).

Treatment of BALB/c mice with rIL-33 inhibits the hepatic recruitment of monocytes and PMN after infection with *L. donovani*. Quantitative analysis of chemokine mRNA in liver lysates showed a significant induction of CCL2 at D15, D30, and D60 and of CXCL2 at D15 and D30 ($P < 0.05$), but the kinetics were surprisingly similar between NT and rIL-33-treated mice for both chemokines (Fig. 7A and B).

The induction of chemokine receptors was also analyzed. A significant induction of CCR2 was observed at all time points ($P < 0.05$ at day 15 and $P < 0.01$ at day 60), with no obvious difference between NT and rIL-33-treated mice (Fig. 7C). CXCR2 was also significantly induced in both NT and rIL-33-treated mice ($P < 0.01$ and $P < 0.05$, respectively), but to a lesser extent at D30 and D60 in rIL-33-treated mice ($P < 0.05$ between both groups of mice at day 60) (Fig. 7D). The discrepancy of reduced CXCR2 but unmodified expression of CXCL2 in rIL-33-treated mice led us to investigate KC expression, since KC-responding cells also express CXCR2. This chemokine was indeed significantly induced in the liver of NT mice at D15, D30, and D60 ($P < 0.05$), whereas it was significantly repressed at D60 in treated mice compared to the level in NT mice ($P < 0.001$) (Fig. 7E).

To analyze the impact of KC and CXCL2 repression in rIL-33-treated mice, infiltrating MPO$^+$ cells were counted on liver sections stained by immunohistochemistry. As expected, significant recruitment of MPO$^+$ cells was observed in NT mice at D30 and D60 ($P < 0.05$). In rIL-33-treated mice, the numbers of MPO$^+$ cells were similar at D15 and D30 but significantly lower at D60 ($P < 0.05$) (Fig. 7F to H). Flow cytometry analysis was performed on infected NT or rIL-33-treated mice to characterize the presence of PMN and macrophages in the total liver during the course of the disease. In agreement with immunohistochemistry, no difference was observed between NT and rIL-33-treated mice at D15 and D30, but the number of CD11b$^+$ GR1^{high} cells was significantly reduced in rIL-33-treated mice at D60, as was the number of CD11b$^+$ GR1^{int} cells (Fig. 7I and J).

Globally, a significant reduction of infiltrate cells was observed on day 60 for other cell types, such as T cells, B lymphocytes, and NK cells (Fig. 8A), and this finding is in agreement with the lower hepatomegaly observed in rIL-33-treated mice (Fig. 8B).

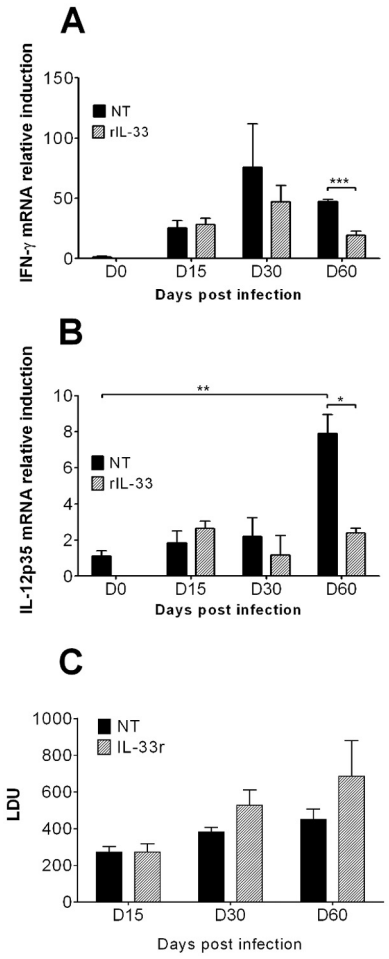

FIG 6 Kinetics of hepatic mRNA induction of IL-12 and IFN-γ and parasite burdens in BALB/c mice infected with *Leishmania donovani* and treated with recombinant IL-33 (rIL-33) or not treated (NT). mRNA induction of IL-12 (A) and IFN-γ (B) was quantified by quantitative PCR in liver extracts at various time points after infection and normalized by comparison to 18S mRNA. Liver parasite burden was determined by microscopic counting of Giemsa-stained tissue sections, and the results are expressed as LDU (no. of parasites/1,000 nuclei × liver weight in mg) (C). Data are means ± SEM for each group of mice (4 to 5 mice per treatment group for each time point; *, $P < 0.05$; ***, $P < 0.001$).

