IMMUNE SIGNATURES OF VIRAL CONTROL IN NONHUMAN PRIMATES

AN ABSTRACT

SUBMITTED ON THE NINTH DAY OF APRIL 2020

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BY

BLAKE SCHOUEST

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ABSTRACT

Immune signatures are patterns of gene and protein expression in immune cells that characterize states of activation and response. As such, signatures indicative of viral control during natural infection may guide vaccine development efforts to achieve similar patterns of protection. Here, we used nonhuman primate (NHP) models of Zika virus (ZIKV) and simian immunodeficiency virus (SIV, as a model for HIV) to explore outcomes of infection in these important human pathogens. We employed a multifaceted approach including high dimensional flow cytometry and RNA sequencing to understand cellular responses to ZIKV generally and during pregnancy, as well as to identify the impacts of infection in astrocytes, a neuroglial target of ZIKV thought to be important in the development of neurologic disease. We found that CD8 T cells may restrict ZIKV persistence in tissues but ultimately have a minimal role in protection to either primary or secondary challenge. However, we showed that immune manipulation, either naturally through pregnancy or artificially through depletion experiments, can skew metabolic and innate immune pathways in unexpected ways. While cellular immunity appeared to minimally impact ZIKV infection, such responses in SIV are important in controlling viral replication, which we inversely showed by tracking patterns of viral mutation to evade CD8 responses. We also identified transcriptional signatures in ZIKV infection that may underlie the development of neurologic diseases and found that different virus lineages have unique impacts on gene expression. Together, these experiments showcase the utility of profiling approaches in understanding the immune complexity that accompanies viral infection.
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<th>Description</th>
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<tbody>
<tr>
<td>ADE</td>
<td>antibody dependent enhancement</td>
</tr>
<tr>
<td>AF</td>
<td>amniotic fluid</td>
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<tr>
<td>AP-2</td>
<td>adapter protein 2</td>
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<tr>
<td>ART</td>
<td>antiretroviral therapy</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<td>C</td>
<td>capsid protein</td>
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<tr>
<td>CBC</td>
<td>complete blood count</td>
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<tr>
<td>CD8TL</td>
<td>CD8 T lymphocyte</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CM</td>
<td>central memory</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>Cyno.</td>
<td>cynomolgus</td>
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<tr>
<td>CZS</td>
<td>congenital Zika syndrome</td>
</tr>
<tr>
<td>DEG</td>
<td>differentially expressed gene</td>
</tr>
<tr>
<td>DENV</td>
<td>dengue virus</td>
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<tr>
<td>DPI</td>
<td>days post infection</td>
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<tr>
<td>E</td>
<td>envelope protein</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ELISpot</td>
<td>enzyme-linked immunospot</td>
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<tr>
<td>EM</td>
<td>effector memory</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FDR</td>
<td>false discovery rate</td>
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<tr>
<td>GBS</td>
<td>Guillain-Barre syndrome</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>GO</td>
<td>Gene Ontology</td>
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<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
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<td>ISG</td>
<td>interferon stimulated gene</td>
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<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
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<tr>
<td>M</td>
<td>membrane protein</td>
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<td>MDM</td>
<td>monocyte-derived macrophage</td>
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<tr>
<td>MFI</td>
<td>median fluorescence intensity</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>NES</td>
<td>normalized enrichment score</td>
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<tr>
<td>NK cells</td>
<td>natural killer cells</td>
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<tr>
<td>NLR</td>
<td>neutrophil-to-lymphocyte ratio</td>
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<tr>
<td>NS1</td>
<td>nonstructural protein 1</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>PCA</td>
<td>principal component analysis</td>
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<tr>
<td>PFU</td>
<td>plaque forming unit</td>
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<td>PRNT</td>
<td>plaque reduction neutralization test</td>
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<td>PRR</td>
<td>pattern recognition receptor</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real time PCR</td>
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<tr>
<td>RLR</td>
<td>RIG-I like receptor</td>
</tr>
<tr>
<td>RM</td>
<td>rhesus macaque</td>
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<tr>
<td>RNAseq</td>
<td>RNA sequencing</td>
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<tr>
<td>RIG-I</td>
<td>RIG-I like receptor</td>
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<tr>
<td>SERINC</td>
<td>serine incorporator</td>
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<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
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<tr>
<td>TBEV</td>
<td>tick-borne encephalitis virus</td>
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<tr>
<td>UPR</td>
<td>unfolded protein response</td>
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<tr>
<td>viSNE</td>
<td>visual t-stochastic neighbor embedding</td>
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<td>vRNA</td>
<td>viral RNA</td>
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<td>WNV</td>
<td>West Nile virus</td>
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<tr>
<td>YFV</td>
<td>yellow fever virus</td>
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<tr>
<td>ZIKV</td>
<td>Zika virus</td>
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CHAPTER 1: INTRODUCTION

What is an immune signature and what can this tell us about an immune response?

Immune responses are inherently complex to accommodate recognition of diverse antigens. Unbiased methods of capturing this complexity have expanded exponentially in recent decades, driven by systems approaches aimed at understanding immune responses in exquisite detail. The pairing of phenotypic and transcriptional profiling, and the more recent advent of single-cell technologies, have revolutionized our understanding of how cells respond to antigens. Immune responses are defined not only by antigen recognition but also by signatures of gene and protein expression that characterize their quality. As such, immune profiling lends itself to many applications, from dissecting responses to viruses and vaccines [1] to tumor neoantigens [2] and autoimmune targets [3], among myriad others.

Cellular immunity in NHP models of viral infection

Nonhuman primates (NHPs) are invaluable models in infectious disease research owing to their phylogenetic relatedness to humans [4], resulting in similarities in innate and adaptive immune function that often makes disease manifestations similar to those seen in humans. A vast array of infections caused by bacterial, viral, fungal, and parasitic pathogens have been successfully modeled in macaques [5], although some models more closely replicate disease course seen in humans.

NHP models have additionally provided fundamental insights into immune mechanisms important in some of these infections. Notably, the macaque model of simian
immunodeficiency virus (SIV) infection is considered among the most informative animal models ever developed to study a human disease, due in part to immune dynamics similar to those that occur in the progression of acquired immune deficiency syndrome (AIDS) in humans [6]. CD8 T cells in particular play a critical role in establishing the set point of HIV-1 and SIV replication in vivo [7] and correlate extraordinarily well with viral control. Further, there are strong associations between certain major histocompatibility class I (MHC-I) alleles and viral control, justifying efforts to produce a vaccine that induces T cell responses [6]. The importance of CD8 cells in SIV infection is further affirmed by the accumulation of mutations in MHC-I epitopes to escape these responses [8]. Macaque studies have shown that CD8 T cells control viral replication through both cytotoxic and non-cytotoxic mechanisms, and depletion of CD8 cells has shown the critical importance of these cells in maintaining viral suppression during antiretroviral therapy (ART) [9]. Technical advances in CD8 manipulation in macaques that were initially applied primarily to study control of SIV have established precedence for the study of CD8 cells in other settings, such as hepatitis B [10], measles [11], Ebola [12], and more recently the neurocognitive disorders that accompany SIV infection [13].

While efficient CD8 T cell responses usually contribute to the elimination of acute viral infections, chronic infections are typically characterized by suboptimal CD8 responses that permit viral persistence. CD8 immunity can be skewed in several ways to result in chronic infections, such as in patterns of immunodominance, tissue distribution of responding T cells, and functional exhaustion and deletion of antigen specific CD8 cells [14]. Despite the critical importance of CD8 cells in controlling SIV infection, several of these functions ultimately subvert the efficacy of the CD8 response to promote chronic
infection. The quick clearance of acute viral infections, on the other hand, may on the surface imply efficacious CD8 responses, but the several mechanisms of protection that are often involved in viral control can obscure the contribution of CD8 cells alone.

*NHP models of ZIKV infection*

In addition to uncovering immune dynamics relevant in immunodeficiency virus pathogenesis and control, the NHP SIV model has also been fruitful in that it catalyzed the development of many reagents for manipulating and interpreting immune responses, resources that can be rapidly deployed to study emerging viral infections. In the midst of the 2015-16 Zika virus (ZIKV) epidemic in the Americas, the virus began to be associated with congenital and neurological complications that were unprecedented for flaviviruses, but the underlying biology of these manifestations was not well understood. The SIV field responded quickly with the development of multiple NHP models across different species, which have since been utilized extensively in basic immunology and preclinical vaccine studies.

Macaques are often considered superior animal models for studying ZIKV immunology since they are a natural host of the virus and also constitute an important zoonotic reservoir [15]. Additionally, immunocompetent mice do not replicate aspects of disease in humans, mostly owing to the inability of ZIKV to antagonize interferon (IFN) signaling in mice as in primates [16]. Among NHPs, Asian macaques have been primarily used for ZIKV studies, including rhesus [17], pigtail [18], and cynomolgus [19] macaque species, which generally capture patterns of viremia observed in human patients. Macaques show viral dynamics similar to those seen in human patients, and ZIKV similarly homes to lymphatic, neural, and reproductive tissues and sheds in semen, saliva, and urine [15].
Opposed to other animal models, NHPs most resemble humans in terms of placental structure and gestational development, validating their use to study ZIKV [16], which has shown an unprecedented ability among flaviviruses for transplacental transmission. Although maternal-to-fetal transmission in macaques appears to produce an uncharacteristically high rate of fetal demise [20], a small proportion of monkey fetuses that have become infected in utero show neuroglial lesions similar to those observed in human fetuses [16, 21, 22]. NHPs also have distinct similarities to humans in nervous system structure and development [23], potentially making them a good model to study other aspects of neurologic disease.

NHPs have also been utilized to understand immune mechanisms of protection in both natural ZIKV infection and vaccination. Vaccine candidates across multiple platforms (purified inactivated virus, DNA plasmid-based, and vector-based, among others) have shown efficacy in preclinical vaccine studies in macaques, and several successful candidates have advanced to clinical trials [24]. NHP challenge studies describe potent innate immune responses characterized by IFN signaling and activation of natural killer (NK) cells and dendritic cells, together with infection of monocytes [18, 19, 25], similar to patterns of activation and tropism seen in humans [26, 27]. Macaques show induction of high antibody titers beginning as early as a week following infection [17, 19, 25], validating the use of humoral immunity as a primary correlate of protection in vaccine studies. T cell activation and proliferation following challenge has also been described in NHPs [17, 25], but whether such responses are essential for controlling infection is less clear.
T cell immunity in flavivirus infections

ZIKV and other flaviviruses cause acute infections that are in many cases self-limiting, and T cell responses are generally an important component of protection in such infections [28]. Humoral immunity is considered among the most important correlates of protection in flavivirus infections, and the efficacy of vaccine candidates is gauged by the induction of neutralizing antibodies [24], but a continually expanding body of literature describes CD4 and CD8 T cell responses that are also key mediators of protection, especially when IFN and humoral immunity is compromised [29]. Cellular responses confer protection in both primary and secondary infections, as well as in vaccination in humans, mice, and NHP models [28, 30].

Although many flaviviruses induce some level of T cell immunity, cell mediated responses are most fully characterized in dengue virus (DENV) infection, a significant global pathogen with close relatedness to ZIKV. DENV comprises four genetically distinct serotypes that have a significant geographic overlap with other flaviviruses such as ZIKV [31]. CD4 and CD8 T cell epitopes are differentially distributed in the viral genome, and particular human leukocyte antigen (HLA) alleles correlate with efficacious CD4 and CD8 responses [32, 33]. The importance of T cell correlates in vaccine-induced immunity is illustrated in that the tetravalent Dengvaxia vaccine (Sanofi Pasteur), which has been associated with safety and efficacy concerns, was not designed to encode regions of the DENV genome important in the induction of CD8 responses, and more recent vaccine strategies that contain these putatively important epitopes show better efficacy in phase 3 trials [31]. T cell responses to DENV are multifunctional and long lasting, capable of limiting the magnitude and duration of subsequent infections [34]. Protective CD4 and
CD8 T cell responses are also described in Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and yellow fever virus (YFV) infection and vaccination [30].

The potential for cross-reactivity to modulate flavivirus immunity in endemic regions is important since these viruses co-circulate in tropical and sub-tropical climates. Among DENV serotypes, the protective versus pathogenic role of cross-reactive immune responses has remained controversial, owing to the enhancement of infectivity by cross-reactive but non-neutralizing antibodies, termed antibody dependent enhancement (ADE), for which there is evidence in cell culture and murine models but remains controversial in humans and NHPs [35, 36]. The potential for cross-reactive T cells to also miscue immunity through original antigenic sin has also been contested, although recent literature suggests that cross-reactivity is protective not only among DENV serotypes but also among DENV and ZIKV [31, 36, 37].

**T cell immunity in ZIKV infection**

Considering the efficacy concerns in DENV vaccination that appear to be attributed to a lack of T cell responses, cellular immunity is potentially an important criterion in evaluating ZIKV vaccine candidates. Studies in mouse models have described protective T cell responses in ZIKV infection, with CD4 and CD8 epitopes mapping primarily to non-structural and structural proteins, respectively [38]. Blocking IFN signaling in mice is necessary to replicate disease manifestations seen in humans, since ZIKV is unable to antagonize IFN signaling in mice as in humans, but IFN signaling also promotes T cell immunity, so immunocompetent mouse models have also been utilized to study these responses [39]. Experiments in IFN deficient mice have shown that in the absence of an intact innate immune response, adaptive responses become critical, with humoral immunity
being particularly important in clearance. However, T cell depletion studies have found that CD4 and CD8 responses are mutually compensating in the loss of either subset, implying some role of T cell immunity in protection [29, 40].

ZIKV-reactive T cell responses are also detected in human cohorts that show patterns of immunodominance similar to those seen in mice [38]. Compared to DENV CD8 T cell responses, which target primarily nonstructural proteins, antigen specific CD8 cells show greater responses to structural proteins [37], responses which appear to be polyfunctional and long lasting [41, 42]. However, whether T cell immunity is a necessary component of protection in ZIKV infection and vaccination is unknown. Some of the virus’s transmission dynamics that are unique among flaviviruses, such as the ability to be spread sexually, are attributed to the persistence of ZIKV in reproductive tissues [25], so T cell immunity may also be important in clearance from these cryptic reservoirs. Thus, additional studies in carefully chosen animal models are required to better understand the roles of cellular immunity in controlling ZIKV infection and transmission.

**Immune signatures of viral immunity**

Here, we describe a series of immune profiling experiments in macaques as a means to understand mechanisms of control of viruses that are significant human pathogens. We explore immune signatures resulting from viral infection and evaluate the corresponding impacts of such responses on viral evolution and fitness. The complexity of the virus-immune system interface requires unbiased approaches to understand responses that correlate with viral control, information relevant in vaccine design by providing a comparison to benchmark protective responses.
Through profiling experiments, we observed that immune manipulation during ZIKV infection, either naturally through pregnancy or artificially through lymphocyte depletion, can ultimately skew metabolic and innate immune pathways in unexpected ways. We identified immune signatures of ZIKV infection that may contribute to neurologic diseases and found that phylogenetically removed isolates of ZIKV produce unique patterns of gene expression. We found a minimal overall impact of CD8 T cell immunity in the control of ZIKV, while in SIV infected animals, we showed that the efficacy of cellular responses drives viral escape. Together, these studies showcase the utility of profiling approaches in understanding the immune complexity that accompanies viral infection.
CHAPTER 2: CD8+ LYMPHOCYTES MINIMALLY IMPACT ZIKA VIRUS
CONTROL IN NONHUMAN PRIMATES

Overview

CD8 T cell responses to Zika virus (ZIKV) are detected in humans and also confer protection in mouse models, but whether similar responses are important in the control of ZIKV in nonhuman primates (NHPs) is unknown. In the present study, we depleted CD8+ lymphocytes in a small group of macaques prior to ZIKV challenge, but the absence of these cells did not meaningfully impact serum viral loads. The collateral loss of natural killer (NK) cells appeared to dysregulate patterns of innate immune cell homing and transcriptional responses in the blood, and CD8-depleted macaques showed evidence of compensatory CD4 and humoral responses. The absence of CD8+ lymphocytes was also associated with an increase in viral persistence in lymphatic tissues, semen, and cerebrospinal fluid (CSF), and neural lesions were evident in two CD8-depleted animals. While our findings suggest that the presence of CD8 T cells or NK cells is important for maintaining patterns of innate antiviral immunity, our data do not support a role for these cells as a critical component of protection to primary ZIKV infection in nonhuman primates.

A number of reports in mouse models have indicated an important role for CD8 T cells in control of Zika virus replication in mice, but these models are limited in that the mice need to be immunocompromised for efficient viral infection. Additionally, CD8 T cell responses against Zika are also described in humans, but whether such responses are a
critical component of protection cannot be ascertained with certainty. We used a nonhuman primate model (both rhesus and cynomolgus macaques) and depleted CD8+ cells prior to infection to assess antiviral immunity and viral dynamics, including viral loads in fluids and viral dissemination into tissues. First and most importantly, we found that CD8 depletion did not meaningfully impact serum viremia. The absence of CD8+ cells appeared to enhance viral dissemination into multiple tissues and fluids following clearance from the blood, but this enhanced persistence ultimately did not worsen disease outcome. Intriguingly, we found that CD8 depletion altered initial responses to the virus in unexpected ways, including a lack of neutrophil recruitment and monocyte and transcriptional responses that were attenuated or absent compared to non-depleted animals, suggesting a role for CD8 cells in orchestrating early immune events during infection.

Introduction

ZIKV has been a known pathogen for over half a century [43], but severe disease manifestations were not directly associated with the virus for most of its history. Although recent outbreaks of ZIKV in the Western hemisphere are notorious for neurological complications including congenital Zika syndrome (CZS) and Guillain-Barré syndrome (GBS), most cases remain asymptomatic, and when symptoms arise, they are usually mild and self-limiting [44]. Differential immune responses to ZIKV infection may dictate the severity of accompanying diseases and underlie clinical outcomes.

As the immunological correlates of protection to ZIKV infection are explored, CD4 and CD8 T cell responses are recognized as important mediators of viral control in mice and humans [38], analogous to adaptive responses mounted in other flavivirus infections [28]. Intriguingly, studies in mice have identified dual protective and deleterious roles of
CD8 T cells in ZIKV infection. CD8+ lymphocyte infiltration appears to reduce viral burdens in the brain, spinal cord, and lymphatic tissue [39, 45], but under certain circumstances, CD8+ influx can promote neural damage and paralysis [46]. While murine studies have pioneered our understanding of T cell responses to ZIKV infection, the mouse model generally requires deficiency in type-I interferon (IFN) signaling to recapitulate pathological manifestations seen in humans [39, 45] owing to the inability of ZIKV to antagonize murine STAT2 [47]. However, IFN signaling is important in the induction of T cell responses, so it is important to evaluate the importance of T cell immunity in a model sufficiently similar to humans immunologically. Utilizing the recently developed rhesus [17, 19, 48-50] and cynomolgus [19, 51] macaque models of ZIKV infection, we carried out CD8+ lymphocyte depletion to ask whether CD8 responses are important in controlling infection. Although CD8+ depletion is an established immune manipulation in nonhuman primates, [52], natural killer (NK) cells are also well known to be collaterally depleted owing to their expression of CD8α. Thus, the absence of NK cells could have implications in acute antiviral responses, while the depletion of CD8 T cells might be more relevant in long term immunity and viral persistence.

