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

Matteo Rossi[a], Patrik Castiglioni[a], Mary-Anne Hartley[a], Remzi Onur Eren[a], Florence Prével[a], Chantal Desponds[a], Daniel T. Utzschneider[b], Dietmar Zehn[c], Maria G. Cusi[d], F. Matthew Kuhlmann[e,f], Stephen M. Beverley[f,1], Catherine Ronet[a], and Nicolas Fasel[a,1]

[a]Department of Biochemistry, University of Lausanne, 1066 Epalinges, Switzerland; [b]Department of Cellular and Molecular Medicine, University of California, San Diego, CA 92093; [c]Division of Animal Physiology and Immunology, Technical University of Munich, 85354 Freising, Germany; [d]Department of Medical Biotechnologies, Policlinico Le Scotte, University of Siena, 53100 Siena, Italy; [e]Division of Infectious Diseases, Washington University School of Medicine, St. Louis, MO 63110; and [f]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110

Contributed by Stephen M. Beverley, March 29, 2017 (sent for review December 30, 2016; reviewed by Barbara A. Burleigh, Paul Martin Kaye, and Robert B. Tesh)

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 IFN (type I IFNs) production by macrophages and signaling in vivo. Moreover, infecting mice with the LRV1-cured *Leishmania 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 finding suggested the possibility that exogenous viral infections could likewise increase pathogenicity, which was tested by coinfecting mice with *L. guyanensis* and lymphocytic choriomeningitis virus (LCMV), or the sand fly-transmitted arbovirus Toscana virus (TOSV). The type I IFN antiviral response increased the pathology of *L. guyanensis* infection, accompanied by down-regulation of the IFN-γ receptor normally required for antileishmanial control. Further, LCMV coinfection of IFN-γ–deficient mice promoted parasite dissemination to secondary sites, reproducing the *LgyLRV1*$^{+}$ metastatic phenotype. Remarkably, LCMV coinfection 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 coinfection 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.

Leishmania RNA virus 1 | Totiviridae | arboviruses | trypanosomatid protozoan parasite | *Leishmania* subgenus *Viannia*

Protozoan parasites of the genus *Leishmania* are 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 *Leishmania guyanensis* (*Lgy*) or *Leishmania braziliensis* (*Lbr*) principally leads to simple cutaneous lesions; however, up to 10% of patients develop disseminated or mucocutaneous leishmaniasis (DCL or MCL). 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 *Leishmania major* and *Leishmania 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 includes 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

Significance

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 *Leishmania guyanensis*-induced murine leishmaniasis, favoring parasite persistence and dissemination resulting in metastasis. Both endogenous and exogenous coinfections were dependent upon type I interferon responses. Strikingly, LCMV coinfection 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.

Author contributions: M.R., D.Z., M.G.C., C.R., 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; D.Z., M.G.C., F.M.K., and S.M.B. contributed new reagents/analytic tools; M.R. and D.T.U. analyzed data; and M.R., S.M.B., and N.F. wrote the paper.

Reviewers: B.A.B., Harvard School of Public Health; P.M.K., University of York; and R.B.T., University of Texas.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. Email: Nicolas.Fasel@UNIL.CH or stephen.beverley@wustl.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1621447114/-/DCSupplemental.

(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 proinflammatory cytokines and chemokines, including 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 proinflammatory cytokines by infected macrophages (19, 20).

The induction of TLR3-dependent IFN-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 antiviral activity, and IFN 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 the parasite species (28, 29). For *L. major*, type I IFNs have been associated with control (30–32), whereas for other species, especially the New World species *L. amazonensis* and *L. braziliensis*, 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 coinfections with exogenous viruses inducing type I IFNs could also worsen the disease outcome. Such coinfections could occur at the site of infection by sand flies carrying both *Leishmania* parasites and *Phleboviruses* [e.g., Toscana virus (TOSV)], or by another virus inducing systemic production of type I IFNs. Thus far, little is known about coinfection 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 coinfecting mice with LRV1-cured *Lgy* (*LgyLRV1*$^-$) and lymphocytic choriomeningitis virus (LCMV), or 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 postinfection, WT mice began to develop lesions that grew until reaching a maximal size at week 5, which then healed 4 wk later, with *LgyLRV1*$^+$ infection inducing significantly larger lesions compared with *LgyLRV1*$^-$-infected mice (Fig. 1*A*). In contrast, *LgyLRV1*$^+$-infected *ifnar*$^{-/-}$ mice developed significantly smaller lesions, similar to those developed by *ifnar*$^{-/-}$ and WT mice infected with *LgyLRV1*$^-$ (Fig. 1*A*). Parasite numbers were also significantly increased in WT mice infected with *LgyLRV1*$^+$ compared with the *LgyLRV1*$^-$-infected counterpart (Fig. 1*B*). 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 at early time points post-*Lgy* infection, to mimic the antiviral response induced by the endogenous dsRNA LRV1 virus (5). In *LgyLRV1*$^-$-infected mice,

