DONOR INFECTIOUS DISEASE TESTING

Zika virus infection studies with CD34+ hematopoietic and megakaryocyte-erythroid progenitors, red blood cells and platelets

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BACKGROUND: To date, several cases of transfusion-transmitted ZIKV infections have been confirmed. Multiple studies detected prolonged occurrence of ZIKV viral RNA in whole blood as compared to plasma samples indicating potential ZIKV interaction with hematopoietic cells. Also, infection of cells from the granulocyte/macrophage lineage has been demonstrated. Patients may develop severe thrombocytopenia, microcytic anemia, and a fatal course of disease occurred in a patient with sickle cell anemia suggesting additional interference of ZIKV with erythroid and megakaryocytic cells. Therefore, we analyzed whether ZIKV propagates in or compartmentalizes with hematopoietic progenitor, erythroid, and megakaryocytic cells.

METHODS: ZIKV RNA replication, protein translation and infectious particle formation in hematopoietic cell lines as well as primary CD34+ HSPCs and ex vivo differentiated erythroid and megakaryocytic cells was monitored using qRT-PCR, FACS, immunofluorescence analysis and infectivity assays. Distribution of ZIKV RNA and infectious particles in spiked red blood cell (RBC) units or platelet concentrates (PCs) was evaluated.

RESULTS: While subsets of K562 and KU812Ep6EPO cells supported ZIKV propagation, primary CD34+ HSPCs, MEP cells, RBCs, and platelets were non-permissive for ZIKV infection. In spiking studies, ZIKV RNA was detectable for 7 days in all fractions of RBC units and PCs, however, ZIKV infectious particles were not associated with erythrocytes or platelets.

CONCLUSION: Viral particles from plasma or contaminating leukocytes, rather than purified CD34+ HSPCs or the cellular component of RBC units or PCs, present the greatest risk for transfusion-transmitted ZIKV infections.

Zika virus (ZIKV), a member of the Flaviviridae, was first isolated 1947 in Uganda and is transmitted by mosquitos of the Aedes species. ZIKV caused several large outbreaks on the Island of Yap (Micronesia) in 2007, French Polynesia in 2013-14 and 2015 in Brazil before it spread through the Americas and the Caribbean. To date, international travel, urbanization, and global warming have allowed for world-wide spread of this arthropod-borne pathogen leading to circulation in 86 countries and territories. The majority of infected people remain asymptomatic or develop mild symptoms like fever, conjunctivitis, myalgia, and rash. However, more severe complications such as Guillain-Barré syndrome in adults and microcephaly and other congenital malformations in the developing fetus and newborns have been associated with ZIKV infection and led to the declaration of ZIKV as a Public Health Emergency of International Concern by the World Health Organization (WHO) in 2016.
Its occasionally severe pathogenicity in combination with the detection of asymptomatic but viremic blood donors in French Polynesia, Martinique, Puerto Rico and the US\textsuperscript{16–22} and three probable cases of ZIKV transfusion-transmission in Brazil\textsuperscript{23,24} have made ZIKV a concern for blood safety. Guidelines and countermeasures have been published by the European Centre for Disease Prevention and Control (ECDC), the WHO, and the American Food and Drug Administration (FDA) to reduce the blood safety risk originating from ZIKV.\textsuperscript{25–27} Yet, the association of infectious virus particles with various cellular blood components such as hematopoietic stem cells, erythrocytes, and platelets, or a potential growth in these cells is still unknown. Several studies indicated clearance of ZIKV viral RNA (vRNA) from plasma within 28 days.\textsuperscript{28,29} However, vRNA was found apparent in red blood cell (RBC) samples for longer than 28 days\textsuperscript{28} and was detected in whole blood for up to 81 days after the onset of infection symptoms.\textsuperscript{28–31} Along these lines, prolonged detection in whole blood samples was recently demonstrated in a study using a Rhesus Macaque animal model.\textsuperscript{32} However, since infectivity of these RNA-positive samples was not tested, the distribution of infectious ZIKV in blood components needs further investigation.\textsuperscript{31–33}

Patient data showed a drastic drop in platelet count (thrombocytopenia), microcytic anemia\textsuperscript{34,35} and a fatal outcome for a patient with sickle cell anemia.\textsuperscript{36} Altogether, these observations raised the question whether ZIKV infects hematopoietic cells, in particular cells of the erythroid and megakaryocytic lineage.

