Title
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Permalink
https://escholarship.org/uc/item/3cq5r867

Journal
Proceedings of the National Academy of Sciences of the United States of America, 115(39)

ISSN
1091-6490

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Publication Date
2018-09-11

DOI
10.1073/pnas.1807690115

Peer reviewed
Deconvolution of pro- and antiviral genomic responses in Zika virus-infected and bystander macrophages

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Contributed by Christopher K. Glass, August 10, 2018 (sent for review May 8, 2018; reviewed by Sumit Chanda and Stephen T. Smale)

Genome-wide investigations of host-pathogen interactions are often limited by analyses of mixed populations of infected and uninfected cells, which lower sensitivity and accuracy. To overcome these obstacles and identify key mechanisms by which Zika virus (ZIKV) manipulates host responses, we developed a system that enables simultaneous characterization of genome-wide transcriptional and epigenetic changes in ZIKV-infected and neighboring uninfected primary human macrophages. We demonstrate that transcriptional responses in ZIKV-infected macrophages differed radically from those in uninfected neighbors and that studying the cell population as a whole produces misleading results. Notably, the uninfected population of macrophages exhibits the most rapid and extensive changes in gene expression, related to type I IFN signaling. In contrast, infected macrophages exhibit a delayed and attenuated transcriptional response distinguished by preferential expression of IFNB1 at late time points. Biochemical and genomic studies of infected macrophages indicate that ZIKV infection causes both a targeted defect in the type I IFN response due to degradation of STAT2 and reduces RNA polymerase II protein levels and DNA occupancy, particularly at genes required for macrophage identity. Simultaneous evaluation of transcriptomic and epigenetic features of infected and uninfected macrophages thereby reveals the coincident evolution of dominant proviral or antiviral mechanisms, respectively, that determine the outcome of ZIKV exposure.

Significance

Interpretation of genome-wide investigations of host-pathogen interactions are often obscured by analyses of mixed populations of infected and uninfected cells. Thus, we developed a system whereby we simultaneously characterize and compare genome-wide transcriptional and epigenetic changes in pure populations of virally infected and neighboring uninfected cells to identify viral-regulated host responses. Using patient-derived unmodified Zika viruses (ZIKV) infecting primary human macrophages, we reveal that ZIKV suppresses host transcription by multiple mechanisms. ZIKV infection causes both targeted suppression of type I interferon responses and general suppression by reducing RNA polymerase II protein levels and DNA occupancy. Simultaneous evaluation of transcriptomic and epigenetic features of infected and uninfected cells provides a powerful method for identifying coincident evolution of dominant proviral or antiviral mechanisms.

Zika virus | macrophage | immune evasion | genomics | transcription

Viruses survive and cause disease by avoiding and suppressing innate and adaptive immune responses. Flaviviruses are small, enveloped, single-stranded positive-sense RNA viruses (1). They replicate using a viral RNA-dependent RNA polymerase that creates viral messenger RNA and new viral genomes using a negative-sense RNA intermediate (2). More than 40 species of flaviviruses cause human disease, some associated with extensive global morbidity and mortality. Most flaviviruses cause acute infections and therefore must inhibit detection and eradication by the innate immune system. Flaviviruses have evolved multiple mechanisms to inhibit innate immune responses. Improved understanding of how these important pathogens subvert innate immune responses could lead to novel strategies for the development of antiviral agents.

Zika virus (ZIKV) is a member of the mosquito-borne group of flaviviruses that are primarily transmitted by Aedes mosquitoes. ZIKV can also be transmitted vertically from mother to fetus and between sexual partners (1, 3–7). Based on the presence of Aedes mosquitoes in nearly all tropical and subtropical areas of the world, it is estimated that ~3.6 billion people worldwide are at risk for ZIKV infection (8, 9). Since 2007, ZIKV has caused outbreaks worldwide with documented transmission in at least 84 countries (10). Although the precise mechanisms are under investigation, there is strong evidence that ZIKV causes life-threatening fetal brain abnormalities and Guilain–Barré syndrome (11–15). ZIKV, like other flaviviruses, naturally infects innate immune cells, including monocytes and macrophages (16). The ability to infect these cells is thought to increase viral dissemination and contribute to pathogenesis. However, how ZIKV infects macrophages and circumvents innate immune activation is not well understood.

Due to the central importance of IFNs in antiviral responses, most pathogenic viruses antagonize IFN production and/or IFN-dependent response pathways directly (17). However, many viruses also employ general host shut-off mechanisms that interfere with RNA transcription, RNA processing, and/or translation to inhibit host gene expression broadly (18, 19). This can both increase cellular resources available for the production of viral products and suppress host antiviral responses. To shut off host transcription, some viruses inhibit RNA polymerase II (RNAPII), a large protein complex responsible for catalyzing the synthesis of mRNAs, most snRNAs, and microRNAs (20–25). Although flaviviruses, including ZIKV, are known to inactivate the IFN pathway at multiple levels, none has been shown to inhibit transcription globally (26).
Genome-wide investigations of ZIKV–macrophage interactions have great potential to identify mechanisms by which ZIKV modulates macrophage response in an unbiased manner. A major limitation to understanding how ZIKV infects macrophages, or other cell types, is that the percentages of infected vs. uninfected bystander cells in cultures vary widely. Thus, population-level analyses are difficult to interpret because they include cells that are not productively infected but could potentially respond to signals from infected cells. Direct characterization of uninfected cells in comparison with infected cells is therefore required to disentangle proviral and antiviral responses and provide insights to viral infections in vivo, in which only a small fraction of cells is infected. Potential options to overcome this limitation include the use of reporter viruses, highly susceptible cell lines, or single-cell sequencing technologies. Reporter viruses help isolate infected cells, but the insertion of reporter proteins into flaviviruses, which are relatively small, may alter their virulence and prohibit studies comparing wild-type patient-derived viruses. Studying susceptible cell lines increases the percentage of infected cells within a population, but these cell lines often support robust viral replication because they lack important antiviral signaling responses. Single-cell profiling technologies are poised to circumvent many of these limitations; however, many methods, including ChIP sequencing (ChIP-seq), cannot yet be reliably applied at the single-cell level. Additionally, single-cell RNA-seq (RNA-seq) technologies that rely on oligo(dT) priming cannot distinguish flavivirus-infected from uninfected cells, as these viruses do not make polyadenylated RNA.

