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TAM receptors are not required for Zika virus infection in mice

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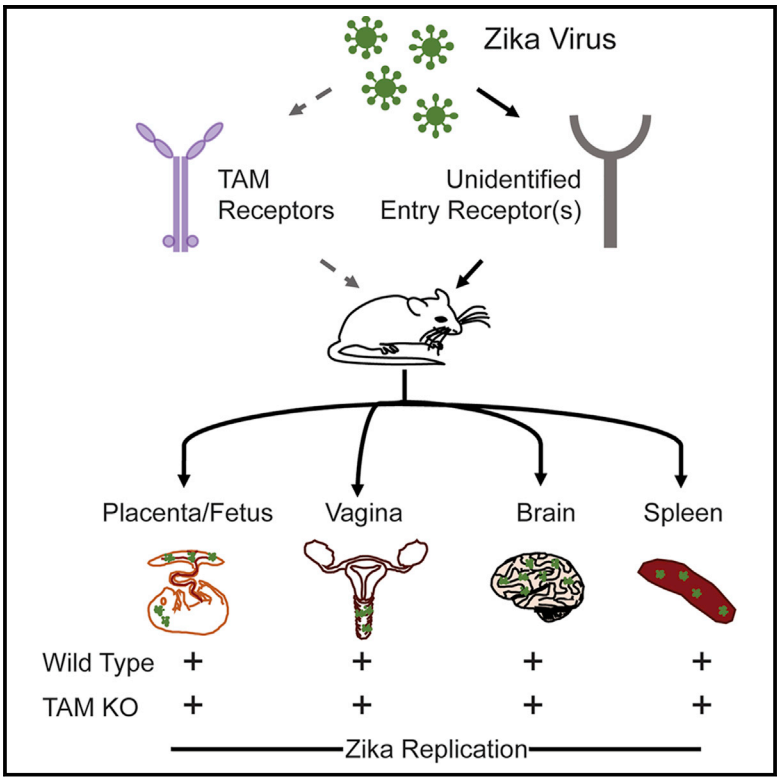
Graphical Abstract



Highlights

- TAM receptors are not essential for ZIKV infection in mice
- TAM receptors are not required for mother-to-fetus transmission in IFNAR-blocked mice
- ZIKV tropism in the placenta and brain does not depend on TAM receptors
- Murine Axl is capable of facilitating ZIKV infection in human cells in vitro

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In Brief

TAM receptors have been implicated as entry receptors for the Zika virus. In this study, Hastings et al. used genetic knockout mouse models to demonstrate that they are not necessary for the infection of mice via multiple routes of viral challenge. These results suggest the existence of redundant entry receptors for ZIKV in mice.

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TAM Receptors Are Not Required for Zika Virus Infection in Mice

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SUMMARY

Tyro3, Axl, and Mertk (TAM) receptors are candidate entry receptors for infection with the Zika virus (ZIKV), an emerging flavivirus of global public health concern. To investigate the requirement of TAM receptors for ZIKV infection, we used several routes of viral inoculation and compared viral replication in wild-type versus *Axl*$^{-/-}$, *Mertk*$^{-/-}$, *Axl*$^{-/-}$*Mertk*$^{-/-}$, and *Axl*$^{-/-}$*Tyro3*$^{-/-}$ mice in various organs. Pregnant and non-pregnant mice treated with interferon-α-receptor (IFNAR)-blocking (MAR1-5A3) antibody and infected subcutaneously with ZIKV showed no reliance on TAMs for infection. In the absence of IFNAR-blocking antibody, adult female mice challenged intravaginally with ZIKV showed no difference in mucosal viral titers. Similarly, in young mice that were infected with ZIKV intracranially or intraperitoneally, ZIKV replication occurred in the absence of TAM receptors, and no differences in cell tropism were observed. These findings indicate that, in mice, TAM receptors are not required for ZIKV entry and infection.

INTRODUCTION

Zika virus (ZIKV) is an emerging, positive-sense, enveloped flavivirus that is primarily transmitted by the *Aedes* species of mosquitoes. Although it was first discovered in Africa in 1947 (Dick, 1952; Dick et al., 1952), ZIKV has gained worldwide attention recently after an outbreak in the Americas and an association with severe birth defects, including microcephaly and congenital malformations. Although mosquito transmission explains most of the ZIKV infections in humans, (Li et al., 2012), it has become apparent during the most recent outbreaks that, in contrast to other flaviviruses, ZIKV can also be transmitted by sexual contact (World Health Organization, 2016).

It is believed that ~80% of ZIKV infections are asymptomatic (Duffy et al., 2009), with the majority of others presenting with a relatively mild self-limiting febrile illness that can be accompanied by rash, myalgia, conjunctivitis, and headache (Bearcroft, 1956; Simpson, 1964). However, the new outbreaks have been linked to more severe disease, including Guillain-Barre syndrome (GBS), marked by subacute flaccid paralysis (Ioos et al., 2014; Oehler et al., 2014) in adults, and congenital malformations and neurological syndromes in newborns. Birth defects have been attributed to this virus in at least 28 countries (Schuler-Faccini et al., 2016; Ventura et al., 2016; World Health Organization, 2016). While many of the acute clinical manifestations of ZIKV are seen with closely related flaviviruses, the ability of ZIKV to persist in semen, transmit through sexual contact, and cause birth defects is unexpected. One possible explanation for the differences in the clinical manifestations of ZIKV compared with related viruses is altered tropism. To this end, identification of the entry receptor(s) for ZIKV is critical to understanding the tropism and pathogenesis of this virus and could promote the development of novel therapies that block or disrupt infection.

Replication of ZIKV has been described in a variety of tissues in the host, including the CNS (Tang et al., 2016a), saliva (Musso et al., 2015), blood (Musso et al., 2016), urine (Zhang et al., 2016), and semen (Atkinson et al., 2016). Tropism of ZIKV for cells in the brain (Li et al., 2016) and the testes (Govero et al., 2016; Ma et al., 2016) leads to apoptosis of critical cell types in these organs. In addition, like other flaviviruses, dendritic cells and macrophages in the skin and other tissues are thought to be a primary target for replication of ZIKV (Hamel et al., 2015; Jurado et al., 2016; Wu et al., 2000). Cell-culture studies investigating the entry factors for other important flaviviruses, including dengue virus (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV), have proposed a variety of proteins, but data supporting their function in vivo are inconclusive or non-existent. Two families

of proteins with the most evidence for augmenting flavivirus infection are the C-type lectins (Dejnirattisai et al., 2011; Tassaneetrithep et al., 2003; Vega-Almeida et al., 2013) and the phosphatidylserine receptors, T cell immunoglobulin and mucin domain (TIM) and Tyro3, Axl, and Mertk (TAM) (Kuadkitkan et al., 2010; Meertens et al., 2012). One member of the TAM family of receptor tyrosine kinases (RTKs), Axl, has been implicated as an entry receptor for ZIKV (Meertens et al., 2017).

