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Cutting Edge: Independent Roles for IRF-3 and IRF-7 in Hematopoietic and Nonhematopoietic Cells during Host Response to Chikungunya Infection

Clémentine Schilte,*† Matthew R. Buckwalter,*† Melissa E. Laird,*† Michael S. Diamond,‡§‖ Olivier Schwartz,‖ and Matthew L. Albert *†

The host response to Chikungunya virus is dependent on the direct action of type I IFN on infected nonhematopoietic cells. Prior studies have demonstrated that multiple host sensors coordinate an antiviral response; however, the tissue source(s) and signaling pathways for IFN production remain unknown. In this study, we demonstrate that IRF-3 and IRF-7 are functionally redundant, but lack of both factors results in lethal infection in adult mice. Reciprocal bone marrow chimeras indicated that IRF-3 or IRF-7 expression in either hematopoietic or nonhematopoietic cell compartments was capable of inducing an antiviral response. Interestingly, redundancy of IRF-3 and IRF-7 was age dependent, as neonatal animals lacking either factor succumbed to infection. We further demonstrate that IPS-1 is essential in nonhematopoietic cells and preferentially required during early life. These results highlight the interplay between nonimmune and immune cells during Chikungunya virus infection and suggest an important role for nonhematopoietic cells as a critical source of IFN-α/β.

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Materials and Methods

**Mice**

C57BL/6 wild-type (WT) mice were purchased (Charles River Laboratories). IFNAR−/− mice on a C57BL/6 background were established at the Institut Pasteur. IRF-3−/− and IRF-7−/− single knock out mice were generated by T. Taniguchi (University of Tokyo, Tokyo, Japan) (6). The IRF-3−/− × IRF-7−/− (DKO) mice were generated in the F3 generation after crossing the single knockout mice (8). MyD88−/− and TLR3−/− mice were generated by S. Akira (Osaka University, Japan) (9, 10). IPS-1−/− mice were obtained from J. Tschopp (Lausanne University, Switzerland) (11). Six- to 8-wk-old animals were inoculated intradermally in the right flank with 10⁶ PFU CHIKV-21 (12). Neonatal mice (9 d old) were infected (2 × 10⁶ PFU). BMcs were generated as previously reported, and 90–98% chimerism was confirmed (2). All infections were performed with the approval of the Institutional Committee on Animal Welfare of the Institut Pasteur.

**Viral titers in mouse tissues and sera**

Mouse sera were obtained after coagulation of blood in T-MG tubes (Terumo) and stored at −80 °C until analysis. Mice were perfused with PBS, and tissues were weighed and homogenized using the TissueLyser II (Qiagen). Virus in individual tissue and sera samples were titrated on Vero cells using standard procedures (2). Results are expressed as median tissue culture infective dose (TCID₅₀)/ml in the serum and TCID₅₀/g for tissue.

**Measurement of IFN-α/β**

Mouse IFN-α and IFN-β were quantified by ELISA (PBL Biomedical) as per the manufacturer’s instructions. Quantitative PCR on mouse embryonic fibroblasts (MEFs) was performed as previously described (2).

**Statistical analysis**

All data were analyzed using Prism software (Graphpad), and specific statistical tests are indicated in the figure legends.

Results and Discussion

**IFR-3** and **IRF-7** are required for efficient host response to CHIKV infection

To characterize the respective requirement for IRF3 and/or IRF7 during CHIKV infection, IRF-3−/−, IRF-7−/−, and IRF-3−/− × IRF-7−/− (DKO) mice were infected with 10⁶ PFU. Survival and viral load were examined, using WT and IFNAR−/− mice as controls for resistant and susceptible hosts, respectively. All mice deficient in either IRF-3 or IRF-7 survived CHIKV infection (Fig. 1A). Whereas low levels of replicating virus were detected in the circulation of IRF-3−/− mice, higher levels were observed in IRF-7−/− mice, and this correlated with increased CHIKV infection in the joint and muscle (5–10-fold; *p < 0.01) at day 3 postinfection (Fig. 1B, Supplemental Fig. 1). In comparison, a combined deficiency of both IRF-3 and IRF-7 caused a more profound phenotype, with 100% of mice succumbing to infection by day 8; this was associated with a greatly enhanced viral burden compared with IRF-7−/− mice.

