Adaptive Immune Responses To Zika Virus Are Important For Controlling Virus Infection And Preventing Infection In Brain And Testes

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Abstract

The recent association between Zika Virus (ZIKV) and neurological complications including Guillain-Barré Syndrome (GBS) in adults and CNS abnormalities in fetuses highlights the urgency to understand the immunological mechanisms controlling this emerging infection. Studies have indicated that ZIKV evades the human type I IFN response suggesting a role for the adaptive immune response in resolving infection. However, the inability of ZIKV to antagonize the mouse IFN response renders the virus highly susceptible to circulating IFN in murine models. Thus, as we show here, although wild type C57BL/6 mice mount both cell-mediated and humoral adaptive immune responses to ZIKV, these responses were not required to prevent disease. However, when the type I IFN response of mice was suppressed, then the adaptive immune responses became critical. For example, when type I IFN signaling was blocked by antibodies in Rag1−/− immunodeficient mice, the mice showed dramatic weight loss and ZIKV infection in the brain and testes. This phenotype was not observed in Rag1−/− mice or mice treated with anti-IFNAR alone. Furthermore, we found that the CD8+ T cell responses of pregnant mice to ZIKV infection were diminished compared to non-pregnant mice. It is possible that diminished cell-mediated immunity during pregnancy could increase virus spread to the fetus. These results demonstrate an important role for the adaptive immune response in control of ZIKV infection, and imply that vaccination may prevent ZIKV-related disease, particularly when the type I IFN response is suppressed as it is in humans.

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**Introduction**

Zika virus (ZIKV) is an emerging infection endemic to the forests of Uganda and South-East Asia that was first discovered in 1947 (1). It has recently spread from these regions to the Pacific and emerged in the Americas in 2015 (2, 3). Infection with ZIKV in humans is often asymptomatic or mild, with clinical signs of a rash, fever, conjunctivitis and joint pain (4, 5). However, recent outbreaks of ZIKV infection since 2007 have been associated with Guillain-Barré Syndrome (GBS) in adults as well as an increase in fetal abnormalities including placental insufficiency, microcephaly, CNS abnormalities and death resulting from mother-to-fetus transmission (6–8). These neurological complications of ZIKV infection prompted declaration of ZIKV infection a global health crisis by the World Health Organization in 2016.

ZIKV is primarily transmitted to humans by the bite of an infected mosquito, although sexual transmission also occurs and is predominantly from male to female (9). Following infection by mosquito, ZIKV produces a short viremia in the blood although ZIKV is detectable in other bodily fluids, including saliva, urine and semen for a longer period of time. Viral RNA and virus have been detected in the brains of fetal microcephaly cases indicating that the virus can infect cells of the central nervous system (CNS) (6, 10). In vitro studies have shown ZIKV infection of human neuroprogenitor cells, fibroblasts, keratinocytes and dendritic cells (10, 11).

Recent studies have demonstrated an important role for type I IFN responses in protection against ZIKV infection. The NS5 protein of ZIKV inhibits human STAT2, suppressing the type I IFN response to ZIKV and allowing for proliferation of virus (12). In contrast, NS5 does not inhibit murine STAT2, allowing for a strong type I IFN response and suppression of virus infection (12). Indeed, wild type mice appear to control ZIKV infection, while mice deficient in the type I IFN receptor 1 (IFNAR1) are susceptible to ZIKV infection, lose weight and develop neurological disease (13). Interestingly, treatment of wildtype C57BL/6 mice treated with anti-IFNAR antibodies develop viremia, but do not lose weight or develop neurological disease (13). This suggests that when the IFN response is suppressed, but not deficient, that other components of the immune system may be able to control ZIKV infection.

Another important component of the anti-viral response is the adaptive immune response including CD4+ and CD8+ T cell and neutralizing antibody (NAb) responses. Relatively little is currently known about the adaptive immune responses to ZIKV infection or their effects on viral pathogenesis. In the current study, we detected CD4+ and CD8+ T cell proliferation and/or activation as well as NAb production in response to ZIKV infection in mice. Furthermore, the effects of these responses on ZIKV pathogenesis were investigated both in the presence and absence of strong type I IFN responses. Finally, since maternal transmission of ZIKV to fetuses is one of the major complications of infection, we determined whether pregnancy affected the generation of adaptive immune responses.
Materials and Methods

Ethics statement

All animal work in this study adhered to the U. S. Government Principles and applicable humane and ethical policies in accordance with the Public Health Service (PHS) Policy, the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Regulations. Animal work was conducted in compliance with the guidelines of and under a protocol approved by the corresponding Institutional Animal Care and Use Committee (IACUC, Protocol #: 2016-015). The Rocky Mountain Laboratories (RML) facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal anesthesia was performed using vaporized isoflurane. Euthanasia, when required, was performed by cervical dislocation only after mice had reached a deep surgical plane of anesthesia.

Infection of mice with ZIKA and disease criteria

Rag1−/− (purchased from Jackson Laboratories) and Unc93b1 3D (obtained from the Mutant Mouse Regional Resource Center) mice were maintained on a C57BL/6 background in a breeding colony at RML. Mavs−/− mice were purchased from Jackson Laboratories on a C57BL/6, 129 mixed background. Wild type (WT) refers to the C57BL/6 strain unless otherwise noted.