During hematopoiesis, multipotent human hematopoietic stem and progenitor cells (HSPCs), characterized by the surface expression of cluster of differentiation 34 (CD34), are able to differentiate into several lineages including common myeloid (CMP) and lymphoid (CLP) progenitors (Fig. S2A, available as supporting information in the online version of this paper).\textsuperscript{37} Myeloid progenitor cells subsequently give rise to megakaryocyte/erythroid (MEP) or granulocyte/macrophage progenitor (GMP) cells. Differentiation of erythroid and megakaryocytic cells (namely erythrocytes, megakaryocytes, and megakaryocyte-derived platelets) upon MEP lineage decision is mainly driven by erythropoietin (EPO) and thrombopoietin (TPO).\textsuperscript{38,39} Whereas HSPCs lose their distinct CD34 marker as differentiation proceeds,\textsuperscript{40} erythroid and megakaryocytic differentiation is characterized by the appearance of the surface markers CD71/Glycophorin A (GLYA) or CD41/CD42b (Fig. S3A, available as supporting information in the online version of this paper) on erythrocytes and megakaryocytes/platelets, respectively.\textsuperscript{41–43}

ZIKV was reported to infect several white blood cells (leukocytes) namely monocytes, macrophages, and dendritic cells.\textsuperscript{44–47} However, so far nothing is known about the infection of CD34+ HSPCs or cells of the MEP lineage by ZIKV.

In this study we investigate whether CD34+ HSPCs and cells of the MEP lineage present a risk for ZIKV transmission either through production of infectious ZIKV progeny or by transmitting attached infectious virus particles.

### MATERIAL AND METHODS

#### Cell lines

African green monkey (Vero) E6 cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM). Chronic myeloid lymphoma cells (K562\textsuperscript{48}), human erythroleukemia cells (HEL\textsuperscript{49}), and megakaryoblast cells (Meg01\textsuperscript{50}) were propagated in RPMI 1640 medium. Both media (Biowest) were supplemented with 2 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin (Thermo Fisher Scientific) and 5% fetal bovine serum (FBS, Biowest). Erythroid KU812E6p6 cells (Fujirebio Inc.\textsuperscript{51,52}) were cultivated in RPMI additionally supplemented with 5 μg/mL erythropoietin (NeoRecormon). All cells were cultured at 37°C and 5% CO\textsubscript{2}.

#### CD34+ HSPC isolation and differentiation

Pseudonymized samples of granulocyte colony-stimulating factor mobilized peripheral or bone marrow human primary CD34+ cells from consenting healthy donors were used, with approval of the ethics committee (permit #329-10). CD34+ cells were purified by immunomagnetic cell sorting (CD34 MicroBead kit; Miltenyi Biotech). Isolated CD34+ cells were expanded for 4 days in SFEM medium (Stemcell Technologies) supplemented with 100 ng/mL human stem cell factor (hSCF), 100 ng/mL Fms-related tyrosine kinase 3 ligand (hFLT3-Ligand), 20 ng/mL interleukin-3 (IL-3) and 20 ng/mL interleukin-6 (IL-6) (Miltenyi Biotech). For erythroid differentiation, CD34+ cells were cultivated in SFEM II medium (Stemcell Technologies) supplemented with 20 ng/mL hSCF, 5 ng/mL IL-3, 2 μM dexamethasone, 0.2 μM β-estradiol (Sigma-Aldrich), and 10 U/mL EPO (Roche). Supplement for megakaryocytic differentiation contained 1 ng/mL hSCF, 7.5 ng/mL IL-6, 13.5 ng/mL IL-9, and 50 ng/mL TPO (Miltenyi Biotech).