In comparison with IFNAR−/− mice, which succumb by day 3 postinfection, DKO mice survived slightly longer, with a median survival time of 5 d (Fig. 1A); this suggested that in vivo, some cells might produce limited amounts of type I IFN through an IRF-3−/− and IRF-7−/−-independent pathway. To investigate the requirement of IRF-3 and IRF-7 for producing type I IFN in response to CHIKV infection, we measured serum levels of type I IFN from adult mice at different time points after CHIKV infection. As in our previous studies, circulating IFN levels were not detected in WT adult mice (Fig. 1C). Interestingly, Gardner et al. (13) detected modest levels of IFN in the serum following s.c. inoculation of the foot, and the kinetics and quantity appear to be CHIKV strain specific. We see no evidence of disseminated viral production (and as a result no plasma IFN) in our model (using intradermal infection to mimic a mosquito bite), which may be a reflection of complete control of CHIKV infection at the inoculation site (2). Despite virus amplification and dissemination in IRF-deficient animals, type I IFNs remained undetectable by ELISA, even in those animals lacking both IRF-3 and IRF-7, supporting a defect in production (Fig. 1C). These data were confirmed by reporter gene assay (data not shown). To further examine the differential sensitivity of DKO mice compared with IFNAR−/− animals, we evaluated in vitro CHIKV infection of MEFs by performing quantitative RT-PCR analysis of IFN-β (as a marker of early IFN) and IFN-α2 (as an indicator of late IFN) mRNA. Notably, we observed that IFN-β mRNA levels were 50-fold decreased in DKO MEF as compared with WT MEF (*p < 0.01); however, production was not completely abolished (Fig. 1D). Indeed, IFN-β was 100-fold higher in CHIKV-infected DKO.

**FIGURE 1.** IRF-3 and IRF-7 transcription factors control CHIKV infection. (A) Survival of WT, IRF-3−/−, IRF-7−/−, DKO, and IFNAR−/− mice infected with CHIKV. Kaplan–Meier plots are shown for indicated groups. (B) Viral titers in the serum were measured by TCID₅₀ at 24, 48, and 72 h postinfection. Due to the rapid death in IFNAR−/− animals, no data were available at 72 h. Data from individual animals are shown, and geometric means are presented (solid black line). Limit of detection is indicated (dotted line). (C) Serum concentrations of IFN-β (top panel) and IFN-α (bottom panel) were measured 24 and 48 h postinfection using ELISA. Data are reported as picograms per milliliter. (D) Low-passage MEFs from the indicated strains were infected using a multiplicity of infection of 0.01 CHIKV. After 24 h, total RNA was extracted, and quantitative RT-PCR analysis was performed. Data are plotted relative to uninfected controls.
Animals were infected with 10^6 PFU of CHIKV and monitored daily. MEF compared with uninfected controls (p < 0.01). Decreased expression of IFN-β also was observed in IRF-3^-/- MEF to a level comparable to DKO cells, suggesting that IRF-3 was the primary transcription factor responsible for IFN-β production in MEF after CHIKV infection (Fig. 1D). From these data, we suggest that although IRF7^-/- cells produce similar levels of IFN-β as compared with WT cells, they are unable to trigger the feed-forward expression of ISGs and the burst of IFN expression required to control viral dissemination. In contrast, IRF3^-/- cells were able to overcome the lack of early signaling events, and late IFNs were induced, likely a result of proinflammatory signals sufficient to upregulate IRF7 (e.g., direct action of RIG-like receptor activation or other cytokines such as IFN-λ). Similar to our findings, a less severe phenotype in DKO mice was observed relative to IFNAR^-/- mice after West Nile virus. Surprisingly, in West Nile virus infection, IFN-α/β production in macrophage and dendritic cells was only partially affected by the absence of IFN-3 and IRF-7 (8).

