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ZIKV NS3 disrupts Nrf2-mediated antioxidant defense and drives vascular smooth muscle cell damage and vascular dysfunction

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ABSTRACT

Zika virus (ZIKV) infection has been linked to vascular complications, yet the basis of vascular injury after apparent clearance of circulating virus remains poorly understood. This study examined the temporal progression of ZIKV-associated vascular damage and defined the contribution of non-structural protein 3 (NS3) to oxidative stress-mediated vascular dysfunction. ZIKV infection was modeled in interferon- α/β receptor-deficient A129 mice and immunocompetent northern pig-tailed macaques (*Macaca leonina*). Vascular pathology and function were evaluated using histopathology, transmission electron microscopy, immunofluorescence, and vascular reactivity assays, and primary murine vascular smooth muscle cells were used for mechanistic analyses. Transcriptomic profiling, antioxidant pathway assessment, and co-immunoprecipitation assays were performed to identify NS3-dependent molecular events. In A129 mice, ZIKV remained detectable in blood and induced progressive vascular injury. In contrast, in immunocompetent *M. leonina*, circulating viral RNA declined to undetectable levels by 15 days post-infection (dpi); however, viral RNA and envelope protein remained detectable in vascular tissues at 63 dpi, indicating sustained vascular viral retention after blood clearance. Across both models, ZIKV infection caused sustained endothelial injury, increased migration of vascular smooth muscle cells, and impaired vascular contractile responses. Mechanistically, NS3

interacted with nuclear factor erythroid 2-related factor 2 (Nrf2), restricted Nrf2 nuclear translocation, and disrupted the Nrf2/Kelch-like ECH-associated protein 1/glutathione peroxidase 4 (Nrf2/KEAP1/GPX4) antioxidant signaling pathway, thereby increasing oxidative stress. Restoration of Nrf2 activity significantly improved antioxidant capacity and attenuated vascular injury. These findings indicate that vascular viral persistence may contribute to post-viremic vascular dysfunction and identify NS3-mediated suppression of Nrf2 signaling as a mechanism underlying ZIKV-induced vascular injury.

Keywords: Zika virus; NS3; Vascular damage; Oxidative stress; Nrf2; Vascular smooth muscle cells

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne virus in the genus *Orthoflavivirus* and family *Flaviviridae* (Michita et al., 2025). Since its initial isolation in the Zika Forest of Uganda in 1947, ZIKV remained a relatively obscure human pathogen for many decades, until the Yap Island outbreak in 2007 and subsequent epidemic expansion across the Pacific and the Americas in 2013–2015 established its global public health significance (Bernardo-Menezes et al., 2022). In 2016, the World Health Organization (WHO) declared clusters of

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microcephaly and other neurological disorders associated with ZIKV infection a Public Health Emergency of International Concern, underscoring the urgent need for coordinated efforts in surveillance, prevention, therapeutic development, and mechanistic research. By 2024, ZIKV transmission had been reported in 92 countries or territories, with low-level transmission persisting in parts of the Americas and other endemic regions. However, given the limited global surveillance capacity in many settings, both infection burden and geographic distribution are likely underestimated, although more than 10 000 new cases were reported in 2024 (Brasil et al., 2025; Rabe et al., 2025; Vajo & Laszlofy, 2026).

Despite extensive investigation, the pathogenic mechanisms and full clinical spectrum of ZIKV infection remain incompletely defined (Creisher & Klein, 2024). Beyond its well-established links to congenital microcephaly and neurological complications, increasing evidence indicates that ZIKV may also affect adult vascular health. The *Chinese Expert Consensus on Zika Virus Disease Prevention and Control* noted that, in addition to neurotropic injury and congenital cerebellar malformation, ZIKV infection may contribute to vascular injury (Zhang et al., 2019a). Experimental studies have further shown that ZIKV disrupts vascular development, promotes endothelial apoptosis, impairs endothelial barrier integrity, and increases vascular permeability (Anfasa et al., 2019; Arias-Arias et al., 2018; Puerta-Guardo et al., 2019). However, the structural and functional vascular consequences of ZIKV infection have not been systematically characterized. In particular, whether vascular damage persists after immune-mediated clearance of circulating virus remains unresolved. The molecular mechanisms that sustain ZIKV-associated vascular pathology, as well as the regulatory targets that could be exploited therapeutically, also remain poorly understood, limiting the development of effective interventions (Siew et al., 2025).

ZIKV contains a positive-sense, single-stranded RNA genome that encodes three structural proteins and seven non-structural (NS) proteins, which coordinate viral replication, particle assembly, immune evasion, and host-cell injury (Alvin Chew et al., 2024). Among these proteins, NS3 is a central enzymatic component of the viral replication complex, with helicase, protease, and RNA-unwinding activities required for polyprotein processing and genome replication (Faye et al., 2020; Mwaliko et al., 2021). Recent evidence from hepatitis C virus (HCV), another member of the *Flaviviridae* family, indicates that NS3 can provoke vascular oxidative stress, promote apoptosis, and impair vascular function (Zhang et al., 2019b). Given that NS3 is evolutionarily conserved across flaviviruses, ZIKV NS3 may similarly couple viral replication machinery to vascular oxidative stress, leading to cellular injury, apoptosis, and vascular dysfunction (Bravo et al., 2024).

Oxidative stress, driven by excessive accumulation of reactive oxygen species (ROS), plays an important role in the pathogenesis of cardiovascular injury and vascular remodeling (Roşian et al., 2025). In the context of ZIKV infection, NS3 has been implicated not only in viral replication but also in host-cell dysfunction and pathogenicity (Cao et al., 2022). In addition to its role in replication, NS3 may induce oxidative stress in host cells and thereby disrupt vascular smooth muscle cell (VSMC) function. ROS-mediated injury can disrupt cell membrane integrity, alter intracellular signaling, and trigger apoptosis, thereby creating a cellular environment that favors vascular dysfunction (Mlynarska et al., 2024; Ni et al., 2025).

Although the mechanisms linking ZIKV infection to cardiovascular injury have not yet been fully clarified, preliminary data implicate NS3 as a direct regulator of VSMC dysfunction. Notably, previous work showed that ZIKV NS3 expression markedly increased VSMC proliferation and migration, consistent with a shift toward a remodeling-associated cellular phenotype. This response was accompanied by redox imbalance, characterized by reduced superoxide dismutase (SOD) activity and nitric oxide (NO) levels, together with increased malondialdehyde (MDA), a marker of lipid peroxidation (Bulboacă et al., 2026; Xu et al., 2019). These observations suggest that NS3 promotes oxidative stress and maladaptive VSMC activation, thereby providing a plausible mechanistic link between ZIKV infection and vascular dysfunction.

In this study, type I interferon-deficient mice were used to investigate vascular injury associated with sustained ZIKV presence in the vasculature, whereas immunocompetent northern pig-tailed macaques (*Macaca leonina*) were used to determine whether vascular structural and functional damage persisted after immune-mediated clearance of circulating ZIKV. Integration of these complementary *in vivo* models with mechanistic analyses in VSMCs enabled systematic evaluation of how ZIKV infection and NS3-driven oxidative stress affect vascular integrity, smooth muscle cell behavior, and vascular reactivity across distinct immunological contexts.

MATERIALS AND METHODS

Virus strains and cell lines

The Asian-lineage Zika virus strain ZIKV-SMGC (GenBank: KX266255), C6/36 cells derived from *Aedes albopictus* larvae, and Vero cells derived from African green monkey kidney epithelium were maintained in the laboratory of Yang-Tang Zheng at the Kunming Institute of Zoology, Chinese Academy of Sciences. All cell lines were authenticated and confirmed to be free of mycoplasma contamination.

Isolation and purification of primary murine VSMCs (mVSMCs): Primary mVSMCs were isolated from the thoracic aortas of C57BL/6 mice euthanized by nitrogen inhalation. Following perfusion with phosphate-buffered saline (PBS), thoracic aortas were dissected longitudinally, and endothelial cells were removed. The medial layer was then enzymatically digested with collagenase types I/II and elastase. The resulting single-cell suspension was filtered, centrifuged (800 r/min, 15 min at 4°C), and cultured at 37°C for 48–72 h in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The culture medium was replaced once obvious spindle-shaped cells were observed under the microscope. mVSMC identity was confirmed by α -SMA immunofluorescence (Zhang et al., 2022).

Cell culture and passage: Cells were cultured in DMEM supplemented with 10% FBS under 5% CO₂ and saturated humidity. C6/36 cells were maintained at 28°C, whereas all other cells were incubated at 37°C. Cells were passaged at 90% confluence. After incubation with 1.25% trypsin dissolved in calcium- and magnesium-free D-Hanks solution at 37°C for 1 min, the trypsin solution was removed, and cells were detached by gently tapping the culture flask. The resulting cell suspension was diluted with two volumes of fresh medium and evenly seeded into two new culture flasks.

Virus amplification and titration

ZIKV was propagated in C6/36 cells at 28°C at a multiplicity of infection (MOI) of 0.1. Culture supernatants were collected at

5 days post-infection (dpi), clarified by centrifugation (12 000 r/min, 10 min at 4°C), passed through 0.22 µm filters, aliquoted, and stored at -80°C. Viral titers were determined by plaque assay in Vero cells. Briefly, Vero cell monolayers were inoculated with serially diluted virus, overlaid with 1.2% low-melting-point agarose in 2× DMEM, and incubated for 4–7 days. Cells were then fixed with 4% paraformaldehyde and stained with 0.5% crystal violet, after which plaques were counted. Viral titers were calculated as plaque-forming units per milliliter (PFU/mL) using the following formula: mean plaque number×dilution factor/inoculum volume (mL) (Barreto-Vieira et al., 2017; Zhou et al., 2022).

Animal models and viral infection

All procedures involving laboratory animals, including non-human primates (NHPs) and A129 mice, complied with Directive 2010/63/EU, the *Guide for the Care and Use of Laboratory Animals*, the *Weatherall Report on the Use of NHPs in Research*, and the *Animal Research: Reporting of In Vivo Experiments (ARRIVE)* guidelines. The NHP study protocol was reviewed and approved by the Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences (Approval No.: IACUC-PE-2023-06-001) (Li et al., 2024). The mouse study protocol was reviewed and approved by the Ethics Committee of Kunming Medical University (KMMU20210029). All ZIKV experiments were conducted under animal biosafety level 2 (ABSL-2) containment. The northern pig-tailed macaques were socially housed with environmental enrichment, *ad libitum* access to food and water, and daily veterinary supervision. A129 mice were maintained under specific pathogen-free (SPF) conditions under a 12 h light/12 h dark cycle (lights on at 0700h and off at 1900h), with controlled temperature and humidity, environmental enrichment, and *ad libitum* access to food and water. All procedures were performed under appropriate sedation or anesthesia, with peri-procedural analgesia provided when required to minimize pain and distress. Humane endpoints were predefined and strictly observed. When euthanasia was indicated, animals were euthanized by barbiturate overdose in accordance with the *AVMA Guidelines for the Euthanasia of Animals* (2020). No procedure was performed without prior ethical approval.

All experimental animals were anesthetized before surgical or invasive procedures in accordance with the principles outlined in the *Guide for the Care and Use of Laboratory Animals, Eighth Edition*. Mice were anesthetized by a single intraperitoneal injection of sodium pentobarbital (36 mg/kg). Procedures were initiated only after stable anesthesia was achieved. Anesthetic depth was monitored by assessing rectal temperature, respiratory rate, and heart rate. When required, an additional intraperitoneal dose of sodium pentobarbital (10 mg/kg) was administered to maintain an adequate anesthetic plane. Macaques were anesthetized by intramuscular administration of ketamine hydrochloride (10 mg/kg) combined with xylazine hydrochloride (1 mg/kg). Anesthetic depth was monitored by evaluating rectal temperature, heart rate, respiratory rate, and blood pressure. Additional ketamine hydrochloride (≤5 mg/kg, intramuscularly) was administered when necessary to maintain an appropriate anesthetic plane. All animals were closely monitored until full recovery from anesthesia. No adverse events were observed, and all animals recovered uneventfully.

A129 mice (IFNAR^{-/-}, 6–8 weeks old, 20±2 g, equal male-to-female ratio) were obtained from the Kunming Institute of Zoology and maintained under SPF conditions at the

Department of Laboratory Animal Science, Kunming Medical University. After 7 days of acclimation, mice were inoculated subcutaneously in the hind footpad with 1×10⁴ PFU ZIKV in 20 µL (Narasimhan et al., 2020). Controls received PBS. Blood was collected from the retro-orbital sinus every 2 days from 0 to 14 dpi and weekly thereafter (15–119 dpi). Viral RNA was quantified by reverse transcription quantitative PCR (RT-qPCR).

