Oligodendrocytes are susceptible to Zika virus infection in a mouse model of perinatal exposure: Implications for CNS complications

Verena Schultz1 | Jennifer A. Barrie1 | Claire L. Donald2 | Colin L. Crawford1 | Margaret Mullin1 | Thomas J. Anderson3 | Tom Solomon4 | Susan C. Barnett1 | Christopher Linington1 | Alain Kohl2 | Hugh J. Willison1 | Julia M. Edgar1

1College of Medical, Veterinary, and Life Sciences, Institute of Infection, Immunity and Inflammation, Glasgow, UK
2MRC-University of Glasgow Centre for Virus Research, Glasgow, UK
3School of Veterinary Medicine, College of Medical, Veterinary and Life Sciences, Glasgow
4Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK

Correspondence
Julia M. Edgar, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, 120 University Place, Glasgow, G12 8TA, UK.
Email: julia.edgar@glasgow.ac.uk

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Abstract
Some children with proven intrauterine Zika virus (ZIKV) infection who were born asymptomatic subsequently manifested neurodevelopmental delays, pointing to impairment of development perinatally and postnatally. To model this, we infected postnatal day (P) 5–6 (equivalent to the perinatal period in humans) susceptible mice with a mammalian cell-propagated ZIKV clinical isolate from the Brazilian outbreak in 2015. All infected mice appeared normal up to 4 days post-intraperitoneal inoculation (dpi), but rapidly developed severe clinical signs at 5–6 dpi. All nervous tissue examined at 5/6 dpi appeared grossly normal. However, anti-ZIKV positive cells were observed in the optic nerve, brain, and spinal cord; predominantly in white matter. Co-labeling with cell type specific markers demonstrated oligodendrocytes and astrocytes support productive infection. Rarely, ZIKV positive neurons were observed. In spinal cord white matter, which we examined in detail, apoptotic cells were observed in the optic nerve, brain, and spinal cord; predominantly in white matter. Co-labeling with cell type specific markers demonstrated oligodendrocytes and astrocytes support productive infection. Rarely, ZIKV positive neurons were observed. In spinal cord white matter, which we examined in detail, apoptotic cells were evident; the density of oligodendrocytes was significantly reduced; and there was localized microglial reactivity including expression of the NLRP3 inflammasome. Together, our observations demonstrate that a clinically relevant ZIKV isolate can directly impact oligodendrocytes. As primary oligodendrocyte cell death can lead later to secondary autoimmune demyelination, our observations may help explain neurodevelopmental delays in infants appearing asymptomatic at birth and commend lifetime surveillance.

KEYWORDS
apoptosis, inflammasome, neurodevelopmental delay, white matter

1 | INTRODUCTION

Zika virus (ZIKV; Flaviviridae, Flavivirus) infection can cause developmental malformations in children born to infected mothers; so-called congenital Zika syndrome (CZS; Araujo, Silva, & Araujo, 2016). Microcephaly is the best-known neurological abnormality associated with CZS (Driggers et al., 2016; Li et al., 2016; Mlakar et al., 2016; Rasmussen, Jamieson, Honein, & Petersen, 2016; Tang et al., 2016). However, a vast range of other neurological signs and symptoms have been reported including visual and hearing deficits, seizure activity,
hypertonicity, spasticity, hyperreflexia, contractures, dysphagia, nystagmus, and feeding difficulties (Walker et al., 2019). In addition, recent reports demonstrate that children with congenital ZIKV infection who appeared asymptomatic at birth, subsequently manifest progressive neurodevelopmental delays (Familiar et al., 2020; Mulkey et al., 2020; Peçanha et al., 2020; Pimentel et al., 2021).

The CNS is susceptible to congenital infection through vertical transmission during the entire gestational period (Brasil et al., 2016); however, infection in the earlier weeks of the embryonic phase generally results in more severe malformations (Chimelli et al., 2017 and reviewed in Saad et al., 2018). This likely reflects the susceptibility of neural progenitor cells (NPCs), resulting in changes in gene expression, impaired proliferation and migration, and apoptotic cell death (Cugola et al., 2016; Garcez et al., 2017; Li et al., 2016; Souza et al., 2016; Tang et al., 2016). Nonetheless, CNS abnormalities have been reported following infection as late as 39 weeks of gestation, demonstrating that later developmental processes are also susceptible (Brasil et al., 2016).

