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**Journal**: Proceedings of the National Academy of Sciences of the United States of America

**Year**: 2017 Apr 24

**DOI:** 10.1073/pnas.1621447114

# Type I interferons induced by endogenous or exogenous viral infections promote metastasis and relapse of leishmaniasis

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**Short title:** Viral infections and exacerbated leishmaniasis

**Keywords:** *Leishmania* RNA virus 1, *Totiviridae*, arboviruses, trypanosomatid protozoan parasite, *Leishmania* subgenus *Viannia.*

## Abstract

The presence of the endogenous *Leishmania* RNA virus 1 (LRV1) replicating stably within some parasite species has been associated with the development of more severe forms of leishmaniasis and relapses after drug treatment in humans. Here, we show that the disease exacerbatory role of LRV1 relies on Type I interferon (Type I IFNs) production by macrophages and signaling *in vivo*. Moreover, infecting mice with the LRV1-cured *L. guyanensis* (*LgyLRV1*$^{-}$) strain of parasites followed by Type I IFN treatment increased lesion size and parasite burden, quantitatively reproducing the LRV1-bearing (*LgyLRV1*+) infection phenotype. This suggested the possibility that exogenous viral infections could likewise increase pathogenicity, which was tested by co-infecting mice with *L. guyanensis* and *Lymphocytic Choriomeningitis* virus (LCMV), or the sand fly-transmitted arbovirus *Toscana* virus (TOSV). The type I IFN anti-viral response increased the pathology of *L. guyanensis* infection, accompanied by down regulation of the IFN-γ receptor normally required for anti-leishmanial control. Further, LCMV co-infection of IFN-γ-deficient mice promoted parasite dissemination to secondary sites, reproducing the *LgyLRV1*+ metastatic phenotype. Remarkably, LCMV co-infection of mice that had healed from *L. guyanensis* infection induced reactivation of disease pathology, overriding the protective adaptive immune response. Our findings establish that Type I IFN-dependent responses, arising from endogenous viral elements (dsRNA/LRV1), or exogenous co-infection with IFN-inducing viruses, are able to synergize with New World *Leishmania* parasites in both primary and relapse infections. Thus, viral infections likely represent a significant risk factor along with parasite and host factors, thereby contributing to the pathological spectrum of human leishmaniasis.


## Significance Statement

Infection with *Leishmania* (*Viannia*) parasites can have different manifestations, ranging from localized cutaneous to disseminated and mucocutaneous leishmaniasis, that are prone to relapse after the healing. We previously described the association of the endosymbiont *Leishmania* RNA virus 1 (LRV1) with increased disease severity. Here we showed that coinfection with the Lymphocytic Choriomeningitis virus (LCMV) or Toscana virus exacerbated the outcome of *L. guyanensis*-induced murine leishmaniasis, favoring parasite persistence and dissemination resulting in metastasis. Both endogenous and exogenous co-infections were dependent upon Type I interferon responses. Strikingly, LCMV

co-infection after the healing of leishmaniasis induced disease reactivation, overriding the protective adaptive immune response. Thus, viral infections may be a significant risk factor contributing to the pathological spectrum of human leishmaniasis.

# Introduction

*Leishmania* are protozoan parasites, transmitted as unicellular promastigote forms by sand flies to their mammalian host (humans and dogs). In the skin, *Leishmania* parasites are phagocytized by tissue resident macrophages, where they survive intracellularly and proliferate as amastigotes. Infection with *Leishmania* parasites may lead to the development of leishmaniasis, affecting over 12 million people worldwide (1, 2). Leishmaniasis may have different outcomes, ranging from localized cutaneous leishmaniasis to visceral leishmaniasis (1, 2). Infection with *L. guyanensis* (*Lgy*) or *L. braziliensis (Lbr)* principally leads to simple cutaneous lesions, however, up to 10% of patients develop disseminated or mucocutaneous leishmaniasis. These latter more severe forms of the disease are characterized by the dissemination of the parasites from the primary infection site. Another complication of *Lgy* or *Lbr* infection can be relapse, which may occur months to years after the healing of the primary lesion, or after a first line drug treatment (3, 4). Recently, we correlated the development of these more severe forms of leishmaniasis with the presence of *Leishmania* RNA virus (LRV1) within different species of *Leishmania* (3-6). Discovered in the 1980s (7, 8), and since then for a long time neglected, *Leishmaniavirus* is a genus of double stranded RNA viruses belonging to the *Totiviridae* family. Like most other viruses in this family, LRV1 is neither shed nor infectious, and thus can be seen as a persistent, endogenous viral element (9). Two species of LRV have been identified. The LRV1 species is principally found in South America within *Lgy* and *Lbr* (10, 11), and the LRV2 species is found within *L. major* and *L. aethiopica* in the Old World (6, 12). The increasing reports of LRVs in different *Leishmania* species could imply a wider role in determining the fate of infection in humans. However, in some instances, metastasis and relapse after drug treatment also occur in the absence of LRV1 (13). The basis for these discrepancies is of considerable interest, the hypothesis put forward include the significance of the presence of other parasite species, microbial or host factors that are known to play an important role in the development of MCL (14-16).

The disease-exacerbatory role of LRV1 relies principally on its modulation of the innate immune system via its dsRNA genome (5). We recently showed that LRV1-dependent

IL-17 promotes the dissemination of the parasite and the consequent formation of metastatic lesions (17). Moreover, we demonstrated that LRV1 increases the life span of *Lgy*-infected macrophages through a Toll-like receptor 3 (TLR3) and *Akt* dependent pathway (18). Further, LRV-containing parasites promote TLR3-dependent secretion of pro-inflammatory cytokines and chemokines, including interferon-β (IFN-β) (5). Interestingly, ablation of LRV1 using the parasite RNAi machinery, or by treatment with compounds selectively inhibiting LRV1, completely abrogated the production of pro-inflammatory cytokines by infected macrophages (19, 20).

The induction of TLR3-dependent interferon-β production following infection with LRV1+ parasites suggested a potential role in the pathway leading to elevated pathogenicity. Type I interferons (Type I IFNs) are mainly known for their anti-viral activity, and interferon therapy is currently used to treat several viral infections, including hepatitis B and C and herpes virus (21-23). The role of Type I IFNs in bacterial and parasitic infection is less clear, as they are known to protect mice from *Plasmodium falciparum* infection, but on the other hand, can promote infection pathology with *Listeria monocytogenes*, *Toxoplasma* and *Trypanosoma* (24-27). During parasite infection, Type I IFNs show more variable effects, being either protective or detrimental for the host, depending on the dose, timing of administration and on the parasite species (28, 29). For *L. major*, Type I IFNs have been associated with control (30-32), whilst for other species, especially the New World species *L. amazonensis* and *Lbr*, IFN-β has been associated with promotion of parasite survival and/or disease (33-35).

The potential significance of IFN signaling during infections with *Leishmania* parasites bearing endogenous dsRNA viruses raises the intriguing possibility that co-infections with exogenous viruses inducing Type I IFNs could also worsen the disease outcome. Such co-infections could occur at the site of infection by sand flies carrying both *Leishmania* parasites and *Phleboviruses* (e.g. TOSV), or by another virus inducing systemic production of Type I IFNs. Thus far little is known about co-infection with *Leishmania* and viruses, with the exception of HIV and the phenotypic change due to its impairment of the adaptive immune response (16, 36).

In this study, we investigated the disease exacerbatory role of viral-induced Type I IFNs in *Lgy* infection, not only with LRV1-bearing *Lgy* (*LgyLRV1+*), but also by co-infecting mice with LRV1-cured *Lgy* (*LgyLRV1*$^{-}$) and *Lymphocytic Choriomeningitis* virus (LCMV), or *Toscana* virus (TOSV).

# Results

### Type I IFNs exacerbate *LgyLRV1+* infection

We first analyzed whether Type I IFNs could modulate the pathogenicity of *Lgy*. C57BL/6 wild type (WT) or Type I IFN receptor deficient (*ifnar*-/-) mice were infected with *LgyLRV1*$^-$ or *LgyLRV1+* parasites. Two weeks post infection, WT mice began to develop lesions that grew until reaching a maximal size at week 5 which then healed 4 weeks later, with *LgyLRV1+* infection inducing significantly larger lesions compared to *LgyLRV1*$^-$ infected mice (Fig. 1A). In contrast, *LgyLRV1+* infected *ifnar*-/- mice developed significantly smaller lesions, similar to those developed by *ifnar*-/- and WT mice infected with *LgyLRV1*$^-$ (Fig. 1A). Parasite numbers were also significantly increased in WT mice infected with *LgyLRV1+* compared to the *LgyLRV1*$^-$ infected counterpart (Fig. 1B). Again, no difference was observed between *ifnar*-/- mice, independent of the presence of LRV1 in the infecting parasite, as the parasite load was similar to *LgyLRV1*$^-$ infected WT mice. Thus, the deleterious effect of Type I IFNs includes worsening of lesion pathology and increased parasite numbers.

To further confirm the deleterious role of Type I IFNs, WT mice were injected with recombinant IFN-β (IFN-β) at early time points post *Lgy* infection, to mimic the anti-viral response induced by the endogenous dsRNA LRV1 virus (5). In *LgyLRV1*$^-$ infected mice, IFN-β showed a dose-dependent effect in increasing the lesion size and the parasite load at the peak of infection. In fact, injection of 100U of IFN-β showed a moderate increase in lesion size and no effect on parasite load, whereas injection of 500U or 1000U caused greater increase of both lesion size and parasite burden, quantitatively reproducing the phenotype of *LgyLRV1+* infection (Fig. 1C-D). In contrast, little effect from IFN-β treatment was seen in *LgyLRV1+* infected mice, (Fig. 1E-F). Similar results were obtained with IFN-α treatment (Fig. S1).

### Viral co-infection increases the severity of *Lgy* leishmaniasis

The data above, in combination with previous findings (5), suggest that the endogenous dsRNA virus LRV1 acts to promote *Lgy* virulence through TLR3 and Type I interferon signaling. This alluded to the possibility that other agents triggering Type I interferon responses might act similarly to promote *Leishmania* virulence, such as co-infections with other viruses. To test this, we co-infected mice with *LgyLRV1*$^-$ parasites and lymphocytic choriomeningitis virus Armstrong (LCMV), an arenavirus which induces a

potent Type I IFN response (37). Two different sites of injection were used, intra-peritoneal injection for LCMV and subcutaneous in the footpad for *Lgy*.