Axl is expressed at high levels in several cell types that are susceptible to ZIKV infection, (Lemke and Burstyn-Cohen, 2010; Ma et al., 2016; Nowakowski et al., 2016; Rothlin et al., 2015; Tabata et al., 2016) and in vitro evidence in cell lines and human primary cells (Hamel et al., 2015; Liu et al., 2016; Meertens et al., 2017; Retallack et al., 2016; Savidis et al., 2016) using gene silencing, ectopic expression, chemical inhibitors, or blocking antibodies supports the hypothesis that the virus uses Axl either to enter cells or as a signaling receptor to enhance infection. However, recent data using CRISPR-Cas9-based gene editing of Axl in human neural progenitor cells (NPCs) and cerebral organoids questioned this conclusion, as loss of Axl expression did not impact ZIKV infectivity (Wells et al., 2016). Moreover, members of our group have shown that ZIKV infection of brain, eyes, and testes (Govero et al., 2016; Miner et al., 2016b) in mice treated with a type I interferon (IFN) receptor blocking antibody, MAR1-5A3 (α-IFNAR [IFN α receptor] antibody), is not dependent on TAM receptors Axl and Mertk. The TAM family of transmembrane proteins contain an extracellular, a transmembrane, and a conserved intracellular tyrosine kinase domain and bind the ligands Gas6 and Protein S, which recognize phospatidylserine moieties on dying cells or on enveloped viruses (Lemke and Burstyn-Cohen, 2010; Shimojima et al., 2007). TAM receptors have many cellular functions, including natural killer (NK) cell differentiation, clearance of apoptotic debris, and innate immune modulation (Bosurgi et al., 2013; Caraux et al., 2006; Carrera Silva et al., 2013; Paolino et al., 2014). TAM receptors are induced through the type I IFN pathway and limit an overabundant inflammatory response by inhibiting signaling downstream of the Toll-like receptors (TLRs) (Rothlin et al., 2007). In some cells (e.g., dendritic cells), TAM receptor anti-inflammatory signaling requires a physical interaction with IFNAR1 (Rothlin et al., 2007). As type I IFN signaling is critical for the control of ZIKV infection (Lazear et al., 2016) and the function and expression of some TAM receptors, an analysis of the role of TAM receptors on ZIKV infectivity must consider the TAM receptor-IFN signaling axis. In this study, we compare ZIKV infection and pathogenesis in wild-type (WT) and TAM-receptor-deficient mice through different routes of infection and analysis.

RESULTS

Axl and Mertk Are Not Required for Transplacental Transmission, Replication in Pregnant Mice, or Replication in Fetuses

A mouse model of transplacental infection of fetuses was described using an α-IFNAR-blocking antibody to facilitate susceptibility of pregnant C57BL/6 wild-type (WT) mice to ZIKV infection. Fetuses from ZIKV-infected dams treated with the IFNAR-blocking antibody become infected and exhibit an intrauterine growth defect compared to control mice (Miner et al., 2016a). We utilized this pregnancy model to determine whether Axl or another TAM receptor, Mertk, was involved in the transmission of ZIKV through the transplacental route or in replication of the virus in fetal tissues. TAM receptors are expressed to varying levels on several relevant cells, including placental trophoblasts, fetal endothelial cells, Hofbauer macrophages, and fetal neuroprogenitor cells (Nowakowski et al., 2016; Tabata et al., 2016). Pregnant WT, $Axl^{-/-}$, $Mertk^{-/-}$, and $Axl^{-/-}Mertk^{-/-}$ dams who had been mated with the respective WT or TAM knockout (KO) sires were treated with α-IFNAR-blocking antibody and inoculated subcutaneously with 10^3 focus-forming units (FFUs) of a Brazilian strain (Paraiba 2015) of ZIKV on embryonic day (E) 6.5, and both maternal tissue and fetal tissue were harvested on E13.5 for analysis of levels of viral RNA by qRT-PCR. In the pregnant dams, we observed no difference in ZIKV RNA levels in the brains and spleens of WT, $Axl^{-/-}$, and $Mertk^{-/-}$ mice; a significant increase in serum ZIKV titer was noted in $Axl^{-/-}$ dams (Figure 1A). Analogously, the levels of ZIKV RNA in the brains of α-IFNAR-treated non-pregnant female $Axl^{-/-}$ mice were similar to those in WT mice (Figure S1).

To assess the role of TAM receptors in promoting transplacental transmission, we analyzed viral RNA in the placenta and the heads of fetal mice. We observed no significant differences in ZIKV infection between WT, $Axl^{-/-}$, and $Axl^{-/-}Mertk^{-/-}$ fetal heads and only a modest increase in viral titers in $Mertk^{-/-}$ placentas as compared to WT (Figure 1B). In addition, we saw no difference in the size of ZIKV-infected fetuses between groups (Figure 1C). To determine whether TAM receptors influenced the tropism of ZIKV infection in the placenta, we performed in situ hybridization (ISH) with ZIKV-specific RNA probes. The placenta is composed of two layers: the junctional zone, composed of spongiotrophoblasts and invasive glycogen cells; and the labyrinth zone composed of cytotrophoblast and syncytial trophoblasts and fetal-derived blood vessels (Coan et al., 2005). At E13.5, ISH for ZIKV RNA revealed similar staining patterns, with scattered positive cells in the junctional zone and a lack of appreciable positivity in the labyrinth zone; similar numbers of positive cells were noted in placentas harvested from WT, $Axl^{-/-}$, and $Axl^{-/-}Mertk^{-/-}$ mice (Figure S2). Together, these data demonstrate that neither Axl nor Mertk are required for ZIKV infection of mice treated with IFNAR-blocking antibody. Additionally, transplacental transmission and replication of the virus in the placenta and the fetus can occur independently of the expression of Axl or Mertk receptors.

Replication of ZIKV in the Vaginal Tract of Virgin Mice Does Not Require Axl Expression

As the expression of AXL is induced by type I IFN signaling (Scutera et al., 2009), and its signaling function in some cells requires IFNAR1 expression (Rothlin et al., 2007), type I IFN signaling may be necessary for Axl to enhance flavivirus infection (Bhattacharyya et al., 2013). Intravaginal infection of ZIKV allows local viral replication in the vaginal tract, even in WT mice (Khan et al., 2016; Yockey et al., 2016). This model allowed us to investigate whether ZIKV infection required Axl expression under conditions when type I IFN signaling remained intact. Virgin WT or $Axl^{-/-}$ mice were treated with Depo-Provera to maintain the diestrus-like phase, when mice are most susceptible to vaginal ZIKV