#### Virus production and titration

An Asian ZIKV strain isolated during the recent outbreak (ZIKV isolate PF12/251013-18; KX369547) was kindly provided by Didier Musso (Institut Louis Malardé).\textsuperscript{53,54} Virus stocks were produced in Vero E6 cells using DMEM supplemented with 2% fetal bovine serum (FBS). Virus containing supernatants were harvested at Days 8 and 9, sucrose-purified (20% sucrose in TNE buffer; 12 mM Tris–HCl [pH 8.0], 120 mM NaCl, 2 mM EDTA) by ultracentrifugation for 2.5 hours at 4°C and 36,000 rpm and stored at −80°C. Infectious virus was quantified by endpoint titration determining the 50% tissue culture infection dose (TCID\textsubscript{50}) on Vero E6 cells.

#### Limiting dilution assay (TCID\textsubscript{50})

Vero E6 cells were seeded to sub-confluency in a 96-well format and six to eight replicate wells were inoculated with 50 μL of serial dilutions. For high titer samples, dilution series with a 1:10 pre-diluted samples and a dilution factor...
of 10 were performed. For low titer samples, undiluted samples were applied to the monolayer and a dilution factor of four was used for the dilution series. To avoid interference of RBC or plasma proteins, low titer samples of the spiking experiments were treated as described and the inoculum was additionally replaced after 2.5 hours with DMEM. After 7 days, the cytopathic effect (CPE) was evaluated using light microscopy and virus titers (TCID₅₀/mL) were calculated by maximum likelihood algorithm as described by Spearman und Kärber.⁵⁵,⁵⁶

ZIKV genome quantification by qRT-PCR

Total RNA was extracted from supernatant or cell pellets using the ExiPrep™ Dx Viral RNA Kit (Bioneer Corporation) on the ExiPrep™ 16 Dx platform according to the manufacturer’s instructions. Viral RNA was quantified using the LightCycler® Multiplex RNA Virus Master kit (Roche). Five µL extracted RNA were added to 15 µL reaction mix containing 4 µL 5x RT-qPCR reaction mix, 0.1 µL RT enzyme solution, 10 pmol of ZIKV NS1 forward primer (5'-CAACCATGCAAGCGGAGG-3'), and reverse primer (5'-GCCCTATCTCCATTCCACT-3'), 5 pmol ZIKV NS1 probe (5'-6FAM-ATGGTGCTGCAGGGA GTGCACAATGC-BBQ-3'), and 5 pmol ZIKV NS4B probe (5'-6FAM-ATGGTGCTGCAGGGA GTGCACAATGC-BBQ-3'). Reactions were performed on a LightCycler 480 II (Roche) as follows: 55°C for 5 minutes, 95°C for 5 minutes and 45 cycles of 95°C for 5 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. ZIKV genome copies were quantified according to a standard curve created from the 1st World Health Organization (WHO) International Standard (IS) for ZIKV RNA (11468.16⁵⁴,⁵⁷). Sensitivity of the assay was determined by end point titration of the ZIKV IS and calculated in relation to the purified sample volume. Data are expressed as international units per mL (IU/mL).

Immunofluorescence microscopy and antibodies

Cells were fixed for 15 minutes with 4% (w/v) paraformaldehyde in PBS, permeabilized with 0.05% Tween 20 in TBS (TBS-T; pH 7.4) for 5 minutes and incubated in blocking buffer containing 5% bovine serum albumin (Sigma-Aldrich) in TBS-T for 20 minutes. Immunostaining was performed by incubation with primary and subsequently secondary antibody diluted in blocking buffer: anti-Glycophorin A (Santa Cruz; 1:250), anti-von-Willebrand-Factor (Abcam; 1:100), anti-Flavivirus-Group-Antigen Antibody (4G2; Merck Millipore; 1:300), anti-NS4B (Genetex; 1:1000). Cells were mounted using Fluoromount G Reagent (Southern Biotech) and fluorescent images were acquired with a Nikon Ti Eclipse microscope.