Infection with LCMV Armstrong was shown to be cleared in 8 days by C57BL/6 WT mice with a robust T cell response (38). Viral titration in mouse serum, following intraperitoneal inoculation, showed only transient and very low viremia (39). As previously reported (40), the presence of *Leishmania* parasites did not increase LCMV infection (Fig. S2). Similarly, the concentration of Type I IFNs in the serum after 24 hours of LCMV infection was comparable between mice infected or not with *Lgy* (Fig. S3). These data demonstrated that the development of the LCMV infection was not affected by the presence of *Lgy* parasites. Interestingly, Type I IFNs were not detected in mouse blood following single infection with *LgyLRV1+*, suggesting that in this particular case the Type I IFN response is only local (Fig. S3).

We subsequently investigated whether LCMV-induced Type I IFNs stimulated gene expression at the site of *Leishmania* infection. We observed that genes known to be stimulated by Type I IFNs (*oas1a*, *oas2*, *oasl2* or *pkr*) were significantly up-regulated in mice infected with *LgyLRV1+,* or with *LgyLRV1*$^{-}$ co-infected with LCMV, compared to *LgyLRV1*$^{-}$ infected mice (Fig. S3).

We then tested the effect of LCMV co-infection on the progression of leishmaniasis. LCMV injection in *LgyLRV1*$^{-}$ infected mice significantly worsened the outcome of leishmaniasis, increasing both pathology and parasite burden, which were very similar to the phenotype induced by *LgyLRV1+* infection (Fig. 2A and C). The LCMV aggravation of *Lgy*-induced leishmaniasis completely relied on Type I IFNs, as no difference was observed when LCMV was injected into *LgyLRV1*$^{-}$ infected *ifnar*-/- mice, neither with regard to footpad swelling nor parasite burden. *ifnar*-/- mice failed to rapidly clear the virus due to the absence of a proper Type I IFN response (Fig. S2) (41). Moreover, this effect could again be related to the Type I IFN level, since, as already shown in the Type I IFNs injection experiment, no significant effect was observed when LCMV was injected in *LgyLRV1+* infected mice (Fig. 2 B and D).

We furthermore asked if another virus could similarly affect the course of leishmaniasis. We focused on *Toscana* virus (TOSV), a phlebovirus transmitted to humans by the same insect vector as *Leishmania* parasites (42). Mimicking the likely biological route of concomitant infection, TOSV was inoculated together with *Lgy* parasites subcutaneously into the footpad. As observed with LCMV, *LgyLRV1*$^{-}$/TOSV co-infection highly increased footpad swelling and parasite burden at the peak of infection (Fig. 2E-F). *ifnar*-/- mice were

susceptible to TOSV infection, with over 50% of mortality after two weeks of infection, precluding further tests on the Type I IFN dependency in this co-infection model.

**Viral co-infection modulates macrophage responsiveness to IFN-γ through Type I IFNs**

The data above suggested that co-infection with exogenous RNA viruses resulted in worsening of leishmaniasis, quantitatively similar to that seen by parasites bearing endogenous LRV1. We thus focused on defining potential mechanisms responsible for these exacerbated phenotypes. The immune system efficiently clears intracellular *Leishmania* through the production of IFN-γ (43), and Type I interferon responses are known to down-modulate IFN-γ responses (24). The early IFN-γ response is fundamental for the determination of the outcome of *Leishmania* infection (44), consistent with our finding showing that injection of Type I IFNs in the first hours of infection is crucial for the development of disease (Fig. 1C-D). As LRV1 or LCMV co-infection did not modulate early IFN-γ production (Fig. S4), we wondered if IFN-γ signaling was modulated during co-infections. To this end, we firstly, measured IFN-γR surface expression on bone marrow derived macrophages. Infection with *LgyLRV1+* parasites induced a significant down-regulation of IFN-γR compared to *LgyLRV1⁻,* in a Type I IFN dependent manner (Fig. S5). In this *in vivo* model of co-infection with LCMV and *Leishmania*, the pathogens were inoculated at two different sites, lowering the chances for the same cell to be infected by both pathogens and arguing for a systemic production of Type I IFNs. Thus, in *in vitro* experiments we replaced LCMV co-infection by treating the cells with increasing doses of recombinant IFN-α or IFN-β subsequent to *LgyLRV1⁻* infection. Both Type I IFNs induced IFN-γR down-regulation in a dose-dependent manner, with IFN-β showing somewhat higher potency (somewhat greater than 2 fold; Fig. S5D). Subsequently, we confirmed these results during *in vivo* infection. Flow cytometric analysis 48 hours post infection showed that macrophages from *LgyLRV1+* infected, or *LgyLRV1⁻*/LCMV infected mice, expressed significantly lower levels of surface IFN-γ-receptor (IFN-γR), compared to infections by *LgyLRV1⁻* alone (Fig. 3A). No significant difference was observed in *ifnar*-/- mice (Fig. 3B), however, the down-regulation of IFN-γR expression was observed when mice were infected with *LgyLRV1⁻* and injected with IFN-α or IFN-β (Fig. 3C). This confirmed that the effect depended on Type I IFNs.

**LCMV – *Lgy* co-infection accelerates parasite dissemination**

The importance of IFN-γ was further demonstrated as a critical component of our murine model for metastatic leishmaniasis, where IFN-γ-/- mice failed to control *Lgy* infection, causing multiple metastatic lesions, usually located on the tail (17). In this model, metastases were accelerated in the presence of the endogenous LRV1 within *Lgy* parasites. Interestingly, IFN-γ-/- mice co-infected with *LgyLRV1*$^{-}$/LCMV developed metastasis earlier than those infected with *LgyLRV1*$^{-}$ alone, thus reproducing the phenotype of *LgyLRV1+* parasites. Therefore, exogenous viral co-infection was equally capable of synergizing parasite virulence and metastasis (Fig. 4A-C).

**LCMV infection induces reactivation of leishmaniasis**

We then asked whether viral infection could lead to reactivation of leishmaniasis following healing, a pathological situation often observed in humans (45). LCMV injection subsequent to lesion healing induced reactivation of the disease, with a reappearance of both lesion pathology and increased parasite numbers, overriding the protective adaptive immune response essential in C57BL/6 mice re-infected with *Lgy* (Fig. S6). Once reactivated, pathology and parasite burden progressed very similarly to that seen in concomitant *LgyLRV1*$^{-}$ /LCMV infections, with the lesion pathology and size peaking around 5 weeks post LCMV injection and healing a few weeks later (Fig. 5A). Further, at the peak of LCMV infection, we found higher parasite burden, a sign of reactivation of parasite proliferation (Fig. 5B). The mechanism of relapse relied on Type I IFNs, since no footpad swelling or increase in parasite load was observed in *ifnar*-/- mice infected with either *LgyLRV1*$^{-}$ or *LgyLRV1+* (Fig. 5A-B). As seen with simultaneous infections, the subsequent delayed post-healing LCMV injection in *LgyLRV1*$^{-}$ infected mice induced down-regulation of IFN-γR surface expression on macrophages in a Type I IFN-dependent manner (Fig. S7A).

Interestingly, *LgyLRV1+* infected WT mice developed only small lesions and parasite number compared to their *LgyLRV1*$^{-}$ counterparts, thus reversing the phenotype observed in simple *Lgy* infection. Indeed, when *LgyLRV1*$^{-}$ infected mice were injected with Type I IFNs during the first two days of infection, they developed less severe relapses when subsequently co-infected with LCMV (Fig. 5C). This result suggests that early Type I IFN production could significantly prevent the reactivation of the disease in mice previously infected with *LgyLRV1+*, thus explaining the difference between the *LgyLRV1+* and *LgyLRV1*$^{-}$ relapsing phenotype. Accordingly, the down-regulation of IFN-γR was not observed in *LgyLRV1+*

infected, nor in *LgyLRV1*$^{-}$/ Type I IFN injected mice (Fig. S7B), suggesting that early Type I IFN signaling may inhibit the effect of a subsequent LCMV co-infection.

## Discussion

In this study we confirmed in an *in vivo* murine model of leishmaniasis that the detrimental role of LRV1 borne by *Lgy* was due to the anti-viral response triggered by viral dsRNA recognition, which culminated in Type I IFN production. Further, we demonstrated that injection of recombinant Type I IFNs during the initial days of infection was sufficient to worsen the outcome of leishmaniasis. Despite the fact that Type I IFNs are mainly known for their anti-viral role, increasing evidence attests the involvement of Type I IFNs in bacterial and parasitic infection (24-27, 29). Our findings are consistent with earlier reports suggestive of a disease exacerbatory role for Type I interferons in New World *Leishmania* species (33-35).

Here we showed that *LgyLRV1*+ infection, or *LgyLRV1*$^{-}$ plus LCMV co-infection, induced the down-regulation of IFN-γR on macrophages, in a Type I IFN dependent manner. In this model both Type I IFNs demonstrated activity, with IFN-β showing greater potency *in vitro*. The importance of IFN-γ production during the first days of infection with *Leishmania* parasites has been widely described (44). In our model of co-infection we did not observe differences in early IFN-γ production, nevertheless the lower expression of the IFN-γ receptor likely acts to promote the development of disease. Recently we have shown that infection with *LgyLRV1*+ highly increased macrophage survival *in vitro* (18). This result, combined with the down-regulation of IFN-γR, suggests that the presence of viral co-infection increased the persistence of *Leishmania* parasites. While the down-regulation of IFN-γR was quantitatively modest, this was also observed in studies of the exacerbatory role of Type I interferons in *Listeria* infections (24). Our studies, however, do not rule out other mechanisms of disease exacerbation induced by Type I IFNs.

