Human neutrophils are not activated by Zika virus but reduce the infection of susceptible cells

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Abstract

Zika virus (ZIKV) emergence highlighted the need for a deeper understanding on virus-host interaction to pave the development of antiviral therapies. The present work aimed to address the response of neutrophils during ZIKV infection. Neutrophils are an important effector cell in innate immunity involved in the host response to neurotropic arboviruses. Our results indicate that human neutrophils were not permissive to Asian or African ZIKV strains replication. Indeed, after stimulation with ZIKV, neutrophils were not primed against the virus as evaluated by the absence of CD11b modulation, secretion of inflammatory cytokines and granule content, production of reactive oxygen species and neutrophil extracellular traps formation. Overall, neutrophils did not affect ZIKV infectivity. Moreover, ZIKV infection of primary innate immune cells *in vitro* did not trigger neutrophil migration. However, neutrophil co-cultured with ZIKV susceptible cells (A549) resulted in lower frequencies of infection on A549 cells by cell-to-cell contact. *In vivo*, neutrophil depletion from immunocompetent mice did not affect ZIKV spreading to the draining lymph nodes. The data suggest human neutrophils do not play a *per se* antiviral role against ZIKV, but these cells might participate in an infected environment shaping the ZIKV infection in other target cells.

1 Introduction

Zika virus (ZIKV) is an enveloped vector-borne RNA virus, member of the genus *Flavivirus* which includes important human pathogens. The epidemic potential of flaviviruses is related to the global distribution of their arthropod vectors (mainly *Aedes* spp.), as well as human population density, mobility and anthropogenic interventions (1). Furthermore, mutation rate in the viral genome and the host immune-status may also impact on viral spread and pathogenesis (2). After decades of sparse reports of infection in Africa and Asia, since 2007 the ZIKV Asian genotype has been implicated in outbreaks in human populations, from Southeast Asia spreading throughout the Americas and reaching Europe (3). In Brazil, ZIKV was first detected in 2015 and recognized as a Public Health Emergency in 2016 (4). More than 220,000 cases were notified and the infection was associated with congenital diseases and to Guillain-Barré Syndrome in adults (5–7).

ZIKV infection has been reported to trigger a rapid recruitment and activation of monocytes, NK cells, plasmacytoid dendritic cells, and lymphocytes, and the upregulation of multiple signaling pathways, like pro-inflammatory cytokines and chemokines in the blood of non-human primates and humans (8–13). Among the innate immune cells, monocytes, dendritic cells and macrophages have been described as targets of ZIKV infection and replication (14–17). It has been suggested that the ensuing innate immune response can be associated with the fate of ZIKV disease. Cell infiltration and inflammation at ZIKV infection sites contributed to placental dysfunctions (18) and encephalitis (19–21). ZIKV affects adhesive properties of monocytes, enhancing their transmigration through endothelial barriers and viral dissemination to neural cells (22). Moreover, neutrophils, Ly6C^{mid-hi} monocytes, and CD45⁺ monocytes from AG129 mice (type I and II IFN receptor deficient), and bone marrow-derived S100A4⁺ macrophages from AG6 mice (type I, II and III IFN receptor deficient) were shown to be essential for ZIKV dissemination and pathogenesis in peripheral organs and testis (23–25). CD45⁺CD11b⁺ monocytes and macrophages play an important role in containing ZIKV spread in the placenta (26), while infected human placental macrophages might gain access to the fetus (27). In this context, the role played by neutrophils during ZIKV infection remains undetermined. Elucidating mechanisms by which neutrophils mediate an antiviral response may enable the development of therapies that retain antiviral functions but limit inflammation-associated damage.

Mature neutrophils are the most abundant granulocytes in the human bloodstream and the major effectors during inflammation and infection. Once in the infection site, neutrophils can rapidly

eliminate intra- and extracellular pathogens by phagocytosis, oxidative burst, multiple granule proteolytic enzymes, antimicrobial peptides and neutrophil extracellular traps (NETs) release (28,29). Neutrophils have been also recognized as multitasking cells capable of cross talking with adaptive responses, for example, presenting antigens during viral infections (30,31). The relevance of neutrophils during flaviviruses infection was demonstrated for *West Nile virus* (WNV) infections, where neutrophils act as Trojan horses carrying the virus into the central nervous system (CNS) enhancing WNV neuroinvasive disease (32). Neutrophil depletion prior to WNV infection resulted in reduced viremia and enhanced host survival (33).

Here, we address the role of neutrophils on ZIKV pathogenesis by the *in vitro* screening of classical human neutrophil defense mechanisms after stimulation with different ZIKV strains. We report that neutrophils are not targets for ZIKV replication nor good responder to ZIKV, yet neutrophils reduce ZIKV infection when in contact with target cells.

2 Materials and Methods

2.1 Cells

Human peripheral blood was obtained by intravenous puncture from healthy volunteers (both genders, aged between 21-50 years old and with no clinical evidence of disease) upon written consent. The procedures were in accordance with the Conselho Nacional de Ética em Pesquisa- CONEP (CAAE 60643816.6.0000.5248). Human neutrophils were isolated from peripheral blood by negative selection with magnetic microspheres MACSxpress Neutrophil Isolation Kit and MACSxpress Separator (Miltenyi Biotec), according to the manufacturer instructions. Cell viability was determined by Trypan blue exclusion assay and the neutrophil purification was confirmed by cytopsin slides (Cytospin 4; Thermo Fisher Scientific) visualized by microscopy (LEICA AF6000 Modular System) (Figure 1A) and flow cytometry.

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque density 1.077 g/mL (Lonza). CD14⁺ cells were sorted with the MACS system (Miltenyi Biotec), according to the manufacturer instructions and seeded at 5x10⁵ cells/mL in RPMI-1640 media with L-glutamine (Lonza) supplemented with 10% fetal bovine serum (FBS; Gibco), 25 µg/mL gentamicin (Gibco), 100 IU/µg/mL penicillin-streptomycin (Sigma-Aldrich), 12.5 ng/mL recombinant human GM-CSF (PeproTech), and 25 ng/mL recombinant human IL-4 (PeproTech). The cells were incubated for 7 days at 37°C, 5% CO₂ and humid atmosphere. On the third day of incubation, fresh supplemented

medium was added to the cell culture. Human monocyte-derived dendritic cells (mdDCs) differentiation was confirmed by flow cytometry (CD11c^{+/high}CD14^{+/low}).