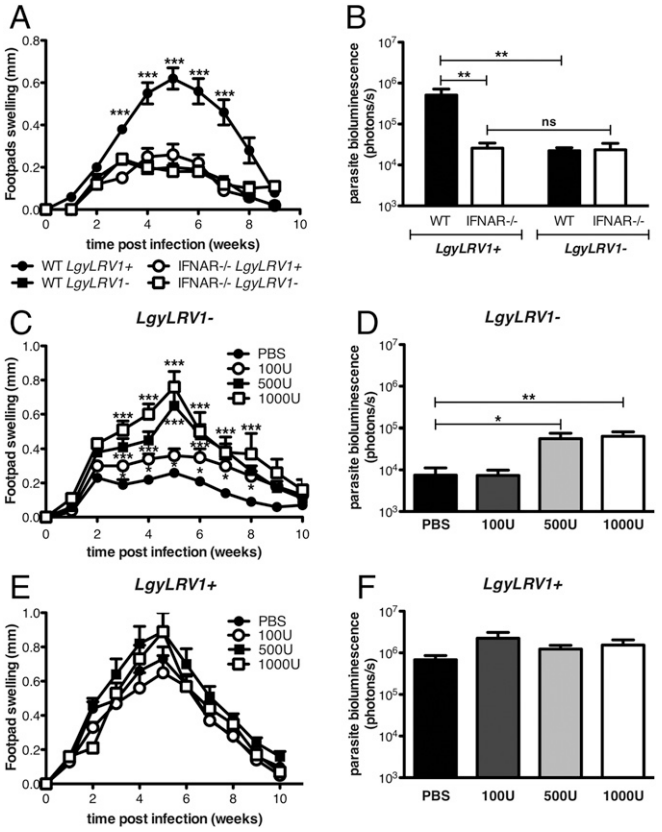

Fig. 1. Type I IFNs increased the severity of *Lgy* infection. (*A* and *B*) WT or *ifnar*$^{-/-}$ mice were infected in the hind footpads with 3×10^6 stationary phase *Lgy* promastigotes. (*C*–*F*) At 6, 24, and 48 h postinfection (p.i.), WT mice were injected with the indicated amount of IFN-β into the footpad. (*A*, *C*, and *E*) Footpad thickness was measured weekly. (*B*, *D*, and *F*) Parasite burden was quantified 5 wk p.i. in vivo by measuring parasite bioluminescence. Results of one representative of three 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*). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, nonsignificant.

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 100 units of IFN-β showed a moderate increase in lesion size and no effect on parasite load, whereas injection of 500 or 1,000 units caused greater increase of both lesion size and parasite burden, quantitatively reproducing the phenotype of *LgyLRV1*$^+$ infection (Fig. 1 *C* and *D*). In contrast, little effect from IFN-β treatment was seen in *LgyLRV1*$^+$-infected mice, (Fig. 1 *E* and *F*). Similar results were obtained with IFN-α treatment (Fig. S1).

Viral Coinfection 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 IFN signaling. This evidence alluded to the possibility that other agents triggering type I IFN responses might act similarly to promote *Leishmania* virulence, such as coinfections with other viruses. To test this possibility, we coinfected mice with *LgyLRV1*$^-$ parasites and LCMV Armstrong, an arenavirus, which induces a potent type I IFN response (37). Two different sites of injection were used: i.p. injection for LCMV and s.c. in the footpad for *Lgy*.

