Type I Interferon Signals in Macrophages and Dendritic Cells Control Dengue Virus Infection: Implications for a New Mouse Model To Test Dengue Vaccines

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ABSTRACT

Dengue virus (DENV) infects an estimated 400 million people every year, causing prolonged morbidity and sometimes mortality. Development of an effective vaccine has been hampered by the lack of appropriate small animal models; mice are naturally not susceptible to DENV and only become infected if highly immunocompromised. Mouse models lacking both type I and type II interferon (IFN) receptors (AG129 mice) or the type I IFN receptor (IFNAR^{-/-} mice) are susceptible to infection with mouse-adapted DENV strains but are severely impaired in mounting functional immune responses to the virus and thus are of limited use for study. Here we used conditional deletion of the type I IFN receptor (IFNAR) on individual immune cell subtypes to generate a minimally manipulated mouse model that is susceptible to DENV while retaining global immune competence. Mice lacking IFNAR expression on CD11c⁺ dendritic cells and LysM⁺ macrophages succumbed completely to DENV infection, while mice deficient in the receptor on either CD11c⁺ or LysM⁺ cells were susceptible to infection but often resolved viremia and recovered fully from infection. Conditional IFNAR mice responded with a swift and strong CD8⁺ T-cell response to viral infection, compared to a weak response in IFNAR^{-/-} mice. Furthermore, mice lacking IFNAR on either CD11c⁺ or LysM⁺ cells were also sufficiently immunocompetent to raise a protective immune response to a candidate subunit vaccine against DENV-2. These data demonstrate that mice with conditional deficiencies in expression of the IFNAR represent improved models for the study of DENV immunology and screening of vaccine candidates.

IMPORTANCE

Dengue virus infects 400 million people every year worldwide, causing 100 million clinically apparent infections, which can be fatal if untreated. Despite many years of research, there are no effective vaccine and no antiviral treatment available for dengue. Development of vaccines has been hampered in particular by the lack of a suitable small animal model. Mouse models used to test dengue vaccine are deficient in interferon (IFN) type I signaling and severely immunocompromised and therefore likely not ideal for the testing of vaccines. In this study, we explored alternative models lacking the IFN receptor only on certain cell types. We show that mice lacking the IFN receptor on either CD11c- or LysM-expressing cells (conditional IFNAR mice) are susceptible to dengue virus infection. Importantly, we demonstrate that conditional IFN receptor knockout mice generate a better immune response to live virus and a candidate dengue vaccine compared to IFNAR mice and are resistant to subsequent challenge.

Dengue virus (DENV, a member of the *Flaviviridae* family, is a mosquito-borne pathogen that infects approximately 400 million people every year (1, 2). Each of the four DENV serotypes causes a spectrum of clinical symptoms ranging from mild fever to potentially fatal manifestations of dengue shock syndrome. DENV causes an acute infection with high fever, which usually resolves after 5 to 7 days. At this time, most patients have cleared the high virus load. Intriguingly, however, this is also the time point when some patients start to develop vascular leakage, which, if untreated, can lead to a collapse of the metabolism and organ failure. The frequency, severity, and geographical spread of cases has increased over the past decades (3, 4), and DENV infection is now considered a leading cause of morbidity in the tropics.

There are no effective treatments for dengue fever, and the development of a vaccine has been hampered by the lack of suitable small animal models. Wild-type (wt) mice are not susceptible to infection with field strains of DENV, and while viral replication

in these animals can be forced by intracranial injections of high-titer mouse-adapted DENV strains, the resulting clinical disease bears little resemblance to dengue fever in humans. Humanized mice, which are engrafted with human progenitor cells, provide a system to study human T-cell responses *in vivo*. However, it has been difficult to generate a sustained antibody production (reviewed in reference 5). Nonhuman primates (NHPs) have been used to test candidate dengue vaccines but do not show clinical

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symptoms upon challenge. In addition, the ethical issues, biocontainment requirements, and high cost of using NHPs serve to highlight the urgent need for a rodent model.