Human A549 lung epithelial cells (ATCC CCL-185) were maintained in RPMI-1640 media supplemented with 10% FBS, 25 µg/mL gentamicin, and 100 IU/µg/mL penicillin-streptomycin at 37°C, 5% CO₂ and humid atmosphere. *Aedes albopictus* mosquito C6/36 cells (ATCC CLR-1660) were grown in Leibovitz's media (L-15; Gibco) supplemented with 5% FBS, 25 µg/mL gentamicin, and 0.26% tryptose (Sigma-Aldrich) at 28°C.

2.2 Zika virus

Viral stocks of the ZIKV Asian strains, the clinical isolate BR 2015/15261 (34) and PE243 (35), and the ZIKV ancestral African isolate MR766 (36) were prepared in C6/36 cells. Seven days post-infection cell culture supernatant was collected, clarified by centrifugation and later titrated by foci-forming immunodetection assay in C6/36 cells (37). In parallel, C6/36 cells were maintained in the same conditions without viral addition. This conditioned supernatant, hereby called mock, was used as a negative control for cell activation and infection.

2.3 Cell interaction with ZIKV

Neutrophils at 2.5x10⁵ cells/200 µL of RPMI-1640 media supplemented with 25 µg/mL gentamicin and 100 IU/µg/mL penicillin-streptomycin were incubated for 2 hours with ZIKV strains (BR 2015/15261, PE243 and MR766) using a multiplicity of infection (MOI) of 1 at 37°C, 5% CO₂ and humid atmosphere under agitation. As a control for cell activation, neutrophils were also incubated in the same conditions with mock, 100 ng/mL of *Escherichia coli* lipopolysaccharide (LPS-EK, InvivoGen), or 16 nM of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich). After the 2h incubation period, neutrophils were washed twice (250 x g; 10 minutes) in non-supplemented media and seeded in 96-well plates in RPMI-1640 media supplemented with 10% FBS and antibiotics at 37°C, 5% CO₂ and humid atmosphere. Supernatant collected right after the wash step was called input and used as a control to account for any remaining viruses in the neutrophil culture after washing out the initial inoculum (Figure 1B). Two (input), 6, 12 and 24 hours after the beginning of the stimulation, neutrophils and the culture supernatant were harvested and analysed. Supernatants were stored at -80°C until further analysis. In some experiments, to exclude non-internalized virus binding in their surface, neutrophils were treated right after the wash steps (2 hours/input) with

0.05% trypsin-EDTA (Gibco) (Figure 1B) for 10 minutes at room temperature, followed by FBS addition, washed and suspended in the new media (38).

Chemokines in PBMCs and mdDCs culture supernatant were quantified 24 and 48 hours after ZIKV stimulation. For that, PBMCs (after isolation) and mdDCs (after 24 hours of resting) at 1×10^6 cells/500 μ L were stimulated with the same protocol described above for neutrophils (Figure 1B), but seeded in 24-well plates.

A549 and C6/36 cells were incubated for 2 hours with ZIKV strains (1 MOI) (or different stimuli when indicated) in 400 μ L of media without FBS. After the incubation period, cells were washed twice with non-supplemented media and kept in media supplemented with 10% FBS and antibiotics during the indicated times.

2.4 Flow cytometry

Neutrophils and A549 cells viability were determined at the indicated time points using Annexin V (ImmunoTools) and 7-Amino-Actinomycin (7-AAD; BD Bioscience) following manufacturer's instructions (PE Annexin V Apoptosis Detection Kit; BD Bioscience). The frequency of ZIKV antigen in neutrophils, A549 cells and C6/36 cells was measured by staining with a flavivirus group specific envelope protein (E) monoclonal antibody 4G2 (ATCC HB-112) (37). Briefly, the cells were recovery, and if necessary were detached from the cell culture flask (A549 cells with trypsin and C6/36 with cell scraper), and blocked (5% FBS and 1% human AB serum in PBS) for 20 minutes at room temperature. Next, cells were fixed and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences), stained with 4G2 FITC-conjugated antibody for 45 minutes at 37°C, and washed twice. Alternatively, these cells were labeled with 4G2 antibody, goat anti-mouse Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific) and Vybrant DyeCycleViolet Stain (Thermo Fisher Scientific), and fixed in slides pre-treated with poly-L-lysine (Sigma-Aldrich) for confocal microscopy imaging (LEICA SP5 AOBS). For surface markers staining, neutrophils were blocked and incubated with fluorochrome-conjugated mouse anti-human monoclonal antibodies specific for CD11b (clone ICRF44), CD16 (clone 3G8), CD62L (clone DREG-56) (BD Biosciences), and Hu Axl (clone D57HAXL) (eBioscience), for 20 minutes at room temperature. To measure intracellular reactive oxygen species generation, neutrophils were labeled with 0.5 μ M of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) probe (Invitrogen) for 45 minutes at 37°C. Cytokines and chemokines in the supernatant

of neutrophils, PBMCs and mdDCs culture supernatants were quantified by the Cytometric Bead Array (CBA) method using the Human Inflammatory Cytokines Kit and Human Chemokine Kit (BD Biosciences), following manufacturer's instructions. Flow cytometry was performed on a FACS Canto II with BD FACSDiva software (BD Biosciences) and the acquired data analyzed on FlowJoV10 (BD Biosciences).

2.5 RT-qPCR

Neutrophils RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RT-qPCR to detect ZIKV was performed in a 20 µL reaction final volume containing GoTaq one-step RT-qPCR master mix (Promega), 25 ng of sample RNA, 500 nM of the ZIKV1086 and ZIKV1162c oligonucleotides, and 200 nM of ZIKV1107-FAM probe according to a previously described protocol (39). RNase P (*RPPH1*) was used as a reference gene (40). All reactions were carried out in a LightCycler 96 System and the fluorescence threshold limit of the probe was automatically set by LightCycler software (Roche). The results were represented as the ΔC_t between ZIKV and RNAase P amplification.

