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Using immunocompromised mice to identify mechanisms of Zika virus transmission and pathogenesis

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Summary

Zika virus (ZIKV) is responsible for a recent global epidemic that has been associated with congenital brain malformations in fetuses and with Guillain–Barré syndrome in adults. Within the last 2 years, a major effort has been made to develop murine models to study the mechanism of viral transmission, pathogenesis and the host immune response. Here, we discuss the findings from these models regarding the role that the innate and adaptive immune responses have in controlling ZIKV infection and pathogenesis. Additionally, we examine how innate and adaptive immune responses influence sexual and vertical transmission of ZIKV infection as well as how these responses can influence the ability of ZIKV to cross the placenta and to induce damage in the developing brain.

Keywords: innate receptors; neuroinflammation; reproductive Immunology; T cell; viral

Introduction

Zika virus (ZIKV) is a flavivirus, first discovered in Uganda in 1947 in a sentinel monkey, which probably contracted the virus via its primary vector, an *Aedes* mosquito. Historically, human cases were rare with symptoms including mild fever, rash, myalgia and conjunctivitis.[1] However, recent outbreaks in French Polynesia in 2013/14[2] and in South America in 2015/16[3] showed an association between ZIKV infection and severe clinical outcomes including Guillain–Barré syndrome (GBS) in adults[4] and microencephaly and congenital pathologies in fetuses and newborns.[5] The congenital defects were associated with the ability of ZIKV to be transmitted vertically across the placental barrier.[6] Additionally, ZIKV can be transmitted human- to- human sexually from males to females.[7] Recent work has focused on the development of small-animal models to better understand ZIKV pathogenesis and the role of the immune response elicited during infection in these processes.

Zika virus and the innate immune response

Developing useful murine models of ZIKV infection has been difficult. Early attempts required significant mouse adaptation through serial viral passaging in brain tissue to consistently observe disease.[8] Even using ZIKV isolates from recent outbreaks demonstrated no obvious signs of disease and little to no detectable virus in tissues in wild-type (WT) strains of mice (C57BL/6, BALB/c or CD-1) following peripheral inoculation (Table 1, part a) suggesting that virus replication is effectively controlled in these animals.

Studies analysing the type I interferon (IFN) responses in human and mouse cells identified a key difference between these species in their ability to control virus infection. In human cells, the virus NS5 protein antagonizes IFN signalling by promoting proteasomal degradation of the human signal transducer and activator of transcription 2 (STAT2) protein.[9,10] STAT2 is a transcription factor that mediates signalling through the IFN-α/β receptor (IFNAR), leading to the production of IFN-stimulated genes.[11] Hence, ZIKV NS5 limits the type I IFN response during human infection. In contrast, ZIKV NS5 does not inhibit mouse STAT2, allowing for an efficient and effective type I IFN response that controls virus replication.[9] Mice deficient in STAT2 are susceptible to ZIKV infection,[12] as are mice deficient in the IFNAR1 gene (*Ifnar1*$^{-/-}$, A129). These mice develop a rapid wasting disease within 5–8 days and have high viral burdens in

Table 1. Influence of innate and adaptive immune responses on Zika infection in mice

