An IRF-3-, IRF-5-, and IRF-7-independent pathway of dengue viral resistance utilizes IRF-1 to stimulate type I and II interferon

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An IRF-3-, IRF-5-, and IRF-7-Independent Pathway of Dengue Viral Resistance Utilizes IRF-1 to Stimulate Type I and II Interferon Responses

Highlights

- An IRF-3, IRF-5, and IRF-7 (IRF-3/5/7)-independent pathway of antiviral immunity exists
- The IRF-3/5/7-independent pathway acts via both type I and type II interferons (IFNs)
- The IRF-3/5/7-independent pathway protects against dengue via IRF-1
- The IRF-3/5/7-independent pathway is predominantly mediated by IL-12/IFN-γ

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In Brief
Carlin et al. identify a non-canonical IRF-3-, IRF-5-, and IRF-7-independent antiviral defense mechanism that mediates protection against severe dengue disease. This alternative pathway utilizes IRF-1, predominantly via IL-12/IFN-γ, enabling survival in the context of reduced type I IFN responses.

Data and Software Availability
GSE104189
An IRF-3-, IRF-5-, and IRF-7-Independent Pathway of Dengue Viral Resistance Utilizes IRF-1 to Stimulate Type I and II Interferon Responses

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SUMMARY

Interferon-regulatory factors (IRFs) are a family of transcription factors (TFs) that translate viral recognition into antiviral responses, including type I interferon (IFN) production. Dengue virus (DENV) and other clinically important flaviviruses are suppressed by type I IFN. While mice lacking the type I IFN receptor (Ifnar1−/−) succumb to DENV infection, we found that mice deficient in three transcription factors controlling type I IFN production (Irf3−/− Irf5−/− Irf7−/−) fail to activate type I IFNs. This pathway signals via IRF-1 to stimulate interleukin-12 (IL-12) production and type I IFN responses. These results reveal a key antiviral role for IRF-1 by activating both type I and II IFN responses during DENV infection.

INTRODUCTION

Interferon-regulatory factors (IRFs) are members of a small group of transcription factors (TFs) that link microbial recognition to production of type I interferons (IFNs) and other antiviral responses (Ikushima et al., 2013; Honda and Taniguchi, 2006). Targeted genetic deletion of IRF transcription factors in mice increases susceptibility to pathogens, particularly viruses (Tamura et al., 2008). IRF-3 and IRF-7 are the primary regulators in the canonical model of type I IFN (IFN-α/β) signaling and thus essential for viral control (Honda and Taniguchi, 2006; Honda et al., 2005). Recognition of viral pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) results in activation and nuclear translocation of IRF-3 and/or IRF-7, which leads to the induction of Ifnb1. Secreted IFN-beta (IFN-β) binds to a type-I-IFN-specific heterodimeric receptor, Ifnar1/Ifnar2 (IFNAR), activating a JAK-STAT signaling cascade, resulting in the induction of hundreds of interferon-stimulated genes (ISGs) that suppress viral pathogens by many mechanisms (Liu et al., 2012; Rusinova et al., 2013; Takaoka and Yanai, 2006; Diamond and Gale, 2012; Schoggins et al., 2011) and create a feed-forward amplification loop of IFN signaling by increasing expression of IRF-7 (Honda and Taniguchi, 2006; Honda et al., 2005). Interferon-gamma (IFN-γ), the only type II IFN, also demonstrates antiviral activity and defends against many infections (Platanias, 2005). Mice with targeted genetic deficiencies in IFN-γ signaling show increased susceptibility to certain intracellular bacterial, protozoan, and viral infections (John et al., 2002; Harty and Bevan, 1995; Dalton et al., 1993; Huang et al., 1993; Jouanguy et al., 1999; Maloney et al., 2012; Schroder et al., 2004; Chesarle and Reiss, 2002). IFN-γ, analogous to type I IFNs, binds a type-specific heterodimeric receptor complex, Ifnγr1/Ifnγr2 (IFNGR), and activates a JAK-STAT signaling cascade, resulting in the induction of a set of ISGs that partially overlaps with the type I IFN response. In contrast to type I IFNs that are secreted directly by virally infected cells, IFN-γ is produced principally by natural killer (NK) cells and activated T cells in response to secreted cytokines such as interleukin-12 (IL-12) and IL-18 (Schoenborn and Wilson, 2007; Okamura et al., 1995, 1998).

