

ORIGINAL ARTICLE

Modeling neuro-immune interactions during Zika virus infection

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Abstract

Although Zika virus (ZIKV) infection is often asymptomatic, in some cases, it can lead to birth defects in newborns or serious neurologic complications in adults. However, little is known about the interplay between immune and neural cells that could contribute to the ZIKV pathology. To understand the mechanisms at play during infection and the antiviral immune response, we focused on neural precursor cells (NPCs)-microglia interactions. Our data indicate that human microglia infected with the current circulating Brazilian ZIKV induces a similar pro-inflammatory response found in ZIKV-infected human tissues. Importantly, using our model, we show that microglia interact with ZIKV-infected NPCs and further spread the virus. Finally, we show that Sofosbuvir, an FDA-approved drug for Hepatitis C, blocked viral infection in NPCs and therefore the transmission of the virus from microglia to NPCs. Thus, our model provides a new tool for studying neuro-immune interactions and a platform to test new therapeutic drugs.

Introduction

Zika virus (ZIKV) is an arbovirus belonging to the genus *Flavivirus* first described in 1947 in Uganda during blood analyses of sentinel Rhesus monkeys (1). Until the 21st century, African and Asian

lineages of ZIKV did not cause substantial human infections. However, in 2007, vectored by *Aedes aegypti* mosquitoes, the first noteworthy epidemic of ZIKV occurred on the island of Yap in Micronesia (2). The recent dramatic increase in newborns with microcephaly and other congenital malformations in Brazil has

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been associated with an outbreak of ZIKV (3,4). The outbreak has further been linked to the autoimmune neurological disorder Guillain-Barré syndrome (GBS) in older children and adults (5). The circulating Asian-lineage strain of ZIKV in Brazil (ZIKV^{BR}) has been detected in the placenta and amniotic fluid of women with microcephalic fetuses (6–8) and in the blood of microcephalic newborns. Curiously, although the African MR-766 strain of ZIKV (ZIKV^{MR766}, strain MR-766) was first identified in the 1950s, no birth defects in newborns or neurological complications in adults have been associated with this strain. Thus, recent publications focused on the mechanisms of transmission and spreading of ZIKV in the Americas (9–12).

Earlier this year, Liu and colleagues reported that ZIKV evolved to acquire a spontaneous mutation in its NS1 (A188V mutation) domain leading to increased antigenaemia (12). This enhancement of NS1 antigenaemia promoted ZIKV infectivity and prevalence in mosquitoes that could ease the transmission and possibly explain the recent ZIKV outbreaks. Following this publication, another group suggested that the A188V mutation on NS1 domain arose in Southeast Asia at the early 2000s and circulated in that region several years before spreading to South Pacific Islands and the Americas (13). Moreover, there is now converging evidence that the African strains lead to higher infection rate and viral production as well as stronger cell death in cellular and immunocompetent adult mouse models leading to the increased mortality while the Asian strains did not (14–16). This feature of the Asian strain might be responsible for causing chronic infections observed in congenital microcephaly cases and explain why no other diseases were observed until recently. Hence, there is an urgent need to develop model systems to examine the complex relationship between ZIKV^{BR} infection and brain malformations.

In order to model ZIKV^{BR} infectivity, we previously showed that ZIKV^{BR} crosses the placenta to infect the fetus *in vivo*, causing microcephaly and birth defects (17). In addition, we showed that ZIKV^{BR} targeted human neural precursor cells (NPCs), growing as neurospheres or organoids, induced cell death and led to a reduction of proliferative zones and a disruption of cortical layers as previously observed *in vivo* (17). Despite advances regarding the causal relationship between the ZIKV^{BR} and birth defects, little is known about the pathogenic interactions of this virus with different cell types and its vertical transmission to fetal brain.

Since the onset of the current Brazilian outbreak in 2015, most ZIKV studies have focused on neurons and glial cells (17,18). However, further examination of ZIKV interaction with fetal circulating monocytes and the interplay between microglia and neural cells are important steps for understanding ZIKV pathophysiology. A recent publication, using the contemporary ZIKV strain PRVABC59 (PR2015), showed that the virus can infect and replicate in Hofbauer cells (HCs), a type of primary human placental macrophage (19). Hence, one possibility for intrauterine transmission allowing ZIKV to gain access to the fetal compartment is by direct infection of cells comprising the placental barrier (19). Yolk sac macrophages invade the brain parenchyma through the blood vessels during embryogenesis to become resident macrophages of the central nervous system (CNS), microglia (20). Considering the timing during embryogenesis of the entry of myeloid precursors, we hypothesized that microglia could also actively participate during ZIKV infection, perhaps acting as a Trojan horse by transporting the ZIKV during CNS invasion (21). Thus, in this study, using a ZIKV strain isolated from a clinical case in northeast of Brazil (17), ZIKV^{BR}, we aimed to mimic those early interactions between NPCs and macrophage/microglia.

Here, we used human-induced pluripotent stem cells (hiPSCs) to model immune interactions in the developing central nervous system of the fetal brain during ZIKV infection by generating human macrophages/microglia and NPCs from the same healthy donor. Then, we established a co-culture system to model the cellular interplay that naturally occurs in the developing human brain. Our data reveal that a similar immune response was elicited in developing human microglia upon infection with the ZIKV^{BR} as was found in the humoral immune response present in ZIKV-infected human tissues (22–25). Upon co-culture, microglia interacted with ZIKV^{BR}-infected NPCs. Our data further show that ZIKV-infected macrophages/microglia transmit the virus to naïve NPCs, increasing the apoptosis of NPCs. These results imply that during embryogenesis, myeloid precursors could be responsible for ZIKV transmission to the CNS during their normal process of brain invasion. Finally, we tested Sofosbuvir (SOF), an FDA-approved drug against Hepatitis C infection, previously tested against ZIKV (26–28), and showed that SOF was able to decrease the cell death of ZIKV-infected NPCs. Our findings reveal that human microglia are profoundly influenced by their microenvironment and can sense ZIKV-infected cells in their immediate surroundings, providing insights on the mechanism of infection. Thus, our co-culture system could be used for studying neuro-immune interactions *in vitro* and testing new therapeutic candidates against ZIKV.

Results

Characterization of hiPSC-derived macrophage/microglia (MΦ)

To understand the immune component of neurologic abnormalities observed in ZIKV-infected newborns, we first generated MΦ from hiPSCs using an edited version by Douvaras and colleagues of the previously published protocol by Yanagimachi *et al.* (29,30). This serum-free, feeder-free method gave rise to CD14⁺ monocytes, precursors of MΦ, in suspension starting at 15 days *in vitro*. CD14⁺ cells were then sorted using magnetic-activated cell sorting (MACS) system, treated with M-CSF and IL-34 for a week as previously published (29), and used in the subsequent experiments (Supplementary Material, Fig. S1A). These monocytes expressed classical MΦ markers such as Iba1 and CD68 (Fig. 1A). The analysis of the cells in suspension, prior to CD14 sorting, revealed that they expressed monocyte, macrophage and myeloid cell markers such as CD45/CD14, CD14/HLA-DRDPDQ, CD14/CD11b and CD14/CD68, (Fig. 1C, Supplementary Material, Fig. S1B). One week after sorting, CD14⁺ cells established a homogeneous macrophage population (Supplementary Material, Fig. S1C) and had similar cellular morphology and expression of cell markers such as CD68 and Iba1 to blood monocyte-derived macrophages (Supplementary Material, Fig. S1C and D). We next assessed whether the MΦ were functional by measuring their capacity for phagocytosis (Fig. 1B). In a classical assay (31–33), hiPSC-derived MΦ engulfed the yeast particle zymosan (Fig. 1B). The MΦ were also able to phagocytose living cells of the leading neonatal bacterial pathogen group B *Streptococcus* (GBS) whereas hiPSC-derived NPCs or 4-week-old neurons did not internalize the bacterium (Fig. 1B); primary human neutrophils were used as positive control of phagocytosis. In addition, we measured cytokine (IL-6, IL-1β, IL12/IL23p40), chemokine (MIP1α, MIP1β, RANTES, Fractalkine, IL8 and IP10) and the growth factor G-CSF release by hiPSC-derived MΦ after activating them with the classical pro-inflammatory TLR-4 ligand lipopolysaccharide (LPS) using