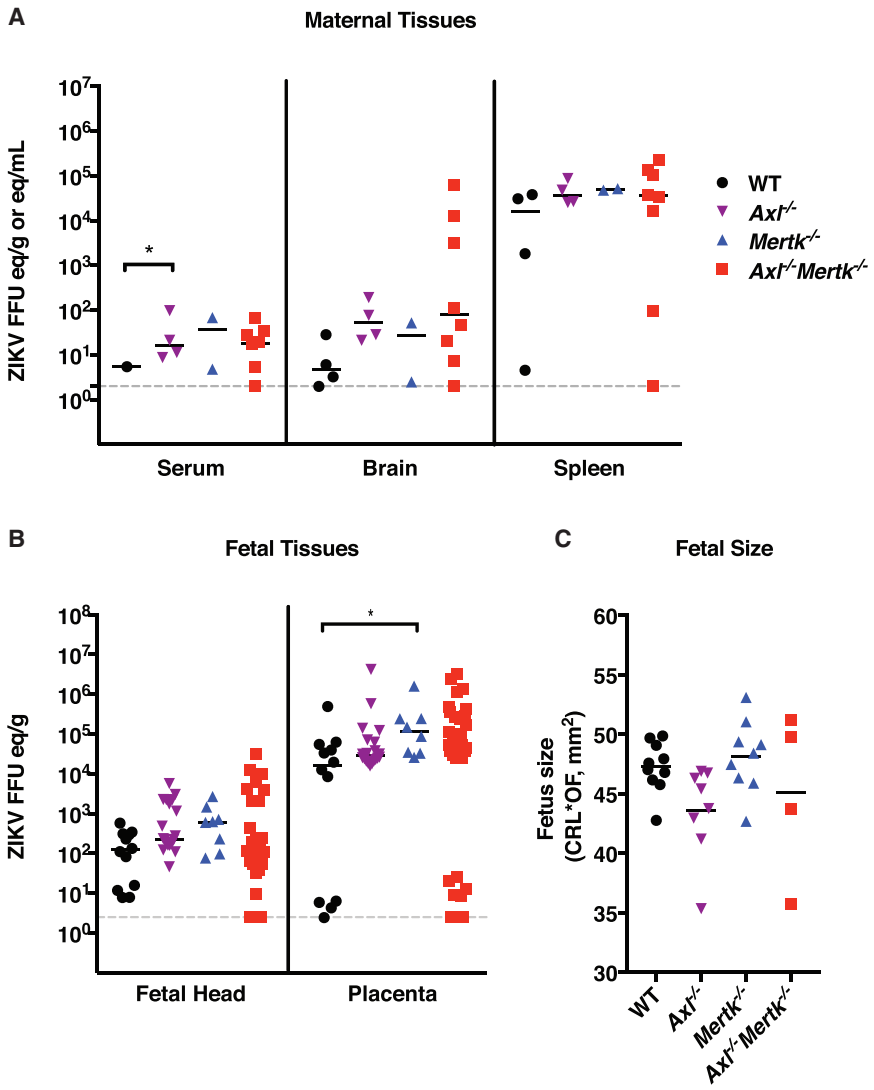

Figure 1. ZIKV Infection of Maternal and Fetal Tissues Is Similar in WT, $Axl^{-/-}$, $Mertk^{-/-}$, and $Axl^{-/-}Mertk^{-/-}$ Mice

Pregnant WT, $Axl^{-/-}$, $Mertk^{-/-}$, and $Axl^{-/-}Mertk^{-/-}$ dams were treated with 2 mg of α-IFNAR-blocking antibody on embryonic day (E) 5.5 and inoculated subcutaneously with 10^3 FFUs of the Brazilian strain of ZIKV on E6.5.

(A) Serum, brain tissue, and splenic tissue from ZIKV-infected pregnant dams were collected at E13.5 and assessed for viral titer by qRT-PCR. ZIKV titers in $Axl^{-/-}$ sera were higher than those in WT dams (15-fold, *p = 0.0372 by Kruskal-Wallis with Dunn's multiple comparisons test), but serum titers in $Mertk^{-/-}$ dams were not significantly different from those in WT. No significant differences in spleen or brain titers were found. n = 4 pregnant dams for WT, 4 for $Axl^{-/-}$, 2 for $Mertk^{-/-}$, and 8 for $Axl^{-/-}Mertk^{-/-}$. eq, equivalent.

(B) Fetal heads and placental tissue were also collected at E13.5 and assessed for viral titer by qRT-PCR. No significant differences in viral burden were observed in fetal heads. In placentas likewise, no significant differences in viral burden were seen, except in $Mertk^{-/-}$ placentas, which exhibited a 7-fold increase in viral burden as compared to WT (*p = 0.0196, Kruskal-Wallis with Dunn's multiple comparisons test). n = 12 for WT, 20 for $Axl^{-/-}$, 8 for $Mertk^{-/-}$, and 32 for $Axl^{-/-}Mertk^{-/-}$. The gray dashed line indicates the limit of detection.

(C) Fetal size at E13.5 was measured as crown-rump length (CRL) × occipita-frontal (OF) diameter expressed as square millimeters. n = 4–10 per indicated group. ns, not significant by Kruskal-Wallis with Dunn's multiple comparisons test.

See also Figures S1 and S2.

infection (Tang et al., 2016b; Yockey et al., 2016). Animals were then inoculated intravaginally with 1.5 × 10^5 plaque-forming units (PFUs) of ZIKV FSS13025 (Cambodia 2010). Vaginal washes were collected daily, and RNA was isolated. ZIKV RNA levels in the vaginal washes persisted through 6 days post-infection in both WT and $Axl^{-/-}$ mice (Figure 2). No significant differences were observed between the groups except on day 4, when ZIKV levels in the $Axl^{-/-}$ mice dropped slightly (p = 0.02); nonetheless, comparable levels of viral RNA were observed in WT and $Axl^{-/-}$ mice at day 5 (Figure 2). These data indicate that Axl is not required for ZIKV infection in the vaginal tract.

ZIKV Replication in 1-Week-Old Neonatal Mice after Intraperitoneal Inoculation Is Axl Independent

Neonatal mice younger than 7 days old are susceptible to ZIKV infection via the intraperitoneal route without IFNAR blockade (Dick, 1952; Lazear et al., 2016; Manangeeswaran et al., 2016). We used this model to determine whether the TAM receptors are involved in replication or trafficking of ZIKV from the periphery to the CNS. Seven-day-old WT, $Axl^{-/-}$, $Mertk^{-/-}$, and $Axl^{-/-}/Mertk^{-/-}$ mice were inoculated with 10^4 PFUs of the Brazilian strain (Paraiba 2015) of ZIKV. After 7 days, these animals were euthanized, spleens and brains were harvested, and ZIKV burden was measured using qRT-PCR. Viral burden was higher in the spleen and brain of $Axl^{-/-}/Mertk^{-/-}$-infected mice, but no other significant differences were detected in the brain or spleen between any of these groups (Figure 3), showing that Axl and Mertk are not required for replication of ZIKV in lymphoid tissue or spread to the brain, even in the presence of an intact type I IFN signaling system.

ZIKV Replication and Tropism after Intracranial Inoculation of ZIKV Is Not Affected by the Absence of Axl, Mertk, and Tyro3

To investigate the requirement of Axl, Mertk, and Tyro3 directly in the brain, again without the need for IFNAR blockade, we used a model of intracranial inoculation of 10-day-old mice (Dick, 1952). This method has an added benefit of bypassing any role that TAM receptors play in replication in other tissues or transmission to the brain (Miner et al., 2015). Ten-day-old WT, $Axl^{-/-}/Tyro3^{-/-}$, and $Axl^{-/-}/Mertk^{-/-}$ mice were inoculated with 10^6 PFUs of ZIKV

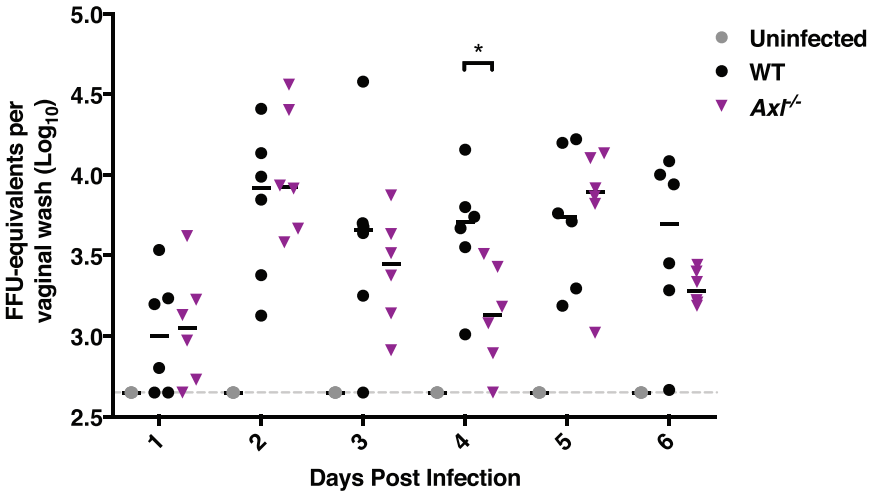

Figure 2. WT and *Axl*$^{-/-}$ Mice Support Similar ZIKV Levels after Intravaginal Infection