DISCUSSION

IL-33 is a recently described cytokine with involvement in many diseases (5, 27–30). Our team and others have contributed to the characterization of IL-33 sources during acute hepatitis (31, 32). During chronic hepatitis, expression of IL-33 and its receptor, ST2, is associated with sustained inflammation and Th2 response leading to liver fibrosis in both mice and humans, with a correlation between IL-33 expression and fibrosis severity (26). As liver fibrosis is a common feature during visceral leishmaniasis (VL) (33), we investigated the expression of IL-33 during human VL. Significant IL-33 release in the serum of VL patients was observed compared with the level in healthy blood donors, and numerous IL-33$^+$ cells were detected by immunohistochemistry in a liver biopsy specimen from a patient. Thus, IL-33 could be either a biomarker of the disease, which could reflect its severity, or a marker among others of the host defense against *Leishmania* parasites. The large variation in the IL-33 levels in the patients (standard error of the mean [SEM], 13.50) compared with healthy donors (SEM, 2.227) suggests a variable release of IL-33, possibly depending on the severity of the disease, the genetic background of the patient, the immunologic status and/or other associated diseases. The place of IL-33 and ST2 as diagnostic or prognostic tools, as proposed for other diseases (34, 35), should be explored in a larger and prospective study of VL patients with different risk factors but also before and after treatment.

To elucidate the role of IL-33 during VL, a BALB/c mouse model was used. As observed in humans, IL-33 was significantly increased in the serum of BALB/c mice infected with *L. donovani* and was detected in the liver by immunohistochemistry. As expected, most endothelial cells were IL-33$^+$ cells (36), but additionally, numerous IL-33$^+$ infiltrating cells were observed in the hepatic tissue, mainly located in granulomas. This led us to investigate the effect of IL-33 on the recruitment of ST2$^+$ immune cells in the livers of BALB/c mice. Immunohistochemistry revealed the presence of ST2$^+$ infiltrating cells in and around the granulomas, suggesting a regulatory role for IL-33 signaling via ST2 in the granulomatous response against *L. donovani*. Flow cytometry analysis of the liver immune cells showed the presence of ST2$^+$ macrophages and ST2$^+$ B lymphocytes in the livers of BALB/c mice, but no significant increase of ST2 MFI was observed after infection. However, the important recruitment of monocytes/macrophages (×5.5) and B lymphocytes compared to other immune cell types could account for enrichment in ST2$^+$ cells in the liver after infection. Interestingly, flow cytometry analysis of the livers of C57BL/6 mice, performed simultaneously, showed lower levels of ST2 MFI (data not shown). Since C57BL/6 mice display a preferential Th1 immune response, whereas BALB/c mice are known to have a Th2-biased genetic background (37), these data are consistent with the widely recognized function of ST2 as a marker associated with Th2 response (38–40). As the balance between Th1 and Th2 responses is critical for VL susceptibility (41–45), we further investigated whether IL-33 could act as a factor of susceptibility associated with Th2 response. It is noteworthy that we also observed a significant increase in IL-33$^+$ cells in the spleens of infected BALB/c mice (data not shown), which can be placed in parallel with the lack of parasite control classically observed in this organ (46, 47).