To overcome these obstacles, we developed an infection model that enables the application of unbiased genome-wide transcriptional and epigenetic analyses to identify how unmodified clinical isolates of ZIKV modulate host responses in pure populations of ZIKV-infected primary human macrophages, major cellular hosts of ZIKV, and other closely related flaviviruses. We observed strikingly divergent transcriptional and epigenetic responses between ZIKV-infected and uninfected bystander macrophages. Many of these differences would not be apparent if the population of cells were studied as a whole. ZIKV infection is associated with minimal gene activation and a nearly complete loss of type I IFN signaling primarily resulting from ZIKV-induced degradation of STAT2. In contrast, noninfected cells exhibit robust activation of the type I IFN response. Additionally, single-cell RNA-seq (RNA-seq) technologies that rely on oligo(dT) priming cannot distinguish flavivirus-infected from uninfected cells, as these viruses do not make polyadenylated RNA.

Divergent Transcriptional Responses in ZIKV-Infected and Bystander Cells. To identify how ZIKV modulates macrophage responses, we assessed genome-wide transcriptional responses in pure populations of uninfected HMDMs. HMDMs were infected with ZIKV using ADE1 conditions and were formaldehyde-fixed at different time points PI. HMDMs were then stained intracellularly for the flavivirus E protein using 4G2 antibody and were separated by FACS into infected ZIKV antigen-positive (ZIKV+) and uninfected ZIKV antigen-negative (ZIKV−) populations. After reverse cross-linking and protease digestion steps, high-quality total RNA was isolated and analyzed using stranded rRNA-depleted total RNA-seq (Fig. 1A). Our approach successfully segregated ZIKV+ from ZIKV− cells, as significant numbers of reads aligning to the ZIKV genome were found only in the ZIKV+ populations (Fig. 1B). As expected, the percentage of reads aligning to the ZIKV genome in ZIKV+ cells increased with time PI. By 24 h PI, more than 40% of all mapped RNA-seq reads in ZIKV+ HMDMs aligned to the ZIKV genome although the ZIKV genome is only ~0.01% the size of all human nonoverlapping exons (Fig. 1B).

We first compared the host transcriptionomes of mock-infected (medium alone), ZIKV+, and ZIKV− HMDMs using principal component analysis (PCA). Even though each of the replicates was performed with cells from different individuals, PCA grouped replicates based on infection status and time point PI (Fig. 1C). This close association among replicates suggests that interindividual differences have less influence on the transcriptional response of HMDMs than infection status and time point PI. Strikingly, the first principal component (PC1) divided the data into two groups, separating ZIKV+ macrophages from ZIKV− and mock-infected HMDMs. This suggested that the transcriptional responses of ZIKV+ macrophages were more similar to mock-infected cells than to ZIKV− bystander cells (Fig. 1C). In fact, when considering only PC1 and PC2, which captured 68% of the overall transcriptional differences between the samples, at 12 h PI, the transcriptionome of ZIKV+ HMDMs largely overlapped with that of mock-infected macrophages. Only at later time points did the transcriptionomes segregate along PC2 based on time PI. This suggests that ZIKV infection of macrophages, especially at early time points PI, does not elicit a strong transcriptional signature.
ZIKV modulates macrophage transcription during infection. (A) Diagram depicting the infection model. HMDMs are infected with ZIKV and stained for ZIKV group antigen followed by FACS isolation of live, productively infected ZIKV− and bystander (ZIKV+) macrophages. (B) Percent of RNA-seq reads aligning to the ZIKV genome in FACS-isolated ZIKV+ and ZIKV− macrophages at the indicated time points PI. Percent was calculated as the total reads aligning to ZIKV alone vs. human + ZIKV genomes. Each data point (mean ± SEM) represents results from HMMDs derived from different human donors. All categories were compared using ANOVA with correction for multiple comparisons. (C) PCA biplot of the first two principal components comparing RNA-seq of FACS-isolated ZIKV+ or ZIKV− HMDMs from three (12 h and 18 h) or five (mock-infected and 24 h) individual donors. (D) Calculated loss of sensitivity in detecting ZIKV-regulated gene expression. Gene-expression levels were calculated using data from ZIKV+, ZIKV−, and mock-infected macrophage RNA-seq experiments 24 h PI performed with macrophages from five different donors. (E and F) Individual gene expression calculated by RNA-seq in pure populations of mock-infected, ZIKV+, and ZIKV− macrophages (24 h PI). Mean (± SEM) IL1A expression (E) and CCR1 (F) expressions of pure populations (black bars) were calculated based on RNA-seq from five different donors. (G) Expressed as in D. (H) Mean (± SEM) EIF2AK2 and IL6 expression of pure populations (black bars) and a 36% mixed population (white bar) calculated as described in E and F.

Transcriptome studies of in vitro infections have typically analyzed mixed populations of infected cells and uninfected bystanders. To determine how a traditional mixed-population transcriptome analysis would differ from our approach of isolating and analyzing pure populations of ZIKV+ and ZIKV− cells, we used our transcriptomic profiles of pure ZIKV+ and ZIKV− HMDM populations derived from five different individuals to simulate the transcriptional profiles of hypothetical ZIKV-infected bulk populations over a range of 100-0% infected cells (Fig. 1D). We used these simulated profiles to calculate the recall rate of significantly changed genes identified in pure ZIKV+ cells. In our analysis, as the fraction of infected cells decreased from 100%, the ability to identify genes specifically repressed, and to a lesser degree induced, by ZIKV was lost rapidly (Fig. 1D). Using our highly efficient macrophage infection system, the average percent of infection at 24 h PI was 36%. Our model estimated that we would identify only 10% of the repressed and 32% of the induced genes in a 36% ZIKV+ HMDM population (Fig. 1D). Examples of genes that were induced by ZIKV that would have not been identified at 36% infectivity included IL1A, EIF2AK3, PERK, ASAP2, and KLF4 (Fig. 1E and SI Appendix, Fig. S1G). Similarly, we would have missed ZIKV-repressed genes such as CCRI, TNFRS11A, and CCL2 (Fig. 1F and SI Appendix, Fig. S1H). In addition to decreased sensitivity in identifying genes regulated by ZIKV, analysis of mixed populations can falsely identify genes as induced or suppressed by ZIKV. At 36% infectivity, we estimated that analysis of a mixed population would misidentify ~600 genes as induced and 300 genes as repressed by ZIKV (Fig. 1G). For example, multiple inflammatory genes, including EIF2AK2/PKR, IL6, and IFITM2 were strongly induced only in ZIKV+ bystander cells. Mixed population analysis would suggest that these genes were strongly up-regulated by ZIKV when in fact these genes were not induced or in many cases were even suppressed in cells productively infected with ZIKV (Fig. 1H and SI Appendix, Fig. S1I).