Infection with LCMV Armstrong was shown to be cleared in 8 d by C57BL/6 WT mice with a robust T-cell response (38). Viral titration in mouse serum, following i.p. 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 h 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*$^{-}$ coinfected with LCMV, compared with *LgyLRV1*$^{-}$-infected mice (Fig. S3).

We then tested the effect of LCMV coinfection 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. 2 *A* 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, because, 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 whether another virus could similarly affect the course of leishmaniasis. We focused on 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 s.c. into the footpad. As observed with LCMV, *LgyLRV1*$^{-}$/TOSV coinfection highly increased footpad swelling and parasite burden at the peak of infection (Fig. 2 *E* and *F*). *ifnar*$^{-/-}$ mice were susceptible to TOSV infection, with over 50% of mortality after 2 wk of infection, precluding further tests on the type I IFN dependency in this coinfection model.

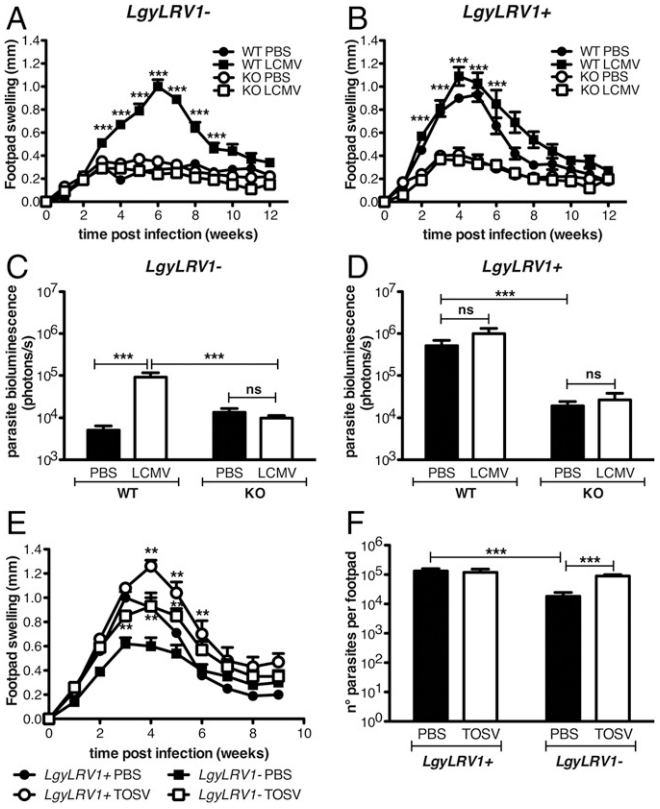

Fig. 2. Viral coinfection increased the severity of leishmaniasis through type I IFNs. (*A*–*F*) WT or *ifnar*$^{-/-}$ mice were infected in the hind footpads with 3×10^6 stationary phase *Lgy* promastigotes. (*A*–*D*) *Leishmania*-infected mice were injected simultaneously with 2×10^5 pfu of LCMV Armstrong or the same volume of PBS as vehicle control intraperitoneally. (*E* and *F*) Alternatively, *Leishmania*-infected mice were simultaneously inoculated with 5×10^5 pfu of TOSV s.c. into the footpad. (*A*, *B*, and *E*) Footpad thickness was measured weekly. (*C*, *D*, and *F*) Parasite burden was measured 5 wk p.i. by measuring parasite bioluminescence or by RT-qPCR. Results of one representative of three 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*). $^{**}P < 0.01$, $^{***}P < 0.001$; ns, nonsignificant.

Viral Coinfection Modulates Macrophage Responsiveness to IFN-γ Through Type I IFNs. The data above suggested that coinfection 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 IFN 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. 1 *C* and *D*). As LRV1 or LCMV coinfection did not modulate early IFN-γ production (Fig. S4), we wondered whether IFN-γ signaling was modulated during coinfections. To this end, we first measured IFN-γ receptor (R) surface expression on bone marrow-derived macrophages. Infection with *LgyLRV1*$^{+}$ parasites induced a significant down-regulation of IFN-γR compared with *LgyLRV1*$^{-}$, in a type I IFN-dependent manner (Fig. S5). In this in vivo model of coinfection 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 coinfection 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 twofold; Fig. S5*D*). Subsequently, we confirmed these results during in vivo infection. Flow cytometric analysis 48 h postinfection showed that macrophages from *LgyLRV1*$^{+}$-infected or *LgyLRV1*$^{-}$/LCMV-infected mice, expressed significantly lower levels of surface IFN-γR, compared with infections by *LgyLRV1*$^{-}$ alone (Fig. 3*A*). No significant difference was observed in *ifnar*$^{-/-}$ mice (Fig. 3*B*); 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. 3*C*). This finding confirmed that the effect depended on type I IFNs.