Interferons (IFNs) are critical mediators of protection against viral infection, and mice lacking the receptors for both alpha/beta IFN (IFN- α/β) (type I IFNs) and IFN- γ (type II IFN) (AG129 mice) have been successfully infected with DENV. The AG129 model has been used to study dengue pathogenesis and to test antiviral compounds and therapeutic antibodies (6–9). However, the complete absence of IFN signaling renders these mice profoundly immunodeficient (reviewed in reference 5), which means data generated in these animals must be interpreted with caution. A recent report proposed an improvement to the AG129 model; mice lacking the IFN- α/β receptor but with intact IFN- γ signaling were highly susceptible to lethal infection with a mouse-adapted dengue virus strain and thus were proposed by the authors to be more useful for vaccine testing than the AG129 mice (10). However, the potential impact of a global lack of type I IFN receptor (IFNAR) signaling on the immune response to both dengue virus infection and vaccination cannot be overlooked.

Studies using knockout mice have revealed that type I and II IFNs play important but distinct roles in host defense against DENV. As well as the AG129 mice (11), mice lacking signaling elements downstream of the IFN receptors (IFNRs) (STAT1/2), and STAT1 IFNAR double knockout mice are all highly susceptible to DENV infection (12, 13). Mice deficient in just the type I IFN receptor are also susceptible to high-titer DENV challenge (14), while mice lacking the type II IFN receptor alone can resist even large doses of DENV without developing significant viremia or pathology (14, 15). Besides being important for the initial clearance of the virus, type I IFNs also enhance B cell responses, Ig class switch (16–18), cell migration (19, 20), cross-presentation (21–24), CD4⁺ T-cell activation (20, 25, 26), and cytotoxic T-lymphocyte (CTL) expansion (27–29). Therefore, any new mouse model for dengue should retain maximal interferon competence while achieving sufficient susceptibility to infection to be of practical use for immunological study of disease and vaccine screening.

In pursuit of this aim, we made use of a series of mice lacking the type I IFNR on different subsets of immune cells and compared their responses to DENV2 infection and immunization with a candidate subunit vaccine. We demonstrated that deletion of the type I IFN receptor on both LysM- and CD11c-expressing cells (predominantly macrophages and dendritic cells, respectively) renders mice susceptible to fatal DENV infection, whereas type I IFN signaling in CD4⁺ cells is dispensable for the control of DENV-2. Intriguingly, mice lacking the type I IFN receptor (IFNAR) on either LysM⁺ or CD11c⁺ cells were susceptible to disease but frequently recovered by 15 days postinfection (p.i.); furthermore, immunization of either of these mice with a dengue virus EDIII-capsid (EDIIC-2) subunit vaccine (30, 31) induced high titers of dengue-specific neutralizing antibodies. Therefore, we have developed a novel model which has revealed that type I IFN receptor signaling in CD11c- and LysM-expressing cells has important and nonredundant roles in determining susceptibility to dengue. Moreover, our minimally manipulative approach renders this model highly appropriate for *in vivo* screening of DENV vaccine candidates, which could facilitate development of effective prophylactic interventions for use in humans.

MATERIALS AND METHODS

Cells and virus. BHK-21 and C6/36 cells were purchased from the American Type Culture Collection (<http://www.atcc.org>). U937 cells expressing DC-SIGN were obtained by lentiviral transfection and subsequent cell sorting. All cells were maintained in minimal essential medium supplemented with fetal bovine serum (5% to 10%).

For challenge experiments, dengue virus TSV01 or D2Y98P produced in C6/36 cells was used. For CD8⁺ T-cell experiments, TSV01 virus was used.