ST2-deficient BALB/c mice showed better control of the hepatic parasite burden than wild-type (WT) congenic controls, which could result from an exacerbated Th1 response known to be of prime importance in the control of VL. Indeed, we observed a switch in favor of Th1 response in the livers of ST2$^{-/-}$ mice, with increased and earlier expression of IFN-γ and IL-12, both cytokines of utmost importance in the control of infection (22, 48–51). The levels of expression of Th2 cytokines such as IL-13, IL-10, and IL-4 did not significantly differ between WT and ST2$^{-/-}$ mice (data not shown). Inversely, rIL-33-treated mice displayed reduced IL-12 and IFN-γ induction, thus strengthening the data obtained with ST2$^{-/-}$ mice. Again, no change in IL-4 and IL-10 expression was observed in the livers of rIL-33-treated mice. These data suggest that IL-33 could act as a repressor of the Th1 response during VL, as observed during CL (20), rather than an inducer of Th2 response, as described during other diseases (17, 52). Additionally, during this study, we have not been able to demonstrate any involvement of IL-33 in the regulation of T regulatory (Treg) cells (data not shown).

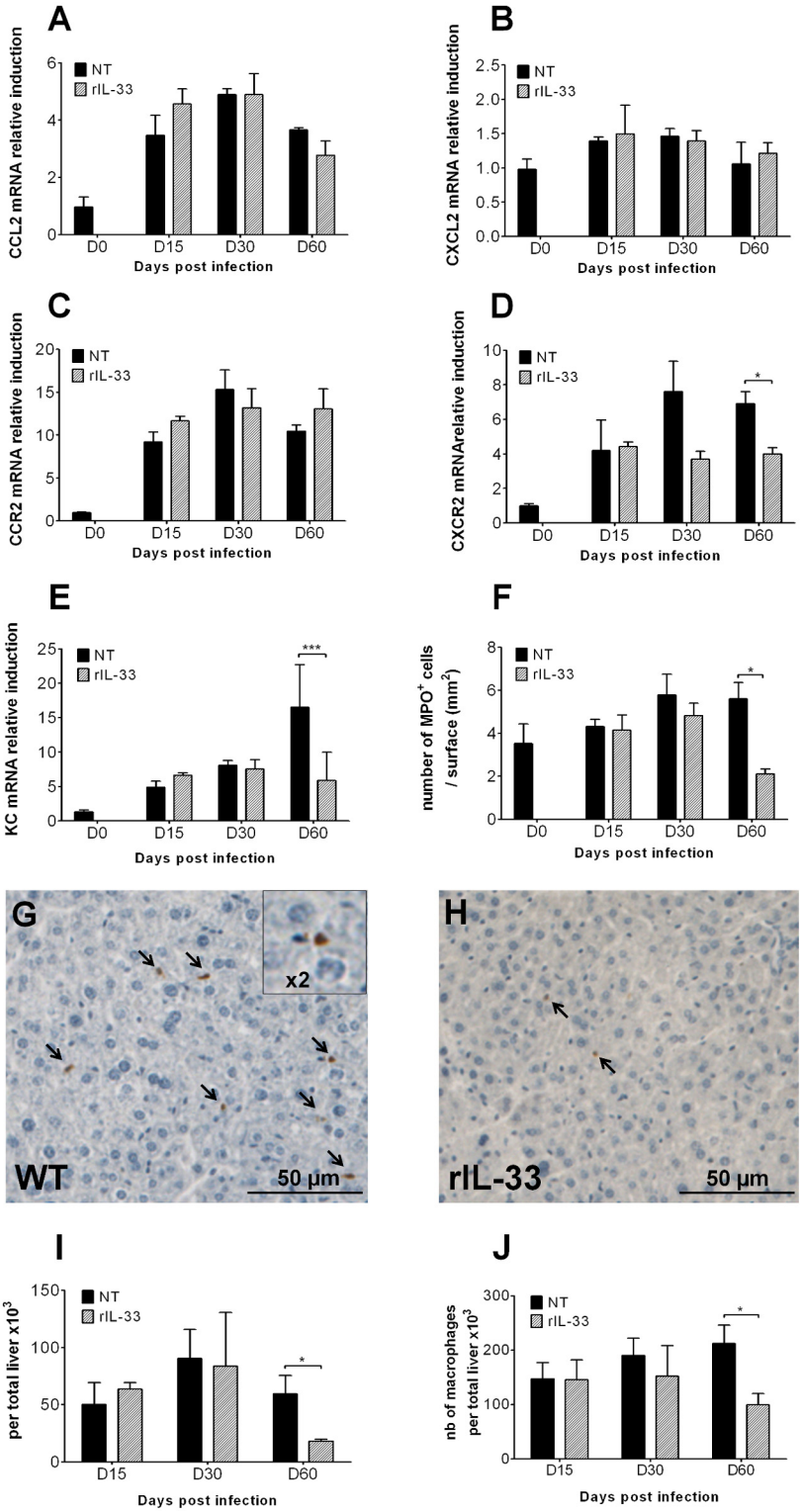

FIG 7 Recruitment of polymorphonuclear neutrophils and monocytes in the livers of BALB/c mice infected with *Leishmania donovani* and treated with recombinant IL-33 (rIL-33) or not treated (NT). Shown is mRNA induction of CCL2 (A) and CXCL2 (B), their respective receptors CCR2 (C) and CXCR2 (D), and