

# Type I IFN controls chikungunya virus via its action on nonhematopoietic cells

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**Chikungunya virus (CHIKV) is the causative agent of an outbreak that began in La Réunion in 2005 and remains a major public health concern in India, Southeast Asia, and southern Europe. CHIKV is transmitted to humans by mosquitoes and the associated disease is characterized by fever, myalgia, arthralgia, and rash. As viral load in infected patients declines before the appearance of neutralizing antibodies, we studied the role of type I interferon (IFN) in CHIKV pathogenesis. Based on human studies and mouse experimentation, we show that CHIKV does not directly stimulate type I IFN production in immune cells. Instead, infected nonhematopoietic cells sense viral RNA in a Cardif-dependent manner and participate in the control of infection through their production of type I IFNs. Although the Cardif signaling pathway contributes to the immune response, we also find evidence for a MyD88-dependent sensor that is critical for preventing viral dissemination. Moreover, we demonstrate that IFN- $\alpha/\beta$  receptor (IFNAR) expression is required in the periphery but not on immune cells, as IFNAR<sup>-/-</sup>→WT bone marrow chimeras are capable of clearing the infection, whereas WT→IFNAR<sup>-/-</sup> chimeras succumb. This study defines an essential role for type I IFN, produced via cooperation between multiple host sensors and acting directly on nonhematopoietic cells, in the control of CHIKV.**

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Abbreviations used: cDC, conventional DC; cDNA, complementary DNA; CHIKV, chikungunya virus; dsRNA, double-stranded RNA; ID, intradermally; IFNAR, IFN- $\alpha/\beta$  receptor; ISG, IFN-stimulated gene; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection; mRNA, messenger RNA; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid DC; PRR, pattern-recognition receptor; RLR, RIG-I-like receptor; SFV, Semliki Forest virus; SMA, smooth muscle actin; ssRNA, single-stranded RNA; TCID<sub>50</sub>, tissue culture infectious dose 50; TLR, toll-like receptor.

La Réunion, an island in the Indian Ocean with a population of ~785,000, experienced an outbreak of chikungunya fever, an arboviral disease transmitted by mosquitoes. In 2005–2006, there were an estimated 300,000 cumulated cases (Simon et al., 2006; Schuffenecker et al., 2006; Gérardin et al., 2008). The epidemic involved India, where estimates approach six million infected people, it emerged in Italy (Mavalankar et al., 2007; Watson, 2007), and ongoing infections exist in South-

east Asia (Ng et al., 2009). The disease was first recognized during an epidemic in East Africa in 1952–1953. The etiologic agent chikungunya virus (CHIKV) is a member of the *Togaviridae* family, genus *Alphavirus* (Johnston and Peters, 1996), which are enveloped, single-stranded positive polarity RNA viruses. In humans, CHIKV typically induces symptoms 2–7 d after infection that are characterized by a rapid onset

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of fever (peaking at 39–40°C) and severe arthralgia and myalgia, which is followed by constitutional symptoms (headache, photophobia, nausea, and abdominal pain) and a rash (Bodenmann and Genton, 2006; Borgherini et al., 2007). It has been reported that viremia peaks at day 2 after the initiation of symptoms, declines sharply during days 3 and 4, and is undetectable by day 5 (Carey et al., 1969). Based on the sharp decline in viremia before the evolution of high-affinity neutralizing antibodies (Carey et al., 1969), we hypothesized that type I IFNs mediate this rapid antiviral response.

IFN was discovered by Alick Isaacs and Jean Lindemann in 1957 as an undefined substance with antiviral activity. Work within the last decades has defined this antiviral substance as type I IFN (IFN- $\alpha/\beta$ ), distinguishing it from type II IFN (IFN- $\gamma$ ) and the more recently described type III IFN (IFN- $\lambda$ ; Sheppard et al., 2003). Leukocytes are the primary producers of IFN- $\alpha$  and fibroblasts are the primary producers of IFN- $\beta$ . Various IFN- $\alpha$  subtypes exist (at least 13; van Pesch et al., 2004) but only one IFN- $\beta$  subtype exists; however, all use a single IFN- $\alpha/\beta$  receptor (IFNAR) and the functional significance of these multiple subtypes is not well understood. Interestingly, in the 1960s and 1970s, CHIKV was used to stimulate IFN production from chick embryo fibroblast-like cells (Friedman, 1964; Wagner, 1964). These were some of the last notable scientific articles that evaluated CHIKV in the context of the IFN pathway before the current epidemic.

The host immune response, and in particular the production of IFN, is triggered by the engagement of receptors that are termed pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) and RNA helicases (referred to as RIG-I-like receptors [RLRs]) represent two classes of PRRs in mammals. Both types of PRRs have an essential role in the initiation of innate immunity by sensing invading pathogens through the recognition of conserved molecular motifs, termed pathogen-associated molecular patterns (PAMPs), which include structures such as surface glycoproteins, single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA), and unmethylated CpG DNA. TLRs constitute a family of 11 members of transmembrane proteins, six of which have been implicated thus far in antiviral immunity (TLR-2, -3, -4, -7, -8, and -9). Viral surface glycoproteins (e.g., the hemagglutinin protein of measles virus) have been reported as agonists for TLR2 and TLR4 (Kurt-Jones et al., 2000; Bieback et al., 2002; Rassa et al., 2002; Compton et al., 2003). ssRNA viruses (e.g., influenza) trigger TLR7 and TLR8 signaling (Diebold et al., 2004), whereas extracellular dsRNA is recognized by TLR3 (Alexopoulou et al., 2001), and unmethylated CpG-containing DNA viruses (e.g., herpes simplex virus) may activate TLR9 (Krug et al., 2004). In this paper, we will refer to these receptors as extracellular sensors, as the agonist binding domains are topologically oriented toward the extracellular space or lumen of the endosome. The family of intracellular sensors includes the RNA helicases Mda5 (melanoma differentiation-associated gene 5) and RIG-I (retinoic acid-inducible gene I), with both of them signaling through Cardif (also known as IPS-1, Visa,

and MAVS; Kawai and Akira, 2006). As CHIKV is an ssRNA virus and likely replicates with a dsRNA intermediary, we predicted engagement of TLR7 and/or TLR3. To our surprise, our results indicate that CHIKV does not directly activate hematopoietic cells expressing these receptors. Instead, direct infection and engagement of a Cardif-dependant pathway seems critical for initiating the host response to CHIKV. In addition, we find evidence for a second sensor pathway mediated by MyD88 that plays an important role in controlling viral dissemination.

Regarding the role of type I IFNs in disease pathogenesis and control of viral infection, there have been several viruses for which IFNAR expression has been shown to be essential. These include DNA (e.g.,  $\gamma$ HV68 and MCMV), dsRNA (e.g., reovirus), positive ssRNA (e.g., Semliki Forest virus [SFV]), and negative ssRNA (e.g., VSV) viruses (Müller et al., 1994; Aricò et al., 2004; Johansson et al., 2007). With that said, many viruses are also known to be controlled, even in the absence of IFNAR signaling, including VV and LCMV (Müller et al., 1994). Nonetheless, in situations in which infection is not lethal, type I IFNs play a role in coordinating an antiviral immune response and IFNAR<sup>-/-</sup> mice have more severe infection with greater histopathology (Müller et al., 1994; Havenar-Daughton et al., 2006). Although there are many gaps in our knowledge, it is important to note that in some cases, type I IFNs are acting primarily via IFN-stimulated genes (ISGs) with direct antiviral activity, whereas for other infections their dominant role is to stimulate humoral and cellular immunity. Our studies demonstrate that CHIKV is critically dependent on type I IFN action on nonhematopoietic cells and, thus, acts as a direct antiviral, likely through the induction of one or more ISGs. This study provides the first detailed information concerning the role of IFN- $\alpha/\beta$  in reemergent CHIKV pathogenesis.

