Dengue virus infects the mouse eye following systemic or intracranial infection and induces inflammatory responses.

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ABSTRACT

Dengue virus (DENV) infection is associated with clinical ocular presentations and here DENV-infection of the eye was assessed in mice. In an AG129 mouse model of antibody-dependent enhancement of DENV-infection, DENV RNA was detected in the eye and vascular changes were present in the retinae. Intraocular CD8 and IFN-γ mRNA were increased in mice born to DENV-naïve, but not DENV-immune mothers, while TNF-α mRNA was induced and significantly higher in mice born to DENV-immune than DENV-naïve mothers. DENV RNA was detected in the eye following intracranial DENV-infection and CD8 mRNA but not IFN-γ nor TNF-α were induced. In all models, viperin was increased following DENV-infection.

Thus, DENV in the circulation or the brain can infect the eye and stimulate innate immune responses, with induction of viperin as one response that consistently occurs in multiple DENV eye-infection models in both an IFN-dependent and independent manner.

IMPACT STATEMENT

Inflammatory eye disease can result from circulating viral infections with acute and potential long-term impact on vision. Dengue is a globally significant disease that can affect the eye but the biology of responses in the eye are ill-defined. Our results demonstrate that DENV can infect the mouse eye and induce immune responses. These findings support the need for vigilance in assessment for eye disease during DENV-infection and provide laboratory models for investigating DENV replication and associated inflammation within the eye.

MAIN TEXT (SHORT COMMUNICATION; UNSECTIONED)

Inflammation within the eye (uveitis) can occur in the context of epidemic systemic infections and clinical data supports post-infectious uveitis and intraocular inflammation during viral infection (1, 2).

47 Arboviruses, such as West Nile Virus (WNV), dengue virus (DENV), zika virus (ZIKV), and chikungunya
48 virus (CHIKV) have been linked with uveitis through clinical studies (3-5) (6) and recently viral RNA for
49 WNV (7), CHIKV (8, 9) and ZIKV (10) have been detected in aqueous humor collected from patients
50 with uveitis and acute systemic viral infection.

51 Dengue is the most common arboviral infectious disease worldwide and there are numerous clinical
52 reports of ocular manifestations associated with DENV infection, largely representing pathology in the
53 retina (11-13). In 2004-2005, an epidemic of DENV retinopathy was documented, with 10% of
54 hospitalised dengue patients affected (14, 15). Our studies have demonstrated replication of DENV *in*
55 *vitro* in human retinal cell lines, primary human retinal pigment epithelial and endothelial cells (16).
56 Importantly, the pigment epithelium and the endothelium are cellular components of the blood-
57 retinal barrier, which protects the posterior segment of the eye. For the present work, an *in vivo*
58 approach was taken to investigate whether DENV can breach the blood-retinal and/or blood-aqueous
59 barriers to infect the eye during a systemic infection or following localised infection of the brain.

60 AG129 mice (129/Sv strain deficient in IFN-α/β and IFN-γ receptors) were infected with D2Y98P-PP1,
61 a DENV-2 patient strain (GenBank accession # JF327392), as described previously (17). In brief, the
62 model utilised a DENV-naïve or DENV-immune model where female AG129 mice were challenged with
63 a non-lethal dose of a DENV-1 and mated one week later. The 5-6-week-old weaned pups born to
64 DENV-naive or DENV-immune mothers were either left uninfected or infected subcutaneously with
65 10^3 plaque forming units (pfu) of DENV-2. As previously reported (17), day 6 post-DENV-2 infection in
66 DENV-naïve mice, resulted in a mean disease score of 2 out of a maximum of 5 (ruffled fur = 1; hunched
67 back =2) and 100% survival, while mice born to DENV-1-immune dams had a mean disease score of 5
68 out of 5. Mice were ethically euthanized at this time point with some animals perfused with phosphate
69 buffered saline (PBS) prior to tissue harvest. Eyes were enucleated, and one eye was collected into

70 Trizol (Ambion Life Technologies) for RNA extraction, and the second eye was fixed in 10% (v/v)
71 buffered formalin for 24 hours for morphological analysis.