The 2015 ZIKA Paraiba strain is a human microcephaly isolate that has been previously described (12) and was kindly provided by Steve Whitehead (NIAID). Mice at 6–8 (adult) weeks of age were inoculated with 10⁴ plaque forming units (PFU) of ZIKA virus intraperitoneally (i.p.) in a volume of 200 μl/mouse. Virus stocks were diluted to the correct concentration in phosphate-buffered saline (PBS) prior to inoculation. Mock infections consisted of a similar dilution of Aedes albopictus clone C6/36 (ATCC) mosquito larval cell culture supernatant diluted into PBS. For experiments characterizing the adaptive immune response to ZIKA infection during pregnancy, adult female mice (8+ weeks) were time-mated with proven males of the same genotype for two days prior to infection. At 7 days post-mating, pregnancy was verified via ultra-sound and/or abdominal palpation and mice were infected as described above and housed individually. Mock infected pregnant mice and age-matched mice that were never mated served as experimental controls.

Infected mice were observed daily for signs of neurological disease that might include hunched posture, seizures, reluctance or inability to move normally or paralysis. Mice that received neutralizing or cell-depleting antibody treatment (described below) were regularly monitored for weight loss, which is a known clinical symptom of ZIKA infection in immune compromised mice(13). Animals with clear clinical signs of disease (including greater than 20% loss of original starting weight) were scored as clinical and euthanized immediately.

Treatment of mice with neutralizing and cell-depleting antibodies

For the depletion of T-cells, anti-CD8 clone 169.4 and anti-CD4 clone 191.1 hybridomas were grown in RPMI media containing 10% FBS. Supernatants were harvested and spun at 500 × g for 10 minutes to remove any cellular debris and then stored at −20°C until use.
Infected mice were injected i.p. with 0.5mL of the supernatant on 1, 3, 5, 12 and 19 days post infection (dpi). Dual CD8 and CD4 T cell depleted mice received 2 injections (a total of 1mL of supernatant) at each indicated time point. This treatment schedule has been shown to deplete ≥95% of either or both T-cell populations (14, 15). Control mice were injected on the same schedules with 10% FBS in RPMI.

For neutralization of signaling through the interferon alpha receptor, mice were treated i.p. with 1mg/mouse of the anti-IFNAR1 clone MAR1-5A3 in PBS on 1-, 3, 7, 11 and 16 dpi. Control mice were treated with an equivalent amount of normal mouse IgG antibody on the same days. This treatment protocol has been shown to effectively impair type 1 interferon signaling during ZIKV infection (13).

Surface and intracellular staining Abs and flow cytometry

Splenocytes were isolated from nonpregnant or pregnant wild type mice by tissue homogenized through a 100μm filter to generate a single-cell suspension. At room temperature (rt) red blood cells were removed using lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 M EDTA) and then cells were washed with PBBS. Cells were surface stained with antibodies for 30–45min at 4°C prior to fixation and permeabilization over night at rt. The antibodies used for cell staining were purchased from BD Pharmingen, eBioscience or BioLegend unless otherwise noted. The following combinations of antibodies were used: CD4-Pacific Blue, CD8-Pacific Blue, CD19-PE/TxRed, CD11a-FITC, CD69-PE, CD27-APC/Cy7, CD43-PerCP/Cy5.5 or -PE/Cy7, CD25-PerCp/Cy5.5, CD62L-PE/Cy7, and CD86-BV605. Intracellular Foxp3 and Ki-67 staining was performed according to the manufacturer’s recommendation using Foxp3-APC (FJK-16s) and Ki-67-AF700 (SolA15) and the Foxp3 staining kit from eBioscience. For intracellular granzyme B staining, the cells were fixed in 4% paraformaldehyde-PBS and then permeabilized with 0.1% saponin-PBS containing 0.1% sodium azide, 0.5% BSA and 50 mM glucose. Cells were then stained using anti-human granzyme B-APC (GB11) (Molecular Probes). Gates were used to exclude cellular debris and doublets and specific gating strategies are outlined in the figure legends. Data were acquired on an LSR II cytometer (BD) and analyzed using FlowJo v10 software (FlowJo LLC).

Immunohistochemistry and In Situ hybridization

At the experimental end point or clinical time point, some mice were perfused transcardially with heparin saline (100 U/ml) followed by 10% neutral buffer formalin. Whole brain or testes was serially sectioned (5μm) and mounted on slides. For immunohistochemistry, sections were blocked (5% BSA, 0.05 % Triton in PBS) at room temperature (rt) for 1 hour (hr) and then primary antibodies against ZIKA NS5 (1:3000, Aves Labs) and NeuN (1:2500, abcam) or active Caspase 3 (1:300, Promega) or Iba1 (1:250 Dako) and were applied and incubated overnight at 4 °C in blocking buffer. Secondary antibodies were used to label these specific primaries (goat anti-chicken AF488 and donkey anti-rabbit AF594). Secondary antibodies were incubated for 1 hr at rt. Slides were cover slipped with Prolong Gold mounting media containing DAPI (Molecular Probes) prior to imaging.
ZIKV replication was detected in tissue sections by in situ hybridization using probes specific for positive sense ZIKV RNA using a previously described method (16). All slides were then imaged using either 1) a Zeiss 710 LSM (Carl Zeiss) with a Plan Apochromat 63× oil immersion objective (NA 1.40) with a pinhole 1AU (airy unit) to generate confocal images (Fig 7) or 2) an Aperio ScanScope FL (fluorescent) slide scanner (Leica Biosystems) to make composite images (S3 Fig) with a UPLSAPO 20× objective (NA 0.75). Representative images were exported to TIFF format and figures built using Canvas 14 (ACD Systems).