## RESULTS

### CHIKV infection results in high levels of type I IFNs but fails to directly trigger its production in hematopoietic cells

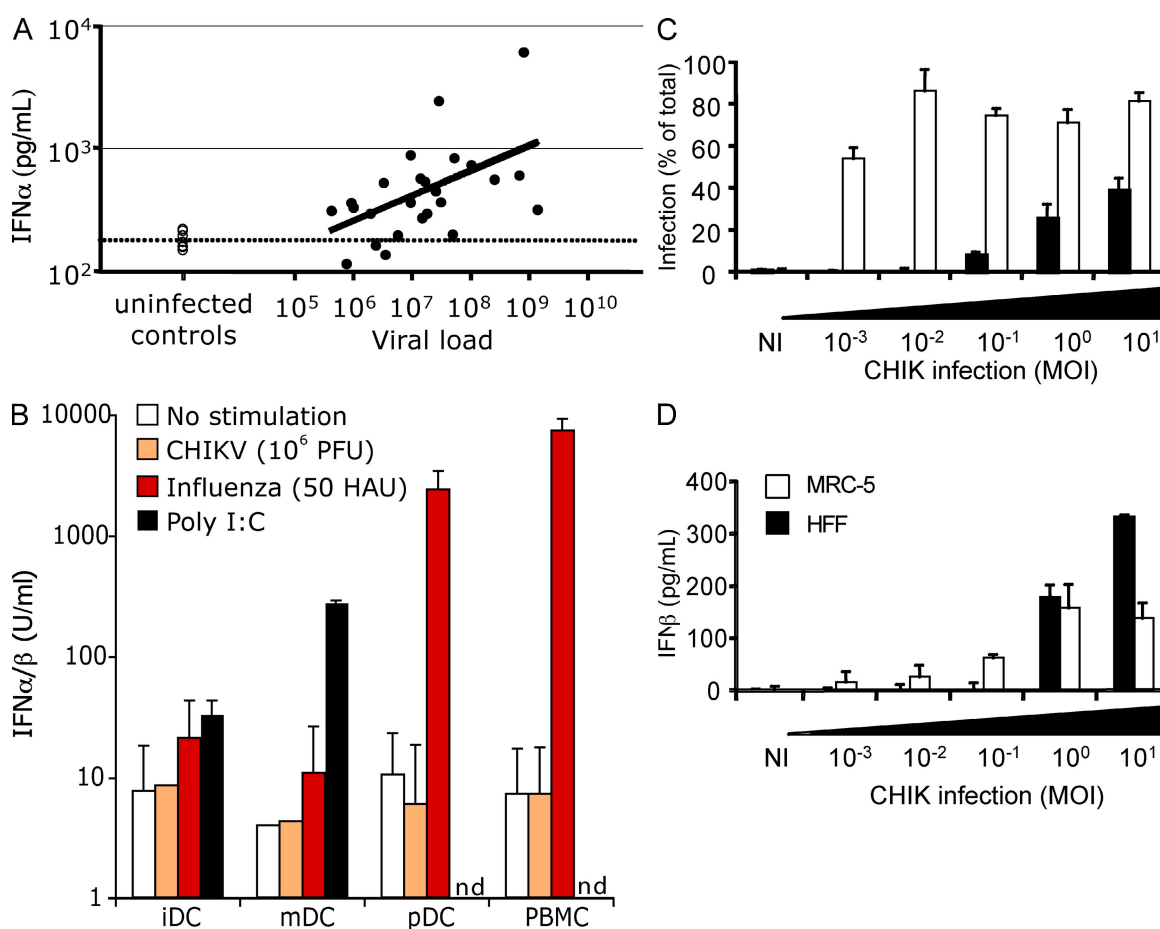
To establish a role for the induction of type I IFNs by CHIKV infection, we analyzed serum from a patient cohort recruited during the 2005–2006 outbreak in La Réunion. Indeed, IFN- $\alpha$  was detected at high levels in the serum of infected individuals with its concentration correlating with viral load (Fig. 1 A). It is of note that plasma concentrations of IFN- $\gamma$  in infected individuals were not statistically different from controls (unpublished data). We thus investigated the mechanisms, accounting for the production of type I IFNs during infection. Primary isolates of CHIKV were obtained and their genome sequence was determined (Schuffenecker et al., 2006). A representative isolate, CHIKV-21, is presented in this study. To avoid laboratory adaptation, viruses were amplified only twice in C6/36 mosquito cells and titrated by plaque assay on BHK cells.

Human plasmacytoid DCs (pDCs) are a robust source of type I IFNs (Siegal et al., 1999). Additionally, it has been shown that conventional DCs (cDCs) may express high levels

of IFN- $\alpha/\beta$  (Diebold et al., 2003). To test CHIKV ability to directly trigger type I IFNs production in these cell types, we generated immature and mature monocyte-derived human cDCs, and we isolated pDCs from PBMCs based on their expression of BDCA-4. In addition, we tested unfractionated PBMCs to evaluate other cell populations (e.g., monocytes). Cells were exposed to  $10^6$  PFU CHIKV (range  $10^5$ – $10^7$  multiplicity of infection [MOI]) for 24 or 40 h, and culture supernatants were evaluated for the presence of type I IFNs. To our surprise, we could not detect IFN- $\alpha$  (9/15 subtypes tested) or IFN- $\beta$  (unpublished data). These data were confirmed functionally using a reporter assay sensitive to IFNAR signaling (Fig. 1 B). As a positive control, influenza A/PR8,

a known agonist of TLR7 in pDCs, or poly I:C, a known agonist of TLR3 in cDCs, was added to the cell cultures. Together, these results suggest that although IFNs are produced during CHIKV disease pathogenesis, hematopoietic cells are not directly stimulated by CHIKV to produce IFN- $\alpha/\beta$ . Although these observations could be explained as being a result of the failure of these cells to get productively infected (Sourisseau et al., 2007), it should be noted that pDCs are capable of producing IFNs in the absence of direct infection (Dalod et al., 2003).

Considering the requirement for direct infection, we next evaluated human fibroblasts, which are known targets of CHIKV infection (Sourisseau et al., 2007; Couderc et al., 2008),



**Figure 1. CHIKV induces IFN- $\alpha/\beta$  in vivo in humans but fails to stimulate production in hematopoietic cells.** (A) Patient sera samples were obtained from consenting CHIKV-infected adults (age = 16–86 yr;  $n = 25$ ) and age-matched controls (age range = 16–89 yr;  $n = 17$ ). IFN- $\alpha$  was monitored by luminex and plotted as a function of viral load, measured by quantitative PCR. Mean IFN- $\alpha$  serum concentration in the control population was 179 pg/ml, indicated by the dotted line. The solid line represents the linear regression between viral load and IFN- $\alpha$  in patients. (B) Monocyte-derived DCs were generated from healthy donors, and  $10^5$  immature DCs (iDCs) or mature DCs (mDCs) were exposed to CHIKV. In parallel,  $3 \times 10^4$  freshly isolated pDCs or  $10^6$  PBMCs were cultured with CHIKV. In the experiment shown,  $10^6$  PFU/ml was used and cells were incubated for 40 h. Culture supernatants were analyzed for IFN- $\alpha/\beta$  production by a reporter assay involving an IFNAR-expressing cell line, HL-116, stably transfected with a plasmid encoding an IFN-inducible luciferase gene (limit of detection, 5 IFN U/ml). 50 HAU influenza A/PR8 or 50  $\mu$ g/ml poly I:C were used as a positive control. Results were identical for three different donors. Similar results were found in monocyte-derived macrophages (not depicted). (C and D) Human foreskin fibroblasts (black) or MRC-5 cell lines (white) were infected at increasing MOI and cultured for 48 h. Cells were harvested and intracellular staining was performed using an anti-CHIKV mAb and analyzed by FACS (C). In parallel, culture supernatant was analyzed for IFN- $\beta$  by Elisa (limit of detection, 20 pg/ml; D). Error bars indicate SD. Data is representative of five experiments.