In addition to IRF-3 and IRF-7, which are considered the master regulators of type I IFN production, both IRF-1 and IRF-5 can induce type I IFN genes (Honda and Taniguchi, 2006; Fujita et al., 1989; Barnes et al., 2001; Yie et al., 1999). IRF-1, IRF-3, IRF-5, and IRF-7 contribute to the suppression of viruses, often in a cell-type- and viral-specific manner (Brien et al., 2011; Chen et al., 2013; Lazear et al., 2013). Because of the central importance of IRF transcription factors in viral defense, many pathogenic viruses have evolved mechanisms to suppress IRF signaling (Barro and Patton, 2007; Dalrymple et al., 2015; Devaraj et al., 2007; Zhu et al., 2002; Robertson et al., 2014). How specific IRFs activate canonical and non-canonical host antiviral defense pathways in response to specific viruses in vivo is still poorly understood but potentially depends on viral tropism and immune evasion mechanisms used by individual viruses.
Type I and II IFNs play key roles in controlling acute dengue virus (DENV) and other flavivirus infections in humans (Navarro-Sánchez et al., 2000). IFN-α/β or IFN-γ protects against DENV infection of human cells in vitro (Diamond et al., 2000; Ho et al., 2005), and humans infected with DENV have high levels of circulating IFN-α (Kurane et al., 1993; Library et al., 2002) and IFN-γ (Chakravarti and Kumaria, 2006; Chen et al., 2005, 2006; Kurane et al., 1991; Nguyen et al., 2004). A critical role for IFNs in the protection against DENV severe disease is supported by studies showing children with dengue shock syndrome have substantially less induction of ISGs than children with milder DENV infection (Clyde et al., 2006). Similarly, adults in Taiwan who survived DENV infection, but not those who succumbed to DENV infection, had significantly higher levels of circulating IFN-γ than controls (Chen et al., 2006). DENV is highly sensitive to type I IFN responses in mice and, accordingly, causes little disease in wild-type (WT) animals but rapid mortality in mice lacking IFNAR expression (Orozco et al., 2012; Makhluf et al., 2013). Remarkably, Ifr3−/− Ifr7−/− double-knockout (DKO) mice survive DENV infection despite developing high levels of viremia (Chen et al., 2013; Shresta et al., 2004), suggesting that an alternative antiviral response is produced in an IRF-3- and IRF-7-independent manner (Chen et al., 2013; Orozco et al., 2012; Shresta et al., 2004). As IRF-5 can contribute to type I IFN signaling and help protect DKO mice from infection with a closely related flavivirus, West Nile virus (WNV) (Schoenemeyer et al., 2005), we hypothesized that IRF-5 also might contribute to the resistance of DKO mice to severe DENV infection.

To test this hypothesis, we infected Ifr3−/− Ifr5−/− Ifr7−/− triple-knockout (TKO) mice with DENV and unexpectedly found that all TKO mice survived, whereas all congenic Ifr1−/− mice died from infection by day 6. Transcriptional profiles of DKO and TKO splenocytes showed activation of the IL-12/IFN-γ/IRF-1 signaling axis that was not present in Ifnar1−/− mice. Blocking IL-12 or IFN-γ signaling or genetic deletion of IRF-1 in TKO mice (Ifr3−/− Ifr5−/− Ifr7−/− quadruple knockout [QKO]) infected with DENV led to severe disease and death, confirming the importance of this alternative pathway in DENV resistance. DENV infection in vitro of TKO macrophages demonstrated selective upregulation of Il12b that encodes the IL-12p40 subunit of the cytokine IL-12, which is known to promote IFN-γ production by NK and T cells. Finally, IFNAR blockade in TKO mice and macrophages revealed a small albeit significant role for type I IFNs in mediating host defense against DENV. Collectively, our results establish an IRF-3, IRF-5, and IRF-7 (IRF-3/5/7)-independent alternative pathway of viral resistance that utilizes IRF-1 to stimulate protective IFN-γ and, to a lesser extent, type I IFN responses against DENV.

**RESULTS**

**Mice Deficient in IRF-3, IRF-5, and IRF-7 Are Resistant to Severe DENV Infection**

To test the hypothesis that IRF-5 is involved in the IRF-3 and IRF-7-independent pathway of DENV resistance, WT, DKO, TKO, and Ifnar1−/− mice were infected with DENV2 (strain 221) and followed for mortality, weight loss, and clinical score (Warfield et al., 2015). Unexpectedly, similar to WT (B6) and DKO mice, all TKO mice survived infection, whereas Ifnar1−/− mice uniformly developed lethal disease by day 6 post-infection (p.i.) (Figure 1A). After the first day of infection, WT mice steadily gained weight and had higher average body weights than either DKO or TKO mice on days 2–4 p.i. (p < 0.0001) (Figure 1B). DKO and TKO mice initially lost weight but recovered after day 2 p.i., whereas Ifnar1−/− mice lost a substantial amount of weight and had lower mean body weights on most days after day 2 p.i. than DKO and TKO mice (p < 0.05 on day 1 and p < 0.0001 on days 3, 4, and 6). Additionally, Ifnar1−/− mice had higher disease scores by day 2 p.i. than WT and higher disease scores by day 4 p.i. than DKO and TKO mice, and these differences were maintained until all Ifnar1−/− mice had succumbed to infection (Figure 1C).

To determine the contributions of IRF-3, IRF-5, and IRF-7 to the kinetics of viral clearance, DENV was measured in blood, spleen (the initial target organ of DENV in this model), kidney, and liver (subsequent target organs of DENV) at 24 and 72 hr p.i. After 24 and 72 hr of infection, DKO, TKO, and Ifnar1−/− mice had significantly higher levels of DENV RNA than WT mice in all tissues examined (Figures 1D–1G). At 24 hr p.i., there was a minimal (4-fold, p < 0.01) increase in infectious virus in DKO compared to Ifnar1−/− mice in the spleen, but no other differences were observed among DKO, TKO, and Ifnar1−/− mice. However, at 72 hr p.i., DKO and TKO mice had lower levels (>10,000-fold, p = 0.0001 and >390-fold, p < 0.03 respectively) of viremia than Ifnar1−/− mice (Figure 1D). In the spleen and kidney, a pattern of progressively higher viral burdens emerged in DKO, TKO, and Ifnar1−/− mice. In the liver, DKO and TKO mice had lower (120-fold, p < 0.0001 and 30-fold, p < 0.002) viral loads than Ifnar1−/− mice (Figure 1G). Collectively, these data demonstrate that IRF-3, IRF-5, and IRF-7 and a functional type I IFN response are necessary to restrict early DENV replication in different organs. These results also demonstrate the presence of a delayed IRF-3, IRF-5, and IRF-7-independent antiviral response that controls DENV infection and dissemination in lymphoid and non-lymphoid tissues and improves clinical disease outcome.