KC/CXCL1 (E) at various time points after infection, normalized by comparison to 18S mRNA. (F) MPO^+ cells were stained by immunohistochemistry and counted per mm^2 of liver section during the course of the disease in infected NT and rIL-33-treated mice. Data are expressed as means ± SEM for each group of mice (4 to 5 mice per treatment group for each time point). Shown are representative fields of MPO^+ cell infiltrates at 60 days postinfection in WT (G) or rIL-33-treated (H) mice at a ×100 magnification. MPO^+ cells are indicated by black arrows. (I and J) Quantification of polymorphonuclear neutrophils (PMN; $CD11b^+$ $GR1^{high}$) (I) and macrophages ($CD11b^+$ $GR1^{int}$) (J) in hepatic infiltrates by flow cytometry on the total livers of NT or rIL-33-treated mice. Data are expressed as means ± SEM for each group of mice (4 to 5 mice per treatment group for each time point; *, $P < 0.05$; ***, $P < 0.001$).

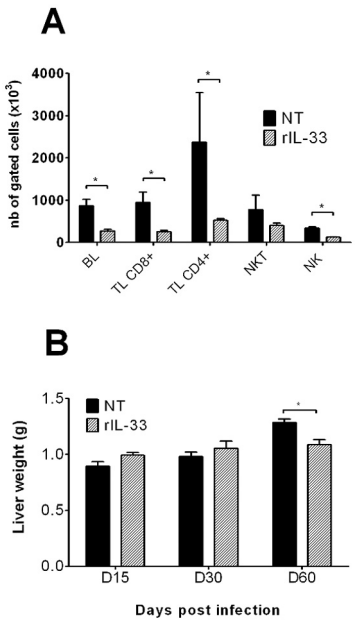

FIG 8 Liver weight and lymphoid cell influx in BALB/c mice treated with recombinant IL-33 (rIL-33) or not treated (NT) after infection with *Leishmania donovani*. (A) Flow cytometry quantification of $CD19^+$ B lymphocytes (BL), $CD3^+$ $NP46^-$ T cells (TL), $NP46^+$ $CD3^+$ T NKT cells, and $NP46^+$ $CD3^-$ NK cells, in the total livers of NT or rIL-33-treated mice. (B) Liver weight over the course of infection in NT and rIL-33-treated mice on day 60 postinfection. Data are the means ± SEM from each group of 4 to 5 mice for each time point (*, $P < 0.05$).