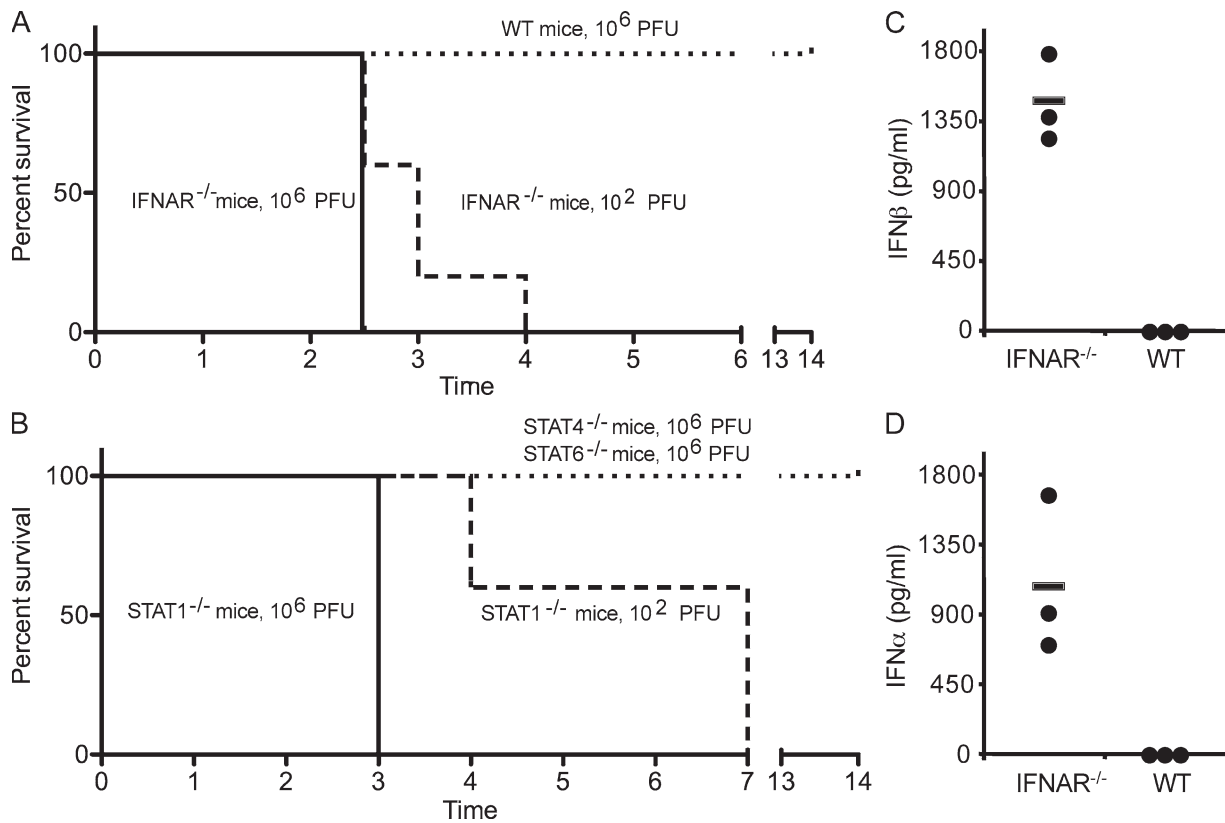
for their ability to produce IFN- $\alpha/\beta$ . Primary human foreskin fibroblasts and MRC-5 were both susceptible to infection at low MOI (Fig. 1 C), which correlated with the induction of high levels of IFN- $\beta$  (Fig. 1 D). Similar results were obtained for mouse hematopoietic cells, which could not be directly infected and did not produce type I IFNs, whereas mouse embryonic fibroblasts (MEFs) were sensitive to infection and produced robust amounts of IFN- $\beta$  (not depicted).

### Type I IFNs acting via a STAT1 signaling pathway are critical for controlling CHIKV

To better define the role of IFN in the control of CHIKV and to identify the viral sensor responsible for its production, we developed a mouse model for CHIKV infection. Using IFNAR $^{-/-}$  mice on the C57BL/6 strain, it was possible to demonstrate a critical role for IFNAR signaling in the control of CHIKV. Remarkably,  $10^2$  PFU injected intradermally (ID) was sufficient to kill the IFNAR $^{-/-}$  mice between days 2.5 and 4 after infection (Fig. 2 A). Injection of  $10^6$  PFU resulted in even faster death with all animals succumbing to infection between days 2 and 3. Similar to what was observed in highly viremic humans (Fig. 1 A; Laurent et al., 2007), the viral load

in IFNAR $^{-/-}$  mice infected with  $10^6$  PFU at 2 d after infection was  $>10^8$  tissue culture infectious dose 50 (TCID $_{50}$ )/ml (not depicted). In contrast, WT animals cleared the infection with undetectable serum viral titers at all time points tested. These data are similar to those reported for IFNAR $^{-/-}$  mice on the 129 strain (Couderc et al., 2008).

Next, we used STAT1 $^{-/-}$ , STAT4 $^{-/-}$ , and STAT6 $^{-/-}$  mice, establishing that STAT1 is critical for the clearance of CHIKV (Fig. 2 B). These data suggest that the canonical STAT1/2 heterodimer is responsible for transducing the signal from the IFNAR to control CHIKV infection. In contrast to the critical role for type I IFNs, mice deficient for IFN- $\gamma$  do not develop viremia at day 2 and successfully cleared CHIKV infection (unpublished data). Although these data suggest a critical role for type I IFNs, no IFN- $\alpha/\beta$  protein could be detected in the serum of WT animals across a time course of 3, 6, 9, 24, 48, 72, and 96 h after infection. In stark contrast, we observed high levels of IFN- $\beta$  as well as IFN- $\alpha$  in both the IFNAR $^{-/-}$  and STAT1 $^{-/-}$  animals (Fig. 2, C and D; and not depicted). These results are likely a reflection of the high viral load in animals that are unable to signal via the IFNAR, as opposed to the rapid clearance of CHIKV in WT animals.

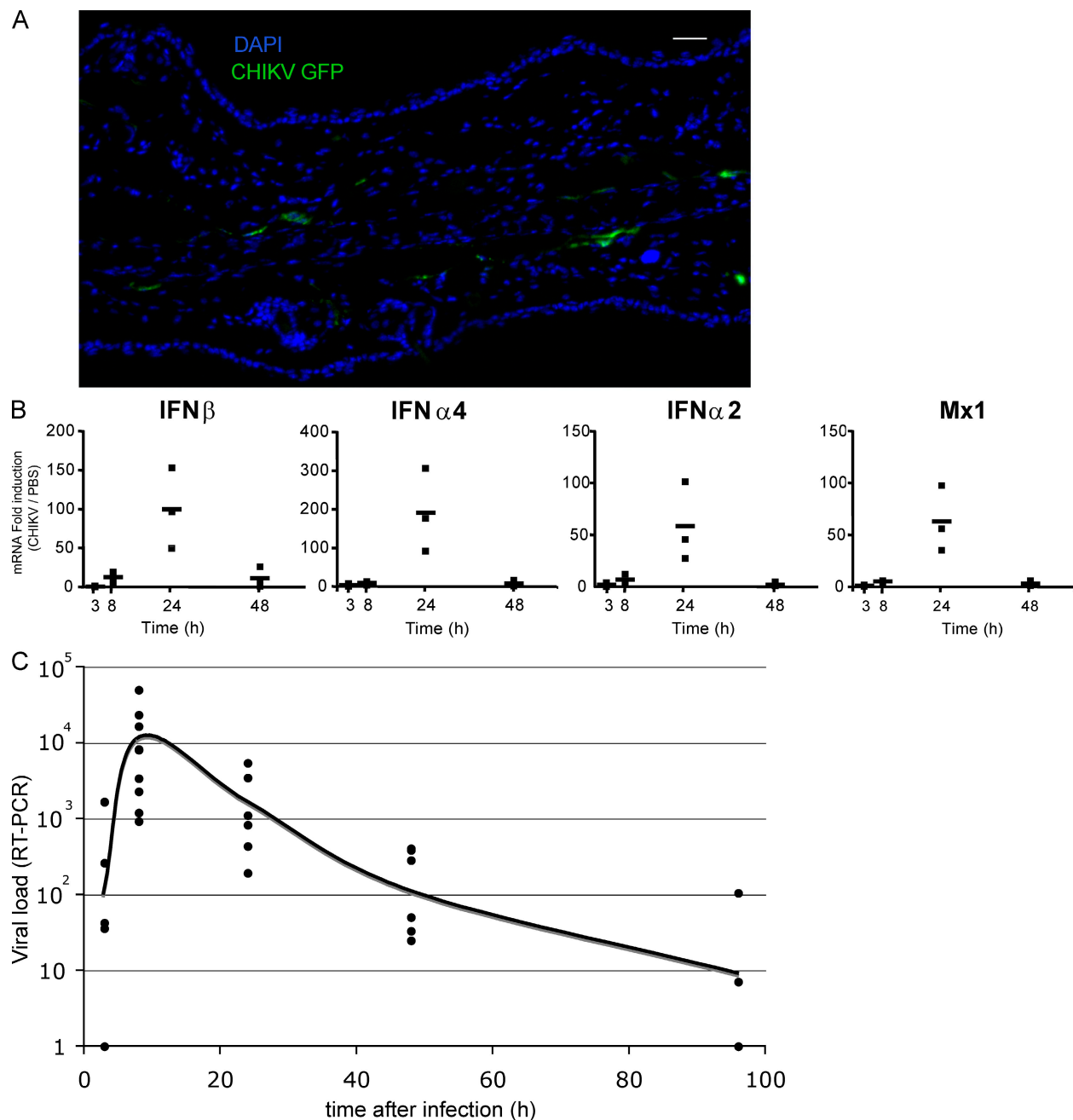


**Figure 2. CHIKV-infected IFNAR $^{-/-}$  mice produce high levels of type I IFNs.** (A) C57BL/6 IFNAR $^{-/-}$  mice were infected ID with  $10^2$  PFU or  $10^6$  PFU CHIKV. Survival was assessed twice a day and Kaplan-Meier survival curves were drawn ( $n = 5$ ). (B) STAT1 $^{-/-}$ , STAT4 $^{-/-}$ , and STAT6 $^{-/-}$  ( $n = 5$ ) mice, along with corresponding WT mice of a similar strain background, were infected ID with  $10^6$  PFU CHIKV. Survival was assessed twice a day and Kaplan-Meier survival curves were drawn. (C and D) WT and IFNAR $^{-/-}$  mice were infected with  $10^6$  PFU CHIKV and serum concentrations of IFN- $\beta$  and IFN- $\alpha$  were monitored at day 2 after infection. Each dot represents an individual mouse, horizontal bars represent the mean. Data shown is combined from two independent experiments.