In developing humans, neurogenesis largely occurs during the first semester (Kostović, Sedmak, & Judas, 2019) and following this, precursors contribute to gliogenesis during which astrocytes and oligodendrocytes are produced (DeAzevedo et al., 2003; Rash et al., 2019). Myelin production by oligodendrocytes is well-underway in the third trimester (Kostović et al., 2019; Poduslo & Jang, 1984) and is largely complete by 2 years of postnatal life (Kinney & Volpe, 2018). Although it is not yet known if ZIKV infection directly affects developmental myelination in humans, other human and/or murine neurotropic viruses including Semliki Forest virus (SFV), Theiler's virus, cytomegalovirus (CMV), and human immunodeficiency virus (HIV-1) are known to induce demyelination (Pohl et al., 2011; Traka et al., 2010). Further, our recent results demonstrated that ZIKV-infected mice had impaired proliferation and migration, and apoptotic cell death (Cugola et al., 2016). Nonetheless, CNS abnormalities have been reported following infection as late as 39 weeks of gestation, demonstrating that later developmental processes are also susceptible (Brasil et al., 2016).

To determine if oligodendrocytes are susceptible to ZIKV during myelination in vivo, we infected P5/6 mice with ZIKV, some days after CNS myelination commences at P1. We mainly used mice lacking the type I interferon receptor (Ifnar1 knockout mice), which recapitulate aspects of human ZIKV infections and disease (Miner & Diamond, 2017), to approximate ZIKV antagonism of the type I interferon (IFN) response in humans (Serman & Gack, 2019). Immunohistochemistry and cell quantification revealed that oligodendrocytes were particularly vulnerable. The functional outcome could not be determined, as mice had to be euthanized due to rapid development of severe clinical disease. However, in other contexts, oligodendrocyte death is followed by some delay, by loss of compact myelin (Pohl et al., 2011; Traka et al., 2010). Further, our data may explain neurodevelopmental delays in some congenitally infected infants and warn of susceptibility to later autoimmune mediated demyelination (Traka, Podoljil, McCarthy, Miller, & Popko, 2016).

2 | METHODS AND MATERIALS

2.1 | Mice

Ifnar1 knockout (KO; type I interferon receptor deficient) and wild type (WT) mice of both sexes, on a 129S7/SvEvBrdBkl-Hprt-m2 background (B&K Universal) were maintained in Tecniplast 1284L Blue line IVC cages, in a 12 hr light/dark cycle and provided with sterile food and water ad libitum. All animal studies were approved by the Ethical Committee of the University of Glasgow and licensed by the UK Home Office (Project Licence numbers PPL P78DD6240 and P9722FD8E). Genomic DNA was extracted from ear biopsies using a modified protocol (Truett et al., 2000). Briefly, ear notches were heated to 95°C for 90 min in 50 mM NaOH. Following neutralization with 10% v/v 1 M Tris pH 5, the resultant solution was vortexed to release DNA and 2 μl was used for PCR as described previously (Cumberworth et al., 2017).

2.2 | Cell lines and Zika virus

As indicated in Section 3, we used both low passage ZIKV, isolated from a patient in Brazil with febrile illness, ZIKV/H. sapiens/Brazil/PE243/2015 (GenBank accession number KX197192; abbreviated to ZIKV PE243) and the prototypic African ZIKV isolate, MR766/1947/UGanda (abbreviated to ZIKV MR766). The origin and history of ZIKV PE243 have been previously described (Donald et al., 2016). ZIKV MR766 was obtained from BEI Resources, NIAID, NIH: Genomic RNA from ZIKV Virus, MR766, NR-50085 (Zmuko et al., 2018). The viral stocks were generated using either the Vero E6 cell line (ATCC, CCL-81), A549/BVDV-NPro cell line (kindly gifted by R.E. Randall, University of St Andrews, UK) or the Aedes albopictus-derived C6/36 cell line. Stocks were titred using the A549/BVDV-NPro cell line as described previously (Donald et al., 2016). The Vero E6 cell line was grown at 37°C with 5% CO2 in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), and penicillin–streptomycin (final concentration 100 units/ml and 100 μg/ml, respectively, Life Technologies). The A549/BVDV-NPro cell line was grown under the same conditions but with the addition of puromycin (2 μg/ml, Invivogen). The C6/36 cell line was grown at 28°C with no additional CO2 in L-15+ Glutamax (Life Technologies) supplemented with 10% FBS, penicillin–streptomycin (final concentration 100 units/ml and 100 μg/ml, respectively) and 10% Trypsine Phosphate Broth (TPB, Life Technologies).