Strain	Route[a]	Age	Virus strains[b]	Dose[c]	Treatment[d]	Clinical disease[e]	Viraemia	Dissemination[f]: Brain	Spleen	Ovaries	Testes	References
(a) Wild-type												
C57BL/6	s.c.	7 weeks	FP2013	10^3	–	None	n.d.				n.d.	18
C57BL/6	s.c.	5–6 weeks	UG1947	10^3	–	None						12
C57BL/6	s.c.	4–5 weeks	FP2013	10^2	–	None		x(R)	x(R)		x(R)	14
C57BL/6	s.c.	5–6 weeks	FP2013, UG1947	10^2	–	None						14
C57BL/6	i.v.	5–7 weeks	CB2010	10^6		None						17
C57BL/6	i.v.	5–6 weeks	FP2013, UG1947	10^2	–	None						14
C57BL/6	i.p.	5–6 weeks	SMGC-1	10^6		None	n.d.	n.d.	x(R)			23
C57BL/6	i.p.	6–8 weeks	PA2015	10^4	–	None	n.d.	n.d.	n.d.		n.d.	16
(b) Innate immune deficiencies												
A129	i.p.	11 weeks	CB2010	10^5	–	None	x (PA)					15
A129	i.p.	3 weeks	CB2010	10^5	–	6–7 dpi	x (PA)					15
A129	i.p.	5 weeks	CB2010	10^5	–	8–9 dpi	x (PA)					15
A129	s.c.	5–6 weeks	UG1962	10^6	–	4–6 dpi	x(R)	x(R)	x(R)	x(R)	x(R)	72
C57BL/6	i.p.	6–8 weeks	PA2015	10^4	anti-IFNAR1	None		n.d.	x		n.d.	16
C57BL/6	i.p.	5 weeks	SN1984	10^6	anti-IFNAR1	7–12 dpi	x(R)	x(R)	x(R)			20
C57BL/6	s.c.	4–5 weeks	FP2013	10^2	anti-IFNAR1	None	3 dpi					14
C57BL/6	s.c.	7 weeks	FP2013	10^3	anti-IFNAR1	None	3 dpi				x	18
C57BL/6	s.c.	5 weeks	SN1984	10^6	anti-IFNAR1	8–19 dpi (40%)	x(R)	x(R)	x(R)			20
Ifnar1$^{-/-}$	i.v.	5–6 weeks	FP2013, UG1947	10^2	–	8–12 dpi						14
Ifnar1$^{-/-}$	s.c.	3–6 months	FP2013	10^3	–	7–18 dpi		x(R)			x(R)	14
Ifnar1$^{-/-}$	s.c.	5–6 weeks	FP2013, UG1947	10^2	–	6–10 dpi						14
Ifnar1$^{-/-}$	s.c.	5–6 weeks	UG1947, SN1984	10^3	–	7–8 dpi	x(R)	x(R)	x(R)	x(R)	x(R)	12
Ifnar1$^{-/-}$ HLA-A*0101	r.o.	5 weeks	CB2010	10^4	–		x(PA)	x(PA)				25
Irf3$^{-/-}$ *Irf5*$^{-/-}$ *Irf7*$^{-/-}$	i.v.	5–6 weeks	FP2013, UG1947	10^2	–	5–10 dpi						14
Irf3$^{-/-}$ *Irf5*$^{-/-}$ *Irf7*$^{-/-}$	s.c.	5–6 weeks	FP2013, UG1947	10^2	–	6–10 dpi						14
Irf3$^{-/-}$	s.c.	5–6 weeks	FP2013, UG1947	10^2	–	None						14
LysM^{CRE+}IFN AR$^{fl/fl}$	i.v.	5–7 weeks	UG1947, CB2010	10^6	–	5–10 dpi						17
Mavs$^{-/-}$	i.p.	6–8 weeks	PA2015	10^4	–	None		n.d.	x(R)		n.d.	16
Mavs$^{-/-}$	s.c.	5–6 weeks	FP2013, UG1947	10^2	–	None						14
Stat2$^{-/-}$	s.c.	5–6 weeks	UG1947, SN1984	10^3	–	5–7 dpi	x(R)	x(R)	x(R)	x(R)	x(R)	12
Unc93b1 3D	i.p.	6–8 weeks	PA2015	10^4	–	None		n.d.	n.d.		n.d.	16
(c) Adaptive immune deficiencies												
C57BL/6	i.p.	6–8 weeks	PA2015	10^4	anti-CD4/CD8	7–14 dpi (<10% wl, recovered)						16
Rag1$^{-/-}$	i.p.	6–8 weeks	PA2015	10^4		None						16
(d) Innate & adaptive immune deficiencies												
AG129	i.d.	3 weeks	CB2010	10^5	–	6 dpi	x(PA)	x	x		x	15

Table 1. *(Continued)*

Strain	Route[a]	Age	Virus strains[b]	Dose[c]	Treatment[d]	Clinical disease[e]	Viraemia	Dissemination[f]: Brain	Spleen	Ovaries	Testes	References
AG129	i.p.	3 weeks	CB2010	10^5	–	6 dpi	x(PA)					15
AG129	s.c.	8 weeks	FB2013	10^5	–	5–8 dpi	x(PA)	x	x			13
CD8$^{-/-}$	s.c.	7 weeks	SN1984	10^5	anti-IFNAR1	12–18 dpi	x(PA)	x(PA)	x(PA)			17
Ifnar1$^{-/-}$	i.p.	5–6 weeks	SMGC-1	10^5	a.t. of niave CD8+ T cells	5–8 dpi		x(R)				23
Ifnar1$^{-/-}$	i.p.	5–6 weeks	SMGC-1	10^5	a.t. of ZIKV-CD8+ T cells	None		n.d.				23
Ifnar1$^{-/-}$ HLA-A*0101	r.o.	5 weeks	CB2010	10^4	anti-CD8		x(PA)	x(PA)				25
Ifnar1$^{-/-}$ HLA-A*0101	r.o.	5 weeks	CB2010	10^4	ZIKV peptide		x(PA)	x(PA)				25
Ifnar1$^{-/-}$ HLA-A*0101	r.o.	5 weeks	CB2010	10^4	ZIKV peptide/ anti-CD8		x(PA)	x(PA)				25
Mavs$^{-/-}$	i.p.	6–8 weeks	PA2015	10^4	anti-CD4/CD8	7–14 dpi (>10% wl, recovered)						16
Rag1$^{-/-}$	i.p.	6–8 weeks	PA2015	10^4	anti-IFNAR1	9–17 dpi	x(PA)	x(R,H)	x(R)		x(R,H)	16

Route of inoculation: [a]i.d., intradermal; i.p., intraperitoneal; i.v., intravenous; r.o., retro-orbital; s.c., subcutaneous.