**Viremia and NAb detection**

For detection of viremia, serially diluted plasma was plated directly to Vero cells as described below. For quantification of NAb serially diluted plasma was mixed with 10^2 (particle forming units) PFU of ZIKA in a final volume of 200μL in DMEM/2% FBS/1% Pen Strep. The mixture was incubated for 1 hr at 37°C for neutralization. After neutralization, the 200μL mixture was added to confluent Vero cells in a 24-well plate and incubated again for 1 hr at 37°C. After incubation, 500 μL of 1.5% carboxymethyl cellulose in MEM was overlaid onto the cells and the cells were incubated undisturbed at 37°C for 5 days. Cells were then fixed by adding 10% formaldehyde to each well until full and allowed to sit for 1hr at room temperature. After fixation, plates were rinsed gently with deionized water wash and stained with 0.35% crystal violet for 15 minutes. Plates were rinsed and allowed to air dry inverted. Viral titers was calculated by dividing the number of plaques per given sample by the plasma dilution factor multiplied by the volume of each well. Neutralizing titer was determined by the dilution that inhibited at least 50% of plaque formation when compared to cells infected with the 10^2 ZIKA.

**Real-time PCR**

Real-time PCR analysis of mRNA expression from brain, spleen and testes was completed as previously described (17). The primers used include: Gapdh.2-152F (TGCACCACCAACTGCTTAGC), Gapdh.2-342R (TGGATGCAGGGATGATGTTC), ZIKAFP8008F (X) and ZIKAFP8121R (X). Primers were subjected to BLAST analysis (NCBI) to ensure detection of only the specified gene and were tested on positive controls to ensure amplification of a single product. Data for each sample were calculated as the percent difference in threshold cycle (ΔC_T) value (ΔC_T = C_T for glyceraldehyde-3-phosphate dehydrogenase [GAPDH] gene − C_T for specified gene). Gene expression was plotted as the percentage of gene expression relative to that of the GAPDH gene.

**Statistical analysis**

All statistical analyses were performed using Prism software Version 7.01 (GraphPad) and is described in the figure legends.

**Results**

**Zika virus infection induces CD4+ T cell proliferation**

To examine the adaptive immune response to ZIKV, we infected adult wild type (C57BL/6) mice with 10^4 plaque forming units (PFU) per mouse. Plasma and spleen samples at 3, 7, 10,
14 or 21 days post infection (dpi) were negative for detectable virus by either plaque assay or real-time PCR, respectively (data not shown). Additionally, we were unable to detect viral RNA in brain tissue or lymph nodes correlating with the ability of wild type to clear virus infection (13). Approximately ½ of each spleen for each time point was used for flow cytometry analysis of CD4+ and CD8+ T cells. CD4+ T cells were gated for CD4+ helper (Foxp3−) and regulatory T cells (Foxp3+) (Fig 1A) and then analyzed for CD69, CD43, Ki67, CD11a and CD25 expression. Representative flow cytometry plots for CD4 T helper cells from naïve (Fig 1B) and ZIKV-infected mice (Fig 1C) are shown for the 7dpi time point. Analysis of helper CD4+ T cells (Fig 1E) and regulatory T cells (Fig 1D) showed no change in the percentage of the cell population over time. Similarly, increased expression of activation markers CD43, CD69 and CD11a was not significant for any of the analyzed time points during ZIKV infection (Fig. 1F–H), but increased proliferation as measured by Ki67 staining was significant at 7 dpi (Fig. 1I). Thus, ZIKV infection resulted in a peak of CD4+ T helper cell proliferation around 7 dpi, despite a lack of detectable virus. Analysis of regulatory CD4+ T cells showed no significant changes in the percentage of CD4+ T cells that were Foxp3+ positive or in any activation nor proliferation markers (S1 Fig).

Zika virus infection induces CD8+ T cell proliferation and activation

CD8+ T cell responses to ZIKV infection were also analyzed. The gating for CD8+ T cells and representative analyses at 7 dpi are shown in Fig 2. No difference was observed in the percentage of CD8+ T cells in the spleen over the course of ZIKV infection (Fig 2D). However, significant proliferation (Fig 2E), activation (Fig 2F, G), and also production of the cytotoxic molecule, granzyme B (Fig 2H) was observed, with peak responses at 7 dpi. At that time point, ~10% of the CD8+ T cells had the phenotype of activated effectors as indicated by CD43 expression and granzyme B production (Fig 2F, H). By 10 dpi, these activation markers had returned to baseline (Fig. 2E–H). These results revealed a strong and rapid CD8+ T cell response to ZIKV infection with most parameters returning to baseline levels by 10 dpi. Thus, wild type mice developed a strong, albeit short-lived, CD8+ T cell response to ZIKV infection, despite undetectable virus replication.