In the present study, we infected adult male rhesus and cynomolgus macaques with a minimally passaged Brazilian ZIKV strain. Prior to infection, two animals of each species were depleted of CD8+ lymphocytes, including CD8 T cells and NK cells. The absence of these cells did not drastically impact serum viral loads or disease outcome but did appear to be associated with enhanced viral persistence in tissues and accumulation of neural damage, along with repressed monocyte-driven virus response patterns in the blood.
Materials and Methods

**Ethics statement.** A total of 4 male Indian-origin rhesus macaques (*Macaca mulatta*; mean age: 9.6 years; range: 6.7-11.9 years; mean weight: 10.62 kg; range: 9.45-12.97 kg) and 5 male cynomolgus macaques (*Macaca fascicularis*; mean age: 8.8 years; range: 8.7-9.2 years; mean weight: 12.02 kg; range: 10.38-13.84 kg) were housed in compliance with the NRC Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. Animal experiments were approved by the Institutional Animal Care and Use Committee of Tulane University (protocol P0367). The Tulane National Primate Research Center (TNPRC) is fully accredited by AAALAC International (Association for the Assessment and Accreditation of Laboratory Animal Care), Animal Welfare Assurance No. A3180-01. Animals were socially housed, indoors in climate-controlled conditions with a 12/12-light/dark cycle. All the animals on this study were monitored twice daily to ensure their welfare. Any abnormalities, including those of appetite, stool, behavior, were recorded and reported to a veterinarian. The animals were fed commercially prepared monkey chow twice daily. Supplemental foods were provided in the form of fruit, vegetables, and foraging treats as part of the TNPRC environmental enrichment program. Water was available at all times through an automatic watering system. The TNPRC environmental enrichment program is reviewed and approved by the IACUC semiannually. Veterinarians at the TNPRC Division of Veterinary Medicine have established procedures to minimize pain and distress through several means. Monkeys were anesthetized with ketamine-HCl (10 mg/kg) or tiletamine/zolazepam (6 mg/kg) prior to all procedures. Preemptive and post procedural analgesia (buprenorphine 0.01 mg/kg or buprenorphine sustained-release 0.2 mg/kg SQ) was required for procedures that would likely cause more
than momentary pain or distress in humans undergoing the same procedures. The above listed anesthetics and analgesics were used to minimize pain or distress associated with this study in accordance with the recommendations of the Weatherall Report. The animals were euthanized at the end of the study using methods consistent with recommendations of the American Veterinary Medical Association (AVMA) Panel on euthanasia and per the recommendations of the IACUC. Specifically, the animals were anesthetized with tiletamine/zolazepam (8 mg/kg IM) and given buprenorphine (0.01 mg/kg IM) followed by an overdose of pentobarbital sodium. Death was confirmed by auscultation of the heart and pupillary dilation. None of the animals became severely ill or died prior to the experimental endpoint. The TNPRC policy for early euthanasia/humane endpoint was included in the protocol in case those circumstances arose. All studies were approved by the Animal Care and Use Committee of the TNPRC (OLAW assurance #A4499-01) and in compliance with animal care procedures. TNPRC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC#000594).

Animal experiments. The four adult male Indian origin rhesus macaques (Macaca mulatta) and five adult male cynomolgus macaques (Macaca fascicularis) utilized in this study were housed at the Tulane National Primate Research Center (TNPRC). The TNPRC is fully accredited by AAALAC International (Association for the Assessment and Accreditation of Laboratory Animal Care), Animal Welfare Assurance No. A3180-01. Animals were cared for in accordance with the NRC Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act Animal experiments were approved by the Institutional Animal Care and Use Committee of Tulane University (protocol P0367).
Two rhesus macaques (R25671 and R64357) and two cynomolgus macaques (C78777 and C18942) were depleted of CD8+ lymphocytes by administration of the anti-CD8a antibody MT807R1 (NHP Reagent Resource; https://www.nhpreagents.org) [52]. The initial subcutaneous administration of 10 mg/kg at 14 days pre-infection was followed by three intravenous administrations of 5 mg/kg at 11, 7, and 5 days pre-infection, as per the distributor’s protocol. C84545 was treated with the irrelevant control antibody anti-desmipramine (NHP Reagent Resource; https://www.nhpreagents.org) at the same dosages and time intervals pre-infection. All animals were subcutaneously infected with 10⁴ plaque forming units (PFU) of a Brazilian ZIKV isolate [53] at 0 days post-inoculation (dpi) (Fig. 1a). As part of a previous study, C46456 (nondepleted) was splenectomized 9 months and 19 days prior to inoculation with ZIKV. For data comparison, we included viral loads and complete blood count (CBC) data from a previous cohort of 4 non-pregnant female rhesus macaques (R32835, R24547, R25508, R22624) that were similarly infected with the same dose of the same Brazilian ZIKV isolate that was used in this study (Fig. S2a-e).

Whole blood, CSF, and semen were obtained from animals at the indicated timepoints (Fig. 1a). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of rhesus macaques using SepMate tubes (Stemcell Technologies) according to the manufacturer’s protocol or from the blood of cynomolgus macaques using Lymphoprep (Stemcell Technologies) for standard density gradient centrifugation. At necropsy, the indicated tissues were collected and snap-frozen.

**Virus quantification.** Viral RNA was extracted from serum and CSF using the High Pure Viral RNA Kit (Roche). Semen, as well as the indicated lymphoid, reproductive, gastrointestinal (GI), and neural tissues were homogenized in Qiazol (Qiagen) using either
disposable tissue grinders (Fisherbrand) or a TissueRuptor (Qiagen), and RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen). Viral RNA from body fluids and tissues was quantified using quantitative real-time PCR (qRT-PCR) as described previously [50].

**Antiviral gene expression assays.** 2.5 ml whole blood was drawn from each animal at 0, 1, 3, and 15 dpi into PAXgene blood RNA tubes (PreAnalytiX) and equilibrated to -80°C as per the manufacturer’s protocol. RNA was extracted from blood samples using the PAXgene blood RNA kit (PreAnalytiX), and complementary DNA (cDNA) was synthesized using the RT2 First Strand Kit (Qiagen). Antiviral transcriptional responses were analyzed by way of qRT-PCR using a rhesus macaque RT2 Profiler PCR Array (Qiagen). Responses within each species and treatment group were analyzed together to identify expression levels at the indicated timepoints relative to pre-infection. Transcriptional profiles of immune signaling were generated using the nCounter NHP Immunology Panel (NanoString Technologies). In whole blood, transcriptional responses were assessed at 3 dpi relative to expression levels pre-infection using nSolver software v4.0 (NanoString Technologies). Fold change data were imported into Ingenuity Pathway Analysis (IPA) (Qiagen) to discern relevant signaling pathways and disease functions. Heatmaps of gene expression and disease-related pathways were generated using Morpheus (https://software.broadinstitute.org/Morpheus). Principal component analysis (PCA) of antiviral gene expression was performed using ClustVis [54].

To identify cell populations contributing to antiviral signaling in blood, the CD14 and CD8 MicroBead kits (Miltenyi Biotec) were used to sort CD14+ monocytes and CD8+ lymphocytes from the PBMCs of cynomolgus macaques at multiple timepoints. Due to limited sample availability, similar experiments could not be performed on the first cohort.
RNA was isolated from cell fractions using the RNeasy Mini Kit (Qiagen) or the Quick-RNA Miniprep kit (Zymo Research), and cDNA was synthesized using the RT2 First Strand Kit (Qiagen). Transcriptional activity in sorted cell populations was probed by qRT-PCR using the RT2 qPCR Primer Assays (Qiagen) for ISG15 and OAS2, as well as the following primers for DDX58: For-5’-GGAAGACCCTGGACCCTACCT-3’; Rev-5’-AAAGCCACGGAACCAGCCCTT-3’. CD14-sorted populations from a representative highly responding nondepleted animal (*C46456) and a representative minimally responding CD8-depleted animal (C18942) were selected for further transcriptional profiling using the nCounter NHP Immunology Panel (NanoString).

To characterize antiviral signaling in myeloid cells, cocultures were set up using lymphocytes isolated from the whole blood of ZIKV-naïve colony rhesus macaques. CD14+ monocytes and CD8+ NK cells were isolated using the CD14 and CD8 MicroBead kits (Miltenyi Biotec) as described above, and neutrophils were captured in the upper red blood cell layer following density gradient centrifugation for PBMCs. NK cell/monocyte and NK cell/neutrophil cocultures were set up using autologous cell populations in the presence or absence of ZIKV Rio-U1, and individual cell types served as negative controls. Direct contact cocultures were carried out in 48-well plates, and contact-independent cocultures were done using Transwell 24-well permeable supports (Corning). Additionally, cocultures involving neutrophils were carried out in the presence of 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) to promote neutrophil survival. qRT-PCR for ISG15 and OAS2 was performed using RT2 qPCR Primer Assays (Qiagen). Antiviral gene induction was assessed using the ddCt method in ZIKV exposed cell
populations at 24 hours post infection relative to unexposed cells, and protein level analysis of neutrophil activation was carried out as described below.

For antiviral gene screening in monocyte-derived macrophages (MDMs), monocytes were cultured at $1 \times 10^6$ cells/ml in RPMI-1640 medium supplemented with 1% human AB serum (Sigma), 20 ng/ml M-CSF (Peprotech), 1% L-glutamine, and 1% penicillin/streptomycin. After 7 days of culture, monocytes were sufficiently differentiated into MDMs and were either infected with Rio-U1 or left uninfected. At 24 hpi, RNA was extracted using the RNeasy Mini Kit (Qiagen), cDNA was synthesized using the RT2 First Strand Kit (Qiagen), and transcriptional signaling was assessed using the rhesus macaque antiviral response RT2 Profiler PCR Array (Qiagen). Antiviral gene expression in ZIKV-infected monocyte-derived macrophages (MDMs) was calculated relative to uninfected controls.

Flow cytometry and gating strategy. For absolute lymphocyte counts, whole blood was stained within 2 hours of blood draw for the surface markers CD45 (PerCP; DO58-1283; BD Biosciences), CD3 (FITC; SP34; BD Biosciences), CD4 (APC; L200; BD Biosciences), and CD8 (V500; SK1; BD Biosciences). Flow cytometry was performed on a BD FACSVerse instrument, and absolute counts were calculated using FACS Suite software.

For immunophenotyping, PBMCs from the indicated timepoints were thawed, washed, and stained using Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen). PBMCs were then stained for the surface markers CD16 (AL488; 3G8; BioLegend), CD169 (PE; 7-239; BioLegend), CD28 (PECF594; CD28.2; BD Biosciences), CD95 (PCP-Cy5.5; DX2; BioLegend), CD3 (PE-Cy7; SP34-2; BD Biosciences), CD8 (PacBlue;
SK1; BioLegend), CD14 (BV605; M5E2; BD Biosciences), HLA-DR (BV650; L243; BioLegend), NKG2A (APC; Z199; Beckman Coulter), and CD4 (APC-H7; L200; BD Biosciences). Cells were subsequently fixed in FluoroFix buffer (BioLegend), permeabilized using Perm/Wash buffer (BioLegend), and stained intracellularly for CD69 (BV711; FN50; BD Biosciences) and Ki67 (AL700; B56; BD Biosciences). Flow cytometry was performed on a BD LSRII instrument, and data were analyzed using FlowJo (vX.10.4.2) and visual t-distributed stochastic neighbor embedding (viSNE) (Cytobank) softwares. For viSNE analysis, live singlet monocytes (CD14+ and/or CD16+) or live singlet CD3+ T cells were gated prior to downsampling at a minimum of 500 cells per animal in FlowJo v. 10.5.3 for computational feasibility. Downsampled files for each animal were then concatenated by group (i.e., species, dpi, and treatment condition). When the number of animals differed per group, concatenated files were further downsampered to achieve an equal number of cells per group. viSNE was conducted using Cytobank with the following settings: Perplexity = 30, Iterations = 1000, Theta = 0.5, Seed = random, Compensation = internal file. For the monocyte viSNE analysis, the following parameters were utilized in the run: Ki67, CD14, HLA-DR, CD69, CD95, CD14, and CD169. For the T cell viSNE analysis, the following parameters were utilized in the run: Ki67, CD4, HLA-DR, CD69, CD95, CD28, CD3, CD8.

For general immunophenotyping analysis, cytometry data were first gated for lymphocytes, singlets, and live cells. NK cells were considered CD8+/CD16+. CD4 T cells (CD3+/CD4+) and CD8 T cells (CD3+/CD8+) were gated into naïve (CD28+/CD95-), central memory (CM, CD28+/CD95+), and effector memory (EM, CD28-/CD95+) subsets. CD3- cells were divided into B cells (DR+/CD14-/CD16-) and monocytes
(classical, CD14++/CD16-; intermediate, CD14+/CD16+; nonclassical, CD14_{low}/CD16+). Cell subsets were analyzed with respect to frequency, proliferation (Ki67+) and activation (CD69+ or CD169+).

For protein level analysis of neutrophil activation following coculture with NK cells, cocultured cell mixtures were stained using Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen) and antibodies for CD66 (FITC), CD123 (PECF594), CD3 (PE-Cy7), CD14 (BV605), HLA-DR (BV650), CD69 (APC), and CD20 (APC-Cy7). Neutrophils were gated on live cells, FSC_{high}/SSC_{dim}, HLA-DR-, CD3-, CD20-, CD123-, and activation (degranulation) was assessed by CD66 positivity.

Intracellular cytokine staining. PBMCs from the indicated timepoints were thawed and rested overnight prior to stimulation with peptide pools comprising ZIKV capsid (C), membrane (M), envelope (E), and nonstructural protein 1 (NS1) (BEI Resources). On peptide stimulation, cells were also treated with brefeldin A (BioLegend), GolgiStop (BD Biosciences), anti-CD28 (NHP Reagent Reference Program, www.nhpreagents.org/), anti-CD49d (9F10; BioLegend), and anti-CD107a (AL700; H4A3; BD Biosciences). 24 hours post-stimulation, cells were stained for the surface markers CD3 (PE-Cy7; SP34-2; BD Biosciences), CD8 (PacBlue; SK1; BioLegend), and CD4 (APC-H7; L200; BD Biosciences). Cells were also fixed and permeabilized as described above and stained intracellularly for perforin (FITC; Pf-344; Mabtech), granzyme B (PE; GB12; Invitrogen), CD69 (PE-CF594; FN50; BD Biosciences), IL-2 (PCP-Cy5.5; MQ1-17H12; BD Biosciences), and IFNγ (AL647; 4S.B3; BioLegend). Flow cytometry was performed on a BD LSRII instrument and data were analyzed using FlowJo software (vX.10.4.2).
Plaque reduction neutralization tests. ZIKV plaque reduction neutralization tests (PRNTs) were conducted according to previously published protocols [55, 56]. Briefly, ZIKV MEX-I-44 isolated in Tapachula, Mexico in 2016 was obtained from The University of Texas Medical Branch, Galveston, TX and cultured to passage 8 in Vero cells. Serum specimens were incubated for one hour at serial dilutions of 1:10, 1:20…1:320 with a previously frozen virus stock of known PFU. Samples were then inoculated in duplicate onto a monolayer of Vero cells grown on 6-well plates and allowed to incubate for an additional hour. Infectious material was then removed and replaced with a 1:1 mixture of Vero media and Avicel® before being incubated for 4 days. To read plaques, the Avicel® layer was fixed with 10% neutral buffered formalin. Finally, the formalin-Avicel® layer was removed and the monolayer was stained with crystal violet, washed with tap water and allowed to dry before plaques were counted manually.

Percent reduction in observed plaques and a PRNT90 cutoff were used for interpretation. A PRNT90 titer is the dilution of a sample at which a 90% reduction in possible plaques is observed. The maximum number of potential plaques was obtained for each run using a corresponding back-titration and a linear model was fit to the observed number of plaques for each dilution. A PRNT90 titer was derived for each sample using the linear model and the equation for a straight line in the statistical program R [57]. For samples that were positive but above the resolution of the PRNT assay the value of the greatest number of possible plaques for that run, as determined by the back titration, was assigned for each dilution for use with the linear model.
Histology. Tissues samples collected at necropsy were fixed in Z-Fix (Anatech), embedded in paraffin and 5 μm thick sections were cut, adhered to charged glass slides, and either stained routinely with hematoxylin and eosin or Prussian blue.

Statistical analysis. Statistical analysis was conducted using GraphPad Prism v8.2.1 (GraphPad Software). Mann-Whitney tests were used to compare viral loads, neutrophil-to-lymphocyte ratios (NLRs), and immunophenotypic patterns among CD8-depleted and nondepleted animals. Area-under-the-curve (AUC) analysis was utilized in time-course measures. Although cohorts 1 and 2 were grouped for statistical analysis where possible, tissue and CSF viral loads were analyzed separately for each cohort owing to differences in duration of infection.