2.3 | Infection of mice with Zika virus

At P5/6, pups of both sexes were removed from the dams and administered an intraperitoneal (ip) injection with 7.5 × 10^4 to 7.5 × 10^5 (Table S1) plaque forming units (PFU) of ZIKV per animal, or with an equivalent volume of vehicle only (cell culture media), using a 1 ml syringe or 5 μl Hamilton syringe. Each litter received both virus and vehicle, providing littermate controls for virally infected animals. Pups were randomly assigned to each of the two groups. Six Ifnar1 knockout litters and two wild type litters were used (Table S1).

2.4 | Examination and assessment of mice

Mice were examined twice daily following inoculation. Their behavior was assessed visually by one or other of two experienced observers while the pups were in the cage together with the dam, or out of the
cage without the dam. Videos of the mice were examined by TJA (veterinary neurologist) who provided clinical descriptions. Pups were humanely killed at timepoints indicated in Table 1, usually being when clinical signs were observed, or as age-matched controls to sick animals. Most pups were immersion fixed in approximately 100 ml 8% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 2 hr. Tissues were permeabilized using 0.1% TritonX in PBS, 10 min. Sections were washed three times in PBS and once in distilled water, then mounted with CitifLUOR (Electron Microscopy Science, Hatfield, Pennsylvania) or Mowiol mounting medium [4.2% glycerol (w/v), 0.4% Mowiol 4–88 (w/v)] (Calbiochem, San Diego, California). 2.1% 0.2 M Tris pH 8.5 (v/v)] with DAPI (1 ng/ml).

### 2.5 Tissue preparation and immunohistochemical staining

The brain, spinal cord, and optic nerve were dissected and placed for further fixation in 4% PFA in PBS overnight. The tissue was then transferred into 20% sucrose in PBS until it sank (usually overnight), embedded in Tissue-Tek OCT medium (Sakura Europe), and rapidly frozen in liquid nitrogen chilled isopentane. Optic nerves were frozen flat between discs of frozen OCT (approximately −23°C). Transverse sections of forebrain and cervical and lumbar spinal cord, and longitudinal sections of optic nerves, were cut at 12-μm thickness and collected on plus charged slides (VWR or Waldemar Knittel) at 96-μm intervals. Sections were stored in a −20°C freezer until required. Slides were allowed to reach room temperature before they were removed from their storage box, then washed in PBS to remove OCT. Tissues were permeabilized using −20°C ethanol, 10 min, −20°C Methanol, 10 min (for CD3), or 0.5% TritonX in PBS, 10 min (for CD68), then sections were washed in PBS, blocked in 10% normal goat serum (NGS) in PBS or 10% horse serum and 1% BSA in PBS (HS-BSA) for 1 hr. Primary antibodies (below) were applied in 10% NGS or HS-BSA overnight at 4°C. After thorough washing in PBS, secondary antibodies (below), diluted 1:1,000 were applied for 1 hr at room temperature. Sections were washed three times in PBS and mounted with CitifLUOR (Electron Microscopy Science, Hatfield, Pennsylvania) or Mowiol mounting medium [4.2% glycerol (w/v), 0.4% Mowiol 4–88 (w/v)] (Calbiochem, San Diego, California). 2.1% 0.2 M Tris pH 8.5 (v/v)] with DAPI (1 ng/ml).

Primary and secondary antibodies were: mouse IgG1 anti-ZIKV Envelope protein (clone 0302156 Aalto Bio; 1 in 500); mouse IgG2a anti-OLIG2 (MABN50, clone 211F1.1, Millipore; 1 in 200); mouse IgG2b anti-APC (clone CC1, NB600-1021 Novus biologicals; 1:200); rabbit IgG anti-NeuN (ABN78 Millipore; 1 in 750), rabbit IgG anti-S100B (PA5 78161, Invitrogen; 1 in 400), rabbit IgG anti-GFAP (Z0334, Dako; 1 in 1,000); rabbit IgG lba1 antibody (O19-19741, Wako/Alpha laboratories; 1 in 500), rat IgG anti-MBP (MCA409S AbD, Serotec; 1 in 500); mouse IgG1 SMI31 anti-phosphorylated heavy (H) and medium (M) chain neurofilament (NF) (NF-M and NF-H; 1 in 1,000); rat IgG anti-CD3 (MCA1477, BioRad; 1 in 200); rat IgG anti-CD68 (MCA1957GA); rabbit IgG anti-cleaved caspase 3 (Ab49822, Abcam, 1 in 200); rabbit IgG anti-NLRP3 (NB1-77080, Novus Biologicals, 1 in 100); goat anti-mouse IgG1 (A21211 and A21125); goat anti-rat IgG (A11006 and A11007); goat anti-rabbit IgG (A11008 and A11037); goat anti-mouse IgG2a (A21135); or goat anti-mouse IgG2b (A21145), all secondaries, conjugated with Alexa 488, Alexa 594, or Alexa 647 (Invitrogen; all 1 in 1,000).