[b]Virus strains were abbreviated as follows: Cambodia/2010 (FSS13025): CB2010; French Polynesia/2013 (H/PF/2013): FP2013; Uganda/1947 (MR-766): UG1947; Paraiba_01/2015: PA2015; Senegal/1984: SN1984; Uganda/1962 (MP1751): UG1962; ZIKA-SMGC-1: SMGC-1.

[c]PFU per mouse.

[d]Treatment of animals: a.t., adoptive transfer.

[e]Reported clnical disease or weight loss (w.l.).

[f]x indicates detected. PA, plaque assay, R, RNA, H, Histology, n.d., not detected.

tissues[13–15] (Table 1, part b). These findings show that the type I IFN response is essential for protection against ZIKV infection and explain, at least in part, disease susceptibility in humans.

Additional studies using knockout mice have further defined the role of type I IFN in regulating ZIKV pathogenesis. Complete deficiency in the IFN response, as observed with *Ifnar1*$^{-/-}$, A129 or *Irf3*$^{-/-}$ *Irf7*$^{-/-}$ *Irf7*$^{-/-}$ mice, results in clinical disease by 5–10 days post infection with virus detection in multiple tissues including brain, spleen, ovaries and testes (Table 1, part b).[14–16] This was also observed when IFNAR1 was specifically knocked out in myeloid cells, suggesting that these cells are critical for protection.[17] The molecules necessary to mediate the type I IFN response to ZIKV infection are not as well defined, as deficiency in *Mavs*, the signalling molecule for RIG-I-like receptors resulted in only a short-term viraemia,[16] whereas deficiencies in *Irf3* or the three-dimensional mutation in *Unc93b1*, which results in deficient endosomal Toll-like receptor responses, had no effect on ZIKV infection.[14,16] Hence, neither cytoplasmic RIG-I-like receptors nor endosomal Toll-like receptors appear to be essential for protection against ZIKV, suggesting that other sensors of virus infection play a role in mediating the type I IFN response to ZIKV.

Partial disruption of the type I IFN response, to more effectively mimic the antagonism of the type I IFN response found in humans, has been achieved by treating mice with anti-IFNAR antibodies. Treatment of WT mice with a single, large bolus of anti-IFNAR1 blocking antibody (MAR1-53A) does not result in clinical signs such as wasting disease, but can result in detectable virus in peripheral tissues.[14,18,19] Furthermore, the concentration of blocking antibody used can influence tissue-specific viral load[14] and result in disease if repeatedly administered.[20] In the future, it may be possible to use this approach as a model of ZIKV-associated GBS because ZIKV infection of *Ifnar1*$^{-/-}$ mice can result in peripheral neuron infection and apoptosis[21,22] (Figure 1c).

Adaptive immune response to ZIKV

Although the innate immune response is clearly necessary for controlling viral replication and preventing disease, the adaptive T-cell and B-cell responses also contribute to protection. Several studies, including work from our laboratory, have demonstrated a short-lived, but strong T-cell response around 7 days post infection in ZIKV-infected WT mice.[16,23,24] CD4$^+$ T cells proliferate rapidly and show a classical T helper type 1 antigen-experienced cytokine profile, expressing IFN-γ, tumour necrosis factor-α (TNF-α) and interleukin-2 (IL-2). Concomitantly, CD8$^+$ T cells are proliferating, are activated and express cytotoxic markers, suggesting a virus-specific cytotoxic T-cell response. However, depletion of T cells using anti-CD4 and anti-CD8 antibodies or deficiency of T cells in