2.6 Neutrophil elastase measurement

Elastase was measured using the Human PMN-Elastase ELISA Kit (Invitrogen) in neutrophil culture supernatant at 6 hours after stimulation. Cell culture supernatant (100 µL) was added to a microwell plate coated with anti-human polymorphonuclear (PMN) elastase polyclonal antibody and incubated at room temperature for 1 hour with a Horseradish peroxidase-conjugated anti- α 1-proteinase inhibitor antibody. The immune complex was detected by adding tetramethyl-benzidine substrate solution and the absorbance was determined at 450 nm on a microplate reader (BioTek Synergy H1 Hybrid).

2.7 Neutrophil extracellular traps assessment

Neutrophils at 2×10^5 cells/200 µL of RPMI-1640 media supplemented with antibiotics were incubated for 5 hours with either mock, ZIKV strains (1 MOI), or 160 nM of PMA in Lab-Tek chamber slides (Thermo Fisher Scientific) pre-treated with poly-L-Lysine at 37°C, 5% CO₂ and humid atmosphere. For NETs visualization, the supernatant was carefully removed and the cells were fixed with 3% paraformaldehyde (Sigma-Aldrich), and stained with an anti-acetyl-histone H3 polyclonal antibody (Sigma) followed by a mouse anti-rabbit Alexa Fluor 488 secondary antibody

(Sigma) and Vybrant DyeCycleViolet Stain (Thermo Fisher Scientific) for 45 minutes at 37°C. The slides were sealed with n-propyl gallate (Sigma-Aldrich) and observed by confocal microscopy (LEICA SP5 AOBS). For NETs quantification, following the stimulation period, neutrophils were treated with 0.04 U/μL of Turbo DNase (Thermo Fisher Scientific) for 10 minutes at 37°C. The enzymatic digestion was stopped with 5 mM EDTA (41). The culture was centrifuged (300 x g for 1 minute), the supernatant collected and 5-fold diluted. Free double-stranded DNA (dsDNA) was quantified using Quant-IT PicoGreen dsDNA kit (Invitrogen) in a Qubit 2.0 fluorometer (Invitrogen) according to the manufacturer's recommendations. To evaluate the effect of pre-formed PMA-induced NETs on viral particles capture, neutrophils were stimulated during 5 hours with media or PMA in the same conditions described above. Then, the culture was stimulated for 1 hour with ZIKV strains (1 MOI). Next, neutrophils were centrifuged (300 x g for 1 minute) and the supernatant (with virus not attached to the traps) collected and quantified by foci-forming immunodetection assay in C6/36 cells.

2.8 Neutrophil chemotaxis assay

Neutrophils at 3×10^5 cells/200 μL of RPMI-1640 media supplemented with antibiotics were seeded on a 3 μm pore Thin Cert insert (Greiner Bio-One) coupled to a 24-well plate. Inducers of neutrophil chemotaxis were added to the bottom well in 600 μL of RPMI-1640 media containing 1,000 or 50,000 pg of recombinant human (rh-) IL-8 (PeproTech) or A549 cells culture previously infected for 48 hours with mock or ZIKV PE243 (1 MOI). For this specific experiment, infected A549 cells were maintained in the absence of FBS. RPMI-1640 media and A549 cells stimulated with mock were used as a negative control for cell migration. After 2 hours at 37°C, 5% CO₂ and humid atmosphere, migratory neutrophils were collected from the bottom well system and the cell concentration determined by Turk dye counting. The chemotactic index was calculated as the ratio of the number of migratory neutrophils in each condition divided by the number of neutrophils that migrated in the negative control (42).

2.9 Co-culture assay

A549 cells (seeded in 24-well plates) at 1×10^5 cells/ 400 μL in RPMI-1640 media supplemented with antibiotics were stimulated for 2 hours with mock or ZIKV strains (1 MOI) in the absence or presence of neutrophils of a ratio of 1:5. Afterwards, neutrophils were assessed for surface markers by flow cytometry as described above, and A549 cells were washed twice and kept in 500 μL of

RPMI-1640 media supplemented with 10% FBS and antibiotics for 16 hours. At the end of the 18 hours of infection, A549 cells were assessed for viability and intracellular ZIKV antigen by flow cytometry. To evaluate if neutrophils were physically interacting with A549 cells through surface proteins, in some experiments, before adding A549 cells culture, neutrophils were treated with trypsin for 10 minutes at room temperature, added FBS, washed and suspended in fresh media. In a different co-culture experiment setting, A549 cells were incubated for 2 hours with mock or ZIKV strains (1 MOI), washed twice and incubated for 24 hours. Then, neutrophils in a ratio of 1:5 were added or not to these cultures for 16 hours. At the end of the 40 hours of infection, A549 cells were evaluated for frequency of ZIKV antigen.

2.10 *In vivo* ZIKV infection model

C57BL/6 mice were obtained from Instituto Carlos Chagas/FIOCRUZ-PR animal facility, and maintained and handled according to the directives of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation. The protocols were approved by the Committee on the Ethics of Animal Experimentation from Fundação Oswaldo Cruz – CEUA/FIOCRUZ (license LW 03-19). Both male and female mice between 8-12-week-old were infected subcutaneously in the hind footpad with ZIKV PE243 (5×10^5 FFU, 10 μ L) to determine systemic ZIKV titers. After 10 minutes, 1, 3, 6 and 24 hours of infection, spleen, kidney and lymph nodes (popliteal (pLN), lumbar aortic (laLN), and sciatic lymph nodes (sLN)) were aseptically removed, homogenized using a tissue grinder, submitted to three freeze-thaw cycles and the viral load measured by foci-forming immunodetection assay in C6/36 cells. Both ipsi- and contralateral lymph nodes per animal were pooled together. Blood was collected through cardiac puncture at the same time points and plasma viremia was titrated in C6/36 cells.