LCMV–*Lgy* Coinfection 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 coinfected with *LgyLRV1*$^{-}$/LCMV developed metastasis earlier than those infected with *LgyLRV1*$^{-}$ alone, thus reproducing the phenotype of

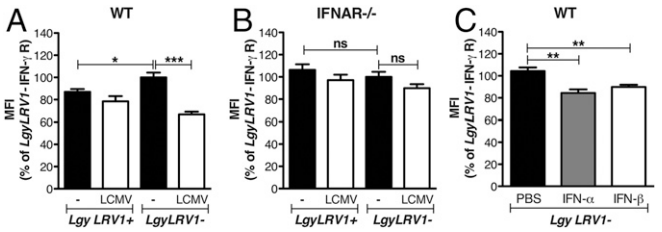

Fig. 3. *Lgy*–LCMV coinfection modulated IFN-γR expression on macrophages through type I IFNs. (*A*) WT or (*B*) *ifnar*$^{-/-}$ mice were infected in the hind footpads with 3×10^6 stationary phase *Lgy* promastigotes. At the same time, where indicated, mice were injected intraperitoneally with 2×10^5 pfu of LCMV Armstrong. (*C*) Alternatively, at 6 h p.i., *LgyLRV1*$^{-}$-infected WT mice were injected with 1,000 units of IFN-α or IFN-β s.c. into the footpad. Forty-eight hours p.i., popliteal lymph node (LN) cells were recovered and IFN-γ receptor expression at the surface of macrophages was measured by flow cytometry. Results of one representative of three independent experiments were expressed as mean ± SEM ($n = 5$). Statistical significance was assessed by two-way ANOVA (*A* and *B*) or Student's *t* test (*C*). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

LgyLRV1^{+} parasites. Therefore, exogenous viral coinfection was equally capable of synergizing parasite virulence and metastasis (Fig. 4 *A*–*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 reinfected 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 wk post-LCMV injection and healing a few weeks later (Fig. 5*A*). Further, at the peak of LCMV infection, we found higher parasite burden, a sign of reactivation of parasite proliferation (Fig. 5*B*). The mechanism of relapse relied on type I IFNs, because no footpad swelling or increase in parasite load was observed in *ifnar*$^{-/-}$ mice infected with either *LgyLRV1*$^{-}$ or *LgyLRV1*$^{+}$ (Fig. 5 *A* and *B*). As seen with simultaneous infections, the subsequent delayed posthealing LCMV injection in *LgyLRV1*$^{-}$-infected mice induced down-regulation of IFN-γR surface expression on macrophages in a type I IFN-dependent manner (Fig. S7*A*).

Interestingly, *LgyLRV1*$^{+}$-infected WT mice developed only small lesions with low parasite number compared with 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 2 d of infection, they developed less severe relapses when subsequently coinfected with LCMV (Fig. 5*C*). 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. S7*B*), suggesting that early type I IFN signaling may inhibit the effect of a subsequent LCMV coinfection.

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 antiviral 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