Three adult macaques were inoculated intramuscularly with 1×10⁶ PFU ZIKV at multiple sites along the inner thigh to promote consistent infection and reproducible model establishment (Yang et al., 2023). Blood was collected at 0 dpi, every other day from 1 to 21 dpi, and weekly thereafter until 63 dpi. The 63-day observation window was selected because it was approximately four times longer than the acute viremic phase, enabling assessment of virus-associated vascular injury during viremia and throughout the post-clearance recovery period. Animals were sacrificed at 63 dpi, and tissues, including aorta, pulmonary artery, vena cava, mesentery, skin, lymph nodes, and pancreas, were harvested for virological and pathological analyses.

Histopathology and transmission electron microscopy (TEM)

Mice and macaques were euthanized at designated time points, and tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 µm. Hematoxylin and eosin (H&E) staining and immunofluorescence staining were then performed. For ultrastructural analysis, vascular specimens were fixed in 2.5% glutaraldehyde and processed for TEM using standard procedures to examine intracellular ZIKV particles.

Immunofluorescence and western blot analysis

Tissue sections and cultured cells were incubated overnight at 4°C with primary antibodies against ZIKV envelope protein (EP), Nrf2, α-SMA, and osteopontin (OPN). Primary antibodies and fluorophore-conjugated secondary antibodies were obtained from Abcam. After incubation with Alexa Fluor-conjugated secondary antibodies, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured using a Leica DM4000 B fluorescence microscope (Leica, Germany).

For western blot analysis, total protein was extracted using RIPA buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and probed with antibodies against Nrf2, KEAP1, Nrf1, GPX4, and GAPDH (Abcam, UK). Protein signals were detected using enhanced chemiluminescence (ECL) substrate (Thermo, USA).

RNA extraction and RT-qPCR analysis

Absolute quantification of ZIKV RNA and viral non-structural RNA was performed using one-step qPCR. Total RNA was extracted from cells or tissues using TRIzol reagent (Takara, Japan) according to the manufacturer's instructions. Each 20 µL RT-qPCR mixture contained RNA template, gene-specific primers and probe for ZIKV EP, DNA polymerase, and RNase-free water. Viral RNA copy numbers were calculated from standard curves. The primer and probe sequences used for absolute quantification were: ZIKV EP, forward 5'-CCGC TGCCCAACACAAG-3', reverse 5'-CCACTAACGTTCTTTTGC AGACAT-3', probe 5'-6-FAM-AGCCTACCTTGACAAGCAGTC AGACACTCAA-TAMRA-3'; ZIKV NS3, forward 5'-AGGAGA TGGAGAGGAGGAAG-3' and reverse 5'-CTCCTCCTCCT

CCTCCTCCT-3'; ZIKV NS2a, forward 5'-GCTGAGGAGAG GAGGAGGAG-3' and reverse 5'-CCTCCTCCTCCTCCTCCT CC-3'; and ZIKV NS5, forward 5'-GGGGGTGGAACAGGAGAG AC-3' and reverse 5'-TTATAGCACTCCAGGTGTGGACC-3'.

For relative quantification in murine cells, total RNA was extracted and reverse-transcribed into cDNA using a Takara Reverse-Transcription Kit (Takara, Japan). qPCR was performed using SYBR Green mix in a 20 μ L reaction system, with GAPDH used as the internal reference gene. Primer sequences used for relative quantification were: Nrf2, forward 5'-TCTTGGAGTAAGTCGAGAAGTGT-3' and reverse 5'-GTT GAACTGAGCGAAAAAGGC-3'; KEAP1, forward 5'-TGCCC CTGTGGTCAAAGTG-3' and reverse 5'-GGTTCGGTTACCG TCCTGC-3'; Nrf1, forward 5'-AGCACGGAGTGACCCAAAC-3' and reverse 5'-TGACGTGGCTACATGGACCT-3'; and GPX4, forward 5'-GATGGAGCCCATTCTGAACC-3' and reverse 5'-CCCTGTACTTATCCAGGCAGA-3'. Relative mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method. PCR cycling conditions were: initial denaturation at 95°C for 5 min, followed by 50 cycles at 95°C for 15 s and 60°C for 45 s.

Cell infection and migration assay

mVSMCs were infected with ZIKV at MOIs of 0.1, 1.0, 5.0, and 10.0. Cells were seeded into culture dishes fitted with two-well wound inserts (Ibidi, Germany), and wound closure was monitored at 0, 24, 48, and 72 h post-infection (hpi). Cell migration was quantified by measuring the distance migrated from the wound edge (Song et al., 2020).

Gene overexpression and knockout

Lentiviral vectors designed for Nrf2 overexpression (RSV-Nrf2) and CRISPR-Cas9-mediated Nrf2 knockout (dual-gRNA vector) were packaged into lentiviral particles and used to transduce mVSMCs at an MOI of 20.0. Each vector contained an mCherry fluorescent marker to verify transduction efficiency. Vector construction and lentiviral packaging were performed by VectorBuilder (China). After transduction, qPCR was performed to confirm changes in gene expression.

Co-immunoprecipitation (Co-IP) and Nrf2 subcellular fractionation

Co-IP: ZIKV-infected mVSMCs were harvested at 48 hpi, washed twice with cold PBS, and lysed in ice-cold RIPA buffer (Beyotime, China) supplemented with protease and phosphatase inhibitors. Lysates were incubated on ice for 30 min and centrifuged at 14 000 $\times g$ for 15 min at 4°C to remove cellular debris. Supernatants were incubated overnight at 4°C with 2 μ g of anti-Nrf2 antibody (Abcam, ab62352, UK), followed by incubation with 30 μ L of Protein A/G magnetic beads (Thermo Fisher Scientific, USA) for 2 h at 4°C with gentle rotation. Beads were then washed five times with cold PBS containing 0.1% Triton X-100, resuspended in SDS sample buffer, and boiled at 95°C for 5 min. Eluted proteins were analyzed by western blotting using anti-NS3 (GeneTex, GTX133308, USA) and anti-Nrf2 antibodies. Input lysates (5%) were loaded to verify total protein levels (Xue et al., 2025). Co-IP was used to confirm the interaction between NS3 and Nrf2.

Nrf2 subcellular fractionation: ZIKV-infected mVSMCs were harvested at 48 hpi to determine the cytoplasmic and nuclear distribution of Nrf2. Nuclear and cytoplasmic proteins were extracted separately using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China; Cat# P0027) according to the manufacturer's instructions. Briefly, treated mVSMCs were washed twice with cold PBS and lysed in

cytoplasmic extraction buffer A supplemented with protease inhibitors. After incubation on ice for 15 min, buffer B was added, and samples were vortexed and centrifuged at 12 000 $\times g$ for 5 min at 4°C. The supernatant was collected as the cytoplasmic fraction, whereas the pellet was resuspended in nuclear extraction buffer, vortexed vigorously, incubated on ice for 30 min, and centrifuged again to collect the nuclear fraction. Protein concentrations were determined using a BCA assay (Thermo Fisher Scientific, USA). Equal amounts of nuclear and cytoplasmic protein (20–30 μ g) were resolved by SDS-PAGE, transferred onto PVDF membranes, and probed with anti-Nrf2 antibody (Abcam, ab62352, UK). Lamin B1 and GAPDH were used as nuclear and cytoplasmic loading controls, respectively. Protein bands were visualized using ECL and quantified using ImageJ software.

Oxidative stress markers

Tissues and cells were homogenized in 10 volumes of cold PBS containing 1 $\times$ protease inhibitor cocktail (Thermo Fisher Scientific, USA) and zirconia beads. Samples were processed using a bead-based tissue grinder with repeated 30 s grinding and 30 s pause cycles for a total processing time of 3 min. Homogenates were centrifuged at 10 000 r/min with a centrifugal radius of 5 cm at 4°C for 15 min, and supernatants were collected for subsequent assays. Superoxide dismutase (SOD) and malondialdehyde (MDA) were quantified using enzyme-linked immunosorbent assay (ELISA)-based kits (Abcam, UK). Nitric oxide (NO) levels were measured using a nitrate reduction assay kit (Beyotime, China). Absorbance was measured using a microplate reader.

NS protein plasmid construction and transfection

Coding sequences for ZIKV NS2a, NS3, and NS5 were synthesized and cloned into the pEGFP-N1 expression vector to generate NS2a-EGFP, NS3-EGFP, and NS5-EGFP fusion plasmids. The enhanced green fluorescent protein (EGFP) tag was used to monitor transfection efficiency. Plasmid construction was performed by VectorBuilder China. mVSMCs were transfected using Lipofectamine 3000 (Invitrogen, USA), and GFP expression was observed at 12, 48, and 72 h post-transfection (hpt). Gene and protein expression levels were analyzed by qPCR and western blotting, respectively.

Statistical analysis

Grayscale intensity analysis was performed using ImageJ (NIH, Bethesda, MD, USA). Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism. For comparisons between two groups, unpaired two-tailed Student's *t*-test was used when the data met the assumptions for parametric analysis. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used for parametric data. For non-parametric data, including NHP datasets where appropriate, the Kruskal-Wallis test or Friedman test, followed by Dunn's multiple comparisons test, was applied. The exact statistical test used for each dataset is specified in the corresponding figure legends. $P < 0.05$ and $P < 0.01$ were considered significant and highly significant, respectively.

RESULTS

ZIKV infection induces persistent vascular damage in A129 mice

Immunocompetent hosts can rapidly clear ZIKV from the

bloodstream through a type I interferon-mediated antiviral response. To evaluate persistent vascular injury under conditions of prolonged viremia, interferon- α/β receptor-deficient A129 mice were inoculated subcutaneously with 1×10^4 PFU ZIKV (20 μ L) through the hind footpad (Hubert et al., 2020). Infected mice began to exhibit visible hair loss at approximately 7 dpi, and this phenotype progressed until approximately 21 dpi, when fur loss was nearly complete across the dorsal, cervical, and shoulder regions (Supplementary Figure S1A). ZIKV-infected mice also displayed a clear reduction in body size relative to mock-infected controls. Body weight began to decline at 10 dpi, with the most pronounced difference observed at approximately 21 dpi, followed by gradual recovery. By 105 dpi, infected mice remained slightly lighter than mock-infected mice, although this difference was no longer significant (Supplementary Figure S1A–C). Progressive hindlimb paralysis was observed from 7 dpi (Supplementary Figure S1B), consistent with neurological manifestations associated with ZIKV infection in adults, including Guillain-Barré syndrome (Kwok et al., 2024). Paralysis incidence and severity peaked at 4 weeks post-infection. Among 60 infected A129 mice, including 30 males and 30 females, mortality began during the second week post-infection and peaked by the fourth week. The 12 surviving mice remained alive until the end of the study (Supplementary Figure S1D).

ZIKV RNA was detected in blood as early as 2 dpi, and viral load increased gradually, reaching a peak of 5.245×10^5 copies/ μ L at 28 dpi and remaining detectable until the end of the experiment (Figure 1A). These data indicated that A129 mice failed to clear ZIKV and developed persistent systemic infection. Because the first 14 dpi generally represent the acute phase of virus-induced injury and coincide with the period during which self-limiting viruses such as ZIKV are typically cleared from the bloodstream by host immunity, vascular pathology was first examined at this time point. Histopathological analysis revealed marked vascular disruption, including cellular detachment into the vascular lumen, disordered VSMC arrangement, pronounced VSMC hyperplasia, and VSMC migration toward the vascular intima, forming distinct protrusions into the lumen (Figure 1B). By 119 dpi, persistent viral injury was associated with substantial thinning of the vascular wall, extensive loss of vascular endothelial cells (VECs), severe VSMC necrosis, and widened intercellular gaps, indicating pronounced destruction of vascular architecture (Figure 1B). TEM revealed abundant ZIKV particles within VSMCs and VECs, primarily localized in the endoplasmic reticulum (ER), with higher particle density in VECs than in VSMCs (Figure 1C). Immunofluorescence staining for ZIKV EP further confirmed viral protein localization in VSMCs and VECs, with stronger fluorescence in VECs, consistent with the TEM observations (Figure 1D). RT-qPCR detected ZIKV RNA in vascular tissues at both 14 and 119 dpi. Viral RNA levels were slightly higher at 119 dpi than at 14 dpi, although the difference was not significant ($P > 0.05$, Figure 1E). Together, these findings show that ZIKV persists in the blood and vascular tissues of A129 mice and induces progressive damage to vascular tissue structure.

Vascular function was next assessed during the acute and chronic phases of ZIKV infection. Isolated vascular functional assays revealed that contractile responses to elevated K^+ and acetylcholine-induced relaxation were significantly impaired at 119 dpi. Compared with mock-infected controls, vessels from infected mice showed significantly attenuated contraction at K^+

concentrations above 0.1 μ mol/L, whereas only a mild, non-significant reduction in K^+ -evoked contraction was observed at 14 dpi (Figure 2A). Endothelium-dependent relaxation in response to acetylcholine was also significantly weakened at 119 dpi at concentrations above 1 μ mol/L, but no obvious difference was detected at 14 dpi. Acetylcholine concentration-response curves exhibited greater variability after infection, suggesting disruption of vascular regulatory control. In contrast, sodium nitroprusside (SNP)-induced endothelium-independent relaxation did not differ among the three groups. These findings indicate that prolonged ZIKV infection causes progressive vascular dysfunction, with marked impairment of both K^+ -induced contraction and acetylcholine-induced relaxation, whereas SNP-induced relaxation remains preserved. In contrast, acute infection produces only mild functional changes, suggesting that major defects in vascular regulation emerge primarily during persistent infection.