To evaluate the neutrophil influence in ZIKV spread to peripheral organs, neutrophils were depleted and the animals infected with ZIKV. Animals were inoculated intraperitoneally with 200 μ L of PBS containing 400 μ g of anti-mouse Ly6G (clone 1A8; BioxCell) or mouse IgG2a isotype control (clone C1.18.4; BioxCell). Control animals received 200 μ L of PBS (Lonza). After 18 hours, the frequency of neutrophils in total blood was evaluated through flow cytometry by surface staining with fluorochrome-conjugated anti-mouse monoclonal antibodies specific for CD11b (clone M1/70), and Ly6C/G (clone RB6-8C5) (BD Biosciences). Neutrophil-depleted animals were inoculated in the hind footpad with PBS only or 1,000 ng of LPS (to induce an inflammatory environment). After 3 hours, mice were inoculated with 10 μ L of ZIKV PE243 (1.3×10^6 FFU) in the same footpad. One

hour later, popliteal lymph nodes (pLNs) were pooled, harvested and the viral title determined as above.

2.11 Statistical Analyses

Analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). Wilcoxon matched-pairs signed rank test (nonparametric paired t-test) was used in the analysis of the *in vitro* experiments with primary human cells to clarify individual patterns. One-way Anova with Tukey's multiple comparison test was used in animal experiments to compare the average of groups. A cut-off of $p < 0.05$ was considered significant.

3 Results

3.1 ZIKV does not establish a productive infection in human neutrophils

We first evaluate whether any of the tested ZIKV strains trigger human neutrophil death until 24 hours after stimulation (Figure 1B). It was observed a similar exposition of phosphatidylserine between mock- and the ZIKV strains-stimulated neutrophils over time and low loss of cellular integrity indicated by 7-AAD uptake (Supplementary Figure 1). The observed neutrophil phenotype concurs with previous knowledge that in the absence of inflammation, neutrophils have a short life span and undergo constitutive apoptosis (43), and LPS, that is a positive control of neutrophil activation, restricted apoptosis at the late time points (44).

Viruses, in addition to subverting the target cell into a reservoir for replication and dissemination, may use the infected cells as a Trojan horse to overcome physiological host defense barriers (32). The infection can also result in the inhibition of important cell signal transduction pathways (17). To address this issue, we sought to understand whether human neutrophils are susceptible to and sustain ZIKV infection. No positive cells for the intracellular staining of the ZIKV E protein (4G2 antibody) were detected 24 hours after stimulation with the three ZIKV strains tested through immunofluorescence or flow cytometry (Figure 1C and 1D). It contrasts with the infection observed in the highly permissive mosquito cell line C6/36 (Figure 1C and 1D). The possibility of an active internalization of a few viral particles by neutrophils via receptors or phagocytosis could not be excluded, even though we reported the absence of AXL in the surface of human neutrophils, which was expressed in the ZIKV susceptible human lineage A549 (Figure 1E). Indeed, ZIKV RNA could be detected in neutrophils incubated with the viruses (Figure 1F). However, we did not observe a

significant increase in ZIKV RNA levels over time (Figure 1F) nor the release of functional viral particles in loads greater than the input (Figure 1G). Treatment of neutrophils with trypsin after the wash steps resulted in the reduction but not in the abolishment of virus RNA levels (Figure 1H). This suggests that the low RNA levels measured cannot be fully attributed to viral inoculum leftover, and at least partially, could be explained by the virus particles that stick at the cell membrane.

3.2 Human neutrophils are mild responsive to direct contact with ZIKV

The recognition of viral elements in the cell cytosol triggers defense mechanisms involved in viral replication control and inflammation. However, a significant part of neutrophil activation mechanisms is coordinated through the signaling of cell surface receptors (43). Following the experimental setting in Figure 1B, the expression of the adhesion integrin CD11b was upregulated over time following LPS stimulation, while the selectin adhesion receptor CD62L was downregulated (Figure 2A-C), indicating priming of neutrophils (45,46). Nevertheless, no differences were observed in the expression of CD11b molecule between mock- and the ZIKV strains-stimulated neutrophils (Figure 2B), and only slight differences were observed in the expression of CD62L (Figure 2C). No significant differences were observed in the IL-8/CXCL8 levels secreted by neutrophils after 24 hours of stimulation between mock and ZIKV (Figure 2D). Similar results were obtained for IL-1 β , IL-6 and IL-10 (data not shown). The low levels of elastase detected in neutrophils culture supernatant after 6 hours of stimulation with ZIKV BR 2015/15261 were similar to mock-stimulated cells (Figure 2E). It was also noted an overall low production of ROS in neutrophils after ZIKV stimulation, measured by the oxidation of chloromethyl-H₂DCFDA (Figure 2A and 2F), in contrast to the oxidative stress generated by PMA stimulation, a well-known ROS inducer. We confirmed that NETs were not induced by any ZIKV strains tested after 5 hours of stimulation through the absence of web-link extracellular structures colocalizing with DNA and histone (Figure 3A) and the absence of free DNA in neutrophil supernatants (Figure 3B). PMA is a robust NET inducer over a 3-4 hour time course via ROS (47), as observed here (Figure 3A-B). Moreover, ZIKV was not trapped by NETs, as the same loads of ZIKV were quantified in the supernatant of neutrophils stimulated or not with PMA (Figure 3C). To confirm that neutrophil does not impact ZIKV particles, neutrophils were stimulated for 6 hours with ZIKV, and the free virus recovered from the supernatant did not have impaired infectivity in a subsequent infection of susceptible cells (Figure 4).

3.3 ZIKV infection does not provide a favorable environment for human neutrophil migration

Although neutrophils are circulatory cells and therefore have the potential to encounter viruses in the bloodstream, their fate is to contribute to the inflammation on the infected tissue. In fact, the priming of neutrophils in the circulation by an isolated stimulus is insufficient, and their complete activation to full capacity is a multistep process achieved after their transmigration through the endothelium following a chemotactic gradient (43). Secretion of IL-8/CXCL8, an important human neutrophil chemoattractant, was measured after ZIKV stimulation of mdDCs and PBMCs for 24 and 48 hours. LPS was used as a positive control of activation and boosted the chemokines production (Figure 5A). A concentration of recombinant human IL-8 (rhIL-8) corresponding approximately to the ones detected in the mdDCs and PBMCs supernatant (1,000 pg) was not enough to induce neutrophil migration in a transwell assay (Figure 5B). Neutrophils migrated with a 50-fold higher rhIL-8 concentration (Figure 5B). Interestingly, a more permissive cell line, A549 cells, when infected with ZIKV PE243 for 48 hours, also did not promote neutrophil migration (Figure 5B).