**Hematopoietic cells produce type I IFN in a MyD88-independent manner after CHIKV infection**

To characterize the cell type-dependent PRR pathways responsible for type I IFN induction after CHIKV infection, we created WT -> DKO as well as DKO -> WT BMCs. After reconstitution, mice were infected with CHIKV and followed for survival and viral dissemination. Remarkably, WT hematopoietic cells were sufficient to protect against CHIKV infection (Fig. 2A), with limited viral replication in tissues of the recipient DKO mice (Fig. 2B). Tissue viral loads in WT -> DKO mice were ~3-log lower (p < 0.001) than that observed in the DKO -> DKO control animals. Our prior studies demonstrated a mildly increased CHIKV replication phenotype in MyD88^-/-, but not TLR3^-/- (2), based on these results, we hypothesized that MyD88 functions predominantly in hematopoietic cells, possibly downstream of TLR7 engagement to induce a protective antiviral response against CHIKV. To further define this pathway of control in hematopoietic cells, we generated MyD88^-/- → DKO and TLR7^-/- → DKO BMCs. Notably, MyD88^-/- or TLR7^-/- hematopoietic cells effectively controlled CHIKV infection in DKO recipient mice (Fig. 2). These data suggest that other PRR signaling pathways are used by bone marrow-derived cells to trigger type I IFN production. We conclude that hematopoietic cells respond to CHIKV via an indirect mechanism, which uses, in part, PRR acting via a MyD88-independent signaling pathway, possibly through Toll/IL-1R domain-containing adapter inducing IFN-β/Toll/IL-1R-containing adaptor molecule-1. These data also suggest that MyD88 signaling may be involved in nonhematopoietic cells, possibly acting downstream of IL-1R or IL-18R. Current efforts are exploring a potential role for cell-to-cell transfer of viral agonists, which may contribute to the activation of host sensors in hematopoietic cells. Future studies will also explore a possible role for the NALP3 inflammasome or other potential nucleotide oligomerization domain-like receptor-mediated pathways (14).

**IPS-1 signaling in nonhematopoietic cells results in a protective response after CHIKV infection**

Fibroblasts are the dominant cell type infected by CHIKV in vivo, and in vitro MEFs produce high levels of type I IFN. Nonetheless, direct investigation of the PRR signaling pathway by which nonhematopoietic cells, including fibroblasts, produce IFN-α/β in vivo has not been studied. Strikingly, DKO → WT mice effectively controlled CHIKV infection, with no evidence of viral dissemination (Figs. 2A, 3A, 3B). Based on the in vitro evidence that IPS-1 is essential for IFN production in MEFs following CHIKV infection (2), we generated reciprocal BMCs from IPS-1^-/- and DKO mice. Although all DKO → IPS-1^-/- mice survived, enhanced viremia (10^4 TCID50 at day 3) and disseminated CHIKV infection (>10^5 TCID50/g) in the tissues was observed (Fig. 3A, 3B). In contrast, the viral titers in IPS-1^-/- → DKO mice were indistinguishable from WT → DKO chimeric mice (all mice survived during the time course of the experiment). Thus, IPS-1 acts primarily in nonhematopoietic cells, presumably in CHIKV-infected cells, as a critical adaptor downstream of RIG-1 and MDA5-induced protective response. Based on our findings in DKO → IPS-1^-/- BMCs, we do not support a major role for an IRF-3- and IRF-7-independent mechanism of type I IFN production; however, we cannot exclude a role for other transcription factors (e.g., IRF-1 or IRF-5) in a subset of nonhematopoietic cells targeted by CHIKV infection.

**An essential role for IPS-1 and IRF-3 during neonatal CHIKV infection**

Age-dependent disease severity has been reported for alphaviruses and reproduced in mouse models of CHIKV (3, 15, 16). Indeed, we previously observed an age-dependent sus-
Survival kinetics of IPS-1 inoculated and monitored for survival. Kaplan–Meier plots are shown. (A) Viral titers in the serum of chimeric animals (IPS-1−/− → WT, WT → IPS-1−/−, IPS-1−/− → DKO, and DKO → IPS-1−/−) were determined at 24, 48, and 72 h postinfection, and TCID₅₀/ml is reported. Individual mice and geometric means are presented (solid black lines), and the limit of detection is indicated (dotted line). (B) Viral titers were determined in select tissues, and TCID₅₀/gram is reported for the 72-h time point. (C) Kinetics of survival for neonatal mice infected by CHIKV. Groups of 8- to 9-d-old, weight-matched pups were monitored daily for survival, and Kaplan–Meier plots are shown. (D) Survival kinetics of IPS-1−/− or WT neonate and adult IPS-1−/− animals. Groups of adult and neonates (IPS-1−/−, blue circles; WT, gray triangles) were inoculated with 10⁶ or 2 × 10⁶ PFU CHIKV, respectively. Animals were monitored daily for survival, and Kaplan–Meier plots are shown.