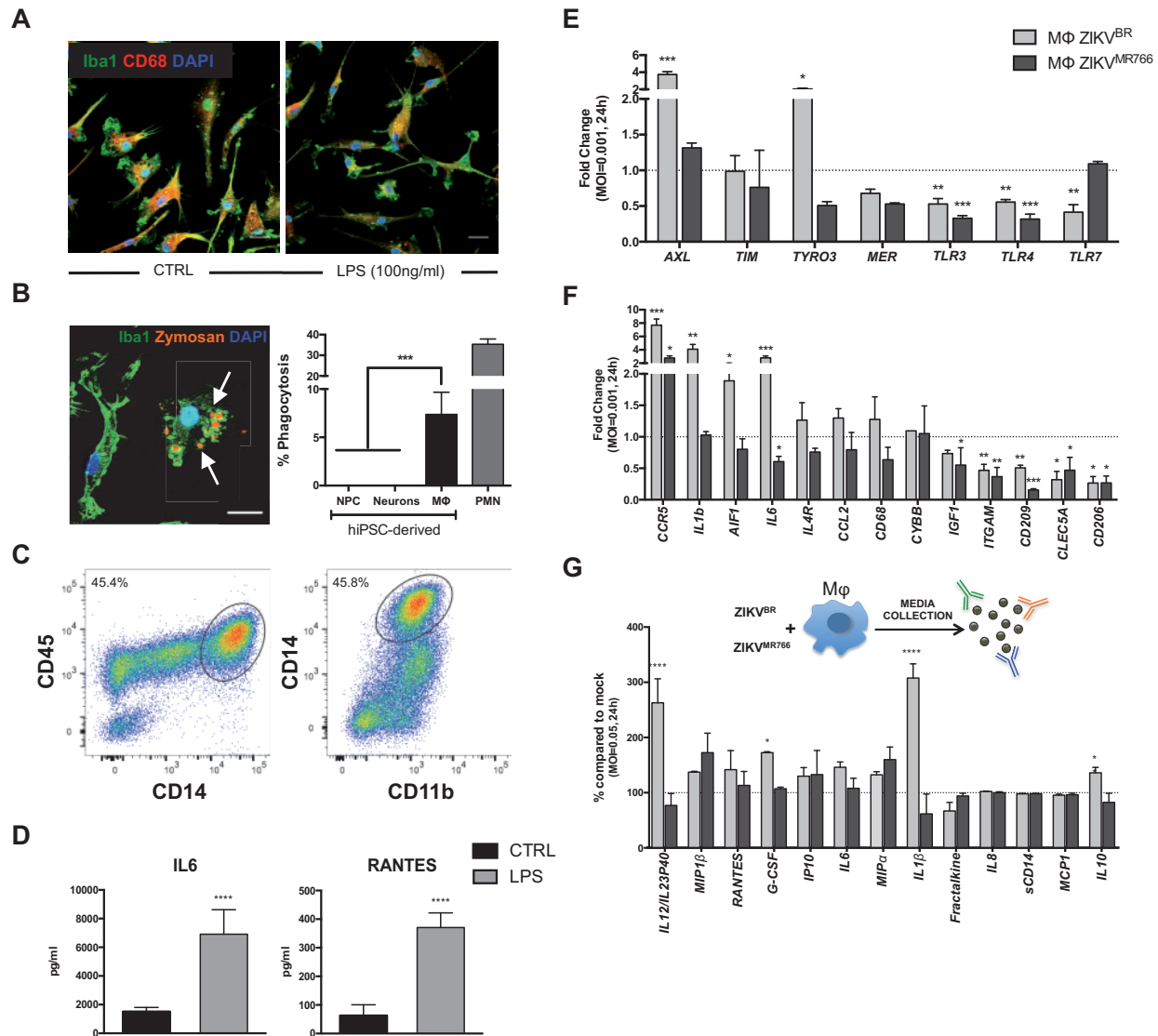


Figure 1. Characterization of functional human induced pluripotent stem cell (hiPSC)-derived macrophages/microglia (M Φ) and their Innate immune system receptors and inflammatory response upon Zika virus (ZIKV) infection. **(A)** Representative images of hiPSC-derived M Φ stained with anti-Iba1 (green), anti-CD68 (red) and fluorescent nuclear DAPI stain (blue): control M Φ untreated CTRL, (left) and treated with lipopolysaccharides (LPS, 100 ng/ml, right). Scale bar: 20 μ m. **(B)** pHrodo red zymosan (orange) particles engulfed by hiPSC-derived M Φ , stained with anti-Iba1 (green). Scale bar: 50 μ m. Phagocytosis percentage determined by group B streptococcus (GBS) internalization. Note that hiPSC-derived neural precursors (NPC) or neurons do not engulf any GBS. Human primary polymorphonuclear neutrophils (PMN) were used as an experimental positive control. One-way ANOVA with Tukey multiple comparison tests were performed, bars represent means of the percentage of phagocytosis \pm SD *** P < 0.001. **(C)** Fluorescent activated cell-sorting (FACS) analysis of monocytic lineage cells derived sequentially from pluripotent stem cells. **(D)** Cytometric Bead Array (CBA) performed on conditioned media from control or 1 μ g/ml LPS-treated M Φ for 24–30 h. Student's t-tests were performed to compare the two groups. Bars represent means of the amount of cytokines/chemokines released in the media (pg/ml) \pm SD, **** P < 0.0001 compared with control (CTRL) untreated cells. **(E)** The expression of potential Zika virus entry receptors including AXL, TIM, TYRO3, MER and several TLR receptors (TLR3, TLR4, TLR7) was measured by qRT-PCR in M Φ infected with either Brazilian (ZIKV^{BR}) or the highly *in vitro* passaged MR766 ZIKV strain (ZIKV^{MR766}) (MOI=0.001). RNA was analysed 24 h p.i. One-way ANOVA with Tukey multiple comparison tests were performed. The bars represent means of the mRNA fold change compared with the mock-infected M Φ , shown by a dashed line on the y-axis at 1 \pm SD, * P < 0.05, ** P < 0.01, *** P < 0.001. **(F)** Inflammatory response was measured by qRT-PCR in hiPSC- M Φ infected with either the ZIKV^{BR} or ZIKV^{MR766} (MOI=0.001). RNA was extracted 24 h after infection. One-way ANOVA with Tukey multiple comparison tests were performed, bars represent means of the mRNA fold change compared with the mock-infected M Φ , shown by a dashed line on the y-axis at 1 \pm SD, * P < 0.05, ** P < 0.01, *** P < 0.001, M Φ ZIKV^{BR} is represented in light grey and ZIKV^{MR766} in dark grey. **(G)** Inflammatory cytokines/chemokines were measured in the media conditioned by M Φ for 24–30 h p.i (MOI=0.05) with ZIKV^{BR} or ZIKV^{MR766}. One-way ANOVA with Sidak multiple comparisons tests were performed. Bars represent average of fold change (in %) compared with the mock-infected M Φ (as shown by the dashed line at 100%) \pm SD, * P < 0.05, **** P < 0.0001, M Φ ZIKV^{BR} is represented in light grey and ZIKV^{MR766} in dark grey.