3.4 Human neutrophils reduce ZIKV infection in A549 by cell-cell contact

To mimic a situation where neutrophils reached an infected environment after migration, we co-cultured neutrophils with A549 cells. Neutrophils were added to A549 cells concomitant with ZIKV infection. Both stimuli were maintained for 2 hours and then removed. The A549 cells infection frequency was evaluated 16 hours after the removal of the viral input by the detection of ZIKV E protein (4G2) (Figure 6A). A significant reduction in A549 cell infection was observed when neutrophils were added during the infection time (Figure 6A and 6B). We did not detect a reduction in the frequency of A549 cells annexin V⁺-AAD⁺ due to neutrophil presence (Figure 6C). We previously hypothesized that a small number of particles might have been internalized by neutrophils and a reduced fraction of the virus particles could be binding to the neutrophils surface. Nevertheless, such a small reduction in the number of free viral particles is not enough to explain the decrease in the infection rate observed in the co-culture. Besides, no decrease in A549 infection was detected when ZIKV was pre-incubated with neutrophils (Figure 4). Neutrophils co-cultured with A549 cells did not modulate CD11b and CD62L receptors (Figure 6D and 6E), indicating the reduction of the infection is not due to neutrophils activation. However, neutrophil treatment with trypsin before addition to A549 cells restored A549 cells infection frequencies and replication (Figure 6A, 6F and 6G). Therefore, we hypothesized that neutrophils were interacting with A459 cells through surface

protein membrane components and impairing ZIKV infection. The treatment with trypsin did not significantly affect the viability of neutrophils (annexin V⁺-AAD⁻) (Figure 7A), nor the expression of CD11b (Figure 7B and C), but significantly reduce the expression of CD62L on neutrophils (Figure 7B and 7D), suggesting an impact on neutrophil surface proteins by trypsin. In an alternative co-culture setting, aimed to mimic the neutrophil role to an established ZIKV infection, A549 cells were infected for 24 hours with ZIKV strains and after this time neutrophils were added to the culture for 16 hours. Even in a scenario where infection was already established, a significant reduction in the frequency of ZIKV infection was observed after neutrophils addition (Supplementary Figure 2).

3.5 Neutrophil depletion does not alter ZIKV titers in the draining lymph node

After subcutaneous infection in the hind footpad, ZIKV PE243 were detected only up to 3 hours after infection in the lymph nodes (popliteal (pLN), lumbar aortic (laLN), and sciatic lymph nodes (sLN)) of C57BL/6 immunocompetent mice (Figure 8A). ZIKV PE243 were not detected in the spleen, kidney or blood of these animals in any of the assessed times. Therefore, we used the pLN 1 hour of ZIKV infection as a viral spread indicator site in our subsequent studies. C57BL/6 mice had neutrophils significantly reduced through pre-treatment with a monoclonal antibody targeting Ly6G (Figure 8B and 8C). Eighteen hours post-neutrophil depletion treatment and 3 hours prior to ZIKV PE243 subcutaneous infection, mice were inoculated subcutaneously in the hind footpad with LPS to stimulate cell migration to the injection site (Figure 8D). The animals treated with the anti-Ly6G antibody (Ly6G x LPS) presented similar titers of ZIKV in the pLN than animals that received no antibody treatment (PBS x LPS) (Figure 8D). The slightly reduced titers in the pLN seen in mice that received LPS in comparison with the ones that received PBS in the footpad were attributed to the inflammatory context. This result suggests that, in this model, the presence of neutrophils was not essential to contain ZIKV spread to the draining lymph node.

4 Discussion

Neutrophil-inflammatory responses triggered by viral infection are necessary for an effective antiviral immunity (48–51), but can also become dysregulated and result in tissue injury (52,53). This concept could be extended to ZIKV pathology, in which neutrophils could be associated with the virus neurotropic nature, the ability to cause injury to the reproductive tract and be sexually transmitted, as well as the long-term viral persistence in some body fluids and tissues (3). In order to

investigate these possibilities, we interrogated the response of human neutrophils to Asian and African ZIKV strains in a diverse experimental setting.

Neutrophils were suggested to be permissive to ZIKV as viral RNA was found in myeloperoxidase⁺ neutrophils present in the lymph nodes of cynomolgus macaques 7 days post-infection (10), and in CD45⁺CD11b⁺ neutrophil-myeloid cells in the placenta of AIR mice (vertical transmission model in a *Rag1*-deficient mouse) (26). In contrast to those previous findings, our results shown that, beside some ZIKV RNA detection, human neutrophils do not appear to support any significant ZIKV replication and do not represent a remarkable ZIKV reservoir. This is in agreement with previous reports demonstrating that ZIKV preferentially targets CD14⁺CD16⁺ monocytes in the blood (16). Also according to our data, AXL, a TAM family tyrosine kinase that has been described as a facilitator of ZIKV infection due to attenuation of type I IFN (54), was found to be absent from neutrophils surface. Nevertheless, ZIKV receptors and co-receptors are still not well characterized and AXL is one of the cell surface molecules that could help mediate ZIKV infection.