Mice. Female or male 6- to 8-week old IFN- $\alpha/\beta/\gamma$ receptor-deficient (AG129) and wt Sv129 mice were purchased from B&K Universal Limited with permission from M. Aguet (ISREC, School of Life Sciences Ecole Polytechnique Fédérale [EPFL]). LoxP-flanked *ifnar1* (*ifnar1*^{fl/fl}) (4) animals were bred with mice that express Cre recombinase specifically in T cells (CD4-Cre), macrophages, (LysM-Cre) (32), or CD11c⁺ dendritic cells (CD11c-Cre) (33). All of these mice, including IFN- α/β receptor-deficient mice (IFNAR^{-/-}), on a C57BL/6 background, were provided by Ulrich Kalinke. All mice were bred and kept under specific-pathogen-free conditions at the Biomedical Resource Centre, Singapore. For the generation of bone marrow chimeras, recipient mice were irradiated with 950 Gy from a Cs source. Twenty-four hours later, femurs and tibiae from donor mice were flushed with a syringe to isolate bone marrow cells. A total of 3×10^6 cells were transferred into irradiated recipient mice intravenously. Mice were kept in individually ventilated cages and used between 6 and 10 weeks of age. The mouse experiments were conducted according to the rules and guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR), Singapore. The experiments were reviewed and approved by the Institutional Review Board of the Biological Resource Center, Singapore (IACUC protocols 100566, 120801, and 060191).

Flow cytometry-based neutralization assay. The flow cytometry-based neutralization assay was performed as described previously (34). Briefly, 4G2 or heat-inactivated serum/plasma was serially diluted, and a constant amount of virus was added. The antibody-virus mixture was incubated at 37°C for 30 min and then added to U937-DC. After incubation overnight, the infected cells were harvested, washed in phosphate-buffered saline (PBS), and fixed and permeabilized with Cytofix/Cytoperm (BD). The percentage of infected cells was quantified by flow cytometry detecting intracellular E protein with Alexa Fluor 647-labeled 4G2 antibody. Data were analyzed with GraphPad Prism software for the calculation of the 50% neutralization titer (NT₅₀).

Immunization and infection of mice. All mice were infected via the intraperitoneal (i.p.) route. AG129, Sv129, and bone marrow chimeras were infected with 5×10^6 PFU of TSV01. IFNAR- and conditional IFNAR knockout mice were infected with 1×10^7 PFU of D2Y98P (35, 36). For survival experiments, the weights of the mice were monitored daily, and mice with loss of 20% of initial weight were considered moribund. EDIIC-2 was expressed in *Escherichia coli* and purified as described previously (31). Twenty micrograms of EDIIC-2 or placebo (preparation containing synthetic oligonucleotide containing unmethylated CpG dinucleotides [ODN] and alum only) was injected per immunization.

IgG ELISA. Ninety-six-well polystyrene plates were coated with concentrated, UV-inactivated dengue virus. Plates were incubated overnight at 4°C. Before use, plates were washed three times in PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T). Nonspecific binding was blocked with 2% nonfat dry milk diluted in PBS (PBS-M) for 2 h at room temperature. After washing, sera were diluted 1:50 in PBS-M and heat inactivated for 1 h at 55°C, and 3-fold serial dilutions were added to the wells. Plates were incubated for 1 h at room temperature, followed by three washes with PBS-T. Peroxidase-conjugated rabbit anti-mouse IgG in PBS-M was added, followed by 1 h of incubation at room temperature and three additional washes with PBS-T. Tetramethylbenzidine (TMB) was used as the enzyme substrate. The reaction was stopped with 1 M HCl, and the optical densities were read at 450 nm using an automatic enzyme-linked

immunosorbent assay (ELISA) plate reader. Endpoint titers were defined as the lowest dilution of plasma in which binding was 2-fold greater than the mean binding observed with the negative controls.

Determination of cytokines and virus in blood and organs. Virus titers in organs were determined from organs frozen in Trizol after weighing and homogenization. Viral RNA was quantified by real-time quantitative reverse transcription-PCR (qRT-PCR) using primers and methods reported previously (37). Virus titers in blood were determined by standard plaque-forming assay using BHK-21 cells. The limit of detection was 100 PFU/ml. Blood was collected in tubes containing sodium citrate, and plasma was subjected to alanine aminotransferase (ALT) measurement. Mouse tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), IFN- γ , IFN- α , IFN- γ -induced protein 10 (IP-10) (eBioscience), and CXCL1 (KC/murine IL-8 homolog) (R&D Systems) protein levels in plasma were measured by ELISA according to the manufacturers' instructions.

T-cell restimulation. Mice were infected i.p. with 5×10^6 PFU of TSV01. Five drops of blood were collected in 3 ml fluorescence-activated cell sorter (FACS) buffer. Cells were collected by centrifugation, and red blood cells were lysed. Single-cell suspensions were stimulated with peptides (34) for 12 h, together with brefeldin for the last 5 h of stimulation, and cells were subsequently stained for intracellular IFN- γ expression.