Vascular permeability was then evaluated by intravenous administration of FITC-labeled 10 kDa dextran followed by whole-body fluorescence imaging (Luo et al., 2024; Nakajima et al., 2024). At 2 h after tail vein injection, fluorescence intensity increased with infection duration, indicating progressive extravasation of dextran into tissues. Mock-infected mice showed only minimal fluorescence in the brain and limbs, whereas mice at 14 dpi exhibited enhanced fluorescence. By 119 dpi, fluorescence was markedly intensified in the brain, upper abdomen, and limbs (Figure 2B). These observations indicate that ZIKV infection increases vascular leakage and that vascular barrier disruption becomes more pronounced during persistent infection.

Although previous studies have reported ZIKV-mediated injury in VECs, whether ZIKV infects and damages VSMCs has not been fully investigated (Chaves et al., 2018; Cox et al., 2018). To determine whether VSMCs participate in ZIKV-associated vascular injury, α -SMA and OPN were examined by immunofluorescence staining as markers of contractile and synthetic VSMC phenotypes, respectively (Sulina et al., 2025). At 119 dpi, α -SMA expression was significantly reduced, whereas OPN expression was markedly increased in vascular tissues compared with mock-infected controls (Figure 2C). Because vascular injury can drive VSMCs from a contractile phenotype toward a synthetic, proliferative, and migratory state involved in tissue remodeling, these findings indicate that persistent ZIKV infection damages VSMCs and promotes phenotypic switching toward a repair-associated synthetic state.

ZIKV infection induces persistent vascular damage in immunocompetent *M. leonina*

Although our previous studies have demonstrated that ZIKV persists and causes tissue injury in immunodeficient A129 mice, circulating virus is typically cleared by intact antiviral immunity in immunocompetent hosts. However, self-limiting viral infections can retain persistent viral components in critical organs after clearance from the bloodstream, thereby sustaining tissue injury beyond the viremic phase (Hanrath et al., 2022; Ndione et al., 2024). To determine whether ZIKV causes persistent vascular damage in an immunocompetent host, ZIKV infection was established in *M. leonina*, an NHP species with immune features closely resembling those of humans and broad utility in antiviral and vaccine research.

Three *M. leonina* individuals (IDs: 15201, 15219, and 15241) were each inoculated intramuscularly with 1×10^6 PFU

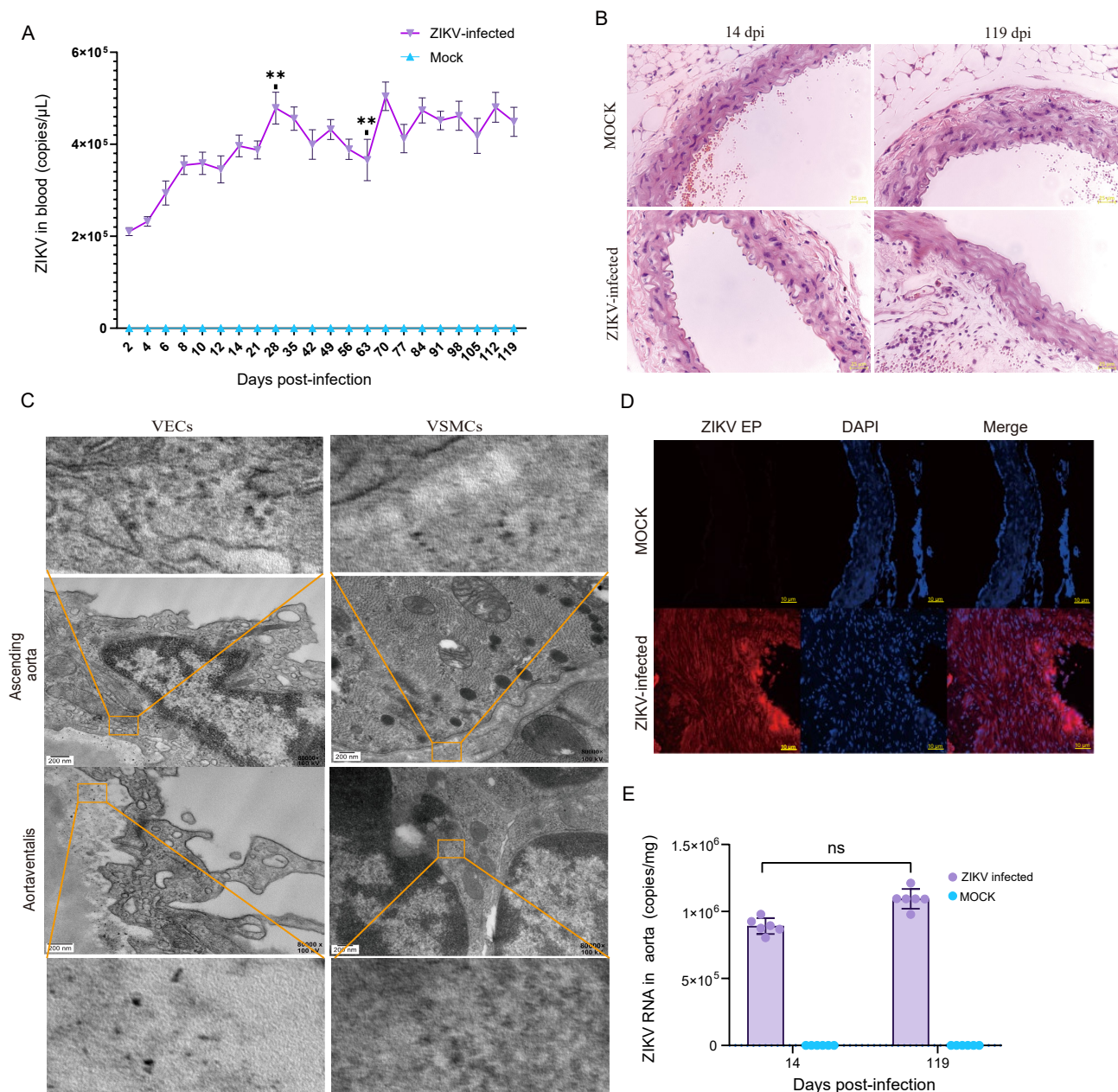


Figure 1 Persistent ZIKV presence induces vascular structural injury in A129 mice

A: Dynamic changes in serum ZIKV RNA levels following infection. $^{*}P < 0.01$ vs. MOCK. **B:** H&E staining showing pathological changes in vascular morphology. Scale bars: 25 μ m. **C:** TEM images of vessels from ZIKV-infected A129 mice showing abundant viral particles in the cytoplasm of VECs and VSMCs, primarily localized in the ER (red arrows). Scale bars: 200 nm. **D:** Immunofluorescence staining of arterial tissues for ZIKV EP (red). Scale bars: 10 μ m. **E:** RT-qPCR analysis of arterial tissues confirming the presence of viral RNA at both 14 and 119 dpi. ns: Not significant.

ZIKV at multiple sites along the inner thigh. Blood samples were collected on the day of inoculation (0 dpi), every other day until 21 dpi, and then every 7 days until 63 dpi to monitor serum viral loads. ZIKV RNA was detectable in serum as early as 1 dpi and peaked at 7–9 dpi, then declined rapidly, reached the detection limit by 15 dpi, and remained undetectable thereafter, indicating effective immune-mediated clearance of circulating virus (Figure 3A). Despite this clearance from blood, ZIKV RNA was detected in the ascending aorta, abdominal aorta, pulmonary artery, inferior vena cava, mesentery, skin, lymph nodes, and pancreas, with the highest copy numbers in the inferior vena cava and lower levels in the skin and pancreas (Figure 3B). These findings indicate that, even in immunocompetent macaques, clearance of circulating ZIKV does not necessarily correspond to complete viral

elimination from the host. Instead, ZIKV components persist in vascular tissues and multiple organs, providing a potential basis for sustained tissue injury after the viremic phase.

Histopathological analysis revealed significant vascular injury across ZIKV-positive vessels. Mock-control tissues were obtained from a co-housed *M. leonina* individual in the mock group of a concurrent study conducted under identical housing and observation conditions (ID: 08237). Mock-control arteries showed a continuous and intact endothelium, well-organized medial VSMCs, and no intimal thickening. Among ZIKV-positive large vessels, the ascending aorta displayed the mildest lesions, characterized by focal endothelial denudation, slight intimal thickening, and largely preserved medial architecture. The abdominal aorta showed moderate injury, with patchy endothelial loss and conspicuous intimal

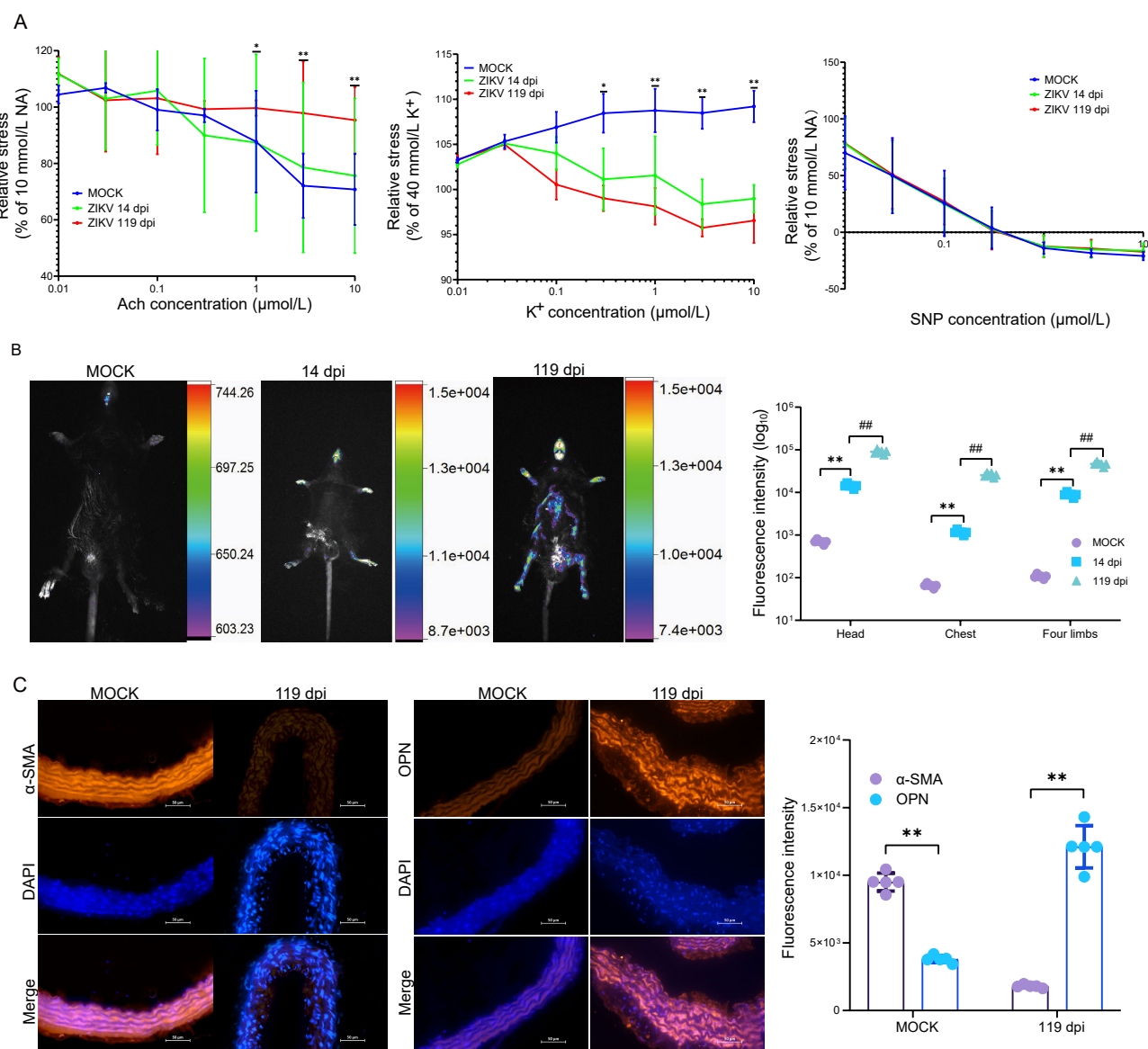


Figure 2 ZIKV infection induces persistent vascular functional impairment in A129 mice

A: Assessment of ZIKV-induced changes in vascular contraction and relaxation. * $P < 0.05$ and ** $P < 0.01$ vs. MOCK. B: *In vivo* assessment of vascular leakage using FITC-labeled 10 kDa dextran. ** $P < 0.01$ vs. MOCK and ### $P < 0.01$ vs. 14 dpi. C: Immunofluorescence staining of vascular tissues for VSMC markers α -SMA and OPN. Scale bars: 50 μ m. ** $P < 0.01$ vs. MOCK.

hyperplasia driven by VSMC proliferation (Figure 3C, orange arrow). The pulmonary artery exhibited the most severe remodeling, including extensive endothelial denudation, marked intimal hyperplasia with inward protrusions, pronounced medial VSMC hyperplasia with disordered arrangement, substantial wall thickening with relative luminal narrowing (green arrow), and focal medial VSMC necrosis or loss (black arrow). Vascular lesions were graded using an internationally accepted semi-quantitative scale for experimental vascular pathology, ranging from 0 to 6, with higher scores indicating greater severity (Gisterå et al., 2022; Stary et al., 1995). Scores were 0 for mock-control vessels, 1 for the ascending aorta, 2 for the abdominal aorta, and 4 for the pulmonary artery (Figure 3C). Similar pathological changes were observed in small vessels of the mesentery, subcutaneous tissue, lymph nodes, and pancreas (Figure 3D).