In addition to improved discovery and accuracy, a major advantage of our infection model is the ability to analyze transcriptional responses in ZIKV-infected cells with unmodified clinical isolates. Using our approach, we observed that transcriptional analyses of mixed populations of ZIKV+ and ZIKV− cells have especially poor sensitivity in detecting down-regulated genes. We estimate that a mixed cell transcriptional analysis with 36% ZIKV+ cells would not identify 36 of the 46 genes specifically down-regulated during FSS infection, including DDX5, DDX17, and DDX42 and the splicing factors SRMR2 and SUGP2 (SI Appendix, Fig. S1N). As shown by our simulation above, transcriptional analyses of mixed populations of ZIKV+ and ZIKV− cells will lack sensitivity in detecting down-regulated genes.

ZIKV Infection Elicits a Limited Transcriptional Response. To evaluate ZIKV–macrophage interactions over time, we compared the transcriptional responses in ZIKV+ and ZIKV− cells at 12, 18, and 24 h PI to mock-infected HMDMs. ZIKV− cells up-regulated a larger number of genes than ZIKV+ cells at 12 h PI (420 genes in ZIKV− cells vs. 100 genes in ZIKV+ cells), 18 h PI (708 in ZIKV− cells vs. 244 in ZIKV+ cells), and 24 h PI (867 in ZIKV− vs. 657 in ZIKV+) (SI Appendix, Fig. S2A). While some inflammatory genes were induced in ZIKV+ cells (examples include CXCL10, RSA2D, IFIT1, IFIT2, CXCL11, IFITM1, IFI44L, CCL8, and TNFSF10/TRAIL), the degree of induction was greater in ZIKV+ bystander cells (SI Appendix, Fig. S2 B–D).
However, as time PI increased, more uniquely up-regulated genes were identified in ZIKV+ cells (SI Appendix, Fig. S2 C and D). For example, at 18 h PI, ZIKV+ cells up-regulated API53 and AS4AP2, involved in Golgi trafficking, and TNFRSF10D, a decoy TNF receptor that protects against TNFSF10/TRAIL-mediated apoptosis, more than ZIKV− cells (SI Appendix, Fig. S2C). After 24 h of infection, ZIKV+ cells up-regulated the gene encoding IFNβ, IFNB1, and the transcription factor KLFL4, involved in cell-cycle control, more than their ZIKV− neighbors (SI Appendix, Fig. S2D).

**ZIKV Suppresses Inflammatory Gene Activation in Macrophages.** To identify signaling pathways affected by ZIKV infection of macrophages, we performed gene ontology and pathway analysis on ZIKV− and ZIKV+ cells and compared them with mock-infected HMDMs. At all time points ZIKV+ HMDMs showed a delayed and diminished induction of genes from multiple inflammation and immune response-related categories including IFN signaling, cytokine signaling, antigen presentation, and pattern recognition receptor (PRR) response pathways (Fig. 2A and B). To identify how ZIKV manipulates the transcriptional responses in HMDMs, we identified the subset of genes uniquely induced in ZIKV+ cells at 12, 18, and 24 h PI. The number of uniquely induced genes at 12 h PI in ZIKV+ cells was limited to only 36 genes. However, these genes were significantly enriched for genes involved in cholesterol biosynthesis, such as HMGC5, MVD, MVK, and SQLE (Fig. 2C). At 24 h PI, genes specifically induced in ZIKV+ macrophages were enriched for those involved in the unfolded protein response, including the endoplasmic reticulum (ER) stress response kinase EIF2AK3/PERK and the transcription factor DDIT3/CHOP (Fig. 2C).

In addition to suppressed inflammatory gene induction, ZIKV+ macrophages also demonstrated increased down-regulation of genes involved in multiple immune pathways, including leukocyte activation/migration and cytokine signaling/secrection (SI Appendix, Fig. S2).

![Image](image-url)

**Fig. 2.** Degradation of STAT2 by ZIKV impairs ISG activation. (A) Heat maps of the top enriched functional annotations of genes significantly up-regulated (fold change ≥ 2, FDR < 0.01) in ZIKV+ and ZIKV− macrophages compared with mock-infected macrophages at the indicated time points. (B) Heat map of the relative expression of selected genes involved in cytokine signaling and antigen presentation in mock-infected, ZIKV+ and ZIKV− macrophages 24 h PI. Data are the average of five experiments. (C) Venn diagrams showing the numbers of unique and shared up-regulated genes in ZIKV+ and ZIKV− macrophages compared with mock-infected macrophages at 12, 18, and 24 h PI. The top significantly enriched functional category for genes uniquely induced in ZIKV+ cells (red) at each time point is shown with examples of genes from that category. (D) Heat map depicting relative transcription of type I and III IFN genes and ISG in ZIKV− and ZIKV+ macrophages vs. control (Mock) over time. Data are the average of three experiments (12 h and 18 h) or five experiments (24 h). (E) Western blot of STAT1, phosphorylated-STAT1, STAT2, phosphorylated STAT2, ZIKV NS2B, and β-actin levels in equivalent numbers of mock-infected, ZIKV−, and ZIKV+ cells at 24 h PI. Data were analyzed by ANOVA with MG132 treatment groups compared with vehicle control (Mock), STAT2 density is relative to β-actin. Data shown (mean ± SEM) are from three infections performed in HMDMs derived from three different individuals at 24 h PI. Data were analyzed by ANOVA with MG132 treatment groups compared with vehicle with correction for multiple comparisons. (G) Relative gene expression in ZIKV− and ZIKV+ cells treated with MG132 compared with DMSO. Data (mean ± SEM) show the relative gene expression by qRT-PCR in at least three independent experiments performed with FACs HMDMs derived from different donors at 24 h PI. Data for each gene were analyzed by ANOVA with MG132-treatment groups compared with vehicle with correction for multiple comparisons. Asterisks indicate statistically significant differences (*P < 0.05).
ZIKV, like other human pathogenic flaviviruses, blocks IFN induction of antiviral IFN-stimulated genes (ISGs). However, the exact mechanisms by which and where in the IFN response pathway ZIKV blocks ISG activation are not fully understood. Moreover, the IFN response during flaviviral infections is highly context-dependent and varies in a virus-, cell type-, and host species-specific manner (30). Previous studies have demonstrated that ZIKV may block the induction and/or translation of IFNβ, thereby inhibiting the secretion of IFNs, and/or degrade STAT2 and inhibit the phosphorylation of both STAT1 and STAT2, which inhibits signaling downstream of the IFN α/β receptor and induction of ISGs (31–33). Therefore, we examined the expression of IFN and ISG genes in ZIKV+ and ZIKV− cells throughout ZIKV infection. ZIKV+ cells express type I and type III IFN genes equivalently to ZIKV− cells at 12 and 18 h PI and higher levels than ZIKV− cells at 24 h PI (Fig. 2D and SI Appendix, Fig. S2G). In contrast, ISG expression is markedly reduced in ZIKV+ cells compared with ZIKV− cells at 12, 18, and 24 h PI (Fig. 2D and SI Appendix, Fig. S2H). During ZIKV infection of human dendritic cells, IFNs are induced at the transcriptional level, but protein levels are not measurable in the supernatants, suggesting impaired IFN protein production (31). To determine if IFNβ is made and secreted following IFNB1 induction during ZIKV infection of macrophages, we measured the IFNβ protein levels in supernatants by ELISA. We consistently detected IFNβ in the supernatants of macrophages infected with ZIKV at 24 h PI (SI Appendix, Fig. S2I). Collectively, these data show that ZIKV broadly suppresses inflammatory gene expression in infected macrophages. Type I and type III IFN genes are induced in ZIKV− macrophages, and IFNβ is produced, but ZIKV+ macrophages demonstrate markedly reduced ISG induction compared with ZIKV− cells. This suggests ZIKV blocks type I IFN signaling rather than type I IFN production in macrophages.