Statistical analysis. Statistical tests were performed with GraphPad Prism software, using Student's *t* test, two-way analysis of variance (ANOVA), or chi-square tests as indicated in the figure legends.

RESULTS

Susceptibility to DENV infection in conditional IFNAR knock-out mice. Dengue virus replicates efficiently in mice lacking type I and II IFN receptors, but it is not known in detail which cell types in these mice are the determinants of DENV susceptibility. Using bone marrow chimeras, we first asked whether type I and II IFNAR deficiency on either hemopoietic or nonhemopoietic cells alone was sufficient to enable viral replication. The chimeric mice were injected intraperitoneally (i.p.) with 5×10^6 PFU of the non-mouse-adapted DENV-2 strain TSV01, and blood samples were taken daily for measurement of serum viremia (Fig. 1A). The lack of IFN receptors on nonhemopoietic cells did not lead to measurable viral titers in serum, whereas mice lacking the IFN receptors on hemopoietic cells were effectively infected. Interestingly, while the positive-control mice (AG129 $\rightarrow$ AG129), which lacked IFN receptors on all cells, remained highly viremic at day 6 postinfection (p.i.), those animals deficient in IFN receptors on hemopoietic cells alone controlled their viremia and cleared the virus. As the majority of human infections with DENV cause a similarly limited viremia, this led us to ask whether further refinements to the specificity of IFN receptor deletion might enable development of an improved model of infection.

To further dissect the impact of type I IFN responsiveness in the hemopoietic cell compartment on susceptibility to DENV infection, we generated a set of conditionally gene-targeted mice. Mice expressing a Cre recombinase under different immune cell-type-specific promoters were crossed with mice with a loxP-flanked IFNAR (IFNAR^{fl/fl}), generating mice lacking the type I receptor on CD4-expressing T cells (CD4-Cre^{+/-} IFNAR^{fl/fl}), on LysM⁺ cells, including macrophages, neutrophils, and some dendritic cells (DCs) (LysM-Cre^{+/-} IFNAR^{fl/fl}), on CD11c⁺ conventional dendritic cells (cDCs) (CD11c-Cre^{+/-} IFNAR^{fl/fl}), or on both LysM- and CD11c-expressing cell populations (CD11c-Cre^{+/-} LysM-Cre^{+/-} IFNAR^{fl/fl}). Because these mice retained type II IFN receptors, they would not be susceptible to infection with the DENV-2 strain used in the bone marrow chimera experiments (TSV01) and therefore were infected i.p. with 1×10^7 PFU of

FIG 1 Importance of type I IFN responsiveness on hematopoietic cells for control of dengue virus infection. (A) Bone marrow-chimeric mice (wt $\rightarrow$ AG129, wt $\rightarrow$ wt, AG129 $\rightarrow$ wt, and AG129 $\rightarrow$ AG129) were infected i.p. with 5×10^6 PFU dengue virus strain TSV01. Blood was drawn at indicated time points, and virus in serum was quantified by plaque assay. One representative experiment of a total of two (with 3 to 5 mice per group) is shown. (B and C) Requirement for IFNAR expression on different leukocyte subsets. LysM-Cre^{+/-} IFNAR^{fl/fl}, CD11c-Cre^{+/-} IFNAR^{fl/fl}, CD11c-Cre^{+/-} LysM-Cre^{+/-} IFNAR^{fl/fl}, CD4-Cre^{+/-} IFNAR^{fl/fl}, wt, and IFNAR^{-/-} mice were infected i.p. with 1×10^7 PFU of DENV strain D2Y98P. Mice were monitored daily, and mice with weight loss of more than 20% were considered moribund. Pooled data from 5 experiments are shown, with totals of $n = 21$ for LysM-Cre^{+/-} IFNAR^{fl/fl} mice, $n = 21$ for CD11c-Cre^{+/-} IFNAR^{fl/fl} mice, $n = 10$ for CD11c-Cre^{+/-} LysM-Cre^{+/-} IFNAR^{fl/fl} mice, $n = 14$ for CD4-Cre^{+/-} IFNAR^{fl/fl} mice, $n = 13$ for wt mice, and $n = 19$ for IFNAR^{-/-} mice.