TEM revealed the presence of ZIKV particles in both VSMCs and VECs, predominantly within the ER. VECs contained more viral particles than VSMCs (Figure 3E).

Immunofluorescence staining for ZIKV EP confirmed viral protein in multiple vascular segments, with stronger fluorescence in endothelial cells than in smooth muscle cells. EP fluorescence was strongest in the pulmonary artery and weakest in the ascending aorta, consistent with the viral RNA levels detected in these vessels (Figure 3F).

RNA sequencing (RNA-seq) revealed suppression of antioxidant signaling in ZIKV-infected vascular tissues

ZIKV infection induced vascular injury in both A129 mice and *M. leonina*, with prominent disruption of VSMC organization and vascular function. To further elucidate the molecular mechanisms and regulatory signaling pathways underlying ZIKV-induced vascular injury, RNA-seq was performed on vascular tissues collected from ZIKV-infected and mock-infected mice at 119 dpi.

Transcriptomic analysis identified 775 differentially expressed genes (DEGs) in ZIKV-infected vascular tissues, including 374 up-regulated and 401 down-regulated transcripts (Figure 4A). KEGG enrichment analysis ranked

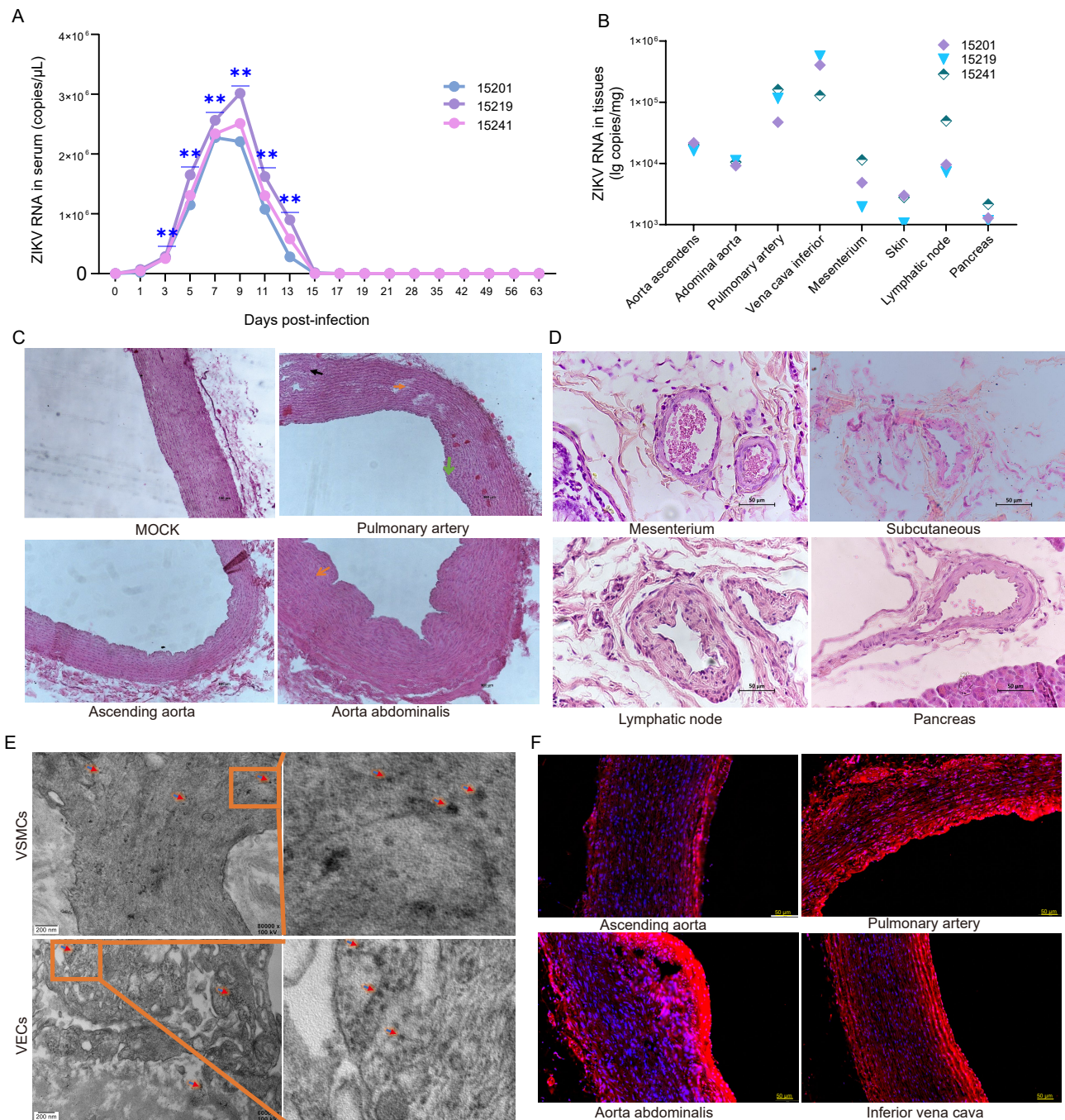


Figure 3 Persistent vascular localization of ZIKV and long-term vascular injury in immunocompetent *M. leonina*

A: Temporal changes in serum ZIKV RNA levels following intramuscular inoculation. $^{**}P < 0.01$ vs. 0 dpi. **B:** Detection of ZIKV RNA in vascular and organ tissues at 63 dpi, indicating persistence of viral RNA after clearance from the bloodstream. **C:** Representative histopathological changes in major blood vessels at 63 dpi. Scale bars: 100 μ m. **D:** Representative histopathological changes in small blood vessels from different tissues at 63 dpi. Scale bar: 50 μ m. **E:** Representative TEM images showing ZIKV particles (arrows) in VECs and VSMCs. Scale bars: 200 nm. **F:** Representative immunofluorescence staining of ZIKV EP in vascular tissues. Red fluorescence indicates ZIKV E protein, and blue fluorescence (DAPI) indicates cell nuclei. Scale bars: 50 μ m.

oxidative phosphorylation as the most significantly altered pathway among the top 15 enriched pathways in ZIKV-infected vessels (Figure 4B), indicating that persistent ZIKV infection markedly perturbed redox metabolism and antioxidant homeostasis.

Unsupervised hierarchical clustering of the top six sentinel genes separated the expression profile into an antioxidant module comprising *GPX4*, *KEAP1*, *Nrf2*, *Nrf1*, and *SOD1* and an inflammatory marker represented by *S100A9*. Relative to the mock-infected tissues, ZIKV-infected vessels showed

coordinated down-regulation of the antioxidant module, with reduced expression of *GPX4*, *KEAP1*, *Nrf2*, *Nrf1*, and *SOD1*, whereas *S100A9* was robustly up-regulated (Figure 4C). These expression patterns indicate simultaneous suppression of the antioxidant/oxidative-stress response alongside activation of vascular inflammation during persistent infection.

Protein-protein interaction analysis centered on *KEAP1*, *Nrf2*, *Nrf1*, and *GPX4* further highlighted a redox regulatory network that controls antioxidant protection and lipid peroxide detoxification. Under basal conditions, *KEAP1* restricts *Nrf2*

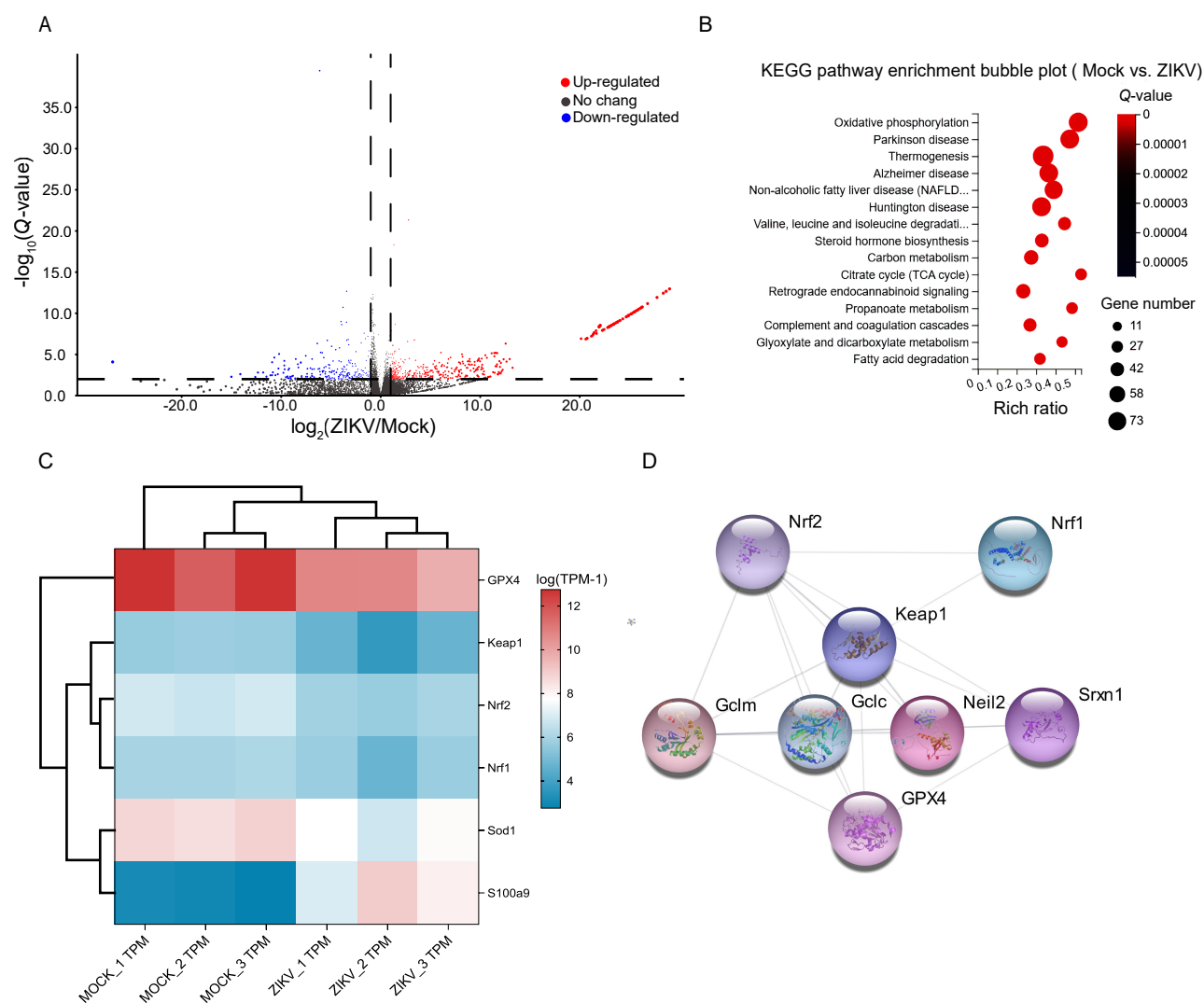


Figure 4 RNA-seq analysis reveals suppression of antioxidant gene expression in ZIKV-infected vessels

A: Volcano plot of differentially expressed genes (DEGs). B: KEGG pathway enrichment analysis. C: Unsupervised hierarchical clustering heatmap of the top six DEGs. D: Protein-protein interaction (PPI) network analysis of the *Nrf2/Nrf1/KEAP1/GPX4* antioxidant pathway.

activity through *CUL3*-dependent ubiquitination, whereas Nrf1 functions through a parallel antioxidant program with overlapping antioxidant response element (ARE)-regulated targets, including *GCLC*, *GCLM*, *GPX4*, and *SRXN1*. *GPX4* serves as a key downstream effector that reduces lipid peroxides through a glutathione-dependent mechanism. Together, this network positions *KEAP1* as a negative regulator of *Nrf2* and supports cooperative regulation of *GPX4* and glutathione synthesis by *Nrf2/Nrf1* to preserve redox balance and membrane integrity (He et al., 2024; Jin et al., 2025). Consistent with this regulatory model, ZIKV infection disrupted the *Nrf2/KEAP1* axis and its downstream antioxidant genes, broadly reducing antioxidant gene expression (Figure 4D). Therefore, dysregulation of the *Nrf2/KEAP1* pathway may be a key contributor to ZIKV-induced vascular damage.