multiplex cytometric bead based-assay (Fig. 1D, Supplementary Material, Fig. S1E). All cytokines/chemokines tested were readily detected in the M Φ -conditioned media. LPS treatment for 24 h significantly increased levels of IL-6, IL-1 β , IL12/IL23p40, Fractalkine, G-CSF, IP10, RANTES, MIP1 α and MIP β (Fig. 1D, Supplementary Material, Fig. S1E).

In sum, hiPSC-derived M Φ displayed monocyte and myeloid markers (Supplementary Material, Fig. S1D), phagocytosed zymosan particles and whole bacteria and responded to a pro-inflammatory stimulus as measured by cytokine/chemokine release similar to primary blood-derived monocytes/macrophages (34).

Immune response of macrophages/microglia (M Φ) upon infection with the ZIKV

Next, we studied the immune response of M Φ upon infection with ZIKV^{BR} as well as with ZIKV^{MR766}, a lab-adapted, highly *in vitro* passaged African MR766 strain, presumably different from the circulating African strain at 12 and 24 h post-infection (p.i) (Supplementary Material, Fig S2A and B). We selected a panel of potential virus entry receptor candidates based on recent publications including AXL, TIM, TYRO3, MER and several TLR receptors previously linked to ZIKV and arboviruses such as DENV (32,33,39); TLR3, TLR4, TLR7, to measure the expression levels by qRT-PCR (17,18,35,36). At 12h p.i., we did not observe any significant changes between the strains when compared with mock-infected samples (Supplementary Material, Fig. S2A). At 24 h p.i., both strains of the virus triggered a TLR7 up-regulation and only ZIKV^{MR766}-infected M Φ expressed higher levels of the MER receptor when compared with mock-infected conditions (Supplementary Material, Fig. S2B). Next, we used a lower MOI aiming to mimic the ZIKV infection *in vivo* and to unveil the most sensitive changes without inducing a dramatic inflammatory response that could mask small and strain-specific changes (37–41). Thus, these ranges of MOIs and 24h p.i. time point were used in the subsequent experiments (Fig. 1E and F).

TYRO3 and AXL were upregulated upon the ZIKV^{BR} infection, with AXL gene expression increased by three-fold compared with the mock-infected M Φ (Fig. 1E). In contrast, expression of MER and TIM did not differ between ZIKV^{BR}-infected and mock-infected M Φ (Fig. 1E). As for the TLR receptors expression, we focused on three relevant for our study: TLR3, a sensor of RNA viruses, which was recently linked to ZIKV infection (35); TLR4, which plays a central role in recognition of LPS from Gram-negative bacteria, which was previously shown to be upregulated in ZIKV-infected human neurospheres (36); and TLR7, a sensor of ssRNA involved in the detection of other arboviruses such as DENV (42). Surprisingly, all were downregulated when compared with mock-infected controls (Fig. 1E). Interestingly, the corresponding changes seen in AXL, TYRO3, and TLR7 gene expression upon infection with the ZIKV^{BR} were absent in parallel infections with the ZIKV^{MR766}, which did not differ from mock-infected M Φ in expression of all three receptor genes (Fig. 1E).

The geographic and symptomatology overlap between ZIKV and other Flaviviruses such as Dengue (DENV) initially complicated diagnosis (42). The vector by which it is propagated (*Aedes aegypti*) is shared by both viruses and clinically presented serological cross-reactivity. In addition, DENV is known to target primary blood cells such as monocytes and macrophages and induces an inflammatory response (42). Thus, we next measured the expression of genes encoding three receptors described for DENV infection: CLEC5A, CD209/DC-SIGN, and CD206/MRC1 (Fig. 1F). All three viral receptors, CLEC5A, CD209/DC-SIGN, and CD206/MRC1, were downregulated in ZIKV^{BR} or ZIKV^{MR766}-infected M Φ compared with mock-infected M Φ , suggesting that ZIKV does not implicate these DENV-related receptors in iPSCs-derived M Φ (Fig. 1F).

Next, we studied the expression of the M Φ markers such as AIF1/Iba1, CD68, and ITGAM/CD11b by qRT-PCR upon infection with the ZIKV strains. AIF1/Iba1 expression was increased in the M Φ infected with ZIKV^{BR} compared with the mock-infected or ZIKV^{MR766}-infected cells, whereas similar changes in CD68 expression did not achieve statistic significance (Fig. 1F). Notably, pro-inflammatory genes encoding IL6, IL1B, and CCR5

were markedly induced upon infection by the ZIKV^{BR}, but not ZIKV^{MR766}. In contrast, the M Φ marker ITGAM/CD11b and the anti-inflammatory factor IGF1 were decreased by infections with either ZIKV strain, whereas expression of CYBB/NOX2 remained unchanged upon infection with the viruses (Fig. 1F). These results indicate that the inflammatory response of M Φ infected with two different ZIKV strains at the same viral MOI elicited different inflammatory responses.

We next compared cytokine/chemokine release by the hiPSC-derived M Φ 24 h p.i. with the ZIKV^{BR} or ZIKV^{MR766}, (Fig. 1G, Supplementary Material, Fig. S4) using the same multiplex cytometric bead array that was used previously (Fig. 1D, Supplementary Material, Fig. S1E). Using several inocula of virus (MOI = 0.05, MOI = 0.01 and MOI = 0.001), we detected changes in release of several pro-inflammatory cytokines/chemokines (Fig. 1G, Supplementary Material, Fig. S4B and C). M Φ release of pro-inflammatory cytokines IL12/IL23p40, IL1 β , IL10 and the growth factor G-CSF was significantly higher in response to ZIKV^{BR} compared with ZIKV^{MR766} infection; whereas MIP1 β , RANTES, IP-10, IL6, and MIP1 α release from ZIKV-infected M Φ were comparable between the two strains (Fig. 1G, Supplementary Material, Fig. S4B and C), arguing for a ZIKV^{BR}-specific response by the hiPSC-derived M Φ .