72 DENV RNA was detected by RT-qPCR in eyes taken from all DENV-infected AG129 mice (Figure 1A).
73 The level of DENV RNA was comparable between eyes regardless of tissue perfusion and therefore
74 not related to circulating virus or virus-infected cells in the circulation. Additionally, DENV RNA was
75 not significantly different in eye tissue from DENV-infected mice born to DENV-naive versus DENV-
76 immune mothers. The latter is a model of antibody-dependent enhancement of disease that is
77 associated with increased circulating viremia and disease severity in the DENV-immune DENV-infected
78 mice (17) and reflects a secondary infection, or infection in infants aged under 1 year who are born to
79 women who are immune to DENV (18, 19). In contrast to the eye, where RNA levels were not different,
80 DENV RNA levels in the liver were significantly higher in the DENV-ADE model, than DENV-infected
81 mice from naïve mothers (data not shown). Thus, DENV RNA level in the eye was not associated with
82 severity of systemic disease.

83 In the AG129 experimental model of DENV-infection, day 6 post-infection is associated with vascular
84 leakage (17). Histopathological examination of eyes demonstrated no major structural changes,
85 although vascular alterations in the retina were noted (Figure 1B), with rouleaux formation by red
86 blood cells (RBCs) in vessels from DENV-infected but not uninfected mice and apparent in the retinae
87 in both superficial vessels and deeper vasculature (Figure 1C). Additionally, RBCs and RBC fragments
88 were found in the vitreous close to the posterior hyaloid face in DENV-infected mice (Figure 1D). These
89 vascular and RBC changes were observed in eyes of DENV-infected mice from both DENV-naïve and
90 DENV-immune mothers. Rouleaux is associated with disseminated intravascular coagulation and the
91 formation of antigen/antibody complexes in the blood, as occurs in severe dengue in humans (20).
92 The RBC fragments present in the vitreous are also suggestive of disseminated intravascular
93 coagulation. Importantly, these intraocular morphological changes were seen in DENV-infected mice

94 born to both DENV-naive and DENV-immune mothers, and thus were not associated with severity of
95 systemic disease. Dengue has been associated with retinal haemorrhage (11), and assessment of
96 patients with dengue maculopathy and acute macular neuroretinopathy by optical coherence
97 tomography-angiography or digital fundus photography has demonstrated pathological microvascular
98 alterations in the retina (21, 22). Consistent with our findings in a the AG129 mouse model, none of
99 these altered retinal vascular measures in humans were specifically associated with severity of dengue
100 disease (22).

101 Scant leukocytes were visualised in Figure 1C and D and next, ocular sections were further examined
102 for the presence of a leukocytic infiltrate. Occasional leukocytes were seen in retinae from both
103 uninfected and DENV-infected mice with potential mononuclear morphology (Figure 2A). CD4 and CD8
104 mRNA, as a measure of CD4+ or CD8+ leukocyte infiltration, was assessed by RT-qPCR. No change in
105 CD4 transcript was detected in eyes from DENV-infected compared to uninfected mice. In contrast, a
106 significant increase in CD8 and IFN-γ mRNA was quantitated in DENV-infected mice, but only in eyes
107 from mice born to DENV-naive mothers. Levels were not different in perfused versus non-perfused
108 eyes suggesting tissue associated cells (Figure 2B). Additionally, results suggest that DENV immunity
109 passed from the mother alters the cell-mediated and IFN-γ response to infection.