### Local production of type I IFNs prevents viral dissemination in WT mice

To gain additional insight into how WT mice control CHIKV dissemination, we investigated the site of injection. Recombinant CHIKV expressing GFP was injected

into the ear and, 24 h later, the injection site was dissected and analyzed by immunohistofluorescence. We observed GFP-positive cells in the injected skin lining sebaceous glands, vessels, and the cartilage (Fig. 3 A). As GFP is not incorporated into the virion in the construct we used



**Figure 3. Local production of type I IFNs control viral dissemination in WT animals.** (A) WT mice were injected with GFP-expressing CHIKV. Immunofluorescence was performed at day 1 after infection on skin at the site of injection (ear). Nuclei were stained with DAPI (blue) and replicating virus was detected using anti-GFP mAbs (green). Bar, 50  $\mu$ m. (B) mRNA was isolated from the infection site at 3, 8, 24, and 48 h after infection. RT was performed and IFN- $\beta$ , IFN- $\alpha_2$ , IFN- $\alpha_4$ , and Mx-1 mRNA expression was determined by quantitative PCR. Individual mice are represented by dots and horizontal bars represent the mean. The experiment presented is representative of three independent experiments. (C) As in B, total RNA was isolated from infected skin at various times and viral load was assessed. Data from three independent experiments are plotted to indicate viral RNA over time. Individual mice are represented.



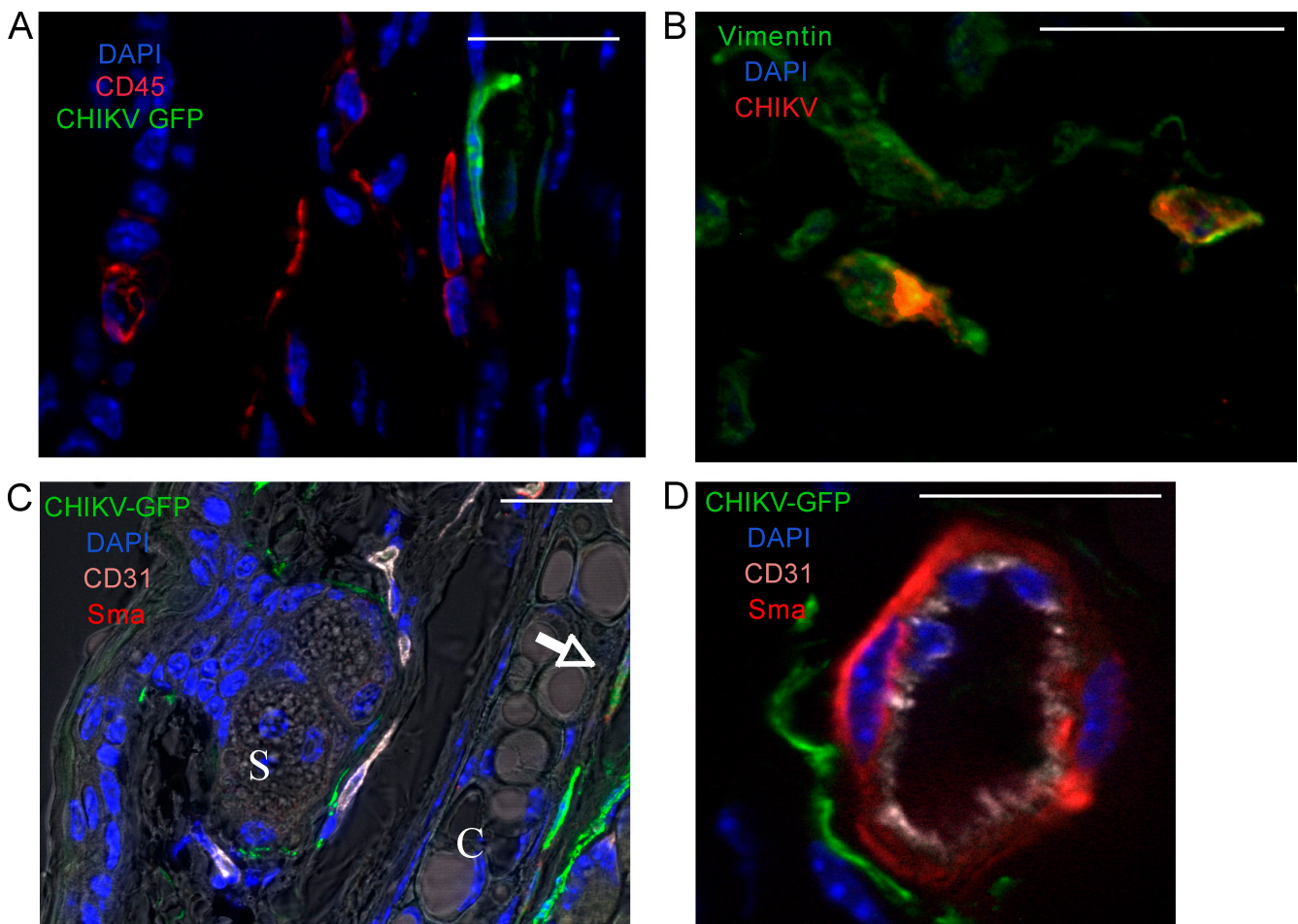
(Vanlandingham et al., 2005), GFP detection provides direct evidence of viral replication.

To determine if the local infection triggered a local immune response, we performed quantitative RT-PCR analysis of the messenger RNA (mRNA) extracted from the injections site. Indeed, we observed a burst of IFN- $\beta$  and IFN- $\alpha_4$  production that peaked at 24 h after infection (Fig. 3 B). In addition, we observed the induction of IFN- $\alpha_2$  and the IFN response gene Mx1, suggesting that the IFN priming loop is intact in CHIKV-infected WT mice (Fig. 3, D and E). Interestingly, the peak of IFN correlated with the decline in CHIKV load (Fig. 3 C). Collectively with the observation that WT mice do not have disseminated infection, we conclude that local IFN is capable of controlling CHIKV.

#### Fibroblasts are the dominant cell type infected with control of CHIKV mediated by a Cardif-dependant pathway

To more precisely define the cell type infected in the WT mice, we first evaluated infection of hematopoietic cells. We

performed double labeling with CD45 and CHIKV-GFP. Screening >25 sections from multiple experiments, we did not observe any GFP-positive CD45-expressing cells. A representative image is presented here (Fig. 4 A). To further characterize the nonhematopoietic cells, we labeled sections with anti-vimentin antibodies, which is a marker for fibroblasts and mesenchymal cells, anti-CD31 antibodies, which is a marker for endothelial cells, and anti-smooth muscle actin (SMA), which is a marker for vascular smooth muscle cell, pericytes, and myofibroblasts. We identified vimentin-positive CHIKV-infected cells (Fig. 4 B) and, in some instances, colocalization in SMA<sup>int</sup> cells, which is suggestive of myofibroblasts (Fig. 4 C, arrow). In most cases, GFP-positive cells were CD31<sup>-</sup> and SMA<sup>-</sup> (Fig. 4, B and C). These data suggest that like the IFNAR<sup>-/-</sup> animals (Couderc et al., 2008), the dominant cell type infected by CHIKV in the skin of WT animals is fibroblasts. These data are compelling, as they constitute the first evidence of a primary viral isolate infecting WT mice using the natural route of infection and



**Figure 4. Skin fibroblasts, but not hematopoietic cells, are infected in WT mice.** Immunofluorescence was performed 1 d after ID infection with CHIKV-GFP (A, C, and D) or with WT CHIKV (B) at the site of inoculation into the ear. Cell nuclei were stained with DAPI and replicating virus was detected using anti-GFP mAbs (A, C, and D) or with anti CHIK antibody (B). CD45 (A), Vimentin (B), or CD31 and SMA (C and D) staining was performed to determine the phenotype of infected cells. S, sebaceous gland; C, cartilage; arrow, infected myofibroblast (SMA<sup>int</sup>). Bars, 20  $\mu$ m. Data are representative of two independent experiments.

are consistent with the observations made in infected human tissue samples (Couderc et al., 2008).