DENV-2 D2Y98P (35, 36). The D2Y98P isolate harbors a mutation in the viral nonstructural protein NS4B, rendering the virus more virulent in mice. Survival (Fig. 1B) and weight (Fig. 1C) of mice were monitored daily over the 13 to 15 days following inoculation. Deletion of the IFNAR on CD4⁺ cells led to only a minimal loss of weight, and all mice survived the infection. In stark contrast, all mice lacking the IFNAR on both LysM⁺ and CD11c⁺ cells rapidly lost weight and died or had to be euthanized by day 5 p.i. However, while the LysM-Cre^{+/-} IFNAR^{fl/fl} and CD11c-Cre^{+/-} IFNAR^{fl/fl} mice rapidly lost weight initially, and some eventually succumbed to the infection, by day 13 or 15, the surviving mice had recovered fully. This showed that the combined action of type I IFN signaling in LysM⁺ and CD11c⁺ cells is responsible for prevention of clinical signs of early DENV infection and that mice lacking the type I IFN receptor in just one of these cell types are susceptible to DENV-2 but maintain sufficient immune competence to resolve the infection.

Dissemination of virus and cytokine production during early dengue virus infection of conditional IFNAR knockout mice. To gain insight into the systemic effects of conditional disruption of type I IFN signaling in immune cells, we infected mice as described above and sacrificed them on day 3 p.i. for measurement of viral titer in organs and serum. Mice lacking the IFNAR on neutrophils and macrophages (LysM-Cre^{+/-} IFNAR^{fl/fl} mice) contained significantly more DENV in their kidneys than CD11c-Cre^{+/-} IFNAR^{fl/fl} mice, while viral titers in blood and other organs were similar (Fig. 2A to G). Surprisingly, the viral titers in blood and organs were also generally similar in groups of mice that would have been significantly more likely to recover from infection (Fig. 1B) (LysM-Cre^{+/-} IFNAR^{fl/fl} and CD11c-Cre^{+/-} IFNAR^{fl/fl}) as they were in groups that would likely have gone on to die by day 5 p.i. (CD11c-Cre^{+/-} LysM-Cre^{+/-} IFNAR^{fl/fl} and IFNAR^{-/-}). This shows that mice lacking IFNAR expression on CD11c- or LysM-expressing cells develop a full systemic viremia in the presence of an otherwise competent immune system, which enables them to resolve infection rather than succumb to disease.

Thus far, we have shown that following DENV-2 inoculation, CD4-Cre^{+/-} IFNAR^{fl/fl} mice have the highest survival rate, followed by CD11c-Cre^{+/-} IFNAR^{fl/fl}, LysM-Cre^{+/-} IFNAR^{fl/fl}, and then CD11c-Cre^{+/-} LysM-Cre^{+/-} IFNAR^{fl/fl} and IFNAR^{-/-} mice. In humans, the factors determining dengue disease severity are undefined, but cytokines are presumed to be important (38–41), and many studies have described increases in inflammatory and regulatory cytokines during DENV infection (33). We therefore asked whether there was any correlation between the levels of specific cytokines in sera at day 3 p.i. and the probability of survival of infected mice. CD4-Cre^{+/-} IFNAR^{fl/fl} and wt mice had low levels of all measured cytokines (Fig. 3A to F), as expected from their low or undetectable viremia (Fig. 2A to G). Accordingly, groups of mice with higher viremia possessed higher overall levels of cytokines in serum. The mice with the highest probability of survival, CD11c-Cre^{+/-} IFNAR^{fl/fl}, had significantly higher serum levels of IL-6 and IFN- γ than both LysM-Cre^{+/-} IFNAR^{fl/fl} and IFNAR^{-/-} mice, suggesting that expression of these cytokines might be associated with survival. Interestingly, sustained IFN- γ levels have been suggested to play a protective role in a human challenge model for dengue virus infection (42). On the other hand, the IFNAR^{-/-} mice, which would all have gone on to die from their infection, had significantly more TNF- α in their sera than CD11c-Cre^{+/-} IFNAR^{fl/fl} mice, confirming previous find-