110 TNF-α mRNA was increased in eyes from DENV-infected mice compared to uninfected animals with
111 significantly higher levels in infected mice born to DENV-immune compared to DENV-naive mothers
112 (Figure 2B). This is consistent with our previous results showing TNF-α induction in DENV infected
113 retinal pigment epithelial cells (16). Additionally, increased TNF-α and IFN-γ are present following
114 secondary heterotypic DENV infection in patients (23), TNF-α is elevated in patients with severe DENV
115 (24-26), TNF-α mediates pathogenesis in mouse models of DENV-vascular leak (27) and is elevated in
116 the circulation in the model here-in of DENV-infected mice born to DENV-immune compared to DENV-

naive mothers (17). The significantly higher TNF-α expression in the eyes of DENV-infected mice born to DENV-immune versus DENV-naive mothers is in line with this role for TNF-α in DENV pathogenesis.

The initial rationale for this study was to assess the ability of DENV to infect the eye *in vivo* either from the circulation across the blood-retinal barrier or from the brain to the eye. Thus, DENV eye infection was next examined in immunocompetent C57BL/6 mice challenged intracranially with DENV. 3-4-week-old C57BL/6 mice were anesthetised and infected by intracranial injection with 80 pfu of DENV-2 Mon601, a DENV-2 New Guinea C strain (28), as previously (29). Animals were euthanised at day 3 and 6, prior to symptomatic infection and eyes harvested into TRIzol, RNA extracted and subjected to qRT-PCR. Following intracranial DENV challenge, viral RNA was found in the eye (Figure 2C). Links between eye and brain infections are well known for viruses such as measles virus, and previously suggested for ZIKV (30, 31). Additionally, in a newborn mouse model of intraperitoneal ZIKV infection, virus is found in the retinal ganglion layer and inner nuclear layer as well as the optic nerve, optic chiasm and visual regions of the brain (31, 32). The reverse occurs with intraocular infection in 4-6-week-old IFNAR$^{-/-}$ mice, leading to infection of the optic nerve, and glial cells of the optic chiasm and optic tract, as well as visual regions of the brain (32). Our findings similarly support that DENV can move between the brain and eye. In this model of intracranial infection, DENV RNA is detected in the brain at day 3 post-infection, increasing at day 6, ahead of the onset of neurological signs at days 8-9 (29). In contrast to the increasing DENV RNA levels in the brain over time, DENV RNA was detected in the eye at day 3 post-infection but was not increased further at day 6 (Figure 2C). These kinetics of detection of DENV RNA in the eye support the conclusion that DENV replication is limited in the eye.

Similar to the systemic infection in the AG129 mouse model, no increase in CD4 but a significant increase in CD8 mRNA was observed in DENV-infected mouse eyes, although only at day 6 post-infection (Figure 2C). In contrast, IFN-γ was not detected in eyes from uninfected or DENV-infected mice, while TNF-α mRNA transcripts were detected but unchanged by intracranial DENV infection

(data not shown). This might be due to a lack of CD8+ T-cell activation and IFN-γ production, or a lack of infiltration of other IFN-γ and TNF-α-producing cells, such as NK cells and macrophages into the eye when infected via the brain (33). The differences in ocular inflammatory responses between the AG129-systemic and intracranial DENV-infection models, present useful comparators to study the roles of these elements in infectious inflammatory eye disease.

Results above suggest DENV can infect the mouse eye but the limited DENV-related pathology in both the AG129 and intracranial intraocular infection models suggests the action of potent anti-viral responses. Viperin is a major contributor to anti-DENV responses in macrophages (34) and endothelial cells (35). Additionally, viperin is anti-viral against other *flaviviruses* such as ZIKV (36), WNV (37, 38) and Japanese encephalitis virus (39) and a broad range of viruses such as human CMV (40), human immunodeficiency virus (41) and CHIKV (42). While induction of viperin has been shown by transcriptome profiling in ZIKV or Ebolavirus infected retinal pigment epithelium (43, 44), the antiviral actions of viperin have not been studied in the eye. RT-qPCR results demonstrate a significant induction of viperin in DENV-infected AG129 mice born to both DENV-naive and DENV-immune mothers (Figure 3A). Similarly, viperin was induced by day 3 and further increased by day 6 post-infection following intracranial infection (Figure 3B). Unexpectedly, one mock-infected mouse returned CD8 mRNA levels of 0.1311 ± 0.037 (see Figure 2C) and viperin levels of 0.50 ± 0.01 (Figure 3B), in the absence of DENV RNA which is unexplained. Thus, viperin mRNA was consistently induced in our DENV eye infection models, including early in the time course and increasing with time in the intracranial infection model, in contrast to DENV RNA. This implies production of viperin is a common response in both models and may be an important contributor to the limited replication that is observed here. Viperin was initially described as an IFN-stimulated gene, but also may be induced by IFN-independent pathways (45). Although the induction of viperin appeared lower in DENV-infected eyes from the AG129 mouse than the immunocompetent mouse infected intracranially, viperin was