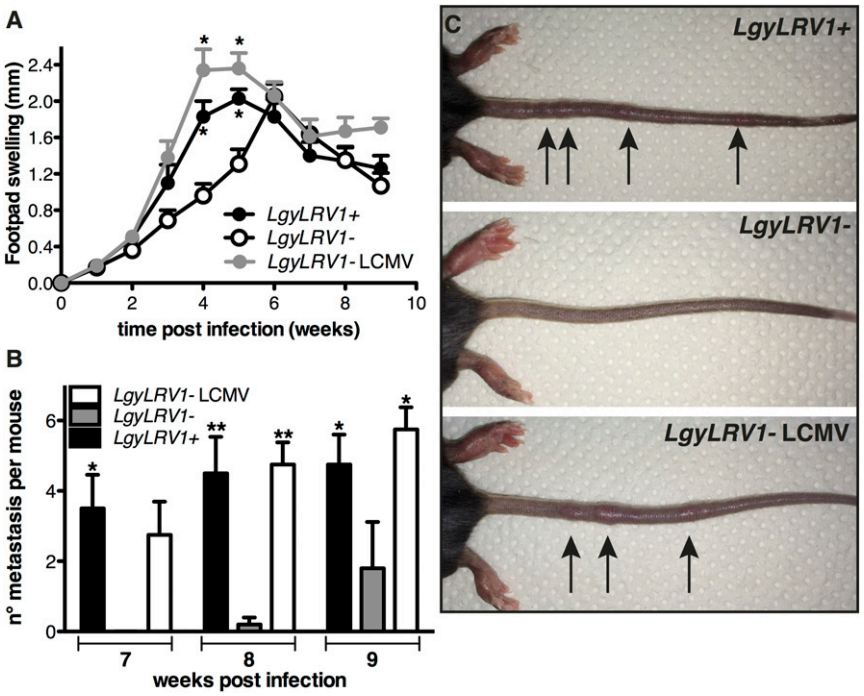

Fig. 4. *Lgy*–LCMV coinfection promoted disease dissemination. (*A*–*C*) IFN-γ$^{-/-}$ mice were infected in the hind footpads with 3×10^6 stationary phase *Lgy* promastigotes. At the same time, where indicated, mice were injected intraperitoneally with 2×10^5 pfu of LCMV Armstrong. (*A*) Footpad thickness was measured weekly. (*B*) Number of metastatic lesions per mouse at week 7, 8, and 9 p.i. (*C*) Photographic image of representative mice showing metastatic lesions on the tail at week 8 postinfection. Results of one representative of three independent experiments were expressed as mean ± SEM ($n = 5$). Statistical significance was assessed by repeated measure ANOVA (*A*) or two-way ANOVA (*B*). $^*P < 0.05$, $^{**}P < 0.01$.

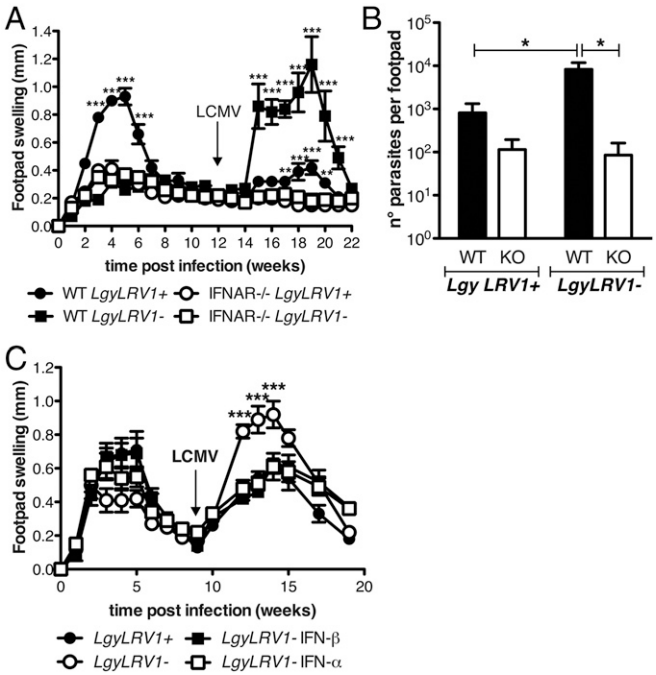

Fig. 5. LCMV late coinfection induced the relapse of leishmaniasis. (*A* and *B*) WT or *ifnar*$^{-/-}$ mice were infected in the hind footpads with 3×10^6 stationary phase *Lgy* promastigotes. (*C*) At 6, 24, and 48 h p.i., *LgyLRV1*$^{-}$-infected WT mice were injected with 1,000 units of IFN-α or IFN-β. Following healing, mice were injected intraperitoneally with 2×10^5 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 three 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*). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