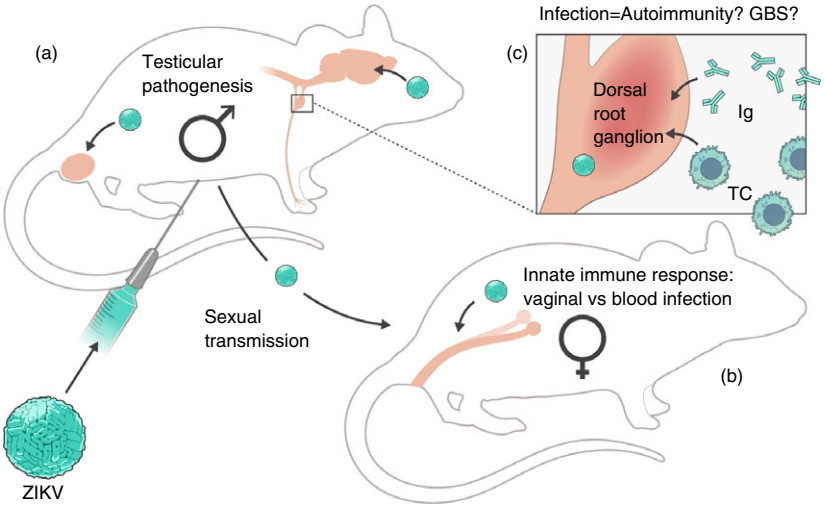

Figure 1. Immune responses to Zika virus (ZIKV) are critical to prevent pathogenesis and transmission but may also contribute to autoimmunity. (a) Models of ZIKV infection in mice have demonstrated that elements of the innate immune response are critical for preventing infection of the testes in males. Experiments with mice deficient in innate immune signalling have shown testicular pathology and prolonged infection of cells in the testes, including sperm, which allow for animal-to-animal sexual transmission of ZIKV. These models are reminiscent of findings in humans. (b) Additionally, sexual transmission or intravaginal inoculation of mice demonstrates that the female reproductive tract is permissive to ZIKV infection. Furthermore, these modes of transmission in mice deficient in innate immune signalling can result in ZIKV-associated disease and vertical transmission to fetuses. (c) In mice with innate immune signalling deficiencies, ZIKV can infect peripheral nerves within the dorsal root ganglia. Such an infection could induce an autoimmune response reminiscent of Guillain–Barré syndrome, which in humans is associated with ZIKV infection. TC, T cell; Ig, immunoglobulin.

Table 2. Models of Zika virus (ZIKV) transmission and influence of infection on central nervous system development

(a) Sexual transmission

Route[a]	Strain	age[b]	Virus strains[c]	Dose	Treatment	Clinical disease[d]	Viraemia	Dissemination[e]: Brain	Spleen	Ovaries	Vagina	Testes	Placenta	References
i.p. to males	AG129 x AG129 or CD-1	13–18 weeks	PR2015	10^3	–	13–28 dpi (transmitter) 9–21 dpi (recipient)		x(R)				x(R)		43
i.p. to males	*Rag1*$^{-/-}$ (m) x *Ifnar1*$^{-/-}$ (f)	6–8 weeks	PA2015	10^4	anti-IFNAR1	16–17 dpi (transmitter) 7–12 dpi (recipient)		x (R/H)	x (R)		x(R)	x (R/H)	x (R/H)	26
i.vag.	C57BL/6	7–22 weeks	CB2010	10^4	Depo-Provera	–	n.d.		n.d.		x(PA)			44
i.vag.	Mx1	7–22 weeks	CB2010	10^4	Depo-Provera	–	n.d.							44
i.vag.	*Rag2*$^{-/-}$	7–22 weeks	CB2010	10^4	Depo-Provera	–	n.d.							44
i.vag.	*Tlr7*$^{-/-}$ *Mavs*$^{-/-}$ Mx1	7–22 wks	CB2010	10^4	Depo-Provera	–	n.d.							44
i.vag.	*Irf3*$^{-/-}$ *Irf7*$^{-/-}$	7–22 weeks	CB2010	10^4	Depo-Provera	weight loss (<10%)	x(R)				x(R)			44
i.vag.	*Ifnar1*$^{-/-}$	7–22 weeks	CB2010	10^4	Depo-Provera	8–9 dpi	x(R)	x(R)	x(R)	x(R)	x(R)			44
i.vag.	AG129, LysM^{Cre+} IFNAR$^{fl/fl}$	8–12 weeks	CB2010	10^5	progest. PMSG	13–22 dpi	x(R)	x(H)	x(H)		x(R/H)			45

(b) Vertical transmission

Route[a]	Strain	Age infected/ analysed	Virus strains	Dose	Treatment	Clinical disease/ pathology	Placenta	Dissemination[e]: fet. CNS	fet. body	fet. lymph	mat. blood	mat. spleen	mat. brain	References
dam i.p.	SJL	E13-15/birth	PA2015	10^{12}	–	FGR, cortical thinning, ocular malformations		x(R)	x(R)					56
dam i.p. pup i.vent	C57BL/6	E13·5/ E17·5-P1	CH2016	10^2	–	cortical thinning, < VZ/SVZ thickness, <ventricular volume and surface area	x(R)	x(R/H)			x(R)			73
dam i.p.	*Rag1*$^{-/-}$	E7/E17-E19 or P0-P2	PA2015	10^4	anti-IFNAR1	–	x (R/H)	x (R/H)	x (R/H)	x (R/H)		x (R/H)	x (R/H)	26
i.vag.	C57BL/6NCrl	E4·5 or E8·5/E18·5	CB2010	10^4	–	modest FGR	x(R)	x(H)						44