We next considered the host sensor responsible for detecting infection and triggering IFN production. For ssRNA viruses such as CHIKV, which replicate via a putative dsRNA intermediary (Strauss and Strauss, 1994), triggers include TLR3, TLR7, PKR, Mda5, and RIG-I (Stetson and Medzhitov, 2006). To date, the host sensor for members of the family of *Togaviridae* remains unknown. We first considered intracellular sensors and tested infection in MEFs derived from *Cardif*<sup>-/-</sup> animals. *Cardif* (also known as MAVS/VISA/IPS-1) is the critical adaptor downstream of RIG-I and Mda5.

*Cardif*<sup>-/-</sup> MEFs were exposed to increasing MOI of virus and CHIKV antigen expression was monitored. *Cardif*<sup>-/-</sup> MEFs were more sensitive to infection than their WT counterparts, especially at a lower MOI (Fig. 5 A). We next evaluated IFN production and, strikingly, the *Cardif*<sup>-/-</sup> MEFs failed to produce IFN- $\beta$  (Fig. 5 B) This was confirmed at the RNA level with the *Cardif*<sup>-/-</sup> expressing 500-fold less IFN- $\beta$  mRNA as compared with the WT MEFs (Fig. 5 C). Based on these data, we suggest that infected fibroblasts respond to CHIKV infection via a *Cardif*-dependant sensor, producing type I IFNs and limiting infection.

#### Multiple viral sensors are involved in the in vivo control of CHIKV infection

To determine the upstream sensor responsible for engaging *Cardif*, we compared RIG-I- and Mda5-deficient MEFs (Fig. S1) and animals (Fig. 6). Viral titers were measured in the joint, muscle, liver, spleen, skin, and serum of infected animals. Although we observed a higher viral titer in the serum of RIG-I<sup>-/-</sup> mice as compared with WT animals, the results were confounded by the increased sensitivity of outbred WT mice to CHIKV infection (Fig. 6). Notably, viral titers were evident in the joint of WT ICR mice. More striking was the fact that the RIG-I<sup>-/-</sup> and Mda5<sup>-/-</sup> mice had such a subtle phenotype.

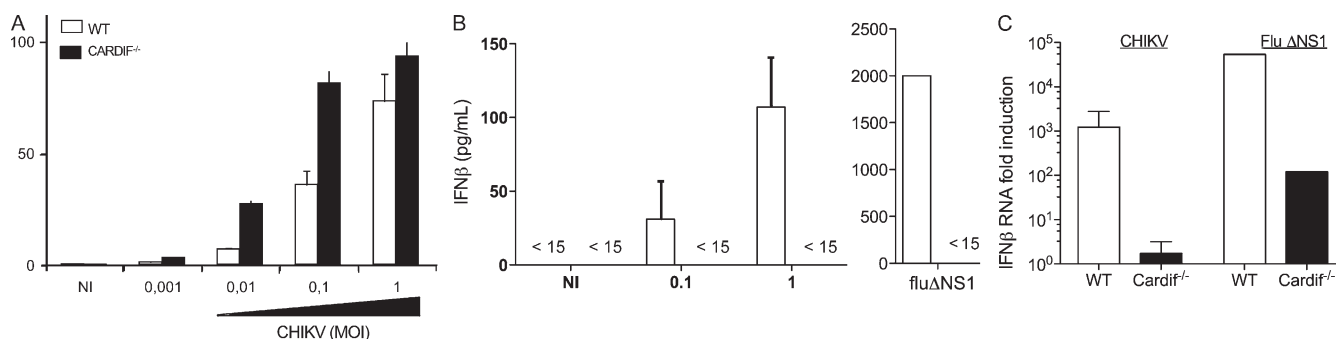
To gain better insight into the in vivo response to CHIKV, we infected *Cardif*<sup>-/-</sup> (C57BL/6 background) animals and

again assessed survival and viral dissemination. Supporting the findings in the RIG-I<sup>-/-</sup> and Mda5<sup>-/-</sup>, none of the *Cardif*<sup>-/-</sup> mice infected with 10<sup>6</sup> PFU of CHIKV died from the infection ( $n = 15$ ). There was, however, a reproducible phenotype. Viral titers in the *Cardif*<sup>-/-</sup> were increased as compared with WT mice at 48 h after infection in the serum (Fig. 7 A), and low but detectable levels of virus were found in the skin, joints, liver, muscle, spleen, and serum at 72 h after infection (Fig. 7 B).

One possibility is that CHIKV is controlled through the sequential actions of two or more sensor pathways. Indeed, there is evidence in SFV that infected cells can trigger TLR-dependant IFN production in phagocytic cells (Schulz et al., 2005). Therefore, we evaluated MyD88<sup>-/-</sup> and TLR3<sup>-/-</sup>. These mice also did not die from CHIKV infection ( $n = 10$ ), but significant viral dissemination could be observed in MyD88<sup>-/-</sup> animals, especially at later time points (Fig. 7 B). Notably, none of the sensor-deficient mice tested were as susceptible to infection as IFNAR<sup>-/-</sup> animals, indicating that host sensor pathways cooperate for the control of CHIKV infection.

#### Type I IFNs act on nonhematopoietic cells for the control of CHIKV infection

Finally, we asked if the type I IFNs induced by viral infection acts directly on infected cells to mediate viral control or if immune cells are the requisite target of the type I IFNs, thus facilitating indirect control of CHIKV infection. To assess these two possibilities, we established bone marrow chimeric mice by lethal irradiation of host CD45.1 WT mice, followed by adoptive transfer of CD45.2 IFNAR<sup>-/-</sup> bone marrow (both mice were on a C57BL/6 background). Mice are referred to as IFNAR $\rightarrow$ WT. Strikingly, the IFNAR $\rightarrow$ WT chimeras were capable of limiting infection, suggesting that host stromal cells alone, receiving IFNAR stimulation, are sufficient for controlling the virus (Fig. 8). Similar results were observed in IFNAR $\rightarrow$ Langerin-DTR mice treated with diphtheria toxin to deplete the radio-resistant Langerhans cells in the skin (unpublished data). Supporting this conclusion, we observe that in the WT $\rightarrow$ IFNAR chimeras, WT



**Figure 5. Cell-intrinsic control of CHIKV in fibroblasts is mediated by intracellular sensors.** (A) *Cardif*<sup>-/-</sup> and the corresponding WT control MEFs were infected with increasing MOI of CHIKV. At 48 h, cells were analyzed by FACS for expression of CHIKV antigens. (B) Culture supernatants were harvested at 48 h and analyzed for IFN- $\beta$  protein. As a positive control, MEFs were exposed to Flu $\Delta$ NS1. (C) IFN- $\beta$  mRNA induction was measured by RT-PCR at 24 h after infection. The mean of three independent experiments is shown. Error bars show SD.

bone marrow was unable to rescue the IFNAR<sup>-/-</sup> mice. These data indicate that IFNAR responsiveness is required in the radio-resistant stromal cells, distinguishing CHIKV from other viruses such as reovirus (Johansson et al., 2007), which also kills IFNAR<sup>-/-</sup> but may be controlled by chimerism with WT BM-derived cells.