Delayed serum viral loads and altered leukocyte kinetics

CD8+ lymphocyte depletion commenced 14 days prior to ZIKV inoculation (Fig. 1a), and CD8 T cells were undetectable in all depleted animals well before challenge (Fig. 1b & Fig. S1a). To achieve CD8 depletion, we used the MT807R1 antibody [52] to target CD8α, effectively depleting CD8 T cells (Figs. 1b & S1a), CD8+/CD4+ double-positive T cells (Fig. S1b), and NK cells (Fig. 1c) but not CD4 T cells (Fig. S1c) from the blood of all treated animals. As MT807R1 targets NK cells in addition to CD8+ lymphocytes, any deficiency in host response following treatment with anti-CD8α could indicate that either or both types of cells are important for acute control of ZIKV. Flow cytometric analysis of NK cell frequency in nondepleted animals revealed expansion early in infection (Fig. 1c). Intriguingly, the CD8-depleted macaque R64357 recovered CD8 T cells and NK cells at later timepoints, between 15 and 21 dpi (Fig. 1b-c).
Figure 1: Serum viral loads and leukocyte kinetics

(A) Study design. Two animals of each cohort were depleted of CD8+ lymphocytes, and all animals were challenged with ZIKV Rio-U1. Viral loads were tracked over 30 days in cohort 1 and 14 days in cohort 2 before necropsy. Colors correspond to cohorts and treatment conditions. C46456 (nondepleted) was splenectomized in a previous study and is indicated by an asterisk (*) throughout. (B) Flow cytometric analysis of CD8 T cell frequencies in PBMCs over time. (Top): cohort 1 (day 0 was assessed for all animals except R25671, for which a pre-depletion timepoint was assessed due to limited sample availability); (Bottom): cohort 2 (consistent throughout). (C) NK cell frequencies, as measured by flow cytometry. (D) Viral RNA in serum during infection (error bars, standard
deviation). A Mann-Whitney test of area-under-the-curve (AUC) analysis was not significant (ns). (E) Neutrophil-to-lymphocyte ratio (NLR), derived using total neutrophil and lymphocyte counts in blood from CBC data. (F) Fold change in NLR from 0 to 1 dpi among 4 rhesus and 4 cynomolgus macaques included in the present study in addition to a previous cohort of 4 ZIKV-infected non-pregnant female rhesus macaques (center line, mean; error bars, standard deviation). (The blood of one CD8-depleted cynomolgus macaque, C18942, clotted prior to CBC analysis, precluding calculation of NLR for this animal.) The significant ($p \leq 0.05$) difference in NLR fold change was determined using a Mann-Whitney test.

Following ZIKV challenge, nondepleted animals and a single CD8-depleted cynomolgus macaque showed rapid induction of viral loads in serum at 3-4.5 logs by 1 dpi (Fig. 1d), consistent with previous reports of ZIKV in rhesus and cynomolgus macaques [17, 19, 51]. Serum viral loads in 3 of 4 CD8-depleted macaques were marginally delayed until 2 dpi (Fig. 1d). Viral RNA was also not detected until 3 dpi in *C46456 (Fig. 1d), a nondepleted cynomolgus macaque that had been previously splenectomized. Perhaps importantly, the spleen is a major site of replication and spread of the related mosquito-borne flaviviruses dengue virus (DENV) [58] and West Nile virus (WNV) [59] and is also a major reservoir of monocytes [60], which are permissive to ZIKV replication in humans [26] and macaques [18]. The lack of a spleen in *C46456 (nondepleted) might have precluded ZIKV replication in this important target organ, thereby delaying viral kinetics. For the remainder of the study, viral kinetics were similar among cohorts and treatment conditions, peaking at 3 dpi and dropping to undetectable levels by 10 dpi and beyond (Fig.
This was again in exception to *C46456 (nondepleted), which showed a small viral rebound at 10 dpi. A previous cohort of female macaques infected with the identical strain of ZIKV demonstrated similar patterns of serum viral loads to those observed in nondepleted animals (Fig. S2a).

The previous female cohort also showed characteristic patterns of innate immune cell recruitment in the blood one day following ZIKV inoculation, summarized by the inflammatory biomarker NLR [61]. These patterns included a spike in neutrophil frequency (Fig. S2c) and a simultaneous drop in lymphocyte frequency (Fig. S2d), resulting in an elevated NLR at 1 dpi (Fig. 2e). These findings were generally consistent in nondepleted animals but not in CD8-depleted animals (Figs. 1e-f & S2c-e), potentially linking NLR and serum viral load, although the trend did not hold in the mock-depleted cynomolgus macaque C84545. This association is strengthened by typical patterns of leukocyte homing in C78777 (Fig. 1e), the only CD8-depleted macaque with viral RNA in serum at 1 dpi (Fig. 1d).

**Differential monocyte-driven transcriptional profiles**

To characterize immune responses that have differentiated patterns of serum viral load and leukocyte mobilization, we used a quantitative real-time PCR (qRT-PCR) array to profile antiviral gene expression in the blood. Nondepleted rhesus and cynomolgus macaques showed strong induction of several RIG-I-like receptors (RLRs) and type-I IFN stimulated genes (ISGs) at 3 dpi (Fig. 2a), with kinetics that peaked at 3 dpi and returned to baseline by 15 dpi, mirroring viral loads in serum (Fig. S3a). The most highly induced genes include the pattern recognition receptors (PRRs) *TLR3, DDX58* (also known as
Figure 2: Differential monocyte-driven transcriptional profiles
(A) Fold regulation of antiviral gene expression in whole blood at 3 dpi relative to 0 dpi, from a qRT-PCR array of 84 genes in the rhesus macaque genome (cyno. = cynomolgus, consistent throughout). (B) NanoString analysis of IFN–I signaling in whole blood at 3 dpi relative to 0 dpi. (C) PCA of antiviral gene expression in whole blood at 3 dpi. (D-E) Antiviral gene induction of representative ISG (*ISG15*) and PRR (*DDX58*) genes in sorted CD14+ monocyte (D) and CD14- (E) fractions from PBMCs at the indicated days post inoculation (x-axis) relative to 0 dpi. Gene induction is normalized to β-actin (consistent throughout). (F) Activation of downstream IFN–I effector molecules in sorted CD14+ monocytes and CD14- PBMCs from representative CD8-depleted (C18942) and nondepleted (*C46456) animals at 3 dpi relative to 0 dpi. (G) Antiviral gene expression in CD8+ and CD8- fractions of PBMCs from a representative nondepleted animal at 3 dpi relative to 0 dpi. (H) Antiviral gene expression in co-cultured CD14+ monocytes and autologous CD8+ cells from ZIKV-naïve PBMC infected with ZIKV *ex vivo* at 1 and 3 dpi. (I) Comparison of antiviral gene induction in cultured, ZIKV-infected MDMs (black) and the whole blood of nondepleted rhesus (blue) and cynomolgus (green) macaques at 3 dpi relative to 0 dpi. Genes included in the qPCR array relate to toll-like receptor (TLR), nod-like receptor (NLR) or IFN-I signaling (resp. = responsive).

*RIG-I*, and *IFIH1* (also known as *MDA5*), as well as the ISGs *ISG15*, *MX1*, and *OAS2*. Strikingly, CD8-depleted macaques in both cohorts showed a global absence of antiviral gene expression at all timepoints tested (Figs. 2a & S3b). To confirm these unexpected results, we used the NanoString platform to compare the expression of macaque immune-related genes at day 3 to expression levels at day 0. Again, there was a virtual absence of
transcriptional responses in CD8-depleted macaques, while nondepleted animals showed strong induction of genes relating to IFN–I signaling (Fig. 2b) and leukocyte homing (Fig. S3c). These transcriptional disparities, summarized by PCA (Fig. 2c), resulted in the enrichment of many disease-related pathways in nondepleted but not CD8-depleted animals (Fig. S3d).

The divergent gene expression signatures among treatment groups raised the question of which cell populations were responding in nondepleted animals and why these cells were failing to respond in animals that were CD8 depleted. Although we suspected monocytes to be driving antiviral gene expression owing to their susceptibility to ZIKV infection [18, 26], an important caveat of probing whole blood is that the identity of the cell populations responding transcriptionally is unknown. To resolve cell populations contributing to antiviral signaling in blood, we sorted CD14+ monocytes from PBMCs at multiple timepoints post-inoculation and probed antiviral activity by performing qRT-PCR for representative ISG (ISG15) and PRR (DDX58) genes. In nondepleted animals, gene induction was highest in the CD14+ fraction (Fig. 2d), whereas CD14- PBMCs were transcriptionally quiescent (Fig. 2e), suggesting that monocytes were driving transcriptional activity. Gene induction peaked around 7 dpi, after serum viral loads had crested. In contrast to nondepleted animals, transcriptional responses in the monocytes of CD8-depleted animals as well as the mock-depleted animal C84545 were comparatively muted for the duration of the study (Figs. 2a & 2d-e). To confirm the observed transcriptional disparities among CD8-depleted and nondepleted macaques, we selected representative minimally responding (C18942) and highly responding (*C46456) animals for further profiling of CD14-sorted populations. Probe hybridization corroborated our
findings from qRT-PCR, showing generalized antiviral gene expression specifically in the monocyte fraction of the nondepleted macaque, while the purified monocytes from the CD8-depleted animal were transcriptionally inert (Fig. 2f). Nonetheless, it remained possible that the lack of a transcriptional response in CD8-depleted macaques could have been attributed in part to an absence of otherwise responding NK cells. Sorting PBMCs from a nondepleted rhesus macaque into CD8+ and CD8- fractions, we found similar levels of gene induction in both populations, although expression was marginally higher in the CD8- subset, and transcription of DDX58 was almost exclusive to CD8- cells (Fig. 2g). Gene induction in CD8+ cells indicates that NK cells may indeed contribute to antiviral signaling, but similar transcriptional activation in the CD8- fraction affirms that the absence of transcriptional activation in CD8-depleted animals was not simply the product of a lack of NK cells.

To further explore the importance of NK cells in initiating transcriptional responses, we set out to recapitulate cell signaling dynamics between NK cells and myeloid cells in vitro. In cocultures of NK cells together with either monocytes or neutrophils, we found that the presence of NK cells was important for promoting antiviral gene expression in both cell types (Figs. 2h, S6a, & S6c-e). Moreover, NK cell/monocyte crosstalk appeared contact dependent, given the lack of transcriptional activation in monocyte/NK transwell cocultures (Fig. S6b). We also cultured monocyte-derived macrophages (MDMs) in vitro, infected the macrophages with ZIKV, and profiled antiviral gene expression using qRT-PCR. We found an overlapping transcriptional fingerprint to those observed in the blood of nondepleted rhesus and cynomolgus macaques at 3 dpi (Fig. 2i), suggesting that myeloid cells may be driving antiviral gene induction in vivo. Although cultured MDMs exhibited
higher induction of several TLR responsive genes (Fig. 2i), this difference might be attributed to cell type.

*Altered monocyte activation and frequency*

Divergent transcriptional patterns in CD8-depleted and nondepleted macaques could be induced by differentially responding monocytes, given that monocytes are known targets of ZIKV infection [18, 62] and contribute to antiviral signaling during ZIKV infection [62]. To expand on our gene expression experiments, we interrogated the immunophenotypic effects of CD8 depletion by developing a multicolor flow cytometry panel to track innate and adaptive immune cells over time. The resulting data were highly dimensional, comprising a variety of surface markers and sampling animals at multiple timepoints and with respect to different treatment groups. To survey general immune responses over time, we used an adaptation of t-distributed stochastic neighbor embedding (tSNE), viSNE [63].

In both rhesus and cynomolgus macaques, CD8 depletion appeared to dysregulate the kinetics of monocyte activation as measured by CD169 (siglec-1) expression [64-66]. Nondepleted rhesus and cynomolgus macaques showed early activation of monocytes, which peaked at 3 dpi and returned sharply to baseline by 14-15 dpi (Fig. 3a-c). Upregulation of CD169 in nondepleted animals was affirmed at the RNA level (Fig. 2a). Although patterns of CD169 induction were consistent in all monocyte subsets (Fig. S4a-c), viSNE analysis indicated that CD169 was most highly upregulated on intermediate and nonclassical monocytes in both cohorts (Fig. 3a-b). Contrasting nondepleted animals, CD8-depleted rhesus and cynomolgus macaques showed less well-defined monocyte activation at 3 dpi, which was accompanied in rhesus macaques by prolonged monocyte
Figure 3: Altered monocyte activation and frequency

(A-B) viSNE analysis of monocyte activation in rhesus (A) and cynomolgus (B) macaques, as measured by CD169 median fluorescence intensity (MFI). Dot plots are concatenated for animals within each treatment condition. The viSNE clustering profile of monocyte subsets (left) correspond to cell populations in the CD169 MFI heatmaps in nondepleted and CD8-depleted animals over time (right). (C) Summaries of CD169 expression in total
monocytes. A Mann-Whitney test reported a significant difference among CD8-depleted and nondepleted macaques of both cohorts at 3 dpi, although AUC analysis did not reach statistical significance. (D) Induction of genes related to myeloid cell activation at 3 dpi relative to pre-infection. (E-G) Frequencies of classical (E), nonclassical (F), and intermediate (G) monocyte subsets in rhesus and cynomolgus macaques over time. Mann-Whitney tests reported non-significant differences in AUC among CD8-depleted and nondepleted animals for each monocyte subset.

Activation beyond 15 dpi (Figs. 3a-c & S4a-d). These findings were consistent transcriptionally, as whole blood from CD8-depleted animals had muted expression of genes related to myeloid cell activation (Fig. 3d). Monocyte subsets showed additional nuances in phenotype that appeared dependent on CD8 depletion: In rhesus macaques, CD95 (Fas) was increased on classical monocytes in CD8-depleted animals (Fig. S4e) and on nonclassical monocytes in nondepleted animals (Fig. S4f), although similar patterns were not observed in cynomolgus monkeys.

CD8 depletion also appeared to modulate the abundance of monocyte subsets in blood. One day following ZIKV infection, classical monocytes expanded immediately in nondepleted animals of both cohorts (Fig. 3e) excluding the mock-depleted cynomolgus macaque C84545. During acute infection (3-7 dpi), the frequency of nonclassical monocytes increased preferentially in CD8-depleted rhesus macaques and in nondepleted cynomolgus macaques (Fig. 3f). Nondepleted rhesus macaques showed an expansion of intermediate monocyte frequency at 3-7 dpi (Fig. 3g), although a CD8-dependent effect on intermediate monocyte frequency was not evident in cynomolgus monkeys.
Possible compensatory adaptive immune responses

Evidence of an adaptive immune response was present in both cohorts, as seen by upregulation of markers for proliferation (Ki67) and activation (CD69) in effector memory (EM), central memory (CM), and naïve CD8 T cell subsets at 7-10 dpi (Figs. 4a-c & S5a-e). CD8 T cell responses in rhesus macaques were antigen-specific and functional, given that CD8 T cells stimulated with ZIKV peptides produced IFNγ and contained perforin by intracellular cytokine staining (ICS) (Fig. 4e). Intriguingly, the CD8-depleted rhesus macaque R64357 also showed evidence of a CD8 T cell response at 21 dpi (Fig. 4c & 4e), concomitant with the recovery of CD8+ lymphocytes in this animal (Fig. S1a). Although tested, antigen-specific T cell responses were not detected by ICS in cynomolgus macaques.

Adaptive immune responses to ZIKV might have been modulated by CD8 depletion, with apparent compensatory CD4 and humoral responses in CD8-depleted macaques in the first cohort. The absence of CD8 T cells in depleted rhesus macaques coincided with the induction of CD4 T cell responses consistent with a Th1 phenotype, characterized by co-positivity for IL-2 and IFNγ, while such responses were not present in nondepleted animals (Fig. 4f). Both cohorts also showed reciprocal activation and expansion of CD4 T cell subsets (Figs. 4a-b, 4d and S5f-j), mirroring the kinetics of CD8 T cell activation in nondepleted animals. To gauge humoral responses to ZIKV, we conducted plaque reduction neutralization tests (PRNTs) using rhesus macaque sera to quantify neutralizing antibody titers. All animals except R20865 (nondepleted) showed
Figure 4: Compensatory adaptive immune responses in CD8-depleted macaques

(A-B) viSNE analyses of T cell activation in rhesus (A) and cynomolgus (B) macaques, as measured by CD69 expression. Dot plots are concatenated for animals within each treatment condition. The viSNE clustering profiles of CD4 and CD8 T cell subsets (left) correspond to cell populations in the CD69 heatmaps in nondepleted and CD8-depleted animals at 1 & 10 dpi (right). (C) Proliferation of EM CD8 T cells in rhesus and
cynomolgus macaques over time. CD8-depleted macaques are excluded owing to the absence of CD8+ lymphocytes in these animals exclusive of R64357 at later timepoints, which also precluded statistical analysis for this and other CD8 expressing populations. (D) Proliferation of EM CD4 T cells in rhesus and cynomolgus macaques over time. AUC analysis revealed a significant difference in EM CD4 T cell proliferation among CD8-depleted and nondepleted animals of both cohorts by a Mann-Whitney test. (E) CD8 T cell responses in rhesus macaques, assessed by ICS of PBMCs stimulated with viral peptides derived from the indicated ZIKV proteins (C = capsid; M = membrane; E = envelope; NS1 = nonstructural protein 1, consistent throughout). CD8 T cell responses were identified by co-positivity for perforin and IFNγ. (F) Th1 responses, determined by ICS for IL-2 and IFNγ co-positivity. (Inset): representative antigen-specific cytometry plots for R64357 (CD8-depleted) at 30 dpi. (G) Serum neutralizing antibody titers in rhesus macaques, represented as PRNT90. (H) Activation of B cells in rhesus macaques over time. (I) Proliferation of B cells in rhesus and cynomolgus macaques over time. AUC analysis was not significant by a Mann-Whitney test.

evidence of neutralizing antibodies at 7 dpi, the earliest post-infection timepoint tested (Fig. 4g). While highly neutralizing titers were detected in all animals at 15 dpi, antibody concentrations declined in nondepleted animals, but not in CD8-depleted animals, beyond this timepoint. Strikingly, depleted rhesus macaques retained highly neutralizing antibody titers until necropsy, a finding consistent with elevated B cell activation (Fig. 4h) and proliferation (Fig. 4i) in these animals.
Figure 5: Enhanced tissue dissemination and neuropathology

(A-E) Viral dissemination in cynomolgus macaques, including lymphatic (A), neural (B) and reproductive (C) tissues, as well as semen (D) and CSF (E) (center line, mean; error
bars, standard deviation of two replicates per sample, LN = lymph node; sub. wt. matter = subcortical white matter, consistent throughout). Mann-Whitney tests reported a non-significant difference in viral load among CD8-depleted and nondepleted animals for each tissue tested and for AUC analysis of CSF. (F-H) Viral dissemination in rhesus macaques, including lymphatic, neural, and reproductive tissues (F), as well as semen (G) and CSF (H). Mann-Whitney tests reported a non-significant difference in viral load among CD8-depleted and nondepleted animals for each tissue tested and for AUC analysis of CSF. (I) Histopathological findings in R25671 (CD8-depleted) brainstem (top) and lumbar spinal cord (bottom). Top: There is an area of encephalomalacia (dotted region, left) adjacent to a vessel that exhibits medial thickening (arrow, left). The area of malacia is characterized by dilated myelin sheaths with swollen axons (arrow, right) or gitter cell infiltration (asterisks, right). H&E, Bar = 100 µm. Bottom: The meninges surrounding the lumbar spinal cord are multifocally infiltrated by aggregates of lymphocytes (arrows). H&E, Bar = 1 mm (left) and 100 um (right). (J) Histopathological findings in R64357 (CD8-depleted) sciatic nerve (top) and brainstem (bottom). Top: Small vessels within the sciatic nerve are surrounded by low numbers of lymphocytes (arrows). Bottom: A focal glial nodule is present within the gray matter of the brainstem (dotted region, left) with dilation of adjacent myelin sheaths and spheroid formation (arrowhead, right). H&E, Bar = 100 µm.