### 2.6 Microscopy and cell quantification

To quantify cells across spinal cord transverse sections, images of immunostained nervous tissue were captured, as illustrated in
Figure 5, at ×40 magnification using an Olympus IX70 microscope with standard epifluorescence optics and Image Pro Plus 6 software. Cell counts were made in areas of interest (AOI) of 44,835 μm² (cell-type specific marker and anti-ZIKV envelope protein) or 5,400 μm² (DAPI +ve nuclei). The experimenter was blinded to the conditions and the field of view was selected in the blue channel (DAPI) to avoid biased selection. One to four sections per spinal cord, at least 50-μm apart, were analyzed (2–10 images/section). To quantify the proportion of ZIKV +ve cells that were CC1 +ve, or cleaved caspase 3 +ve cells that were ZIKV and/or CC1 +ve, images were captured in four channels at sites where ZIKV +ve cells were present, at ×20 magnification, using a Zeiss Axio Imager M.2 with an AxioCam MRm and Zen 2012 blue edition (version 1.1.2.0). Up to three sections, 50-μm apart, were imaged of both cervical and lumbar spinal cord, from two animals (1–3 AOI per section). Representative images for illustration were obtained using the Olympus BX51 fluorescence microscope and Ocular software (QImaging); a Zeiss Axio Imager M.2 with an AxioCam MRm and Zen 2012 blue edition (version 1.1.2.0); or a Zeiss LSM 880 inverted confocal microscope and Zen Black software.

2.7 | Quantification of myelin and axon volumes

Fluorescence images of myelin basic protein (MBP; labeled in green) and phosphorylated H-neurofilament and M-neurofilament (NF; labeled in red) were captured of the cervical and lumbar spinal cord white matter (input images). These were transformed in Cell Profiler (Jones et al., 2008) to binary images (output images) and the total pixels per AOI, red pixels per AOI and green pixels per AOI were quantified to provide a readout of the relative volumes occupied by myelin or axons; being red or green pixels as a percentage of all pixels, as described previously (Bijland et al., 2019).

2.8 | Resin sections and electron microscopy

At 4 dpi, mice were rapidly perfusion fixed in 4% paraformaldehyde, 5% glutaraldehyde in cacodylate buffer and tissue was processed, stained, and imaged as described (Edgar et al., 2020).

2.9 | Statistical analysis

Analyses were performed using Graphpad Prism 8.3.0 software (GraphPad Software Inc., San Diego, California). The p values ≤0.05 were considered significant. An unpaired two-tailed Student’s t-test was used to compare cell densities between mock-infected (n = 3 independent animals) and PE243 ZIKV-infected (n = 3 independent animals) Ifnar1 knockout animals at 5 days post-infection with ZIKV.

3 | RESULTS

3.1 | Neonatally infected mice develop rapidly progressing disease

Experimental studies on ZIKV infection in mice have used a variety of mouse strains, ages of infection, viral strains, and viral propagation protocols. Here we infected P5/6 (equivalent to the perinatal period in humans, with respect to myelination) Ifnar1 knockout mice, in which ZIKV replicates efficiently (Lazear et al., 2016), with clinically relevant ZIKV PE243. We used mammalian cell-propagated virus to emulate viral replication in somatic cells following mosquito bite. For comparison with other studies, we also examined a small number of identically housed wild type mice on the same A129 background, and a small number of animals of both genotypes infected with mammalian cell-propagated ZIKV MR766, a mouse brain-passaged isolate (Dick, 1952) from a sentinel monkey in the Zika Forest (Table S1).

Ifnar1 knockout mice infected with ZIKV PE243 developed clinical signs and died or had to be euthanized at 5 or 6 dpi. Signs included wide stance and failure to bear body weight, flaccid tail, urinary retention, weakness or paralysis of hindlimbs, or failure of righting (Figure 1). The progression of clinical signs occurred rapidly, where some mice showing no, or only very mild signs in late afternoon, were found moribund or dead the following morning. Over and above the clinical neurological phenotype, an overwhelming viral infection and systemic antiviral inflammatory response may have contributed to the moribund state and early death in some mice, but this was not investigated further.