Flow cytometry analysis

For fluorescence-associated cell sorting (FACS), intracellular NS4B staining was performed with the eBioscience™ Foxp3/ Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturers protocol using anti-NS4B (Genetex; GTX133311-25; 1:500) and goat F(ab')2 Anti-Rabbit IgG Fc FITC (Abcam; ab6018; 1:200) antibodies. Surface marker staining in FACS buffer (PBS, 5 mM EDTA pH 7, 2% FBS) with anti-CD34 PE and anti-CD71 APC (Miltenyi Biotec), anti-CD235a BV412 (BD Biosciences), anti-CD45 FITC, anti-CD41 Pacific Blue, anti-CD41 APC, anti-CD62P BV421, and anti-CD42b APC (all BioLegend) was performed for 20 minutes at 4°C. Data were acquired on a LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar).

Electron microscopy

Cells were fixed with 2.5% glutaraldehyde in PBS for 45 minutes and embedded in Epon resin.⁵⁶ Ultrathin slices were stained with 2% uranyl acetate followed by 0.2% lead citrate and examined in an electron microscope (Model EM902, Zeiss).

RESULTS

Human hematopoietic K562 and KU812Ep6EPO cells promote ZIKV propagation

Human cell culture models mimicking features of hematopoietic cells are commonly used for in vitro studies. For ZIKV infection experiments, we employed chronic myeloid lymphoma K562 cells,⁴⁸ human erythroid leukemia cells (HEL),⁵⁰ megakaryoblast Meg01 cells⁵⁰ and the Ep6 subclone of KU812 cells which is constantly cultivated with EPO (KU812Ep6EPO) for erythroid differentiation.⁵¹,⁵² Cell line characteristics were confirmed by FACS analysis of the erythroid and megakaryocytic lineage differentiation markers Glycophorin A (GLYA) and CD41, respectively (Fig. S1A, available as supporting information in the online version of this paper). Cells were infected with ZIKV at a multiplicity of infection (MOI) of 0.01 TCID₅₀ per cell and viral replication was analyzed by qRT-PCR over 7 days. There was an ~2.5 fold logarithmic increase in ZIKV genome copies in the cellular fraction and supernatant of infected K562 and KU812Ep6EPO cells indicating ZIKV replication (Fig. 1A), whereas HEL or Meg01 cells did not support ZIKV RNA amplification (Fig. S1B, available as supporting information in the online version of this paper). Viral translation was visualized by immunofluorescent (IF) staining of ZIKV non-structural protein 4B (NS4B) in ZIKV-infected K562 and KU812Ep6EPO cells at 96 hours post infection (Fig. 1B). Monitoring ZIKV NS4B on single cell level revealed that only a minor subpopulation of K562 and KU812Ep6EPO cells permitted ZIKV protein synthesis. Data were confirmed by staining against the ZIKV structural envelope protein (ENV) (Fig. S1C, available as supporting information in the online version of this paper). Remarkably, the maximum of NS4B-positive K562 and KU812Ep6EPO cells identified by IF analysis was reached at an MOI of 5 TCID₅₀ per cell and could not be augmented by infection at higher MOIs (data not shown). As indicated by the IF analysis, FACS analysis revealed that only small subsets with about 27% and 8% cells
Fig. 1. K562 and KU812Ep6EPO cells support ZIKV replication, translation, and particle formation. (A) K562 and KU812Ep6EPO were infected with ZIKV (MOI 0.01; \( \sim 5 \times 10^8 \) IU/mL) for the indicated time period. Total RNA was extracted from cellular fraction (cells) and supernatant and quantified by qRT-PCR. Shown are mean absolute genome copies per mL (IU/mL) ± SD of one exemplary experiment (n = 3). Dotted line indicates limit of detection (LOD). (B) K562 and KU812Ep6EPO were infected with ZIKV (MOI 1), fixed at 96 hours p.i. and immunostained using ZIKV NS4B antibody (green). Nuclei were visualized by DAPI staining (blue) (n = 4). Scale bars, 25 μm (C) Naïve and ZIKV-infected (MOI 5) K562 and KU812Ep6EPO were stained with ZIKV NS4B antibody and analyzed by FACS. Living cells were gated and expression of NS4B was plotted against cell size (forward scatter; FSC). Percentage of live, NS4B-positive cells comparing naïve and infected cells are shown (box area) (n = 3). (D) Infectious particle production of ZIKV-infected (MOI 0.01; \( \sim 5 \times 10^3 \) TCID50/mL) K562 and KU812Ep6EPO cells was analyzed by TCID50 assay over a time course of infection. Shown are mean TCID50 values per mL ± SD of one exemplary experiment (n = 3). Dotted line indicates LOD. (E) Electron microscopy analysis of ZIKV-infected (MOI 5, 96 h p.i.) KU812Ep6EPO cells. Magnification 20000x and 30000x; Scale bars 500 and 200 nm.
were found NS4B-positive when evaluating ZIKV-infected K562 and KU812EpEPO cells, respectively (Fig. 1C). Since the amount of detected vRNA or protein does not provide information about the formation of infectious viral particles, TCID_{50} analysis of cellular fraction and supernatant of ZIKV-infected K562 and KU812EpEPO cells over a 7-day period was performed. ZIKV particle production was demonstrated for both cell lines by increasing TCID_{50}/mL values reaching a plateau after 3 days post infection (Fig. 1D). HEL and Meg01 cells did not support infectious particle production (Fig. S1D, available as supporting information in the online version of this paper). By using electron microscopy, ZIKV particles were visualized in the cytoplasm of infected KU812Ep6EPO cells organized in a disorderly fashion (Fig. 1E).