The influx of both monocytes and PMN is necessary for functional granuloma formation and final parasite clearance in the liver and has been highlighted in several studies (21, 53). Previous studies by our team and others showed a key role of CCL2, CXCL2, and KC/CXCL1 in the recruitment of these myeloid cells and the efficacy of disease control (53–55); thus, we focused on these chemokines. We observed an earlier and stronger induction of CCL2 and CXCL2 in $ST2^{-/-}$ mice than in WT mice, associated with a higher induction of their corresponding receptors CCR2 and CXCR2 until day 15. Similarly, rIL-33-treated mice displayed dramatically impaired induction of CXCR2, although this was mostly perceptible from day 30 postinfection. This apparent difference in kinetics is probably linked to these experimental models, with an early impact of genetic ST2 deficiency on microenvironmental factors but later effects of long-term treatment with low rIL-33 dosages. However, no difference in CCL2 and CXCL2 was observed between rIL-33-treated and nontreated mice; thus, we explored KC/CXCL1 induction, which is also involved in $CXCR2^+$ cell attraction and was indeed dramatically reduced in rIL-33-treated mice. Overall, these results suggest that besides CCL2 and CXCL2, KC/CXCL1 is also implicated in the recruitment of MPO^+ cells in the livers of BALB/c mice and can be downregulated by IL-33. The decrease in monocytes and PMN influx in the liver in both models was associated with higher hepatic parasite burdens in WT compared to $ST2^{-/-}$ mice ($P < 0.05$), as well as in rIL-33-treated mice compared to nontreated ones, although the result was statistically not significant because of a lack of power (*P* not significant). This apparent lack of statistically significant difference in parasite loads could be also related to the design of our model, using repetitive injections of low doses of rIL-33 (0.5 μg). A treatment using higher rIL-33 dosages and/or more frequent injections might have allowed the observation of higher parasite loads in the livers of treated mice. Anyway, the whole data are in full agreement with our previous observation of the important role of myeloid cells in the efficacy of parasite clearance (53).

Divergent conclusions can be found in the literature regarding the role of IL-33 in monocyte and neutrophil recruitment according to the model shown in references 14, 28, 56, and 57, but it is frequently associated with cell attraction during inflammatory diseases. In contrast, our results are consistent with a recent study showing that IL-33 is associated with the repression of neutrophil recruitment, thereby limiting liver damage and disease severity in an experimental model of liver ischemia and reperfusion (57).

The signaling pathways underlying the downregulation of Th1 effectors in our model remain to be determined. As IL-33 is known to be a regulator of the NF-κB pathway (58), and NF-κB is a key regulator of expression of cytokines, chemokines, and receptors, including IL-12 (59, 60), IFN-γ (61), KC (62), CCL2 (63), CXCL2 (64), and CXCR2 (65), which have been identified as being of major interest in our study, the study of NF-κB regulation by IL-33 in infected cells should be undertaken to better understand the mechanism of action of IL-33 during VL. As a first approach, we analyzed the NF-κBp65 induction by qPCR on liver extracts, which revealed significant overexpression in $ST2^{-/-}$ BALB/c mice and significant downregulation in rIL-33-treated mice compared to WT untreated mice (data not shown). These results suggest that IL-33 could repress the NF-κBp65 expression, as stated in a previous study (58), in order to counterbalance the NF-κB induction observed in response to *Leishmania* infection (66, 67), thus leading to downregulation of chemokines and Th1 cytokines. As IL-33 signaling via ST2 is usually associated with NF-κB activation (2), the apparent downmodulation of NF-κBp65 expression by IL-33 during *L. donovani* VL needs further investigation to better characterize the posttranslational regulation of NF-κB pathways in this model.

In conclusion, our results showed that in BALB/c mice, the IL-33/ST2 pathway does not control *L. donovani* infection but instead is associated with the downregulation of the Th1 response and poorer outcome. The role of IL-33 as a prognostic marker during VL in humans should be further explored.

MATERIALS AND METHODS

Ethics statement. The study on mouse models was carried out in accordance with the French institutional guidelines (Direction des Services Vétérinaires, agreement no. 35 to 135) and with EC directive 86/609/CEE. The use of $ST2^{-/-}$ mice in our animal facilities was approved by the Commission Génie Génétique (Ministère de l'Enseignement Supérieur et de la Recherche, agreement no. 5387-CA-1), and the protocol was approved by the local ethical committee (R-2012-JPG-01). Human blood samples were collected after written informed consent was obtained from the patients.

Patients. The sera from patients with VL ($n = 6$) and healthy donors ($n = 21$) were collected for IL-33 dosage. A specimen from a liver biopsy performed during the diagnosis of one case of VL was also available for histological analysis with IL-33 staining.