Enhanced tissue dissemination and neuropathology

Given the persistence of high neutralizing antibody titers in CD8-depleted rhesus macaques, we suspected that virus might be lingering in the peripheral tissues of these animals. Although the duration of infection before necropsy differed among the cohorts,
potentially complicating our ability to identify patterns of viral dissemination and clearance over time, previous reports of ZIKV tropism in macaques [19, 25, 49] suggested that the virus can persist in lymphoid, neural, GI, and reproductive tissues, as well as in semen and CSF well past the clearance of serum viremia, so we evaluated viral distribution in these sites. Relative to nondepleted animals, CD8-depleted cynomolgus macaques had markedly higher levels of ZIKV RNA in the inguinal, mesenteric, and colonic lymph nodes, as well as in the spleen and jejunum (Fig. 5a & 5c), although the small sample size precluded statistical significance by the non-parametric Mann-Whitney test. All cynomolgus monkeys except C91638 (nondepleted) harbored virus in the rectum without an obvious difference among treatment groups. Notably, the trend of higher viral burdens in the lymphatic tissues of CD8-depleted animals was consistent in rhesus macaques (Fig. 5f). CD8 depletion also appeared to promote ZIKV dissemination in the semen, with both CD8-depleted cynomolgus macaques presenting semen viral loads and no viral RNA detected in nondepleted animals of the same cohort (Fig. 5d). Intriguingly, the nondepleted macaque C84545 (mock-depleted) showed the highest level of viral RNA in the prostate and was the only animal to present virus in the testes (Fig. 5c), yet no ZIKV was detected in the semen of this animal (Fig. 5d). Similar trends were evident in rhesus macaques, with viral RNA detected in the semen (Fig. 5g) and seminal vesicle (Fig. 5f) of a CD8-depleted animal and only a miniscule quantity of virus detected in the semen of a nondepleted animal (Fig. 5g).

ZIKV RNA was detected in the brainstem and subcortical white matter of C84545 (mock-depleted) (Fig. 5b), and this animal also presented a high magnitude viral load in the CSF early in infection, which persisted until necropsy (Fig. 5e). Exclusive of C84545
(mock-depleted), CD8-depleted cynomolgus and rhesus macaques manifested CSF viral loads at least an order of magnitude greater than nondepleted animals (Figs. 5e and 5h). Although ZIKV was not detected in the central nervous system of any animal in the first cohort, R25671 (CD8-depleted) and R64357 (CD8-depleted) manifested neural lesions at necropsy that were not present in nondepleted animals. Most strikingly, the brainstem of R25671 (CD8-depleted) had an area of severe multifocal to coalescing encephalomalacia which showed evidence of Wallerian degeneration, characterized by vacuolation, swollen axons, and infiltration by lymphocytes and phagocytic gitter cells (Fig. 5i). Gitter cells were occasionally found within dilated myelin sheaths. Scant brown granular pigment (presumed hemosiderin) and a proliferative cerebral vessel adjacent to the malacia may indicate that the malacia was the result of a vascular event (thromboembolism, infarct, ischemia, etc.). Additionally, lymphocytic infiltrate was present in the meninges surrounding the lumbar spinal cord (Fig. 5i). No gross abnormalities were noted in R64357 (CD8-depleted), although the sciatic nerve exhibited mild lymphocytic perivasculitis. The sciatic nerve is a known site of ZIKV replication in mice depleted of CD8 cells [39]. Further, the brainstem contained a localized area of gliosis, an indicator of CNS damage [67], and dilated myelin sheaths (Fig. 5j). A cause for these neural inflammatory lesions was not apparent by histology.

Discussion

Owing to the importance of CD8+ T cells in the control of ZIKV in mice and in humans [38, 39, 45], and given the recent development of a nonhuman primate model for ZIKV infection, we asked whether CD8 cells are similarly important in controlling ZIKV infection in macaques. Without question, the small sample size of the present pilot study
limited meaningful statistical analysis. In contrast to similar CD8-depletion studies with simian immunodeficiency virus, the absence of CD8+ lymphocytes did not overtly affect the control of virus in serum [68, 69]. However, nuances in innate immune responses emerged in the absence of CD8+ lymphocytes that seemed to be consistent in rhesus and cynomolgus macaques.

The marginal delay in serum viral loads in CD8-depleted macaques contrasted patterns observed by our own group (Fig. S2a) and others [17], but this anomaly did not turn out to meaningfully impact viral kinetics beyond 2 dpi. A mechanism underlying this shift remains obscure, but it is possible that a lack of NK cell stimulation in CD8-depleted animals could have misfired viral replication in what would otherwise be readily permissive monocytes. Monocytes are known to engage in intercellular crosstalk with NK cells [70, 71], and IFNγ supports ZIKV replication [72]. NK cell derived IFNγ might have activated ZIKV infected myeloid cells in nondepleted animals, promoting an inflammatory milieu that favored early viral replication. Alternatively, NK cells are shown to be minor reservoirs of ZIKV RNA in infected humans [26] and pigtail macaques [18], so the absence of this potential target cell could have contributed to the delayed serum viral loads in CD8-depleted animals. Additional co-culture assays may elucidate intercellular dynamics important for maintaining patterns of innate immune regulation.

CD8 depletion also appeared to impact the mobilization of leukocyte populations acutely following infection, again contrasting patterns observed by our own group (Fig. S2c-e) and others [19]. Depleted animals showed unusually little fluctuation in the biomarker of inflammation NLR, possibly indicating altered innate immune responses immediately following infection. In line with these observations, mice lacking NK cells
exhibit altered neutrophil recruitment in a variety of infectious and noninfectious conditions [73]. Neutrophil effector functions are modulated by NK cell-derived cytokines [73], a signaling axis which might have been disrupted by the depletion of NK cells in macaques.

In support of miscommunication within the innate immune system of CD8-depleted macaques, these animals presented largely muted transcriptional activity in key virus response pathways during acute infection. In nondepleted macaques, antiviral gene expression appears to be driven principally by circulating monocytes, contrasting the transcriptional void evident in CD8-depleted animals. Notably, the one control animal that received an irrelevant antibody prior to infection exhibited relatively muted transcriptional responses, similar to the depleted animals, suggesting the possibility that the presence of the antibody itself caused the attenuated responses. However, these antiviral response patterns were replicated ex vivo when monocytes engaged in direct contact with NK cells (or another CD8+ lymphocyte population), implying that this cell signaling dynamic might have been disrupted in CD8-depleted macaques. Indeed, intercellular crosstalk between monocytes and NK cells is known to affect transcriptional responses to ZIKV infection [62], but the possibility that NK cells additionally engage neutrophils, another significant cytokine producing population, warrants further investigation.

Consistent with a model of CD8-dependent monocyte response patterns during ZIKV infection, CD8-depleted and nondepleted animals showed striking phenotypic differences in monocyte activation. CD16-expressing monocyte subsets showed the greatest evidence of activation in both cohorts, agreeing with recent findings that these populations are primary targets of ZIKV in the blood [26, 27, 74]. CD8 depletion also
impacted the activation of monocytes temporally, further underscoring dysregulated innate responses in depleted animals. CD169 (siglec-1) is a sialic acid-binding lectin previously found to be upregulated during acute ZIKV infection in rhesus macaques [25, 64]. CD169 has important roles in virus capture by myeloid cells [75] and in the mounting of CD8 T cell responses in viral infection [76], so the robust induction of CD169 in nondepleted animals might have promoted sufficient CD8 T cell responses. CD8 depletion also might have affected monocyte frequency, possibly contributing to differential transcriptional responses. Both cohorts showed a transient increase in classical monocytes, perhaps analogous to the monocytosis that accompanies acute ZIKV replication in human patients [26]. The increase in CD16+ nonclassical monocytes in CD8-depleted rhesus macaques is an outcome also observed in ZIKV infection of human blood [27], and the expansion of intermediate monocytes in nondepleted animals resembles ZIKV infection in Nicaraguan patients [26]. These trends were not consistent in cynomolgus macaques, resulting in species differences that in several cases precluded statistical significance. Nonetheless, our data suggest perturbations in monocyte phenotype in the absence of CD8+ lymphocytes, implying the existence of crosstalk within the innate immune system that orchestrates patterns of activation.

While we detected antigen-specific T cell responses in rhesus macaques, the loss of CD8 cells did not overtly impact ZIKV clearance from the serum, suggesting a limited role of these cells in viral control. In mice, CD8 responses are important in conferring protection and promoting survival [39, 45, 77], but their role in ZIKV infection in NHPs appears to be more subordinate to innate immunity. The antigen-specific CD8 T cell responses we detected were sparse, but there was evidence of compensatory Th1 and
humoral responses in CD8-depleted rhesus macaques, suggesting that CD8 responses may have some role during ZIKV infection in this species. Protective CD4 T cell responses are reported in murine studies of ZIKV infection [77, 78], which exist in a dynamic balance with CD8 immunity [38], a model which may be consistent in NHPs. However, we failed to detect antigen specific CD8 T cell responses in cynomolgus monkeys, precluding the conclusion that such responses are important in all NHP species.

The persistence of high neutralizing antibody titers until necropsy in CD8-depleted rhesus macaques suggested that there might be virus lingering in the peripheral tissues of these animals, and indeed, ZIKV RNA was generally more abundant in the lymphatic tissues, semen and CSF of CD8-depleted rhesus and cynomolgus macaques relative to nondepleted animals. Although the limited sample size negated significance, these trends may imply the importance of CD8+ lymphocytes in limiting ZIKV dissemination and persistence in tissues. Among the cohorts, lymphatic tissue viral loads were higher in cynomolgus compared to rhesus macaques, possibly reflecting the abbreviated time of infection before necropsy. Despite the detection of viral RNA in semen, ZIKV was scarce in reproductive tissues, in line with an absence of gross pathological lesions including atrophy of the testes. Although testicular atrophy is reported in murine models of ZIKV [79], such manifestations have not been observed in macaques or in clinical cases [80]. In rhesus monkeys, it is possible that viral RNA in neural and reproductive tissues might have been only transiently present due to viral clearance given that the study lasted as long as 30 days. Previous reports in rhesus [81] and pigtail [18] monkeys have also shown that ZIKV persists in lymphatic tissues well beyond the clearance of virus from the serum. It remains unclear whether the ZIKV present in lymph nodes is replication competent, but
our data are consistent with a model where the absence of CD8+ lymphocytes seems to facilitate ZIKV persistence. Although CD8 depletion in the context of ZIKV infection may produce interesting and unexpected immunophenotypic patterns, these observations did not reach statistical significance even when animals in both cohorts were analyzed together. The small sample size of each cohort precluded meaningful statistical analysis within each species individually, and differences in study design prevented significance due to the ranking method of nonparametric statistical tests when the cohorts were combined for analysis. Nonetheless, CD8 depletion produced interesting deviations in host immunity that appeared to be species independent.

CD8-depleted rhesus macaques also presented gross neural lesions at necropsy not seen in nondepleted animals. The most severe lesion occurred in the brainstem of a depleted animal that never recovered CD8+ lymphocytes, and similar manifestations of encephalomalacia and axon degeneration have been reported in ZIKV infection of human fetal brain tissue [82-84]. Perhaps complementarily, neural lesions in the CD8-recovering rhesus macaque were less severe. Although it is tempting to speculate that the absence of CD8+ lymphocytes in R25671 (CD8-depleted) and R64357 (CD8-depleted) allowed neural dissemination of the virus which thereby promoted neuropathy, our inability to detect ZIKV RNA in brain sections from these animals precludes this conclusion. Because ZIKV was cleared from the CSF of rhesus monkeys within 15 dpi, it is possible that virus could have also cleared from the CNS by necropsy and that these lesions were virus associated even if viral RNA was not detectable late in infection. Supporting this argument, CSF viral loads appear to be associated with ZIKV dissemination in neural tissue, given that the mock-depleted cynomolgus macaque C84545 showed the highest and most
persistent level of viral RNA in CSF and was also the only animal with ZIKV RNA identified in the brain. Despite the presence of CD8+ lymphocytes in C84545 (mock-depleted), this animal was the sole example of neural dissemination and occasionally produced responses more similar to CD8-depleted animals in key immune measures including NLR and classical monocyte frequency. Although it remains possible that off-target antibody effects produced these effects in C84545, this animal ultimately aligned more closely with nondepleted animals in terms of serum viral load, antiviral gene induction, and adaptive immune activation. The general absence of immune surveillance and IFN signaling in CD8-depleted animals might have permitted initial infection of neural tissues, which could have been transient due to the eventual priming of compensatory adaptive responses. Additionally, ZIKV localizes as discrete foci in macaque tissues [64], complicating the detection of sparse viral lesions within organs. It is worth noting that CNS localization of ZIKV has been observed as early as 5 dpi in acutely infected macaques [19], and a separate study in rhesus monkeys failed to identify ZIKV RNA in the CNS at 14 dpi, despite diffuse patterns of viral dissemination [49]. These findings, together with our own, suggest a model where viral dissemination to the CNS occurs soon after ZIKV challenge in nonhuman primates, but the virus may not persist later in infection.

In conclusion, the present study illustrates a pliable dynamic between ZIKV and its hosts. CD8 T cells and NK cells do not overtly impact serum viral loads or disease outcome but do appear capable of establishing innate immunity and limiting the extent of viral persistence in tissues, findings that implicate these cells in the long-term control of ZIKV in nonhuman primates.
Supporting Information

Supplementary Figure 1: MT807R1 depletes CD8+ lymphocytes with variable recovery

(A-C) Absolute counts of CD8 T cells (A), CD4/CD8 double-positive T cells (B), and CD4 T cells (C) in blood, as determined by CBC.

Supplementary Figure 2: Comparison of virus and immune cell dynamics to a previous female cohort
Data from a previous cohort of ZIKV-infected non-pregnant female rhesus macaques is shown in gray, and data from rhesus macaques of the present study is overlaid. (A) Serum viral loads (error bars, standard deviation). (B) CSF viral loads. (C-D) Frequencies of neutrophils (C) and total lymphocytes (D) in whole blood over time, determined by CBC. (E) NLR, derived using total neutrophil and lymphocyte CBC data.
**Supplementary Figure 3: Transcriptional profiling in blood**

(A-B) Patterns of antiviral gene induction at 1, 3, and 15 dpi in the whole blood of nondepleted (A) and CD8-depleted (B) rhesus macaques relative to pre-infection. (C-D) Pathway analysis of gene expression in whole blood at 3 dpi relative to pre-infection revealed the induction of genes relating to leukocyte homing (C) as well as differentially activated biological functions and disease-related pathways among CD8-depleted and non-depleted animals (D).

**Supplementary Figure 4: CD8 depletion modulates monocyte phenotype during ZIKV infection**
Flow cytometric analysis of monocyte activation, as measured by CD169 expression in classical (A), intermediate (B), and nonclassical (C) subsets in rhesus and cynomolgus macaques. (D) Overall monocyte activation, as measured by CD69 expression. (E-F) Expression of CD95 in classical (E) and nonclassical (F) subsets. Mann-Whitney tests of AUC analyses failed to reveal statistical differences among CD8-depleted and nondepleted animals for each parameter shown.

Supplementary Figure 5: Reciprocal T cell responses

(A-E) Immunophenotyping of CD8 T cells in rhesus and cynomolgus macaques, including EM CD8 activation (A), CM CD8 activation (B) and proliferation (C), and naïve CD8 activation (D) and proliferation (E). The absence of CD8+ T cells in depleted animals
precluded statistical analysis among treatment groups. (F-J) Immunophenotyping of CD4 T cells, including EM CD4 activation (F), CM CD4 activation (G) and proliferation (H), and naïve CD4 activation (I) and proliferation (J). Mann-Whitney tests of AUC analyses reported a significant difference in the proliferation of CM CD4 T cells among CD8-depleted and nondepleted animals, although significance was not reached for other subsets.
Supplementary Figure 6: NK cell crosstalk with monocytes and neutrophils

(A-B) Antiviral gene induction monocyte/NK cell cocultures either with direct contact (A) or spatial separation by a transwell (B). In (A), cocultures exposed to ZIKV were compared to monocytes exposed to ZIKV in the absence of NK cells at the indicated timepoints. qRT-PCR for the ISGs ISG15 and OAS2 represent a readout for antiviral gene induction (C-D). Antiviral gene induction in neutrophil/NK cell cocultures compared to neutrophils exposed to ZIKV in the absence of NK cells at 24 hours post infection including an NK cell only control (D). (E) Protein-level activation of neutrophils when cocultured with NK cells in the presence of ZIKV.
CHAPTER 3: IMMUNE SIGNATURES OF ZIKA VIRUS INFECTION DURING PREGNANCY

Overview

T cell immunity is an important component of the adaptive immune response to Zika virus (ZIKV) infection in humans and in nonhuman primate (NHP) models, and ZIKV infection in NHPs produces immune responses that are sufficient to protect from secondary challenge by homologous and heterologous viral isolates. However, the strong associations between in utero ZIKV infection and congenital syndromes suggest that immune responses that occur during pregnancy are insufficient to protect from infection of the fetus. Whether immune responses mounted during pregnancy sufficiently protect from secondary infection, and whether CD8 T cells are an important role mediator of this control, remains unknown. Here, we tested the capacity of CD8 lymphocytes to protect from secondary challenge in four macaques, two of which were depleted of CD8 cells prior to rechallenge with a heterologous ZIKV isolate. The initial challenge during pregnancy produced transcriptional signatures consistent with metabolic reprogramming and low levels of immune activation, but following rechallenge, virus was not detected in the serum or cerebrospinal fluid of any animal. Rechallenge produced an expansion of humoral responses and activation of several innate and adaptive immune cell subsets, suggesting a brief period of infection prior to clearance. These data confirm that ZIKV infection during pregnancy induces sufficient immunity to protect from secondary challenge and suggest that this protection entails multiple arms of the immune system.
Introduction

ZIKV was first isolated nearly seventy years prior to the Brazilian outbreak of 2015 [43, 85], but the recent epidemic came to be associated with vertical transmission dynamics and congenital syndromes that were unprecedented for ZIKV or any other flavivirus. [44]. Although infrequent neurological manifestations, including Guillain-Barre syndrome, meningitis, and meningoencephalitis, became linked to infection in adults [86-89], the most severe neurological consequences were documented in infants born to mothers infected during pregnancy [90-92]. Referred to as congenital Zika syndrome (CZS) [93], this collection of manifestations has provided the greatest justification to develop prophylactic and therapeutic countermeasures. Several murine and NHP models have been developed to understand mechanisms of maternal-to-fetal transmission and to develop and test antiviral therapies and vaccines [16-18, 25, 50, 51, 94-96], but NHPs may provide a superior model to study vertical transmission and congenital hazards due to the similarities in placental structure and gestational development to humans [16].