FIGURE 1 Ifnar1 knockout mice developed clinical signs 5–6 dpi ZIKV PE243. Sequential frames from videos of one mock-infected animal and its three ZIKV PE243 infected littersmates. Clinical signs included failure to bear weight, weakness or paralysis of hindlimbs, flacid tail and failure to right. Black arrows in the lower images indicate abnormal positioning of the back, abdomen, tail and hindlimbs. White arrows indicate failure of righting [Color figure can be viewed at wileyonlinelibrary.com]
In contrast, only one of four wild type mice infected with ZIKV PE243 displayed signs of disease, occurring at 9 dpi. Three of three Ifnar1 knockout mice (two litters) infected with ZIKV MR766 became severely clinically compromised and were found dead or had to be euthanized at 4–5 dpi. Two of four wild type mice (two independent litters) infected with the same isolate showed clinical signs at 6 or 7 dpi and were euthanized. No mock-infected littermate of either litters) infected with the same isolate showed clinical signs at 6 or 7 dpi. Clinical observations in infected mice are summarized in Table S1. Numbers of mice, strains, viral isolates, and PFUs are summarized in Table S1.

In summary, all infected Ifnar1 knockout mice maintained beyond 4 dpi, succumbed to disease, independent of the viral isolate. In wild type mice, the response to infection was variable, irrespective of the viral isolate.

### 3.2 Zika virus positive cells are present as clusters in the CNS

Following infection at P5/6 with ZIKV PE243 (19 mice; six litters), seven infected and six mock-infected Ifnar1 knockout littersmates (two independent litters) were culled at 4 dpi without overt clinical signs. Using immunohistochemistry on CNS tissue of three infected and three control mice, we found small clusters of ZIKV +ve cells in all CNS areas examined, being cervical spinal cord, cerebral cortex (Figure 2), lumbar spinal cord, and cerebellum (Figure S1). ZIKV +ve cells were observed most frequently in white matter regions including the ventrolateral columns (Figure 2a–c) and dorsal columns of the spinal cord, the cerebellum, and the brain stem. Less frequently, we observed ZIKV +ve cells in gray matter (Figure 2d). Mock-infected littersmates appeared negative for anti-ZIKV (Figure 2a,b).

### 3.3 The CNS appears grossly normal in clinically affected ZIKV-infected animals

To determine whether CNS changes might contribute to clinical signs, we used immunohistochemical staining of nervous tissue of clinically affected mice (5 or 6 dpi) to examine localization and frequency of infected cells and overall tissue integrity. As at 4 dpi, ZIKV +ve cells were distributed heterogeneously throughout the nervous system, particularly as clusters in white matter of the spinal cord (Figure 3a,b and Figure S1) and cerebellum (Figure S1). Due to these observations and because clinical signs (urinary retention and hindlimb weakness) suggested spinal cord involvement, we examined the cervical and lumbar cord in more detail. Grossly, the overall appearance of the spinal cord at 5/6 dpi, including axons, myelin and astrocytes was comparable to that of mock-infected animals (Figure 3a,b). Furthermore, quantification of immunohistochemical staining revealed similar relative volumes of myelin (MBP staining), axons (neurofilament staining), and astrocytes (GFAP staining), compared with mock-infected controls (Figure 3c–e).

### 3.4 Zika virus-infection leads to cell death

Although the CNS appeared grossly normal despite the severe clinical signs, we next asked whether ZIKV-infection caused localized cell death. At 4 dpi, when mice appeared clinically unremarkable, we found cleaved caspase 3 +ve cells localized to regions containing ZIKV +ve cells (magenta arrowheads Figure 4a). To determine if the dying cells were oligodendrocytes, which are highly abundant in white matter, we co-labeled tissue with antibody clone CC1 [a marker that labels oligodendrocytes but not oligodendrocyte progenitor cells (OPCs)], anticleaved caspase 3 and anti-ZIKV (Figure 4b, showing single z plane confocal images of dorsal and ventrolateral spinal cord white matter). We found cells labeled only with anti-cleaved caspase 3 (Figure 4 b, magenta arrowhead); some also with anti-ZIKV [52 (±27.94 SD) % of cleaved caspase 3 +ve cells; 63 cells observed in sections of cervical and lumbar cord from 2 animals; Figure 4b, green arrowhead], rare cells positive only for CC1 and cleaved caspase 3 (Figure 4b, white arrowheads); and some positive for all three markers (Figure 4b, yellow arrowhead [16 (±4.82 SD) % of cleaved caspase 3+ ve cells, 63 cells observed in sections of cervical and lumbar cord from two animals]. Some ZIKV +ve cells (green arrowheads) and pyknotic or karyorrhxic nuclei (white arrows) were not co-labeled with either anticleaved caspase 3 or antibody CC1. In resin sections obtained from a separate litter, we observed pyknotic nuclei in spinal cord white matter of infected mice (Figure 4c, white arrows) interspersed amongst healthy-appearing cell nuclei. Electron microscopy confirmed the presence of dying cells (Figure 4d, cell 1) interspersed amongst healthy-appearing cells (Figure 4d, cells 2–4), including healthy-appearing (Figure 4e) and dying (Figure 4f–h) oligodendrocytes. In summary, dying cells, including early myelinating oligodendrocytes, were observed in white matter of ZIKV PE243 infected mice.