Significantly, exogenous viral co-infection with LCMV or TOSV worsened the outcome of murine leishmaniasis caused by *LgyLRV1*$^{-}$, reproducing the phenotype of *LgyLRV1*+ infected mice (Fig. 2). It was recently reported that mice infected with LCMV present increased lesions when subsequently infected with *L. major* (46). This phenotype was due to an increased inflammatory response induced by memory T cells not accompanied by an increased parasite burden. Similar results were observed when mice were firstly infected with *L. major* and only two weeks later co-infected with LCMV (40). In this latter case, a

transiently decreased anti-*Leishmania* immune response was observed one week following LCMV co-infection. In our experiments, mice were infected at the same time with parasites and LCMV, leading not only to increased footpad lesions, but also to higher parasite burden, compared to infection with parasites alone. This suggests that there was a decrease of macrophage anti-parasitic activity, consistent with the lower IFN-γR expression on macrophages, this observation, however, did not exclude other possible systemic effects of LCMV. Interestingly, LCMV co-infection was able to promote the metastatic phenotype in *LgyLRV1*$^{-}$ infected IFN-γ-deficient mice (Fig. 4). Further, we showed that disease exacerbation of LRV1 and LCMV co-infection depended completely on Type I IFNs (Fig. 2A-B).

These findings may explain why the tight correlation between disease severity and metastasis with *LgyLRV1+* in animal models (5) may be more variable in humans (13, 17, 47, 48). Potentially, co-infection with viruses or other pathogens inducing a sufficient amount of Type I IFNs could increase the severity of *Lgy* infection, contributing to the development of more severe disease manifestations. Certainly, many viral diseases are found in *Leishmania* endemic regions which could contribute to increased disease severity (49). A number of arboviruses are transmitted by the sand fly vectors also transmitting *Leishmania*, including the Massilia and TOSV Phleboviruses (50, 51). Co-infection was reported in dogs, while seropositivity to TOSV was associated with *L. infantum* in humans (52, 53), however, the clinical relevance of the co-infection is unknown. Since our results suggest that events early in infection were crucial to determine the fate of the disease (Fig 1C-D), having the virus transmitted simultaneously with *Leishmania (Viannia*) could magnify the impact of coinfection. However, intraperitoneal LCMV infections which induce a systemic Type I IFN response strongly exacerbated disease, suggesting that co-infections do not require the same entry site.

Finally, we showed that LCMV infection following the resolution of the primary lesion induced relapses of leishmaniasis, overriding the memory immune response of the host. Surprisingly, relapses were more severe in *LgyLRV1*$^{-}$ infected mice compared to *LgyLRV1+*. This result correlated with a Type I IFN dependent down-regulation of IFN-γR observed in *LgyLRV1*$^{-}$ infected mice compared to *LgyLRV1+*, confirming the role of IFN-γR in determining the outcome of the disease. Identification of the mechanism(s) underlying difference will require future investigation. In humans, relapses are observed following infection with different species of *Leishmania* and can have varying outcomes, ranging from a

simple cutaneous lesion, to DCL or MCL in the case of *Lgy* or *Lbr* infection, or post-kala-azar dermal leishmaniasis, in the case of *L. donovani* infection (3, 54, 55). Here we suggest that viral co-infection and prior exposure to Type I IFNs could not only be a risk factor for relapses of leishmaniasis, but could be the trigger of parasite reactivation.

In total, our findings establish a major role for simultaneous or subsequent viral infection in determining the severity of *Leishmania (Viannia)* infection in animal models and that viral co-infections could contribute towards metastasis and relapse in human patients suffering from leishmaniasis.

# Material and Methods

For additional information please refer to the SI text.

**Mice**

C57BL/6 WT mice were purchased from Harlan Laboratories (Netherlands), type-1 IFN receptor deficient (*ifnar*-/-) mice were obtained from M. Aguet, Swiss Institute of Experimental Cancer Research (Epalinges, Switzerland), IFN-γ-/- mice were purchased from Jackson laboratories. Experimentation procedures were undertaken with strict adherence to ethical guidelines set out by the Swiss Federal Veterinary Office and under inspection by the Department of Security and Environment of the State of Vaud, Switzerland.

**Parasites and viruses**

Matched *LgyLRV1+* and *LgyLRV1*$^{-}$ parasites expressing luciferase obtained following limited treatment with anti-LRV1 inhibitors were used in all studies (20). *In vivo* parasites were quantified by luciferase bioluminescence imaging as described previously (17). LCMV and TOSV were provided by D. Zehn and M. G. Cusi, respectively.

**Macrophages**

Bone marrow derived macrophages were generated as described previously (18).

**Author contributions**

M.R., C.R., D.Z., M.G.C., S.M.B. and N.F. designed research; M.R., P.C., M.A.H, R.O.E., F.P., C.D. and D.T.U. performed research; F.M.K., D.Z. and M.G.C. provided key reagents;

M.R. and D.T.U. analyzed data; M.R., S.M.B. and N.F. wrote manuscript; D.Z., F.M.K. and M.A.H. revised manuscript.

**Acknowledgments:** We thank S. Masina for critical reading of the manuscript and J. Boon for comments on an early draft of the manuscript. This work was funded by the Swiss National fund for research (FNRS 310030-153204 and IZRJZ3_164176), the Institute for Arthritis Research (iAR), the Pierre Mercier Foundation, and the NIH (R56AI099364 and R01AI029646).

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# Figures

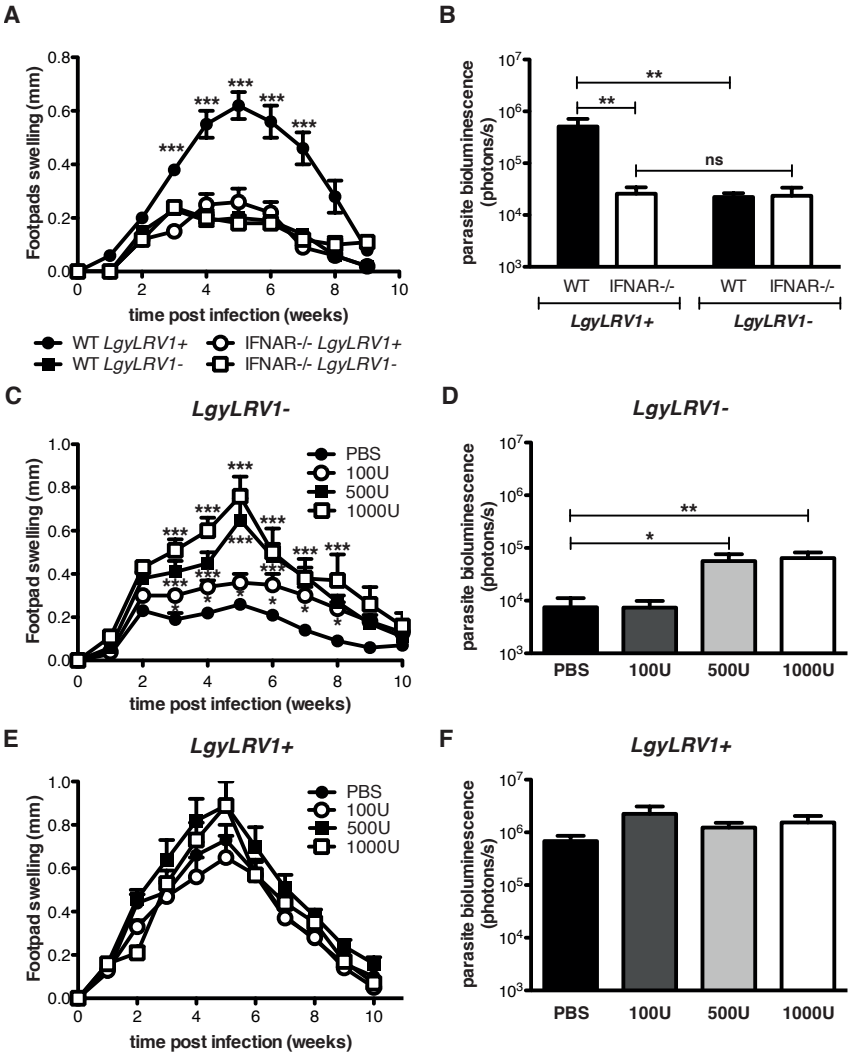


**Figure 1. Type I IFNs increased the severity of *Lgy* infection.**

**(A-B)** WT or *ifnar*-/- mice were infected in the hind footpads with 3 x $10^6$ stationary phase *Lgy* promastigotes. (**C**-**F**) At 6, 24 and 48 hours post infection (p.i.) WT mice were injected with the indicated amount of IFN-β into the footpad. (**A**, **C** and **E**) Footpad thickness was measured weekly. (**B**, **D** and **F**) Parasite burden was quantified 5 weeks p.i. *in vivo* by measuring parasite bioluminescence. Results of one representative of 3 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by Repeated measure ANOVA (**A**, **C** and **E**), *Two-way ANOVA* (**B**) or *Student's t-test* (**D** and **F**) *p<0.05, **p<0.01, ***p<0.001.