ZIKV Degradation of STAT2 to Inhibit Type I IFN Signaling in Macrophages.

To explore ZIKV inhibition of type I IFN signaling in macrophages, we first asked at which time point PI ZIKV inhibits IFN signaling by treating ZIKV-infected HMDM cultures with exogenous type I IFN before infection or at multiple time points PI. Exogenous IFN inhibited ZIKV infection in the HMDM cultures when given at or before 4 h PI but not after 8 h PI (SI Appendix, Fig. S3A). We next measured the kinetics of type I IFN and ISG gene expression during ZIKV infection to determine how rapidly these genes are up-regulated. Compared with mock-infected cells, IFNB1 and ISGs, such as MX1, were consistently up-regulated only at 8–12 h PI in ZIKV-infected HMDM cultures (SI Appendix, Fig. S3 B and C). In contrast to IFNB1, which is induced similarly in ZIKV+ and ZIKV− cells at 12 h PI, there was no induction of MX1 in ZIKV− cells (SI Appendix, Fig. S3 D and E). These findings demonstrate that ZIKV blocks type I IFN signaling in productively infected macrophages before IFNs can stimulate ISG production.

To identify the potential mechanisms by which ZIKV inhibits type I IFN signaling in macrophages, we examined levels of total and phosphorylated STAT1 and STAT2 proteins in ZIKV+, ZIKV−, and mock-infected control HMDMs. Western blots demonstrated decreased STAT2 levels in ZIKV+ cells compared with ZIKV− and mock-infected cells, consistent with STAT2 degradation (Fig. 2E and SI Appendix, Fig. S3F). We did not detect STAT2 phosphorylation in either ZIKV+ or mock cells. Levels of STAT1 were lower in ZIKV− than in ZIKV+ cells but were not statistically different from levels in mock-infected HMDMs (SI Appendix, Fig. S3G). Phosphorylated STAT1 was detectable in both ZIKV+ and ZIKV− cells but not in mock-infected cells (Fig. 2E and SI Appendix, Fig. S3F). Consistent with the data described above suggesting blockade of downstream IFN signaling early in infection, we observed (i) suppressed induction of STAT1, an ISG, and STAT1 phosphorylation by 12 h PI and (ii) up-regulation of STAT1 protein by 18 h PI in ZIKV− cells compared with ZIKV+ cells (SI Appendix, Fig. S3 H–J).

Proteasome Inhibition Rescues STAT2 Degradation but Minimally Restores ISG Induction. Studies using cell lines have shown that ZIKV induces STAT2 degradation in a proteasome-dependent manner and that treatment with a proteasome inhibitor can restore STAT2 levels (32, 33). Given that STAT2 is degraded by ZIKV in human macrophages, we wanted to confirm that inhibiting proteasome function would block the degradation of STAT2 and additionally determine if this was sufficient to protect macrophages from ZIKV infection. We infected HMDMs with ZIKV and allowed the virus to establish infection for 12 h. We then added the proteasome inhibitor MG132 or DMSO control and isolated ZIKV+ and ZIKV− macrophages 12 h later (24 h PI) (SI Appendix, Fig. S3K). Addition of MG132, even at 12 h PI when ZIKV has inhibited IFN signaling, significantly, increased STAT2 protein levels (Fig. 2F and SI Appendix, Fig. S3L). However, while restoration of STAT2 levels by MG132 led to significantly increased IFIT3 gene expression in ZIKV+ cells, it did not increase MX1, CXCL10, IFI27, IFI16, and OAS3 expression (Fig. 2G).

ZIKV Degradation of STAT2 Suppresses the Genomic Activation Landscape of Infected Cells. Signal-dependent transcription factors (SDTFs) play major roles in the regulation of gene expression by binding to distal enhancers (34). The activities of transcription factors at enhancer elements can be inferred by changes in H3K27ac, which correlates with transcriptional activity (35). Therefore, motif analysis of genomic regions exhibiting gain or loss of H3K27ac can be used to infer the activity states of the corresponding transcription factors in an unbiased manner. To implement this approach, we performed ChIP-seq for H3K27ac utilizing pure populations of ZIKV+ and ZIKV− cells 24 h PI as well as mock-infected controls from three independent infections using HMDMs derived from three different individuals (Fig. 3A).