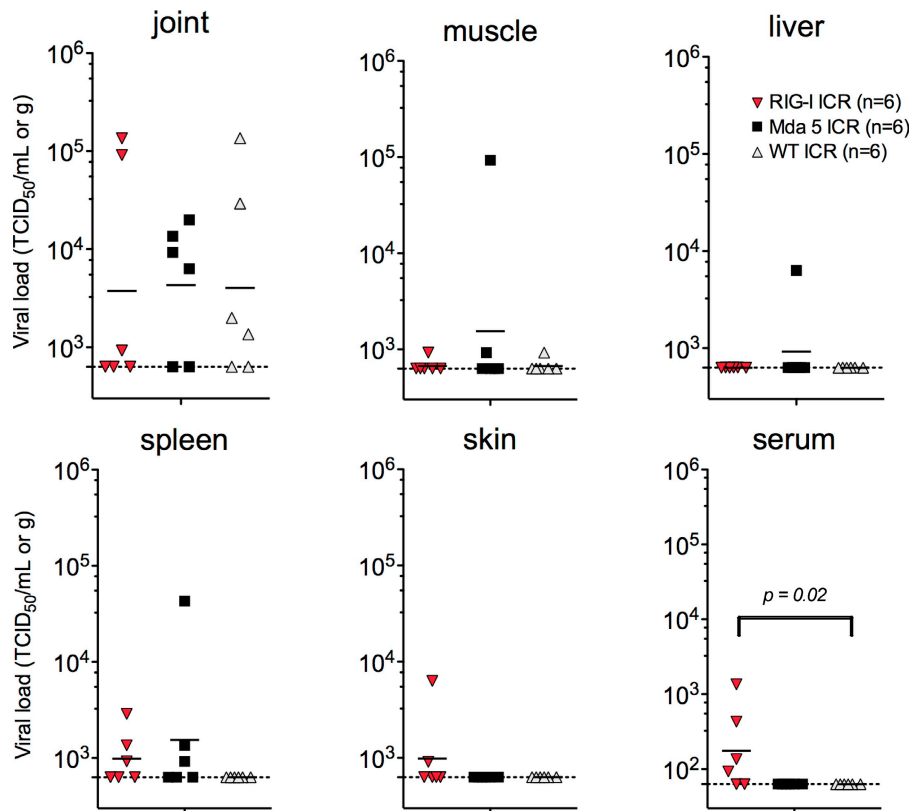
**DISCUSSION**

In this paper, we have characterized the mechanisms of viral control in CHIKV infection and we demonstrate a critical role for type I IFNs. We provide evidence in infected human subjects that CHIKV infection triggers the production of type I IFNs (Fig. 1 A). It has previously been reported that CHIKV does not directly infect primary leukocytes (Sourisseau et al., 2007). Nonetheless, we expected that an ssRNA virus would be capable of directly activating hematopoietic cells. Remarkably, our in vitro stimulation of human PBMC, as well as human and mouse DC subsets, indicates that CHIKV is not capable of directly engaging PRRs for the induction of type I IFNs (Fig. 1 B and data not depicted). Instead, we suggest that infected fibroblasts are the source of type I IFN. This conclusion is based on in vitro experiments and investigation of the injection site of WT animals (Fig. 1 D and Fig. 3). The use of defined knockout mice allowed us to further characterize

the production and target of type I IFNs in CHIKV pathogenesis. Specifically, we report that fibroblast production of type I IFNs is under the control of Cardif (Fig. 5). Furthermore, we demonstrate that IFN acts on nonhematopoietic cells to achieve viral clearance (Fig. 8).

Although these data suggest a central role for fibroblasts in the production and reception of IFN signals, the host response to CHIKV is more complex. Notably, adult Cardif<sup>-/-</sup> mice infected with CHIKV had only a subtle phenotype. In addition to a role for the RLR pathway, we find that mice lacking MyD88 also became viremic (Fig. 7). Alone, neither of the pathways account for the phenotype observed in the IFNAR<sup>-/-</sup> mice, suggesting that there is cooperation between RLR and TLR recognition, acting together to mediate rapid clearance of CHIKV.

Based on the tissue tropism (Fig. 4), we argue that Cardif is engaged as a result of fibroblast and stromal cell infection. We attempted to define the upstream sensor, namely RIG-I or Mda5. As reported for the Cardif<sup>-/-</sup>, neither of the sensor-deficient animals had a strong phenotype, although both demonstrated increased viral load in selected tissues as compared with control animals (Fig. 6). One caveat is that the RIG-I and Mda5 knockouts are of a mixed ICR/C57BL/6/129 background as a result of problems of lethality

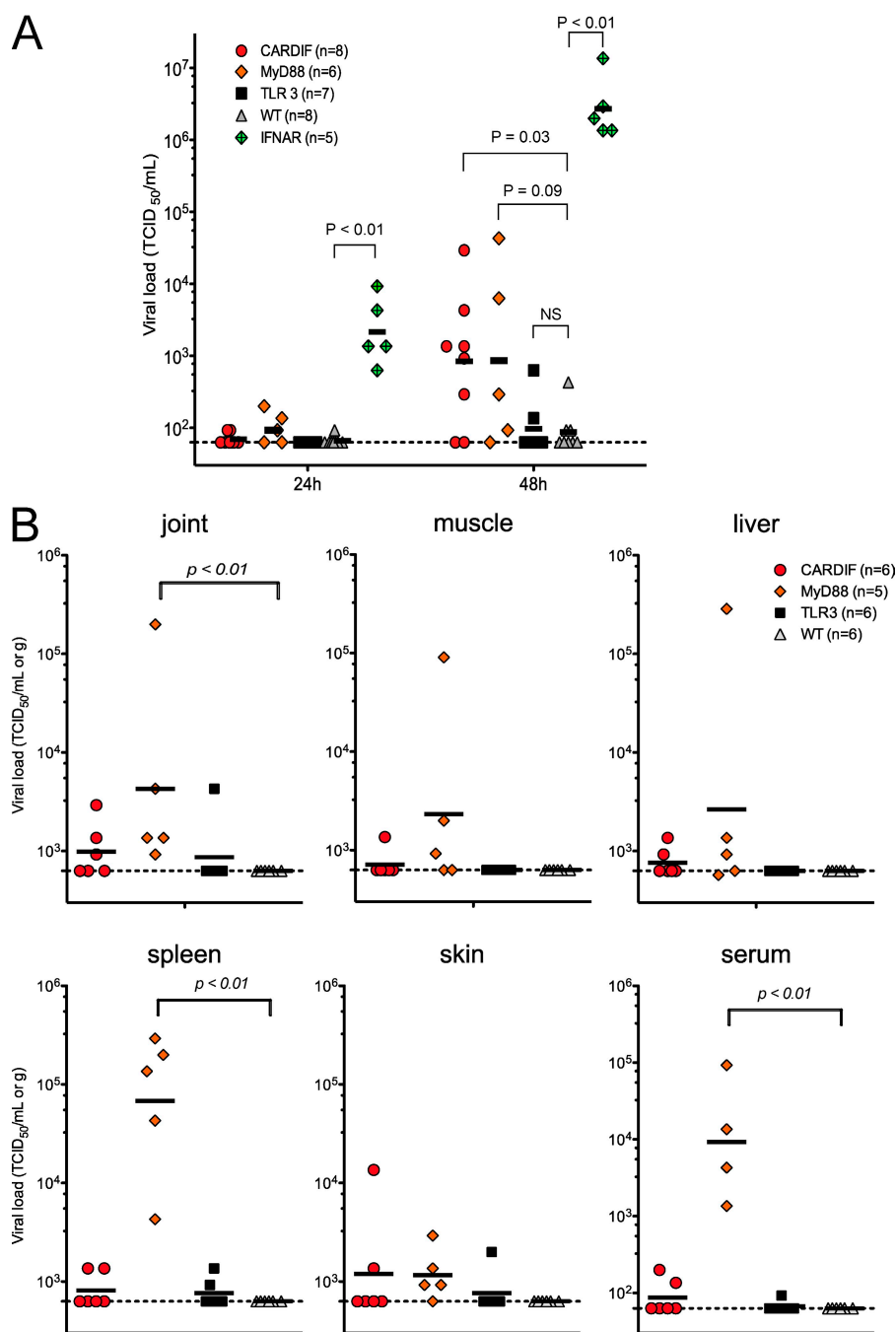


**Figure 6. Redundancy between RIG-I and Mda5 pathway.** Mda5<sup>-/-</sup>, RIG-I<sup>-/-</sup>, and littermate WT controls were infected. Tissues and serum were collected 72 h after infection and viral titers were determined using TCID<sub>50</sub>. The limits of detection are indicated by dotted lines. Individual mice are represented and the geometric means are indicated by bars. A Mann-Whitney U test was used and, where significant, p-values are reported. Data shown is combined from two independent experiments.



(Kato et al., 2006). In contrast to inbred animals used in our studies, the outbred WT controls showed CHIKV replication in the joint, making the data in the knockout strains harder to interpret. Additionally, MEFs isolated from these animals showed high variability, indicating that other genetic loci may be impinging on the Cardif-mediated sensor path-

ways (Fig. S1). Based on these observations, we suggest that there is a shared role for both RIG-I and Mda5, as has been reported for West Nile virus (Fredericksen et al., 2008). In addition to these two sensors, it will also be important to explore a role for PKR and NOD2, both of which have now been implicated in the response to ssRNA viruses and may