leishmaniasis. Despite the fact that type I IFNs are mainly known for their antiviral role, increasing evidence attests to 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 coinfection, 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 coinfection, 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 coinfection increased the persistence of *Leishmania* parasites. Whereas 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 coinfection 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 first infected with *L. major* and only 2 wk later coinfected with LCMV (40). In this latter case, a transiently decreased anti-*Leishmania* immune response was observed 1 wk following LCMV coinfection. 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 with infection with parasites alone. This finding suggests that there was a decrease of macrophage antiparasitic activity, consistent with the lower IFN-γR expression on macrophages; this observation, however, did not exclude other possible systemic effects of LCMV. Interestingly, LCMV coinfection 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 coinfection depended completely on type I IFNs (Fig. 2 *A* and *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, coinfection 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). Coinfection was reported in dogs, whereas seropositivity to TOSV was associated with *Leishmania infantum* in humans (52, 53); however, the clinical relevance of the coinfection is unknown. As our results suggest that events early in infection were crucial to determine the fate of the disease (Fig. 1 *C* and *D*), having the virus transmitted simultaneously with *Leishmania (Viannia)* could magnify the impact of coinfection. However, i.p. LCMV infections which induce a systemic type I IFN response strongly exacerbated disease, suggesting that coinfections 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 with *LgyLRV1*$^{+}$. This result correlated with a type I IFN-dependent down-regulation of IFN-γR observed in *LgyLRV1*$^{-}$-infected mice compared with *LgyLRV1*$^{+}$, confirming the role of IFN-γR in determining the outcome of the disease. Identification of the mechanism(s) underlying this 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 postkala-azar dermal leishmaniasis, in the case of *Leishmania donovani* infection (3, 54, 55). Here, we suggest that viral coinfection and prior exposure to type I IFNs not only could 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 coinfections could contribute toward metastasis and relapse in human patients suffering from leishmaniasis.

Materials and Methods

For additional information please refer to *SI Text*.

Mice. C57BL/6 WT mice were purchased from Harlan Laboratories; *ifnar*$^{-/-}$ mice were obtained from M. Aguet, Swiss Institute of Experimental Cancer Research, Epalinges, Switzerland; IFN-γ$^{-/-}$ mice were purchased from The Jackson Laboratory. 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.Z. and M.G.C., respectively.

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

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 (Grants 310030-153204 and IZRJZ3_164176), the Institute for Arthritis Research, the Pierre Mercier Foundation, and the NIH (Grants R56AI099364 and R01AI029646).