Differential expression of TAM/TIM and TLR receptors in human NPCs

Human NPCs were shown to be targeted by ZIKV^{BR} (17). Similar to M Φ , we first infected NPCs at an MOI of 0.1 with either strains and studied the expression of different viral entry receptors at 12 and 24 h p.i. by qRT-PCR (Supplementary Material, Fig. S2C and D), however, we did not detect any differences between the two different strains at 12h p.i. (Supplementary Material, Fig. S2C). At 24h p.i., TYRO3 and TLR4 were both upregulated in NPCs upon infection with both ZIKV^{BR} and ZIKV^{MR766} strains (Supplementary Material, Fig. S2D). Given that we did not see major differences in gene expression in both of the strains tested, we focused on the same MOI (0.001) determined from the M Φ expression analysis and the time points (24 and 96 h p.i.) (Fig. 1A and B, Supplementary Material, Fig. S2A and B) for the NPCs (Supplementary Material, Fig. S3A and B). Of four receptors analysed, only the MER was increased in the ZIKV^{BR} 24 h p.i. compared with mock but returned to mock levels by 96 h p.i. (Supplementary Material, Fig. S3A). Expression of TYRO3 was downregulated in both mock- and ZIKV^{BR}-infected NPCs at 96 h p.i. compared with 24 h p.i. (Supplementary Material, Fig. S3A). TLR4 was also decreased as in M Φ , while TLR7 expression was increased compared with the mock-infected controls at 24 h p.i. (Supplementary Material, Fig. S3B). The expression of TAM/TIM receptors and TLRs observed in NPCs at 96 h p.i. were accompanied by a decreased expression of cell markers such as NES/Nestin and PAX6 in both mock- and ZIKV^{BR}-infected NPCs (Supplementary Material, Fig. S3C).

To summarize, we did not observe great changes in ZIKV^{BR}-infected NPCs except for MER and TLR7, which were significantly upregulated compared with the mock-infected NPCs at 24-h post-infection.

The interplay between NPCs with macrophages/microglia (M Φ) during ZIKV infection

To investigate interactions of hiPSC-derived M Φ with ZIKV^{BR}-infected NPCs we established a co-culture experimental

platform. NPCs were infected (MOI = 0.1) for 2 h with the ZIKV^{BR} or ZIKV^{MR766}, media replaced, and MΦ overlaid 2 h after the media change. MΦ and NPCs were maintained in co-culture for 96 h, and the fraction of cells undergoing cell death examined by DNA fragmentation using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Fig. 2A and B, Supplementary Material, Fig. S5). We initially seeded the same amount of MΦ and NPCs, however, the proliferation rate of the NPCs was higher and thus the percentage of MΦ in the co-culture with NPCs was around 2% at 96 h p.i. (Supplementary Material, Fig. S5C). In the absence of infection, addition of MΦ in co-culture atop NPCs did not influence the number of TUNEL⁺ cells (~10%, see Fig. 2C). However, as previously shown (17), ZIKV^{BR} infection at MOI = 0.1 doubled the percentage of TUNEL⁺ NPCs (Fig. 2B and C). No increase in TUNEL⁺ was detected in NPCs infected with ZIKV^{BR} at MOI = 0.01 for 96 h (Supplementary Material, Fig. S5A). Infection with ZIKV^{MR766} had similar effect by increasing the amount of TUNEL⁺ NPCs (Supplementary Material, Fig. S5B). In addition, the amount of MΦ undergoing apoptosis (TUNEL⁺) remained under ~5% in presence or absence of ZIKV^{BR} indicating that the ZIKV^{BR} was not cytotoxic to MΦ under our experimental conditions (Fig. 2B and C, Supplementary Material, Fig. S5D). Interestingly, adding MΦ to ZIKV-infected NPCs reduced the percentage of TUNEL⁺ cell levels similar to mock-infection (Fig. 2B and C, Supplementary Material, Fig. S5B). Thus, while adding naïve MΦ to uninfected NPCs did not increase cell death, infection of NPCs with the ZIKV^{BR} increased cell death, and the presence of MΦ reduced cell death in ZIKV^{BR}-infected NPCs (Fig. 2B and C). To elucidate the mechanism by which MΦ presence could decrease TUNEL⁺ ZIKV^{BR}-infected NPCs, whether it is through phagocytosis or through protection by release of neurotrophic factors by MΦ, we used conditioned media (CM) from mock or ZIKV^{BR}-infected MΦ and added on top of NPCs (Fig. 2D). Mock MΦ CM did not have any effect on the basal level of apoptosis in mock-infected NPCs whereas ZIKV^{BR} MΦ CM addition tended to increase the level of TUNEL⁺ NPCs (Fig. 2D). Although the addition of mock or ZIKV^{BR} MΦ CM on top of ZIKV^{BR}-infected NPCs had a small tendency to decrease the amount of TUNEL⁺ cells, there was no statistically significant change compared with ZIKV^{BR}-infected NPCs in the absence of any CM (Fig. 2D). Thus, MΦ most likely need to be in contact with NPCs to decrease the amount of TUNEL⁺ NPCs, most likely by phagocytosing the apoptotic cells.

Finally, to assess whether ZIKV-infected MΦ could transmit the virus to the NPCs, we first infected MΦ with ZIKV (ZIKV^{BR} or ZIKV^{MR766}, Supplementary Material, Fig. S5), washed and added on top of NPCs the next day (Fig. 2E). We co-cultured ZIKV-infected MΦ with NPCs for 96 h and analysed the amount of TUNEL⁺ cells (Fig. 2E and F, Supplementary Material, Fig. S5E). Importantly, upon addition of ZIKV-infected MΦ on top of NPCs, the amount of TUNEL⁺ cells increased significantly (Fig. 2F and G, Supplementary Material, Fig. S5E). Keeping in mind that MΦ represent around 2% of the cells in total (Supplementary Material, Fig. S5C) and that ZIKV infection did not increase the MΦ in apoptosis (Supplementary Material, Fig. S5D), the increase in TUNEL can be attributed to the increase of cell death of NPCs (Fig. 2G, Supplementary Material, Fig. S5E). More importantly, we tested an FDA-approved drug, SOF, which was previously shown efficient against ZIKV infection in our model (26–28).

Next, in a following experiment, we added 20 μM SOF at the same time as the addition of ZIKV-infected MΦ on top of uninfected NPCs (Fig. 2E, G and H, Supplementary Material, Fig. S5E

and F). Importantly, addition of SOF decreased significantly the amount of TUNEL⁺ cells in the case of ZIKV^{BR}-infected MΦ addition (Fig. 2F and G) and showed a tendency to decrease upon infection with ZIKV^{MR766}-infected MΦ (Supplementary Material, Fig. S5E). In addition, this decrease was accompanied by a decrease the amount of the flavivirus NS1⁺ domain specific staining, suggesting that SOF was able to limit the ZIKV^{BR} replication (Fig. 2F–H). As for the MR766, SOF had only a tendency to decrease the amount of NS1⁺ cells (Supplementary Material, Fig. S5F). Thus, SOF was able to block the increased cell death of NPCs due to the infection by ZIKV-infected MΦ.

Altogether, our model was able to mimic neuro-immune interactions, which is likely to occur during human neurodevelopment and was proven to be useful for testing new therapeutic drugs to block ZIKV-associated phenotypes.