### 3.5 Oligodendrocytes are particularly vulnerable following Zika virus infection

As shown in Figures 2–4, most ZIKV +ve cells appeared to be in white matter. We confirmed this by quantifying the proportion of ZIKV +ve cells in cervical and lumbar cord white or gray matter (Table 2). Next, we used co-labeling with anti-ZIKV and cell type specific markers to determine which cell types support productive ZIKV infection. We found all major neural cell types, being neurons (Figure 5a), oligodendrocytes (Figure 5b), astrocytes (Figure 5c), and microglia/macrophages (Figure 5d) could be productively infected. We did not examine OPCs because the fixation required to inactivate ZIKV was not compatible with antibody staining for NG2, a classical marker for OPCs.

To determine if cell death observed at 4 dpi (Figure 4) led to a significant reduction in cell densities, we quantified individual cell types in cervical and lumbar spinal cord sections in animals at 5/6 dpi. The density of NeuN +ve neurons was similar in mock-infected and ZIKV-infected animals (gray bars, Figure 5e,f). In cervical cord white matter,
densities of OLIG2 +ve oligodendroglia (oligodendrocytes and OPCs) tended to be reduced in infected mice compared with mock-infected controls, although the difference was not significant (Figure 5g; \( p = .058 \)). However, the density of CC1 +ve oligodendrocytes (Bhat et al., 1996; Bin, Harris, & Kennedy, 2016; Kuhlmann, Remington, Maruschak, Owens, & Brück, 2007) was significantly decreased in
FIGURE 3  The spinal cord appears grossly normal in clinically affected mice. Immunofluorescence images of transverse sections of (a) cervical and (b) lumbar spinal cord of mock and ZIKV PE243 infected Ifnar1 knockout mice at 5 or 6 dpi, when infected mice displayed severe clinical signs. Spinal cord overviews are widefield epifluorescence images and higher magnification images are confocal maximum intensity projections (MIPs) of the ventrolateral columns and adjacent dorsal horn gray matter. ZIKV +ve cells were present, particularly in white matter, indicating productive infection. Grossly, myelin (MBP staining) and axons (NF staining) appeared intact at this time point. GFAP staining of astrocytes, appeared similar in mock-infected and ZIKV-infected animals. The inset in b, upper right panel, shows a higher magnification view of ZIKV +ve cells. The inset in b, middle right panel, shows a single Z plane to illustrate MBP +ve myelin rings. (c–e) Quantification of myelin (MBP), axons (NF), and astrocytes (GFAP) staining in white matter revealed no significant differences in the volume of myelin, axons, or astrocytes in ZIKV-infected mice compared with mock-infected controls. In c–e, Y-axes values are indicated on the graph on the left. Each data point represents the average value from one independent animal. Bars indicate mean ± SEM [Color figure can be viewed at wileyonlinelibrary.com]
infected animals (Figure 5g; \(p = .02\)). Similar trends were observed in the lumbar cord, although the differences were not significant (Figure 5h). Densities of microglia or astrocytes in white matter of cervical and lumbar cord were unchanged in ZIKV-infected animals compared with mock-infected controls (Figure 5g,h).

To determine if the vulnerability of oligodendrocytes reflects susceptibility to infection, we quantified the proportion ZIKV +ve cells that were CC1 positive. In spinal cord white matter at 4 dpi, 57.5\% (±3.08 SD) of ZIKV +ve cells were also positive for CC1 (green arrowheads). Pyknotic or karyohexic nuclei (white arrows) were also observed in close proximity to ZIKV +ve cells. (c) Resin section of the ventrolateral columns (tissue edge delineated by dashed line) demonstrates that pyknotic nuclei (white arrows) are interspersed with healthy-appearing nuclei. (d) Electron micrograph of white matter (tissue edge delineated by dashed line) confirms that a dying cell (1) is interspersed with healthy appearing cells (2–4). (e) Normal-appearing oligodendrocyte, wrapping axons (purple overlays), has a dark cytoplasm and contains many mitochondria (M) and endoplasmic reticulum (ER). (f–h) Dying oligodendrocytes, still wrapping axons (purple overlays), have a densely labeled cell nucleus and very dark cytoplasm which appears vacuolated [Color figure can be viewed at wileyonlinelibrary.com]
In summary, in Ifnar1 knockout mice, within the time frame examined, all neural cell types could be productively infected with ZIKV PE243, but only a small proportion of post-mitotic neurons was targeted. Although the proportions of oligodendrocytes and astrocytes that were ZIKV +ve were similar, oligodendrocytes appeared most vulnerable, resulting in decreased densities in white matter.
3.6 Perinatal Zika virus infection causes mild neuroinflammation