1. Alvar J, et al.; WHO Leishmaniasis Control Team (2012) Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7:e35671.
2. Kaye P, Scott P (2011) Leishmaniasis: Complexity at the host-pathogen interface. *Nat Rev Microbiol* 9:604–615.
3. Bourreau E, et al. (2016) Presence of Leishmania RNA virus 1 in Leishmania guyanensis increases the risk of first-line treatment failure and symptomatic relapse. *J Infect Dis* 213: 105–111.
4. Adaui V, et al. (2015) Association of the endobiont double-stranded RNA virus LRV1 with treatment failure for human leishmaniasis caused by Leishmania braziliensis in Peru and Bolivia. *J Infect Dis* 213:112–121.
5. Ives A, et al. (2011) Leishmania RNA virus controls the severity of mucocutaneous leishmaniasis. *Science* 331:775–778.
6. Zangger H, et al. (2014) Leishmania aethiopica field isolates bearing an endosymbiontic dsRNA virus induce pro-inflammatory cytokine response. *PLoS Negl Trop Dis* 8:e2836.
7. Tarr PI, et al. (1988) LR1: A candidate RNA virus of Leishmania. *Proc Natl Acad Sci USA* 85:9572–9575.
8. Stuart KD, Weeks R, Guilbride L, Myler PJ (1992) Molecular organization of Leishmania RNA virus 1. *Proc Natl Acad Sci USA* 89:8596–8600.
9. Hartley MA, Drexler S, Ronet C, Beverley SM, Fasel N (2014) The immunological, environmental, and phylogenetic perpetrators of metastatic leishmaniasis. *Trends Parasitol* 30:412–422.
10. Salinas G, Zamora M, Stuart K, Saravia N (1996) Leishmania RNA viruses in Leishmania of the Viannia subgenus. *Am J Trop Med Hyg* 54:425–429.
11. Saiz M, et al. (1998) Short report: Detection of Leishmaniavirus in human biopsy samples of leishmaniasis from Peru. *Am J Trop Med Hyg* 58:192–194.
12. Scheffter SM, Ro YT, Chung IK, Patterson JL (1995) The complete sequence of Leishmania RNA virus LRV2-1, a virus of an Old World parasite strain. *Virology* 212:84–90.
13. Pereira LdeO, et al. (2013) Severity of tegumentary leishmaniasis is not exclusively associated with Leishmania RNA virus 1 infection in Brazil. *Mem Inst Oswaldo Cruz* 108: 665–667.
14. Castellucci LC, et al. (2014) Host genetic factors in American cutaneous leishmaniasis: A critical appraisal of studies conducted in an endemic area of Brazil. *Mem Inst Oswaldo Cruz* 109:279–288.
15. Schriefer A, Wilson ME, Carvalho EM (2008) Recent developments leading toward a paradigm switch in the diagnostic and therapeutic approach to human leishmaniasis. *Curr Opin Infect Dis* 21:483–488.
16. Parmentier L, et al. (2016) Severe cutaneous leishmaniasis in a human immunodeficiency virus patient coinfected with Leishmania braziliensis and its endosymbiotic virus. *Am J Trop Med Hyg* 94:840–843.
17. Hartley MA, et al. (2016) Leishmaniavirus-dependent metastatic leishmaniasis is prevented by blocking IL-17A. *PLoS Pathog* 12:e1005852.
18. Eren RO, et al. (2016) Mammalian innate immune response to a leishmania-resident RNA virus increases macrophage survival to promote parasite persistence. *Cell Host Microbe* 20:318–328.
19. Brettmann EA, et al. (2016) Tilting the balance between RNA interference and replication eradicates Leishmania RNA virus 1 and mitigates the inflammatory response. *Proc Natl Acad Sci USA* 113:11998–12005.
20. Kuhlmann FM, et al. (2017) Antiviral screening identifies adenosine analogs targeting the endogenous dsRNA Leishmania RNA virus 1 (LRV1) pathogenicity factor. *Proc Natl Acad Sci USA* 114:E811–E819.
21. Cooksley WG (2004) The role of interferon therapy in hepatitis B. *MedGenMed* 6:16.
22. Shepherd J, Waugh N, Hewitson P (2000) Combination therapy (interferon alfa and ribavirin) in the treatment of chronic hepatitis C: A rapid and systematic review. *Health Technol Assess* 4:1–67.
23. Wilhelmus KR (2010) Antiviral treatment and other therapeutic interventions for herpes simplex virus epithelial keratitis. *Cochrane Database Syst Rev* (12):CD002898.
24. Rayamajhi M, Humann J, Penheiter K, Andreasen K, Lenz LL (2010) Induction of IFN-alphabeta enables Listeria monocytogenes to suppress macrophage activation by IFN-gamma. *J Exp Med* 207:327–337.
25. Miller JL, Sack BK, Baldwin M, Vaughan AM, Kappe SH (2014) Interferon-mediated innate immune responses against malaria parasite liver stages. *Cell Reports* 7:436–447.
26. Abou-Bacar A, et al. (2004) Role of gamma interferon and T cells in congenital Toxoplasma transmission. *Parasite Immunol* 26:315–318.
27. Chessler AD, Caradonna KL, Da'dara A, Burleigh BA (2011) Type I interferons increase host susceptibility to Trypanosoma cruzi infection. *Infect Immun* 79:2112–2119.