ZIKV vaccine efforts have been successful thus far, with a number of candidate vaccines having advanced to clinical trials, but an underappreciated consideration in vaccine design thus far is whether protective responses can be attained in context of pregnancy. Complex interactions between sex hormones and the immune system make pregnant women more susceptible to a host of infections [97], so an important question for ZIKV vaccine design is whether immunity induced during pregnancy is sufficient to prevent subsequent infections and if this protection extends to infants born to women infected during pregnancy.
A recent study showed that NHPs infected during pregnancy establish long-term immune responses that are sufficient to protect against secondary challenge [98], a finding that is also true in non-pregnant macaques [94]. Similar to other flaviviruses, ZIKV infection results in rapid neutralizing antibody titers [49] suggesting that humoral immunity may be the most important correlate of protection. However, ZIKV-specific T cell responses have been described in mice [39, 45, 77], macaques [17], and humans [37, 41, 99, 100], so cell-mediated immunity might also have role in protection from secondary infection. Here, we used the rhesus macaque model to confirm whether ZIKV infection during pregnancy induces sufficient immunity to protect from rechallenge, and we also assessed whether CD8 lymphocytes are an important component of this protection.

Materials and Methods

CD8 depletion. Approximately nine months after initial challenge, as described previously [50], two of the dams were depleted of CD8α+ lymphocytes (primarily NK cells and CD8+ T cells) using the M-T807R1 antibody with a standard four-dose regimen over 10 days. CD8+ cell counts in blood were monitored by complete blood count (CBC), and animals were screened for adverse events after each administration and none were observed.

Viral challenge and viral load quantification. Primary ZIKV inoculations of the primary ZIKV isolate Rio-U1 were described previously [50]. The challenge virus was isolated in 2015 from the urine of a pregnant women in a region in Rio de Janeiro [53]. Briefly, four Indian rhesus macaques were initially challenged with ZIKV Rio-U1 at 10^4 plaque forming units (PFU) during the third trimester of pregnancy, resulting in serum viremia that peaked at 3 days post infection (dpi) with 5-6 logs of viral RNA/ml in plasma.
that cleared between 14-28 dpi. One animal, JA38, had detectable virus in amniotic fluid just prior to full term fetal harvest, between 35 and 40 dpi. Two infants were sacrificed for tissue harvest at birth, but no evidence for in utero infection was present. The other two infants were kept alive for viral challenge and behavioral observation, as described previously [101].

Secondary inoculations of the heterologous Puerto Rican isolate PRVABC-59 were carried out at the same dose (10⁴ PFU), and route (subcutaneous) as the primary challenge. Blood and cerebrospinal fluid (CSF) were drawn on days 0, 3, 5, 7, 14, and 28 post challenge. Viral RNA was isolated from serum and CSF using the Roche High Pure Viral RNA Kit followed by quantification as described previously [50]. Animals were euthanized 28 dpi (n=2) or 30 dpi (n=2) after secondary challenge.

Anti-ZIKV binding antibody titers. Serum was tested for reactivity to ZIKV antigen using the commercial enzyme-linked immunospot assay (ELISA, Xpressbio) from before the initial infection, 28 days after the initial infection, on the day of reinfection, and 30 days after rechallenge. Responses during primary infection, as optical density (OD), were tested at a 1:50 dilution. This dilution proved too concentrated for the rechallenge, so a 1:200 dilution was used.

Flow Cytometry. Cryopreserved PBMCs were thawed and labeled with the following antibodies: CD16 AL488, CD169 PE, CD28 PE-CF594, CD95 PCP-Cy5.5, CD3 PE-Cy7, CD8 Pacific Blue, CD14 BV605, HLA-DR BV650, CD69 BV711, NKG2A APC, and CD4 APC-H7, followed by fixation, permeabilization and labeling with an antibody against Ki67 AL700. Flow cytometry data was collected on a BD LSR II instrument and analyzed using FlowJo v10.
RNA-sequencing and analysis. Using the Zymo Quick-RNA Miniprep kit, total RNA was extracted from PBMC pellets of all four animals at the day of initial challenge and three days post-challenge. RNA was purified using the Zymo RNA clean & concentrator-25 kit and quantitated using the Qubit RNA BR assay kit (Thermo Fisher). A beta release of the Collibri 3’ mRNA Library Prep Kit (Invitrogen) was used to prepare libraries, and sequencing was carried out at the Tulane NextGen sequencing core using an Illumina NextSeq instrument with 150 cycles. Sequencing data were aligned and mapped to the rhesus macaque genome (Mmul_10 assembly) using STAR [102] with default settings in gene quantification mode. Differential expression was calculated using DESeq2 [103], and pathway analysis was carried out using gene set enrichment analysis (GSEA) [104] and Ingenuity Pathway Analysis (Qiagen). Differential expression data from a previous cohort of male rhesus and cynomolgus macaques infected with the identical ZIKV isolate (Rio-U1) [105] is shown in Fig. S7.

CD8 lymphocyte depletion and rechallenge

CD8α lymphocyte depletion (targeting primarily CD8 T cells and NK cells) resulted in a rapid decrease in CD8+ cell counts to an undetectable level (Fig. 6a). Given that ZIKV can persist in tissues long past the clearance of virus from the serum, viral loads were determined prior to rechallenge to ensure CD8 depletion did not result in recrudescent viremia from a cryptic reservoir, and none was detected (data not shown). Following rechallenge, viral RNA was not detected by qRT-PCR in any sample at any time point, suggesting complete immunity to the Puerto Rican strain (Fig. 6b).
Sustained anti-ZIKV antibody titers expand after rechallenge

Initial challenge resulted in the induction of binding antibodies, which were detected at 28 dpi using a 1:50 dilution of blood plasma, the only post-infection timepoint tested (Fig. 7a). Binding antibodies were re-assessed on the day of rechallenge, again using a 1:50 dilution of blood plasma, to provide a reference for determining humoral responses to secondary infection. At this dilution, antibody responses were outside of the detection

Figure 6: CD8 depletion and rechallenge

(A) CD8α+ cells were depleted using a 10-day protocol. Absolute counts of CD8+ cells in blood dropped to near zero before ZIKV rechallenge. (B) Nine months after initial infection during pregnancy, four macaques, including the two CD8 depleted animals, were challenged subcutaneously with 10^4 PFU of the Puerto Rican strain (PRVABC-59) of ZIKV and viral loads monitored for one month.
Figure 7: Anti-ZIKV humoral responses

(A-B) ELISA was used to assess anti-ZIKV humoral immunity after primary infection (A) and rechallenge (B). Antibody titers measured at a 1:50 dilution rose between the day of primary infection and day 28 (A). Anti-ZIKV antibody responses also expanded following rechallenge (B), which were tested at a 1:200 dilution as 1:50 proved too concentrated for the dynamic range of the assay.

range (OD > 3.5) (data not shown), suggesting they had continued to rise since 28 dpi of the initial infection. We then repeated the assay using a 1:200 plasma dilution and found that the concentration of binding antibodies expanded after rechallenge in 3 of 4 animals, while antibodies in the fourth animal remained above the limit of detection (Fig. 7b).

Immune activation following secondary challenge

Given that viral RNA was not detected in the serum of any animal following rechallenge, we assessed the activation of innate and adaptive immune cells as a surrogate of infection. Using a multicolor flow cytometry panel that we adapted from a previous
ZIKV study [105], we evaluated the proliferation (Ki67) and activation (CD69 or CD169) of T cells and monocyte subsets cells before and after rechallenge.

Classical and intermediate monocytes showed no discernable changes in frequency or activation (Fig. 8a-f). However, nonclassical monocytes (CD14^low, CD16^+) expanded following inoculation at 3 dpi selectively in CD8-depleted animals (Fig. 8g). Although nonclassical monocytes showed no increase in CD169 expression following rechallenge (Fig. 8h), there was an increase in activation as measured by CD69 expression in nondepleted animals at 5 dpi (Fig. 8i).

Following rechallenge, central memory CD4 T cells expanded in frequency primarily in CD8-depleted animals (Fig. 9a), and these cells also showed increases in activation (CD69 expression, Fig. 9b) and proliferation (Ki67 expression, Fig. 9c) between 3-5 dpi in the same animals. Nondepleted animals also showed an increase in central

![Figure 8: Monocyte changes after ZIKV rechallenge](image-url)
Classical (CD14+ , CD16-) (A-C), intermediate (CD14+ , CD16+) (D-F), and non-classical (CD14low/+, CD16+) (G-I) monocytes were assessed for changes in frequency (A, D, G) and activation as measured by CD69 expression (B, E, H) and CD169 expression (C, F, I), after ZIKV rechallenge.

memory CD4 T cell proliferation during the same period (Fig. 9c), but the magnitude of this increase was marginal compared to that of the CD8-depleted animals. Effector memory CD4 T cells showed a modest increase in frequency in CD8-depleted animals at 3-5 dpi (Fig. 9d) but no clear patterns in activation or proliferation occurred following rechallenge (Fig. 9e-f). Naïve CD4 T cells showed a striking drop in frequency between 3-5 dpi, which was most pronounced in CD8-depleted animals (Fig. 9g). The decline in naïve CD4 T cell frequency was concomitant with an increase in activation (CD69 expression, Fig. 9h) and proliferation (Ki67 expression, Fig. 9i) primarily in 3 of 4 animals.

Interestingly, phenotypic patterns in the CD8 T cell subsets of nondepleted animals generally mirrored those that occurred in the CD4 T cells of CD8-depleted animals. Although central memory CD8 T cells did not show appreciable changes in frequency (Fig. 10a) or activation (Fig. 10b) after rechallenge, there was a clear increase in proliferation between 3-5 dpi (Fig. 10c). Effector memory CD8 T cells clearly expanded between the day of rechallenge and 5 dpi (Fig. 10d), but these cells did not become activated (Fig. 10e) and showed an increase in proliferation in only 1 of 2 nondepleted animals (Fig. 10f). In similar fashion to naïve CD4 T cells, naïve CD8 T cells dropped in frequency following rechallenge until 5 dpi (Fig. 10g) and showed marked increases in activation (Fig. 10h) and proliferation (Fig. 10i).
Figure 9: CD4 T cell changes after ZIKV rechallenge

Central memory (A-C), Effector memory (D-F), and naïve (G-I) CD4 T cells were assessed for changes in frequency (A, D, G), activation as measured by CD69 expression (B, E, H), and proliferation as measured by Ki67 expression (C, F, I), after ZIKV rechallenge.
Figure 10: CD8 T cell changes after ZIKV rechallenge

Central memory (A-C), Effector memory (D-F), and naïve (G-I) CD8 T cells were assessed for changes in frequency (A, D, G), activation as measured by CD69 expression (B, E, H), and proliferation as measured by Ki67 expression (C, F, I), after ZIKV rechallenge.
Figure 11: Transcriptome analysis following primary challenge

(A) Volcano plot showing differentially expressed genes (DEGs) in PBMCs during initial challenge at day 3 relative to day 0. (B) Gene sets from GSEA that were significantly enriched at a false discovery rate (FDR) < 10%. NES: normalized enrichment score. (C-E)
Heatmaps of read count data for genes responsible for core enrichment of genes sets relating to oxidative phosphorylation (C), fatty acid (FA) metabolism (D), and autophagy (E). Read counts are log2-transformed. *Left*: pre-infection; *Right*: 3 dpi.

Transcriptome analysis following primary challenge

Finally, we carried out transcriptomic analysis of PBMC following the initial challenge during pregnancy to assess the quality of immune responses in this potentially altered physiological state. Initial challenge resulted in the up- and downregulation of many genes at 3 dpi (Fig. 11a), but interestingly, very few of these differentially expressed genes pertained to interferon (IFN) signaling. The IFN response is among the most important components of the immune system that limits acute ZIKV infection [106], and we have previously detected strong IFN responses in male macaques infected with the same strain of ZIKV (Rio-U1) at 3 dpi (Fig. S7a). Although this previous analysis was more restrictive than RNA-seq in that we used the NanoString platform to quantify gene expression, it was striking that none of the IFN related genes that were highly induced in the previous male cohort were upregulated in the pregnant females. Instead, we noted downregulation of genes such as ERAP2, which is involved in antigen presentation and the induction of MHC-I restricted immune responses, along with downregulation of genes relating to chemokine signaling (CXCL17) and neuron development and differentiation (TLX3, RBM11). Through pathway analysis, we detected an immunoregulatory phenotype characterized by a lack of immune cell recruitment and activation that was driven by a decrease in a core set of chemokines (IL2) and their receptors (CCR7, IL12RB1), together with downregulated adhesion proteins (SELP, CD48, SELL, ICOS, CD40LG), signaling molecules (IRF1,
NFATC2), and activation markers (CD69, CD48) (Fig. S7b). Among the generalized suppression of pathways relating to immune cell activation and homing, pathways relating to actin cytoskeleton remodeling, integrin signaling, and leukocyte extravasation were among the most significantly affected (Fig. S7F), possibly highlighting a deficiency in immunologic synapse formation. IPA also showed that FGF signaling was affected by ZIKV infection (Fig. S7f), which has been shown to support ZIKV infection by suppressing IFN signaling [107]. This finding was supported by the induction of FGF2 as well as the predicted activation of FGF2 as an upstream regulator (Fig. S7e).

In line with IPA results, GSEA similarly predicted immunomodulation through TGFβ signaling (Fig. 11b), but enrichment analysis further suggested that infection during pregnancy resulted in metabolic reprogramming, characterized by changes in cell respiration (oxidative phosphorylation, glycolysis) and lipid metabolism (adipogenesis, fatty acid metabolism, cholesterol homeostasis, peroxisome) (Fig. 11b). Gene-level analysis of oxidative phosphorylation and fatty acid metabolism showed that activation of these pathways occurred primarily in only 2 of 4 animals (Fig. 11c-d). Autophagy signaling was also significantly enriched (Fig. 11e), which might have contributed to the observed changes in cell respiration and lipid metabolism. Autophagy in placental trophoblasts is shown to promote maternal-to-fetal transmission of ZIKV in mice [108], and interestingly, one of the animals that showed upregulation of autophagy signaling also had virus detectable in the amniotic fluid at multiple timepoints (Fig. S7c). GSEA additionally detected generalized cell stress, with induction of gene sets relating to hypoxia and the unfolded protein response, together with indications of cell cycle arrest (G2M checkpoint, p53 pathway) (Fig. 11b). Consistent with a hypoxia response, we identified evidence of
oxidative stress (Fig. S7f), marked by activation of several antioxidant and stress response proteins as well as phase I and II metabolizing enzymes (Fig. S7d). We previously observed induction of similar oxidative stress pathways in macaque astrocytes infected with the identical strain of ZIKV (Chapter 4), underscoring the stress responses that likely result from massive replication of this virus.

Discussion

ZIKV has been a known teratogen for some time, but the impacts of pregnancy on the quality of virus-specific immune responses have yet to be fully understood. The decrease in ZIKV incidence in the Western hemisphere since the peak of the outbreak in 2015 is of little reassurance until effective vaccines and therapeutics are mobilized. Although ZIKV vaccine candidates have performed well in preclinical settings, the congenital risks associated with ZIKV infection introduce a set of challenges to vaccine development that require special consideration and carefully chosen animal models. Pregnancy presents a substantially altered immunologic state to facilitate fetal development and protect the mother and developing fetus from infectious agents [97], so it follows that immune correlates of protection during pregnancy might differ from mechanisms that are important in otherwise nonpregnant individuals.

Whether immunity induced during pregnancy, due to either infection or vaccination, is sufficient to protect from subsequent infection has begun to be examined in murine and NHP models. A recent study in IFN-deficient mice showed that a live-attenuated vaccine protected pregnant dams from infection and also prevented in utero transmission, and this protection appeared to be mostly dependent on neutralizing antibodies [109]. The report cautioned that higher antibody titers were required to protect
pregnant animals compared to nonpregnant animals, and pregnancy appeared to negatively impact the potency of the T cell response induced by the vaccine, which are important considerations in the evaluation of future vaccine candidates. A separate study in NHPs showed that animals initially challenged during pregnancy mount immune responses similar to non-pregnant animals, and these responses adequately protect against secondary challenge [98]. Again, antibodies appeared to be the most important correlate of protection, as cell-mediated responses were not detected following reinfection. Our data similarly demonstrate that primary infection during pregnancy provides sufficient immunity to protect from secondary challenge, and this protection also appeared dependent primarily on the humoral response. The high concentration of serum antibodies that continued to rise following rechallenge suggests that a humoral response might have limited reinfection before serum viremia could take hold. Challenge of NHPs with ZIKV reliably produces a rapid serum viremia [17, 50], so the absence of serum viral RNA in either the present study or a similarly designed NHP study [98] suggests that ZIKV immunity induced during pregnancy sufficiently protects from reinfection.

Together, these reports are not entirely surprising, as several studies have shown efficient generation of immunity by vaccines administered during pregnancy [110-113]. Outdated models portray pregnancy as a global suppression of immunity [114], but these perspectives are no longer generally accepted, as it has become clear that pregnancy is rather a complex alteration of particular immune subsets to balance fetal development and protection from infection [97]. Indeed, pregnancy is a progressive biological process that requires a progressively adapting immune microenvironment [114].
Our data show phenotypic changes in immune cell populations in both CD8-depleted and nondepleted animals, indicating that some level of cellular immune involvement conferred resistance to rechallenge. Moreover, the expansion and activation of several memory T cell subsets suggests that immune memory might have been an important mediator of this protection. Nondepleted animals showed preferential expansion of effector memory CD8 T cells, while CD8-depleted animals showed greater increases in memory CD4 T cell subsets, possibly suggesting a compensatory CD4 response as we have observed previously in a cohort of male macaques that were similarly CD8 depleted prior to ZIKV challenge [105]. Antibody-mediated depletion experiments in mice have also illustrated the redundancy of adaptive responses to ZIKV, with the depletion of individual immune cell populations resulting in alternative compensatory responses [40]. Together, these studies begin to reveal the plasticity of immune responses to ZIKV that may coordinate to maintain overall immune integrity.