Discussion

In this study, we modeled immune interactions between NPCs and macrophages/microglia (MΦ) that would occur in the developing brains of the ZIKV^{BR}-infected fetuses/newborns, using an induced pluripotent stem cell experimental platform. A fundamental strength of induced pluripotent stem cells is the possibility of deriving different cell types from individuals *in vitro*. Generation of MΦ using this technology will circumvent the need for MHC-matched bone marrow-derived MΦ from a healthy donor. In addition, by generating NPCs and MΦ from the same donor, we established an autologous approach to the study of immune interactions between different cell types derived from the same genetic background. The novelty of our study relies on the establishment of an *in vitro* platform, able to mimic human neuro-immune interactions. In particular, these interactions in human cells were studied by co-culturing hiPSC-derived NPCs together with hiPSC-derived MΦ. To our knowledge this is the first study enabling the analysis of human neuro-immune interactions, unlike the studies using 3D organoids, where the mesoderm-derived immune cells such as MΦ of the central nervous system are lacking, which is a major limitation of this technology (17,35,43–45). Thus, we strongly believe that our platform could be further used in other studies implicating MΦ to model the interplay among the different cell types and their impact on neurodevelopmental diseases.

In this study, using low MOI infections to mimic the ZIKV infection *in vivo*, we analysed the cytokine and immune receptor responses to infections with the ZIKV^{BR} or ZIKV^{MR766} strains. We then investigated cellular receptors implicated in ZIKV cell entry in developing human MΦ, beginning with C-type lectin receptors known to bind DENV on the surface of macrophages and dendritic cells and modulate inflammatory responses (42,46). Unlike in DENV infections, CLEC5A, CD209/DC-SIGN and CD206/MRC1 all had reduced expression in ZIKV^{BR} and ZIKV^{MR766}-infected MΦ compared with controls (42,46). TAM/TIM receptors and TLRs have been recently implicated in ZIKV infection (18,35). We found that MΦ and NPCs had differential expressions of the genes encoding these receptors upon infection with ZIKV^{BR}: TYRO3 and AXL were upregulated in MΦ infected with ZIKV^{BR} unlike NPC, which had only an upregulation of MER, suggesting that AXL does not have a central role in ZIKV infections in NPCs. Indeed, several publications focused on AXL receptors over the last year since it was suggested as a potential viral entry receptor (18), however, two independent studies showed that blocking AXL does not prevent ZIKV infection of NPCs (46,47). But a recent publication claims that selective expression of Musashi in NPCs could explain their

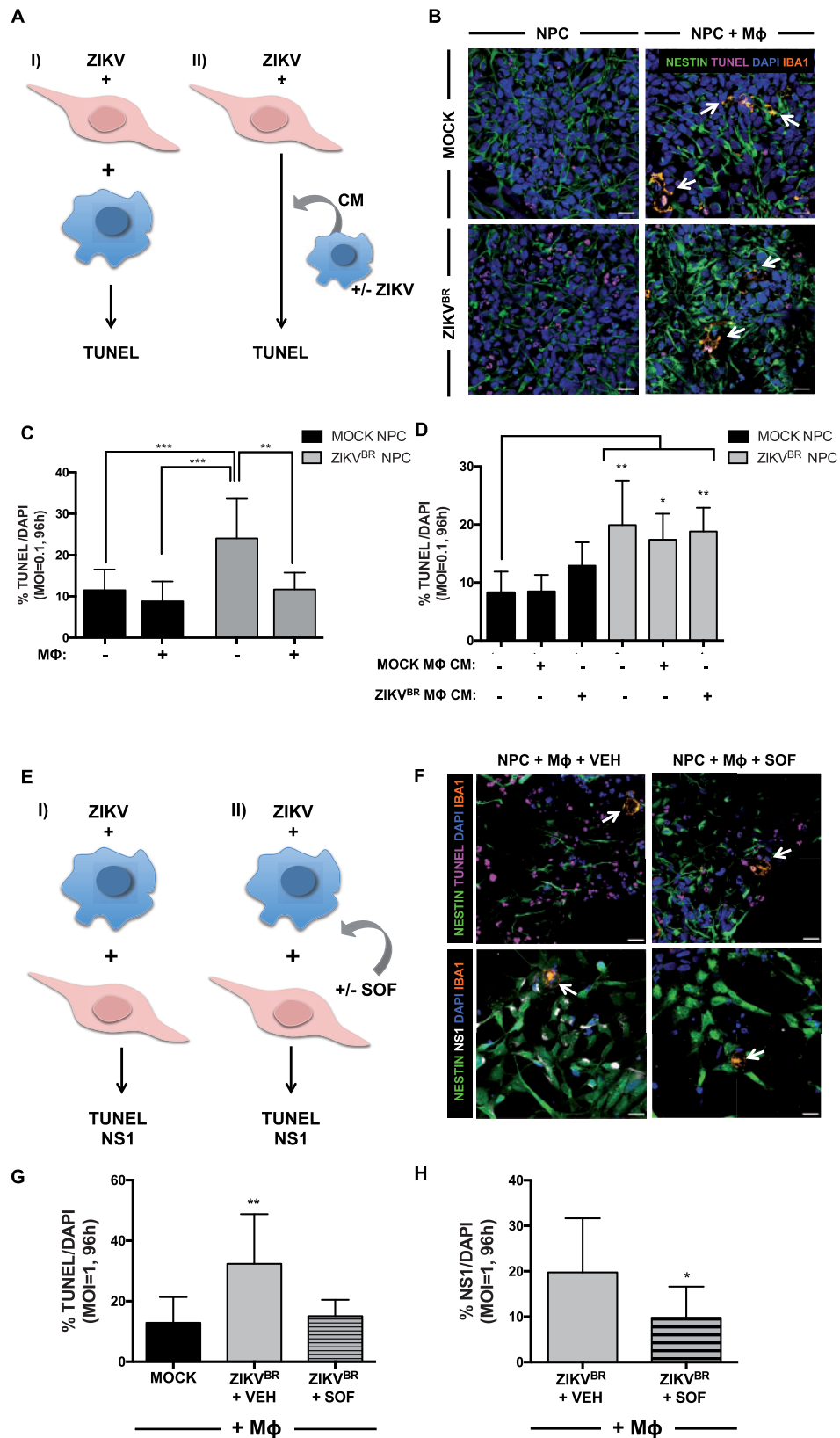


Figure 2. The impact of ZIKV on macrophage/microglia (MΦ) and NPCs co-culture. (A) Schematic of the experimental design. ZIKV-infected NPCs are co-cultured with either naïve MΦ (I), or mock or ZIKV^{BR}-infected MΦ-conditioned media (MΦ CM) (II), followed by an analysis of cell death measured by the percentage of TUNEL positive cells. (B) Images of mock and ZIKV^{BR}-infected NPCs stained with Nestin (green), TUNEL (pink) and fluorescent nuclear staining, DAPI (blue), and MΦ stained with Iba1 (orange) in control (upper left), ZIKV^{BR}-infected NPCs (bottom left), in control NPCs co-cultured MΦ (upper right) and in ZIKV^{BR}-infected NPCs co-cultured with MΦ

increased vulnerability to ZIKV (48). In the future, experiments such as RNAseq on ZIKV-infected M Φ and NPCs could be more informative to comprehend mechanisms underlying ZIKV-infections. Thus, more research is needed to unveil the different mechanisms underlying ZIKV infection of immune and neural cells.