still significantly induced in the AG129 mouse, supporting IFN-independent mechanisms of induction of viperin within the eye during DENV infection.

Despite a number of approaches, our study could not conclusively demonstrate the presence of DENV antigen or dsRNA positive cells in the eye. This, combined with the lack of association between DENV RNA level and severity of systemic disease, plus the absence of major structural change in the eye, suggests there are very few DENV-infected cells in the eye. This may reflect potential host restriction of DENV replication to preserve the integrity of the organ - even in the AG129 mouse, in which type I IFN-stimulated responses are lacking. While systemic infection of young mice leads to the presence of infected retinal cells and the optic nerve (30, 31) with widely disseminated dsRNA and antigen-positive cells in the retina (46), other studies are consistent with ours and suggest that the retina is protected from virus infection. Voigt et al., 2018, demonstrate the presence of cytomegalovirus (CMV) in the anterior eye, with a lack of CMV but apparent inflammatory responses in the retina (47). Similarly, in the adult AG129 mouse ZIKV causes a panuveitis but no major structural abnormalities or retinal pathology (30).

In conclusion, DENV may cause clinical eye pathology, particularly in the retina (12, 13), and our studies have shown that DENV can replicate and cause functional changes in human retinal cells (16). Here we show that DENV may infect the mouse eye, following either systemic infection in an IFN-receptor-deficient AG129 mouse or following intracranial inoculation of an immunocompetent C57BL/6 mouse. Thus the virus may move from the circulation or brain to the eye during DENV-infection. In both cases, DENV replication appears to be restricted and accompanied by common responses of increases in CD8+ cells and induction of the antiviral viperin. These models of intraocular DENV infection will have future value for investigating DENV-infection and inflammatory responses in the eye.

AUTHOR STATEMENTS

Conflicts of interest

The author(s) declare that there are no conflicts of interest.

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Ethical approval

Animal experiments in Singapore were performed in accordance with the guidelines of the National Advisory Committee for Laboratory Animal Research (NACLAR) under licence to operate in accredited animal facilities with IACUC/NUS protocol approval number R13-4751. Animal experiments in Australia were undertaken in accordance with the South Australian Animal Welfare Act, 1985 with approval from the Flinders University Animal Welfare Committee, approval number 870/14. The use of infectious genetically modified organisms (GMO) was approved by the Flinders University Institutional Biosafety Committee (protocol approval number: NLRD 2011-13).