Similar to the RNA-seq results that showed suppressed gene induction, ZIKV+ macrophages had far fewer significantly up-regulated H3K27ac peaks than ZIKV− cells (547 vs. 2,049 significant peaks) (Fig. 3B). In contrast to ZIKV− cells, ZIKV+ macrophages had more significantly down-regulated H3K27ac peaks than up-regulated peaks (Fig. 3B). Comparing H3K27ac in ZIKV+ and ZIKV− cells identified many H3K27ac regions at promoters and enhancers of genes that were specific to ZIKV− cells. These included ISGs, such as OAS2 and members of the IFITM family, as well as other inflammatory genes (Fig. 3 C and D). Cis-regulatory elements marked exclusively by H3K27ac in ZIKV− cells were associated with genes involved in cell cycle, cell differentiation, delayed senescence, and apoptosis inhibition such as CDK6, BCL2, and KLF4 (Fig. 3C and SI Appendix, Fig. S4A). Genome-wide analysis of promoter-distal H3K27ac peaks demonstrated strikingly reduced enrichment of IFN regulatory factor (IRF) IFN-stimulated response element (ISRE) motifs in
**Fig. 3.** ZIKV suppresses the activation of genomic regions containing ISRE/IRF motifs. (A) Diagram depicting the infection model for H3K27ac ChIP-seq. HMDMs are infected with ZIKV and stained for ZIKV group antigen followed by FACS isolation of productively infected ZIKV+/− and bystander (ZIKV−) macrophages and then followed by H3K27ac ChIP-seq. (B) The number of regions with significantly increased or decreased H3K27ac (fold change >2 and FDR < 0.01) in ZIKV+ or ZIKV− cells compared with mock-infected cells. (C) Scatter plot of H3K27ac tag counts at genomic regions marked by significant H3K27ac in ZIKV+ and ZIKV− macrophages 24 h PI. Regions with significantly elevated or decreased H3K27ac in ZIKV+ (red) and ZIKV− (blue) are colored. (D) UCSC browser visualization of H3K27ac near the IFITM gene locus in control, ZIKV+, and ZIKV− cells. The upper panel displays transcription as defined by RNA-seq. The lower panel displays H3K27ac abundance in control (Mock, black), ZIKV+ (blue), or ZIKV− (red) macrophages. Regions with significantly up-regulated H3K27ac in ZIKV+ cells are marked with blue shading. (E) Comparative motif enrichment at promoter-distal active chromatin regulatory regions as defined by H3K27ac in ZIKV+ (blue bar) vs. ZIKV− (red bar) HMDMs.

ZIKV+ cells (Fig. 3E). In contrast, although enriched to a much lesser extent, NF-κB and STAT1 motifs were equally represented in promoter-distal H3K27ac peaks in ZIKV+ and ZIKV− macrophages. Motifs for PU.1 and C/EBP, two macrophage lineage-determining transcription factors (LDTFs), also showed increased representation in distal H3K27ac peaks in ZIKV− cells, likely reflecting their general requirement for binding of SDFTs (34). Collectively, these results provide evidence that the dominant transcriptional response of ZIKV+ cells results from activation of type I IFN signaling and suggest that this response is impaired in ZIKV− cells. STAT2 degradation in ZIKV+ cells leads to inhibition of genomic activation of ISRE/IRF-containing enhancers and promoters, thereby broadly suppressing inflammatory and ISG activation. These findings confirm and extend the literature by demonstrating that, in human macrophages, the type I IFN system is the dominant anti-ZIKV mechanism and that type I IFN signaling, rather than type I IFN production, is the major target of ZIKV antagonism.

**ZIKV Infection Reduces RNApol2 Protein Levels and DNA Occupancy, Particularly at Genes Required for Macrophage Identity.** The RNA amounts recovered from ZIKV+ HMDMs were reproducibly lower than the amounts recovered from the corresponding ZIKV− cells [mean ZIKV+/-to-ZIKV− ratio = 0.63, 95% CI (0.47–0.80)] (SI Appendix, Fig. S4F). Although the majority of cellular RNA is rRNA synthesized by RNApol1, we focused on RNApol2 because this polymerase produces the majority of protein-coding transcripts involved in antiviral responses. To determine the location and relative quantities of RNApol2 genome-wide, we performed ChIP-seq for RNApol2 in equivalent numbers of pure populations of ZIKV+ and ZIKV− cells 24 h PI as well as in mock-infected controls (Fig. 4A). In three independent infections, using HMDMs derived from three different individuals, we consistently detected less RNApol2 at many locations across the genome in ZIKV+ macrophages than in ZIKV− macrophages (Fig. 4A and SI Appendix, Fig. S4C). To ensure that the lower RNApol2 ChIP levels in ZIKV+ cells were not related to general problems performing ChIP-seq in ZIKV+ cells, we performed ChIP-seq for the CCCTC-binding factor (CTCF) transcription factor that is involved in regulating chromatin structure. In contrast to RNApol2, the levels of CTCF were roughly equivalent in both ZIKV+ and ZIKV− cells (SI Appendix, Fig. S4D). RNApol2 levels were increased at IFNB1 and decreased at MX1, MX2, CXLC10, CCL8, and CCL2 in ZIKV+ cells compared with ZIKV− cells, as expected (Fig. 4A and SI Appendix, Fig. S4E). However, RNApol2 was also significantly decreased at unexpected sites, such as CEBPB, and many mRNAs, such as RNU4-2 and RNU4-1 (Fig. 4A–C). Additionally, ZIKV+ cells exhibited significantly lower levels of RNApol2 at many genes commonly associated with core macrophage functions such as the LDTFs SPI1, CEBPB, and MAFF, peptides MIP9, CTSD, and CTSZ, lysozyme LZ7, ferritin light and heavy chain genes FTH1 and FTL, cell-surface receptors CD14 and CD68, and genes involved in antigen processing, i.e., IFIT3 and CD74 (Fig. 4D and SI Appendix, Fig. S4F). Many of these genes are associated with superenhancers (SEs) in HMDMs. SEs are regions of disproportionately high densities of active chromatin regulatory marks and transcription factor binding close to genes that play essential roles in the identity and function of cell types (35). While the majority of genes have lower RNApol2 levels in ZIKV+ cells than in mock-infected macrophages, genes associated with SEs demonstrated a disproportionate reduction in RNApol2 signal (Fig. 4E).