Although limited sample availability precluded analysis of antigen specific CD8+ T cell responses, the potential for CD8+ lymphocytes to aid in protection from rechallenge is intriguing. Vaccine induced CD8 T cells are important in protection from Ebola virus challenge [12, 115, 116], and the lack of a cell-mediated response may contribute to some of the safety and efficacy risks associated with the currently licensed dengue virus (DENV) vaccine (Dengvaxia, Sanofi Pasteur) [31]. These findings affirm the importance of CD8 responses in protection from these viruses underscores that T cell immunity should not be overlooked in vaccine design. ZIKV-specific CD8 T cells are described in multiple species [39, 41, 45, 77] and may be important for viral clearance in mouse models. CD8 T cells have active roles in controlling infections caused by other flaviviruses including West Nile
virus [41, 117-121], DENV [34, 122-126], and yellow fever virus [127-130], which, given their relatedness, suggests that CD8 cells may be similarly important in limiting ZIKV infection. Nonetheless, our data show that CD8 T cells play at most a minor role in protection from secondary infection.

Phenotypic changes among monocyte subsets were minimal, but monocytes are primary targets of ZIKV in the blood [18, 26, 27], so any alterations in frequency or activation of these cells are potentially interesting. The increases in nonclassical monocyte frequency and activation in CD8-depleted and nondepleted animals are intriguing because among monocytes, the nonclassical subset is preferentially targeted by ZIKV infection [18]. Moreover, Asian-lineage ZIKV selectively infects nonclassical monocytes in the blood of pregnant women has been shown to induce cell proliferation that associates with an M2-skewed immunosuppressive phenotype [27]. Why nonclassical monocytes responded differently among CD8-depleted and nondepleted animals is unclear, but similar patterns occurred in a previous study from our group that also used CD8 depletion in a cohort of male rhesus macaques [105]. In that study, the collateral depletion of NK cells in CD8-depleted animals appeared to skew patterns of monocyte activation among treatment groups, which might have also occurred here. The previous male cohort also showed strong upregulation of the activation marker CD169 on monocytes during acute infection, but in contrast, the pregnant animals showed little fluctuation in monocyte CD169 expression. CD169 is a sialic-acid binding lectin that serves as a biomarker of inflammation [65, 66] with expression levels that closely track ZIKV serum viremia in macaques [64, 105]. In the present study, CD169 expression was unaffected by rechallenge, which is consistent with the absence of viremia in these animals. Together, these cellular immune data suggest
some involvement of innate and adaptive cellular immune responses to the rechallenge virus, but the complete absence of viremia in both groups confirms that protection from rechallenge was robust.

Although pregnancy did not seem to outwardly impair immune responses to ZIKV infection in that all animals efficiently controlled rechallenge even in the absence of CD8 cells, transcriptome analysis offered a more nuanced perspective of immune activation during the primary challenge. Namely, we detected a general suppression of immune cell homing and activation at the height of infection, which contrasts sharply with gene expression patterns we previously observed in a group of male macaques infected with ZIKV [105]. Transcriptional analysis in the male cohort at the same timepoint post-infection indicated strong induction of immune cell homing and adhesion, including a robust IFN response. Meanwhile, the pregnant animals lacked evidence of an IFN response but instead showed FGF signaling, which is known to promote ZIKV infection by suppressing IFN responses [107]. Regarding the lack of lymphocyte migration and activation, maternal tolerance might have promoted a T-regulatory phenotype, consistent with the observed activation of TGFβ, but it also remains possible that these nuances in immune activation are due not to pregnancy but to generalized sex differences among the cohorts. However, any immunoregulatory phenotype that might have occurred during pregnancy ultimately did not compromise fetal health. Infants from two dams were born healthy and quickly cleared postnatal ZIKV challenge, and no adverse effects on nervous system development or behavior were noted, as we have described previously [101].

In addition to attenuated immune activation, the pregnant female cohort also showed evidence of metabolic reprogramming during acute infection that appeared to
center on autophagy. Although autophagy generally aids in pathogen degradation and in the induction of immune responses during microbial infection [131], ZIKV and DENV, like other viruses, are known to interact directly with autophagy pathways to promote their own replication [132]. Moreover, it has been shown in mice that ZIKV activates autophagy in placental trophoblasts to enhance vertical transmission [108], so it was fascinating that a pregnant macaque with evidence of autophagy signaling also had virus cross the placental barrier. Since autophagy is at its core a degradative process that frees biomolecules such as lipids to enter energy producing pathways, functions we detected in pregnant ZIKV infected animals, further metabolomic experiments should address whether autophagic flux is related to ZIKV infection generally or ZIKV infection during pregnancy specifically.

Together, our data confirm findings from a recent study in NHPs suggesting that pregnancy does not overtly impair immune responses to ZIKV infection, a finding with potential implications for vaccine design. We caution that the cohort of animals used in this study were initially infected during the third trimester of pregnancy, so it remains possible that infection or vaccination during earlier stages of pregnancy may be less protective. But ultimately, our findings add to a growing body of data describing the correlates of ZIKV-induced immunity in models of pregnancy, justifying vaccine research efforts in this unique subpopulation.
Supplementary Figure 7: Immunomodulation and cell stress following primary challenge

(A) Volcano plot showing DEGs from a previous cohort of male rhesus and cynomolgus macaques infected with ZIKV Rio-U1 [105]. DEGs were calculated from NanoString transcriptional analysis of immune responses in whole blood at 3 dpi relative to 0 dpi. (B) Regulator effects pathway from IPA, showing the predicted activation states of upstream
regulators and canonical pathways based on observed gene expression data. (C) Viral loads in serum and amniotic fluid (AF) during primary challenge in one pregnant animal. (D) Pathway layout for the NRF2-mediated oxidative stress response; red overlay represents upregulation and green represents downregulation of molecules at day 3 relative to day 0. (E) Upstream biological regulators that are predicted to be significantly affected by infection; z-score represents predicted activation of the molecule, while experimental log ratio (expr_log_ratio) overlay represents the observed induction of the molecule. Molecules with gray overlay were not found to be differentially expressed experimentally. (F) Canonical pathways from IPA that are predicted to be significantly affected by infection; z-score overlay represents prediction activation of the pathway; gray overlay represents modulation of the pathway without an associated z-score.
CHAPTER 4: TRANSCRIPTIONAL SIGNATURES OF ZIKA VIRUS INFECTION IN ASTROCYTES

Overview

Astrocytes are an early and important target of Zika virus (ZIKV) infection in the developing brain, but the impacts of infection on astrocyte functions remain controversial. Given the recent development of nonhuman primate (NHP) models of ZIKV infection that replicate aspects of vertical transmission and neurologic disease seen in human infections, we established an *ex vivo* model using primary macaque astrocytes to explore targeted outcomes of infection in a cell type relevant to brain development. Following infection by either Asian or African lineage ZIKV strains, transcriptomic analysis revealed typical virus response patterns, although the magnitude of antiviral signaling was greater in response to the African lineage virus. Both viruses promoted hypoxic stress, but the Asian lineage strain additionally reprogrammed metabolic and lipid biosynthesis pathways in a manner similar to other disease causing flaviviruses. Together, these findings highlight unique transcriptional signatures that accompany Asian and African lineage ZIKV infection in primary astrocytes that imply lineage specific mechanisms of neuropathogenesis.

Introduction

For years following its initial isolation in 1947, ZIKV was not considered a significant human pathogen; the virus caused only mild and sporadic symptoms in localized populations in sub-Saharan Africa and southeast Asia [133]. The scarcity of reported cases within the first 60 years of its discovery are likely due to the virus being
unrecognized or misdiagnosed as yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV) or a host of other infections caused by viruses, bacteria, or parasites. In 2007, ZIKV rapidly spread among the Yap islands of the Federated States of Micronesia, changing the perception that ZIKV could not cause epidemics, and neurological sequelae including Guillain-Barre syndrome (GBS) were not associated with ZIKV infection until the 2013 French Polynesian outbreak [134]. ZIKV was likely transmitted from French Polynesia and other Pacific islands to Brazil in 2014 [134], resulting in an epidemic in 2015-16 that became associated with a sharp rise in congenital manifestations unprecedented among related flaviviruses that cause human disease.

Among the animal models that rapidly emerged to study viral dynamics and antiviral immune responses, the NHP model held particular interest due to the similarities to humans in terms of placental structure and gestational development, which have historically made NHPs preferred models of congenital diseases. Macaque fetuses that become infected in utero replicate some aspects of neurologic disease observed in human fetuses, including neutrophilic infiltration, the development of calcifications, and reduced head circumference [21, 96]. Maternal ZIKV infection in macaques is also associated with prolonged serum viremia [21, 50, 96], together with miscarriage and stillbirth [48], outcomes also associated with infection in pregnant women [135, 136]. Given these correlates of disease, we asked whether the transcriptional signatures that accompany ex vivo infection would elucidate mechanisms of pathophysiology that occur in vivo. We further explored whether ZIKV lineage variation governs these outcomes, since Asian lineage viruses are principally associated with neurologic disease.
Contrasting ancestral African-lineage ZIKV strains that have circulated for at least decades without producing major outbreaks, modern Asian-lineage viruses, including isolates from Brazil, are responsible for recent epidemics and show associations with congenital and neurological manifestations. Despite these epidemiological patterns, comparative analyses of Asian and African lineage viruses have incongruously detected higher virulence in African viruses in cell culture and animal models [137]. Although phylogenetic disparities contribute to some of these lineage dependent effects, such as the microcephaly enhancing S139N mutation that arose in French Polynesia before the virus spread to Brazil [138], host factors likely impact clinical outcomes as well. Existing ZIKV immunity in sub-Saharan Africa may limit vertical transmission and contribute to the perception that African-lineage viruses are not neurotropic [139]. Likewise, cross-reactive immunity to DENV in regions where ZIKV is not endemic may skew clinical outcomes from those that would otherwise occur in flavivirus-naïve populations [36]. Thus, carefully designed experiments in well-chosen models are necessary to compare the neurovirulence of different ZIKV strains.

The tropism of Asian-lineage ZIKV for neural tissues is well documented, and these findings have raised the question of which cell types are permissive to infection and whether these cells are also susceptible to infection by African-lineage viruses. Studies using induced stem cell and organoid models suggest that neural progenitor cells are important targets in the etiology of virus induced neuropathology [140, 141], but identification of AXL as a putative entry factor implicates a number of other potentially permissive cell types, including glial cells such as astrocytes and microglia [93, 142]. Flaviviruses characteristically have broad cellular tropisms, so it is not surprising that
ZIKV replicates in diverse cell types such as monocytes [18, 26, 27], multiple skin-resident populations including keratinocytes, fibroblasts, and dendritic cells [143], as well as cells of the nervous system. Astrocytes are among the earliest cells in the developing brain that become infected with ZIKV [144, 145], and primary human astrocytes are also permissive to infection by both Asian and African strains [142, 146]. Given the crucially supportive roles of astrocytes in synaptogenesis and central nervous system homeostasis, infection of these cells may have multifaceted and detrimental outcomes on brain development. Thus, we asked whether infection of astrocytes produces transcriptional signatures that may promote disease, and we further explored whether these effects occur in a lineage dependent manner.

Materials and Methods

Primary astrocyte culture and infection. Brain tissue was collected from infant rhesus macaques of Indian origin that were being culled from the breeding colony due to chronic diarrhea or injury. The animals were not infected with ZIKV. The procedure used for euthanasia was consistent with the recommendations of the American Veterinary Medical Association's Panel on Euthanasia. Cortical tissue was collected into Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) and immediately processed as follows: Tissue was mechanically dissociated and treated with 0.25% trypsin and 0.1% DNase for 1 hour at 37°C with agitation every 10 minutes. After centrifugation at 1,600 rpm for 5 minutes, cells were resuspended in DMEM/F-12 and passed through a 40 µm filter. Cells were centrifuged at 1,600 rpm for 5 minutes, resuspended in DMEM/F-12 supplemented with 1X Antibiotic-Antimycotic and 0.5 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), and cultured at 37°C and 5% CO₂. After 21 days, the
cultures consisted almost entirely of astrocytes with minimal microglia present. Astrocyte cultures were infected with either Rio-U1 (KU926309), an Asian-lineage isolate from Rio de Janeiro that was minimally passaged in Vero cells (ATCC CCL-81), or MR766 (KU963573; obtained through BEI Resources, NIAID, NIH, as part of the WRCEVA program), the prototypical 1947 African-lineage strain that has been passaged extensively in mouse brain. Separate astrocyte cultures were left uninfected as a control. Viral stocks were quantified using an in-house qRT-PCR assay described previously [50], and infections were carried out at by adding 3.2*10^9 copies of Rio-U1 or 9.4*10^8 copies of MR766 to each T75 flask for 24 hours at 37°C and 5% CO2.

RNA isolation and sequencing. Among three biological replicates and three conditions each (uninfected or infected with Rio-U1 or MR766), astrocyte cultures were lysed, and total RNA was extracted using the Qiagen RNeasy Mini Kit. Poly-A mRNA enrichment, library preparation and paired-end mRNA sequencing (RNAseq) were carried out by BGI on a BGISeq instrument.

Bioinformatic analysis. Paired-end RNAseq reads were analyzed using an in-house pipeline. Briefly, reads were deduplicated using the BBMap package clumpify and pseudoaligned to the rhesus macaque genome (Ensembl Mmul_10 assembly) using kallisto [147]. DESeq2 [103] was used with default parameters to calculate differentially expressed genes (DEGs) with a multiple testing adjusted p-value less than 0.05 in astrocytes infected with either virus relative to uninfected controls, and volcano plots were generated using the EnhancedVolcano Bioconductor package. ReactomePA [148] was used for exploratory pathway analysis of DEGs, and Gene Set Enrichment Analysis (GSEA, [104]) was also utilized using a gene set permutation of 1000 to evaluate the enrichment of gene sets in the
hallmarks, Reactome, and Gene Ontology (GO) collections. Heatmaps were generated on log2-transformed normalized read counts using the pheatmap package in R.

Lineage-dependent impacts on host gene expression

Relative to uninfected controls, astrocytes infected with MR766 showed a greater degree of differential gene expression compared to astrocytes infected with Rio-U1 (Fig. 12). Infection by either virus resulted in the upregulation of genes relating to the interferon (IFN) response, and among the most highly upregulated IFN stimulated genes (ISGs), many appeared to be responsive to either type-I IFN or both type-I and type-II IFN (Fig. 12a-b) [149]. Although Rio-U1 infected astrocytes showed induction of a few cytokine and interleukin related genes (CXCL10, CCL8, IL6), chemokine signaling was generally more highly represented in cells infected with MR766 (Fig. 12a). MR766 also resulted in the modulation of genes relating to lipid movement (APOL2, APOL6, FABP4, ABCA8) as well as lipid metabolism (HELZ2, PLAAT4, PDK4) and protein metabolism (LY6K) (Fig. 12a). Interestingly, HELZ2 is a known restriction factor of the related flavivirus DENV that may mediate antiviral effects by modulating lipid metabolism [150]. Rio-U1 infection also affected genes relating to lipid metabolism (PLIN2, ANGPTL4) and genes encoding structural proteins (MYH2, ACTG2) (Fig. 12b), as well as TGFBI, possibly pointing to an immunomodulatory function.
Figure 12: Lineage dependent impacts on host gene expression

(A-B) Volcano plots showing differentially expressed genes (DEGs) identified by RNA-seq analysis in astrocytes infected with MR766 (A), or Rio-U1 (B). (C) Venn diagram depicting overlapping and unique DEGs in astrocytes infected with either virus. (D) Bar graph showing the fold change values of DEGs similarly affected by either virus.

Direct comparison of differentially expressed genes (DEGs) showed a greater number of genes affected by MR766 infection (134 in MR766 compared to 38 in Rio-U1), however, 17 genes were similarly modulated by both viruses (Fig. 12c). Interestingly, genes that were differentially expressed by both viruses were regulated in the same
direction and to a similar magnitude (Fig. 12d). Some of the genes that were similarly modulated by MR766 and Rio-U1 related to cytokine signaling (CXCL10, CCL8) and the IFN response (ISG15, OAS2, DDX58, IFIT1, MX1, IIF44) (Fig. 12d), suggesting that antiviral signaling was not exclusive to astrocytes infected with MR766.

*Overlapping but distinct transcriptional signatures*

Exploratory pathway analysis of DEGs was carried out using ReactomePA software, which revealed an orchestrated IFN response in MR766 infected astrocytes (Fig. 13a). The most heavily induced pathways included IFN functions with a high degree of overlap, seen in the upregulation of several shared IFN effector genes (Fig. 13b). MR766 infection also appeared to promote gene expression patterns consistent with cytokine signaling, together with pathways relating to phagosome-mediated protein degradation, antigen presentation, and cell death (Fig. 13a).

Meanwhile, the limited number of DEGs in Rio-U1 infected astrocytes resulted in a less integrated gene expression signature. Although IFN signaling pathways were also detected (Fig. 13c), they shared a limited degree of overlap (Fig. 13d). Instead, Rio-U1 appeared to promote a separate set of functions relating to the maintenance of structural proteins, including collagen biosynthesis and tubulin folding (Fig. 13c-d). There was also evidence that Rio-U1 infection affected the expression of molecules with important roles in the maintenance of homeostasis, including metal ion transporters, connexon molecules, and the extracellular matrix (Fig. 13c).
Figure 13: Lineage dependent virus response patterns

Reactome pathway analysis of DEGs in MR766 (A-B) and Rio-U1 (C-D) infected astrocytes. Enrichment maps (A & C) show interrelatedness of top ranking Reactome pathways, and network maps (B & D) show overlap of the top enriched pathways at the gene level.
We next turned to gene set enrichment analysis (GSEA) to discern nuances in gene expression and avoid the exclusion of genes based on an arbitrary p-value cutoff. GSEA largely confirmed patterns previously observed in DEGs alone, but this more inclusive analysis additionally detected subtle patterns of gene expression that may discriminate infection caused by either virus. MR766 and Rio-U1 again showed evidence of inducing an antiviral response, with enrichment of gene sets relating to IFN signaling and inflammation (Figs. 14a & S8a-b). Although antiviral responses were detected in astrocytes

![Figure 14: Overlapping but distinct transcriptional signatures](image)

(A) Bar graph showing gene sets in the Hallmarks collection that were significantly enriched at FDR<0.1 (NES, normalized enrichment score). Gene sets were either similarly enriched in both viruses (top) or enriched only in MR766 (middle, yellow) or Rio-U1 (bottom, blue) infected cells. (B-I) Boxplots showing normalized read counts of representative genes in MR766 (yellow) or Rio-U1 (blue) infected cells and uninfected controls (white) relating to the IFN response (B-C), inflammation (D-E), hypoxia signaling (F), the unfolded protein response (G), cell cycle arrest (H), and immunomodulation (I).
infected with either virus, the magnitude of these responses was greater in response to the African lineage virus, represented by the higher enrichment of IFN related gene sets and activation of additional gene sets relating to acute inflammation (Figs. 14a & S8a-b), together with the induction of key IFN responsive genes including ISG15 and MX1 and the chemokines CXCL10 and TNF (Fig. 14b-e). Infection by MR766 resulted in apoptosis signaling (Fig. 14a) due to the upregulation of several caspases including CASP4 (Fig. S8c). Both viruses also upregulated AXL, a putative entry receptor for ZIKV (Fig. S8d).