Neutralizing antibodies are produced in response to ZIKV, but are not essential for protection

B cell responses to ZIKV infection were analyzed by expression of activation and proliferation markers as well as neutralizing antibody (NAb) production. Analysis of CD19+ B cell markers by flow cytometry showed no significant changes in response to ZIKV infection with the parameters tested (S2 Fig). However, NAb was detected at low levels by 7 dpi and at higher levels by 14 and 21 dpi, indicating a B cell response (Fig 3). Thus, ZIKV infection elicited both humoral and cellular adaptive immune responses in mice, despite limited detection of virus in wildtype mice. Comparison between sexes demonstrated no difference between males and females in any of the adaptive immune responses (data not shown).

T cell depletion in wildtype (WT) mice results in minor weight loss following ZIKV infection

Since CD4+ and CD8+ T cell proliferation and/or activation were observed following ZIKV infection, we examine whether T cells were essential for protection in mice. We treated wild
type mice with anti-CD4 and anti-CD8 monoclonal antibodies, which have previously been described to deplete both cell populations (14, 15). Depletion was confirmed by flow cytometry (data not shown). Zika-infected mice treated with anti-CD4 and anti-CD8 T cell antibodies had a slight, but significant reduction in body weight beginning at 11 dpi that persisted for the majority of recorded time points until the experimental end point (Fig 4). No effect was observed by anti-CD4/anti-CD8 treatment in uninfected mice (Fig. 4). While these findings imply T-cell depleted modestly influenced ZIKV infection, the anti-CD4/anti-CD8 treatment group did regain weight throughout the experiment, (Fig. 4) suggesting depletion did not result in uncontrolled virus replication. Furthermore, ZIKV infection of Rag1−/− mice, which lack both T cell and B cell responses, did not result in significant weight loss or neurological disease (data not shown). Collectively, these data suggest that the innate immune response is sufficient to control ZIKV infection in mice, even in the absence of adaptive immune responses.

**CD4 and CD8 depletion affects ZIKV-induced weight loss in Mavs−/− mice**

The ZIKV NS5 protein antagonizes STAT2 in human cells to suppressing the type I IFN response to ZIKV and allow a more productive infection (12). However, STAT2 is not inhibited by NS5 in mouse cells, allowing for a strong type I IFN response and suppression of ZIKV replication. The type I IFN response to flaviviruses is primarily initiated following activation of cytosolic RIG-I like receptors (RLRs) signaling to the adaptor mitochondrial anti-viral protein (MAVS) as well as endosomal toll-like receptors (TLRs) signaling through MyD88 (18, 19). We compared viral RNA levels in Mavs−/− mice (deficient in RIG-I like receptor responses) or in Unc93b1 3D mice (with a mutation in Unc93b1 that prevents trafficking of TLR3, TLR7 and TLR9 to the endosome). Mice with the Unc93b1 3D mutation were similar to WT mice, with low to undetectable levels of virus present in the spleen (Fig 5A). However, ZIKV replicated 100-fold higher in the spleen of Mavs−/− mice at 3 dpi followed by rapid control of replication by 7 dpi (Fig 5A). Thus, Mavs−/− mice develop an acute infection reminiscent of that seen in humans suggesting these mice could provide a model in which to examine the role of the adaptive immune response to ZIKV.

To examine if T cell responses were necessary for control of the increased virus replication in Mavs−/− mice, we completed a T cell depletion study in these mice. Mavs−/− mice that were mock infected and T cell depleted or ZIKV infected and vehicle-control treated did not exhibit significant weight loss over time (Fig 5B). In contrast, ZIKV infection of T cell-depleted Mavs−/− mice resulted in approximately 10% weight loss starting at 8–9 dpi that was sustained until the end of the experiment at 24 dpi (Fig 5B, black squares). This weight loss did not increase over time and animals did not develop clinical signs of neurological disease (Fig 5B). Thus, T cell responses were necessary for prevention of weight loss induced by ZIKV infection. Interestingly, no viral RNA was observed in the brain or spleen tissue at the end of the experiment by real-time PCR analysis or immunohistochemistry (data not shown) suggesting infection was controlled by mechanism not involving MAVS signaling or T cells. Analysis of plasma from ZIKV infected Mavs−/− mice, demonstrated high levels of NAb, independent of T cell depletion (Fig. 5C) Interestingly, Mavs−/− mice had higher levels of NAb at both time points analyzed (Fig. 5C) than comparable time points.
in WT mice (Fig. 3) correlating with the higher level of early virus infection in Mavs<sup>−/−</sup> mice (Fig. 5A).