**An IRF-3, IRF-5, and IRF-7-Independent Pathway Contributes to Cell-Intrinsic Antiviral Resistance in Bone-Marrow-Derived Macrophages**

DENV infects macrophage populations in the spleen and other lymphoid tissues before spreading to resident and infiltrating macrophages in non-lymphoid tissues (Prestwood et al., 2012). To determine whether the differences in DENV phenotypes between TKO and Ifnar1−/− mice in vivo could reflect disparate cell-intrinsic antiviral responses, bone-marrow-derived macrophages (BMDMs) from WT, TKO, and Ifnar1−/− mice were infected with DENV, and yield of secreted virus in the culture supernatant was measured by focus-forming unit (FFU) assay. DENV established productive infection in BMDMs derived from TKO and Ifnar1−/−, but not WT, mice (Figures 2A and S1A). Although DENV replicates in TKO BMDMs, less virus was produced compared to Ifnar1−/− cells at 24, 48, and 72 hr p.i. (12-fold, p < 0.0003; 4-fold, p < 0.05; and 10-fold, p < 0.001,
Moreover, there was substantially less virus produced during infection of DKO macrophages than TKO macrophages at 24 and 48 hr p.i. (70-fold, \( p < 0.0002 \) and 25-fold, \( p < 0.007 \), respectively; Figure 2B). The progressive increase in susceptibility to DENV productive infection in DKO, TKO, and Ifnar1-/C0/C0 macrophages was similar to that seen in vivo at 72 hr p.i. To further compare the relative resistance of DKO and TKO BMDMs to DENV infection, we measured the expression of Ifnb1 and the ISGs Mx1 and Ifit1. Although we did not detect a statistically significant upregulation of Ifnb1 in DKO or TKO BMDMs, we did measure a significantly greater induction of both Mx1 and Ifit1 in DENV-infected DKO BMDMs than in DENV-infected TKO BMDMs (Figures 2C and S1B). Additionally, TKO BMDMs were infected with DENV in the presence or absence of an IFNAR blocking antibody (IFNARAb) that inhibits type I IFN signaling. IFNAR blockade significantly increased the susceptibility of TKO BMDMs to DENV infection (Figure 2D). These results show that IRF-3, IRF-5, and IRF-7 contribute to macrophage resistance to DENV infection and that despite the lack of all three of these transcription factors, BMDMs still make low levels of type I IFN that contribute to DENV resistance.

**DKO and TKO Mice Upregulate IRF-1- and IFN-γ-Associated Transcriptional Programs**

To determine the levels of type I IFN signaling in DKO and TKO mice and potentially identify alternative mechanisms for the delayed IRF-3, IRF-5, and IRF-7-independent pathway of immune response to DENV infection in vivo, we infected WT, Ifnar1-/-, DKO, and TKO mice and prepared total splenocytes at 6 and 24 hr p.i. for genome-wide transcriptional sequencing (RNA sequencing [RNA-seq]). Gene expression profiles determined by RNA-seq from independent biological replicates respectively). Moreover, there was substantially less virus produced during infection of DKO macrophages than TKO macrophages at 24 and 48 hr p.i. (70-fold, \( p < 0.0002 \) and 25-fold, \( p < 0.007 \), respectively; Figure 2B). The progressive increase in susceptibility to DENV productive infection in DKO, TKO, and Ifnar1-/- macrophages was similar to that seen in vivo at 72 hr p.i. To further compare the relative resistance of DKO and TKO BMDMs to DENV infection, we measured the expression of Ifnb1 and the ISGs Mx1 and Ifit1. Although we did not detect a statistically significant upregulation of Ifnb1 in DKO or TKO BMDMs, we did measure a significantly greater induction of both Mx1 and Ifit1 in DENV-infected DKO BMDMs than in DENV-infected TKO BMDMs (Figures 2C and S1B). Additionally, TKO BMDMs were infected with DENV in the presence or absence of an IFNAR blocking antibody (IFNARAb) that inhibits type I IFN signaling. IFNAR blockade significantly increased the susceptibility of TKO BMDMs to DENV infection (Figure 2D). These results show that IRF-3, IRF-5, and IRF-7 contribute to macrophage resistance to DENV infection and that despite the lack of all three of these transcription factors, BMDMs still make low levels of type I IFN that contribute to DENV resistance.

**Figure 1. Lethality and Clinical Disease Manifestations after DENV Infection**

(A–C) WT, DKO, TKO, and Ifnar1-/- mice were infected with \( 5 \times 10^6 \) FFU of DENV2 strain 221 with antibody-dependent enhancement (ADE), and lethality (A), percent weight loss (B), and clinical disease scores (C) were assessed daily for 10 days. Survival was compared between all groups and Ifnar1-/- mice showed less survival than all other groups tested (log-rank test, \( p < 0.0001 \)). Data are expressed as mean percent weight loss and clinical scores that were compared daily by one-way ANOVA or non-parametric (Kruskal-Wallis) ANOVA with multiple correction, respectively.

(D–G) Levels of viral RNA in the serum (D), spleen (E), kidney (F), and liver (G) at 24 and 72 hr p.i. were determined by qRT-PCR. Data are presented as mean log_{10} viral GE per mL of serum or per copy of 18S ribosomal RNA of tissues for six to eight mice from two independent experiments. The lower limit of detection is denoted by a dotted line. Viral titers between the gene-deficient and WT mice were compared by ANOVA with Tukey’s multiple comparisons tests. Asterisks indicate differences that are statistically significant (**p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05).
revealed substantial differences in gene expression patterns among mouse genotypes (Figures 3A and 3B). A chord diagram that depicts connections between individual genes contained in multiple groups demonstrates a broader scope of gene upregulation at 6 hr p.i. (fold change [FC] >1.5 and false discovery rate [FDR] <0.05) in splenocytes from WT (262 genes) and DKO (472 genes) mice than in those from TKO (55 genes) and FFR (2 genes) mice, with minimal overlap in induced genes between genotypes (Xia et al., 2014, 2015) (Figure 3A). A broader and more overlapping gene signature (FC >2 and FDR <0.05) was evident after 24 hr of infection in all genotypes (Figure 3B).