ZIKV infection inhibits the *Nrf2/Nrf1/KEAP1/GPX4* antioxidant pathway in VSMCs and promotes VSMC migration

To investigate whether ZIKV infection induces oxidative stress in VSMCs, a ZIKV infection model was established in primary mVSMCs. Cells were infected with ZIKV at MOIs of 0.1, 1.0, 5.0, and 10.0, with Vero cells serving as a positive control. ZIKV successfully infected mVSMCs at all four MOIs and

significantly affected cell growth and viability. In GFP-expressing infected cells, increasing viral dose was accompanied by greater cellular debris and progressively weaker GFP fluorescence, indicating that ZIKV infection disrupts mVSMC growth and protein expression in a dose-dependent manner (Supplementary Figure S2A).

Immunofluorescence staining for ZIKV EP confirmed strong viral protein signals across all infection doses, and qPCR confirmed the presence of viral RNA in infected cells (Supplementary Figure S2B, C). Infected cells were serially passaged three times, and viral RNA was measured in both cells and culture supernatants 24 h after each passage. Viral RNA remained detectable after each passage, indicating that mVSMCs supported sustained ZIKV replication and release (Supplementary Figure S2C). These findings indicate that ZIKV effectively replicates in mVSMCs and forms infectious viral particles.

TEM analysis at 72 hpi revealed abundant ZIKV particles in both Vero cells and mVSMCs, with particles primarily localized within the ER. Vero cells harbored more viral particles than mVSMCs. Infection induced significant ER remodeling, including swelling, expansion, and formation of ER-derived vesicular structures (Figure 5A), consistent with viral exploitation of host membranous compartments for replication

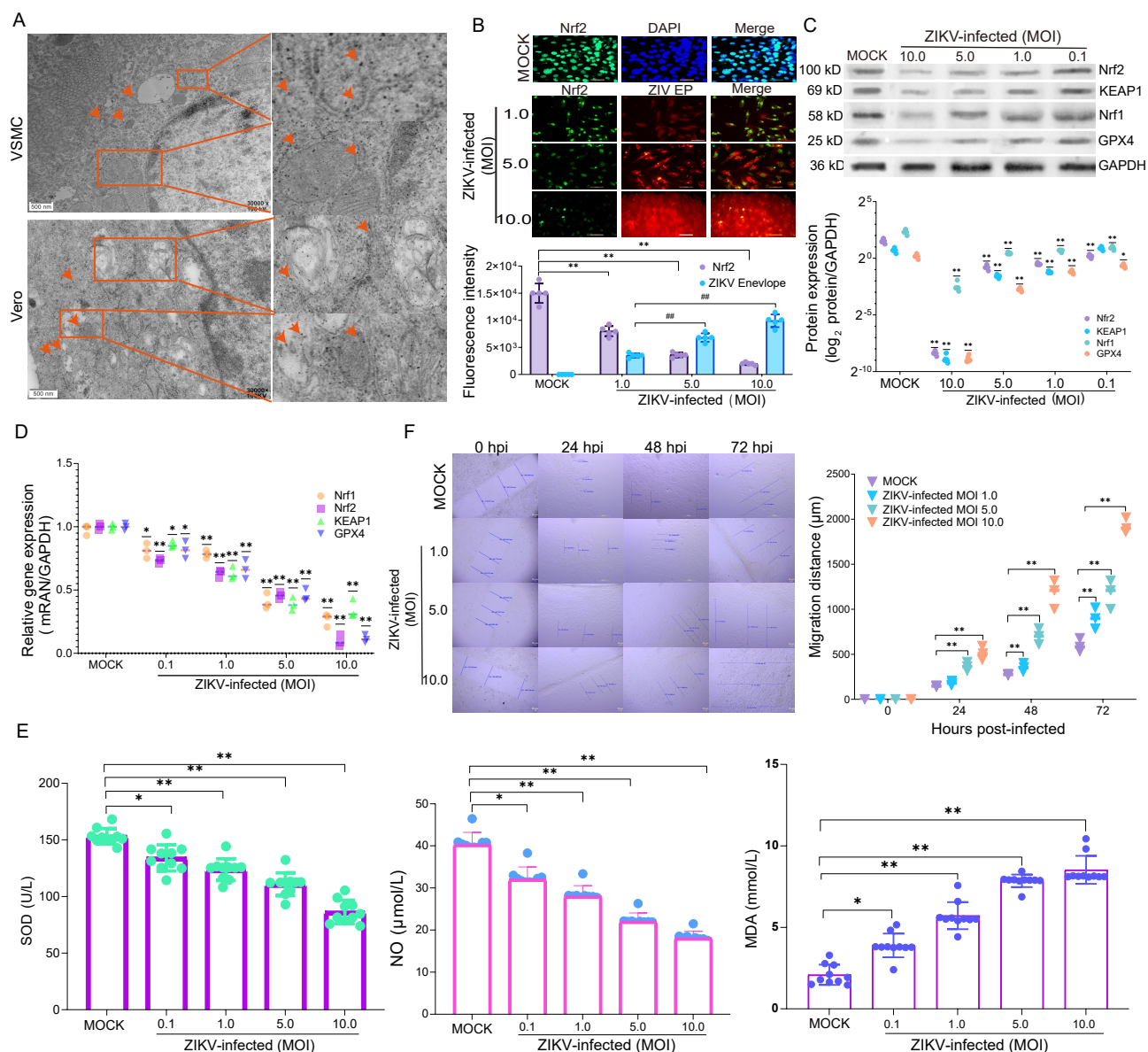


Figure 5 ZIKV infection inhibits the Nrf2/Nrf1/KEAP1/GPX4 antioxidant pathway and promotes VSMC migration

A: Ultrastructural images of ZIKV-infected mVSMCs and Vero cells. Scale bars: 500 nm. B: Immunofluorescence staining for Nrf2 (green) and ZIKV EP (red) in ZIKV-infected mVSMCs. Scale bars: 50 μm. $^{**}P<0.01$ vs. MOCK, $^{###}P<0.01$ vs. 1.0 MOI. C: Protein expression of key antioxidant regulators in the Nrf2/Nrf1/KEAP1/GPX4 pathway post-ZIKV infection. $^{*}P<0.05$ and $^{**}P<0.01$ vs. MOCK. D: mRNA expression of key antioxidant regulators in the Nrf2/Nrf1/KEAP1/GPX4 pathway following ZIKV infection. $^{*}P<0.05$ and $^{**}P<0.01$ vs. MOCK. E: ZIKV suppresses intracellular antioxidant factors and promotes accumulation of oxidative byproducts in mVSMCs. $^{*}P<0.05$ and $^{**}P<0.01$ vs. MOCK. F: ZIKV infection promotes mVSMC migration. Scale bars: 50 μm. $^{**}P<0.01$ vs. MOCK at the same time point.

and assembly, disrupting ER homeostasis. ZIKV infection also caused severe mitochondrial damage, including mitochondrial swelling, outer membrane disruption, and cristae fragmentation and dissolution (Figure 5A). Damage to the mitochondrial membrane compromises the electron transport chain, leading to electron leakage into the cytoplasm and exacerbating oxidative stress.

To further explore the relationship between ZIKV infection and antioxidant regulation, Nrf2 and ZIKV E protein were analyzed by immunofluorescence staining in mVSMCs (Zhang et al., 2018). In mock-infected cells, Nrf2 exhibited strong fluorescence with predominant nuclear localization. As infection dose increased, Nrf2 fluorescence progressively decreased and nuclear localization became less distinct. Cytoplasmic Nrf2 remained visible in the 1.0 MOI group, whereas Nrf2 fluorescence was markedly diminished in the

5.0 and 10.0 MOI groups (Figure 5B). These findings suggest that ZIKV infection not only down-regulates Nrf2 abundance but also disrupts its nuclear translocation, thereby compromising its antioxidant transcriptional regulation.

Western blotting and RT-qPCR were then used to assess key components of the Nrf2/KEAP1 antioxidant pathway, including Nrf2, KEAP1, Nrf1, and GPX4. ZIKV infection significantly down-regulated this pathway in mVSMCs. Nrf2 and Nrf1 were markedly suppressed across all infection doses (0.1, 1.0, 5.0, and 10.0 MOI), with significant differences from mock-infected cells ($P<0.01$). GPX4 and KEAP1 were also reduced, although the magnitude of suppression was less pronounced. At 0.1 MOI, several Nrf2/KEAP1 pathway components were significantly altered compared with mock-infected cells ($P<0.05$); however, the reduction in GPX4 protein abundance did not reach statistical significance

($P>0.05$; Figure 5C, D). These results show that ZIKV infection suppresses Nrf2/KEAP1-associated antioxidant signaling in a dose-dependent manner.

The Nrf2/KEAP1 pathway regulates antioxidant mediators, including SOD and NO (Chen et al., 2020; Makino et al., 2016), and disruption of this pathway can promote accumulation of oxidative byproducts such as MDA (Xia et al., 2020). To evaluate the oxidative status of ZIKV-infected mVSMCs, SOD, NO, and MDA were measured in cell lysates. ZIKV infection caused a dose-dependent decrease in SOD and NO levels, together with a corresponding increase in MDA levels. Except for the MOI 0.1 group, which showed a significant difference compared with the mock-infected cells ($P<0.05$), all higher-dose infection groups exhibited highly significant differences ($P<0.01$; Figure 5E). These findings demonstrate that ZIKV infection inhibits antioxidant capacity and induces oxidative stress in mVSMCs.

Because VSMCs are central regulators of vascular remodeling and oxidative stress can enhance VSMC migration during vascular repair responses, the effect of ZIKV infection on mVSMC migration was next evaluated (Lehners et al., 2025; Wang et al., 2024). mVSMCs were infected at MOIs of 1.0, 5.0, and 10.0, and migration was monitored over time. ZIKV infection significantly increased mVSMC migration in a dose-dependent manner. The 10.0 MOI group showed markedly enhanced migration at 24, 48, and 72 hpi compared with mock-infected cells ($P<0.01$). The 5.0 MOI group showed significant increases at 48 and 72 hpi ($P<0.01$), whereas the 1.0 MOI group exhibited a significant increase only at 72 hpi ($P<0.01$) and no significant difference at 24 hpi ($P>0.05$; Figure 5F). These results indicate that ZIKV infection promotes mVSMC migration in a dose-dependent manner, consistent with oxidative stress-associated vascular injury and remodeling.

ZIKV NS3 inhibits Nrf2 nuclear translocation and induces oxidative injury in mVSMCs

To define the contribution of ZIKV NS proteins to VSMC injury, expression constructs encoding NS2a, NS3, and NS5 were introduced into mVSMCs, and the resulting cellular responses were assessed. EGFP-tagged plasmids encoding the three ZIKV NS proteins were generated (Figure 6A) and transfected into mVSMCs using Lipofectamine 3000, producing NS2a-, NS3-, and NS5-expressing mVSMCs. At 48 hpt, EGFP fluorescence was readily detected, confirming successful plasmid delivery and protein expression (Figure 6B). Total RNA from transfected cells was analyzed by qPCR using viral RNA standards to quantify NS gene expression. NS2a, NS3, and NS5 transcripts were detected at comparable levels, ranging from 3.5×10^8 to 4.1×10^8 copies/ 10^5 cells, with no significant differences among the three groups (all $P>0.05$; Figure 6C). Western blot analysis further confirmed the intracellular expression of all three NS proteins, with comparable normalized protein abundance among NS2a-, NS3-, and NS5-transfected cells ($P>0.05$; Figure 6D). These results verify the efficient and comparable expression of ZIKV NS2a, NS3, and NS5 in mVSMCs.

The effects of these NS proteins on antioxidant regulation were then evaluated by measuring mRNA levels of Nrf1, Nrf2, KEAP1, and GPX4, together with the antioxidant mediators SOD and NO and the lipid peroxidation product MDA. Among the three viral proteins tested, NS3 produced the strongest disruption of antioxidant signaling. NS3-mVSMCs showed marked down-regulation of Nrf2, Nrf1, and GPX4 compared with mock-transfected cells ($P<0.01$), with KEAP1 expression

also significantly reduced but to a lesser extent ($P<0.05$; Figure 6E). In contrast, empty-vector-transfected mVSMCs and mVSMCs expressing NS2a or NS5 showed no significant changes in Nrf2, Nrf1, or GPX4 expression relative to the mock-transfected group (Figure 6E). Consistent with selective impairment of antioxidant defense by NS3, MDA levels were significantly increased in NS3-expressing mVSMCs, whereas SOD and NO levels were significantly reduced ($P<0.01$; Figure 6F). No significant changes in MDA, SOD, or NO were detected in empty-vector-transfected cells or in cells expressing NS2a or NS5 ($P>0.05$; Figure 6F). These findings identify ZIKV NS3 as the principal NS protein responsible for inhibiting antioxidant defense and driving oxidative stress in mVSMCs.