Regarding TLR receptors, we show that all three TLRs, TLR3, TLR4 and TLR7 were downregulated in ZIKV^{BR}-infected M Φ . On closer examination of the inflammatory response, we showed that ZIKV^{BR}-infected M Φ expressed higher levels of AIF1, IL6, IL1 β and CCR5 and released higher levels of IL12/IL23p40, IL1 β , IL10 and G-CSF upon infection with ZIKV^{BR} but not with ZIKV^{MR766} suggesting that the Brazilian strain might induce a distinct inflammatory response at lower MOIs. In line with this, a recent publication showed that the Asian-lineage ZIKV infection of pregnant women's blood led CD14⁺ monocytes to a M2 phenotype, with a notable increase in IL-10 production (49), suggesting that our ZIKV^{BR}-infected M Φ can, at least partially, recapitulate the strain-specific features of ZIKV infection. Noteworthy, as stated previously, the African strain MR766 of the ZIKV has been passaged multiple times *in vitro*, hence the differences seen in inflammatory response could be attributed to the artificial nature of MR-766 strain (50). Thus, this platform could be used to distinguish subtle differences between the several viruses. Interestingly, Manageswaran and colleagues found increased levels of IL1 β in the CNS of IFNAR KO mice upon infection with an Asian strain of the ZIKV (24). Another study investigating the cytokine kinetics upon ZIKV infection of travelers from Thailand, Tahiti, Malaysia and Brazil, found also elevated levels of IL1 β and IL10 in the blood (23). A Brazilian patient who developed with encephalomyelitis upon ZIKV infection, showed elevated levels of G-CSF in the cerebrospinal fluid (25). Finally, a recent study that evaluated the humoral response during ZIKV infection in the amniotic fluid of ZIKV-positive pregnant women with neonatal microcephaly reported elevated IP-10, IL6, IL8, MCP-1 and G-CSF (22). Altogether, we show that the Brazilian ZIKV-induced similar inflammatory response by hiPSC-derived M Φ , thus resembling the immune activation observed in patients, including pregnant women infected with ZIKV. Therefore, hiPSC-derived M Φ could be used as potential targets for drug development.

Finally, in order to mimic the immune interactions occurring between macrophages of the central nervous system, microglia, and NPCs in the fetal brain during ZIKV infection, we derived hiPSC-derived M Φ and NPCs from the same donor. We then established a co-culture system to study the multi-cellular interactions that will occur during infection, such as between naïve M Φ and ZIKV^{BR}-infected NPCs. In our co-culture system, we sought to mimic the microglia percentage *in vivo* which in

mouse cortex is around 5% (51–54). In our experiments, we obtained 2% M Φ after 96 h of co-culture both in ZIKV^{BR} or mock-infection conditions, similar to the percentage of microglia found *in vivo* (51–54). When we added naïve M Φ to infected NPCs, the percentage of dying NPCs decreased significantly. Moreover, by using conditioned media from mock or ZIKV^{BR}-infected M Φ , we explained our results mechanistically by identifying M Φ phagocytosis of apoptotic NPCs, demonstrating that hiPSC-derived M Φ are functional in their ability to recognize virus-infected cells. Furthermore, we showed that conditioned media from ZIKV^{BR}-infected M Φ alone is not sufficient to cause apoptosis of NPCs in our experimental conditions and that physical contact is necessary. The M Φ inflammatory response to ZIKV infection together with the phagocytosis of ZIKV-infected NPCs by M Φ , could contribute to microcephaly in newborns.

Next, we hypothesized that ZIKV-infected M Φ could transmit the virus to the NPCs since the timing of microglial CNS invasion during neurodevelopment corresponds to the timing of ZIKV antigen detection in placental tissues (20,55). Indeed, our data show that ZIKV-infected M Φ transmit ZIKV to NPCs, increasing apoptosis. Importantly, an FDA-approved drug, SOF, was able to block the cell death of NPCs in this context.

Finally, one of the classic pathological findings in GBS, which has been linked to the ZIKV outbreak (5), is inflammatory infiltrates, mainly T cells and macrophages (56). Thus, our *in vitro* model using hiPSC-derived M Φ could be used to further investigate the inflammatory machinery involved in GBS. Our study sheds light on the immune implications of ZIKV infection and might give insights on the risk of developing ZIKV-associated GBS. Thus, we propose that M Φ could be a therapeutic target to limit the virus spreading amongst the most vulnerable and relevant cell types involved in microcephaly, such as NPCs, and that our co-culture model could be used for drug discovery against ZIKV infection.

Experimental procedures

Cell culture

hiPSC-derived macrophages/microglia (M Φ) differentiation protocol was adapted from (29) and (30). Briefly, iPSCs cell lines were generated as previously described, by reprogramming fibroblast from two healthy donors (57). The iPSC colonies were plated on Matrigel-coated (BD Biosciences) plates and maintained in mTESR media (Stem Cell Technologies). The protocol of myeloid cell lineage consisted of 4 sequential steps. In the first step, primitive streak cells were induced by BMP4 addition, which in step 2, were differentiated into hemangioblast-like hematopoietic precursors [VEGF (80 ng/ml, Peprotech), SCF (100

(bottom right). White arrows point to M Φ (orange). Scale bar: 20 μ m. (C) TUNEL images of NPCs infected with the ZIKV^{BR} (MOI=0.1) were acquired, and the percentages of dying cells were calculated, averaged, and graphed accordingly. One-way ANOVA tests with Tukey multiple comparison were performed to compare different groups. The presented values are means of TUNEL⁺/DAPI⁺ percentage \pm SD, **P < 0.01, ***P < 0.001. Note that infected NPCs have increased TUNEL⁺/DAPI⁺ percentage compared with the mock and the addition of M Φ decreases TUNEL⁺/DAPI⁺ percentage. (D) TUNEL images of NPCs infected with the ZIKV^{BR} and treated with mock or ZIKV^{BR}-infected M Φ conditioned media (M Φ CM) (MOI=0.1) were acquired, and the percentages of apoptotic cells were calculated, averaged, and graphed accordingly. One-way ANOVA with Tukey multiple comparisons tests were performed to compare different groups. The presented values are means of TUNEL⁺/DAPI⁺ percentage \pm SD, *P < 0.05, **P < 0.01. (E) Schematic of the experimental design: ZIKV-infected M Φ are co-cultured with NPCs (I), treated with either vehicle (VEH) or Sofosbuvir (SOF) (II) for 96 h before quantification of TUNEL and NS1 positive cells. (F) Images of ZIKV^{BR}-infected cells stained with Nestin (green), TUNEL (pink), Iba1 (orange) and DAPI (blue) (upper), and images of ZIKV^{BR}-infected cells stained with Nestin (green), NS1 (white) and Iba1 (orange) (lower). M Φ were treated with either VEH (left), or 20 μ M SOF (right) after ZIKV-infection and co-cultured with NPCs. White arrows point to M Φ (orange). Scale bar: 20 μ m. (G) TUNEL images of cells infected with the ZIKV^{BR} (MOI =1) were acquired, and the percentages of dying cells were calculated, averaged, and graphed accordingly. One-way ANOVA tests with Tukey multiple comparison were performed to compare different groups. The presented values are means of TUNEL⁺/DAPI⁺ percentage \pm SD, **P < 0.01. Note that M Φ treated with SOF and co-cultured infected NPCs have decreased TUNEL⁺/DAPI⁺ percentage compared with the vehicle (VEH). (H) NS1 images were acquired, and the percentages of NS1 positive cells were calculated, averaged, and graphed accordingly. One-way ANOVA with Tukey multiple comparisons tests were performed to compare different groups. The presented values are means of NS1⁺/DAPI⁺ percentage \pm SD, *P < 0.05.

ng/ml, Gemini) and basic fibroblast growth factor (bFGF, (25 ng/ml, Life Technologies)]. Then, in the third step, the hematopoietic precursors were pushed towards myeloid differentiation [FLT-3 ligand (50 ng/ml, HumanZyme), IL-3 (50 ng/ml, Gemini), SCF (50 ng/ml, Gemini), Thrombopoietin, TPO (5 ng/ml), M-CSF (50 ng/ml)] and finally into the monocytic lineage in step 4 [FLT3-ligand (50 ng/ml), M-CSF (50ng/ml), GM-CSF (25 ng/ml)]. Cells produced in suspension in step 4 were recovered, sorted by using anti-CD14 magnetic microbeads (MACS, Miltenyi), treated with 50ng/ml M-CSF and 50ng/ml IL-34 for a week before being used in different experiments.