RNApol2 is a large protein complex made up of 12 subunits. The largest RNApol2 subunit, CBP, contains the DNA-binding domain of RNApol2 and a C-terminal domain that is essential for regulating polymerase activity and associated processes. To determine if the decreased RNApol2 ChIP-seq levels in ZIKV+ cells were associated with decreased RNApol2 protein levels, we measured RBPI levels in mock-infected, ZIKV+, and ZIKV− cells. ZIKV+ cells had significantly lower levels of RBPI protein than ZIKV− and mock-infected control macrophages (Fig. 4F and G and SI Appendix, Fig. S4G). In contrast to RBPI protein levels, transcription of the gene POLR2A that encodes RBPI was significantly up-regulated in mock+ cells compared with ZIKV+ and mock-infected control macrophages (Fig. 4H). While this observation was similar to the discrepancy in STAT2 RNA and protein levels induced by ZIKV, RBPI levels did not normalize after the addition of proteasome inhibitor, suggesting that ZIKV employs a different mechanism to lower host cell RBPI levels. Collectively, these data demonstrate that productive ZIKV infection reduces RNApol2 DNA occupancy globally but that this effect is amplified at core macrophage genes, many of which are associated with SEs.

**Discussion**

In these studies, we identify the genome-wide signaling networks in primary human macrophages infected with ZIKV. We demonstrate that neighboring ZIKV-infected and uninfected cells have differing, often opposite, transcriptional responses that obscure analyses of mixed populations in which uninfected cells make up even a small percentage of the total population. Our transcriptomic and epigenomic profiles of pure populations of infected macrophages thus provide a more accurate map of the human macrophage signaling response during ZIKV infection. By comparing the transcriptomes and epigenomic features in neighboring ZIKV+ and ZIKV− macrophages that are exposed to identical environmental signals, the mechanisms employed by ZIKV to subvert macrophage immunity are revealed. Importantly, our findings demonstrate that studying a mixed culture of ZIKV-infected HMDMs would lead to the reasonable but erroneous
conclusion that infection induces a type I IFN response when, in fact, this response is restricted to cells that do not become productively infected. The direct comparison of ZIKV<sup>+</sup> and ZIKV<sup>−</sup> cells thus provides important insights into the balance of pro- and antiviral mechanisms and an unbiased assessment of the dominant regulatory pathways involved. This approach does not require viral infections in any cell type. During infection with any virus and to directly compare host RNApol2 recruitment. (A) Scatter plot of log<sub>2</sub> FPKM RNApol2 tag counts at genomic regions marked by significant RNApol2 in ZIKV<sup>+</sup> vs. ZIKV<sup>−</sup> macrophages at 24 h PI. Color coding: gray, all genomic regions; blue, protein-coding regions; red, snRNA-coding regions. (B) UCSC browser visualization of RNA-seq (first panel), RNApol2, H3K27ac (third panel), and CTCF (fourth panel) near the CEBPB gene locus in control (Mock, black), ZIKV<sup>+</sup> (blue), or ZIKV<sup>−</sup> (red) macrophages. (C) UCSC browser visualization of RNApol2 (Upper) and H3K27ac (Lower) near two snRNA genes, RNU4-2 and RNU4-1, in control (Mock, black), ZIKV<sup>+</sup> (blue), or ZIKV<sup>−</sup> (red) macrophages. (D) Heat map depicting relative RNApol2 levels at 24 h PI. (E) Scatter plots to show the fraction of genes overlapping SE as a function of their change in RNApol2. (F) Western blot of RPB1, β-actin, and ZIKV-NS2B levels extracted from FACS-isolated equivalent numbers of mock-infected, ZIKV<sup>+</sup>, and ZIKV<sup>−</sup> cells at 24 h PI. (G) Relative quantitation of Western blot RPB1 levels. RPB1 density is relative to β-actin with control samples set to 1. Relative levels (mean ± SEM) of RPB1 in control (Mock), ZIKV<sup>+</sup>, and ZIKV<sup>−</sup> cells are shown for three infections in different individuals at 24 h PI. (H) Log<sub>2</sub>-transformed FPKM RNA-seq counts for POLR2A in control (Mock), ZIKV<sup>+</sup>, and ZIKV<sup>−</sup> cells 24 h PI. Data represent expression from RNA-seq performed in five different individuals. Data for F and H were analyzed by ANOVA with all-group comparison with correction for multiple comparisons. Asterisks indicate statistically significant differences (****p < 0.0001; **p < 0.01).
by blocking proliferation and inducing cell death (37, 38). Conceivably, loss of RNApol2 in these cell types could contribute to cell death in these tissues.

Our genome-wide studies of ZIKV infection in primary human macrophages demonstrate targeted suppression of ISRE/IRF-dependent signaling networks that can be explained by STAT2 degradation and are consistent with previous studies in cell lines demonstrating that ZIKV NS5 protein degrades STAT2 in proteasome-dependent manner (32, 33). In macrophages, proteasome inhibitor treatment given more than 4 h after ZIKV has disabled cellular IFN signaling partially restored induction of IFIT3 but not of other ISGs, such as MX1, CXCL10, IFI27, IFI6, and OAS3. While all six of these ISGs have at least one ISRE motif in their promoters [−300 to +100 bp from the transcription start site (TSS)], only CXCL10 and OAS3 have NFκB motifs in their promoters (SI Appendix, Fig. S4H). These and other antiviral ISGs that are not partially rescued by MG132 may also be regulated by NFκB at distal enhancer elements. Thus, although these results could reflect gene-specific requirements for NFκB, which is inhibited by proteosome blockade, they are also consistent with the inhibitory effects of ZIKV on RNApol2.

ZIKV was discovered in 1947 and has caused sporadic human infections for more than 50 y, but only during the recent outbreaks in French Polynesia and South America was ZIKV associated with congenital ZIKV syndrome and Guillain–Barré syndrome (13, 14, 39, 40). Although the specific mechanisms underlying this increased human pathogenicity are still under investigation, it has been suggested that evolution of the ZIKV genome may contribute to this increasing virulence (41, 42). In support of this hypothesis, experimental evidence has demonstrated strain-specific differences in viral pathogenesis and neurologic disease in a STAT2-deficient mouse model and single-amine acid substitutions that increase infectivity of Aedes aegypti mosquitoes and human and mouse neural progenitor cells (43–45). Here, we performed genome-wide transcriptomic analysis comparing human macrophage responses to two patient-derived Asian ZIKV subtypes, SD001 and FSS, and the specificity of our system is demonstrated by the fact that when bystander ZIKV+ cells from SD001 and FSS infections are compared, we detect no significant differences in gene regulation. In contrast, when ZIKV+ cells infected with SD001 or FSS are compared, 110 genes are identified as being regulated in a strain-specific manner. The similar transcriptional response in bystander ZIKV+ cells during SD001 and FSS infections suggests that the bystander ZIKV+ cells principally respond to secreted environmental signals such as IFNs and cytokines and that these environmental signals are largely the same during FSS and SD001 infections. Indeed our transcriptional analysis shows that the majority of cytokines/chemokines are made in ZIKV+ bystander cells. ZIKV+ cells also respond to environmental stimuli, but their response is modified by viral activation and repression of cellular response pathways. In the ZIKV+ populations, strain-specific differences can influence how the virus activates or represses these responses. In ZIKV+ cells, we identified differentially expressed genes with functional enrichment for RNA processing and RNA splicing and include multiple DEAD box RNA helicases (DDX5, DDX17, and DDX42) that are down-regulated in a strain-specific manner. Members of this protein family can act as PRRs that recognize viral RNA and initiate antiviral responses. Japanese encephalitis virus, another flavivirus, has been shown to prevent DDX42 from activating type I IFN signaling (46, 47). Additionally, RNA splicing is modulated by many human pathogenic viruses, and ZIKV was recently shown to cause alternative splicing events in infected neural progenitor cells (48). Given the accuracy of our method and its ability to study differences between unmodified patient-derived viruses in primary human cells, we anticipate that future studies comparing numerous ZIKV strains could identify key virulence mechanisms associated with viral genome evolution.