Clustering of differentially expressed genes (FC >2 across time points or genotypes) identified three clusters (clusters 1–3) showing temporal and genotype-specific patterns of expression (Figure 3C). Cluster 3 is composed of genes strongly induced by 6 hr p.i. and throughout the response of WT mice, whereas clusters 1 and 2 are composed of genes strongly (cluster 1) or moderately (cluster 2) induced in DKO and TKO mice in a more delayed manner. Gene ontology and pathway analysis of cluster-3-containing genes were enriched for terms related to classical antiviral responses, including viral PRRs and type I IFN stimulation (Figure 3D). In contrast, clusters 1 and 2 demonstrated enrichment for ontologies and pathways related more generally to inflammatory processes such as immune responses, response to wounding, apoptosis, and cytokine signaling.

Individual gene analysis of upregulated genes in cluster 3 identified type I IFN genes (Ifnb1, Ifna4, and Ifna2) in addition to well-characterized ISGs involved in viral recognition and restriction (Mx and Oas genes, Ddx58/RIG-I, Isg15, Eif2ak2/PKR, and Rsad2/Viperin) (Figure 3E). Within cluster 3, type I IFN genes (Ifnb1, Ifna4, and Ifna2) were strongly induced in Ifnar1−/− mice, but there was little to no induction of ISGs, as expected. Additionally, there was mild upregulation of some ISGs (Ddx58, Isg15, Eif2ak2, and Oas1b) in cluster 3 in DKO and TKO mice at 24 hr despite no detectable upregulation of type I IFN encoding genes.

Cluster-1-containing genes were strongly upregulated in DKO and TKO mice, but not in WT or Ifnar1−/− mice, at 24 hr p.i.; these included AP-1 transcription factors (Fos andJun) and signal-dependent transcription factors (SDTFs) (Stat1 and Irf1) that regulate innate immune responses and the pro-inflammatory cytokines IL-6, tumor necrosis factor (TNF-α), IL-1β, and IFN-γ. Additionally, many components of the IL-12/IFN-γ signaling axis involved in macrophage host defense, including Irf1, Ifng, Il12b, and Il12rb1, as well as multiple IFR1 target genes (Ij12b, Il12rb1, Nos2, Tap1, Cdkn1a, Gbp2, Socs1, Tnf, and Il6), were present in cluster 1 (Honda and Taniguchi, 2006; Kano et al., 2008; Maruyama et al., 2003; Brien et al., 1995; Madonna et al., 2010; Sancéau et al., 1995; Vila-del Sol et al., 2008). These data are consistent with prior findings in DENV-infected DKO mice that showed higher levels of circulating IFN-γ, IL-6, IL-12p70, and TNF-α after 24 hr (Chen et al., 2013). University of California Santa Cruz (UCSC) browser shots of genes from clusters 3 (Mx1) and 1 (Ifng and Irf1) demonstrate the distinct patterns of expression in WT compared to DKO and TKO mice (Figures 3F, S2B, and S2C).
De novo motif enrichment analysis of promoters from cluster 1 genes identified motifs known to bind IRF-1 and nuclear factor κB (NF-κB) as the most enriched sequences (Figure 3G). In contrast, analysis of cluster 3 genes identified the IFN-stimulated response element (ISRE) motif, where ISGF3 binds and promotes ISG expression following type I interferon (IFN-α/β) activation.
IFN signaling, as the most enriched sequence in promoter proximal regions.

A first-order interaction network based on genes significantly upregulated (FC > 2 and FDR < 0.05) in both DKO and TKO at 24 h.p.i. identified Irf1, Irf8, Jun, Fos, Cebpb, Stat3, and Myd88 as hub nodes with high degrees of connectivity within the network (Figure S3A). Analysis of upregulated genes within the network showed enrichment for the reactome categories immune response (57 nodes, p = 5.18e-18) and IFN signaling (14 nodes, p = 1.13e-8). The immune response module contained many of the same hubs as the original network, with the addition of NFKB1 and RELA showing high degrees of centrality in this subnetwork (Figure S3B). Similar to the cluster-based analysis, the IFN response subnetwork contained many genes involved in the IL-12/IFN-γ signaling axis, including Irf8, Irf1, Ifng, Il12rb1, and Il12b (Figure S3C).

In addition to the RNA-seq analysis, we measured Ifnb1, Mx1, Ifng, Irf1, and Nos2 expression in splenocytes from mock- and DENV-infected WT and TKO mice by qRT-PCR. We detected a small but inconsistent upregulation of Ifnb1 in splenocytes from DENV-infected TKO mice (Figure 3H). Consistent with the RNA-seq results, we detected significantly more induction of Mx1 in DENV-infected splenocytes from WT mice than TKO mice but greater upregulation of Ifng, Irf1, and Nos2 in splenocytes from TKO mice relative to WT animals (Figure 3I).