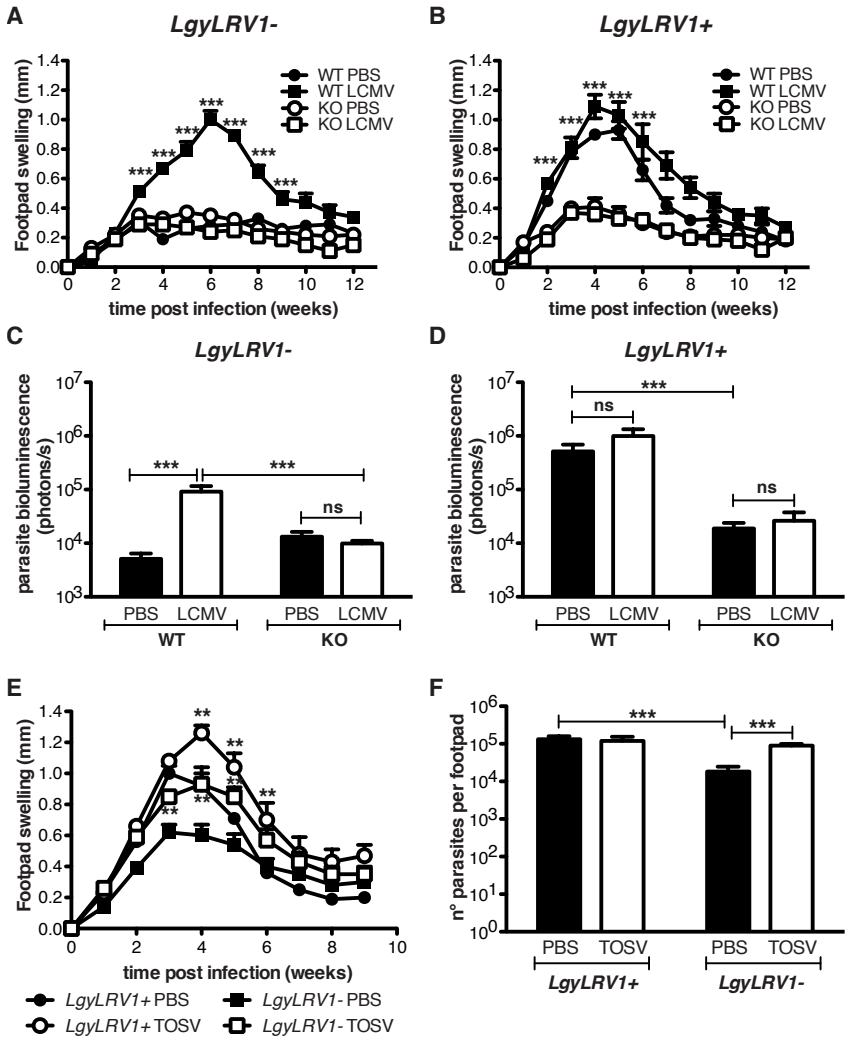


**Figure 2. Viral co-infection increased the severity of leishmaniasis through Type I IFNs.** (**A**-**F**) WT or *ifnar*-/- mice were infected in the hind footpads with 3 x $10^6$ stationary phase *Lgy* promastigotes. (**A**-**D**) *Leishmania* infected mice were injected simultaneously with 2 x $10^5$ PFU of LCMV Armstrong or the same volume of PBS as vehicle control intra-peritoneally. (**E**-**F**) Alternatively, *Leishmania* infected mice were simultaneously inoculated with 5 x $10^5$ PFU of TOSV subcutaneously into the footpad. (**A**, **B** and **E**) Footpad thickness was measured weekly. (**C**, **D** and **F**) Parasite burden was measured 5 weeks p.i. by measuring parasite bioluminescence or by RT-qPCR. Results of one representative of 3 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by Repeated measure ANOVA (**A**, **B** and **E**) or *Two-way ANOVA* (**C**, **D** and **F**) **p<0.01, ***p<0.001.

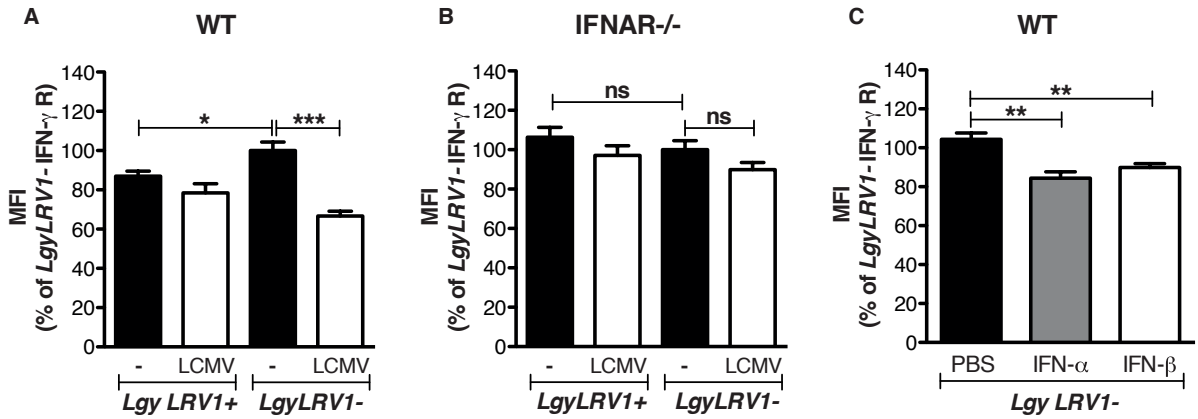


**Figure 3. *Lgy* - LCMV co-infection modulated IFN-γR expression on macrophages through Type I IFNs.**

(**A**) WT or (**B**) *ifnar*-/- mice were infected in the hind footpads with 3 x $10^6$ stationary phase *Lgy* promastigotes. At the same time, where indicated, mice were injected intra-peritoneally with 2 x $10^5$ PFU of LCMV Armstrong. (**C**) Alternatively, at 6 hours p.i., *LgyLRV1*$^-$ infected WT mice were injected with 1'000U of IFN-α or IFN-β subcutaneously into the footpad. Forty-eight hours p.i., popliteal LN cells were recovered and IFN-γ receptor expression at the surface of macrophages was measured by flow cytometry. Results of one representative of 3 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by Two-way ANOVA (**A**-**B**) or Student's t-test (**C**) *p<0.05, **p<0.01, ***p<0.001.

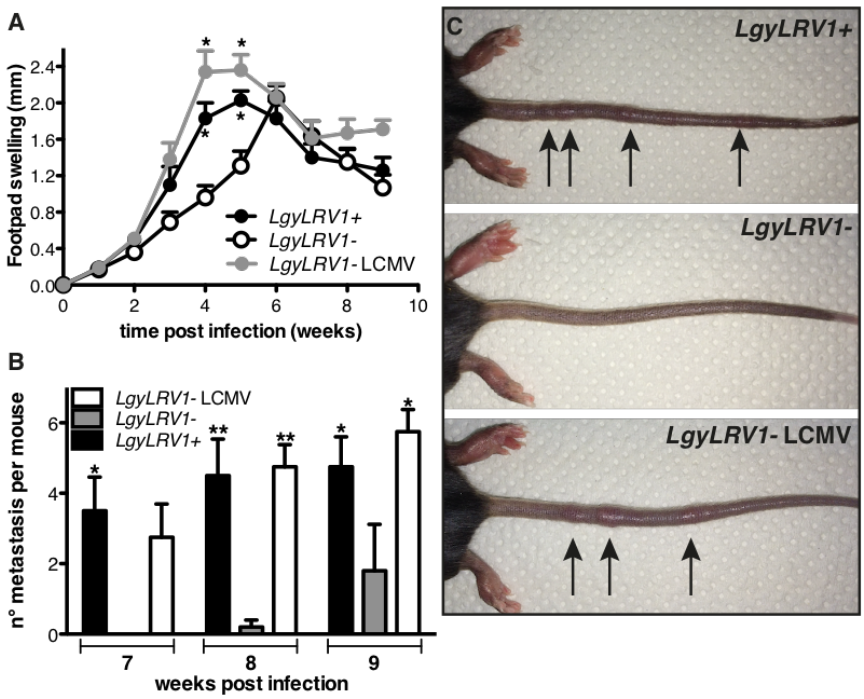


**Figure 4. *Lgy* - LCMV co-infection promoted disease dissemination.**

(**A**-**C**) IFN-γ-/- mice were infected in the hind footpads with 3 x $10^6$ stationary phase *Lgy* promastigotes. At the same time, where indicated, mice were injected intra-peritoneally with 2 x $10^5$ PFU of LCMV Armstrong. (**A**) Footpad thickness was measured weekly. (**B**) Number of metastatic lesions per mouse at week 7, 8 and 9 p.i.. (**C**) Image of photo of representative mice showing metastatic lesions on the tail at week 8 post infection. Results of one representative of 3 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by Repeated measure ANOVA (**A**) or Two-way ANOVA (**B**), *p<0.05, **p<0.01.

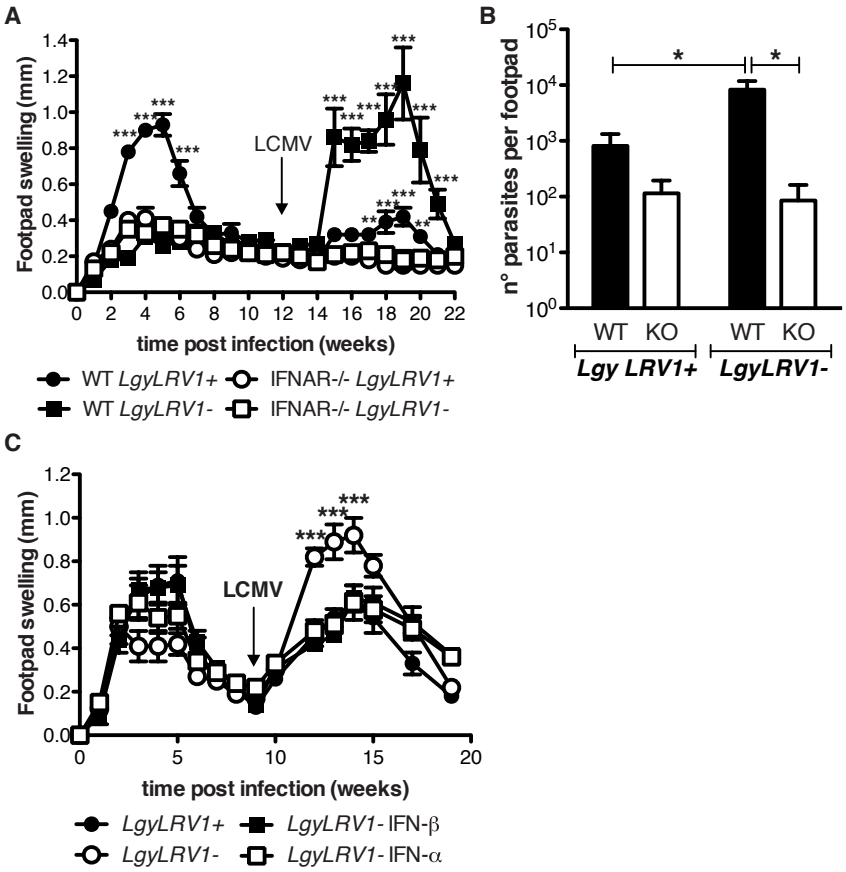


**Figure 5. LCMV late co-infection induced the relapse of leishmaniasis.**

(**A-B**) WT or *ifnar*-/- mice were infected in the hind footpads with 3 x $10^6$ stationary phase *Lgy* promastigotes. (**C**) At 6, 24 and 48 hours p.i., *LgyLRV1*$^-$ infected WT mice were injected with 1'000U of IFN-α or IFN-β. After the healing, mice were injected intra-peritoneally with 2 x $10^5$ PFU of LCMV Armstrong. (**A** and **C**) Footpad thickness was measured weekly. (**B**) Parasite burden was measured at week 17 p.i. by RT-qPCR, measuring *kmp11* gene expression. Results of one representative of 3 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by Repeated measure AN*OVA* (**A** and **C**) or Student's t-test (**B**) *p<0.05, **p<0.01, ***p<0.001.