Mice. Female BALB/c wild-type mice were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France) and acclimatized for at least 10 days before challenge. BALB/c ST2 knockout ($ST2^{-/-}$) mice (17) were backcrossed for at least 10 generations. Mice were bred and housed in our

animal facilities. Mice were 7 to 12 weeks old when challenged with *L. donovani*. Naive congenic mice, matched according to age, were used as noninfected controls. The results were obtained in three independent experiments, with a total of 7 to 13 mice per time point.

Parasites and infection of mice. The *L. donovani* strain (MHOM/SD/97/LEM3427, typed as Zym MON-18 by the Center National de Référence des Leishmanioses, Montpellier, France) used in this study was maintained *in vivo* by serial murine passages and grown *in vitro* on Novy-McNeal-Nicolle (NNN) blood agar at 27°C. Prior to infection, amplification of promastigotes was carried out by culture in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin, for 6 days at 27°C, until they reached stationary phase. Animals were infected on day 0 (D0) by intraperitoneal injection of 10^8 promastigotes, and groups of 7 to 13 mice were sacrificed on D15, D30, or D60. Prior to sacrifice, blood was collected by retro-orbital puncture, and the serum was stored at −80°C. The liver was recovered and weighed, cut into pieces, and then used for immune cell typing by flow cytometry or formalin fixed and paraffin embedded or snap-frozen in isopentane-liquid nitrogen for mRNA extraction.

Treatment with recombinant IL-33. Recombinant IL-33 (rIL-33) was purchased from PeproTech. Mice (4 to 5 animals per time point) were infected with *L. donovani* at D0 as previously described and treated by intraperitoneal injection of 0.5 μg of rIL-33 per mouse twice a week until the mice were sacrificed at D15, D30, or D60. Nontreated BALB/c mice were used as controls and infected with the same parasite inoculum.

Serum IL-33 quantification. IL-33 was quantified in the serum of both humans and mice at a 1:2 dilution using, respectively, the specific human and mouse Duoset enzyme-linked immunosorbent assay (ELISA) development systems (R&D Systems), according to the manufacturer's instructions, except for the reveal step, in which orthophenyl-dianisidine was used instead of tetramethylbenzidine. Absorbance was determined at 490 nm using a spectrophotometer, and the results were determined from a 10-point standard curve, and expressed as pg/ml.

Quantification of liver parasitic burden. Parasite burden was determined by microscopic examination of Giemsa-stained smears, with the results expressed as *L. donovani* units (LDU) (i.e., number of amastigotes per 1,000-cell nuclei × liver weight in mg) (68).

Immunohistochemical characterization of immune cells in the liver. Immunohistochemical studies were performed as previously described by our team (26, 32, 53). Briefly, mouse myeloperoxidase (MPO) was stained using polyclonal rabbit anti-MPO antibody diluted 1/1,000 (DakoCytomation), mouse ST2 receptor was stained by immunochemistry of liver sections using a rat anti-mouse ST2 antibody diluted 1/100 (DJ8; MB Bioproducts), mouse IL-33 was stained using goat anti-mouse IL-33 diluted 1/50 (R&D Systems), and human IL-33 was stained using the Nessy-1 monoclonal antibody diluted 1/50 (Enzo Life Sciences). All immunohistochemical experiments were performed with the Ventana Discovery XT robot, using the Ventana DABMap detection kit (Ventana Medical Systems, Tucson, AZ) with a biotinylated goat anti-rabbit antibody diluted 1/700 (Vector Laboratory), a biotinylated donkey anti-rat antibody diluted 1/100 (Jackson Immune Research), a horse anti-goat antibody diluted 1/700 (Vector Laboratory), and a horse anti-mouse antibody diluted 1/700 (Vector Laboratory), respectively. The sections were then counterstained with hematoxylin. Appropriate controls were made to validate the antibodies: no staining was observed without primary antibodies, and $ST2^{-/-}$ and $IL\text{-}33^{-/-}$ mice showed no ST2 or IL-33 staining, respectively. The number of MPO^+ cells was counted after microscopic examination and is reported per mm^2 using a Zeiss Primo Star optical microscope. Pictures were obtained with a Nikon 80i optical microscope equipped with a numerical camera.