Table 2. *(Continued)*

(b) Vertical transmission

Route[a]	Strain	Age infected/ analysed	Virus strains	Dose	Treatment	Clinical disease/ pathology	Placenta	Dissemination[e]: fet. CNS	fet. body	fet. lymph	mat. blood	mat. spleen	mat. brain	References
i.vag.	*Irf3*$^{-/-}$ *Irf7*$^{-/-}$	E4·5 or E8·5/E18·5	CB2010	10^4	–	FGR	x(R)		x(R)		x(R)			44
i.vag.	*Ifnar1*$^{-/-}$ x C57BL/6NCrl	E4·5 or E8·5/E18·5	CB2010	10^4	–	resorption, FGR	x(R)		x(R)		x(R)			44
dam i.v.	FVB/NJ, C57BL/6J	E5 or E7·5-9·5 or 12·5/various to E18·5	BZ2016	10^5	–	dysraphia, hydrocephalus, FGR	x	x	x (W)			x	n.d.	57
dam s.c.	*Ifnar1*$^{-/-}$ (dam) x C57BL/6 (sire)	E6·5 or 7·5/E13·5 or 15·5	FP2013	10^3		FGR, fetal CNS apoptosis	x(FA)	x(FA)			x(FA)	x(FA)	x(FA)	19
dam s.c.	C57BL/6	E6·5 or 7·5/E13·5 or 15·5	FP2013	10^3	anti-IFNAR1	Modest FGR	x(FA)	x(FA)			x(FA)	x(FA)	x(FA)	19

(c) Developmental pathogenesis

dam i.p.	SJL	E13-15/birth	PA2015	10^{12}	–	FGR, cortical thinning, ocular malformations		x(R)	x(R)					56
i.c.	BALBc	E13·5/E18·5 or P1/clinical	VZ2016	10^1	–	cortical thinning (E13·5) 4-25 dpi (P1)		x (PA, H)						74
s.c.	Swiss	P3/clinical	PA2015	10^6	–	9–18 dpi		x(H)						59
i.c., i.p., s.c.	Swiss	P1 or 2 wks/ clinical	BZ2015	10% BS	–	6 dpi (i.c.), 12 dpi (s.c.), gliosis, infiltration, neuronal death, WM damage		x(H)						75
i.c.	C57BL/6	P7 or P21/4dpi	UG1974	10^7	–	decreased brain volume, gliosis, < NPC proliferation, neuronal death		x(H)						76

Table 2. *(Continued)*

(c) Developmental pathogenesis

Route[a]	Strain	Age infected/ analysed	Virus strains	Dose	Treatment	Clinical disease/ pathology	Placenta	Dissemination[e]: fet. CNS	fet. body	fet. lymph	mat. blood	mat. spleen	mat. brain	References
i.c.	CD-1	E13·5/P3-5	CH2016	10^5	–	cortical thinning, >cell death, >progenitor cell cycle exit		x(H)						[77]
i.c.	C57BL/6	E14·5/P3-5	MX2016	10^6	–	FGR, <brain size, cortical thinning, neuronal death, <NPC proliferation, gliosis, blood–brain barrier leakage		x(H)						[78]

[a]Route of transmission: i.c., intracerebral; i.p., intraperitoneal; i. vag, intravaginal; i.vent, intraventricular; s.c. subcutaneous.
[b]Age at infection, E: embryonic day; P: post birth day.
[c]Virus strains: BZ2015: Brazil/2015 (HS-2015-BA-01); CB2010: Cambodia/2010 (FSS13025); CH2016: China 2016; FP2013: French Polynesia/2013 (H/PF/2013); MX2016: Mexico/2016(MEX1-44); PA2015: Paraiba_01/2015; PR2015: Puerto Rico/2015 (PRVABC58); UG1947: Uganda/1947 (MR-766); VZ2016: Venezula/2016.
[d]BS, brain suspension; FGR, fetal growth restriction; NPC, neural progenitor cell; VZ/SVZ, ventricular zone/subventricular zone; WM, white matter.
[e]FA, focus forming assay; H, Histology; n.d., not detected; PA, plaque assay; R, RNA; w, Western blot.

Rag1$^{-/-}$ mice did not result in clinical disease following ZIKV infection[16] (Table 1, part c). Hence, a functional innate response appears to be sufficient to control ZIKV infection in mice, even in the absence of functional T cells.

Combined influence of innate and adaptive immune responses

Combined deficiencies in components of the innate and adaptive immune response have shown that both arms of the immune system influence ZIKV pathogenesis (Table 1, part d). AG129 mice, which lack both IFN type I (α/β) and type II (γ), develop disease in an age-dependent manner with younger mice being more susceptible.[13,15] These mice have similar infection kinetics to A129 mice but with exaggerated disease signs. Depletion or deficiency of T cells in IFN-antagonized mice results in high ZIKV titres and associated disease, demonstrating that the adaptive immune response is critical to controlling infection, when the type I IFN response is suboptimal.[16,17] Furthermore, adoptive transfer of ZIKV-specific CD8 T cells also prevents disease in *Ifnar1*$^{-/-}$ mice, suggesting that a vaccination strategy could be effective at preventing disease, even in the absence of strong IFN responses[17,23,25] (Table 1, part d).