Monocyte-derived macrophages were isolated from the peripheral blood of a healthy donor as previously described (34). PBMCs were isolated from the blood by density gradient centrifugation. CD14⁺ monocytes were then purified by MACS using CD14 microbeads.

For the generation of NPCs, cell were differentiated and maintained as previously described (57–59). Two iPSCs lines obtained from healthy patients maintained in mTSE media were switched to N2 [DMEM/F12 media supplemented with 1xN2 NeuroPlex Serum-Free Supplement (Gemini) with the dual SMAD inhibitors 1 μ M of dorsomorphin (Tocris) and 10uM of SB431542 (Stemgent) daily, for 48 h. After two days, colonies were scraped off and cultured under agitation (95rpm) as embryoid bodies for seven days using N2 media with dorsomorphin and SB431542. Media was changed every other day. Embryoid bodies were then plated on Matrigel-coated dishes, and maintained in DMEM/F12 supplemented with 0.5x of N2 supplement, 0.5x Gem21 NeuroPlex Serum-Free Supplement (Gemini), 20ng/ml basic fibroblast growth factor (bFGF, LifeTechnologies) and 1% penicillin/streptomycin (P/S). After 7 days in culture, rosettes that arose from the plated EBs were manually selected, gently dissociated with StemPro Accutase (LifeTechnologies) and plated onto 10 μ g/ml poly-L-ornithine (Sigma)/5 μ g/ml Laminin-coated (LifeTechnologies) plates. Neuronal precursor cells (NPCs) were maintained in DMEM/F12 with N2, Gem21, bFGF and P/S. The medium was changed every other day. NPCs were split as soon as confluent, using StemPro Accutase for 5min at 37 °C, centrifuged and replated with NGF with a 1: 3 ratio in poly-L-ornithine/Laminin-coated plates.

Virus culture and amplification

Both ZIKV^{BR} and ZIKV^{MR766} were obtained and amplified as in (17): lyophilized ZIKV^{BR}, which was isolated from a clinical case in Brazil, was provided by the Evandro Chagas Institute in Belém, Pará and was reconstituted in 0.5 ml of sterile DEPC water. The African-lineage MR-766 (ZIKV^{MR766}), a reference strain isolated in Uganda in 1947, used here as a control, was provided by the Institute Pasteur in Dakar, Senegal. C6/36 *Aedes albopictus* mosquito cells were used to culture both viruses. The culture medium used for C6/36 cells was Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) (Gemini), 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Gibco), 1% penicillin/streptomycin (Gibco) and 0.05% amphotericin B (Gibco). The cells were kept at 27 °C. When cells reached around 70% of confluency, 50 μ l of viral sample was added. The cells were subcultured three times before the supernatant was harvested, titrated and used for experimental inoculation.

Virus titration

The virus titration was performed as previously described (17). Briefly, porcine kidney epithelial (PS) cells were used for virus

titration in L15 medium with 5% FBS. Serial dilution ranging from 10⁻¹ to 10⁻¹¹ for each virus stock were added onto 24-well plates, followed by PS cells seeding into each well of a 24-well plate for 3 h at 37 °C. Next, each well was overlaid with complete carboxymethyl cellulose medium (0.6% in L15 supplemented with 3% FBS). Following 5 days of incubation in 37 °C, the plaque assay was performed using 0.05% crystal violet solution. The viral dilution was estimated to determine the amount of infected cells (plaque forming units or PFU.ml⁻¹).

In vitro infection

NPCs were infected with commercially available ZIKV^{MR766} (MR-766, obtained from ATCC) and ZIKV^{BR}, which was isolated from a febrile case in the state of Paraíba (Brazil-ZKV2015) as previously described (17). Briefly, NPCs were seeded in 24-well, infected at different MOIs for an hour at 37 °C, then were washed and their media was changed. For NPC and M Φ , both of the viruses were used at MOIs of 0.001 for RNA, 0.001, 0.01 and 0.05 for CBA experiments, 0.1 for the first co-culture experiment and MOI of 1 for the second co-culture experiment.

Cytometric bead array (CBA)

The bead-based immunoassay was performed following manufacturer's instructions (BD Biosciences). Briefly, iPSCs-derived M Φ or monocyte-derived macrophages were seeded at a density of 5x10⁴ cells/well. Media was conditioned with or without 1 μ g/ml lipopolysaccharides (LPS, Sigma Aldrich) for 24–30h. 50 μ l of conditioned media per sample were analysed by CBA. A customized array of cytokines and chemokines were measured in the conditioned media, including IL1 β , IL-6, IL-8, IL-10, IL12/23p40, G-CSF, IP10, sCD14, RANTES, Fractalkine, MIP1 α , MIP1 β and MCP1, using a BD fluorescence activated cell sorting (FACS) Canto II instrument followed by an analysis on FCAP Array software (BD Biosciences).

Phagocytosis assay

For internalization experiments, hiPSCs-derived M Φ were seeded to 2 x10⁴ cells per well. Human neutrophils (PMNs), used as positive controls, were isolated from healthy donors using PolyMorphPrep (Axis-Shield) in accordance with the University of California, San Diego, (UCSD) Human Research Protections Program, and seeded to 2 x10⁴ cells per well.

Group B Streptococcus (GBS) strain COH1 was grown overnight at 37 °C in Todd-Hewitt broth (Difco), diluted in PBS to MOI = 10, and spun onto cell 167 g for 3 min. After 90 min, the media was supplemented with 100 μ g/ml gentamicin to kill extracellular bacteria. After 30 min, the cells were washed 1x in PBS and lysed with 0.05% Triton-X100 (Sigma) for dilution plating and CFU enumeration on Todd-Hewitt agar plates (Difco).

Co-culture assay

For the first co-culture experiment, 2 x 10⁴ NPCs per well of a 96-well plate were plated 24 h prior to the ZIKV infection. After ZIKV infection for an hour at 37 °C, cells were washed and fresh media was added. Two hours later, 2 x 10⁴ naïve hiPSCs-derived M Φ per well were plated on top of the NPCs. Cells were fixed 96 h post-infection for further analysis with 4% paraformaldehyde, for 20 min at room temperature. For the second co-culture experiment, 2 x 10⁴ NPCs were plated. M Φ were infected at an MOI of 1 for an hour at 37 °C and the media was changed. The next day, M Φ were lifted off by adding accutase for 5 min, washed with PBS and centrifuged at 300g for 5 min. The cells

were counted and 2×10^4 cells/well of M Φ were added onto NPCs with 20 μ M of SOF (Acme Bioscience AB3793) or vehicle (DMSO). The dose of 20 μ M of SOF used on NPCs was optimized in our laboratory (Mesci *et al.* manuscript in revision). The cells were fixed after 96 h of incubation. For mock controls, the same volume of supernatant was added to each experiment.