Fourteen- to 16-week-old WT and *Axl*$^{-/-}$ female mice were treated with Depo-Provera and inoculated intravaginally with 1.5 × 10^5 PFUs of Cambodia strain ZIKV and daily vaginal washes were collected. Titers were determined by RNA isolation of vaginal washes, and levels were determined by qRT-PCR. ZIKV levels on day 4 were significantly lower in *Axl*$^{-/-}$ mice (*p = 0.026) but were not significantly different between WT and *Axl*$^{-/-}$ at any other time point. Data shown are pooled from two independent experiments (n = 6 per group for infected; n = 5 per group for uninfected). Gray dashed line indicates the limit of detection.

MEX2-81 (Mexico 2016) via an intracranial route and monitored for 7 days. Brains were harvested at day 5 and day 7 post-infection, the peak of viral infection, and assessed for viral replication using both qRT-PCR and plaque assays. Groups showed no significant differences in growth rates, until day 7 when all three infected groups exhibited a decline in weight as compared to uninfected controls (Figure 4A). Viral titers in the brain were not significantly different in the WT and KO groups as measured by RNA levels (Figure 4B) or infectious virus (Figure 4C). To determine whether TAM receptor expression affected the cellular tropism of ZIKV infection in the brain, we performed immunohistochemical analysis. We co-stained for ZIKV infection with antigenic markers for neurons (neuronal nuclei; NeuN), astrocytes (glial fibrillary acidic protein; GFAP), and microglia (ionized calcium-binding adapter molecule 1; Iba1) at days 5 and 7 post-infection. Our data suggest that the majority of ZIKV infection localizes to neurons on day 5 (Figure 5), whereas at day 7, some microglia also co-stain with ZIKV antigen (Figure 5). All images shown are from the cortex, but staining was also seen in the hippocampus for most samples and in the cerebellum in some samples. Notably, no differences were observed in ZIKV antigen staining or co-localization with cells from WT and TAM-receptor-deficient mice. These data show that pathogenesis and viral replication is equal in WT, *Axl*$^{-/-}$/*Tyro-3*$^{-/-}$ and *Axl*$^{-/-}$/*Mertk*$^{-/-}$ mice after intracranial infection of ZIKV.

Mouse Axl Is Sufficient to Restore ZIKV Infection of Human Cells Lacking Axl

One possibility for the discrepancy between our studies, which show no role for TAM receptors in mouse models of ZIKV infection, and the previously published in vitro studies showing a requirement for TAM receptors in infection of different human cell types, is that there may be a species-specific incompatibility between ZIKV's interaction (via Gas6) with human and mouse Axl. To directly address this, we used transcomplementation studies to investigate whether mouse Axl is sufficient to restore ZIKV infection in human cells lacking Axl. Consistent with previous studies (Hamel et al., 2015; Liu et al., 2016; Meertens et al., 2017; Retallack et al., 2016; Savidis et al., 2016), we used fluorescent microscopy to show that HeLa cells in which Axl has been gene edited using CRISPR-Cas9 were markedly less susceptible to ZIKV infection (Figures 6A and 6B), and we validated these findings using flow cytometry (Figure 6C). Expression of mouse Axl using a retroviral vector restored ZIKV infection of human Axl-deficient cells (Figures 6A–6C). The presence of human and mouse Axl was confirmed by western blotting (Figure 6D). These data show that mouse Axl is capable of mediating ZIKV infection in human cells and that the dispensability of Axl in mice is unrelated to its ability to serve as an entry receptor.

DISCUSSION

TAM receptors are considered candidates for a ZIKV entry receptor, based largely on studies in cell culture or co-localization analysis in vivo. By performing comparative infection studies in WT and TAM receptor KO mice, we demonstrated that TAM receptors are not required for ZIKV infectivity through subcutaneous, transplacental, vaginal, or intracranial routes of infection. ZIKV replication is unaffected by the lack of these receptors in tissues including the spleen, placenta, vagina, and brain. We also show that the cellular targets in the brain and placenta are similar, regardless of the absence of the TAM receptors. These findings indicate that, in mice, TAM receptors are not required for ZIKV infection.

Several previous studies show that inhibition of Axl in vitro in human cells blocks ZIKV infection (Hamel et al., 2015; Liu et al., 2016; Meertens et al., 2017; Retallack et al., 2016; Savidis et al., 2016). Specifically, CRISPR KO of Axl results in reduced ZIKV infection in human cervical adenocarcinoma cells (HeLa) (Savidis et al., 2016), the human glioblastoma line (U87) (Retallack et al., 2016), the human microglial cell line (CHME3) (Meertens et al., 2017), and human embryonic kidney cells (293T) (Liu et al., 2016); and small interfering RNA (siRNA) knockdown of Axl results in reduced ZIKV infection in human alveolar basal epithelial carcinoma cells (A549) (Hamel et al., 2015). Based on these results, we speculated that the mechanisms that underlie the dispensability of TAM receptors in ZIKV infection in mice in vivo include the following: (1) the inability of mouse TAM to serve as ZIKV entry receptors; (2) the existence of other entry receptors in mice that are not

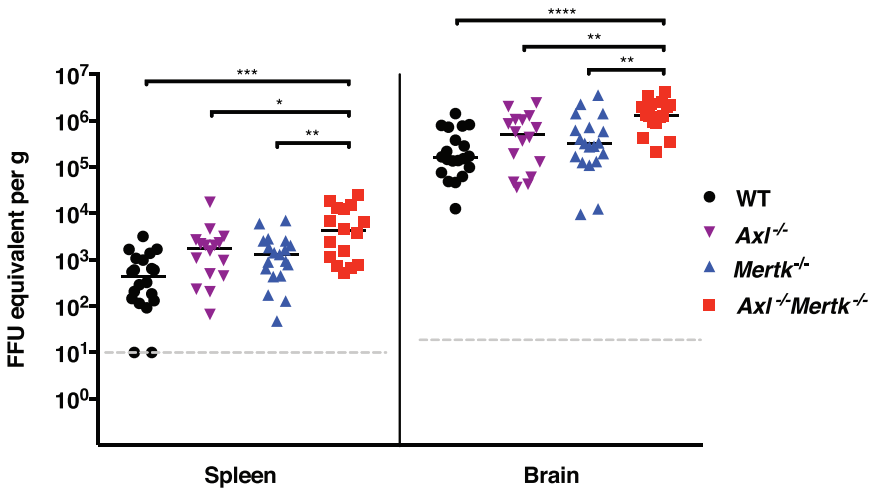

Figure 3. ZIKV Titers in Intraperitoneal Infection of 7-Day-Old WT, $Axl^{-/-}$, and $Mertk^{-/-}$ Mice Are Similar and Higher in $Axl^{-/-}Mertk^{-/-}$ Mice