At the point of euthanasia, our mice showed no gross pathological changes that would explain the neurological signs. We therefore asked if these signs might reflect a neuroinflammatory process. Using antibody to IBA1, we demonstrated a propensity for microglia to cluster around ZIKV +ve cells (Figure 6a and Figure S2), however overall microglial densities in white matter were similar to control (Figures 5g, h and 6a). Using anti-CD68 (a marker of activated microglia/macrophages) and anti-CD3 (a T lymphocytes marker), we observed a localized activation of microglial/macrophages (Figure 6b), but only very rare T cells in the CNS parenchyma (Figure 6c). As the NLRP3 inflammasome was recently implicated in white matter injury in the perinatal and neonatal period in children (Holloway et al., 2021), we co-stained spinal cord and optic nerve sections with anti-NLRP3. We found NLRP3 +ve cells mainly in association with ZIKV +ve cells (Figure 6d). NLRP3 is likely expressed by microglia, however, as antibodies to NLRP3 and IBA1 are raised in the same species, we were unable to co-stain. Comparing Figure 6a,d (right panel), the densities of IBA1 +ve cells and NLRP3 +ve cells in the proximity of ZIKV +ve cells, appear similar. We conclude that neurological signs might be the consequence of neuroinflammation. The reason for the rapid clinical course, culminating in death of the Ifnar1 knockout mice, is unlikely to be related to CNS pathology.

4 DISCUSSION

ZIKV was first isolated in 1947; however, it was only during recent outbreaks in Micronesia (2007), French Polynesia (2013), and Brazil (2015) that it became clear that congenital infection with circulating Asian ZIKV is associated with a wide range of neuropathology and clinical signs and symptoms referred to as CZS (reviewed in Walker...
et al., 2019). The reason why some congenitally infected children appear asymptomatic at birth but subsequently experience transient or longer-lasting postnatal neurodevelopmental delays (Familiar et al., 2020; Mulkey et al., 2020; Peçanha et al., 2020; Pimentel et al., 2021), is obscure. Here, using a mouse model of perinatal infection, we confirmed our earlier observations in cell culture (Cumberworth et al., 2017; Schultz et al., 2021), that postmitotic neurons are rather refractory to infection, whilst newly generated CNS glia are considerably more susceptible, leading to a reduction in the density of oligodendrocytes. Our data might help predict pathological changes in humans infected perinatally and explain the emergence of neurodevelopmental delays postnatally.

In our mouse model, we found most ZIKV +ve cells in the CNS were located in white matter, and the majority co-labeled with antibody CC1, which labels Quaking (QKI) 7, an RNA-binding protein highly upregulated in myelinating oligodendrocytes (Bin et al., 2016). We found significantly reduced densities of CC1 +ve cells in white matter and confirmed by electron microscopy that oligodendrocytes were dying. However, by immunostaining, only approximately 16% of cleaved caspase 3 +ve cells, were positive for CC1. We speculate this reflects that QKI7 is downregulated in apoptotic oligodendrocytes. Nonetheless, it is likely that some of the cleaved caspase 3 +ve/CC1 –ve cells are OPCs, although we were unable to confirm this as the fixation required to inactive ZIKV (8% paraformaldehyde) is not compatible with tissue staining with antibody to NG2, the classical OPC marker. Certainly, we previously showed in cell culture that OPCs are vulnerable to ZIKV infection (Figure 3b, Cumberworth et al., 2017). In the current study we showed that NLRP3 is expressed in proximity to ZIKV +ve cells. Recently, Holloway et al. (2021) showed that microglial activation of the NLRP3 inflammasome drives developmental hypomyelination through dysregulation of Activin A signaling, which promotes developmental myelination (Geebels et al., 2017; Miron et al., 2013). Consequently, both cell death and impaired signaling are likely to impact developmental myelination following ZIKV infection.