FIGURE 3. Nonhematopoietic cells act via an IPS-1–independent pathway and play a critical role in neonatal mice. (A) Viral titers in the serum of chimeric animals (IPS-1−/− → WT, WT → IPS-1−/−, IPS-1−/− → DKO, and DKO → IPS-1−/−) were determined at 24, 48, and 72 h postinfection, and TCID₅₀/ml is reported. Individual mice and geometric means are presented (solid black lines), and the limit of detection is indicated (dotted line). (B) Viral titers were determined in select tissues, and TCID₅₀/gram is reported for the 72-h time point. (C) Kinetics of survival for neonatal mice infected by CHIKV. Groups of 8- to 9-d-old, weight-matched pups were inoculated and monitored for survival. Kaplan–Meier plots are shown. (D) Survival kinetics of IPS-1−/− or WT neonate and adult IPS-1−/− animals. Groups of adult and neonates (IPS-1−/−, blue circles; WT, gray triangles) were inoculated with 10⁶ or 2 × 10⁶ PFU CHIKV, respectively. Animals were monitored daily for survival, and Kaplan–Meier plots are shown.

susceptibility to CHIKV infection in mice. To evaluate the function of IRF-3 and IRF-7 in controlling CHIKV infection during neonatal life, we challenged 9-d-old animals and evaluated disease progression. WT mice responded as previ-ously described; ∼50% of animals succumbed to infection by day 15 (Fig. 3C). Mice deficient in only IRF-3 were highly vulnerable to infection, displaying rapid viral dissemination (data not shown), and all animals died by day 5 postinfection (Fig. 3C). IRF-7−/− animals were even more susceptible to CHIKV infection and, surprisingly, showed a survival pattern similar to the DKO mice, with death occurring 2 to 3 d postinfection. These data convincingly demonstrate an essential role for IRF-3 and IRF-7 in the control of neonatal CHIKV infection and highlight an age-dependent redundancy for IRF-3 or IRF-7 in adult but not newborn animals; whereas IRF-3 or IRF-7 signaling is sufficient to control CHIKV in adults, both transcription factors are required in neonates.

Based on the increased sensitivity of neonatal animals to CHIKV infection and the requirement for IRF-3, we speculated that there may also be a nonredundant role for nonhematopoietic cell-derived type I IFN. Although it is not technically feasible to generate BMCs in neonatal mice, we hypothesized that IPS-1 would function principally in stromal cells based on studies in adult mice (Fig. 3A, 3B). As such, we infected 9-d-old weight-matched IPS-1−/− and WT mice and evaluated morbidity and mortality. Whereas ∼50% mortality rate was observed in WT mice, IPS-1−/− showed enhanced mortality and a shorter survival time (p < 0.0001). IPS-1−/− also demonstrated a failure to thrive as suggested by lack of weight gain (data not shown). In comparison, no lethality was observed in adult IPS-1−/− after CHIKV infection. Together, these data demonstrate that IPS-1–dependent signaling is critical for the antiviral response in newborn mice and suggests a crucial role for nonhematopoietic cells in mediating the innate response to infection.

Dissecting the host response to CHIKV infection has provided new insight into the central role of type I IFNs. In this study, we provide new information regarding the cellular sources of IFN-α/β and begin to map the respective host–sensor pathways that participate in the clearance of CHIKV infection. These studies permitted the characterization of two independent mechanisms for IFN-α/β production. First, hematopoietic cells produce type I IFN via at least one pathway, which acts independently of TLR7 or MyD88 signaling. As previously observed, hematopoietic cells are neither directly infected nor directly stimulated by live CHIKV, thus supporting the observation that in vivo infection does not engage TLRs in a conventional manner. The second mechanism focuses on the production of type I IFNs by nonhematopoietic cells via an IPS-1–dependent signaling pathway. These data are consistent with a role for RIG-I and/or MDA5 as host sensors for CHIKV RNA in infected fibroblasts (2, 17). The most surprising aspect of these studies was that nonhematopoietic cell-derived IFN-α/β was sufficient to control CHIKV infection. Indeed, viral titers were indistinguishable between CHIKV- infected DKO → WT and WT animals. To our knowledge, these data provide the first in vivo evidence for nonimmune cells controlling viral pathogenesis. These data also provide important insight into age-dependent innate immune and antiviral responses and may influence strategies for modulating host immune responses to control CHIKV and other viral infections.
Disclosures
The authors have no financial conflicts of interest.

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