Increased VSMC migration is a characteristic response to oxidative stress-induced injury (Wang et al., 2024). To identify the ZIKV NS protein responsible for this phenotype, mVSMCs expressing NS2a, NS3, or NS5 were subjected to wound-healing assays. No significant differences in migration were observed among groups at 12 hpt ($P>0.05$). At 24 hpt, NS3-expressing mVSMCs showed a modest increase in migration distance compared with the other groups, although the difference did not reach statistical significance ($P>0.05$). At 36 hpt, migration was higher in NS3-mVSMCs than in other groups, with a significant difference compared with the mock-infected group ($P<0.05$). From 48 to 72 hpt, NS3-mVSMCs showed markedly increased migration compared with all other groups, with highly significant differences relative to mock cells ($P<0.01$). In contrast, migration distances among EVP-mVSMCs, NS2a-mVSMCs, and NS5-mVSMCs did not differ significantly from those in mock cells at any time point ($P>0.05$; Figure 6G). These findings indicate that ZIKV NS3 selectively promotes mVSMC migration, recapitulating the migratory activation observed during ZIKV infection and identifying NS3 as a major viral determinant of oxidative stress-induced VSMC injury.

Nrf2 regulates antioxidant defense after translocation into the nucleus, where it binds to AREs and activates cytoprotective gene expression. The NS3 protein from HCV, another member of the family *Flaviviridae*, has been reported to interact with Nrf2 and inhibit its nuclear translocation, thereby promoting oxidative stress (Chen et al., 2013; Tseng et al., 2018). To determine whether ZIKV NS3 interferes with Nrf2 through a similar mechanism, Co-IP assays were performed in ZIKV-infected mVSMCs. Nrf2-associated protein complexes were precipitated with anti-Nrf2 antibody and probed with anti-NS3 antibody. No NS3 signal was detected in Nrf2 immunoprecipitates from mock cells. In contrast, NS3 was detected in Nrf2-associated complexes from cells infected with ZIKV at MOIs of 0.1, 1.0, 5.0, and 10.0, and the abundance of Nrf2-associated NS3 increased with viral dose. Cytoplasmic and nuclear fractionation further demonstrated progressive reductions in nuclear, cytoplasmic, and total Nrf2 protein levels with increasing ZIKV dose, with nuclear Nrf2 exhibiting the most pronounced reduction after infection ($^*P<0.05$, $^{**}P<0.01$; Figure 6H). These results suggest that ZIKV NS3 associates with Nrf2 and impairs Nrf2 nuclear translocation, thereby suppressing Nrf2-dependent antioxidant responses in mVSMCs.

Nrf2 knockout exacerbates oxidative injury and promotes migration in ZIKV-infected mVSMCs

To model ZIKV-induced inhibition of *Nrf2* and determine whether *Nrf2* contributes to antioxidant protection and ameliorates ZIKV-induced vascular injury, Nrf2 expression

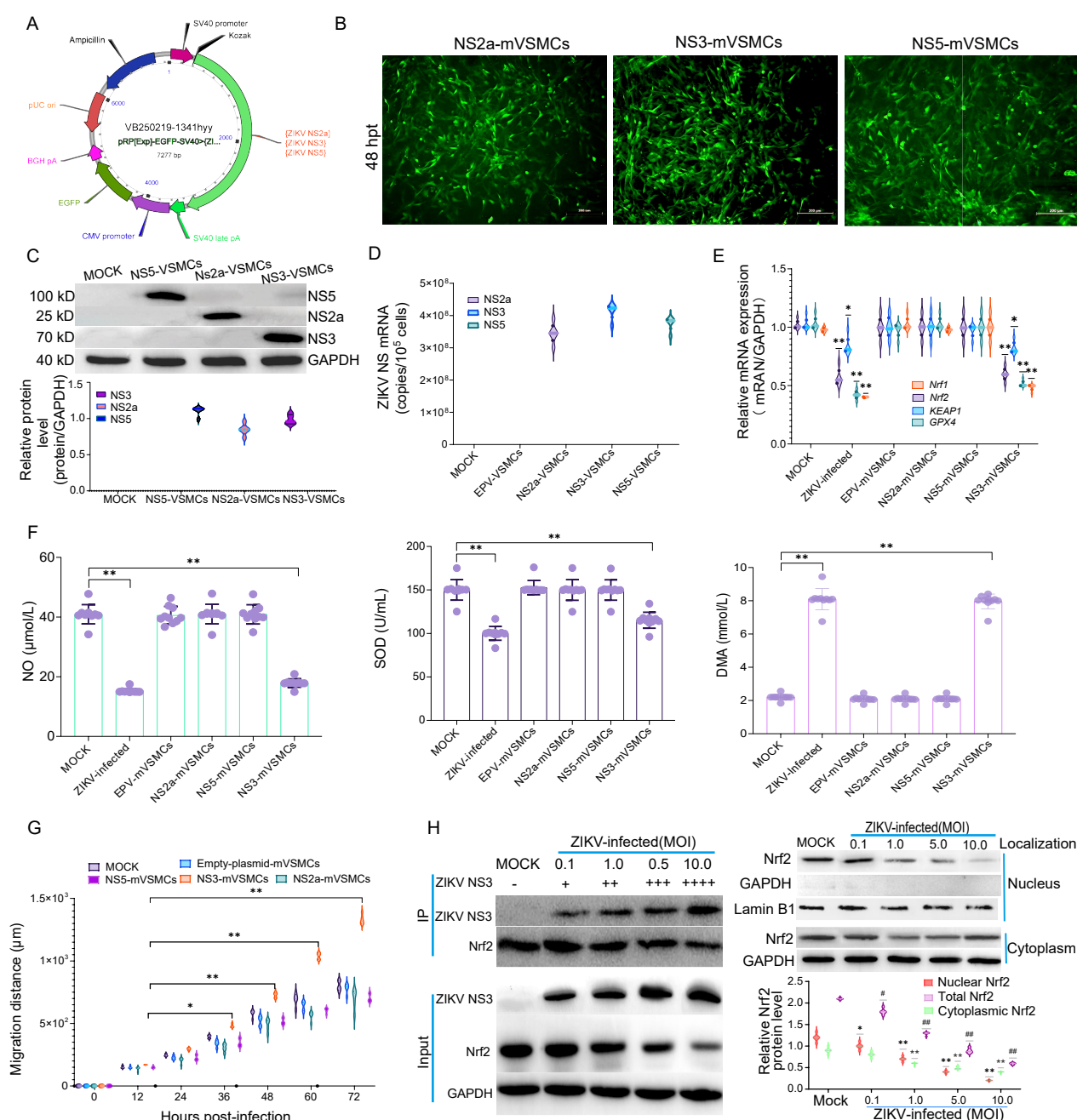


Figure 6 ZIKV NS3 inhibits Nrf2 nuclear translocation and induces oxidative stress injury in mVSMCs

A: Lentiviral constructs encoding ZIKV NS proteins NS2a, NS3, and NS5, each tagged with EGFP to visualize transduction efficiency. B: Green fluorescence in NS2a-, NS3- and NS5-mVSMCs at 48 hpt. Scale bars: 200 μ m. C: Western blot analysis of ZIKV NS protein expression in NS2a-, NS3- and NS5-mVSMCs. D: RT-qPCR analysis of ZIKV NS mRNA expression in NS2a-, NS3- and NS5-mVSMCs. E: Effects of ZIKV NS proteins on key antioxidant regulatory genes in the Nrf2/Nrf1/KEAP1/GPX4 pathway. * P <0.05 and ** P <0.01 vs. MOCK. F: Oxidative stress markers in mVSMCs expressing NS2a, NS3, and NS5. ** P <0.01 vs. MOCK. G: Migration distance changes in NS2a-, NS3- and NS5-mVSMCs. ** P <0.01 vs. MOCK. H: Co-IP analysis of the interaction between Nrf2 and ZIKV NS3, together with western blot analysis of nuclear and cytoplasmic distribution of Nrf2. Cytoplasmic Nrf2 levels were normalized to GAPDH, and nuclear Nrf2 levels were normalized to Lamin B1. * P <0.05 and ** P <0.01 vs. nuclear Nrf2 in MOCK; # P <0.05 and ## P <0.01 vs. total Nrf2 in MOCK, and * P <0.05 and ** P <0.01 vs. cytoplasmic Nrf2 in MOCK.

was knocked down in mVSMCs. The CRISPR-Cas9 *Nrf2* knockout vector contained an RSV promoter, two guide RNAs (gRNAs) targeting upstream and downstream regions of the *Nrf2* gene, a Cas9 coding sequence, and an *mCherry* reporter for tracking lentiviral transduction. mVSMCs were transduced at an MOI of 20.0. Weak red fluorescence was first detected at 24 hpt, with fluorescence intensity increasing at 48 hpt and peaking at 72 hpt. Nearly all cells exhibited red fluorescence, similar to the empty vector control (Figure 7A), confirming efficient delivery and expression of the knockout construct and

successful establishment of an *Nrf2*-deficient mVSMC model (Nrf2⁻mVSMCs).

RT-qPCR confirmed significant suppression of *Nrf2* transcription in Nrf2⁻mVSMCs, with transcript levels reduced to less than 50% of those in mock controls (** P <0.01). Correspondingly, the expression levels of key antioxidant regulatory genes *KEAP1* and *GPX4* were also significantly down-regulated (** P <0.01), reflecting downstream effects of *Nrf2* loss (Figure 7B). ZIKV infection at MOIs of 1.0, 5.0, and 10.0 further weakened antioxidant gene expression in

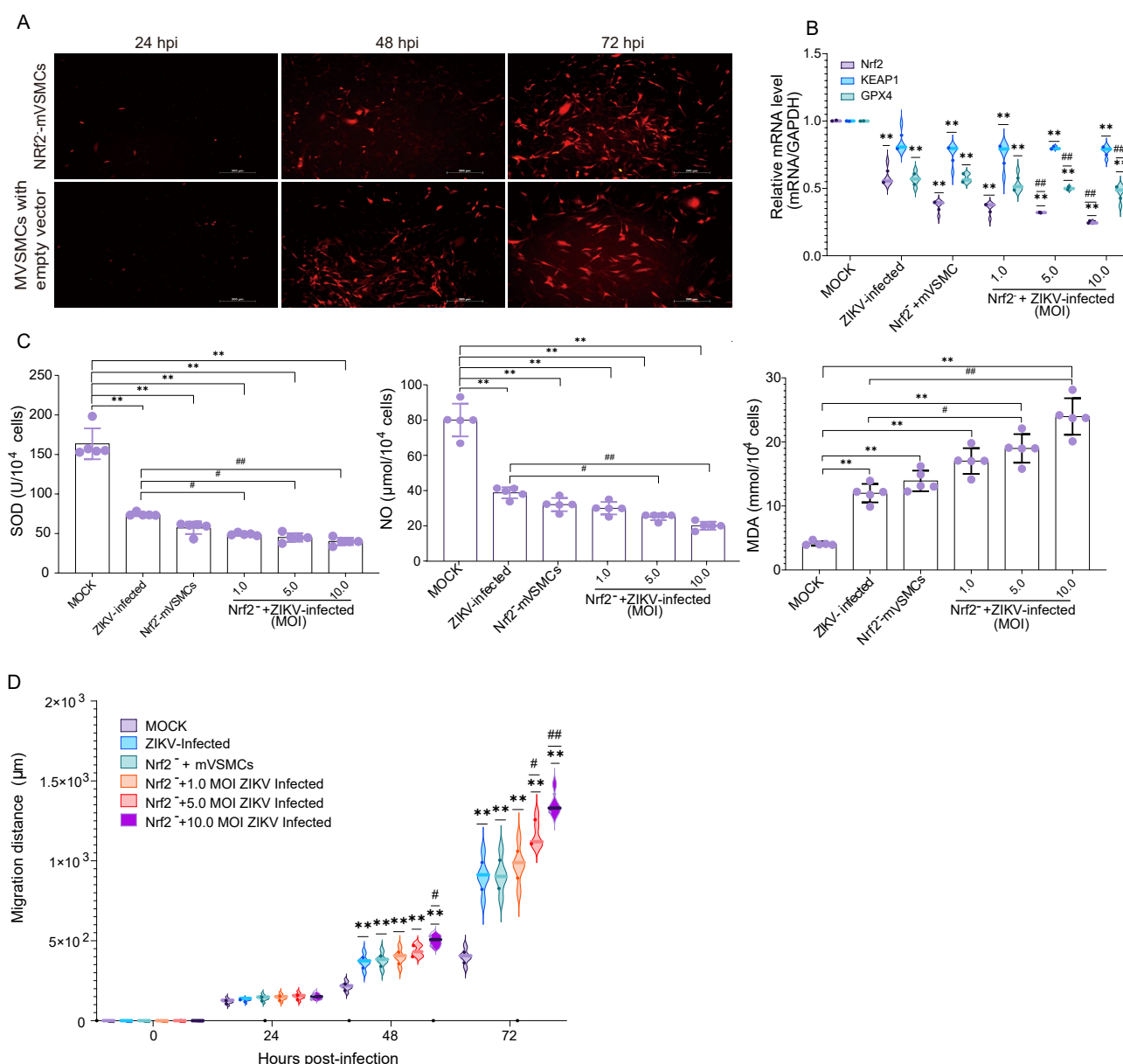


Figure 7 *Nrf2* gene knockout enhances oxidative damage and promotes migration in ZIKV-infected mVSMCs