In addition to the cellular adaptive response, there is some evidence from mouse models implicating the humoral response in preventing ZIKV-associated disease. Although B cells are not activated during ZIKV infection in WT animals,[16] a strong neutralizing antibody response is correlated with recovery from ZIKV infection in highly susceptible *Ifnar*$^{-/-}$ mice.[26] Additionally, monoclonal antibodies as well as antibodies derived from convalescent patient serum can inhibit disease in ZIKV-susceptible mice.[27–30] These antibodies target the envelope (E) glycoprotein, which is required for flavivirus entry into the cell.[31] Multiple candidate ZIKV vaccine platforms are currently being developed to prevent human disease.[32–34] So far, all effective candidates have demonstrated induction of a potent sterilizing neutralizing antibody titre and a robust T-cell response, further demonstrating the importance of the adoptive immune response to controlling ZIKV infection.

Influence of immune responses on sexual transmission

Although ZIKV is primarily transmitted to humans by the bite of a mosquito, human-to-human transmission can be observed sexually, from infected males to females,[7] and vertically, from a pregnant woman to her fetus.[1,35,36] Mouse models have shown that both innate and adaptive immune responses influence both sexual and vertical transmission. For example, *Ifnar1*$^{-/-}$ male mice or anti-IFNAR1 treatment of WT male mice develop infection in the testes, which is associated with apoptotic cells following ZIKV infection (Fig. 1a, Table 2, part a).[18,37,38] Similar findings were observed in BALB/c mice treated with dexamethasone.[39] These findings, in association with reports of long-lived infectious virus in human male testes,[40–42] suggest that ZIKV infection could impact male reproductive health (Fig. 1a). Additionally, several studies have shown sexual transmission of ZIKV from infected immune-compromised males to naive immune-compromised females (Fig. 1b).[26,43] Direct animal-to-animal sexual transmission results in infection of reproductive organs in the female (Fig. 1b).[26] Enhanced infection of female reproductive tissues by intravaginal inoculation has also been shown in several studies in mice with suppressed type I IFN responses (*Irf3*$^{-/-}$ *Irf7*$^{-/-}$, *Ifnar1*$^{-/-}$ and LysM^{Cre+} *Ifnar*$^{fl/fl}$ mice).[44,45] In contrast, WT mice or mice without severe IFN suppression (*Mx1*, *Rag2*$^{-/-}$) more readily controlled vaginal infection. Hence, sexual transmission of ZIKV requires an impairment of the type I IFN response in mice and infection of reproductive tissue in both males and females is enhanced in this context.

Immune-mediated mechanisms of ZIKV transmission across the placental barrier

Vertical transmission of ZIKV from infected dam to the fetus has also been successfully modelled in mice (Fig. 2, Table 2, part b). This includes infection of immune-competent and immune-compromised mice, by intraperitoneal, subcutaneous or intravaginal routes at either early (E4–5–E7) or late (E13–15) stages of fetal development. Virus has been detected in the fetal central nervous system (CNS) by RNA, histological and focus-forming assay with pathology demonstration of fetal growth restriction, as well as cortical thinning and apoptosis of neurons in the fetal brain. The timing of infection during pregnancy may greatly influence this response as younger embryos/placentas in infected mice are more likely to develop fetal insufficiency rather than develop microcephaly.[46]

These models allow investigators to address an important question in terms of the ZIKV pathogenesis, how does ZIKV cross the placental barrier? This barrier is established with in several days of conception[47] and is necessary for maintaining the pregnancy. A critical function of the placenta is to connect the maternal and fetal blood (Fig. 2a,c). In both humans and mice, this critical interface is maintained and controlled by syncytiotrophoblasts,[47,48] suggesting that the mouse may be a useful model to study transmission. ZIKV may enter the fetal blood through various mechanisms involving both the maternal and fetal immune systems. Maternal immune cells may become infected and be transported across the syncytiotrophoblast layer into close proximity to the fetal