**Suppression of type I IFN responses in the absence of humoral and cellular immune responses leads to CNS disease and widespread virus infection**

To further examine the role of the adaptive immune response, we used Rag<sub>1</sub><sup>−/−</sup> mice, which lack both functional T and B cell responses. We treated WT or Rag<sub>1</sub><sup>−/−</sup> mice with anti-IFNAR antibodies starting at −1 dpi and again on days 1, 3, 7, 11 and 15 dpi to mimic conditions of an insufficient type I IFN response. Previous studies have shown that anti-IFNAR treatment in mice increased virus replication but did not result in disease as indicated by body weight loss or neurological signs (13). Similarly, in our study WT mice treated with anti-IFNAR did not lose weight or develop neurological disease, although increased virus replication occurred in the spleen compared to Ig-treated controls (Fig 6A, B). However, anti-IFNAR treated Rag<sub>1</sub><sup>−/−</sup> mice lost significant weight by 9 dpi, and reached criteria for euthanasia (≥ 20% weight loss) at 17 dpi (Fig 6A). Tissue analysis showed a five-log<sub>10</sub> increase in viral RNA in spleen compared to controls (Fig 5B), as well as high levels of viral RNA in the lymph nodes (Fig 6C) and brain (Fig 6D). Thus, adaptive immune responses were required to control ZIKV replication and spread in anti-IFNAR treated mice.

**Virus infection of neurons, but not astrocytes or microglia, in the brains of anti-IFNAR treated Rag<sub>1</sub><sup>−/−</sup> mice**

We analyzed virus infection in both the brain and testes of anti-IFNAR treated Rag<sub>1</sub><sup>−/−</sup> mice by immunohistochemistry. Virus, as detected by ZIKV NS5 staining (green fluorescence), was readily detected in the hippocampus and cortex (S3 Fig) in NeuN-positive neurons (red fluorescence) of the CA1 and CA3 and dentate gyrus regions (Fig. 7A–D). Staining of astrocytes with GFAP (Fig. 7E–H), or microglia/macrophages with Iba1 (Fig. 7I–L), indicated that these cells were not productively infected. However, astrocytes and microglia did display morphological characteristics consistent with activation such as cell body enlargement, process ramification and in the case of microglia, cellular engulfment of infected neurons (Fig. 7I–L, inserts). Costaining of infected neurons for active caspase 3 demonstrated that some of the infected neurons, particularly those in the CA1 and CA3 regions, were undergoing apoptosis (Fig. 7M–P, arrows). Thus, ZIKV infection of the CNS of Rag<sub>1</sub><sup>−/−</sup> mice was associated with the infection and apoptosis of neurons and the activation of glial cells.

**ZIKV infection of polygonal cells in the testes**

As ZIKV has also been detected in the testes, we also analyzed tissues from anti-IFNAR treated Rag<sub>1</sub><sup>−/−</sup> mice by immunohistochemistry. Virus (green fluorescence) was not detected in Ig-treated wildtype control mice (Fig 8A), but was readily detected in polygonal cells in anti-IFNAR-treated Rag<sub>1</sub><sup>−/−</sup> mice (Fig 8B). The location and structure of infected cells indicate that they are likely germinal spermatogonia or primary spermatocytes and the lack of co-localization with vimentin staining suggested they were not stromal or sertoli cells (Fig 8C). Co-localization with active-caspase 3 (red fluorescence) indicated apoptosis of both infected and uninfected cells (Fig. 8C–E), that was not observed in control mice (Fig 8A). These immunohistochemistry results demonstrated ZIKV infection in two cell types in...
different tissues of anti-IFNAR treated *Rag1*−/− mice. Furthermore, ZIKV infection was associated with cellular apoptosis in both tissues.

**Pregnancy reduces CD4 and CD8+ T cells responses, but not NAb**

One the most concerning pathologies associated with ZIKV infection is microcephaly in the fetus following infection of a pregnant individual. Pregnant women are at increased risk for certain infectious diseases, a phenomenon linked to a unique immunological condition associated with pregnancy (20). To investigate if the adaptive immune response was influenced by pregnancy, CD4+ and CD8+ T responses were measured at 7 dpi in pregnant wildtype mice representing the peak of response for both of these cell types (Figs. 1–2) Since gestation in a C57BL/6 mouse is approximately 18 days long, we infected mice at 7 days of gestation, during the early-mid stages of pregnancy. Analysis of CD4+ and CD8+ T cell responses revealed demonstrable reductions in the responses of both subsets in pregnant mice compared to non-pregnant controls (Fig. 9A–F). In pregnant mice the percentages of proliferating (Ki67+) CD4+ and CD8+ T cells were both reduced (Fig. 9A,B) and significantly fewer CD8+ T cells appeared activated (CD11a+ and CD62L−, CD43+) (Fig. 9C–E). In addition, there were also significantly fewer granzyme B positive CD8+ T cells in pregnant mice infected with ZIKV (Fig 9F), suggesting a reduction in cytolytic effector function. These results indicated that pregnancy influenced the cellular arm of the adaptive immune response. In contrast, no significant differences in NAb responses in pregnant mice was observed compared to non-pregnant females (S4 Fig), suggesting that pregnancy may not significantly affect the humoral immune response to ZIKV.

**Discussion**

In the current study we examined the adaptive immune response to ZIKV infection and its potential role in regulating virus infection and preventing disease. We found a detectable T cell response and NAb response in WT mice, despite undetectable levels of virus in multiple tissues. These adaptive immune responses were not necessary to control virus in mice with fully competent type I IFN responses, as *Rag1*−/− mice did not lose weight or shown any signs of clinical disease following ZIKV infection. However, when *Rag1*−/− mice were treated with anti-IFNAR antibodies, to more closely recapitulate the suppressed anti-IFN response in human ZIKV infection, the mice developed severe disease with ≥20% weight loss and high levels of virus in both the brain and the testes. Thus, when the type I IFN response is not fully competent, the adaptive immune response has an important role in regulating ZIKV infection and preventing the spread of virus to the brain and testes.