Taken together, our RNA-seq and qRT-PCR analyses of differential gene expression provided the anticipated result that WT mice upregulate type I IFN genes and activate a robust and early classical ISG response, whereas Irfar −/− mice induce genes encoding type I IFNs but cannot produce ISGs. In comparison, DKO and TKO mice appeared to mount a low-level type I IFN response with a robust induction of an alternative inflammatory transcriptional program that includes the IL-12/IFN-γ/IRF-1 signaling axis and NF-κB signaling pathways. Since DKO and TKO mice seemed to activate these alternative programs and Irfar −/− mice do not, we hypothesized that the IL-12/IFN-γ/IRF-1 responses could contribute to protection against lethal DENV infection.

**IL-12 and IFN-γ Contribute to DENV Resistance in TKO Mice**

Activated macrophages and dendritic cells can secrete IL-12, which promotes the development of Th1 responses and induces IFN-γ secretion by NK and T cells. As the genes encoding the IL-12p40 subunit, IL-12 receptor, and IFN-γ were upregulated in splenocytes from DENV-infected DKO and TKO mice, we hypothesized that IL-12 contributed to IFN-γ production and resistance to severe DENV disease in TKO mice. To determine the contribution of IL-12 to DENV resistance in TKO mice, we injected IL-12p40 neutralizing antibody (IL12p40Ab) or isotype control antibody (Ctrl Ab) into TKO mice infected with DENV and monitored mortality, weight loss, and clinical disease score (Figures 4A–4C). In two experiments, 50% of TKO mice injected with IL12p40Ab died (p = 0.055) and manifested significantly increased weight loss starting at day 5 p.i. and a worse clinical score at day 8 compared to isotype-control-injected TKO mice (Figures 4B and 4C), demonstrating a role for IL-12 in protection against DENV infection in TKO mice.

To test the hypothesis that IFN-γ has a key role in TKO resistance to DENV-induced mortality, we injected Irfar −/− mice and two groups of TKO mice, one injected with an IFN-γ neutralizing antibody (IFNγAb) and the other with Ctrl Ab, with DENV and monitored mortality, weight loss, and clinical score (Figure 4D–4F). All TKO mice injected with IFNγAb succumbed to DENV infection, whereas those injected with Ctrl Ab survived (Figure 4D). Additionally, TKO mice injected with IFNγAb showed markedly increased weight loss (after day 2) and higher clinical disease scores (after day 4) than TKO mice injected with Ctrl Ab (Figures 4E and 4F). Although 100% of TKO mice treated with IFNγAb and Irfar −/− mice died following DENV infection, there was a difference in the survival curves, with Irfar −/− mice dying more rapidly (p < 0.0001, median survival: 4 days for Irfar −/− and 7 days for TKO + IFNγAb). Moreover, IFNγAb-administered TKO mice showed a small delay in weight loss (day 4, p < 0.0001) and in clinical disease score (days 3 and 4, p < 0.01 and p < 0.05, respectively) compared to Irfar −/− mice (Figures 4E and 4F), suggesting the existence of another pathway besides IFN-γ in protection against DENV infection in TKO mice.

As splenocytes from DENV-infected TKO mice showed modest upregulation of typical type I ISG genes by RNA-seq at 24 h, we next evaluated whether type I IFN signaling contributed to DENV resistance in TKO mice. We infected Irfar −/− and TKO mice that were injected with IFNARAb alone or in combination with IFNγAb and measured DENV-associated morbidity and mortality (Figures 4G–4I). Treatment with IFNARAb alone did not increase DENV-associated mortality in TKO mice, but the combination of IFNARAb and IFNγAb resulted in 100% mortality, with a survival curve identical to Irfar −/− mice (Figure 4G). Although TKO mice injected with IFNARab alone survived infection, they manifested similar degrees of weight loss as Irfar −/− and TKO mice receiving both IFNARAb and IFNγAb for 3 days after infection before showing differential weight gain (Figure 4H). Similarly, IFNARab-administered TKO mice demonstrated clinical disease comparable to TKO mice receiving both IFNARab and IFNγAb throughout the experiment (Figure 4I). Collectively, these data indicate that IFN-γ and, to a lesser degree, type I IFNs are key components in the IRF-3, IRF-5, and IRF-7-independent resistance pathway against severe DENV infection.

**IRF-1 Is Required for Resistance to Severe DENV Infection in TKO Mice and II12b Induction in DENV-Infected Macrophages**

The transcription factor IRF-1 can contribute to both type I and II IFN signaling and is an important regulator of the immune response to many viral infections (Brien et al., 2011; Saikowsky et al., 2000; Kimura et al., 1994; Dutia et al., 1999; Taniguchi et al., 2001). Based on the RNA-seq analysis that identified Irf1 and many IRF-1 target genes as significantly upregulated in TKO mice during DENV infection, we hypothesized that IRF-1 was an important component of the IRF-3-, IRF-5-, and IRF-7-independent pathway of resistance to severe DENV infection. To test this idea, Irf1 −/−, Irf3 −/−, Irf5 −/−, or Irf7 −/− QKO mice were generated for in vitro and in vivo DENV infection experiments. DENV infection in vitro demonstrated...
that QKO macrophages sustain higher viral replication than TKO cells at 24, 48, and 72 hr p.i., which approaches but does not completely recapitulate the permissiveness seen in Ifnar1−/− cells (Figure 5A). WT and Ifnar1−/− macrophages infected with DENV upregulated Ifnb1 and Ifna4 gene expression significantly more than TKO or QKO macrophages (Figures 5B and S4A). Although WT cells induce Nos2, a typical IRF-1 target gene, TKO macrophages expressed more Nos2 during DENV infection than Ifnar1−/− or QKO macrophages (Figure 5C). Upregulation of Nos2 appeared to be completely IRF-1 dependent in the TKO background, as QKO cells expressed little to no Nos2 transcript. In contrast to all other genes tested, macrophages from TKO mice expressed more Il12b than WT, Ifnar1−/−, and QKO macrophages in response to DENV infection (Figure 5D).