# Supporting Information

# SI text

### *LgyLRV1+* infection induced Type I IFN expression *in vivo*

We previously demonstrated that macrophages produce IFN-β upon infection with *LgyLRV1+* parasites *in vitro* (5). To verify if similar production was occurring *in vivo*, C57BL/6 WT mice were inoculated with *LgyLRV1+* or *LgyLRV1-* parasites in the hind footpad. At 6 or 12 hours post infection, mice were sacrificed, and the footpads and draining lymph nodes were recovered and used for RT-qPCR analysis. *ifn-β* transcription was up-regulated at early time points post *LgyLRV1+* infection in both footpads and draining lymph nodes (Fig. S8). The increase of transcription was found to be only transient, as 48 hours post infection, Type I IFNs were not detectable neither in the lymph node, nor in the footpad. Similar results were obtained for *ifn-α* (Fig. S8). Further, *in vivo* macrophage depletion using clodronate-containing liposomes completely abrogated the induction of *ifn-α* and *ifn-β*, demonstrating that macrophages are responsible for Type I IFN production in *LgyLRV1+* infection (Fig. S8 and S9).

# SI Material and methods

### Mice

Four to 5 week old C57BL/6 mice were purchased from Harlan Laboratories (Netherlands), Type I IFN receptor deficient (*ifnar*-/-) mice were obtained from M. Aguet, Swiss Institute of Experimental Cancer Research (Epalinges, Switzerland), IFN-γ-/- mice were purchased from Jackson laboratories. Mice were kept and bred at the animal facility of the Center of Immunity and Immunology, Lausanne (Switzerland) in a pathogen-free environment. All animal protocols were approved by the Swiss Federal Veterinary Office (SFVO), under the authorization numbers 2113.1, 2113.2a and 2113.2b. Animal experimentation procedures were undertaken with strict adherence to ethical guidelines set out by the SFVO and under inspection by the Department of Security and Environment of the State of Vaud, Switzerland.

### Parasites

LRV1-bearing and LRV1-cured *L. guyanensis* parasites (*Lgy*LRV+ and *Lgy*LRV- respective) were used in all studies. These parasites were obtained following brief drug treatment of the LRV1+ strain of *L. guyanensis* M4147 containing a firefly luciferase (ffLUC) gene integrated stably into the small subunit gene of the ribosomal RNA locus (LgM4147/SSU:IR2SAT-LUCb LRV1+) as described previously (20).
Parasites were cultured at 26°C in Schneider's medium (Sigma-Aldrich) supplemented with 10% Fetal Calf Serum (FCS), 1% penicillin/Streptomycin (P/S), 1% HEPES (Sigma-Aldrich), 0.6 μg/ml of Biopterin (Sigma-Aldrich) and 5 μg/ml of Haemin (Sigma-Aldrich).

**Viruses**

Lymphocytic choriomeningitis virus (LCMV) 53b Armstrong was provided by Prof. Dietmar Zehn (Technical University of Munich, Germany). The LCMV 53b Armstrong strain was propagated in baby hamster kidney cells and titrated on Vero African green monkey kidney cells according to an established protocol (56). Frozen stocks were diluted in PBS; $2x10^5$ plaque-forming units (PFU) of LCMV Armstrong were injected intra-peritoneally. Blood samples from LCMV-infected mice were 'shock frozen' to release the virus. Diluted samples were used for infection of Vero cells, and viral titers were determined by the LCMV focus-forming assay.

Toscana virus strain 1812 (TOSV) was provided by Maria Grazia Cusi (University of Siena, Italy). TOSV was propagated and titrated on Vero cells according to an established protocol (57). $5x10^5$ PFU of TOSV in 50μl of PBS was inoculated subcutaneously into the footpad.

**Mice infection**

Stationary phase parasites were injected into the hind footpads of mice at a concentration of $3x10^6$ parasites per footpad in 50μl of PBS. Footpad thickness was measured weekly post infection (p.i.) using a Vernier caliper. Where required, mice were injected in the footpad at 6, 24 and 48 hours p.i. with the indicated amount of murine recombinant interferon beta (IFN-β, CellScience), or interferon alpha (IFN-α, CellScience) in 50μl of PBS, or with the same volume of PBS as vehicle-control.

**Macrophage extraction and culture**

In order to obtain bone marrow derived macrophages (BMDMs), bone marrow was extracted from tibias and femurs of C57BL/6 or *ifnar*-/- mice. Macrophages were cultured at

37°C and 5% $CO_2$ in 10ml of Dulbecco's modified eagle medium (DMEM, Gibco®) supplemented with 10% of Fetal Calf Serum (FCS), 1% of P/S, 1% of HEPES (Sigma-Aldrich) and 50ng/ml of murine recombinant M-CSF (Immunotools). After 3 days, 5ml of fresh DMEM medium was added to the culture and 3 days later BMDMs were used for stimulation assays.

**IFN-γ receptor measurement**

5 x $10^5$ BMDMs in 200µl of DMEM supplemented with 10% FCS, 1% P/S, and 1% HEPES (DMEM complete medium) were seeded in a 48 well plate and incubated at 37°C and 5% $CO_2$ O/N. BMDMs were subsequently infected with *L. guyanensis* parasites (*Lgy*) (multiplicity of infection (moi) 3:1) in 200µl of DMEM complete medium. At 6 hours p.i., BMDMs were treated with 50, 100, 250, 500 or 1000U/ml of murine recombinant IFN-α or IFN-β (CellScience). Forty-eight hours p.i., BMDMs were detached with PBS/5mM EDTA and IFN-γR surface expression was measured by FACS.

To measure IFN-γR expression *in vivo*, mice were infected with *L. guyanensis* parasites and co-infected with LCMV, or treated with IFN-α, or IFN-β as described above. Mice were sacrificed at 48 hours p.i. and popliteal lymph node cells were recovered. IFN-γR expression on macrophages was measured by flow cytometry using the following antibodies: IFN-γR1-PE (clone 2E2, dilution 1:100), CD11b-FITC (clone M1/70, 1:500), CD11c-PECy7 (clone N418, 1:500) and F4/80-APC (clone BM8, 1:300) (ebioscience). Flow cytometry was performed with a BD Accuri™ C6 cytometer and results were analyzed using FlowJo Software (Tree Star).

**IFN-γ measurement *in vivo***

WT mice were infected with *L. guyanensis* parasites and co-injected with LCMV or PBS as control as described above. Mice were sacrificed at 48 hours p.i., popliteal lymph node cells were subsequently recovered and intracellular expression of IFN-γ within NK, CD4T and CD8T cells was measured by flow cytometry, using the following antibodies: CD3-APC-Cy7 (clone 17A2, dilution 1:1000), CD4-Pacific blue (clone GK1.5, 1:1000), CD8-PerCP (clone 53-6.7, 1:1000)(BioLegend), NK1.1-FITC (clone PK136, 1:500), IFN-γ-PE (clone XMG1.2, 1:500)(ebioscience). Flow cytometry was performed with a BD™ LSR-II flow cytometer (BD Biosciences) and results were analyzed using FlowJo Software (Tree Star). Alternatively, popliteal lymph node cells were cultured for 72 hours in complete

DMEM medium. After 72 hours, cells were pelleted and supernatant was recovered. IFN-γ protein concentration was measured by ELISA using an IFN-γ ELISA kit (ebioscience) following manufacturer's instructions.

**Type I Interferon and Interferon stimulated gene measurement**

Transcript levels of *ifn-α* and *ifn-β* were measured *in vivo* in footpads and draining lymph nodes (LNs), at 6 and 12 hours p.i., respectively. Alternatively, transcript levels of ISGs were measured *in vivo* in draining LNs at 24 hours p.i.. To allow RNA extraction, LNs were stored in RNA*later* RNA stabilization solution (Quiagen) whereas footpads were frozen and then homogenized in TRIzol (Life Technologies). Total RNA was extracted from LNs and footpads using the ZR-96 RNA Clean & Concentrator™ (Zymo research), and cDNA was generated using SuperScript II Reverse Transcriptase (Invitrogen™). The cDNA obtained was purified with the ZR-96 DNA Clean & Concentrator™-5 (Zymo research). Real-Time Quantitative PCR (qRT-PCR) was performed using the LightCycler 480 (Roche Applied Science) to measure SYBR green (Lightcycler®480 SYBR Green I Master, Roche) incorporation. The following primers were used: *ifn-α*: 5'-GGACTTTGGATTCCCGCAGGAGAAG and 5'-GCTGCATCAGACAGCCTTGCAGGTC; *ifn-β*: 5'-AACCTCACCTACAGGGC, and 5'-CATTCTGGAGCATCTCTTGG; *l32*: 5'-AAGCGAAACTGGCGGAAAC and 5'-TAACCGATGTTGGGCATCAG; *oas1a*: 5'-CCCATCTGCATCAGGAGGTG and 5'-GGATCAGGCTTGCTGGAAGT; *oas2*: 5'-TGAAGAAGCGAAGGAGTGGC and 5'-GGGTCTGCATTACTGGCACTT; *oasl2*: 5'-CCGCGACATCTGCATCTACT and 5'-CGGTCTCCCTTCAGCTGTTT; *pkr*: 5'-CAGAAACTTTGGCCACTGGGA and 5'-CCGTGCATCTGGCGGTATT. The results were analyzed using the $C_T$ method ($2^{-\Delta\Delta Ct}$) for relative quantification of gene expression and normalized to l32. The fold induction was calculated according to the relative expression in naive mice.

Type I interferon protein levels were measured 24 hours p.i. in mice serum, using the Verikine mouse IFN-α ELISA Kit and Verikine-HS Mouse IFN-β Serum ELISA Kit (PBL assay science), following manufacturer's instructions.