Surprisingly, stimulation of neutrophil with ZIKV did not promote a strong cell activation or any of the classical neutrophil microbicidal mechanisms as noted by the absence of CD11b modulation, secretion of inflammatory cytokines and elastase, production of reactive oxygen species and NETs. These results indicate neutrophils that presented a non-responsive phenotype in our experimental settings and would not be the major source of inflammatory mediators during ZIKV infection. Other viruses, such as *Human immunodeficiency virus-1*, induced activation on neutrophils by modulating the expression of several Toll-like receptors, CD11b and CD62, promoting the secretion of IL-6 and TNF- α , and altering ROS production (55). According to our results, ZIKV and *Dengue virus* type 2 were previously shown not be able to provoke NETs induction in mice (56). Interestingly, our data pointed out that ZIKV particles are not captured by NETs in a context of the DNA web induced by a secondary stimulus. Zanluqui *et al.* preprint manuscript (57), also addresses the role played by neutrophils during ZIKV infection. Corroborating our finds, the authors shown the lack of ZIKV interference in human neutrophil viability and NETs release, as well as mice neutrophils did not display a pro-inflammatory profile and ROS production against the virus (57). A viral escape from innate immune components could result in delayed immune responses favoring virus spread. Furthermore, we cannot rule out a possible suppression of neutrophil action by ZIKV, as already shown for primary monocytes, mdDCs and plasmacytoid DCs that have their maturation and

activation impaired during ZIKV replication (14,16,17,58,59). Moreover, a cohort of rhesus monkeys produced minimal systemic cytokine response to ZIKV infection (9).

Furthermore, several reports have indicated that neutrophils migrate to different tissues during ZIKV infection in IFN receptor-deficient mouse, such CNS, spleen, spinal cord, epididymis, and testis (19,20,24,60,61), and in humans and non-human primates mucosa, placenta, and fetus (11,62,63). Patients during acute or recovery disease phase, THP-1 cells and monocytes secrete increased amounts IL-8/CXCL8 following ZIKV infection (12,13,16,64). Moreover, myeloid cells in AG129 mice challenged with ZIKV were responsible for the production of cytokines involved in leukocyte recruitment and viral dissemination to peripheral organs (23). Despite these reports, in our settings, 48 hours of ZIKV infection of mdDCs and PBMCs did not induce sufficient levels of IL-8 to promote neutrophil migration *in vitro*. Even in ZIKV infected A549 cells, that endure high levels of viral replication and can generate a more complex environment, the neutrophil migration is not promoted. Frumence *et al.* (65) shown the secretion of soluble IL-8 in ZIKV infected A549 cells, however at low levels, confirming that the *in vitro* assay we used presents limitations in order to promote neutrophil migration.

In an attempt to assess the putative impact of neutrophil migration to infected tissues *in vitro*, we used a co-culture system with A549 cells. Our results show a reduction in the rate of ZIKV infection of A549 cells when neutrophils are present at the moment of the infection or in a pre-established infection. This infection impairment seems not to be related to neutrophil activation, but due to a physical interaction between surface molecules in both cells. It has been shown that cell-to-cell contact between neutrophils and A549 cells leads to a pro-proliferative effect on these cells involving the release of elastase and COX-2 products by neutrophils (66). An increase in the production of IL-6 and IL-8 by A549 cells (67), and induction of A549 cell death by apoptotic neutrophils by soluble Fas ligand (68) have also been reported. However, it was not the case in our model, where neutrophil presence did not affect A549 cell viability. The loss of CD62L in neutrophils after treatment with trypsin, a condition in which the frequency of ZIKV infection were restored in A549 cells, might bear a connection to the pathways involved in the neutrophil modulation of ZIKV infection in A549 cells. Herbert *et al.* (69) have reported that the β 2- integrin ligand LFA-1 on neutrophils binds to the ICAM-1 receptor on epithelial cells and mediate, at least in part, epithelial damage, neutrophil degranulation and reduction of *Respiratory syncytial virus* (RSV) load. It remains to be defined what molecules and pathways might be involved during these cell-cell interactions that potentially

contribute to the reduction of viral infection and proliferation and that could be explored in the future as targets against flavivirus infection. Secondary action of neutrophils in the site of infection is linked to the mosquito saliva components. During *Semliki Forest virus* infection, the mosquito bite induced a neutrophil influx at the site of the bite, and these cells helped coordinate the entry of susceptible myeloid cells that are permissive to viral infection (70).

Finally, in order to gain a better understanding on the role played by neutrophils in ZIKV clearance at the inflammation site, we depleted neutrophils from C57BL/6 mice. In this scenario, we did not observe a direct action of neutrophils in preventing ZIKV spread to the lymph nodes in the first hour after ZIKV inoculation in a setting where an inflammatory environment had been previously induced by LPS injection. Interestingly, other authors reported that besides an extensive neutrophil recruitment to the inflammation site after certain virus infections (RSV, *Herpes simplex virus type 1* and *Coxsackievirus B3*), neutrophils did not play an important role in viral replication and disease susceptibility, which in turn was exerted by monocytes and macrophages (71–74).

Immunocompetent mice, like C57BL/6 strain, readily resolve ZIKV infection and might be a limited model to answer long-term questions. However, IFN pathway deficient models, despite being valuable tools to study ZIKV pathology, could bear differences in the kinetics of neutrophil recruitment. Intracranial ZIKV infection in C57BL/6 WT or Rag1^{-/-} mice (deficient in mature T and B cells) resulted in a lethal encephalitis with infiltration of macrophages and NK cells (19). Neonatal immunocompetent mice challenged subcutaneously with ZIKV elicit CD8⁺ T cells to the CNS (20). In contrast, IFNAR^{-/-} mice (IFN type I and II receptor deficient) infected by both routes, shown an accelerated ZIKV spread to peripheral organs and to the CNS, where it elicits an inflammatory response characterized by neutrophils infiltration (19,20). In the context of infection with the pulmonary bacteria *Francisella tularensis* and *Influenza virus*, the absence of IFN-I results in higher neutrophil recruitment (75,76). It would be of considerable interest to assess the production of neutrophil chemoattractants following ZIKV infection in the CNS of humans *pos mortem*, since ZIKV antagonizes human IFN-I (77).

In conclusion, the results indicate that human neutrophils are slightly activated by direct contact with ZIKV. However, the direct interaction between ZIKV and neutrophils does not contribute to the viral replication or to the inflammatory disease associated with the virus infection. Conversely, human neutrophils are able to reduce ZIKV infection and replication on A549 cells. It is not yet clear though, what is the mechanistically role of neutrophils in this context. Finally, despite not being a

target cell for ZIKV infection, our data suggest that, *in vitro*, neutrophils play a role in shaping ZIKV infection in other target cells.