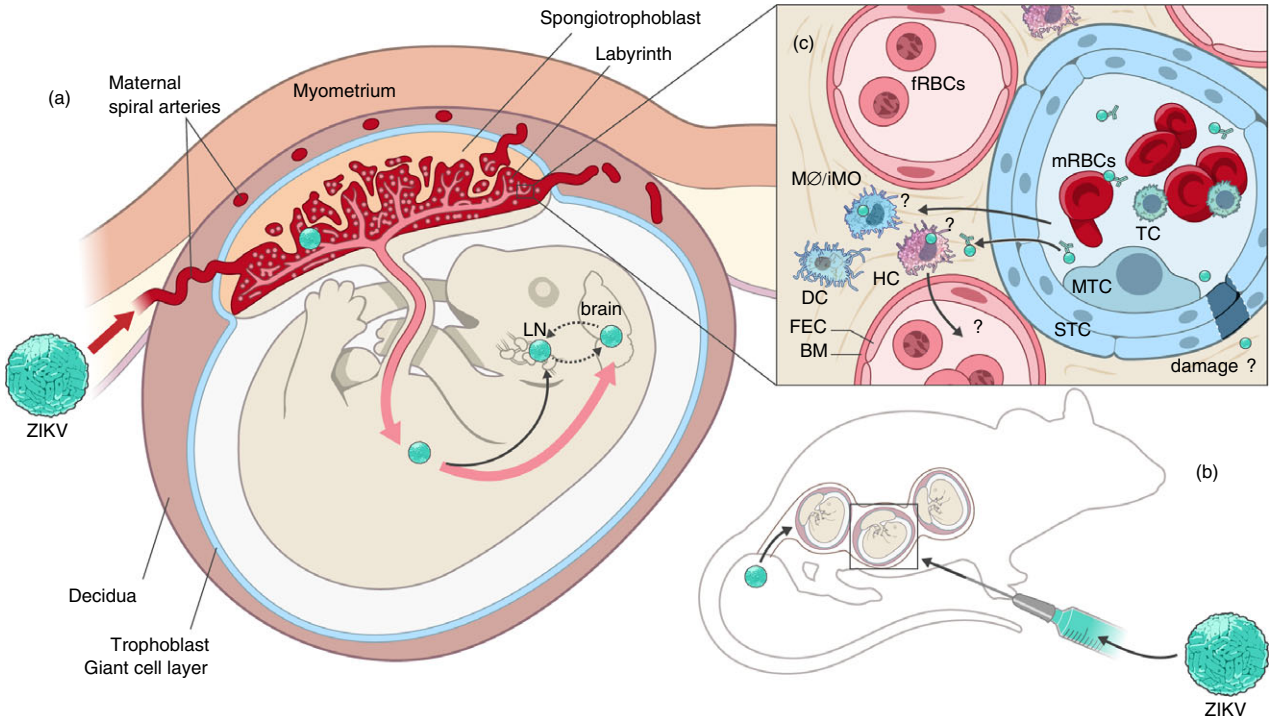

Figure 2. Zika virus (ZIKV) vertical transmission has been demonstrated in murine models, but further study is required to determine the specific mechanism of transmission. (a) In both mice and humans, the developing fetus and placenta are separated from the maternal myometrium by the decidua and the trophoblast giant cell layer. The placenta serves as the primary interface between the fetal and maternal blood where nutrient exchange occurs. In several mouse vertical transmission models, ZIKV has been shown to heavily infect the placenta. If infection occurs early in development, fetuses typically are reabsorbed or are not viable at birth. Infection later in development can result in vertical transmission and infection of the fetal brain and lymphatic tissue. (b) Vertical transmission in mice can occur if virus is inoculated intravaginally or through a peripheral route and is not necessary transmitted to all fetuses in a litter. Further experimentation with these models is required to determine the rate and timing of transmission. (c) ZIKV must cross the placental barrier, which is formed by syncytiotrophoblasts (STC), in order to be vertically transmitted. Multiple crossing mechanisms are possible. These include damage to STC cells by maternal or fetal immune cells, antibody-dependent viral transcytosis, infection of maternal immune cells that cross the STC and or infection of HCs that cross back into fetal blood. FEC, fetal endothelial cells, BM, basement membrane, HC, Hoffbauer cells, DC, dendritic cells, MO/iMO, monocyte/macrophage, fRBCs, fetal red blood cells, MTC, mononuclear trophoblast cell, STC, syncytiotrophoblast cells.

blood supply (Fig. 2c). Alternatively, syncytiotrophoblasts may be damaged by maternal immune cells through direct or indirect cytotoxic mechanisms leading to breakdown of the placental barrier (Fig. 2c). It is also possible that ZIKV may be trafficked across the placental barrier via immunoglobulin-mediated transcytosis, as has been suggested for other placenta-invasive viruses (Fig. 2c).[49] This mechanism could be facilitated by dengue-specific cross-reactive antibody, which has been shown to enhance ZIKV infection.[50]

On the fetal side of the barrier, resident macrophage Hofbauer cells are known to be activated by ZIKV infection.[51] These cells may cross the fetal endothelium and become infected before returning to the fetal blood or may damage the fetal endothelium in response to infection (Fig. 2c). Although any of these mechanisms may contribute to ZIKV crossing the placenta, caution must be applied when drawing conclusions from murine models. Mice are haemotrichorial in that three layers of syncytiotrophoblasts separate maternal from fetal blood whereas humans are haemochorial with a single layer of syncytiotrophoblasts. Hence, findings from murine models should be verified in other haemochorial systems such as guinea pigs.[52]