**Parasite quantification**

*In vivo* parasites were quantified at the peak of infection in mouse footpads by injecting 15mg/kg of luciferin (Invivo Imaging) intra-peritoneally and measuring the luminescence produced in the footpads with a Xenogen IVIS Lumina II. The following

parameters were used: exposure 10 minutes, binning medium, F/stop 1.2. Alternatively, the Brucker Extreme II machine was used with the following parameters: exposure 5 minutes, binning 4, F/stop 1.2.

**Macrophage depletion**

To deplete macrophages from the draining lymph nodes, WT mice were injected subcutaneously into the ankle with 50µl of PBS- or Clodronate-containing liposomes (ClodronateLiposomes.org). Three weeks post-treatment, mice were infected with $3x10^6$ stationary phase *L. guyanensis* parasites in each footpad. Mice were sacrificed 12 hours p.i., draining LNs were taken and macrophage presence was measured by FACS using the Accuri® cytometer C6 and data analyzed with FlowJo (version 10.0.7). The following antibodies were used: CD169-PE (clone 3D6.112), CD11b-FITC (clone M1/70) and CD11c-PEcy7 (clone N418) (ebioscience).

**Statistical analysis**

Data obtained from Xenogen and FACS were analyzed using either two-way ANOVA or Student's t-test. Data obtained from qRT-PCR and ELISA were analyzed using a Student's t-test. Data obtained from footpad swelling measurements were analyzed using a *repeated measure ANOVA test.* When the curves were non-parallel (i.e. the treatment-time interaction was statistically significant), statistical significance of difference between treatments at different time points was assessed using Bonferroni's multiple test correction.

Statistical significance was set at a p-value lower than 0.05. All data are represented with the mean ± the standard error of the mean (SEM). All analyses were performed using GraphPad Prism® software.

# SI Figures

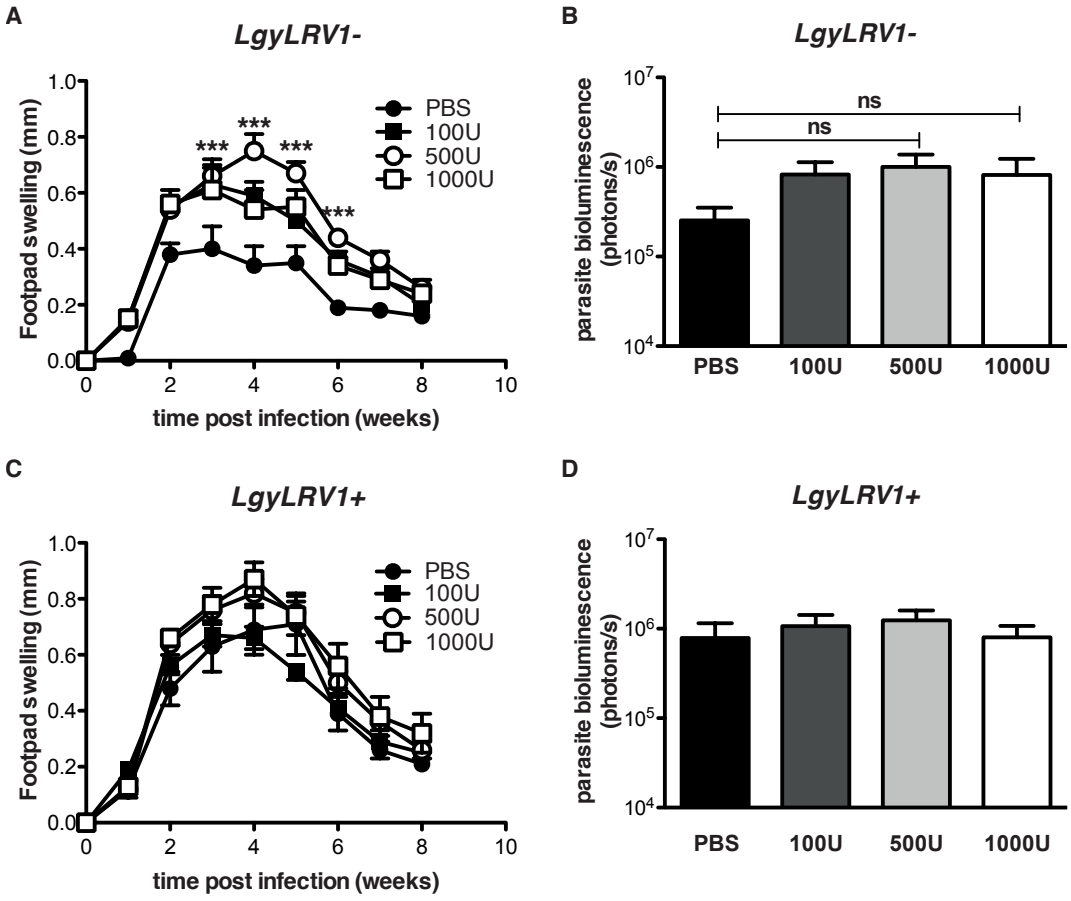


**Figure S1. IFN-α injection increased the size of the lesions of *LRV1-Lgy* infected mice.**
WT mice were infected in the hind footpads with 3 x $10^6$ stationary phase *Lgy* promastigotes. At 6, 24 and 48 hours p.i., mice were injected with increasing doses of IFN-α subcutaneously into the footpad. (**A** and **C**) Footpad thickness was measured weekly. (**B** and **D**) Parasite burden was quantified 4 weeks p.i. *in vivo* by measuring parasite bioluminescence. Results of one representative of 2 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by *Repeated measure ANOVA* (**A** and **C**) or *Student's t-test* (**B** and **D**) ***p<0.0001.

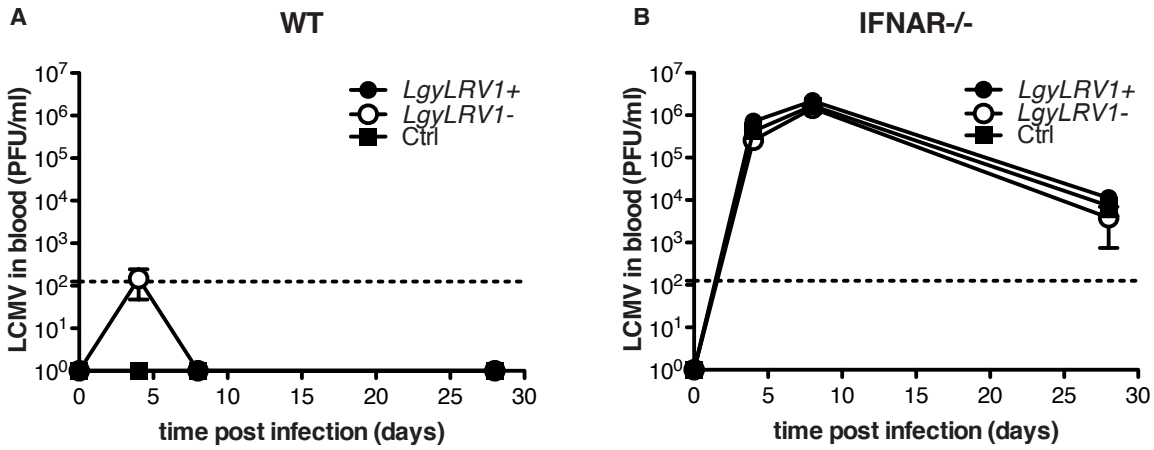


**Figure S2. *Leishmania* co-infection did not affect LCMV clearance.**

(**A**) WT or (**B**) *ifnar*-/- mice were infected intra-peritoneally with 2 x $10^5$ PFU of LCMV Armstrong. At the same time, where indicated, mice were injected in the hind footpad with 3 x $10^6$ stationary phase *Lgy* promastigotes. LCMV titer in the blood was measured at different times p.i. by plaque assay. Results of one experiment were expressed as mean ± SEM (n=5). Statistical significance was assessed by *Repeated measure ANOVA*.

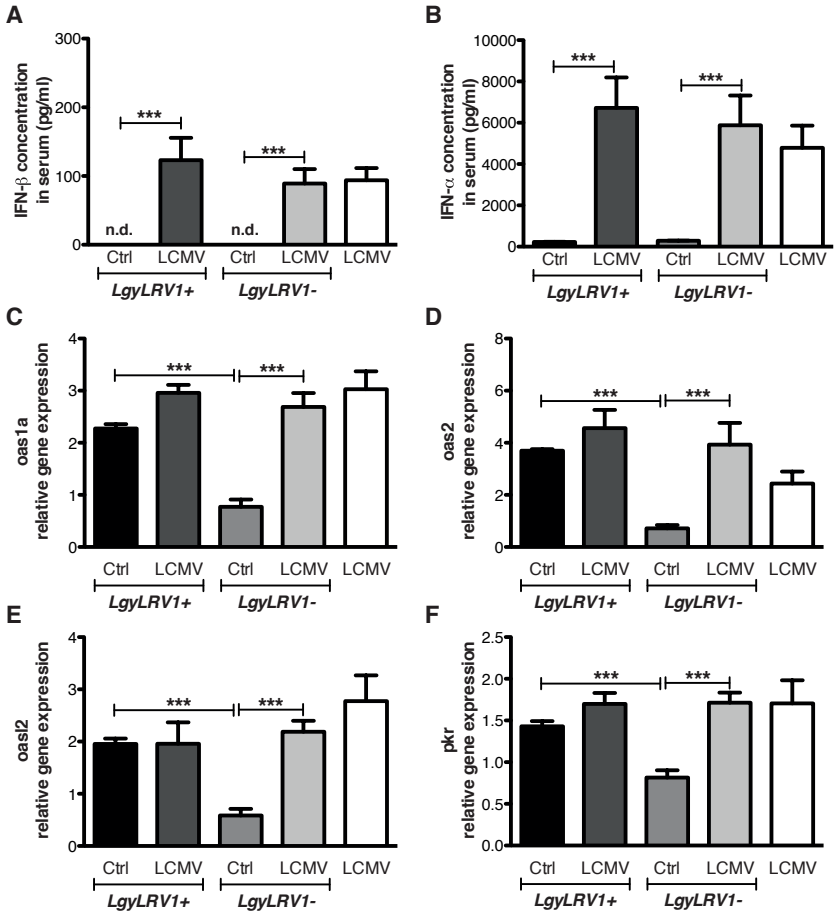


**Figure S3. LRV1 and LCMV co-infection induced a similar interferon response *in vivo*.**
WT mice were infected in the hind footpad with 3 x $10^6$ stationary phase *Lgy* promastigotes, or PBS as control. At the same time, where indicated, mice were injected intra-peritoneally with $2*10^5$ PFU of LCMV Armstrong. (**A**) IFN-β and (**B**) IFN-α concentration in serum was measured by ELISA 24 hours p.i.. (**C-F**) Popliteal LNs were recovered 24 hours p.i. and used for RT-qPCR analysis. Results of one representative of 2 independent experiments were expressed as mean ± SEM (n=5) of protein concentration (**A-B**) or as mean ± SEM (n=5) of transcript increase relative to *LgyLRV1-* infected mice, and normalized to housekeeping gene l32 (**C-F**). Statistical significance was assessed by *Student's t-test,* ***p<0.001.