One-week-old mice were infected with 10^4 FFUs of Brazil strain ZIKV by intraperitoneal injection. Viral replication in spleen (left) and brain (right) was quantified 1 week post-infection by qRT-PCR. 10-fold more ZIKV RNA was found in $Axl^{-/-}Mertk^{-/-}$ spleens than in WT spleens, and 4-fold more was found in $Axl^{-/-}Mertk^{-/-}$ brains than in WT. Viral burdens in $Axl^{-/-}Mertk^{-/-}$ spleens and brains were also higher than in $Axl^{-/-}$ and $Mertk^{-/-}$ mice. Symbols represent focus-forming equivalents per gram of ZIKV RNA for individual animals; solid line represents median, and gray dashed line represents limit of detection. For spleen: *p = 0.0161; **p = 0.002; ***p = 0.0001. For brain: **p = 0.0038 ($Axl^{-/-}Mertk^{-/-}$ versus $Mertk^{-/-}$); **p = 0.0074 ($Axl^{-/-}Mertk^{-/-}$ versus $Axl^{-/-}$); and ****p < 0.0001 ($Axl^{-/-}Mertk^{-/-}$ versus WT) as calculated using one-way ANOVA with Tukey's multiple comparisons test.

expressed by human cells; and (3) human cells in vivo also have redundant entry receptors, although human cell lines in vitro fail to recapitulate the expression of the entire spectrum of viral entry receptors present in their in vivo counterparts.

In order to determine whether mouse Axl is capable of serving as a viral entry receptor, we performed a transcomplementation study in HeLa cells. We found that endogenous human Axl is required for ZIKV infection, as previously reported (Savidis et al., 2016), and that mouse Axl was sufficient to restore infection in HeLa cells lacking human Axl. Thus, these results eliminated the possibility that mouse Axl fails to serve as a viral receptor for ZIKV. The second possibility, as discussed earlier, is that key target cells for ZIKV infection in mice, but not humans, express additional receptors (e.g., TIM-1 or other phosphatidylserine receptors such as CD300a; Carnec et al., 2015), allowing infection to occur in the absence of TAM receptors due to functional redundancy. This possibility requires generating multigene KO mice in the future. The third possibility is that the human cells in vivo also have multiple entry receptors for ZIKV but that human cell lines in vitro fail to recapitulate the expression of the entire spectrum of viral entry receptors. Many of the experiments suggesting a key entry role for ZIKV were performed in cell lines that may not fully represent the patterns of receptor expression in vivo on primary cells (Liu et al., 2016; Retallack et al., 2016; Savidis et al., 2016). It is also possible that the differences in type I IFN signaling after ZIKV infection in mice versus humans may be masking an important role for Axl in infection in mice. Meertens et al. suggest that ZIKV exploits Axl's ability to suppress the type I IFN response after infection (Meertens et al., 2017), and most mouse models for ZIKV infection require the ablation of IFN signaling due to an impaired ability of the virus to suppress mouse, but not human, IFN response (Grant et al., 2016). To address this, we included models that do not require suppression of the type I IFN response among the models that we tested. However, ZIKV may preferentially be infecting cells that have an impaired ability to produce type I IFN, potentially minimizing the effects of Axl (Khan et al., 2016; Kreit et al., 2014).

It remains possible that Axl, Mertk, or Tyro3 are entry receptors in specific cells that we did not capture with the infection routes that were tested in our study. We consider this unlikely, given the various routes of infection used including subcutaneous, transplacental, intravaginal, intraperitoneal, and intracranial ZIKV administration. We did not observe any differences in viral titers or tropism in the mouse in various organs tested. Particularly, in our intracranial infection model, neurons were infected, but astrocytes were not. In the human developing brain, Axl is required for ZIKV infection in astrocytes but not in NPCs (Meertens et al., 2017). In the present study, intracranial infection of young WT mice showed no infection of astrocytes. However, our results showing no differences in infection of neurons in WT versus Axl KO mice are consistent with a lack of role of Axl in infection of NPCs or brain organoid cultures (Meertens et al., 2017; Wells et al., 2016).

Independent of its possible function as an entry receptor, TAM receptors can have important roles in the pathogenesis of viral infections through additional mechanisms. For example, Mertk and Axl promote blood-brain-barrier integrity and protect mice against pathology after neuroinvasive WNV infection (Miner et al., 2015). $Axl^{-/-}$ mice have increased lethality and delayed virus clearance after influenza and WNV infection due to impaired priming of the adaptive immune response by DCs (Schmid et al., 2016). Mertk and Axl are also essential for many aspects of microglial function and the ability of microglia to clear apoptotic cells in the adult brain (Fourgeaud et al., 2016). Our results indicate that Axl and Mertk may not be necessary for the clearance of ZIKV-infected cells, as we showed that mice deficient in Axl and Mertk had similar colocalization between ZIKV antigen and microglia as that of the WT mice, suggesting phagocytosis by the microglia, at later time points of infection. However, it is unclear whether this represents infection of these cells or the clearance of dead cell debris from infected neurons.

Overall, the data presented here suggest that other, as yet unidentified, receptors may have redundant roles for ZIKV entry and infection. Finding the relevant entry receptors will be