Effect of fetal CNS inflammation on brain development and pathogenesis

Important questions remain regarding ZIKV infection and vertical transmission such as how the virus mediates damage to the developing brain and the role of the immune response in mediating this damage. Direct intravaginal inoculation of the dam or intracerebral injection of ZIKV into fetuses at late stages of embryonic development or early points post birth have shown clear damage to the developing brain, including cortical thinning, decreased brain size, decreased neuroprogenitor cell numbers and gliosis (Fig. 2, Table 2). However, the role of the immune

response in mediating this damage is only starting to be examined. Exposure to virus *in utero* can elicit a strong local immune response within the fetus, which is mediated by pro-inflammatory cytokines such as TNF, IL-1β, IFN-β and IFN-γ.[53] Such exposure can cause both behavioural and CNS structural pathology in offspring, which is correlated with changes in developmental gene expression within the CNS.[54] Experiments using poly(I:C) injected into pregnant mice to mimic viral infection have demonstrated that the cytokine profile induced in the fetal CNS is dependent on the gestational age when exposed to the stimulus.[55] Fetuses exposed at earlier embryonic time-points express higher levels of pro-inflammatory cytokines in their brain, which correlates with increased CNS pathology and worsened behavioural outcomes. Hence, immune responses generated in the developing fetal brain caused by early exposure to ZIKV may account for the more severe phenotypic outcomes seen in these models of ZIKV vertical transmission, such as fetal abortion/resorption, (Table 2, part b).[19,44,56,57] In contrast, CD8 T cells may play a role in clearing virus from the brain as they are the predominant infiltrate in a model of ZIKV infection in neonatal immunocompetent mice.[58]

Fetal mice exposed to ZIKV later in development generally present with neurodevelopmental pathologies such as cortical thinning, neuronal death, reduced neural progenitor proliferation, white matter damage and gliosis (Table 2, part c). One study found that neonatal mice injected intracerebrally with ZIKV have increased levels of *Tnf*, *Il6*, *Il1b*, *Nos2* and *Ccxcl1* mRNA in their brains at the peak of viral replication, which are associated with increased incidence of seizure.[59] Hence, virus-associated neuroinflammation may contribute to these neuropathologies. Similar behavioural abnormalities are observed when neonatal mice are directly injected intracerebrally with TNF-α, indicating impaired CNS development.[60] Furthermore, IL-6 when applied to neural progenitor cells *in vitro* decreases their differentiation into mature neurons and increases programmed cell death, suggesting that pro-inflammatory cytokines may be detrimental to neurodevelopment.[61,62] Interleukin-1β and TNF have similar effects when applied to rat embryonic primary cortical neurons.[63] In this way, virus-associated pro-inflammatory cytokines produced in the CNS during development could, in part, account for the reduced brain volumes and impaired cortical patterning observed in ZIKV-associated cases of microcephaly.[64]

In related studies, intracerebral injection of TNF and IL-1β into neonatal rat brain results in increased astrogliosis and microgliosis,[65] suggesting that these cytokines could contribute to the reactive gliosis observed in models of ZIKV infection in developing brains (Table 2, part c). Likewise, these cytokines, regardless of whether they are directly injected or are induced by hypoxic injury in the neonatal brain, have been shown to induce oligodendrocyte death and hypomyelination of axons.[65,66] Hence, pro-inflammatory cytokines may also contribute to the white-matter injury and hypomyelination associated with ZIKV infection in the developing brain (Table 2, part c).[67,68] Furthermore, microglia have been associated with synaptic pruning during development,[69] which is a necessary process for cellular patterning and function. Stimulation of developmentally immature microglia with poly(I:C) can shift these cells toward a more mature, reactive phenotype.[70] Hence, ZIKV infection may predispose the developing brain to abnormal synaptic pruning, as is the case with Fragile-X syndrome.[71] Collectively these data suggest that activation of glial responses during CNS development will probably have a negative outcome during fetal development.

Conclusions and future directions

The ability to use a large variety of knockout mice and antibody treatments have provided the tools to gain a basic understanding of the key innate and adaptive immune responses that are essential for controlling ZIKV infection and preventing both pathogenesis and transmission. As one of the most harrowing outcomes of ZIKV infection is microcephaly, the use of vertical transmission models and CNS developmental models to dissect the mechanisms by which ZIKV induces damage to the developing CNS is essential. Therapeutic studies have indicated a potential for improving CNS developmental outcomes during fetal viral infection. For example, administration of anti-TNF antibodies reduced the incidence of seizures in neonatal mice injected intracerebrally with ZIKV.[59] Hence, measures taken to minimize the pro-inflammatory response in the CNS of fetuses with ZIKV infection may improve developmental outcomes. However, further studies are needed to determine the efficacy of any such intervention and should ensure that the innate immune viral clearance mechanisms within the CNS cells are not impaired.

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