ings that higher levels of TNF- α are associated with disease severity (41, 43). We also measured serum levels of the murine IL-8 homologue CXCL1/KC, IFN- α , and IP-10. However, no significant differences between the conditional IFNAR and IFNAR^{-/-} mice were observed (Fig. 3D to F). Why double knockout mice did not show the same cytokine profile as IFNAR^{-/-} mice remains to be further studied. The presence of viremia in mice correlated with elevated levels of alanine transaminase (ALT), which is indicative of liver damage (Fig. 3G). In summary, CD11c- and LysM-targeted IFNAR knockout mice developed full systemic viremia following DENV-2 inoculation, which was accompanied by prolific cytokine production. Significantly higher levels of IFN- γ and IL-6 and lower levels of TNF- α were evident in sera from CD11c-Cre^{+/-} IFNAR^{fl/fl} mice and thus warrant further investigation as potential correlates of protection.

Stronger CD8⁺ T-cell responses to dengue virus infection in conditional IFNAR knockout mice compared to IFNAR^{-/-} mice. Type I IFNs are key drivers of T cell responses (22, 25). We therefore sought to test the ability of conditional IFNAR mice to generate DENV-specific CD8⁺ T cells. To this end, we infected mice intraperitoneally with TSV01 and measured IFN- γ ⁺ CD8⁺ T cells in the blood at days 3, 6, and 8 postinfection. Surprisingly, IFNAR^{-/-} mice showed minimal expansion of DENV-specific CD8⁺ T cells. In contrast, wild-type mice and CD11c-Cre^{+/-} IFNAR^{fl/fl} and LysM-Cre^{+/-} IFNAR^{fl/fl} mice responded with a swift increase of virus-specific CD8⁺ T cells, peaking at day 6 p.i. (Fig. 4). Of note, the peak of the CD8⁺ T-cell response correlated with the gain of weight and recovery of surviving mice (Fig. 1C), highlighting the importance of CD8⁺ T cells for viral clearance.

Immune responses of conditional IFNAR knockout mice to a dengue subunit vaccine candidate. While mice lacking the IFNAR on all cells have been used as models of dengue virus infection, the fact that type I IFNs are essential drivers of B- and T-cell responses (18, 22, 25, 40) renders these mice of questionable use in the assessment of potential vaccine candidates. We therefore asked whether our immunocompetent mice with conditional deletion of the IFNAR in either CD11c- or LysM-expressing cells would be able to generate more effective B-cell responses after vaccination with a recombinant EDIIC-2 subunit vaccine candidate (31). Groups of mice were immunized subcutaneously three times, each separated by 2 weeks, with 20 μ g of EDIIC-2 vaccine. All mice generated high levels of DENV-2 virion-specific antibodies as measured by ELISA (Fig. 5A) and also showed comparable neutralizing titers (Fig. 5B). Surprisingly, however, CD11c-Cre^{+/-} IFNAR^{fl/fl} mice were best protected against subsequent viral homologous challenge with DENV-2 strain D2Y98P (Fig. 5C) and showed the highest survival rate, followed by LysM-Cre^{+/-} IFNAR^{fl/fl} mice (Fig. 5D). To compare the neutralizing effect of antibodies produced by wt mice with those produced by conditional knockout mice, we immunized mice with EDIIC-2 as described above and transferred 100 μ l of serum into dengue virus-susceptible AG129 mice. All mice succumbed to infection within 4 to 5 days postinfection. While the difference in viral titers was not statistically significant, antibodies produced by the most susceptible mice (IFNAR^{-/-}) induced the highest antibody-dependent enhancement (ADE) when transferred into wt mice, suggesting a lower protective capacity of the antibodies produced in IFNAR^{-/-} mice compared to those produced in wt and conditional IFNAR mice