Oligodendrocyte death is followed after some delay by loss of compact myelin (Pohl et al., 2011; Traka et al., 2010). However, the regenerative properties of CNS myelin are well known (Franklin & ffrench-Constant, 2017) and impaired developmental myelination can recover in both humans (Yan et al., 2019 and reviewed in Malik, Muthusamy, Mankad, Shroff, & Sudhakar, 2020), and animal models (Câmara et al., 2009; Yool et al., 2001). Consequently, if the vascular and neuronal “scaffoldings” are intact, and microglia downregulate the NLRP3 inflammasome, then ZIKV-associated oligodendrocyte injury might represent a temporary pathology. Certainly, it is one that might be difficult to detect by clinical examination in new-borns. Indeed, MRI observations in clinically unremarkable congenitally infected infants revealed high signal in T2 weighted images of the white matter (Brasil et al., 2016), potentially reflecting otherwise silent myelin changes. Further, in a study of infected mothers in Rio de Janeiro, 29% of pregnancies with third trimester infection had adverse outcomes including dysphagia, clonus, hyperreflexia, hypertonicity, and irritability (Table S2, Brasil et al., 2016), suggesting potential white matter involvement, as in the hypomyelinating leukodystrophies (Adang et al., 2017). Thus, myelin changes should not be ruled out in apparently asymptomatic new-borns.

Earlier experimental studies also provided support for myelin involvement, including reports of ZIKV-related disruption to OPC development and myelin deposition in mouse models of direct CNS ZIKV inoculation, embryonically (E15.5) or postnatally (P0; Zhang et al., 2017; Li et al., 2018). In contrast to the current study, these studies involved ZIKV inoculation at timepoints prior to the differentiation of OPCs into myelinating oligodendrocytes. Non-human primate studies have also provided support for myelin involvement in the pathogenesis of ZIKV infection of the CNS. Oligodendroglial development was impaired in fetuses of mid-gestation olive baboons infected systemically with a French Polynesian ZIKV isolate (H/PF/2013; Gurung et al., 2019) and white matter hypoplasia were reported in preterm pigtail macaque following systemic infection of the pregnant dam with a Cambodian isolate (FSS13025; Adams Waldorf et al., 2016). As in the current study, these non-human primates were killed before the long-term consequences were known and it will be important for future studies to address later outcomes, within the limitations of animal welfare issues.

ZIKV-related myelin changes might not be limited developmental aspects. Acute myelitis and meningoencephalitis (Neri et al., 2018; Mécharles et al., 2016; Carteaux et al., 2016; Brito Ferreira et al., 2020), have also been described and may indicate transient focal demyelination due to ZIKV-related oligodendrogial injury.

Whilst myelin can be restored following dysmyelination and/or demyelination (Franklin & ffrench-Constant, 2017), it has been shown using an experimental mouse model of primary oligodendrocyte cell death, that demyelination and repair are followed months later by fatal secondary disease characterized by extensive myelin and axonal loss (Traka et al., 2016). These data demonstrate that primary oligodendrocyte death is sufficient to trigger an adaptive autoimmune response against myelin (Traka et al., 2016) and raise the concerning possibility that apparently minor consequences of ZIKV infection could lead later, in genetically susceptible individuals (International Multiple Sclerosis Genetics Consortium et al., 2011; Manet et al., 2020), to autoimmune-mediated demyelination.

5 | CONCLUSION

The relation between ZIKV infection in pregnancy and adverse neurological outcomes will become clearer when longitudinal studies are published (Wilder-Smith et al., 2019). Our data highlight the need for lifetime monitoring in susceptible individuals, particularly in relation to development of autoimmune-mediated demyelination. They may also help explain postnatal neurodevelopmental delays in children who appeared asymptomatic at birth; although alterations in other developmental processes in the perinatal and early postnatal period, aside from myelination, might also contribute (Kostović et al., 2019).
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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
Verena Schultz, Jennifer A Barrie, Julia M. Edgar: Conducted experiments and analysed data. Claire L Donald: Prepared and provided viral stocks. Margaret Mullin, Julia M. Edgar, Colin L. Crawford: Conducted electron microscopy. Thomas J. Anderson: Described clinical signs in mice. Hugh J. Willison, Julia M. Edgar, Alain Kohl, Susan C. Barnett, Christopher Lintington, Tom Solomon: Obtained funding and contributed discussion. Julia M. Edgar, Verena Schultz: Designed study and wrote the article, which was edited and agreed by all authors.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Verena Schultz https://orcid.org/0000-0002-8172-1525
Colin L. Crawford https://orcid.org/0000-0001-7154-5698
Susan C. Barnett https://orcid.org/0000-0003-1009-0575
Julia M. Edgar https://orcid.org/0000-0002-3869-0962

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