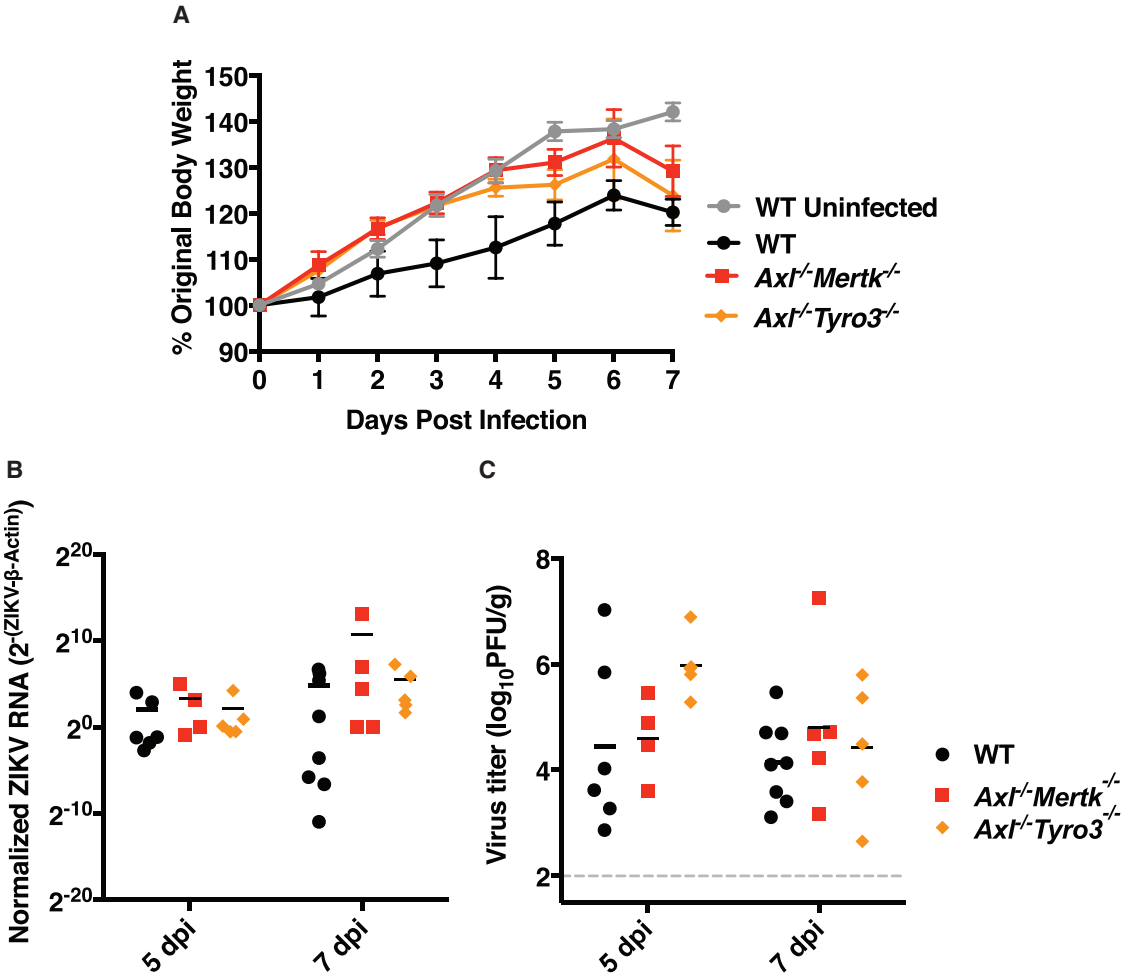

Figure 4. Intracranial Infection of 10-Day-Old WT, *Axl*$^{-/-}$*Mertk*$^{-/-}$, and *Axl*$^{-/-}$*Tyro3*$^{-/-}$ Mice Results in Similar Weight Loss and Viral Titers

Ten day-old WT, *Axl*$^{-/-}$*Mer*$^{-/-}$, and *Axl*$^{-/-}$*Tyro3*$^{-/-}$ mice were inoculated intracranially with 10^6 PFUs of Mexico strain ZIKV.

(A) Weights were measured daily after infection with ZIKV. Data are expressed as percentages of the average original body weight; error bars represent the SEM (n = 4–12 per group).

(B and C) At days 5 and 7 post-infection (5 and 7 dpi, respectively), brains were harvested and assessed for ZIKV replication using (B) qRT-PCR and (C) plaque assays. Data shown are the result of two pooled independent experiments (n = 4–8 per group). Significance was calculated using a one-way ANOVA with a post hoc Tukey test. Error bars represent the SEM. n.s., not significant.

essential for a more complete understanding of ZIKV biology and tropism. Given the reported overlap between Axl expression and ZIKV tropism, it is possible that the entry receptor expression is coincident to Axl expression in such cell types. These findings pose the question as to whether there is similar redundancy in ZIKV entry in humans in vivo. Pharmacological agents that disrupt ZIKV binding to a bona fide entry receptor could be an effective strategy to prevent or minimize infection.

EXPERIMENTAL PROCEDURES

Ethics Statement

All experiments were performed in accordance with guidelines from the Guide for the Care and Use of Laboratory Animals of the NIH. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University School of Medicine (assurance number A3230-01) and Washington University School of Medicine (assurance number A3381-01). Every effort was made to reduce distress in animals.

Viruses, Cell Lines, and Titration

Vero cells (ATCC) were maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotics at 37°C with 5% CO_2 and have been routinely confirmed to be mycoplasma free. *Aedes albopictus* midgut C6/36 cells were grown in DMEM supplemented with 10% FBS, 1% tryptose phosphate, and antibiotics at 30°C with 5% CO_2 air atmosphere. Mexico and Cambodia strains of ZIKV were obtained from the University of Texas Medical Branch at Galveston's World Reference Center for Emerging Viruses and Arboviruses and propagated in C6/36 insect cells or Vero cells. Brazil ZIKV (Paraíba 2015) was obtained from Steve Whitehead (NIH/National Institute of Allergy and Infectious Diseases). Viral titers were determined by utilizing plaque assays as previously described (Jurado et al., 2016).

Mouse Experiments

Axl$^{-/-}$, *Mer*$^{-/-}$, *Axl*$^{-/-}$*Mer*$^{-/-}$, and *Axl*$^{-/-}$*Tyro3*$^{-/-}$ mice have previously been published (Lu and Lemke, 2001), and we confirm by western blot on the brain

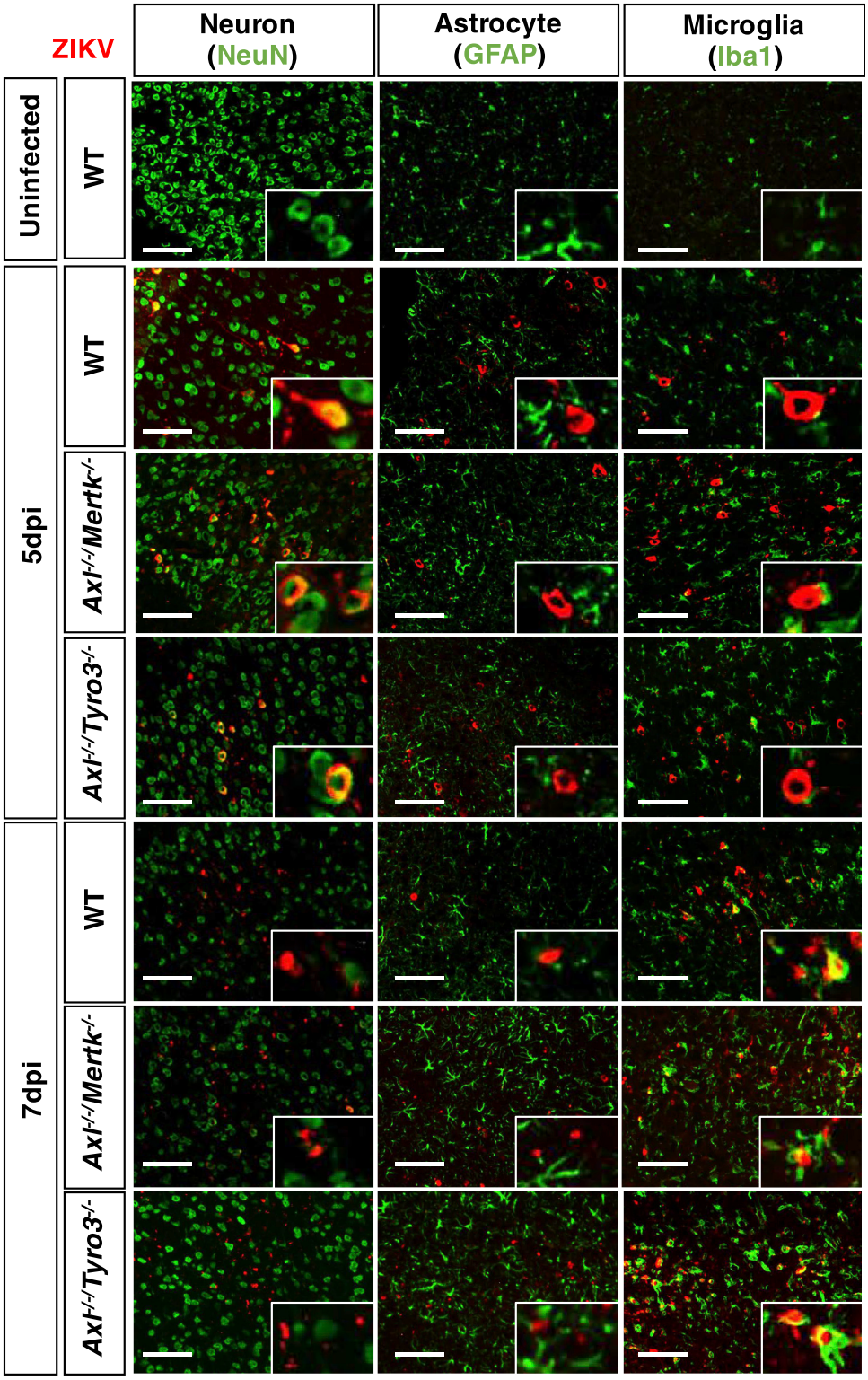

Figure 5. WT, *Axl*$^{-/-}$*Mertk*$^{-/-}$, and *Axl*$^{-/-}$*Tyro3*$^{-/-}$ Mice Show Similar ZIKV Tropism in the Brain after Intracranial Infection