28. Stifter SA, Feng CG (2015) Interfering with immunity: Detrimental role of type I IFNs during infection. *J Immunol* 194:2455–2465.
29. Beiting DP (2014) Protozoan parasites and type I interferons: A cold case reopened. *Trends Parasitol* 30:491–498.
30. Mattner J, et al. (2004) Protection against progressive leishmaniasis by IFN-beta. *J Immunol* 172:7574–7582.
31. Diefenbach A, et al. (1998) Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* 8: 77–87.
32. Favila MA, et al. (2014) Human dendritic cells exhibit a pronounced type I IFN signature following Leishmania major infection that is required for IL-12 induction. *J Immunol* 192:5863–5872.
33. Khouri R, et al. (2009) IFN-beta impairs superoxide-dependent parasite killing in human macrophages: Evidence for a deleterious role of SOD1 in cutaneous leishmaniasis. *J Immunol* 182:2525–2531.
34. Pereira RM, et al. (2010) Novel role for the double-stranded RNA-activated protein kinase PKR: Modulation of macrophage infection by the protozoan parasite Leishmania. *FASEB J* 24:617–626.
35. Xin L, et al. (2010) Type I IFN receptor regulates neutrophil functions and innate immunity to Leishmania parasites. *J Immunol* 184:7047–7056.
36. van Griensven J, Zijlstra EE, Hailu A (2014) Visceral leishmaniasis and HIV coinfection: Time for concerted action. *PLoS Negl Trop Dis* 8:e3023.
37. Teijaro JR, et al. (2013) Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* 340:207–211.
38. von Herrath M, Whitton JL (2001) Animal models using lymphocytic choriomeningitis virus. *Curr Protoc Immunol*, 10.1002/0471142735.im1910s36.
39. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77:4911–4927.
40. Crosby EJ, Clark M, Novais FO, Wherry EJ, Scott P (2015) Lymphocytic choriomeningitis virus expands a population of NKG2D+CD8+ T cells that exacerbates disease in mice coinfected with Leishmania major. *J Immunol* 195:3301–3310.
41. Müller U, et al. (1994) Functional role of type I and type II interferons in antiviral defense. *Science* 264:1918–1921.
42. Cusi MG, Savellini GG, Zanelli G (2010) Toscana virus epidemiology: From Italy to beyond. *Open Virol J* 4:22–28.
43. Decker T, Stockinger S, Karaghiosoff M, Müller M, Kovarik P (2002) IFNs and STATs in innate immunity to microorganisms. *J Clin Invest* 109:1271–1277.
44. Scott P (1991) IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J Immunol* 147:3149–3155.
45. Netto EM, et al. (1990) Long-term follow-up of patients with Leishmania (Viannia) braziliensis infection and treated with glucantime. *Trans R Soc Trop Med Hyg* 84:367–370.
46. Crosby EJ, Goldschmidt MH, Wherry EJ, Scott P (2014) Engagement of NKG2D on bystander memory CD8 T cells promotes increased immunopathology following Leishmania major infection. *PLoS Pathog* 10:e1003970.
47. Cantanhêde LM, et al. (2015) Further evidence of an association between the presence of Leishmania RNA virus 1 and the mucosal manifestations in tegumentary leishmaniasis patients. *PLoS Negl Trop Dis* 9:e0004079.
48. Adaui V, et al. (2016) Association of the endobiont double-stranded RNA virus LRV1 with treatment failure for human leishmaniasis caused by Leishmania braziliensis in Peru and Bolivia. *J Infect Dis* 213:112–121.
49. Tsai TF, Halstead SB (1998) Tropical viral infections. *Curr Opin Infect Dis* 11:547–553.
50. Es-Sette N, et al. (2014) Phlebotomus sergenti a common vector of Leishmania tropica and Toscana virus in Morocco. *J Vector Borne Dis* 51:86–90.
51. Faucher B, et al. (2014) Presence of sandflies infected with Leishmania infantum and Massilia virus in the Marseille urban area. *Clin Microbiol Infect* 20:O340–O343.
52. Dincer E, Gargari S, Ozkul A, Ergunay K (2015) Potential animal reservoirs of Toscana virus and coinfections with Leishmania infantum in Turkey. *Am J Trop Med Hyg* 92: 690–697.
53. Bichaud L, et al. (2011) Epidemiologic relationship between Toscana virus infection and Leishmania infantum due to common exposure to Phlebotomus perniciosus sandfly vector. *PLoS Negl Trop Dis* 5:e1328.
54. Passos VM, et al. (2000) American cutaneous leishmaniasis: Use of a skin test as a predictor of relapse after treatment. *Bull World Health Organ* 78:968–974.
55. Zijlstra EE, Musa AM, Khalil EA, el-Hassan IM, el-Hassan AM (2003) Post-kala-azar dermal leishmaniasis. *Lancet Infect Dis* 3:87–98.
56. Battegay M, et al. (1991) Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. *J Virol Methods* 33:191–198.
57. Gori Savellini G, et al. (2011) Toscana virus induces interferon although its NSs protein reveals antagonistic activity. *J Gen Virol* 92:71–79.