Figure 4. Effects of Blocking IL-12p40 or Type I or II IFN Signaling on TKO Resistance to DENV Infection

(A–C) TKO mice were infected with 5 × 10⁶ FFU of DENV2 strain 221 with ADE and treated with IL-12p40 blocking or isotype control antibodies and lethality (A), percent weight loss (B), and clinical scores (C) assessed daily for 10 days.

(D–I) TKO and Ifnar1−/− mice were infected with 5 × 10⁶ FFU of DENV2 strain 221 and treated with IFN-γ or isotype control monoclonal antibodies. Lethality (D), percent weight loss (E), and clinical disease scores (F) were assessed daily for 10 days. TKO and Ifnar1−/− mice were infected as above and treated with IFNAR blocking, IFNAR and IFN-γ blocking, or isotype control monoclonal antibodies. Lethality (G), percent weight loss (H), and clinical disease scores (I) were assessed daily for 10 days. Survival was compared between all groups by the log-rank test, and significance between groups is indicated by asterisks. Data are expressed as mean percent weight loss and clinical scores that were compared daily by Student’s t test with Holm-Sidak correction (B and C) for multiple comparison or one-way ANOVA (E and H) or non-parametric (Kruskal-Wallis) ANOVA with multiple correction (F and I). Asterisks indicate statistically significant differences (****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05).
with survival curves, weight loss, and clinical scores virtually identical to Ifnar1−/− mice (Figures 5E–5G). These results demonstrate that IRF-1 is essential for the IRF-3, IRF-5, and IRF-7-independent pathway responsible for resistance to severe DENV infection. DENV-infected WT mice produced significantly more serum IFN-β and expressed higher levels of Mx1 in splenocytes at 8 and 24 hr p.i. than TKO and QKO mice (Figures 5H and S4C). In the majority of TKO and QKO mice, IFN-β was not detectable during DENV infection. In contrast, significantly higher levels of circulating IL-12p70 and IFN-γ as well as splenocyte upregulation of Ifng, Il12b, Irf1, and Nos2 were found at 24 hr p.i. in TKO mice (Figures 5I, 5J, and S4D–S4G).
DISCUSSION

This study demonstrates that mice deficient in key SDTFs regulating type I IFN secretion (IRF-3, IRF-5, and IRF-7) remain resistant to severe DENV infection. Splenocytes from DKO and TKO mice, but not the highly susceptible Ifnar1−/− mice, predominantly activate a transcriptional program compatible with the IL-12/IFN-γ/IFN-1 signaling pathway following DENV infection, despite similar levels of baseline IRF-1 gene expression in these three mouse strains. Consistent with the importance of this pathway in the response to DENV, we found that inhibiting IL-12 or IFN-γ signaling with blocking antibodies or removal of IRF-1 genetically was sufficient to increase the severity of DENV disease in TKO mice. In vitro, DENV infection in TKO BMDMs caused an IRF-1-dependent increase in the production of IL-12, a gene that encodes for the IL-12p40 subunit of IL-12, which is secreted by macrophages and promotes IFN-γ production by NK and T cells. Additionally, both TKO mice and macrophages in vitro activate a low-level type I IFN response that acts in concert with the IL-12/IFN-γ/IFN-1 pathway to protect against DENV infection. Collectively, our data demonstrate that IRF-1 stimulates both an IL-12/IFN-γ and, to a lesser extent, a type I IFN pathway in macrophages that protects against lethal DENV infection in the absence of the canonical transcription factors IRF-3, IRF-5, and IRF-7.

As Ifnar1−/− mice are highly susceptible to DENV infection, TKO mice were not expected to survive DENV challenge. However, previous viral infections in TKO mice did not show uniform results. Whereas WNV infection of TKO and Ifnar1−/− mice produced similar mortality, murine norovirus (MNoV) infection showed delayed mortality in TKO compared to Ifnar1−/− mice (Lazear et al., 2013). It remains unclear whether a similar IL-12/IFN-γ alternative pathway was stimulated in TKO mice infected with WNV and MNoV. DKO mice infected with chikungunya virus (CHIKV), another positive-sense, single-stranded RNA virus, produced massive quantities of IFN-γ (Rudd et al., 2012), demonstrating that stimulation of IFN-γ responses in DKO mice is not unique to DENV. In the case of MNoV, both IRF-1 and IFN-γ contribute to macrophage resistance to infection, and thus, if activated, the alternative pathway of IFN-γ activation may explain the difference in MNoV-induced mortality in TKO and Ifnar1−/− animals (Maloney et al., 2012). We speculate that the susceptibility of TKO mice to viral infections will depend on part whether the alternative IFN-γ response is activated and the relative susceptibility of different viruses to type I and II IFN responses.