Ten-day-old mice of indicated genotypes were inoculated intracranially with 10^6 PFUs of ZIKV Mexico. Brains were harvested 5 and 7 days post-infection (dpi). ZIKV was stained using immune rat sera, as indicated in red, and co-stained for neurons (NeuN), astrocytes (GFAP), or microglia (Iba1), as indicated in green. All images shown are from the cortex. Representative images from two to three brains per time point per group are shown. Scale bars, 100 μm.

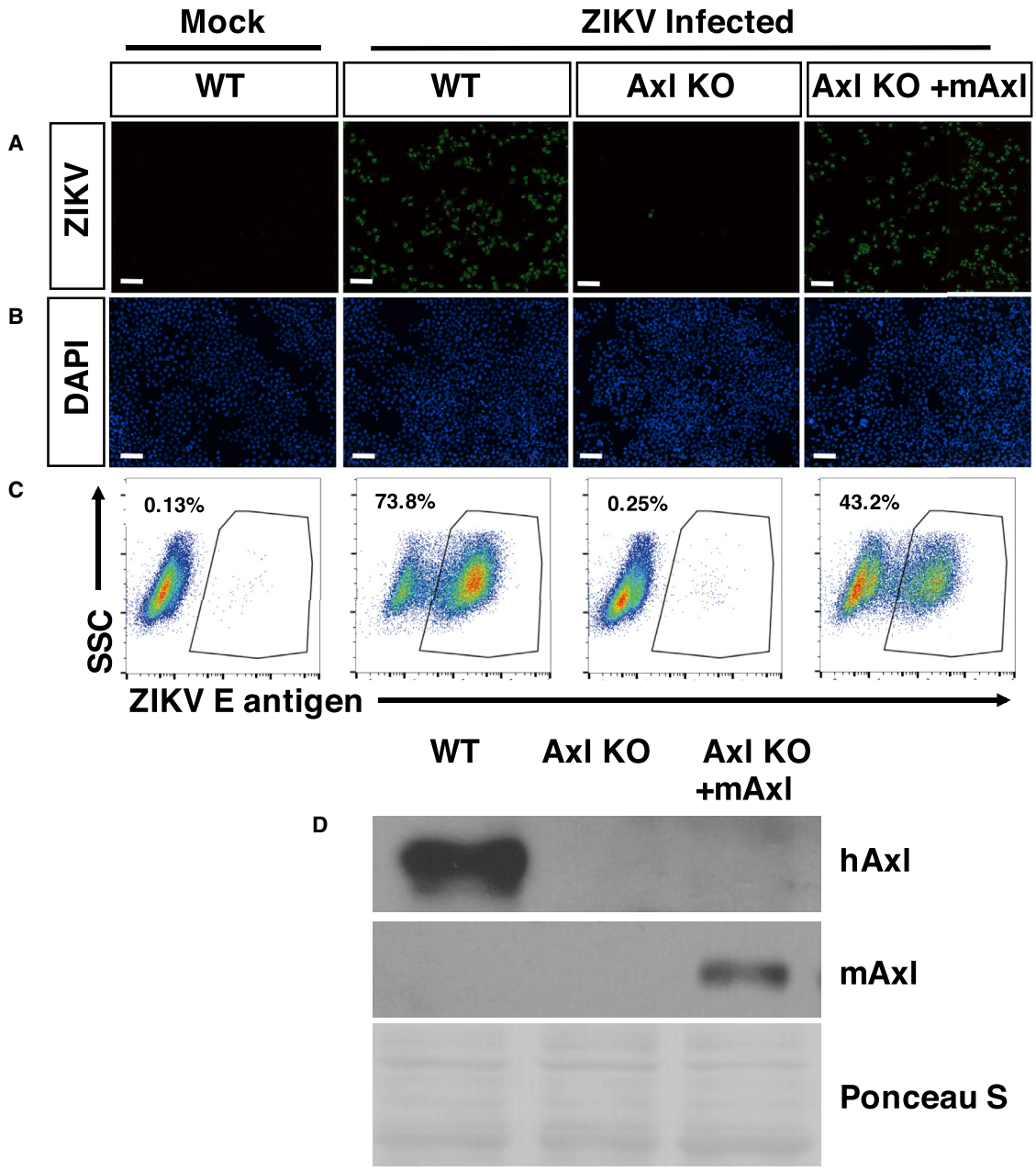

Figure 6. Mouse Axl Is Sufficient to Restore ZIKV Infectivity in HeLa Cells Lacking Human Axl

(A–D) WT HeLa, Axl KO HeLa, and Axl KO transcomplemented with murine Axl HeLa were infected with ZIKV (MOI = 1) or mock infected. At 24 hours post-infection (hpi), cells were either fixed and probed with the anti-flavivirus E protein (4G2) antibody and AF488 secondary antibody and DAPI before imaging by fluorescent microscopy (A and B) or harvested for flow cytometry analysis and probed using the same antibodies (C). Western blot using anti-Axl human- and mouse-specific antibody to show presence or absence of Axl in each cell line (D). hAxl, human Axl; mAxl, mouse Axl. Scale bars, 100 μm. SSC, side scatter.

and spleen that our $Axl^{-/-}$ mice do not express Axl (Figure S3). Mice were bred in a specific-pathogen-free facility at Yale University, Washington University, or purchased (WT animals) from Jackson Laboratory. For the pregnancy model, adult WT, $Axl^{-/-}$, $Mer^{-/-}$, and $Axl^{-/-}Mer^{-/-}$ dams were treated with 2 mg of an anti-Ifnar1 blocking mouse monoclonal antibody (mAb) (MAR1-5A3) (Sheehan et al., 2006) by intraperitoneal injection prior to infection with ZIKV. For intravaginal infection, 14- to 16-week-old female mice were injected with Depo-Provera (GE Healthcare) 5 days prior to infection. Mucous from the vaginal lumen was removed using a Calginate swab (Fisher Scientific), and 1.5×10^5 PFUs of Cambodian ZIKV in a volume of 10 μL was inoculated into the vaginal lumen using a pipette (Yockey et al., 2016). Daily vaginal washes were collected 1–6 days after infection by pipetting 50 μL PBS into the vaginal lumen as previously described (Yockey et al., 2016). One-week-old suckling mice were infected with 10^4 FFUs of Brazil ZIKV in 50 μL of PBS diluent by intraperitoneal injection. One week after infection, mice were euthanized per animal study protocol, and brains and spleens were dissected

and flash frozen prior to virological analysis as described in the following text. To determine TAM receptors' role in ZIKV replication in the brain, 10-day-old mice were infected intracranially with 10^5 PFUs of MEX2-81 ZIKV in a volume of 10 μL as previously described (Dick, 1952). Weights were measured daily for 1 week, and brains were harvested at day 5 and day 7 after infection. For intraperitoneal (IP) and intracranial (IC) infections, an equal mix of male and female mice were used.