FIG 2 Virus loads in organs and blood of mice infected with D2Y98P. Mice were inoculated i.p. with 1×10^7 PFU of D2Y98P, and organs were harvested 3 days postinfection. Viral loads were determined by real-time PCR in spleen (A), liver (B), lung (C), brain (D), kidney (E), and inguinal lymph nodes (LN) (F) and by plaque assay in serum (G). Each symbol represents one mouse. Data are presented as means $\pm$ standard deviations (SD). Statistical analysis of differences between LysM-Cre^{+/+} IFNAR^{fl/fl} and CD11c-Cre^{+/+} IFNAR^{fl/fl}, CD11c-Cre^{+/+} IFNAR^{fl/fl} and IFNAR^{-/-}, and LysM-Cre^{+/+} IFNAR^{fl/fl} and IFNAR^{-/-} mice was performed using Student's *t* test. ***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05. Nonsignificant differences are not indicated.

(data not shown). Similar results have been published by others (44) with the conclusion that antibodies alone can cause ADE, while the combination of antibody and T-cell response seems key for protection. This highlights once again the urgent need for better, immunocompetent mouse models that are able to

generate functional antibody and T-cell responses and demonstrates that the conditional IFNAR knockout mice not only are suitable models to study DENV infection biology but also are sufficiently immunocompetent to raise effective antibody and T-cell responses to an adjuvanted subunit vaccine.

FIG 3 Systemic levels of soluble mediators in infected mice. Mice were infected i.p. with 1×10^7 PFU of D2Y98P, and sera were harvested 3 days postinfection. Levels of IL-6 (A), IFN- γ (B), TNF- α (C), IP-10 (D), CXCL1/KC (E), and IFN- α (F) were determined by ELISA. Alanine transaminase (ALT) levels (G) were quantified using the Cobas C111 chemistry analyzer (Roche). Each symbol represents one mouse. Data are presented as means $\pm$ SD. Statistical analysis of differences between LysM-Cre $^{+/-}$ IFNAR $^{fl/fl}$ and CD11c-Cre $^{+/-}$ IFNAR $^{fl/fl}$, LysM-Cre $^{+/-}$ IFNAR $^{fl/fl}$ and IFNAR $^{-/-}$, and CD11c-Cre $^{+/-}$ IFNAR $^{fl/fl}$ and IFNAR $^{-/-}$ mice was performed using one-way ANOVA with Bonferroni's posttest. ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$. Nonsignificant differences are not indicated.

FIG 4 T-cell response of mice infected with DENV. Mice were infected with 5×10^6 PFU dengue virus strain TSV01, and blood was drawn on the indicated days postinfection. Cells were collected by centrifugation and restimulated with peptides for the quantification of IFN- γ production in CD8⁺ cells. (A) Intracellular IFN- γ was measured in blood CD8⁺ cells of infected LysM-Cre^{+/-} IFNAR^{fl/fl}, CD11c-Cre^{+/-} IFNAR^{fl/fl}, IFNAR^{-/-}, and wt mice. Representative FACS graphs gated on CD8⁺ lymphocytes for each group at day 6 postinfection are shown. (B) Quantitative analysis of IFN- γ production. Bars are means $\pm$ standard errors of the means (SEM) from two independent experiments with a total of 4 to 8 mice.

DISCUSSION

Despite huge efforts over the last 70 years and a growing clinical need, there remains no treatment or vaccine for dengue. Dengue vaccine research has faced numerous challenges, including uncertainties on immune correlates of protection and enhancement and the lack of suitable animal models in which to dissect disease pathways and test novel therapeutics or vaccines. The first animal models established were immunocompetent mice in which a high titer of DENV was delivered intracranially (45, 46). This method caused neurotropic disease and paralysis, neither of which is typically observed in humans. More recently, immunocompromised mice lacking type I and/or type II IFN receptors have been generated (10, 11). Dengue infection in these mice recapitulates some aspects of the human disease, such as cytokine storm, vascular leakage, TNF- α production, hemorrhage, and nonparalytic death (36). However, valid concerns have been raised regarding the extrapolation of data generated in these mice to the human setting. In fact, it is known that type I IFN receptor triggering is required for maximal T-cell expansion (47), cross-priming of CD8⁺ T cells (22), and promotion of clonal expansion and memory T-cell generation (29); additionally, type I IFNs directly stimulate naive T cells (48, 49) and enhance B-cell responses (18, 25, 50, 51). Thus, mice defective in these pathways are unlikely to recapitulate many features of a natural immune response to dengue virus infection.