Conflict of Interest

The authors have no financial conflicts of interest.

Author Contributions

JBA and PFW conceptualized, designed, and performed the experiments and data analysis. BNP and ALMP contributed to the interpretation and discussion of the data. CNDS contributed with her expertise in virology. All authors prepared the manuscript.

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Figure Captions

Figure 1. ZIKV do not replicate in human neutrophils. **(A)** Neutrophils morphological features after isolation shown by flow cytometry and microscopy (bright-field, bar = 25 μ m, magnification = 100x.). **(B)** The cells were stimulated *in vitro* during 2 hours with ZIKV BR 2015/15261, ZIKV PE243, ZIKV MR766 (1 MOI), or mock (C6/36 cells conditioned media in equivalent volume of ZIKV strains). Then, neutrophils were washed to remove the stimuli (indicated by ▼), and evaluated right after that (2 hours), and at 6, 12 and 24 hours after stimulation. When indicated, neutrophils were treated with trypsin after the wash step at 2 hours of stimulation with ZIKV strains. **(C)** Immunostaining of neutrophils with 4G2 (E protein; green) at 24 hours after ZIKV PE243 (a-d) or mock (e-h) stimulation. Nuclei (blue) were stained with Vybrant DyeCycleViolet. Merge of these stainings and the bright-field colocalization are also shown. C6/36 cells (i-p) were used as an infection positive control (bar = 50 μ m, magnification = 40x.) **(D)** Histograms showing 4G2 intensity of fluorescence in neutrophils and C6/36 cells at 24 hours after mock and ZIKV PE243 stimulation as measured through flow cytometry. Infected-unstained cells were used as a negative fluorescence control. **(E)** Histograms showing AXL receptor intensity of fluorescence in neutrophils and A549 cells. An isotype control antibody was used as a negative fluorescence control. One representative result of three independent experiments is shown. **(F)** RNA levels of ZIKV strains in neutrophils at 2, 6, 12 and 24 hours after stimulation. **(G)** ZIKV loads on neutrophil culture supernatant at the same time after stimulation with ZIKV strains. The dashed line named input represents the mean of the titers of the three ZIKV strains still detected on the supernatant after the stimuli removal after 2 hours of stimulation. **(H)** RNA levels of ZIKV PE243 detected in neutrophils treated or not with trypsin after 2 hours of stimulation. Bars indicate standard error of the mean (SEM). Three-four independent experiments are shown (n = 9-15). The asterisk (*) denotes statistical difference between 6 and 12 hours of the ZIKV PE243 stimulation (F).

Figure 2. ZIKV mildly regulates CD62L expression in human neutrophils, but does not stimulate cytokines, elastase, and reactive oxygen species production by these cells. **(A)** Contour plots depicting the frequency of neutrophils CD16⁺CD11b⁺ and CD16⁺CD62L⁺ and the fluorescence intensity of CD11b and CD62L molecules inside these populations at 6 hours after stimulation with mock, LPS (100 ng/mL) or ZIKV PE243 (1 MOI). This time point and strain were chosen as a representative of these results. Isotype control antibodies were used as a negative fluorescence control to set the gates. A representative result of chloromethyl-H₂DCFDA (CM-H₂DCFDA)

fluorescence in the total neutrophil population at 6 hours after stimulation with mock, PMA (16 nM) or ZIKV PE243 is also shown. **(B)** Frequency of neutrophils CD16⁺CD11b⁺ and the mean fluorescence intensity (MFI) of CD11b in that population at 2, 6 and 12 hours after stimulation with mock, LPS or ZIKV strains (1 MOI). The dashed line represents the measurements right after neutrophil obtention from blood (0 hours). **(C)** Same analysis as in **(B)** applied to the CD62L molecule. **(D)** IL-8 levels in neutrophil culture supernatant at 6 and 12 hours after stimulation. **(E)** Elastase levels in neutrophil culture supernatant at 6 hours after stimulation. **(F)** Frequency of neutrophils CM-H₂DCFDA⁺ at 2 and 6 hours after stimulation. Bars indicate SEM. Two-three independent experiments are shown (n = 6-12). The asterisk (*) denotes statistical difference between mock and LPS (**B-E**) or PMA (**F**), and the number sign (#) between mock and all the three ZIKV strains in that time point (**B-C**).

Figure 3. ZIKV do not induce NETs, and are not captured by the DNA trap. **(A)** Immunostaining of neutrophils DNA (Vybrant DyeCycleViolet, blue) and acetyl-histone H3 (green) after 5 hours of stimulation with mock (a-d), PMA (160 nM) (e-h) or ZIKV PE243 (1 MOI) (i-l). ZIKV PE243 was chosen as representative of the ZIKV strains' effects. Merge of these stainings and the bright-field colocalization are also shown (bar = 50 µm, magnification = 60x). One representative of three independent experiments is shown. **(B)** Free double stranded DNA (dsDNA) on neutrophil culture supernatant after 5 hours of stimulation with mock, PMA or ZIKV strains (1 MOI). **(C)** ZIKV strains loads on neutrophil culture supernatant after 1 hour in the presence or absence of NETs induced by PMA stimulation during 5 hours. Bars indicate SEM. Two-three independent experiments are shown (n = 6-9). The asterisk (*) denotes statistical difference between mock and PMA (**B**).

Figure 4. Human neutrophil does not impair ZIKV infectivity. Frequency of 4G2⁺ A549 cells at 36 hours after infection with ZIKV strains (1 MOI) previously incubated 6 hours in the absence (ZIKV) or presence (ZIKV + NØ) of neutrophils. ZIKV PE243 is shown as a representative of the ZIKV strains' effects on the flow cytometry plots. The mock condition was used as a negative fluorescence control. Bars indicate SEM. Three independent experiments are shown (n = 9). (▼) indicates the moment A549 cells were washed to remove the virus. NØ = neutrophils.