Staining for ZIKV in the testes of anti-IFNAR treated *Rag1*−/− mice indicated that the primary cell type infected in the testes is polygonal cells, most likely spermatogonia or primary spermatocytes. This is similar to a recent study by Govero et al., which found ZIKV infected spermatogonia, primary spermatocytes and Sertoli cells in the testes, and induced apoptosis of these cells (21). This was observed in anti-IFNAR treated wild type or *Rag1*−/− mice, most notably when using a mouse adapted strain of ZIKV, Dakar. The Dakar strain of ZIKV induced more apoptosis of ZIKV than the Asian ZIKV strain (H/PF/2013). Analysis with caspase 3 in our studies with the Paraiba strain of ZIKV demonstrated that the majority
of infected cells were not undergoing apoptosis suggesting that spermatogonia function as a cellular reservoir of ZIKV. Since ZIKV is sexually transmitted (9), it is possible that one of the mechanisms of sexual transmission is through the transfer of infected spermatozoa (21).

ZIKV infection of anti-IFNAR treated 

\[ \text{Rag1}^{-/-} \]

mice also showed high levels of virus present in the CNS (Fig 6), with the focal areas of ZIKV infection being neurons in the cortex and the hippocampal region (S3 Fig). The primary cells infected by ZIKV were neurons, while astrocytes and microglia did not show obvious signs of infection (Fig 7). However, astrocytes and microglia did display morphological characteristics consistent with activation such as cell body enlargement, process ramification and in the case of microglia, cellular engulfment. Microglia were recently shown to contribute to WNV-mediated neuronal damage through a mechanism of complement-driven synapse loss (22). Astrocytes can also contribute to CNS damage through recruitment of inflammatory cells and breakdown of the blood brain barrier (BBB) (23). Thus, the activation of these glial cells in areas of virus infection may contribute to CNS damage caused by ZIKV infection.

Neuronal infection was primarily found in the hippocampus, including the dentate gyrus and in the cortex of 

\[ \text{Rag1}^{-/-} \]

anti-IFNAR treated mice (S3 Fig). Analysis of these cells with active caspase 3 and ZIKV NS5 staining showed areas of heavy neuronal loss particularly in the CA3 region, but also in the CA1 and cortex regions, suggesting that these regions may be particularly susceptible to ZIKV infection and neuronal damage. Infection of the hippocampus and/or dentate gyrus has been noted for two other flaviviruses, WNV and tick-borne encephalitis virus (TBEV) (24–27). Thus, in adults, ZIKV may have similar neuronal cellular tropism as other flaviviruses. These data indicate that ZIKV can readily infect neurons in the adults, if peripheral responses are suppressed sufficiently to allow virus entry into the CNS and suggest that immunocompromised individuals may be susceptible to ZIKV-induced encephalitis even as adults.

One condition that may lead to reduced immune responses to ZIKV is pregnancy. Studies with other viruses have suggested that pregnancy can reduce the immune response to pathogen infections, including viruses (20). Our current studies demonstrate reduced CD4+ and CD8+ T activation and proliferation in infected pregnant dams compared to non-pregnant controls (Fig 9). Interestingly, while the cellular response to ZIKV was suppressed by pregnancy, the production of NAbs was not. We did not see an increase in virus RNA in the spleens of pregnant vs non-pregnant mice, indicating that decreased T cell responses were not sufficient to allow uncontrolled virus replication (data not shown). This is similar to the anti-CD4/anti-CD8 studies in both WT and 

\[ \text{Mavs}^{-/-} \]

mice, where weight loss was observed, but viral RNA or viremia remained undetectable. Possibly, early innate responses coupled with the NAb responses in both cases is sufficient to control virus replication even in the absence of either CD4 or CD8 T cell responses.

The antiviral type I IFN response in human cells is antagonized by ZIKV. In our effort to understand the contribution of adaptive responses in this context, we examined peripheral viral replication in the type I IFN inhibited 

\[ \text{Mavs}^{-/-} \]

and 

\[ \text{Unc93b1} \]

3D mouse strains. We found ZIKV RNA was 100-fold higher in the spleen of 

\[ \text{Mavs}^{-/-} \]

mice relative to wild type controls whereas viral RNA was unchanged in mice deficient in 

\[ \text{Unc93b1} \]