IFN-γ is less effective at mediating viral clearance from both lymphoid and nonlymphoid tissues than type I IFNs yet still protects mice from lethal DENV infection. The finding that IFN-γ can protect TKO mice against DENV infection is consistent with previous studies demonstrating that mice with combined deficiency of IFNAR and IFNGR are more susceptible to DENV-associated mortality than animals with either defect alone (Shresta et al., 2004). Blocking IFN-γ signaling in DENV-infected TKO mice resulted in 100% mortality, whereas blocking IL-12 led to 50% mortality. Although IL-12 is a major stimulator of NK and T cell IFN-γ production, IL-12 blockade may produce a less dramatic phenotype than IFN-γ inhibition, because other cytokines (e.g., IL-2, IL-15, IL-18, and type I IFNs) can stimulate an IFN-γ response (Schoenborn and Wilson, 2007; Okamura et al., 1995, 1998). Despite the essential role of IFN-γ for survival of TKO mice during DENV infection, type I IFNs still contribute significantly to DENV resistance, as anti-IFNAR blockade in combination with IFN-γ blockade in TKO mice exacerbated mortality to levels comparable to Ifnar1−/− mice. Additionally, blocking IFNAR alone during DENV infection of TKO mice resulted in significant disease, as evidenced by the degree of weight loss and increased clinical scores. Residual activation of type I IFNs in TKO macrophages was detected in vitro by monitoring expression of IIfb1 and Mx1, a classical ISG. As we did not detect significant upregulation of IFN-γ in TKO BMDMs infected with DENV, we suspect that low-level type IFN activation, along with potential direct activation of IRF-1 target genes such as Nos2, may explain the difference in resistance to DENV infection in vitro. This is supported by in vitro experiments showing that the addition of IFNARAb increased the susceptibility of TKO BMDMs to DENV infection.

We observed a shift from robust type I IFN production in WT and Ifnar1−/− mice to a significant type II IFN response in DKO and TKO mice. This finding is consistent with previous publications showing that IFR-3 can act as a molecular switch regulating Ifnar1−/− and a type II IFN response. Many clinically important viruses, including DENV, herpes viruses, and the filoviruses Ebola and Marburg, suppress IFR-3 and/or IRF-7 to antagonize IFN signaling and inhibit antiviral immunity (Barro and Patton, 2007; MacMicking, 2012; Dalrymple et al., 2015; Devaraj et al., 2007; Zhu et al., 2002; Angleró-Rodríguez et al., 2014; Cloutier and Flamand, 2010; Ronco et al., 1998; Wang et al., 2009; Chang et al., 2009; Hahn et al., 2005; Basler and Amarasinghe, 2009). Future studies will be needed to investigate whether the IFR-3, IRF-5, and IRF-7 alternative pathway of type II IFN production is activated during these infections in humans and how it may impact pathogenesis and clinical disease manifestations.

In summary, the present study revealed the existence of a late-acting IRF-3-, IRF-5-, and IRF-7-independent pathway of innate immunity that protects against lethal viral infection. This pathway is mediated by IRF-1 activation of both type I and II IFNs, with a predominant role for the IL-12/IFN-γ signaling axis. Our work is consistent with recent studies revealing that multiple transcription factors exist to regulate the IFN system, which operates in a time-, cell-type-, host-species-, or virus-specific manner. Because of the effectiveness of immune evasion mechanisms by specific viruses, these non-canonical pathways of the IFN system may be important depending on the virus and the host cell type or species.

EXPERIMENTAL PROCEDURES

Cells and Viruses

C6/36 Aedes albopictus mosquito cells were maintained in Leibovitz’s L15 medium (Invitrogen,) supplemented with 10% fetal bovine serum (FBS; Gemini
Bio Products), penicillin, streptomycin, and HEPES (all from Invitrogen) at 28°C in the absence of CO₂. S221 is a plaque-purified virus strain derived from the Taiwanese DENV2 clinical isolate PLO46 (obtained from Dr. Huan-Yao Lei, National Cheng Kung University, Taiwan). The virus was propagated in C6/36 cells and quantified based on genomic equivalent (GE) by real-time RT-PCR and FFUs as previously described (Yauch et al., 2009).

Mice and Infections

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DKO and TKO mice have been described previously (Lazzeri et al., 2013; Daffis et al., 2009) and were derived from single-knockout mice provided by Dr. Tadatsugu Taniguchi (The University of Tokyo). Irf1−/− mice (Matsuyama et al., 1993) were also obtained from Dr. Taniguchi. Irf1−/− mice were obtained from Dr. Wayne Yokoyama (Washington University, St. Louis, MO) via Dr. Carl Ware (La Jolla Institute for Allergy and Immunology). All knockout mice were on the C57BL/6 genetic background. Male and female mice age 5 to 6 weeks were infected intravenously with 5 × 10⁶ FFU of DENV S221 under conditions of antibody-dependent enhancement (ADE) (Zellweger et al., 2010). For ADE, mice were injected with 10 μL immune serum 1 hr prior to DENV infection. Immune serum was generated by inoculating Irf1−/− mice with DENV2 strain PLO46 (1 × 10⁶ plaque-forming units [PFU]), followed by harvesting of serum on day 30 p.i. The presence of DENV-specific immunoglobin G (IgG) was checked via ELISA and western blotting, and the absence of DENV by qRT-PCR and FFA. DENV2 strain PLO46 replicates but does not cause disease in these mice; virus is cleared by day 5–7 p.i. Mice were weighed and scored for development of clinical signs on a daily basis. All animal experiments were approved by the Animal Care Committee at La Jolla Institute for Allergy and Immunology.

Measurement of DENV Burden in Mice

Mice were euthanized via isoflurane inhalation at different time points after infection and perfused with 30 mL PBS. Blood was collected via cardiac puncture, and viral RNA in serum was extracted using the QiAamp viral RNA Mini kit (QIAGEN). Tissues were harvested into RNAlater (QIAGEN) and homogenized in RLT lysis buffer (QIAGEN) for 3 min using TissueLyser (QIAGEN) according to the manufacturer’s instruction. Total RNA in tissues was extracted using the RNeasy Mini kit (QIAGEN). Extracted RNA was stored at −80°C until analysis. qRT-PCR of DENV viral RNA in serum and tissues was performed as previously described (Yauch et al., 2009). A standard curve was generated with known concentrations of DENV2 or 18S RNA. Virus titers in serum were expressed as the log₁₀ DENV2 GE/mL, and those in tissues were expressed as log₁₀ DENV2 GE relative to 18S.