Although ZIKV largely suppresses inflammatory gene transcription activation, genes associated with specific pathways are selectively up-regulated in ZIKV+ cells. Genes involved in cholesterol biosynthesis (HMGCSS1, MVD, MVK, and SQLE), ER/Golgi trafficking (API53 and ASAP2), and cell survival (TNFRSF10D and KLFR) are all induced in ZIKV+ macrophages and, based on previous data, have the potential to increase ZIKV pathogenicity. Cholesterol synthesis plays critical roles in flavivirus innate immune evasion and replication (49). Flavivirus buds into the ER lumen and requires transport through the trans-Golgi to form mature infectious particles and viruses (50). Developing treatments that target these pathways could prove efficacious, as evidenced by the fact that treatment with the HMG-CoA reductase inhibitorlovastatin increased survival rates in a mouse model of DENV-2 infection (51).

In summary, our work demonstrates that ZIKV manipulates macrophage transcription at multiple levels. ZIKV specifically inhibits type I IFN signaling and suppresses global transcription by decreasing RNApol2 levels. Our method for identifying and comparing genome-wide transcriptional and epigenetic changes in neighboring infected and uninfected primary human macrophages using unmodified patient-derived viruses provides an approach to allow rapid deconvolution of complex host–pathogen interactions and directly compare the pathogenicity of clinical viral isolates.

Methods

**HMDM Isolation and Differentiation.** Human blood for HMDM isolation was obtained from healthy volunteers and was unidentified under the La Jolla Institute Internal Review Board Protocol VD-057-0217. Donors were HIV, hepatitis B, and hepatitis C negative. Human peripheral blood was separated using Histopaque 1077 (catalog no. 10771; Sigma) spun at 400 × g for 30–60 min at 4 °C. Theuffy coat was washed once, and then the red blood cells were lysed with molecular-grade water. Monocytes were then negatively selected from the buffy coat using the pan monocyte isolation kit (catalog no. 130-096-537; Miltenyi Biotech) as described by the manufacturer. Cells were seeded onto tissue-coated plates and differentiated for 7 d in complete macrophage medium [macrophage serum-free medium (catalog no. 12065; Gibco) supplemented with 1% penicillin/streptomycin, 1% Nutridoma-SP (catalog no. 11011375001; Roche), 1% fungizone (catalog no. 15290-018; Gibco), and 100 ng/mL human macrophage-colony-stimulating factor (M-CSF) (catalog no. 300-25; PeproTech)] at 37 °C, 5% CO2. The medium was changed every 2–3 d.

**ZIKV Infection and Treatments.** On day 7 of culture, macrophages were infected with Zika virus clinical isolates SD001 or FSS13025 at the indicated MOI with or without ADE [MOI = 1 + 0.6% (vol/vol) DENV human immune serum]. The virus was incubated with HMDMs for 2 h at 37 °C, 5% CO2 with rocking every 30 min. The supernatants were removed, and cells were washed three times with 1× PBS, before the addition of fresh warm complete macrophage medium. Both RNA-seq and flow cytometry confirmed that no infectious DENV was present in the human immune serum. When indicated, HMDMs were treated with 1,000 U of universal type I IFN (PBL Assay Science), 10 μM MG132, or carrier controls at the indicated time points.

**Quantification of Infected HMDMs by Flow Cytometry.** HMDMs were differentiated for 3 d. Baby hamster kidney cells (BHKs) were seeded at 1–2×10^5 cells per well in a 24-well plate overnight. Supernatants harvested from infection experiments were serial diluted, added to BHKs, and incubated for 2 h with rocking. Supernatant was aspirated, and cells were washed twice with MEM-Wash Buffer followed by resuspension in MEM-Wash buffer and were analyzed by flow cytometry on a BD LSRII flow cytometer.

**Quantitation of Virus Production by FFU.** Baby hamster kidney cells (BHKs) were seeded at 1–2×10^5 cells per well in a 24-well plate overnight. Supernatants harvested from infection experiments were serial diluted, added to BHKs, and incubated for 2 h with rocking. Supernatant was aspirated, and cells were washed twice with MEM-Wash Buffer followed by resuspension in MEM-Wash buffer and were analyzed by flow cytometry on a BD LSRII flow cytometer.
permeabilized with 1% Triton X-100, washed, blocked with 10% PBS, and stained with 4G2 primary antibody (1 μg/ml) supplemented with 1% PBS. Wells were washed with 1× PBS and stained with HRP-conjugated secondary antibody supplemented with 1% BSA at room temperature. Cells were washed three times with 1× PBS, and 0.2 mL of TrueBlue (SeraCare) was added and incubated for 20 min at room temperature or until foci became apparent. Plates were then washed and dried, and FUs were counted.

**Virucal RNA Quantitation by qR-PCR.** Total RNA was isolated using the RNeasy Mini Kit (74106, Qiagen) as described by the manufacturer. A one-step qPCR was performed using ZIKV primers 853F: 5′-TGTGCTGATGACTGTGGTCAC-3′ and 911R: 5′-CTCTCACAAGTGCTCATTTG-3′ and 18S rRNA primers R: 5′-GCTGGAATTACCGCGGCT-3′ and F: 5′-GGCTGACCACATTCCAAGAAGA-3′ using a Bio-Rad qR-PCR Thermomycer. Virucal RNA concentration was determined based on an internal standard curve composed of five 100-fold serial dilutions of in vitro-transcribed RNA from ZIKV strain F551302S and normalized to 185 rRNA.