RNA isolation and analysis of hepatic gene expression. Total cellular RNA was extracted and purified from liver samples using Trizol reagent (Invitrogen) and then treated with DNase (10 U DNase I/μg total RNA) and reverse transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative PCR amplifications were carried out in duplicate using Power SYBR green PCR master mix (Applied Biosystems), 3 μM primers, and cDNA corresponding to 30 ng of total RNA input in a final volume of 10 μl, in 384-well optical plates, using a 7900HT fast real-time qPCR system (Applied Biosystems). The PCR primers were designed using Primer express 3 software and synthesized by Qiagen or Sigma-Aldrich (Lyon, France). Expression levels of target genes were normalized by comparison to expression of 18S rRNA. Results are expressed as $2^{-\Delta\Delta CT}$, referring to the fold induction in relation to the mean threshold cycle (C_T) obtained with noninfected WT mice.

Flow cytometry analyses. For flow cytometry analyses, livers were perfused with phosphate-buffered saline (PBS) to remove blood cell contamination prior to dissection. After homogenization of liver tissue and elimination of hepatocytes by sedimentation, immune cells were purified using 35% Percoll (GE Healthcare), and red blood cells were lysed. A total of 10^6 leukocytes were incubated with anti-CD16/32 (BD Pharmingen) to block nonspecific binding and washed. Cells were then incubated for 30 min with appropriate dilutions of anti-GR1-Pacific Blue (PB), anti-CD11b-phycoerythrin (PE)-Cy7, anti-CD3-Pacific Blue, anti-CD8-allophycocyanin (APC)-Cy7, anti-CD4-PE, anti-NP46-peridinin chlorophyll protein (PerCP)-Cy5.5, and anti-CD19-APC antibodies, all purchased from BD Pharmingen. The staining of ST2 was assessed with a rat monoclonal anti-mouse ST2-fluorescein isothiocyanate (FITC) antibody (clone DJ8; MB Bioproducts). Cells were washed, fixed in PBS containing 2% fetal calf serum (FCS), 0.01 M sodium azide, and 2% formaldehyde, and analyzed by fluorescence-activated cell sorter (FACS) on an Aria II flow cytometer using BD FACS Diva software (BD Bioscience), and the data were processed using CXP software (Beckman Coulter). Dead cells and doublet cells were excluded on the basis of forward and side scatter. The different immune cell types were identified and gated as follows: B lymphocytes were $CD19^+$, NK cells were $NP46^+$ $CD3^-$, NKT cells were $NP46^+$ $CD3^+$, T $CD8^+$ lymphocytes were $NP46^-$ $CD3^+$ $CD8^+$ $CD4^-$, and T $CD4^+$ lymphocytes were $NP46^-$ $CD3^+$ $CD4^+$ $CD8^-$. PMN were $GR1^{high}$ $CD11b^+$, and macrophages were $GR1^{int}$ $CD11b^+$ (see Fig. S1 in the supplemental material). The macrophage gating strategy was validated using an F4/80 antibody as previously described (53). Cell numbers per liver were calculated as follows: (no. of gated cells/no. of living cells) × no. of infiltrating cells purified from the whole liver. ST2 expression and induction were quantified by the ratio of mean fluorescence intensities (MFI) of ST2 to the MFI of control Ig-FITC in each gated cell type.

Statistical analysis. Data are expressed as means ± standard errors of the means (SEM) for each group of mice (4 to 11 mice per group from 2 to 3 independent experiments). Differences between groups were analyzed using the Student *t* test (human data) or the nonparametric Mann-Whitney test (mouse data). A one-sample *t* test was used to confirm the expression of ST2 observed by flow cytometry on different cell types as follows: the MFI ratios were compared to 1, which is the value theoretically expected if there were no difference between a specific antibody and its control isotype. Statistical analysis was performed using GraphPad Prism 5.02 software. Differences were considered significant when the *P* value was <0.05 and are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00383-13/-/DCSupplemental.

Figure S1, TIF file, 7.6 MB.

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