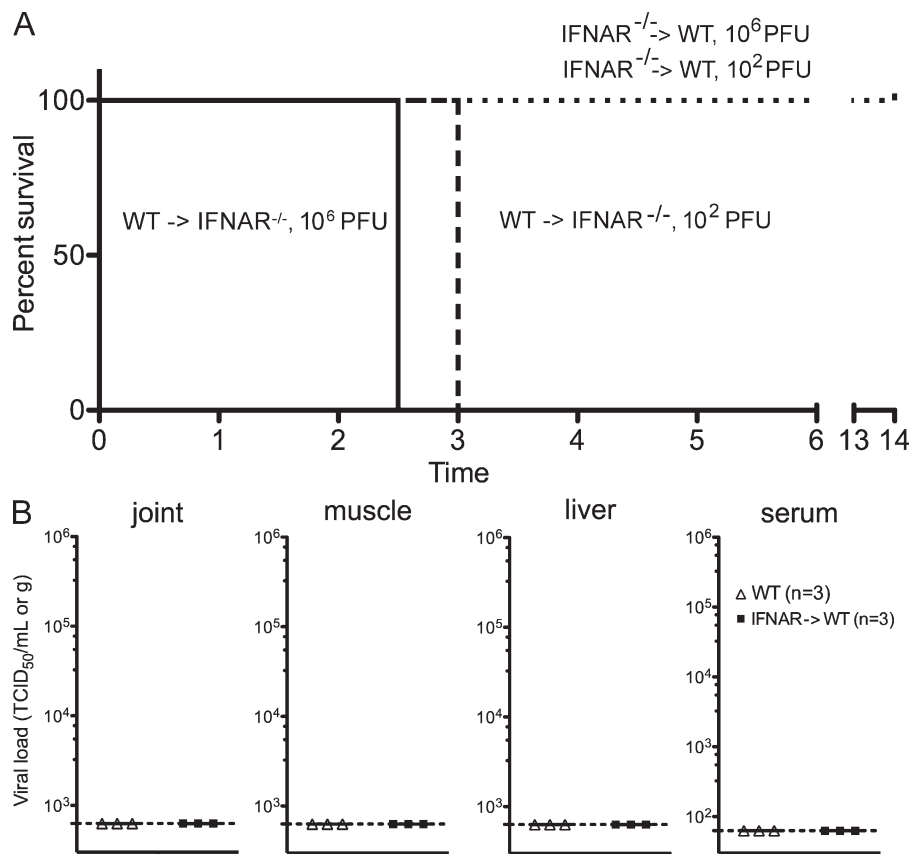
**Figure 7. Cross talk between Cardif- and MyD88-dependant pathways for efficient clearance of CHIKV.** *Cardif*<sup>-/-</sup>, *MyD88*<sup>-/-</sup>, *TLR3*<sup>-/-</sup>, and the corresponding WT controls were infected with 10<sup>6</sup> PFU. (A) Viral titer was measured at 24 and 48 h after infection. As a control we show infection of an *IFNAR*<sup>-/-</sup> animal. (B) Tissues were collected 72 h after infection and viral titers were determined using TCID<sub>50</sub>. The limits of detection are indicated by dashed lines. Individual mice are represented and geometric means are indicated by bars. It is of note that *IFNAR* mice are dead at that time point. Mann-Whitney *U* test was used and, where significant, p-values are reported. Data shown is combined from two independent experiments.

signal via Cardif (Ryman and Klimstra, 2008; Barry et al., 2009; Sabbah et al., 2009).

Regarding the role for MyD88, two possible pathways may account for its role in the control of CHIKV infection. As previously stated, hematopoietic cells are not directly stimulated, suggesting that CHIKV does not engage TLRs in a conventional manner. There is, however, the possibility that endosomal TLRs are engaged as a result of hematopoietic cells phagocytosing infected cells, the latter being a source of viral PAMPs. For example, cells infected by SFV, also a member of the *Alphavirus* genus, results in the generation of dsRNA that may engage TLR3 on CD8<sup>+</sup> DCs upon engulfment (Schulz et al., 2005). Although TLR3 utilizes TRIF and thus does not help explain the phenotype in MyD88<sup>-/-</sup> mice, this mechanism of PAMP transfer may generalize for other host sensors, and future studies will explore a role for TLR7 or TLR8. A second pathway for engaging MyD88 concerns its role as an adaptor of the IL-1 $\beta$  and IL18 receptors (Muzio et al., 1997). There has been a surge of new information regarding the role of the inflammasome, which is well recognized as critical for IL-1 $\beta$  production after bacterial infection. Indeed, this pathway also seems to be participat-

ing in the control of viruses. Specifically, IL-1R activation enhances survival in a model of influenza infection (Schmitz et al., 2005). Supporting this observation, mice deficient in Caspase-1, ASC, and NALP3 have been shown to have a defect in the control of influenza (Ichinohe et al., 2009). As such, IL-1 $\beta$  or IL18 produced by CHIKV-infected cells may participate in viral control by stimulating noninfected cells in a MyD88-dependant manner.

Regarding the role of type I IFNs, we provide evidence that IFNAR signaling in radioresistant stromal cells is critical for clearance of CHIKV. This was demonstrated using WT $\rightarrow$ IFNAR<sup>-/-</sup> bone marrow chimeras, which died with a similar kinetic as the IFNAR<sup>-/-</sup> animals (Fig. 8). Using the IFNAR<sup>-/-</sup> $\rightarrow$ WT chimeras, we also demonstrated that the IFN amplification loop in hematopoietic cells is not required for the control of CHIKV dissemination. To our knowledge, there is only one prior study that has taken a similar approach. In the case of reovirus infection, it is known that infection kills IFNAR<sup>-/-</sup>, and the gut epithelium, but not hematopoietic, cells are the target of infection (Fleeton et al., 2004). Distinguishing our findings with CHIKV, it is interesting that Johansson et al. (2007) find that IFNAR<sup>-/-</sup> $\rightarrow$ WT succumb to



**Figure 8.** IFNAR<sup>-/-</sup> $\rightarrow$ WT bone marrow chimeras maintain their ability to clear CHIKV infection. WT and IFNAR<sup>-/-</sup> mice were lethally irradiated and reconstituted with bone marrow from IFNAR<sup>-/-</sup> or WT mice, respectively. After 2–3 mo, chimeric mice were inoculated with 10<sup>2</sup> or 10<sup>6</sup> PFU CHIKV. (A) Survival was monitored twice a day for 2 wk and Kaplan-Meier curves are shown ( $n = 8$  mice). Data represents two independent experiments. (B) At day 3 after infection, virus titer was determined using TCID<sub>50</sub> in IFNAR<sup>-/-</sup> $\rightarrow$ WT and WT controls ( $n = 3$  mice). Dashed lines represent the limit of detection. Horizontal bars represent mean.

infection, whereas WT→IFNAR<sup>-/-</sup> chimeras control reovirus. Although we fully acknowledge that IFN may be acting as both a direct antiviral and a proimmune cytokine in both cases, the former seems to be critical in CHIKV infection, whereas the latter is required for clearance of reovirus.

In addition to the importance of studying CHIKV pathogenesis because of the threat to public health, from a fundamental level, we have identified features that make CHIKV unique as compared with other alphaviruses. Notably, Ross River, Sindbis, SFV, and Venezuelan Equine Encephalitis are known to infect and stimulate DCs (Hidmark et al., 2005; Ryman et al., 2005; Shabman et al., 2007). It should be noted that human and mouse DCs are not similar in their sensitivity to these viruses (Shabman et al., 2007); however, in the case of CHIKV, neither human nor mouse DCs are directly infected. Recent data would thus indicate that in this regard, CHIKV is more similar to Eastern Equine Encephalitis Virus (Gardner et al., 2008). Extending it to other arboviruses, we also note that infection in the WT mice failed to demonstrate evidence for direct infection of CD45<sup>+</sup> cells, including Langerhans cells, thus distinguishing CHIKV from other vector borne infections such as Dengue (Wu et al., 2000). This is relevant, as it indicates that the viral sensor as well as the host response in CHIKV pathogenesis may be distinct from other alphaviruses (MacDonald and Johnston, 2000; Ryman et al., 2005; Shabman et al., 2007). Also unique may be the ISG responsible for antiviral activity. For SFV, MxA confers resistance (Landis et al., 1998), whereas ISG15 appears critical for controlling Sindbis virus (Lenschow et al., 2007). Future work will be aimed at defining the ISG in fibroblasts that actively controls CHIKV replication.

In sum, we believe our study provides important insight into developing preventive measures and therapeutic strategies for controlling CHIKV infection. Our results also suggest a role for nonhematopoietic cells as important players for IFN production and viral control.

## MATERIALS AND METHODS

**Cytokine analysis.** Sera were harvested and conserved at -80°C for analysis. IFN- $\alpha$  was measured by Luminex (25-plex kits; Invitrogen) according to the manufacturer's instructions. Human IFN- $\beta$  and mouse IFN- $\alpha/\beta$  levels were quantified by Elisa (PBL InterferonSource and Fujirebio Diagnostics, Inc.). Human IFNAR activation was quantified using HL-116 lines (provided by G. Uzé, université Montpellier, Montpellier, France) expressing luciferase under an ISRE element. In brief, supernatant were treated for at least 48 h at pH 2.0 to destroy the virus, the pH was then equilibrated, and supernatants were added on the HL-116 cells. 6–8 h later, cells were lysed and luciferase activity was read by luminometer. Intrun A (Scherring Plough) was used to generate the standard curve.