Imaging analyses

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Next, samples were permeabilized in 1x-PBS containing 0.1% (v/v) Triton X-100 for 10 min. Cells were next incubated with blocking solution [1% fetal bovine serum, (Life Technologies) in 1xPBS]. After 1 h, the primary antibody was added (diluted in blocking solution) and samples were incubated overnight at 4 °C. Slides were then washed two times with 1x-PBS, and incubated with the secondary antibody for 30 min at 4 °C. Secondary antibodies (all conjugated to Alexa Fluor 488, 555 and 647) were purchased from Life Technologies and used at a 1: 1000 dilution. After the 30 min incubation, samples were washed twice (1x-PBS), incubated for 5 min with and fluorescent nuclear DAPI stain, and mounted with Prolong gold anti-fade reagent (Life Technologies). Samples were imaged using an Axio Observer Z1 Microscope with ApoTome (Zeiss). Captured images were analysed with Zen software from Zeiss. Antibodies and dilutions used: Monoclonal Mouse Anti-Human CD68, (1: 500, Dako); Polyclonal Rabbit Anti-Iba-1, (1: 500, Wako). For TUNEL analysis, NPCs were plated, infected after 24 h, and fixed 96 h p.i with 4% paraformaldehyde for 20 min. Samples were permeabilized with 0.25% Triton X-100 for 15 min, and stained for fragmented DNA using TUNEL (Click-iT TUNEL 647 assay kit from Life Technologies). The cells were then blocked 1% bovine serum albumin for 60 min. Cells were incubated in primary antibodies: Monoclonal Mouse Nestin, Polyclonal anti-Chicken or Polyclonal Rabbit Nestin (1: 500, Abcam); Polyclonal Rabbit Anti-Iba-1, (1: 500, Wako) and monoclonal mouse NS1 (1: 250, Millipore) overnight at 4 °C and stained with secondary antibodies conjugated to Alexa Fluor and DAPI the following day prior to mounting. Images were blindly collected using an Axio Observer Z1 Microscope with ApoTome (Zeiss) and blindly analysed with ImageJ software.

Fluorescence-activated cell sorting analyses

Using previously mentioned protocol to generate monocytes/macrophages (29), we have collected the cells in the supernatant at the step 4 and labeled them with the appropriate antibodies (CD45 BV786, CD14 PE, CD11b APC, CD68 PE-Cy7, HLA-DRDPDQ FITC all from BD Biosciences). Briefly, cells were harvested and counted, then washed twice with the stain buffer, containing PBS and 1% heat inactivated FBS, at 300g for 5 min. The antibodies were added in the cell mixture and incubated in RT in the dark for 20–30 min. The cells were then washed twice with the stain buffer. The cells were resuspended in 500 μ l stain buffer and the viability dye, 7-AAD was added. The data were acquired in BD LSRForteSSA instrument and the plots were generated using the software FlowJo.

RNA extraction and expression analyses by qPCR

Total RNA was extracted from cells using RNeasy Micro and Mini kit for iPSC-derived M Φ and NPCs respectively (Qiagen), following manufacturer's instructions. Next, 900 ng of total RNA was treated RNase-free DNaseI (Qiagen), and was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen). Approximately, 15 ng of cDNAs was used per reaction and PCRs

Table 1. List of primers

AXL-F	GTTTGGAGCTGTGATGGAAGGC
AXL-R	CGCTTCACTCAGGAAATCCTCC
CCR5-F	CCTGCTGCTTTGCCTACATTC
CCR5-R	ACACACTTGGCGGTCTTTCCGG
CD11b-F	GGAACGCCATTGTCTGCTTTCCG
CD11b-R	ATGCTGAGGTCATCTCCGGCAGA
CD206-F	AGCCAACACCAGCTCCCAAGA
CD206-R	CAAAACGCTCGGCAGTATGTCCA
CD209-F	GCACTCTCCAGAAGTAAACCCG
CD209-R	GCTCTCCTCTGTCCAATACTGC
CD68-F	CGAGCATCATTCTTTACCAGCT
CD68-R	ATGAGAGGCAGCAAGATGGACC
CLEC5A-F	TTGTCAACACGCCAGAGAAACT
CLEC5A-R	CAACGCCACCTTTTCTCTTCAGC
IBA1-F	CCCTCCAACTGGAAGGCTTCA
IBA1-R	CTTTAGCTCTAGGTGAGTCTTGG
IGF1-F	CTCTTCAGTTGCTGTGTGGAGAC
IGF1-R	CAGCCTCCTTAGATCAGAGCTC
IL1B-F	CCACAGACCTTCCAGGAGAATG
IL1B-R	GTGCAGTTCAGTGATCGTACAGG
IL6-F	AGACAGCCACTCACCTTCCAG
IL6-R	TTCTGCCAGTGCCTCTTTGCTG
MCP1-F	AGAATCACCAGCAGCAAGTGTCC
MCP1-R	TCCTGAACCCACTTCTGCTTGG
MERTK-F	CAGGAAGATGGGACCTCTCTGA
MERTK-R	GGCTGAAGTCTTTTCATGCACGC
NOX2-F	CTCTGAACTTGAGACAGGCAAA
NOX2-R	CACAGCGTATGACAACTCCAG
TIM-F	CTTCACCTCAGCCAGCAGAAAC
TIM-R	GCCATCTGAAGACTCTGTCCAG
TLR3-F	GCGCTAAAAAGTGAAGAACTGGAT
TLR3-R	GCTGGACATTTTCAGAAAAGAGG
TLR4-F	CCCTGAGGCATTTAGGCAGCTA
TLR4-R	AGGTAGAGAGGTGGCTTAGGCT
TLR7-F	CTTTGGACCTCAGCCACAACCA
TLR7-R	CGCAACTGGAAGGCATCTGTAG
TYRO3-F	GCAAGCCTTTGACAGTGTCTATG
TYRO3-R	GTTTCATCGCTGATGCCAAGCT
NES-F	CCATAGAGGGCAAAGTGGTAA
NES-R	TTCTTCCATATTTCTGTGTC
PAX-F	TGTCCAACGGATGTGTGAGTA
PAX-R	CAGTCTCGTAATACCTGCCCA
GAPDH-F	TGCACCACCAACTGCTTAGC
GAPDH-R	GGCATGGACTGTGGTCATGAG

were carried out in a final volume of 10 μ l. Triplicate samples were analysed in a CFX384 Touch Real-Time PCR Detection System (Bio-rad) using iQTM SYBR[®] Green Supermix (Bio-rad). GAPDH was used as internal normalization control. For the complete list of primers, please refer to Table 1. The run method was as follows: 3min at 95 °C, 40 cycles of 10s at 95 °C followed by 30s at 58 °C, and a melting curve was performed to confirm the identity of the amplified product.

Statistical analysis

One-way ANOVA or two-way ANOVA (if two or more variables) tests followed by a Tukey or Sidak multiple comparison tests were used to compare groups and Student's t-test to compare means of two groups.

Supplementary Material

Supplementary Material is available at HMG online.

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