3D mice (Fig 5A).
This finding is consistent with an earlier study in a ZIKV vaginal infection model that showed higher viral replication in Mavs<sup>−/−</sup> but not TLR7<sup>−/−</sup> mice (28). NAb responses were also higher in Mavs<sup>−/−</sup> mice (Fig. 5C) compared to WT mice (Fig 3) at both 1 and 3 weeks post infection, suggesting a more active infection in Mavs<sup>−/−</sup> mice. Furthermore, depletion of T cells in Mavs<sup>−/−</sup> mice (Fig 5B), resulted in significant weight-loss that was not observed in T cell depleted wild type mice (Fig 4). Collectively these findings indicate that RLR-MAVS signaling may contribute to the early type I IFN-mediated suppression of ZIKV replication. However, this MAVS-dependent antiviral response is clearly not solely responsible for early viral clearance as Mave<sup>−/−</sup> mice alone did not lose weight or succumb to infection (Fig 5B and (13)) as did our anti-IFNAR1 treated Rag1<sup>−/−</sup> (Fig 6) mice. We did find that T cell depleted Mavs<sup>−/−</sup> mice generated a robust NAb response that is likely involved in controlling viral replication (Figure 5C), however we cannot exclude the possibility that the remaining type-I IFN response is also involved. Thus, both RLR-MAVS-dependent and -independent antiviral signaling is important for early control of ZIKV infection but requires a robust adaptive response to prevent disease if it is suppressed.

In conclusion, we found that ZIKV infection does induce a strong adaptive immune response, which provides protection against ZIKV-induced weight loss as well as virus spread to the brain and testes. These results support the development of vaccines that drive a strong T cell and humoral antibody response, which could protect against ZIKV spread, even in the case of weakened or suppressed type I IFN responses. Future studies on how pregnancy affects the development of these responses and whether pregnancy suppresses already formed anti-ZIKV responses will be important in determining how to best generate strong adaptive immune responses in terms of protecting pregnant mothers from ZIKV infection.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


Fig. 1. ZIKV infection induces CD4+ T cell proliferation, but not an increase in activation markers

Adult wild type mice were infected with 10^4 PFU of ZIKV. At 3, 7, 10, 14, and 21 dpi, spleens were removed and splenocytes analyzed by flow cytometry as described in the methods. (A) CD4+ T cells were separated into helper (Foxp3−) and regulatory (Foxp3+) cells. Examples of splenocytes from (B) mock and (C) ZIKV infected mice at 7 dpi labeled for CD43/CD69 and CD11a/Ki67 are shown. (D, E) CD4+ T cells were separated into helper (Foxp3−) and regulatory (Foxp3+) cells. The overall percentages of (E) helper T cells or (D) regulatory T cells did not change over time following ZIKV infection. (F–I) Average percentage of helper CD4+ T cells expressing (F) CD43, (G) CD69, (H) CD11a and (I) Ki67 are plotted for each time point. Mock-infected mice are shown as 0 dpi. Dotted line represents the average for mock-infected controls. Data are mean +/- SD 3–6 mice per time point and are the combined data of two experiments. Statistical analysis was completed using a One-way ANOVA with a Dunnett’s multiple comparison post-test. ***P<0.001 compared to mock.
Fig. 2. ZIKV infection induces CD8+ T cell proliferation and activation

Mice described in Fig 1 were also analyzed for CD8+ T cell activation. Cells were gated for (A) CD8+ expression. Examples of splenocytes from (B) mock and (C) ZIKV infected mice at 7 dpi labeled for Ki67/CD43 and CD11a/Granzyme B are shown. The overall percentages of (D) CD8+ T cells did not change over time following ZIKV infection. (E–H) Average percentage of CD8+ T cells expressing (E) Ki67, (F) CD43, (G) CD11a and (H) GranB are plotted for each time point. Mock-infected mice are shown as 0 dpi. Data are mean ± SD 3–6 mice per time point and are the combined data of two experiments. Statistical analysis was completed using a One-way ANOVA with a Dunnett’s multiple comparison post-test. * P<0.05, ** P<0.01, ***P<0.001 compared to mock.
Fig. 3. Neutralizing antibody (NAb) response to ZIKV
Plasma from mice described in Fig 1 were also analyzed for NAbs. Diluted plasma was mixed at with virus prior to plating on Vero cells in a NAb assay described in the Methods. Data are plotted as the highest dilution of plasma that inhibited virus infection by 50%. Data are shown as individual animals at 7, 14 and 21 dpi from two independent experiments. Plasma from mock-infected mice showed no inhibition of virus and were scored as 0 on the graph.
Fig. 4. T cell depletion results in ZIKV-induced weight loss in wildtype mice
Wild type mice were infected with $10^4$ PFU of ZIKV or mock supernatant and treated with anti-CD4 and anti-CD8 antibodies as described in the methods. Mice were individually weighed every 2 days. Data are plotted as the average increase/decrease in weight over time of 6 mice per group per strain. Statistical analysis was done using a two-way ANOVA with a Tukey’s multiple comparison analysis. * P<0.05, ** P<0.01, ***P<0.001 compared to vehicle control.
Fig. 5. T cell depletion results in ZIKV-induced weight loss in Mavs<sup>−/−</sup> mice