Infection by either virus promoted cell stress pathways consistent with low oxygen availability, including hypoxia, the unfolded protein response (UPR), mTOR signaling, and cell cycle arrest (Figs. 14a & S8a). HIF1α, which is a key mediator of transcriptional signaling in hypoxic conditions [151], was upregulated by infection with either virus (Fig. 14f), as were HSP90B1 and DBF4 (Fig. 14g-h), which are key mediators of the unfolded protein response [152] and cell cycle arrest [153], respectively. Rio-U1 infection induced the immunomodulatory cytokine TGFβ (Fig. 14i) together with gene sets relating to cell respiration and lipid metabolism (Figs. 14a, S8a, & S9a-c). Interestingly, the Asian lineage virus appeared to promote glycolysis and downregulate lipid catabolism, metabolic signatures in line with a hypoxic response [151].

Gene-level analysis of biological functions identified by GSEA revealed highly ranking genes responsible for core enrichment. Inflammation and IFNγ signaling, which were among the most highly enriched gene sets in MR766 infected cells, were driven by increases in the expression of chemokines (CXCL10, CXCL9, IL6, CCL2) and their receptors (OSMR, IL15RA), together with molecules involved in cell adhesion (ICAM1, SELL), pattern recognition (TLR2, MYD88) and IFN cascades (IRF7/9, IFIT1/2, STAT1)
Hierarchical clustering revealed a lineage-dependent effect on gene expression, where MR766 and Rio-U1 infected astrocytes each produced signatures differentiating them from uninfected cells, but the most drastic gene induction was seen in MR766. GSEA identified the activation of hypoxia by both viruses, which was driven by an increase in the expression of transcription factors (ATF3, ETS1) and genes relating to intracellular homeostasis (HK2, MT2A, SLC2A3) (Fig. 15c). Both viruses also appeared to have an effect on cell proliferation, with the induction of genes relating to cell cycle checkpoints such as proteasome subunits (PSMB8, PSMA2, PSMB9), DNA damage
sensors (NBN, HUS1, EXO1), and microtubule/kinetochore regulators (GTSE1, CENPI) (Fig. 15d). Although GSEA identified the activation of glycolysis only in Rio-U1 infected astrocytes, gene-level analysis revealed a similar signature in MR766 infected cells, with induction of glycolytic enzymes such as PGK1, HK2, and LDHA (Fig. S10a). MR766 and Rio-U1 also produced similar signatures in the expression of genes relating to UPR (Fig. S10b) and mTor signaling (Fig. S10c).

Discussion

Astrocytes are an early and important target of ZIKV infection in the developing brain [145], but the full impacts of infection on cellular processes remain unclear. Conflicting reports argue that ZIKV infection either increases [154], or decreases [142, 155] the production of proinflammatory cytokines by astrocytes, and phylogenetic differences among ZIKV strains used in these experiments further complicate analysis [156-158]. While modern Asian-lineage strains are primarily linked to neurologic diseases, studies in cell culture and in animal models have more commonly found that infection by African-lineage ZIKV results in greater infectivity, virus production, and antiviral responses (reviewed in [137]). Thus, we asked whether transcriptomic analysis of primary astrocytes from a highly relevant animal model would elucidate lineage-dependent effects of ZIKV infection.

Previous reports in human neural cells have suggested that African lineage ZIKV produces stronger proinflammatory responses compared to Asian lineage viruses, a finding that is consistent at the protein and RNA levels [137, 154, 159]. Additionally, transcriptomic analysis of primary human astrocytes has outlined the crucial role of the IFN response in restricting ZIKV infection [155]. In line with prior findings, IFN signaling
was the most highly induced function by either strain included in the present study, although the magnitude of the IFN response was greater in astrocytes infected with the African lineage virus. IFN functions are indispensable in controlling acute infection, as evidenced by the lethality of ZIKV infection in mice when IFN signaling is impaired [16], so it was not surprising to observe induction of IFN by either virus. Differences in infectivity among the strains might have impacted the corresponding magnitude of antiviral signaling, although the present study did not address infectivity directly. MR766 is neurologically adapted owing to its extensive passage history in mouse brain [160], perhaps making this virus uniquely predisposed to neurotropic and transcriptional outcomes. Nonetheless, similar experiments using minimally passaged African isolates maintain that African viruses have greater infectivity and antiviral signaling in astrocytes [159], a finding which has been replicated in multiple models [137]. It is possible that a lower infectivity of Asian lineage strains might permit these viruses to avoid immune detection and persist in neural tissues where they have damaging effects, while overt infection by African lineage viruses might more readily promote immune cell infiltration and viral clearance.

Infection by either lineage also appeared to induce hypoxia and cell stress, which was driven in part by HIF1α, an oxygen sensitive transcription factor that is known to be activated in a number of viral infections [161]. Oxygen stress enhances the infectivity and replication of several important human RNA viruses such as human immunodeficiency virus, hepatitis B and C, and the related flavivirus DENV [162-164], so a similar mechanism may exist in ZIKV infection. UPR and mTor signaling, other important hypoxia response pathways [151], were among the most highly enriched gene sets by infection with either virus, and metabolic pathways also underwent reprogramming in a
manner consistent with low oxygen availability. Hypoxia has been shown to contribute to the development of neurologic lesions in infant NHPs infected with ZIKV [22], so astrocytes may be an important target cell where ZIKV induced oxygen stress leads to detrimental outcomes. However, it is worth noting that HIF1α is also strongly activated during general inflammation [165], so further metabolomic analysis should address whether hypoxia signaling is collaterally activated due to an innate immune response.

In addition to its role in hypoxia signaling, UPR is a common feature of ZIKV infection given the massive remodeling of the endoplasmic reticulum (ER) to form lipid complexes that support viral replication. Although considered a homeostatic mechanism to relieve ER stress, UPR can also halt cell replication and promote apoptosis in cases of prolonged ER stress, pathways occurring in ZIKV infected neural cells that are thought to contribute to microcephaly on the gross scale [166]. Indeed, transcriptome analysis of human astrocytes found that ZIKV modulates microRNA expression to promote UPR and support viral replication [167]. Our findings of UPR and cell cycle arrest in astrocytes infected with either MR766 or Rio-U1 are consistent with viral replication induced cell stress that might represent an important pathophysiologic manifestation \textit{in vivo}.

Despite the similarities among MR766 and Rio-U1 in promoting IFN signaling and cell stress, infection by the Brazilian isolate alone perturbed metabolic pathways regulating lipid catabolism and cell respiration, autophagy related functions that are known targets of flavivirus infection [132, 168]. Autophagy generally functions to induce innate and adaptive immune responses to microbial infections [168], and flaviviruses such as DENV and ZIKV interact directly with autophagy machinery to enhance their infectivity [132, 168] by manipulating lipid content [169]. In our analysis, this mechanism appeared to be
more effectively utilized by the Brazilian ZIKV isolate. However, it is worth noting that autophagy and lipid metabolism are also affected by mTor signaling and hypoxic stress [151, 168], pathways also upregulated in the present analysis, highlighting the interrelatedness of these pathways and supporting a model where Asian lineage ZIKV infection causes cell stress and generalized metabolic reprogramming in astrocytes.

An important contribution of the present study was the finding that previously identified transcriptional signatures in ZIKV infection are consistent in primary astrocytes from infant macaques. For example, African lineage ZIKV is known to induce stronger antiviral responses [137], and altered lipid metabolism through autophagy is a common feature of flavivirus infection [169]. The extension of these findings in an important cell type from a relevant animal model affirms the broader relevance of these signaling patterns. The NHP model faithfully captures aspects of ZIKV infection in humans, such as maternal-to-fetal transmission and neurologic diseases [21, 96, 170], and circumstantial evidence in infant macaques has found astrogliosis together with brain lesions following ZIKV infection [170]. But to our knowledge, the direct impacts of ZIKV infection on macaque astrocytes, which is an important target cell in other animal models, remain unknown. Here, we show that macaque astrocytes respond to ZIKV infection through gene expression patterns that are partly dependent on the lineage of the infecting strain. Studies directly comparing Asian and African lineage ZIKV infection in NHPs have been limited to date; one such report found that animals challenged with an African lineage virus had an almost undetectable serum viral load compared to animals infected with Asian lineage strains [171]. Despite the scarcity of comparative analyses in the NHP model, our findings are consistent with some of the lineage-dependent transcriptional signaling patterns observed
in mice and in cell culture models [137], suggesting that phylogenetic differences among viruses ultimately skew transcriptional outcomes.

Although the transcriptional signatures in our *ex vivo* astrocyte model complement patterns observed in other models, a couple of limitations in the present study warrant further investigation. First, our analysis does not address the impact of infection kinetics on transcriptional responses. A previous report found that ZIKV strains affect antiviral gene expression in a time dependent manner, with infection by an African lineage virus having a more potent response at 24 hours post infection and an Asian lineage strain dominating prior to this timepoint [146]. Since we assessed gene expression signatures only at 24 hours, it remains possible that a more varied temporal analysis might have revealed additional nuances among MR766 and Rio-U1. Also, while the present analysis uncovered transcriptional responses in a single glial cell population, the milieu of other infectable cell types in the brain complicates the extrapolation of these findings to signaling patterns that might occur *in vivo*. We focused on astrocytes given that astrocytes are important cellular targets of ZIKV in the developing brain, but other cell types in the brain are also permissive to infection, so further analyses should address the impacts of ZIKV infection in mixed neural cell cultures.

In conclusion, we found that ZIKV infection of primary astrocytes results in common antiviral gene expression patterns, although the strain of infecting virus appears to impact the magnitude of these responses and skew metabolic processes in a way that may ultimately influence disease outcomes.
Supplementary Figure 8: Enriched gene sets

(A-B) Bar graphs showing gene sets in the Reactome (A) and Gene Ontology (GO) (B) collections that were significantly enriched at FDR<0.1. The top 10 gene sets that were either similarly enriched in both viruses (top) or enriched only in MR766 (middle, yellow) or Rio-U1 (bottom, blue) infected cells are displayed. (C-D) Boxplots showing normalized read counts for CASP4 (C) and AXL (D) in MR766 (yellow) or Rio-U1 (blue) infected cells and uninfected controls (white).
Supplementary Figure 9: Downregulated gene sets

(A-C) Bar graphs showing gene sets in the Hallmarks (A), Reactome (B), and Gene Ontology (GO) collections (C) that showed negative enrichment at FDR<0.25. Gene sets that were enriched in both viruses (top) or enriched only in MR766 (middle, yellow) or Rio-U1 (bottom, blue) infected cells are displayed.
Supplementary Figure 10: Gene-level analysis of auxiliary biological functions

(A-C) Heatmaps showing read counts of genes involved in glycolysis (A), UPR (B), and mTor signaling (C). Log2-transformed read counts for the 20 most highly ranking genes in each of the indicated sets from GSEA are displayed.
CHAPTER 5: IMMUNE DRIVEN ESCAPE MUTATIONS IN SIMIAN IMMUNODEFICIENCY VIRUS

Overview

Nef-specific CD8+ T lymphocytes (CD8TL) are linked to extraordinary control of primate lentiviral replication, but the mechanisms underlying their efficacy remain largely unknown. The immunodominant, Mamu-B*017:01+-restricted epitope Nef195-203MW9 in simian immunodeficiency virus (SIV)mac239 partially overlaps a sorting motif important for interactions with host adapter protein 2 (AP-2) and, hence, downmodulation of several host proteins, including tetherin (CD317/BST-2), CD28, CD4, SERINC3, and SERINC5. We reasoned that CD8TL-driven evolution in this epitope might compromise Nef’s ability to modulate these important molecules. Here, we used deep sequencing of SIV from nine B*017:01+ macaques throughout infection with SIVmac239 to characterize the patterns of viral escape in this epitope and then assayed the impacts of variants on Nef-mediated modulation of multiple host molecules. Acute variation in multiple Nef195-203MW9 residues compromised the downregulation of surface tetherin, CD4, and CD28 by Nef and also reduced antagonism of SERINC5 but did not impact downregulation of CD3 or major histocompatibility complex class I (MHC-I), implying the selective disruption of Nef functions that depend on AP-2 interactions. We also found that one such variant that negatively impacted Nef functions, H196Q, was present with surprising frequency in published SIVmac251 stocks. However, an accompanying upstream mutation, E191R, rescued key Nef functions, implying a compensatory role. Together, our
data illuminate a pattern of viral escape dictated by a selective balance to maintain AP-2-mediated downregulation while evading epitope-specific CD8TL responses. These findings may shed light on mechanisms of both CD8TL-driven viral control generally and on Mamu-B*017:01-mediated viral control specifically.

Introduction

Virus-specific CD8TL play a critical role in establishing the set point of HIV-1 and SIV replication in vivo, and there are strong correlations between expression of particular MHC-I alleles and extraordinarily low or high viral set points [7], further implicating CD8TL as causative agents in viral control. However, the control of HIV and SIV replication by antiviral CD8TL is complicated by the immense capacity of the viruses to evolve to escape these immune pressures [8]. In fact, CD8TL are likely dominant selective forces driving viral sequence variation in individuals and on population scales during infection with HIV-1 or SIV [172, 173], but escape from CD8TL may ultimately exact a cost to viral fitness [174]. The balance among these variables—the efficacy of antiviral CD8TL, the propensity of the virus to evolve to evade them, and the specific fitness costs associated with particular escape mutations—is likely critically important in determining the viral set point in a given individual, so understanding immune-mediated control of HIV/SIV replication necessitates understanding all components of this process.

Nef represents an intriguing and potentially important target for CD8TL. In both SIV and HIV-1, Nef is a pleiotropic protein whose myriad functions are focused on immunomodulation, including the downregulation of several cell surface proteins that are involved in host immunity, such as TCR-CD3 (in most SIVs but not HIV-1), CD4, CD8αβ, CD28, tetherin (in most SIVs but not HIV-1), MHC-I, MHC-II, CD1d, CD80/CD86, and
likely others, as well as enhancing viral infectivity by preventing virion incorporation of host serine incorporator 3 (SERINC3) and SERINC5 proteins [175]. Importantly, modulation of several of these molecules, including CD4, CD28, tetherin, and SERINC3 and SERINC5 is effected via interactions between Nef and AP-2 complexes [176].

The Nef protein in both SIV and HIV-1 is highly immunogenic and harbors epitopes restricted by MHC-I alleles associated with control in both rhesus macaques (RM) and humans [177, 178]. Interestingly, one of two dominant Mamu-B*017:01-restricted epitopes in Nef, Nef195-203MW9, spans the region between two characterized sorting motifs, the first of which is a “dileucine” motif (ExxxLM in SIVmac viruses) known to be important for modulation of host molecules [176], likely via direct interactions with host AP-2 proteins, and the other is a diacidic motif which represents a novel AP-2 binding motif in HIV-1 Nef [179]. Given the importance of Nef-mediated immunomodulation to viral replication and pathogenesis in vivo, we asked whether viral escape in this epitope compromised Nef's ability to downregulate host molecules that rely on interactions between Nef and host AP-2. We were also intrigued to find that one such damaging mutation, H196Q, was prevalent in the sequences of many published SIV stocks, so we explored whether an associated mutation, E191R, rescued key functions of Nef.

Materials and Methods

Viral RNA isolation, sequencing, and analysis. Viral RNA (vRNA) was isolated as described previously [180] using the QIAamp viral RNA minikit (Qiagen, Valencia, CA) or the QIAamp UltraSens virus kit for low viral load samples (specifically from week 60 from RM r03007). We reverse transcribed and PCR amplified SIV vRNA from select time points as described previously [181] using Superscript III one-step RT-PCR (Life
Technologies) and four overlapping PCR amplicons that, together, span the genome. RT-PCR-amplified products were isolated using agarose gel electrophoresis, followed by purification of fragments using a Qiagen MinElute gel extraction kit. Fragments were quantified using Qubit reagents (Life Technologies, Carlsbad, CA). All amplicons from a single viral genome were pooled in equimolar amounts. Approximately 1 ng of DNA was subjected to tagmentation (simultaneous fragmentation and adaptor ligation) using the Nextera XT DNA prep kit. After cleaning of the DNA using an Agencourt AMPure system, the samples were PCR amplified to add Illumina-compatible adaptors onto each fragment, followed by further cleanup. DNA fragments were then sequenced by using an Illumina MiSeq instrument. Data analysis was performed using Geneious software version 10.0.2 (created by Biomatters). After pairing bidirectional sequence reads, sequence reads were trimmed and mapped to the SIVmac239 genome. Nonsynonymous polymorphisms were pursued in subsequent assays if they were represented multiple times and at a minimum frequency of 1% of sequences at a given nucleotide. We used this cutoff previously with this same sequence data set [180].

Site-directed mutagenesis and mutant virus and plasmid production. Mutations identified via deep sequencing were engineered into a plasmid encoding the 3′ end of the SIVmac239 genome, as well as the pCGCG-Nef plasmid, as described previously [182] using a Stratagene QuikChange site directed mutagenesis kit (Agilent, Santa Clara, CA) and according to the manufacturer’s protocol with primers that harbored the mutant residue near the center of the primer. Virus was produced by transfecting plasmids into Vero cells using Lipofectamine 2000 (Life Technologies). Twenty-four hours after transfection, Vero cells were overlaid with CEMx174 cells, and virus was harvested at the peak of syncytium
formation (typically 10 to 14 days post transfection). The viruses were then sequenced to verify maintenance of the mutations through production. At 24 hours after complete medium replacement, virus stocks were obtained by freezing filtered culture media from the infected CEMx174 cells at −80°C for short-term storage and in vapor-phase liquid nitrogen for longer-term storage. Introduced mutations were verified by Sanger sequencing of both the plasmid DNA and the harvested viral stocks prior to use in assays.