Figure 5. ZIKV infection in human primary cells does not induce high levels IL-8 to promote migration of human neutrophils. **(A)** IL-8 levels in mdDCs and PBMCs culture supernatant at 24 and 48 hours after stimulation with mock, LPS (100 ng/mL), or ZIKV strains (1 MOI). **(B)** Chemotactic index of neutrophils after 2 hours of stimulation with 1,000 or 50,000 pg of rhIL-8, or the supernatant

of A549 cells culture pre-infected with ZIKV PE243 for 48 hours. Bars indicate SEM. Two-three independent experiments are shown (n = 6-9). The asterisk (*) denotes statistical difference between mock and LPS, and the number sign (#) between mock and all the three ZIKV strains in the time point.

Figure 6. Human neutrophils reduce ZIKV infection in A549 cells by contact. Flow cytometry plots depicting the frequency of 4G2⁺ A549 cells at 18 hours post-infection with ZIKV PE243 (1 MOI). Cells were infected for 2 hours in the absence of neutrophils (ZIKV), presence of neutrophils (ZIKV + NØ), or presence of trypsin pre-treated neutrophils (ZIKV + NØ-trypsin). ZIKV PE243 is shown as a representative of the ZIKV strains' effects. Mock condition was used as a negative fluorescence control. **(B)** Frequency of 4G2⁺ A549 cells at 18 hours post-infection with ZIKV strains (1 MOI) in the absence or presence of neutrophils. **(C)** Frequency of viable A549 cells (annexin V⁻7-AAD⁻) after 18 hours of infection with ZIKV PE243 (1 MOI) in the absence or presence to neutrophils during the infection. **(D)** Frequency of neutrophils CD16⁺CD11b⁺ and the mean fluorescence intensity (MFI) of CD11b in that population after the 2 hours of interaction with A459 cells stimulated with mock or PE243. **(E)** Same analysis as in (D) applied to the CD62L molecule. **(F)** Frequency of 4G2⁺ A549 cells at 18 hours post-infection with ZIKV PE243 in presence of trypsin pre-treated neutrophils. **(G)** ZIKV PE243 loads on A549 cells culture supernatant at 18 hours post-infection in presence of trypsin pre-treated neutrophils. Bars indicate SEM. Three-four independent experiments are shown (n = 7-15). The asterisk (*) denotes statistical difference between the absence and presence of neutrophils (B, F-G) or neutrophils pre-treated with trypsin (F) in the conditions. (▼) indicates the moment A549 cells were washed to remove the stimuli. NØ = neutrophils.

Figure 7. Trypsin treatment affects the expression of CD62L in neutrophils. **(A)** Frequency of viable neutrophils (annexin V⁻7AAD⁻) after treatment with trypsin. **(B)** Contour plots depicting the frequency of neutrophils CD16⁺CD11b⁺ and CD16⁺CD62L⁺ and the fluorescence intensity of CD11b and CD62L molecules inside these populations right after isolation from blood (NØ) or in trypsin pre-treated neutrophils (NØ-trypsin). Isotype control antibodies were used as a negative fluorescence control to set the gates. **(C)** Frequency of neutrophils CD16⁺CD11b⁺ and the mean fluorescence intensity (MFI) of CD11b in that population after treatment with trypsin. **(D)** Same analysis as in (C) applied to the CD62L molecule. Bars indicate SEM. Two independent experiments are shown (n = 6-7). The asterisk (*) denotes statistical difference between neutrophils treated or not with trypsin (D). NØ = neutrophils.

Figure 8. Mice neutrophil depletion does not restrict ZIKV relocation to draining lymph nodes. **(A)** ZIKV loads detected in the pool of both ipsi- and contralateral popliteal (pLN), sciatic (sLN) or lumbar aortic (laLN) lymph nodes per C57BL/6 mice after 10 minutes, 1 and 3 hours of ZIKV PE243 (5×10^5 FFU) injection in the footpad. **(B)** Flow cytometry plots depicting the frequency of PMN leukocytes CD11b⁺Ly6C/G⁺ in the total blood of mice 18 hours post-treatment with PBS or anti-Ly6G antibody (400 μ g). **(C)** Frequency of CD11b⁺Ly6C/G⁺ cells in the PMN population in mice treated with PBS, anti-Ly6G, or an isotype antibody for 18 hours. **(D)** After neutrophil depletion, mice received a footpad subcutaneously injection of PBS or LPS (1000 ng) and 3 hours later, ZIKV PE243 (5×10^5 FFU). One hour later, ipsi- and contralateral pLNs from each mouse were harvested and pooled to determine ZIKV loads. Bars indicate SEM. Three animals per group were used in each experiment. One of three independent experiments is shown. The asterisk (*) denotes statistical difference between PBS and anti-Ly6G groups.

Supplementary Figure 1. ZIKV does not affect human neutrophil viability. **(A)** Contour plots depicting the frequency of annexin V⁺ and 7-AAD⁺ in the neutrophils gated population at 6 hours of stimulation with mock, LPS (100 ng/mL), or ZIKV PE243 (1 MOI), as a representative of the results. Mock-unstained condition was used as a negative control of fluorescence to set the gates. **(B)** Frequency of annexin V⁺ and annexin V⁺7-AAD⁺ neutrophils at 2, 6, 12 and 24 hours of stimulation with mock, LPS, or ZIKV strains (1 MOI). The dashed line represents annexin V⁺ and annexin V⁺7-AAD⁺ frequency right after neutrophil purification from blood (0 hours). Bars indicate SEM. Three independent experiments are shown (n = 11). The asterisk (*) denotes statistical difference between mock and LPS and the number sign (#) between mock and all the three ZIKV strains in that time point.

Supplementary Figure 2. Human neutrophils reduce ZIKV infection in A549 cells previously infected with ZIKV. Frequency of 4G2⁺ A549 cells at 40 hours post-infection with ZIKV strains (1 MOI) when neutrophils were added or not to the previously infected A459 cells at 24 hours post-infection and left in contact with the culture for additional 16 hours. Bars indicate SEM. Three-four independent experiments are shown (n = 7-15). The asterisk (*) denotes statistical difference between the conditions in which neutrophils were present or absent. NØ = neutrophils.

FIGURE 1

FIGURE 2

5h of stimulation

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8