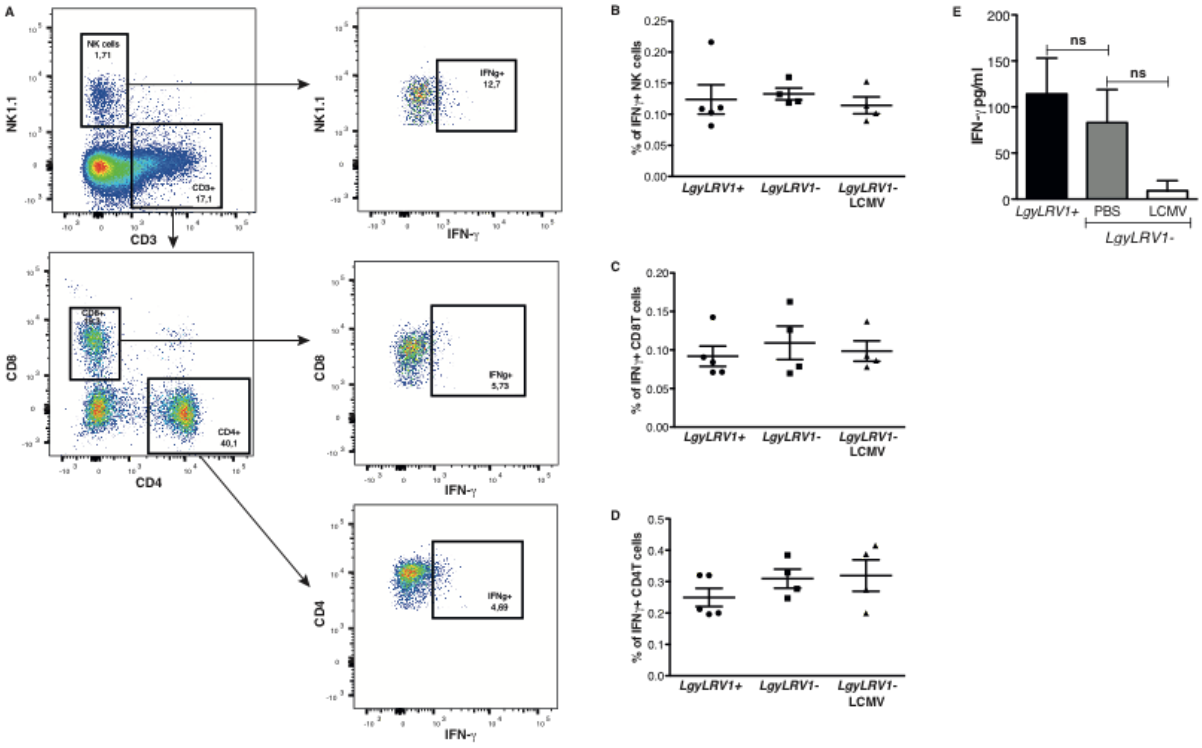


**Figure S4. LRV1 or LCMV co-infection did not modulate early IFN-γ production *in vivo*.**

WT mice were infected in the hind footpad with 3 x $10^6$ stationary phase *Lg*y promastigotes. At the same time, where indicated, mice were injected intra-peritoneally with 2*$10^5$ PFU of LCMV Armstrong, or PBS as control. Popliteal LN cells were recovered 48 hours p.i.. (**A**) Flow cytometry dot plots showing intracellular IFN-γ expression in different cell populations. (**B-D**) Frequency of IFN-γ-expressing NK, CD8T and CD4T cells, respectively. Results of one experiment were expressed as mean ± SEM (n≥4). (**E**) IFN-γ secretion by lymph node cells was measured by ELISA. Results of a pool of two independent experiments were expressed as mean ± SEM (n=8). Statistical significance was assessed by *Student's t-test*, results were non-significant.

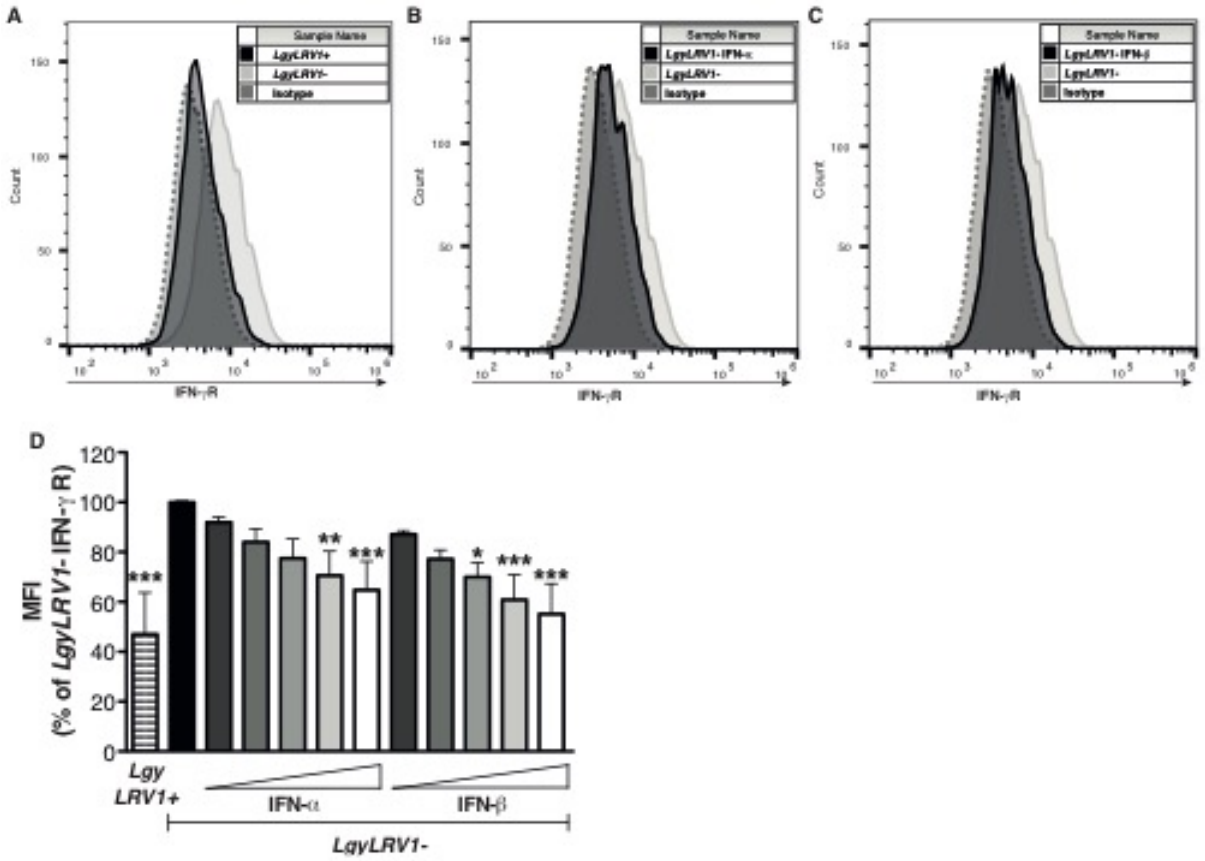


**Figure S5. *LgyLRV1+* infection or Type I IFN treatment down-regulated IFN-γR expression on macrophages.**

WT BMDMs were infected with *Lgy* parasites. Six hours p.i., *LgyLRV1*- infected macrophages were treated with 1'000U/ml of IFN-α or IFN-β. Forty-eight hours p.i., IFN-γR expression was measured by flow cytometry. Results of one representative of 3 independent experiments showed IFN-γR surface expression on *LgyLRV1+* infected (**A**), IFN-α (**B**), or IFN-β (**C**) treated macrophages compared to *LgyLRV1-* infection. (**D**) WT BMDMs were infected with *Lgy* parasites. Six hours p.i., *LgyLRV1*- infected macrophages were treated with increasing doses of IFN-α or IFN-β (50, 100, 250, 500 or 1'000U/ml). Forty-eight hours p.i., IFN-γR expression was measured by flow cytometry. Results of a pool of 2 independent experiments were expressed as mean ± SEM of IFN-γR MFI. Statistical significance was assessed by Student's t-test (**D**) *p<0.05, **p<0.01, ***p<0.001.

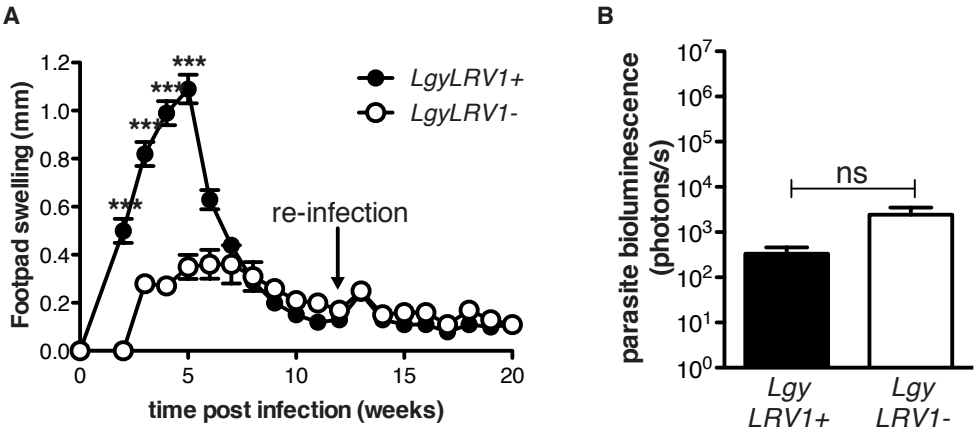


**Figure S6. C57BL/6 WT mice are protected against *Lgy* re-infection.**

WT mice were infected in the hind footpad with 3 x $10^6$ stationary phase *Lg*y promastigotes. Twelve weeks p.i. mice were re-infected with *Lgy* promastigotes. (**A**) Footpad thickness was measured weekly. (**B**) Parasite burden was quantified 15 weeks p.i. *in vivo* by measuring parasite bioluminescence. Results of one representative of 2 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by *Student's t-test*, results were non-significant.