Organ Collection

ZIKV-infected animals were euthanized on day 5 or day 7 after infection. To collect brain tissue in young mice, after euthanasia, heads were removed, skin was split from the base of the skull and peeled away, sharp scissors were inserted at the base of the skull, carefully cut dorsally over the top of the head, and bone was opened to reveal brain. Scissors were slid under the brain to sever brain from ligaments and spinal cord and lifted out. The right and left hemispheres of the brain were separated, one was placed in 4% paraformaldehyde (PFA) for histology, and the other was separated into two equal parts, weighed, and placed in media for plaque assay or TRIzol for RNA purification. For pregnancy harvests, pregnant dams were euthanized, and maternal and fetal tissues were flash frozen and then weighed in preparation for later homogenization and extraction as described in the following text.

Viral Burden Analysis

Tissues were homogenized using ceramic beads in either 10% DMEM (plaque assay), TRIzol, or PBS (qRT-PCR); homogenized tissue was centrifuged for 10 min at 13,000 × rpm; and supernatant was transferred to new tubes. For plaque assays, supernatant was incubated on Vero cell monolayers in 10-fold serial dilutions for 1 hr at 37°C and overlaid with a mixture of 2% agarose and 2× media. 3 to 4 days post-infection, cells were fixed by 10% formalin and stained with 0.005% amido black, and PFUs were counted. For tissues, total RNA was extracted using the QIAGEN RNeasy Mini Kit and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's protocol. For pregnancy experiments, total RNA was analyzed using one-step qRT-PCR on an Applied Biosystems (ABI) 7500 Fast instrument, as per previously published protocols and primer set, with quantification accomplished via fit to concurrently run standard curve (Lanciotti et al., 2016; Lazear et al., 2016). IQ SYBR Green Supermix (Bio-Rad) was used along with ZIKV-specific primers, and ZIKV replication was calculated using the $2-\Delta\Delta CT$ method normalized to β actin RNA. For vaginal washes, ZIKV was detected using primers designed to NS5 (forward: GGCCACGAGTCTGTACCAAA; reverse: AGCTTCACTGCAGTCTTCC), and FFU-equivalent was determined by normalization to RNA prepared from virus stock (Yockey et al., 2016).

RNA ISH

RNA ISH was performed using the RNAscope 2.5 HD-BROWN assay (Advanced Cell Diagnostics) according to the manufacturer's instructions and as previously described (Govero et al., 2016). 4% PFA-fixed, paraffin-embedded tissue sections were deparaffinized by incubating for 60 min at 60°C, and endogenous peroxidases were quenched with H_2O_2 for 10 min at room temperature. Slides were then boiled for 15 min in RNAscope Target Retrieval Reagents and incubated for 30 min in RNAscope Protease Plus Reagent prior to ZIKV RNA (Advanced Cell Diagnostics, catalog #467771), positive control *Mus musculus Ppib* gene (#313911), or negative control bacterial gene *dapB* (#310043) probe hybridization and signal amplification. Sections were counterstained with Gill's hematoxylin and visualized by bright-field microscopy.

Brain Sectioning and Immunostaining

Brains from intracranially infected mice were collected in 4% PFA (Electron Microscopy Sciences) and fixed overnight at 4°C. After fixation, brains were dehydrated in a sucrose gradient up to 30% sucrose, cut sagitally, and embedded in OCT (optimal cutting temperature) compound. Sections were washed with PBS, permeabilized, and blocked in buffer containing 2% donkey serum. The following primary antibodies were incubated overnight: for ZIKV staining, ZIKV-immune rat serum was used at 1:2,000 (van den Pol et al., 2017). For costaining, the following primary antibodies were used: anti-Iba1 (1:500; Wako Pure Chemicals Industries, #019-19741), GFAP (1:500; Dako, #Z0334), and NeuN (1:50; Cell Signaling, #24307). After rinsing with PBS, goat anti-rat secondary antibody conjugated to A594 (1:1,000; Life Technologies) and goat anti-rabbit antibody conjugated to Alexa 488 (1:1,000; Life Technologies). Samples were mounted in Prolong Gold containing DAPI (4'6'-diamidino-2-phenilindole) (Life Technologies).

Axl CRISPR KO of HeLa Cells and Murine Axl Transcomplementation

The single-guide (sgRNA) sequence GGAGGTTACGGGGCTGCTGG was cloned into lentiCRISPR v.2 (Addgene, #52961) (Sanjana et al., 2014), and the resulting plasmid was transiently transfected into HeLa cells and selected in puromycin (1 μg/mL) for 4 days. Clones were screened for Axl KO by immunoblot analysis, and a single clone was carried out for in vitro experiments. For retroviral transcomplementation with murine Axl, a full-length open reading frame (ORF) of mAxl was cloned into the EcoRI and BamHI restriction sites of pLXSN. COS-1 cells were co-transfected with pLXSN-mAxl, pUMVC (Addgene, #8449), and pCMV-VSV-G (Addgene, #8454), and the retrovirus-containing supernatant was collected at 48 hr post-transfection. HeLa Axl KO cells were transduced with retrovirus in the presence of polybrene (10 μg/mL) and selected with G418 (750 μg/mL) for 10 days to obtain a pooled population of mAxl-expressing cells. Axl protein expression was confirmed by human-specific (R&D Systems, #AF154) and mouse-specific (R&D Systems, #AF854) Axl antibodies.

ZIKV Infection and Analysis of Axl KO and Murine Axl Transcomplemented HeLa Cells

WT, Axl KO, and mouse Axl transcomplemented HeLa cells were infected with ZIKV at an MOI of 1 and incubated at 37°C. After 24 hr, cells were trypsinized and resuspended in ice-cold methanol to fix and permeabilize. Cells were probed with 4G2, anti-flavivirus mouse monoclonal antibody, at 6.2 ng/mL, followed by an Alexa Fluor 488 secondary antibody (1:2,000; Thermo Fisher #A-11001), before analyzing on a 13-color Stratedigm flow cytometer. Flow plots were generated using FlowJo.

Data Analysis

GraphPad Prism software was used to analyze all data. Log_{10}-transformed titers used for plaque assays, and either β-actin-normalized viral RNA or tissue-weight-normalized values were analyzed using one-way ANOVA and post hoc Tukey test for multiple comparisons or Kruskal-Wallis and Dunn's multiple comparisons test, where appropriate, as indicated in figure legends. A p value < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.058.

AUTHOR CONTRIBUTIONS

Conceptualization and Methodology, A.K.H., L.J.Y., B.W.J., J.H., R.U., I.M., C.V.R., E.F., M.S.D., and A.I.; Investigation, A.K.H., L.J.Y., B.W.J., J.H., R.U., H.F.G., B.C., and L.A.P.; Formal Analysis, A.K.H., L.J.Y., B.W.J., J.H., R.U., E.F., M.S.D., and A.I.; Resources, C.V.R.; Writing – Original Draft, A.K.H. and L.J.Y.; Writing – Review & Editing, A.K.H., L.J.Y., B.W.J., J.H., C.V.R., E.F., M.S.D., and A.I.; Funding Acquisition, I.M., E.F., M.S.D., and A.I.; Supervision, C.V.R., I.M., E.F., M.S.D., and A.I.

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