**STAT1 Flow Cytometry Analysis.** Macrophages were lifted by gentle cell scraping (scraping no. 83.1830; Sarstedt) into PBS, washed once with PBS, and incubated with Zombie live/dead stain (BioLegend) at 4 °C. After 4 h, cells were fixed in 4% formaldehyde, washed, and permeabilized in 90% ice-cold methanol. Samples were washed and blocked with Human Fc Block for 10 min at 4 °C followed by 30-min incubation at 4 °C with directly conjugated 4G2-AF647, AF488-Phospho-Stat1 (Tyr701) (Sty601), PE-Stat1 (D1K9Y), or isotype controls (Cell Signaling). Samples were centrifuged, washed twice with PBS + 0.5% BSA, and analyzed by flow cytometry on a BD LSR II flow cytometer.

**Separation of Infected and Uninfected Cells by FACS for RNA-Seq.** Macrophages were washed once with 1× PBS, gently scraped off in 1× PBS, pelleted at 4 °C, and stained with Zombie Violet viability dye (BioLegend) in the dark. Cells were treated once with FBS buffer (1× PBS, 3% FBS, and 1 mM EDTA) and were fixed and permeabilized with 4% formaldehdy containing 0.1% saponin (51705-5; Electron Microscopy Sciences) and 0.1% saponin (47036; Sigma-Aldrich) in molecular-grade PBS supplemented with 1:100 RNasin Plus RNase Inhibitor (N2615; Promega) for 30 min at 4 °C. Cells were washed in buffer wash (1× PBS containing 0.2% BSA, 0.1% saponin, and 1:100 RNasin Plus RNase inhibitor) and were blocked for 10 min with human Fc Block (1:500) (BioLegend) in staining buffer (1× PBS containing 1% BSA, 0.1% saponin, and RNasin Plus RNase inhibitor). HMDDM were then stained with 4G2 antibody (BioXcell) conjugated to AF647 (catalog no. A20186; Thermo Scientific) for 30 min at 4 °C, washed twice, resuspended at 5×10^6 cells/ml in sort buffer (PBS containing 0.5% BSA and 1:25 RNasin Plus RNase inhibitor) and sorted into ZIKV+ and ZIKV− cells on a FACsAria cell sorter (BD Biosciences) at the La Jolla Institute for Allergy and Immunology. Gates were set with reference to negative controls.

**RNA isolation and Library Preparation.** After sorting, cells were pelleted at 4 °C, the supernatant was discarded, and total RNA was isolated using the RecoverAll Total Nucleic Acid Isolation Kit (AM1975; Ambion); starting at the TruSeq stranded total RNA-seq kit (Illumina) per the manufacturer’s instructions. RNA was treated with in-column DNase per the manufacturer’s recommendations with the following modification. Cells were incubated in digestion buffer for 3 h at 50 °C supplemented with RNase Plus RNase inhibitor. RNA was treated with in-column DNase per the manufacturer’s instructions and was eluted; RNA quality was determined by BioAnalyzer using the Eukayto Total RNA Pico Chip. Samples with RNA integrity number (RIN) values greater than 8.0 were used for library preparation. RNA libraries were generated using the Truseq stranded total RNA-seq kit (Illumina) per the manufacturer’s instructions and were single-end sequenced for 51 cycles on an Illumina HiSeq 2000 or NextSeq 500 system per the manufacturer’s instructions.

**ChIP.** For ChIP-seq experiments, HMDDMs were cross-linked with 1% formaldehyde for 15 min in the presence of 1 mM sodium butyrate and were quenched with 0.125 M glycine. Preparation of HMDDMs for FACs was performed as described above for RNA-seq except that 1× Complete protease inhibitors (Roche) and 1 mM sodium butyrate (Sigma) were included in all buffers instead of RNase Plus RNase inhibitor. Following FACs, cells were washed, pelleted, and snap-frozen. ChIP for histone modification H3K27ac was performed as previously described (52). Sequencing libraries were prepared from recovered DNA (ChIP) using the NEBNext Ultra II DNA library prep kit (New England Biolabs) using NEBNext DNA Barcodes (Bio Scientific). ChIP-seq libraries were single-end sequenced for 51 cycles on an Illumina Hiseq 4000 or Nextseq 500 system according to the manufacturer’s instructions.

**Western Blot.** HMDDMs were processed as described in the ChIP-seq protocol above except that cComplete protease inhibitors (Roche) and PhosSTOP phosphatase inhibitors (Sigma) were included in all buffers. Following FACs, equal numbers of cells were aliquoted for each condition and were lysed using RIPA Buffer. Samples were then boiled in Laemmli Sample Buffer (Bio-Rad) for 5–10 min. Protein lysates were separated by 10% SDS/PAGE, electrophoretically transferred to a nitrocellulose membrane, and immunoblotted at 4 °C overnight with antibodies (1:1,000) against STAT1, STAT1-P, β-actin (Cell Signaling Technology), and β2M (GTX133308; GeneTex). Membranes were then incubated with an HRP-conjugated secondary antibody (1:10,000) for 1 h at room temperature followed by detection by enhanced chemiluminescence (Bio-Rad).
calling, and peaks overlapping GENCODE-defined promoter regions were excluded during this step. RNApol2-defined expression levels were calculated via ChIP-seq data and quantifying gene-body FPKM values using HOMER's analyzePeaks.pl script.

Statistical Analysis. Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software) using the recommended multiple comparison test. Values of P < 0.05 were considered significant.

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ACKNOWLEDGMENTS. We thank Dr. Aravinda de Silva (University of North Carolina School of Medicine, Chapel Hill) for providing dengue-immune serum. This research was supported by National Institute of Diabetes and Digestive and Kidney Diseases/NIH Grants R01 DK091183 and PPG DK074868 (to C.K.G.), National Institute of Allergy and Infectious Diseases/NIH Grants R01AI16813, R21NS100477, and R21AI127988 and an Interactive Fund Grant from Kyowa Kirin Pharmaceutical Research (to S.J.), and NIH KL2 Grant 1KL2TR001444 of the Clinical and Translational Science Awards (CTSA) Program and a Career Award for Medical Scientists from the Burroughs Wellcome Fund (to A.F.C.).