**Quantitative RT-PCR for quantification of viral load and IFN mRNA.** For determination of patient viral load, total nucleic acid extraction was performed in a MagNa Pure automate using the Total Nucleic Acid kit (Roche). For analysis of cells, an RNeasy mini kit with DNase treatment (QIAGEN) was used. For tissues, samples were homogenized in Trizol, mixed with phenol-chloroform, and the aqueous phase was treated with the RNeasy mini kit. CHIKV RNA was detected with specific taqman probes using a one-step RT-PCR (Master RNA hybridization probes; Roche) performed on a Chromo 4 machine (Bio-Rad Laboratories). The 20- $\mu$ l reaction mix contained

2  $\mu$ l of extracted RNA, 7.5  $\mu$ l of LightCycler RNA Master Hyb-Probe, 3.25 mmol/liter Mn<sub>2</sub>, 450 nmol/liter CHIKV forward primer (5'-AAGCTCC-GCGTCCTTTACCAAG-3'), 150 nmol/liter CHIKV reverse primer (5'-CCAAATTGTCCGGGTCCTCCT-3'), and 150 nmol/liter CHIKV probe (5, 6-carboxyfluorescein-3 TAMRA [5'-CCATGTCTTCAGCCTGGAC-ACCTTT-3']; TIB MOL-BIOL). The thermal cycling consisted of a reverse transcription at 61°C for 20 min followed by 45 cycles at 95°C for 5 s and 60°C for 15 s. The fluorescence was measured at 530 nm. CHIKV load is measured using a synthetic RNA calibrator (Laurent et al., 2007). For mouse IFN- $\beta$ , mouse IFN- $\alpha_2$ , mouse IFN- $\alpha_4$ , and Mx1, RT was performed with random primers (Roche) and complementary DNA (cDNA) was detected using Applied Biosystem Taqman probes (Mm00439546-s1, Mm00833961\_s1, Mm00833969\_s1, and Mm00487796-m1).

**Mice.** C57BL/6 mice were obtained from Charles River. Mice lacking the IFNAR1 were backcrossed >10 $\times$  onto the C57BL/6 background (referred to as IFNAR<sup>-/-</sup>). Stat-4<sup>-/-</sup> and Stat-6<sup>-/-</sup> (BALB/c) were provided by A. Freitas (Institut Pasteur, Paris, France), and Stat-1<sup>-/-</sup> (129) was purchased from Taconic. TLR3<sup>-/-</sup> was provided by Pasteur animal facility, Cardif<sup>-/-</sup> (B6) was generated by J. Tschopp, and MyD88<sup>-/-</sup> (C57BL/6 background), RIG-I<sup>-/-</sup>, and Mda5<sup>-/-</sup> mice (ICR/129/C57BL/6 mixed background) were generated and provided by S. Akira (Osaka University, Osaka, Japan; Kato et al., 2006). Langerin-DTR transgenic mice were provided by B. Malissen (Centre d'Immunologie Marseille-Luminy, Marseille, France; Kissenpennig et al., 2005). For bone marrow chimera, hosts were irradiated with 1,050 Rad and 3  $\times$  10<sup>6</sup> donor bone marrow cells were injected intravenously. Bone marrow chimerism was assessed after 8 wk with 90–98% chimerism for all animals. Mice were inoculated ID with 50  $\mu$ l of viral suspension diluted with PBS. The principles of good laboratory animal care were followed all through the experimental process. Infections were performed in containment isolators under SPF conditions. All protocols were approved by the Institutional Committees on Animal Welfare of the Institut Pasteur.

**Cells.** Human blood components were obtained from the EFS (établissement français du sang). PBMCs were isolated using Ficol-Paque PLUS (GE Healthcare). pDCs were isolated from 250  $\times$  10<sup>6</sup> PBMCs using the anti-BDCA-4 magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol (90–95% purity was achieved). Immature DCs were prepared from the T cell-depleted fraction by culturing cells in the presence of 1,000 U/ml GM-CSF (Bayer HealthCare) and 500–1,000 U/ml IL-4 (R&D Systems) for 6 d (Bender et al., 1996; Romani et al., 1996). To generate mature DCs, cultures were stimulated on day 6 with 50 ng/ml TNF (Enzo Life Sciences, Inc.) and 0.1  $\mu$ M PGE<sub>2</sub> (Sigma-Aldrich) for 36–48 h (Rieser et al., 1997). At days 6–7, >95% of the cells were CD14<sup>+</sup>CD83<sup>+</sup>HLA-DR<sup>lo</sup> DCs. After maturation, on days 8–9, 70–95% of the cells were of the mature CD14<sup>+</sup>CD83<sup>+</sup>HLA-DR<sup>hi</sup> phenotype. HFF and MRC5 were obtained from American Type Culture Collection. MEFs were generated from pregnant knockout mice 13 d after mating.

**Antibodies, flow cytometry, and immunohistochemistry.** To detect CHIKV viral proteins, we used a mouse mAb directed against a conserved region of the *Alphavirus* nucleocapsid protein (Anti-C), a gift of I. Greiser-Wilke (Institute of Virology, Hanover, Germany; Greiser-Wilke et al., 1989), and mouse polyclonal anti-CHIKV antibodies (Schuffenecker et al., 2006). For detection of intracellular CHIKV proteins, cells were permeabilized before labeling and analyzed by flow cytometry using a FACSCanto (BD) and FlowJo software (Tree Star, Inc.).

For immunohistochemistry with CHIKV-GFP, tissues were fixed in 4% PFA for several hours, rinsed, and frozen in OCT. Slides were stained overnight at 4°C with primary antibody and 1 h with secondary antibody at room temperature, and then slides were counterstained with DAPI (InvivoGen). The following antibodies were used: rabbit anti-GFP (clone A11122; 1/1,000; Invitrogen), PE-conjugated mouse anti-CD45.2 (clone 104; 1/200; BD), APC-conjugated rat anti-mouse CD31 (clone MEC13.3; 1/150; BD), cy3-conjugated mouse monoclonal anti- $\alpha$ -SMA (clone 1A4;

1/600; Sigma-Aldrich). For staining of vimentin, tissues were not fixed and, instead, flash frozen in liquid nitrogen as previously described (Couderc et al., 2008). A chicken polyclonal anti-vimentin antibody was used (Abcam; 1/200). Images were acquired with an Axiovert 200 fit with an ApoTome and AxioVision 4.6 software (Carl Zeiss, Inc.).

**CHIKV titer in mouse tissues and serum.** Mice were perfused with 40 ml of saline buffer. Tissues were homogenized. Viral samples were titrated as TCID<sub>50</sub> endpoint on Vero cells using a standard procedure. Serial 10-fold dilutions (100 µl) of tissue homogenate or serum were added in six replicates in 96-well plates seeded with 10<sup>4</sup> Vero cells. The cytopathic effect was scored 5 d after infection and the titer was calculated by determining the last dilution giving 50% of wells with cells displaying a cytopathic effect. Results were expressed as TCID<sub>50</sub>/ml in the serum and TCID<sub>50</sub>/g of tissue.

**CHIKV infection.** The preparation of CHIKV from clinic clinical samples has been previously described (Schuffenecker et al., 2006). CHIKV-21 strain was propagated in C6/36 cells, and supernatants were harvested and frozen at -80°C before titration and further use. Adherent cells (plated at ~50% confluence in 24-wells plates) and nonadherent cells were exposed to the indicated viruses for 2–4 h at 37°C, extensively washed (except for PBMC and for the RNA experiment on MEF), and cultivated for various periods of time before further analysis. The MOI was defined as the amount of CHIKV infectious units (calculated on BHK cells as PFU) per one target cell. 3'CHIKV-EGFP was generated using a full-length infectious cDNA clone provided by S. Higgs (Vanlandingham et al., 2005). Infectious virus was obtained by transfection of BHK21 cells with RNA-derived cDNA, as described. Viral titers in IFNAR<sup>-/-</sup> mice at day 2 were equivalent to WT CHIKV (Fig. S2). As positive control, pIC (InvivoGen) with or without DOTAP (Roche) was used. Alternatively, cells were stimulated with influenza virus strain A/PR8/1976 (Charles River) or with FluΔNS1 (given by S. Diebold; King's College, London, UK) at 10 HAU/ml.

**Online supplemental material.** Fig. S1 shows that IFN-β production in RIG-I<sup>-/-</sup> and Mda5<sup>-/-</sup> MEFs is strain dependant. Fig. S2 shows viral load in IFNAR<sup>-/-</sup> mice infected with CHIKV-21 and CHIKV-GFP. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090851/DC1>.

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