Wildtype C57BL/6, Unc93b1<sup>3D</sup>, and Mavs<sup>−/−</sup> mice were infected with 10<sup>4</sup> PFU of ZIKV and followed for either (A) splenic viral RNA, (B) weight loss or (C) NAb production. (A) At 3 and 7 dpi, spleens were removed and analyzed for viral RNA levels. Data are from 5–6 mice per group per time point. ***P<0.001 compared to wildtype control at each time point. (B) Mock and Zika-infected Mavs<sup>−/−</sup> mice were treated with anti-CD4 and anti-CD8 antibodies as described in the methods. Mice were individually weighed every 2 days. Data are plotted as the average increase/decrease in weight over time of 6 mice per group per strain. Statistical analysis was done using a two-way ANOVA with a Tukey’s multiple comparison analysis. * P<0.05, compared to vehicle control. (C) Plasma from Mavs<sup>−/−</sup> mice at 7 dpi or at the end of the weight loss experiment (25 dpi) were analyzed for neutralizing antibody as described in the Methods. Data are plotted for individual mice on a log 2 scale. No inhibition was observed in mock-infected mice indicated on the graph as a dotted line at the undiluted fraction.
Fig. 6. Treatment with anti-IFNAR in Rag1−/− mice results in clinical disease and high levels of virus

(A–D) Wild type and Rag1−/− mice were infected with 10⁴ PFU of ZIKV and followed for either (A) weight loss or (B–D) measured for viral RNA in the spleen, lymph nodes or brain. Mice were treated with anti-IFNAR1 (MAR1-5A3) on −1, 1, 3, 7, 11 and 15 dpi. Control mice were treated with an equivalent amount of normal mouse IgG antibody. Mice were individually weighed every 2 days. Data are plotted as the average increase/decrease in weight over time of 9 mice per group per strain. Statistical analysis was done using a two-way ANOVA with a Tukey’s multiple comparison analysis. * P<0.05 compared to wildtype B6 mouse IgG. Rag1−/− mice treated with anti-IFNAR1 were euthanized at 17 dpi due to a 20% loss in weight (τ). (B–D) At 17 dpi for anti-IFNAR1 treated Rag1−/− mice or 21 dpi for the other 3 groups, (B) spleen, (C) lymph nodes and (D) brain tissue was removed and analyzed for viral RNA by real-time PCR. Individual mice are plotted using the same symbols as for (A) with the mean average shown as a bar. Filled circles represent Ig treated
mice. Filled triangles represent anti-IFNAR1 treated mice. Statistical analysis was completed by One-Way ANOVA with a Tukey multiple comparison post-test. * P<0.05, ** P<0.01, ***P<0.001
Fig. 7. Zika infection of neurons, but not astrocytes or microglia in brain tissue from anti-IFNAR in Rag1−/− mice

(A–P) Brain tissue from anti-IFNAR treated Rag1−/− mice described in Fig 6, was processed for histology and stained for ZIKV NS5 protein (green fluorescence). Samples were co-stained with red (pseudo colored magenta) fluorescent label for (A–D) NeuN to detect neurons, (E–H) GFAP to detect astrocytes (I–L) Iba1 to detect microglia/macrophages and (M–P) active caspase 3 to detect apoptotic cells. Representative sections from the (A, E, I, M) CA1, (B, F, J, N) CA3 and (C, G, K, O) dentate gyrus regions of the hippocampus and (D, H, L, P) layers II/III of the cortex are shown. Samples with active caspase 3 costaining were also counterstained with DAPI (blue fluorescence) to indicate nuclei. (M–P) Arrows demonstrate active caspase 3, NS5 dual positive cells. Scale bar is the same for all images and is shown in A.

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Fig. 8. Zika infection of polygonal cells of the testes from anti-IFNAR treated Rag1−/− mice
(A–H) Testes from mock-treated (A, G) wildtype and (B–F, H) anti-IFNAR treated Rag1−/−
mice described in Fig 6, was processed for histology and stained for (A, B and D–F) ZIKV
NS5 protein (green fluorescence) and active Caspase 3 or (C) Vimentin (red fluorescence,
pseudo colored magenta). Sections were also stained by in situ hybridization for (G and H)
ZIKV sense RNA. ZIKV-positive cells localized primarily to (B) regions of polygonal cells
in the testes and to a lesser extent (C) stromal and Sertoli cells and induced high levels of
(B) apoptosis in anti-IFNAR treated Rag1−/− mice, but not in (A) mock-treated wildtype
mice. (D–F) Higher resolution image of square in (B) showing some co-localization (white
arrows) between ZIKV NS5 protein and active caspase 3 positive cells. Images are
representative between mice. (G and H) Detection of ZIKV RNA is undetectable in (G)
control animals, but the polygonal area is heavily positive for viral RNA in (H) anti-IFNAR
treated Rag1−/− mice suggesting that a large numbers of cells are infected with virus. The
scale bar in A is relevant to images shown in A–F. The scale bar shown in G is relevant to
images shown in G and H.
Fig. 9. Pregnancy suppresses CD4+ and CD8+ T cell response to ZIKV infection
Non-pregnant and pregnant mice were infected with $10^4$ PFU of ZIKV at 7 days post-mating as described in the methods. At 7 dpi, spleens were removed and analyzed for flow cytometry. Cells were gated for (A) CD4+ or (B–F) CD8+ expression and then analyzed for (A–B) Ki67, (C) CD11a, (D) CD62L, (E) CD43 and (F) Granzyme B. Symbols indicate individual animals with lines shown as the mean per group. Data are the combined results of two experiments. Statistical analysis was completed using a One-way ANOVA with Tukey’s multiple comparison post-test. * P<0.05, ** P<0.01, ***P<0.001.