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FIGURES AND TABLES

Figure 1. DENV RNA is present in eyes from DENV-infected AG129 mice with RBC and vascular changes. 5-6-week-old AG129 mice born to either DENV-1 naïve (naïve), or DENV-1 immune (immune) mothers, were challenged with DENV-2 (DENV, n=5) or mock-infected (Ui; n=3) and at day 6 pi eyes collected either with or without prior perfusion. **A.** Total RNA was extracted from whole eyes, DNase I-treated (Zymo Research), and 0.5 µg was reverse-transcribed and subjected to real-time quantitative (q)PCR as described previously (29). Quantitative DENV copy number was calculated from a standard curve generated from a known concentration of Mon601 DNA with a limit of quantitative detection of 0.3pg/µg GAPDH (dashed line). A value of zero represents no valid melt curve detected. Results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values represent single animals with duplicate PCR measurements. *=$p<0.05$, Students unpaired *t*-test with Welch's correction compared to Ui group; **B-D.** Fixed whole mouse eyes were embedded in paraffin, sectioned at 5 µm thickness, stained with hematoxylin and eosin and imaged under bright-field microscopy (BX50, Olympus); **B.** 100X magnification of retina from Ui and DENV-infected eyes. Arrows highlight areas of vascular congestion; **C.** 40X (upper panel) and 100X (lower panel) magnification of DENV-infected eyes demonstrating rouleaux formation in deep retinal and inner retinal layers. NFL=nerve fibre layer, RGL=retinal ganglion layer, IPL=inner plexiform layer, INL=inner nuclear layer, OPL=outer plexiform layer, ONL=outer nuclear layer, R/C=rods and cones, PL=pigmented layer. Boxed region demonstrates a computational zoom. Arrows indicate vascular congestion and rouleaux. Open ended arrow indicates an infiltrating leukocyte; **D.** 40X (upper panel) and 100X (lower panel) magnification of DENV-infected eyes demonstrating RBC fragments close to the posterior hyaloid face, as indicated by arrows. Open ended arrow indicates an infiltrating leukocyte. Representative images are shown.

354

355 **Figure 2. Eyes from DENV-infected mice show evidence of inflammatory responses.** Eyes were
356 enucleated and processed, as in Figure 1. **A.** 100X magnification of retina from Ui and DENV-infected
357 eyes. Arrows indicate rare infiltrating leukocytes; **B.** RNA was subjected to RT-qPCR for CD8, as
358 previously (29), mouse TNF-α (F CATCTTCTCAAAATTCGAGTGACAA; R
359 TGGGAGTAGACAAGGTACAACCC) and IFN-γ (F ACTGGCAAAAGGATGGTGAC; R
360 TGAGCTCATTGAATGCTTGG) (48). Values represent single animals with duplicate PCR measurements,
361 with relative mRNA level calculated by ΔCt method and normalised against GAPDH; **C.** C57BL/6 mice
362 were inoculated intracranially with DENV. Ipsilateral eyes were harvested from DENV-infected mice
363 at day 3 (n=3) and 6 pi (n=8) or uninfected (mock-infected with PBS) at day 6 pi (n=3). RNA was
364 extracted and subjected to RT-qPCR for DENV RNA or CD8 mRNA in the eye and normalised against
365 GAPDH. Values represent single animals with mean ± SD from duplicate PCR measurements. Limit of
366 quantitative detection was defined by the dashed line at 0.3pg/µg GAPDH (DENV) or C_t>32,
367 corresponding to 0.002-0.004 for CD8, IFN-γ and TNF-α. A value of zero represents no valid melt curve
368 detected. *=p<0.05, one-way ANOVA with Dunnett's multiple comparison test. #=outlier, mock-
369 infected animal lacking DENV RNA by RT-qPCR but with elevated CD8.

370

371 **Figure 3. Viperin mRNA is increased in DENV-infected mouse eyes.** Mice were DENV-infected: **A.**
372 AG129 mice, as in Figure 1 or **B.** C57BL/6 mice, as in Figure 2C. RNA was extracted and subjected to
373 RT-qPCR for viperin mRNA. Values represent single animals with duplicate PCR measurements, with
374 relative mRNA level calculated by ΔCt method and normalised against GAPDH. Limit of quantitative
375 detection was defined by the dashed line at C_t>32, corresponding to 0.002. A value of zero represents
376 no valid melt curve detected. *=p<0.05, Students *t*-test compared to Ui groups, **=p<0.05, one-way

377 ANOVA compared to mock-infected animals. #=outlier, mock-infected animal lacking DENV RNA by

378 RT-qPCR but with elevated viperin mRNA.

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