A: Red fluorescence in mVSMCs transfected with the *Nrf2* knockout plasmid at 24, 48, and 72 hpt. Scale bars: 200 μm. B: RT-qPCR analysis showing down-regulation of antioxidant-related genes in both *Nrf2*-knockout mVSMCs and ZIKV-infected mVSMCs. ^{*}*P*<0.01 vs. MOCK, and [#]*P*<0.05 and ^{##}*P*<0.01 vs. ZIKV-infected group. C: *Nrf2* knockout reduces SOD and NO antioxidant activity and increases MDA accumulation. ^{*}*P*<0.05 and ^{**}*P*<0.01 vs. MOCK, and [#]*P*<0.05 and ^{##}*P*<0.01 vs. ZIKV-infected group. D: *Nrf2* knockout promotes mVSMC migration. ^{**}*P*<0.01 vs. MOCK, and [#]*P*<0.05 and ^{##}*P*<0.01 vs. ZIKV-infected group.

Nrf2⁻-mVSMCs. Notably, *Nrf2* and *GPX4* transcripts were significantly lower after infection at 5.0 and 10.0 MOI than after infection at 1.0 MOI (^{##}*P*<0.01), whereas *KEAP1* transcription remained largely unchanged (*P*>0.05; Figure 7B).

Loss of *Nrf2* markedly altered the redox state of mVSMCs. Compared with mock controls, *Nrf2*-deficient mVSMCs showed a significant reduction in intracellular SOD and NO levels and increased MDA accumulation (^{**}*P*<0.01; Figure 7C). ZIKV infection further exacerbated this oxidative imbalance. At 1.0 MOI, ZIKV significantly reduced SOD activity in *Nrf2*-deficient mVSMCs compared with ZIKV-infected control mVSMCs ([#]*P*<0.05), and stronger suppression was observed at 5.0 and 10.0 MOI (^{##}*P*<0.01). Similar trends were observed for NO levels, with significant reductions after infection at 5.0 and 10.0 MOI ([#]*P*<0.05 and ^{##}*P*<0.01, respectively). The loss of antioxidant capacity was accompanied by increased lipid

peroxidation. MDA levels were significantly higher in ZIKV-infected *Nrf2*⁻-mVSMCs than in ZIKV-infected control mVSMCs at 1.0 MOI ([#]*P*<0.05), with larger increases observed at 5.0 and 10.0 MOI (^{##}*P*<0.01; Figure 7C).

Migration assays showed that *Nrf2* knockout significantly enhanced mVSMC migration. *Nrf2*⁻-mVSMCs migrated significantly farther than mock controls at 48 hpt (^{*}*P*<0.05), and this difference became more pronounced at 72 hpt (^{**}*P*<0.01; Figure 7D). ZIKV infection further enhanced migration in *Nrf2*⁻-mVSMCs. At 48 hpi, *Nrf2*⁻-mVSMCs infected with ZIKV at 10.0 MOI showed significantly greater migration than ZIKV-infected control mVSMCs ([#]*P*<0.05). At 72 hpi, infection at 5.0 and 10.0 MOI continued to promote migration in *Nrf2*-deficient mVSMCs ([#]*P*<0.05 and ^{##}*P*<0.01, respectively; Figure 7D). These findings indicate that *Nrf2* loss weakens antioxidant defenses, amplifies ZIKV-induced

oxidative stress, and promotes migratory activation of mVSMCs.

Nrf2 overexpression alleviates ZIKV-induced oxidative damage in mVSMCs

Nrf2 is a key mediator of cellular antioxidant defense, suggesting that reinforcement of *Nrf2* may counteract ZIKV-induced oxidative injury in mVSMCs. To test this possibility, *Nrf2* was overexpressed in mVSMCs, and antioxidant signaling, redox balance, and migratory responses were assessed after ZIKV infection. The *Nrf2* lentiviral vector contained an RSV promoter, a full-length mouse *Nrf2* cDNA, and an *mCherry* reporter to verify successful transduction of mVSMCs. After lentiviral transduction at an MOI of 20.0, weak

red fluorescence was detected at 24 hpt, increased at 48 hpt, and peaked at 72 hpt, indicating near-complete transduction. Control cells transduced with EVP showed slightly stronger fluorescence at 24 and 48 hpt, but there was no significant difference at 72 hpt (Figure 8A), confirming successful generation of *Nrf2*-overexpressing mVSMCs (*Nrf2*⁺mVSMCs).

RT-qPCR analysis demonstrated that *Nrf2* and *GPX4* transcripts increased significantly in *Nrf2*⁺mVSMCs, by approximately 45% and 100%, respectively, compared with mock controls (***P*<0.01). ZIKV infection suppressed the expression of both genes; however, after infection at 1.0 and 5.0 MOI, *Nrf2* and *GPX4* levels in *Nrf2*⁺mVSMCs remained significantly higher than those in mock controls and ZIKV-infected control mVSMCs (##*P*<0.01). Only high-dose infection

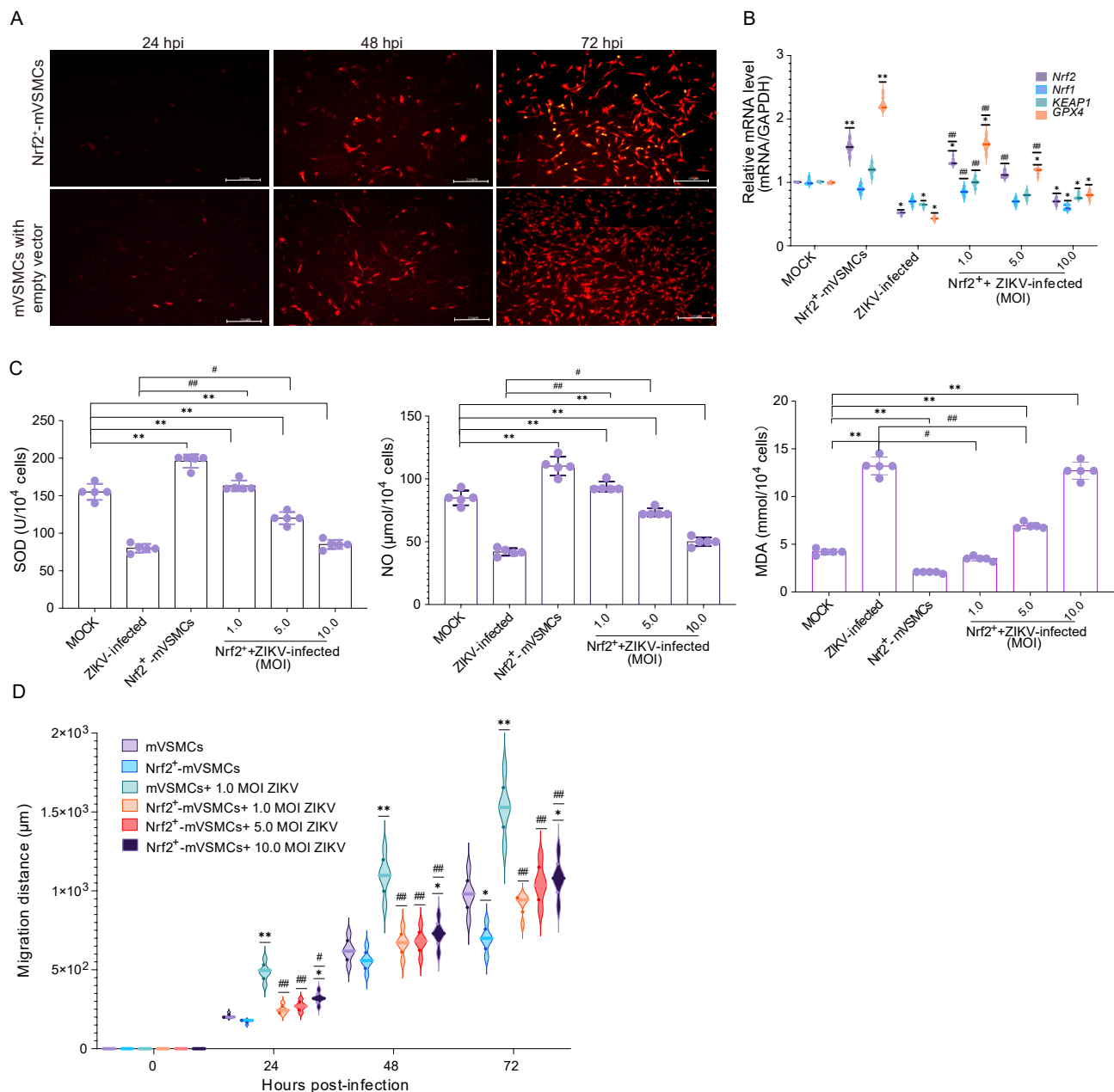


Figure 8 *Nrf2* overexpression alleviates ZIKV-induced oxidative damage in mVSMCs

A: Red fluorescence in mVSMCs transfected with the full-length *Nrf2* expression plasmid at 24, 48, and 72 hpt. Scale bars: 200 μm. B: RT-qPCR analysis showing up-regulation of antioxidant-related genes in *Nrf2*⁺mVSMCs and ZIKV-infected mVSMCs. ***P*<0.01 vs. MOCK, and #*P*<0.05 and ##*P*<0.01 vs. ZIKV-infected group. C: Overexpression of *Nrf2* increases SOD and NO antioxidant activity and decreases MDA accumulation. **P*<0.05 and ***P*<0.01 vs. MOCK, and #*P*<0.05 and ##*P*<0.01 vs. ZIKV infected. D: Overexpression of *Nrf2* significantly reduces mVSMC migration. ***P*<0.01 vs. MOCK, and #*P*<0.05 and ##*P*<0.01 vs. ZIKV-infected group.

at 10.0 MOI reduced *Nrf2* and *GPX4* expression below mock-control levels ($P < 0.05$), although expression remained higher than that in ZIKV-infected mVSMCs, albeit not significantly ($P > 0.05$; Figure 8B). *Nrf2* overexpression also alleviated ZIKV-induced inhibition of *Nrf1* and *KEAP1*. At 1.0 MOI, transcript levels of both genes in *Nrf2*⁺mVSMCs were significantly higher than in ZIKV-infected control cells ($P < 0.05$), with their expression levels only falling below mock levels after infection at 10.0 MOI ($P < 0.05$; Figure 8B).

Biochemical assays further showed that *Nrf2* overexpression strengthened intracellular antioxidant capacity. SOD and NO levels were significantly higher in *Nrf2*⁺mVSMCs than in mock controls ($P < 0.01$). Following ZIKV infection, SOD and NO levels remained higher in *Nrf2*⁺mVSMCs infected at 1.0 and 5.0 MOI than in ZIKV-infected controls ($P < 0.05$ and $^{***}P < 0.01$, respectively). Only infection at 10.0 MOI reduced these antioxidant mediators to levels comparable with those in ZIKV-infected control cells ($P > 0.05$). Conversely, accumulation of the oxidative product MDA was significantly reduced in *Nrf2*⁺mVSMCs. MDA levels were lower than those in mock controls under basal conditions ($P < 0.01$) and remained significantly lower in *Nrf2*⁺mVSMCs after ZIKV infection at 1.0 and 5.0 MOI than in ZIKV-infected control cells ($P < 0.05$ and $^{***}P < 0.01$, respectively). At 10.0 MOI, MDA levels became comparable between ZIKV-infected *Nrf2*⁺mVSMCs and ZIKV-infected control cells ($P > 0.05$; Figure 8C). Collectively, these findings indicate that *Nrf2* overexpression reinforces the *Nrf2*/KEAP1-ARE antioxidant axis, partially preserves *Nrf1*, *KEAP1*, and *GPX4* expression during ZIKV infection, restores SOD and NO levels, and limits lipid peroxidation. Collectively, these findings indicate that *Nrf2* overexpression reinforces the *Nrf2*/KEAP1-ARE antioxidant axis, partially preserves *Nrf1*, *KEAP1*, and *GPX4* expression during ZIKV infection, restores SOD and NO levels, and limits lipid peroxidation.