During viral infections, macrophages and cDCs play an important role in both early innate immunity and in instructing the development of the adaptive immune response. Our data demonstrate that protection of mice from DENV-2 infection depends on IFN signaling in LysM-expressing cells, including macrophages and neutrophils and CD11c-expressing cDCs. Mice lacking the

IFNAR on either LysM⁺ or CD11c⁺ cells were susceptible to DENV-2 but frequently began to recover from day 5 p.i. As the CD8⁺ T-cell response to DENV also begins around this time (52), this leads us to speculate that these cells may be important in resolving DENV-2 infection in conditional IFNAR knockout mice. In line with this, conditional IFNAR knockout mice showed higher DENV-specific CD8⁺ responses than IFNAR^{-/-} mice, which succumbed to infection. Further investigation will reveal to what extent CD8⁺ and CD4⁺ T cells are necessary for recovery and how type I IFNs are involved in this process.

Our immunization studies using an alum- and ODN-adjunct EDIIIC-2 subunit vaccine highlighted the importance of IFN signaling in macrophages for the development of an effective adaptive immune response. A commonly used readout of vaccine efficacy is serum titer of dengue-specific and neutralizing antibodies. Our data demonstrate that neutralizing antibody titers alone are of limited value in predicting the ability of a vaccine to protect from viral challenge in mice, which is consistent with previous findings in humans (53, 54). Although all mouse strains tested produced comparable amounts of DENV-2-specific IgG and similar titers of neutralizing antibodies, CD11c-Cre^{+/-} IFNAR^{fl/fl} mice were significantly more protected against challenge. Unexpectedly, although viral titers in serum of untreated and placebo-treated CD11c-Cre^{+/-} IFNAR^{fl/fl} mice at day 3 p.i. were comparable (Fig. 2G and 5C), the placebo control group succumbed to the infection (Fig. 5D), whereas untreated mice mostly survived (Fig. 1B). One reason for this unexpected result might be that the adjuvant in the placebo activated immune cells and rendered the mice more susceptible to immunopathology-related disease outcome. Not only does this reemphasize the finding that peak virus

FIG 5 Immune response of mice vaccinated with a dengue virus subunit vaccine candidate. Mice were immunized 3 times fortnightly subcutaneously (s.c.) with 20 μ g of EDIII-C vaccine or placebo. Forty-five days after the first immunization, blood was drawn prior to challenge with 1×10^7 PFU D2Y98P. (A) IgG antibody titers against DENV-2 were measured by ELISA. (B) Neutralizing antibodies against DENV-2 were measured using U937-DC-SIGN cells as target cells. (C) Three days postchallenge, viral titers in serum were determined by plaque assay. (D) Survival of vaccinated and placebo-treated mice was monitored daily over the course of 10 days postinfection. Mice exhibiting more than 20% weight loss were considered moribund and were euthanized. Each symbol represents one mouse. (A to C) Data are presented as means $\pm$ SD. Statistical analysis was performed using Student's *t* test. (D) Survival curves were generated using the Kaplan-Meier method, and the significance of differences was calculated by the log rank test. **, $P < 0.01$; *, $P < 0.05$. ns, not significant; nd, not detected.

titers are not necessarily predictive of final disease outcome during DENV infection of mice (Fig. 1 and 5) but highlights the fact that the delicate immunological balance determining recovery versus death from dengue depends on currently unidentified factors which are likely linked to IFNAR expression on CD11c⁺ and/or LysM⁺ cells. Taken together, our data reveal that the conditional IFNAR knockout mice are appropriate models for the study of dengue virus immunology and pathology. They have begun to provide insight into the association of peak viremia, cytokine production, and the role of type I IFNs in determining disease severity and outcome, and further study may advance the search for the elusive immune correlates of protection. Moreover, CD11c-Cre^{+/−} IFNAR^{fl/fl} mice are a more relevant model than either AG129 or global IFNAR knockout models with which to screen dengue vaccine candidates. The development and study of these conditional IFNAR knockout mice should facilitate progress toward the development of effective dengue treatments and vaccines for clinical use.

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