Blockade of Cytokines and Chemokines In Vivo

A dose of 0.5 mg functional grade (azide-free, sterile filtered, and with endotoxin levels <0.2 EU/mg) anti-mouse IFN-γ antibody (clone XMG1.2), anti-mouse IFNAR1 antibody (clone MAR1-5A3), anti-IL-12p40 (clone C17.8), or isotype-matched control antibodies (all from Bio X Cell, West Lebanon, NH) was administered via an intraperitoneal (i.p.) route and survival monitored for 10 days after infection.

BMDM Culture and Infection

To generate BMDM, bone marrow from femur and tibia of 5- to 7-week-old mice were isolated and cultured for 6–8 days in RPMI 1640 (10% fetal bovine serum, penicillin, streptomycin, HEPEX, and sodium pyruvate) in the presence of 25 ng/mL murine macrophage-stimulating factor (M-CSF) (PeproTech). On day 6–7 of culture, cells were re-plated at 500,000 cells per well in a 12-well plate and infected with DENV2 strain S221 under ADE conditions (MOI = 0.5 + 0.083% [v/v] immune serum from DENV-infected Irf1−/− mice). Cells were harvested at 0, 4, 24, 48, and 72 hr p.i. Infectious titer was determined by focus forming assay (FFA) on BHK21 cells. For IFNAR blocking studies, an IFNARAb (clone MAR1-5A3) or isotype-matched control antibody was added to BMDMs at 25 μg/mL 12 hr prior to infection.

Serum Cytokine quantification by ELISA: Mice were sacrificed and blood removed by cardiac puncture into Z-Gel tubes that were then centrifuged at 13,200 RPM for 15 min. Serum was aliquoted into single use vials and stored at −80°C. Serum IL-12p70 (mouse IL-12p70, 433604, BioLegend), IFN-γ (mouse IFN-γ, 430804, BioLegend), and IFN-β (high sensitivity mouse IFN-β PBL 42140 L) were measured using ELISA per manufacturer instructions and quantified by generating standard curves in GraphPad Prism (GraphPad Software).

pQCR

Total RNA was isolated by (Zymo) Quick-RNA MiniPrep treated in column with DNase. RNA was then converted to cDNA by reverse transcription using SuperScript III First-strand synthesis kit (Invitrogen, 18080051). qPCR (SYBR GreenER, 172-5125, Bio-Rad) analysis was performed on an Applied Biosystems 7300 real-time PCR system (Invitrogen).

RNA-Seq Library Preparation and Sequencing

For RNA sequencing analysis, total RNA was extracted from spleen tissue using an RNeasy minikit (QIAGEN, 217004). Polyadenylated RNA was isolated using the Poly(A) Purist MAG kit (Ambion, AM1922) and subsequently fragmented using RNase III. 25 ng of each sample was used in the SOLiD Total RNA-Seq Kit (Ambion, 4453374), per the manufacturer’s instructions, to generate barcoded, strand-specific SOLiD libraries. The libraries were then converted into SOLiD Wildfire compatible fragments using the 5500 WF Conversion Primer Kit (PN 4478020) and the protocol supplied by Life Technologies (PN 4477188). Sequencing was performed with a SOLiD 5500FW sequencer (Life Technologies), producing between 36 and 190 million single-end reads with 35 nt lengths.

Data Analysis

Fastq files from RNA-seq experiments were mapped to mm10 using spliced transcripts alignment to a reference (STAR) (Dobin et al., 2013; Heinz et al., 2010) with default parameters and gene expression analyzed using hypergeometric optimization of motif enrichment (HOMER) (Heinz et al., 2010) with the parameter analyzeRepeats ma mm10 ~count exons ~noad ~pc 3 ~condenseGenes followed by edgeR normalized abundance measurements. Subset-specific expression was defined with a 2-fold difference in expression between two experimental groups or time points. Genes with less than 16 tag counts in all groups were defined as not expressed. To map subset-specific peaks to gene expression, only expressed genes were considered and used for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation analysis with Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang et al., 2009a, 2009b). Cluster analysis of all differentially expressed genes was performed using Cluster 3 (de Hoon et al., 2004) and Java Tree View. Number of clusters to be used for k-means clustering was determined by analyzing significant expression pattern changes using edgeR. To identify enriched transcription-factor-binding motifs, findMotifs.pl in HOMER was used with the standard background from position −300 to +50 relative to transcription start site. Chord diagrams and network analysis was performed on significantly upregulated genes at 6 hr p.i. (FC >1.5 and FDR <0.05) and 24 hr p.i. (FC >2 and FDR <0.05) compared to baseline in each genotype using NetworkAnalyst (Xia et al., 2014, 2019).

Statistical Analysis

Survival curves were analyzed using the log-rank (Mantel-Cox) test. Ordinary one-way ANOVA with Tukey’s correction for multiple comparison was used to compare genotypes on individual days for percent weight loss and tissue and cell culture viral loads. Non-parametric (Kruskal-Wallis) ANOVA with multiple correction was used to analyze in vivo clinical scores and in vitro qPCR data. Statistical analysis was conducted using GraphPad Prism (GraphPad Software) or Python. Values of p < 0.05 were considered significant.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq data reported in this paper is GEO: GSE104189.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.10.054.

AUTHOR CONTRIBUTIONS


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