Migration assays revealed that *Nrf2* overexpression also reduced ZIKV-induced migratory activation. At 24, 48, and 72 hpi, migration distances were consistently lower in ZIKV-infected *Nrf2*⁺mVSMCs than in ZIKV-infected control mVSMCs across 1.0, 5.0, and 10.0 MOI ZIKV infection ($P < 0.05$ and $^{***}P < 0.01$, respectively; Figure 8D). These results indicate that *Nrf2* overexpression mitigates ZIKV-induced oxidative injury and restrains the promigratory VSMC phenotype through enhanced antioxidant defense.

DISCUSSION

Cardiovascular disease remains a leading cause of morbidity and mortality worldwide, yet many pathogenic drivers of vascular and myocardial injury have not yet been fully elucidated (Bredow et al., 2024; Moreno-Manuel et al., 2024). Viral infection has emerged as an important contributor to cardiovascular pathology, with effects that extend beyond acute inflammation and may persist after resolution of overt infection (Bork et al., 2024; Dong et al., 2025; Nguyen et al., 2025; Weinert et al., 2013). This delayed or sustained injury is particularly relevant for viruses traditionally classified as “self-limiting”, for which long-term cardiovascular consequences may have been underestimated in both clinical and basic research settings. SARS-CoV-2, HPV, influenza virus, and Coxsackievirus B have been linked to cardiovascular pathology, with evidence ranging from epidemiological associations with vascular disease risk to acute myocardial injury, thromboinflammatory complications, myocarditis, and longer-term cardiac remodeling (Dutta et al., 2024; Oseghale

et al., 2024; Sardari et al., 2021; Zhou et al., 2024). ZIKV has also been linked to cardiovascular abnormalities in adult patients and experimental murine models (Lim et al., 2020; Rossi et al., 2020). However, the vascular consequences of ZIKV infection and the molecular mechanisms that sustain ZIKV-associated vascular injury have not been systematically defined.

Self-limiting viral infections such as ZIKV involve two biologically distinct phases: an acute viremic phase characterized by direct vascular involvement and a post-clearance phase in which injury may persist despite loss of detectable circulating virus. To examine ZIKV-induced vascular damage across these phases, type I interferon receptor-deficient A129 mice were used to model injury under sustained viremia, while immunocompetent *M. leonina* macaques were used to evaluate vascular injury after clearance of circulating viral RNA. Results showed that ZIKV infection induced persistent and progressive vascular injury in both immunodeficient A129 mice and immunocompetent *M. leonina*. Although ZIKV is generally regarded as a self-limiting infection and circulating viral RNA can be cleared after the acute phase in immunocompetent hosts, viral RNA and protein remained detectable in vascular tissues. These findings indicate that retained viral components in the vasculature may provide a basis for prolonged vascular damage after apparent systemic viral clearance and highlight persistent vascular complications as a potential consequence of ZIKV infection.

Across both large and small vessels, ZIKV infection was associated with loss of vascular endothelial integrity, as well as VSMC hyperplasia and necrosis, disrupted medial organization, and phenotypic remodeling. VSMCs retain substantial plasticity and can transition between contractile and synthetic states in response to vascular injury. In healthy vessels, the α -SMA-positive contractile phenotype predominates, whereas injured vessels show expansion of the OPN-positive synthetic phenotype (Xiang et al., 2024). In the present study, ZIKV-injured vessels exhibited reduced α -SMA expression and increased OPN expression, indicating a shift from a contractile phenotype toward a synthetic, remodeling-associated state. These structural changes are consistent with functional deterioration, including impaired vascular reactivity and increased vascular permeability, as shown by reduced vasoconstrictive and vasodilatory responses *in vitro* and increased FITC-dextran accumulation *in vivo*. Notably, the severity of injury differed among vascular territories, with the pulmonary artery showing the most pronounced remodeling and the ascending aorta displaying only mild involvement. This regional pattern may reflect local hemodynamic differences, as higher shear stress and laminar flow in the ascending aorta may limit viral adhesion, endothelial entry, and subsequent tissue injury. These findings suggest that local hemodynamic conditions may influence ZIKV-vessel interactions and contribute to regional differences in vascular injury.

Transcriptome profiling of ZIKV-injured vessels identified antioxidant defense as a major disrupted functional axis. Among the most significant DEGs, antioxidant-related components, including *GPX4*, *KEAP1*, *Nrf2*, *Nrf1*, and *SOD1*, were markedly down-regulated, whereas the inflammation-related gene *S100A9* was up-regulated. These findings indicate that, in addition to inflammatory injury, disruption of antioxidant defense, especially the *Nrf2*/KEAP1 pathway, is a key contributor to ZIKV-induced vascular damage. Consistent

with this, TEM analysis revealed prominent mitochondrial abnormalities in vessels from ZIKV-infected A129 mice and *M. leonina*, as well as in ZIKV-infected mVSMCs, including mitochondrial swelling, cristae loss, and outer membrane disruption. Because mitochondria represent a major intracellular source of ROS, mitochondrial injury may further amplify oxidative stress during ZIKV-associated vascular damage. However, this study primarily focused on how ZIKV perturbs host antioxidant defense, particularly the Nrf2/KEAP1/GPX4 pathway, rather than defining the hierarchical relationship between mitochondrial dysfunction and antioxidant pathway inhibition. Therefore, although the ultrastructural findings support the presence of mitochondrial damage during ZIKV infection, the precise contribution of mitochondrial ROS and its mechanistic connection with impaired Nrf2 signaling require further investigation.

To further define the role of antioxidant dysregulation in ZIKV-associated vascular injury, primary mVSMCs were used to establish an *in vitro* infection model. At 72 hpi, TEM analysis detected abundant virions within mVSMCs, predominantly localized to the ER, which displayed marked dilation and proliferation. Immunostaining demonstrated robust intracellular ZIKV E protein signals, and RT-qPCR confirmed the presence of viral RNA in infected cells. These findings indicate that ZIKV effectively infects mVSMCs and induces substantial ultrastructural injury. Infection also enhanced mVSMC migration in a viral load-dependent manner, suggesting that ZIKV promotes a remodeling-associated vascular smooth muscle phenotype. Consistent with this phenotype, ZIKV infection suppressed Nrf2, KEAP1, Nrf1, and GPX4 at both mRNA and protein levels, reduced intracellular SOD and NO levels, and increased MDA accumulation. Together, these data indicate that ZIKV disrupts the Nrf2/KEAP1/GPX4 antioxidant axis and drives oxidative stress in mVSMCs, thereby contributing to vascular dysfunction.

Among the ZIKV NS proteins (NS2a, NS3, and NS5) examined, NS3 exerted the strongest inhibitory effect on intracellular antioxidant regulation. NS3 expression in mVSMCs reduced Nrf2-related antioxidant gene expression, decreased SOD and NO levels, increased MDA accumulation, and enhanced mVSMC migration, closely recapitulating the effects of whole-virus infection. Thus, these findings identify NS3 as a major viral determinant of oxidative stress-associated VSMC injury. Similar effects have been reported for HCV NS3, suggesting that NS3-mediated redox disruption may represent a conserved pathogenic feature among members of the family *Flaviviridae*. Based on the dominant effect of NS3 in this model, subsequent mechanistic analyses focused on NS3 rather than the other viral proteins.

Oxidative stress-induced cellular injury is a well-recognized pathogenic mechanism in viral infection, and the Nrf2/KEAP1/GPX4 axis is a principal pathway governing cellular antioxidant defense (Heo et al., 2024). Nrf2 activation requires nuclear translocation and binding to AREs, which initiate transcription of antioxidant genes. Under physiological conditions, KEAP1 retains Nrf2 in the cytoplasm and promotes ubiquitination-dependent degradation, whereas under oxidative stress, Nrf2 dissociates from KEAP1 and translocates to the nucleus to activate antioxidant responses (Cai et al., 2023). To determine whether ZIKV NS3 interferes with this regulatory process, Co-IP was used to assess the association between NS3 and Nrf2. NS3 was detected in Nrf2 immunoprecipitates, and the amount of Nrf2-associated NS3 increased with viral MOI, indicating a positive association

between infection level and NS3-Nrf2 interaction. Because nuclear Nrf2 is required for ARE-dependent transcription, cytosolic and nuclear fractions were analyzed separately. ZIKV infection caused a marked dose-dependent reduction in nuclear Nrf2, accompanied by decreases in cytosolic and total Nrf2. These findings suggest that NS3 associates with Nrf2 and restricts Nrf2 nuclear accumulation, thereby suppressing Nrf2-mediated antioxidant responses in mVSMCs.

These findings identify a mechanistic link between a viral NS protein and disruption of host antioxidant defense. Notably, total Nrf2 abundance also decreased as viral load increased, indicating that ZIKV infection may affect Nrf2 regulation beyond impaired nuclear translocation. Under physiological conditions, cytoplasmic Nrf2 is tightly regulated by KEAP1-dependent ubiquitination and degradation; under oxidative stress, Nrf2 stabilization enables nuclear translocation and activation of antioxidant gene transcription. The present data raise the possibility that, following ZIKV infection, NS3-associated impairment of Nrf2 nuclear translocation may promote cytoplasmic retention of Nrf2 and thereby enhance its degradation. In parallel, the dose-dependent decline in Nrf2 mRNA suggests that transcriptional down-regulation may also contribute to the reduction in total Nrf2 abundance. Further work is required to distinguish the relative contributions of impaired nuclear trafficking, enhanced protein degradation, and transcriptional suppression to ZIKV-mediated Nrf2 loss.

The functional importance of Nrf2 in ZIKV-induced mVSMC injury was further supported by complementary loss- and gain-of-function analyses. Nrf2 deficiency reduced the expression of Nrf2 and key components of the Nrf2/KEAP1 antioxidant axis and intensified ZIKV-induced redox imbalance, as shown by stronger depletion of SOD and NO, greater accumulation of oxidative byproducts, and increased cell migration. In contrast, Nrf2 overexpression strengthened antioxidant pathway activity, attenuated ZIKV-mediated repression of antioxidant genes, restored intracellular SOD and NO levels, reduced oxidative byproduct accumulation, and limited the migratory response after ZIKV challenge. These results identify Nrf2 as a pivotal determinant of antioxidant protection in ZIKV-infected VSMCs. Loss of Nrf2 weakens the Nrf2/KEAP1 axis, amplifies oxidative stress, and increases susceptibility to ZIKV-mediated injury, whereas Nrf2 overexpression strengthens antioxidant capacity and mitigates cellular damage.

In summary, ZIKV induces vascular injury during viremia and after clearance of circulating viral RNA, likely through retention of viral components in vascular tissues. Mechanistically, ZIKV NS3 associates with host Nrf2, restricts nuclear accumulation of Nrf2, and suppresses ARE-dependent transcription, thereby reducing the expression of key antioxidant genes within the Nrf2/KEAP1 axis. This impairment compromises antioxidant defense, lowers intracellular SOD and NO levels, increases oxidative byproduct accumulation, and promotes sustained oxidative stress, ultimately contributing to VSMC dysfunction and vascular structural and functional impairment. In the present study, transcriptomic, biochemical, and functional analyses consistently implicated the Nrf2-dependent antioxidant pathway in this process. Nevertheless, oxidative vascular injury is likely multifactorial, and involvement of additional oxidative stress-related or inflammatory pathways, including NF- κ B and NLRP3 signaling, cannot be excluded. Because these pathways were not directly examined in the present study, their contribution to ZIKV-induced VSMC migration and vascular injury warrants further investigation. These findings

broaden current understanding of ZIKV pathogenesis and suggest that vascular sequelae may persist even after resolution of acute infection. Further studies are needed to define the role of mitochondrial ROS, clarify the precise molecular basis of the NS3-Nrf2 interaction, and determine whether similar mechanisms occur in other vascular beds and clinically relevant settings.

Significance and future directions

This study identified NS3-mediated oxidative stress as a novel mechanism through which ZIKV promotes vascular and systemic damage. Given that oxidative injury is a common feature of viral pathogenesis, these findings not only provide new therapeutic avenues for ZIKV-related diseases but may also serve as a conceptual basis for developing treatments against other flaviviruses, including dengue virus and West Nile virus.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

R.H.Y. designed the experiments and was involved in all experimental procedures. W.C.G. performed vascular function assays and contributed to molecular biology experiments. L.Z. was responsible for additional molecular analyses. T.Z.S. and R.H.L. carried out the *M. leonina* experiments. T.Z. and L.M.Y. conducted transcriptomic and bioinformatics analyses. R.H.Y. drafted the initial manuscript and revised subsequent versions. Y.T.Z. and P.C. supervised the experimental design and manuscript preparation and critically revised the manuscript. P.C., Y.T.Z., and C.B.Z. provided financial support for the study. All authors read and approved the final version of the manuscript.

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