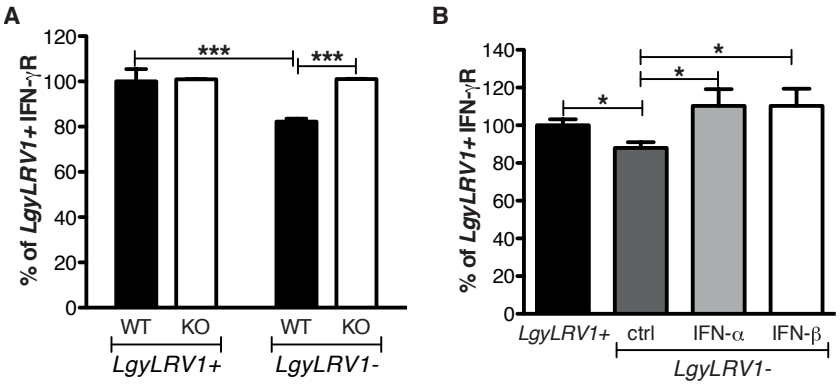


**Figure S7. Early Type I IFNs inhibited IFN-γR down-regulation following LCMV co-infection.**

(**A**) WT or *ifnar*-/- mice were infected in the hind footpads with 3 x $10^6$ stationary phase *Lgy* promastigotes. (**B**) At 6, 24 and 48 hours p.i., *LgyLRV1*$^-$ infected WT mice were injected with 1000U of IFN-α or IFN-β. After the healing, mice were injected intra-peritoneally with 2 x $10^5$ PFU of LCMV Armstrong. (**A-B**) Forty-eight hours after LCMV infection, popliteal LN cells were recovered and IFN-γ receptor expression on macrophages was measured by FACS. Results of one representative of 2 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by *Two-way ANOVA* (**A**) or *Student's t-test* (**B**) *p<0.05, ***p<0.001.

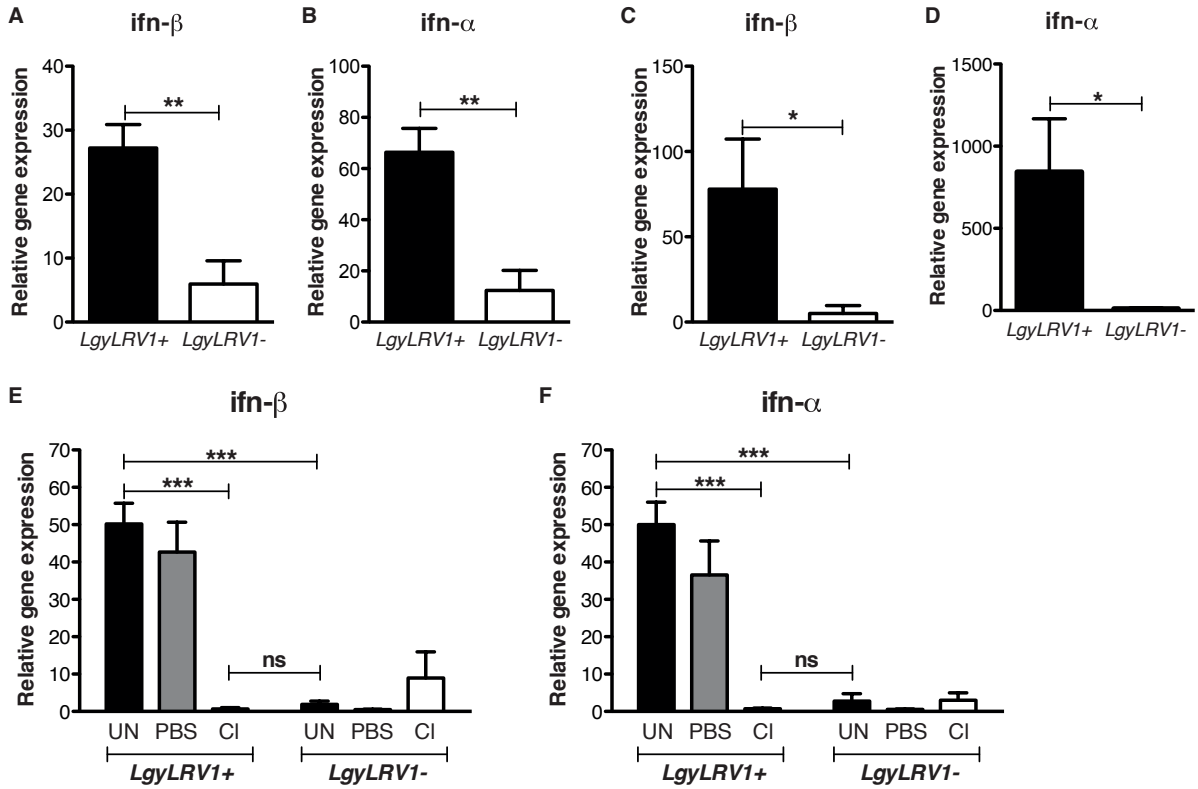


**Figure S8. *LgyLRV1+* parasite infection induced Type I IFN up-regulation *in vivo*.**

WT mice were infected in the hind footpads with 3 x $10^6$ *Lgy* stationary-phase promastigotes. *ifn-β* and *ifn-α* transcript levels were quantified by RT-qPCR at 6 or 12 hours p.i. in footpads (**A**-**B**) or draining LNs (**C**-**D**). (**E**-**F**) Three weeks prior to infection, mice were injected subcutaneously into the ankle with 50µl of PBS- or clodronate-containing liposomes. *ifn-β* and *ifn-α* transcript levels were quantified by RT-qPCR in draining LNs 12 hours p.i..

Results of one representative of 3 independent experiments were expressed as mean ± SEM (n=5) transcript increase relative to a naïve mouse and normalized to housekeeping gene l32. Statistical significance was assessed by *Student's t-test*, *p<0.05, **p<0.01, ***p<0.001 .

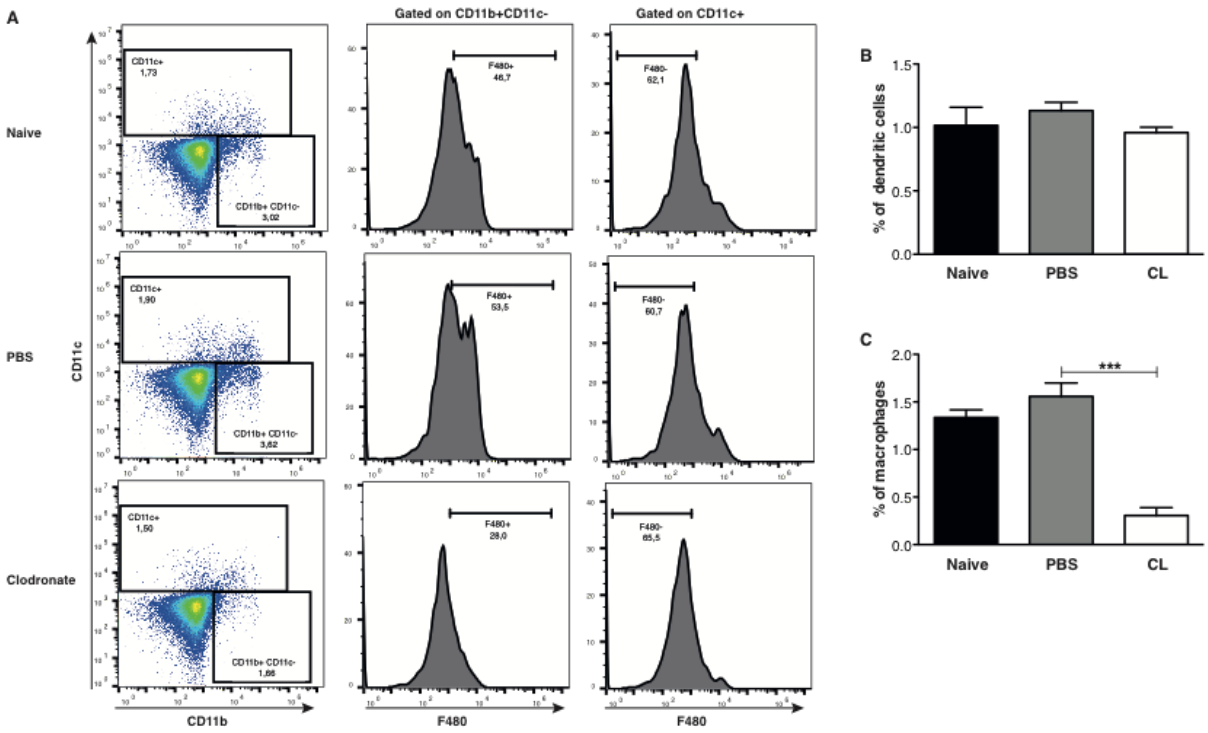


**Figure S9. Clodronate depletion of macrophages *in vivo*.**

WT mice were injected subcutaneously in the ankle with 50µl of PBS- or clodronate-containing liposomes. Three weeks post-treatment, mice were infected in the hind footpads with 3 x $10^6$ *Lgy* stationary-phase promastigotes. LNs cells were recovered 12 hours p.i.. (**A**) FACS plots showed the frequency of macrophages and dendritic cells in LNs. (**B-C**) Graphs showed the percentage of dendritic cells and macrophages in total LNs cells. Results of one representative of 3 independent experiments were expressed as mean ± SEM (n=5). Statistical significance was assessed by *Student's t-test,* ***p<0.001.