TZM-bl CD4 downregulation assay. TZM-bl cells were obtained from the NIH AIDS Reagent Program. TZM-bl cells were cultured in Dulbecco’s modified Eagle medium with 10% fetal bovine serum (FBS). For the CD4 downregulation assays, cells were plated in 48-well plates and transfected with 1 μg of each pCGCG vector using the GenJet transfection reagent for HeLa cells (SignaGen). At 48 hours after transfection, cells were removed from the plate using trypsin and labeled with a BV605-labeled anti-CD4 antibody (clone OCT-4; BioLegend) for 30 min. The cells were then washed once using phosphate-buffered saline supplemented with 10% FBS and fixed. Data were acquired on a BD LSRII instrument and analyzed using FlowJo software (vX.07). Preliminary analysis showed that Nef and green fluorescent protein (GFP) were expressed in a 1:1 ratio, so gating on GFP-positive cells served as a surrogate for Nef expression. The median fluorescence intensity (MFI) values for CD4 expression were compared among GFP-positive and -negative cells within each sample. Data were analyzed using GraphPad Prism and are displayed as the n-fold downregulation relative to untransfected (GFP-negative) cells. Downregulation was compared between each mutant pCGCG Nef vector and wild-type pCGCG Nef using a two-tailed t-test, and the differences were considered significant at p<0.05. For rescue mutation experiments, a similar strategy was used, except the Sup-
T1 cell line was selected for transfection due to the stable surface expression of rhesus macaque CD4. Sup-T1 cells were transfected with Nef variants (wildtype, H196Q, and H196Q/E191R) using the Amaxa 4D-Nucleofector Protocol with the CA-137 program and SF cell line reagent in 20 µl Nucleocuvette strips. Briefly, 2*10^5 cells were transfected with 1 µg plasmid DNA for each reaction. Average transfection efficiencies were as follows: 33.7% for wildtype (n=3), 33.5% for H196Q (n=6), and 36.9% for H196Q / E191R (n=6) compared to 4.3% for the untransfected control (n=3). N-fold downmodulation was calculated as described above.

**CD4+ T cell isolation, infection, and functional assays.** Primary PBMC were harvested from SIV-naive Indian rhesus macaques from the Tulane National Primate Research Center's specific-pathogen-free breeding colony. CD4+ T cells were isolated from PBMC using nonhuman primate CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4+ T cells were then activated using ConA (Sigma, St. Louis, MO) for 3 days in RPMI 1640 medium (Cellgro; Corning, Corning, NY) containing 15% FCS (Atlas Biologicals, Fort Collins, CO) and 50 U/ml IL-2 (Peprotech, Rocky Hill, NJ). Activated cells were infected using the spinoculation technique [183] with virus purified through 20% sucrose as described previously [184]. At 36 h after infection, the cells were surface labeled for the following molecules: CD4 (Qdot605, NHP Reagent Reference Program, [www.nhpreagents.org](http://www.nhpreagents.org/); BV421, BD Biosciences, Franklin Lakes, NJ), CD3 (PE-Cy7; BD Biosciences), tetherin (PE; BioLegend, San Diego, CA), and MHC-I (W6/32, Alexa 647; BioLegend). After fixation, the cells were washed and permeabilized, followed by intracellular staining with an FITC-labeled antibody against the Gag p27 molecule (NIH
AIDS Reagent Program and conjugated with FITC at the Wisconsin National Primate Research Center).

**Sequence analysis and alignments.** Nef sequences from a broad array of SIV isolates were identified from a published report [185] and downloaded from NCBI for amino acid alignments using Geneious Prime 2019.1.3 using the built-in Geneious Alignment algorithm with default settings. SIVmac251 sequences available from published reports [186, 187] were downloaded from NCBI into Geneious Prime 2019.1.3 and mapped to SIVmac239, used as the reference genome, followed by identification and quantification of variations using the Find Variations/SNPs function. Sequences published in the Lamers et al report [187], were first divided into those extracted from the inoculum and from individual tissues, which were analyzed separately.

**Structural analysis.** Structures showing interactions between SIVsm Nef, tetherin, and AP-2 subunits were recently published [188]. We used UCSF Chimera [189] and PISA (Proteins, Interfaces, Structures, and Assemblies) softwares [190] to examine how variations in Nef residues at positions 191 and 196 affected potential interactions with host AP-2 and tetherin proteins.

*Sequence variation in Nef195-203MW9 throughout infection*

As described previously [191], we detected CD8TL responses in all 6 acutely infected RM included in the present study. The responses were Nef195-203MW9-specific and generally increased between 4 and 8 weeks post-infection. To evaluate viral sequence evolution arising from Nef195-203MW9-directed CD8TL pressure, we used deep sequencing of plasma virus at multiple time points during infection. Nonsynonymous variation at ≥1% was found at every residue in the Nef195-203MW9 epitope (Fig. 16a),
while nonsynonymous variation above 0.5% was never detected in nucleotides encoding highly conserved amino acids near the Nef195-203MW9 epitope, including in the diacidic

![Image of viral evolution in the Nef195-203MW9 epitope]

**Figure 16: Viral evolution in the Nef195-203MW9 epitope**

(A) Heat map generated from virus deep sequencing data showing the frequency of nonsynonymous variation at each residue in the epitope at each sampled timepoint. Only nonsynonymous mutations that represented more than once and ≥1% of nucleotides at each location are shown. (B) Pie charts showing the frequency of specific amino acid substitutions at each residue in the epitope from each sample are shown. The identity of the amino acids is shown by the pie slices below each residue.
motif. We identified a limited number of frequently occurring variants within the Nef195-203MW9 epitope (Fig. 16b), such as at M195 (p1 of the epitope), which nearly always showed a change from methionine (M) to isoleucine (I) or valine (V) and frequently was a mixture of both mutants, along with residual wildtype virus. Similarly, nonsynonymous variation at H196 nearly always encoded an H196Q change. Variations at other positions in the epitope were limited; in both acutely infected and chronically infected cohorts, variants generally failed to become fixed in the viral population as late as 17 weeks (Fig. 16b, RM r02105) and 35 weeks post-infection (RM rh1937), respectively, although RM rh1937 showed nearly complete fixation of the M195I mutant at 167 weeks post-infection.

Variants impact cell surface tetherin, CD28, and CD4 expression

To identify the functional consequences of escape in the Nef195-203MW9 epitope, we introduced the most common Nef195-203MW9 variants identified from our cohorts into both the SIVmac239-encoding plasmid, which was used to produce infectious SIV stocks bearing the introduced mutations, as well as in an expression construct (pCGCG) that expresses both Nef and GFP from the same bicistronic mRNA. Whenever possible, we measured the Nef-mediated modulation of specific host proteins in primary CD4 T cells infected in culture with SIVmac239 and our Nef variants. However, SIV also modulates surface CD4 expression even in the absence of Nef, possibly due to interactions between Env and CD4 at the cell surface (Fig. 18), so to assess the impacts of Nef195-203MW9 variants on CD4 expression, we used an alternate strategy where we introduced the mutations into a pCGCG Nef expression plasmid to measure CD4 expression in transfected cells.
Using differential gating of p27- and p27+ cells to identify uninfected and infected primary CD4 T cells (Fig. 17a), we found that several Nef195-203MW9 variants impacted CD28 and tetherin expression. While neither variant detected at M195 (M195I and

Figure 17: Specific variants of Nef195-203MW9 compromise tetherin and CD28 modulation but not CD3 or MHC-I

(A) Schematic showing how the downregulation of surface molecules was detected. (B) N-fold decrease in the MFI values of surface tetherin expression in Gag p27+ cells relative to Gag p27− (uninfected) cells in the same samples. Cells from at least four RM were infected with each virus. (C-E) Same as in panel B but surface CD28 (C), CD3 (D), and
MHC-I (E) levels were measured. Above each bar graph is a representative histogram showing the strategy for assessing surface downregulation of each molecule. For each tested molecule, the N-fold downregulation was compared between each variant and wild-type SIVmac239 using a paired t-test (*, p < 0.05).

M195V) had a significant impact on downregulation of any of the tested molecules, the variants tested at all other residues in the epitope (H196Q, P197T, A198D, Q199R, T200A, S201A, Q202K, and W203C) reduced the downregulation of tetherin by Nef (Fig. 17b), and most also negatively impacted CD28 downregulation (Fig. 17c). Importantly, the variant with the greatest impact on Nef-mediated tetherin and CD28 downregulation was H196Q. H196 is the N-terminal anchor residue, critical for binding of the peptide to the Mamu-B*017:01 molecule, while variation of the C-terminal anchor residue (W203C) also reduced both Nef-mediated tetherin and CD28 downregulatory capacity. We found that mutations in this epitope had no effect on AP-2 independent functions including downregulation of CD3 or MHC-I (Fig. 17d-e).
Figure 18: Impact of mutations in the Nef195-203MW9 epitope on surface CD4 downregulation

(A) Surface CD4 expression in primary CD4 T cells infected with SIVmac239 or SIVmac239Δnef. (B) Surface CD4 expression in TZM-bl cells (CD4 expressing HeLa cells) transfected with pCGCG vectors that express wild-type SIV Nef or the indicated mutants. (C) N-fold decreases in surface CD4 expression in GFP+ cells relative to GFP− cells. N-fold values were compared between each variant and wild-type SIV Nef. *, p < 0.05 for each comparison tested using a two-tailed t-test. The G2A variant of Nef, which lacks a myristoylation signal and is defective for most Nef activities, was included as a negative control in these experiments.

To detect the impacts of the variants on CD4 downregulation, we transfected TZM-bl cells (CD4-expressing HeLa cells) with the pCGCG Nef vectors. Forty-eight hours later, we labeled the cells for surface CD4 and analyzed expression in Nef-expressing cells, identified by the coexpression of GFP in transfected cells (Fig. 18b). H196Q had the most significant impact on CD4 downregulation (Fig. 18c), and the pattern of variants that impacted CD4 downregulation generally mirrored that of variants that impacted CD28 downregulation, with the exception of P197T, which impacted CD28 but not CD4 downregulation.

A compensatory mutation restores key Nef functions that are lost in Nef195-203MW9 variants

We next scanned publicly available sequences from a recent study that used single genome amplification to extensively examine SIVmac251 challenge stocks [186]. In this
report, a Q196 residue was detected in a large fraction of sequences from SIVmac251 stock viruses from several labs. Interestingly, there was a perfect linkage between the Q196 residue and an upstream R191 residue, which is E191 in SIVmac239 (Fig. 19a). Of 38 total sequences that contained the region of interest, derived from three different challenge stocks, 25 sequences contained both R191 and Q196 while Q196 was never found in the absence of R191. Other nearby variants relative to SIVmac239 were detected, but only R191 co-occurred with Q196 in all sequences.

Figure 19: A compensatory mutation restores Nef functions

(A) SIVmac251 stock sequences from a published study [186] show a variant amino acid upstream of Q196 (R191) that was always associated with Q196. Alignments were performed using Geneious Prime 2019.1.3. (B-C) Representative flow cytometric analysis of surface expression of tetherin on primary CD4 T cells infected with SIVmac239 variants
(B) or CD4 on Sup-T1 cells transfected with pCGCG Nef variants (C). Nef was either wildtype or contained the H196Q mutation alone or in combination with the E191R variant. Cells were identified as infected by intracellular Gag p27 staining (B) or transfected by GFP positivity (C) as described previously [180, 191]. Surface expression of tetherin or CD4 between infected/transfected cells and uninfected/untransfected cells was used to calculate N-fold downregulation, which was then analyzing using a two-tailed t-test.

Given the strong linkage between the R191 (E191 in SIVmac239) variant and Q196, we tested whether R191 would rescue AP-2 dependent functions, such as tetherin and CD4 downregulation, in the presence of Q196. Introducing the E191R variant along with the H196Q either into the SIVmac239 backbone or into the pCGCG Nef expression plasmid, we infected primary CD4 T cells or transfected Sup-T1 cells (stably expressing rhesus CD4) to assess tetherin and CD4 downregulation, respectively. As expected, Nef harboring H196Q alone was largely deficient in tetherin and CD4 downregulation, but the introduction of E191R along with H196Q restored competency in tetherin and CD4 downregulation, similar to wildtype SIVmac239 (Fig. 19b-c). N-fold analysis of tetherin downregulation in cells from multiple animals demonstrated significant loss of downregulation in the virus harboring only H196Q, while the addition of E191R restored this ability to wildtype levels. Restoration of CD4 downregulation approached but did not reach statistical significance.

The structure of SIVsm Nef bound to simian AP-2 was recently published [188], allowing us to speculate on the spatial consequences of these variants. Using UCSF Chimera and PISA softwares, we found that the H196Q mutation is predicted to disrupt a
salt bridge between H196 and the tetherin residue D15, suggesting that disruption of a direct interaction between Nef and tetherin may contribute to the selective disadvantage of this change. Position 191 is a T in SIVsm, as opposed to E191 in SIVmac239. While this residue does not contact AP-2 (Fig. 20a), intriguingly, it does interact directly with the K18 residue in the DIWK motif of the tetherin protein itself via a hydrogen bond (Fig. 20b). Replacement of T191 with an E (as in SIVmac239) maintained the predicted hydrogen bond with K18, suggesting this interaction holds true between SIVmac239 and tetherin.

![Image of Nef, AP-2, and tetherin interactions](image.png)

**Figure 20: Structural insights into Nef, AP-2, and tetherin interactions**

(A) The flexible loop of Nef (containing Nef195-203MW9) in complex with tetherin and AP-2. H196 in Nef is positioned away from AP-2 and toward tetherin. (B) T191 in SIVsm interacts directly with K18 in the DIWK motif of tetherin via a hydrogen bond.

**Discussion**

We identified patterns of variation in the Nef195-203MW9 epitope suggesting that viral evolution in this epitope is a result of an interplay of selective forces to effectively escape CD8TL immunity while maintaining optimal modulation of host molecules. Nef195-203MW9 completely encompasses the region spanning the dileucine and the
diacidic motifs, both of which are sorting signals important for interaction with host adaptor proteins, which Nef leverages to facilitate clathrin-mediated endocytosis and modulation of multiple surface proteins [192]. Hence, immune targeting of the Nef195-203MW9 epitope might be important for viral control due to fitness costs associated with viral escape in this critical region of Nef. Interestingly, CD8TL targeting of nearly the same epitope in Mauritian cynomolgus macaques has been associated with control of SIV, further demonstrating the importance of this region of Nef [193].

Several mutations we identified in the Nef195-203MW9 epitope impaired Nef's modulation of tetherin, CD4, CD28, and SERINC5, while others only impacted modulation of a subset of these molecules, suggesting genetic separation of these Nef functions and that the loss of direct binding between Nef and AP-2 likely does not fully explain the compromised functions in this study. Thus, viral evolution in Nef195-203MW9 likely impacts direct interactions between Nef and AP-2 but may also impact other interactions as well. For instance, the P197T variant significantly impacted Nef's ability to modulate tetherin, CD28, and SERINC5 but did not impact CD4 downregulation. Several of the residues we identified as being important for tetherin and/or CD4 modulation were recently shown to impact the same molecules through the use of alanine substitutions [194], while our data demonstrated overlapping but not identical impacts. For instance, Serra-Moreno et al. reported that positions Q199 and T200 in Nef (p5 and p6 in the epitope) were important for tetherin but not CD4 downregulation [194], while our results show that these residues are important for both tetherin and CD4 downregulation. It is not entirely surprising that our approach of using mutants selected in vivo would have results that differed from those using alanine scanning, and these findings highlight the need to address
Nef functions with multiple approaches, particularly given the immunogenicity of Nef and its propensity to evolve to escape CD8TL responses.

It is intriguing that the M195 residue, in the first position of the epitope, was among the most variable residues and these changes appeared to have only a minimal impact on Nef functions. This residue forms part of what is typically characterized as a dileucine motif (ExxxLL in HIV-1; ExxxLM in SIVmac239) that interacts directly with host AP-2 proteins. M195I was the most commonly selected mutation in the epitope, but this variant ultimately did not provide complete escape from CD8TL [191], and the M195I and M195V variants also did not disrupt Nef functions that depend on interactions with AP-2, such as downmodulation of tetherin, CD28, and CD4, together with SERINC3/5 antagonism. M195I and M195V were the most dominant residues selected for in our cohort, underscoring the importance of achieving a balance to provide escape with a minimal loss of function. In contrast, the N-terminal anchor residue in the epitope, H196, was critical for all tested functions. This amino acid is conserved across nearly all sequenced primate lentiviral Nef proteins [185], suggesting strong stabilizing selection. Interestingly, the recently published three-dimensional structure of the flexible loop of SIV Nef [188] shows that H196 does not interact with host AP-2 directly but rather forms a salt bridge with tetherin, which was predicted to be disrupted in the H196Q variant. But the other AP-2 dependent Nef functions that were also ablated in H196Q suggests that disruption of a direct interaction with tetherin likely does not fully explain the functional deficits identified in this variant.

CD8TL that target specific regions of HIV-1 Gag are correlated with viral control, likely due to fitness costs associated with viral evolution in targeted epitopes, and many CD8TL escape mutations in Gag are associated with extraepitopic mutations that
compensate, or partially compensate, for the functional consequences of variation within the epitope [174]. Our data suggest that similar mechanisms could be important for CD8TL that target the viral Nef protein, which is inherently more variable than Gag and, as such, is often ignored as an important CD8TL target. We tested whether any of the variants we characterized were commonly associated with any specific variants outside the epitope. Since variation at every residue in the Nef195-203MW9 epitope negatively impacted Nef's ability to perform at least one of the tested AP-2 dependent functions, extraepitopic variants might compensate to restore or partially restore these functions. Indeed, we identified one such variant, E191R, which was genetically linked to H196Q and compensated for two key AP-2 dependent functions of Nef, affirming the importance of these functions and underscoring the virus’s dependence on the Nef195-203MW9 epitope in orchestrating these functions. Intriguingly, T191 in SIVsm interacts directly with the lysine in the DIWK motif (K18) in the tetherin protein [188], suggesting variation at this residue may impact tetherin downregulation via a direct effect on this interaction. E191 in SIVmac239 may also interact with K18 as these two amino acids are well known to form hydrogen bonds, although we cannot confirm without structural data.

Together, our findings describe the impacts of immune driven viral escape on viral fitness, which ultimately restricted viral evolution in SIV infected macaques. These data link the molecular immunology of MHC-associated lentiviral control with specific functions and motifs of the SIV Nef protein, suggesting that Nef-specific CD8TL can control viral replication by targeting epitopes in which viral escape is associated with significant loss of critical functions. Hence, particular regions of the Nef protein might be beneficial components of vaccines designed to induce potent CD8TL responses.


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BIOGRAPHY

Blake Schouest was born in Kenner, Louisiana on January 12, 1994, the son of Brent and Becky Schouest. He attended the Louisiana Scholars’ College at Northwestern State University beginning in 2012 and received Bachelor of Arts and Bachelor of Science degrees in 2016. He entered the Biomedical Sciences Training Program at Tulane University School of Medicine in August 2016 and was awarded the Doctor of Philosophy degree in May 2020. After graduation, he will begin a postdoctoral fellowship at La Jolla Institute for Immunology studying epitope specific T cell responses in viral infections.