In summary, our experiments demonstrate ZIKV permissiveness of myeloid-like K562 and erythroid-like KU812Ep6EPO cells.

**ZIKV replication and particle formation in KU812Ep6 cells is independent of erythropoietin**

Given that only a minor subset of the KU812Ep6EPO cells was permissive for ZIKV infection, we examined if EPO-driven, erythroid differentiation of the Ep6 subclone contributes to viral infection. KU812Ep6 cells were cultured without EPO for 4 weeks and GLYA expression (as an indicator for erythroid differentiation) was monitored by FACS determining the percentage of GLYA-positive versus GLYA-negative cells in the whole population. Even though EPO-deprivation resulted in a significant reduction of GLYA-positive KU812Ep6 cells by about 25% (Fig. 2A and B), ZIKV infection kinetics revealed no difference in viral replication or particle production comparing EPO-treated versus untreated KU812Ep6 cells (Fig. 2C and D). Further, we aimed to determine whether subsets of the KU812Ep6EPO cells with either high or low GLYA expression promote ZIKV propagation. ZIKV-infected KU812Ep6EPO cells were evaluated for GLYA and ZIKV NS4B by IF analysis (Fig. 2E). However, both high and low GLYA expressing KU812Ep6EPO cells were found positive for NS4B indicating no preferential ZIKV propagation in either type of cells.

Thus, ZIKV propagation in the KU812Ep6 subclone appears to be independent of EPO-driven, erythroid differentiation indicated by GLYA expression.

**CD34+ HSPCs are non-permissive for ZIKV**

We complemented our analysis by conducting infection studies in primary CD34+ HSPCs (Fig. S3A, available as supporting information in the online version of this paper). To this end, hematopoietic CD34+/CD45+ HSPCs were enriched from mobilized healthy donor apheresis samples by magnetic-associated cell sorting (MACS) with preparations generally achieving a purity of more than 96% as determined by FACS analysis (Fig. S2B, available as supporting information in the online version of this paper). Cells were subsequently cultivated in expansion medium for 4 days and infected with sucrose-purified ZIKV (Fig. 3A). TCID_{50} analysis of the inoculum verified application of infectious virus (Fig. 3C, red cross), vRNA levels remained stable in all fractions over the 7-day period and low amounts of ZIKV infectious particles were detected at Days 0 and 2 in the supernatant only (Fig. 3B and C). However, no significant increase of either ZIKV RNA (Fig. 3B) or infectious particles (Fig. 3C) was observed indicating the absence of ZIKV propagation. The results were confirmed by IF analysis showing no signal for ZIKV NS4B or ENV protein in CD34+ HSPCs after inoculation with ZIKV (data not shown).

Thus, the data indicate that primary CD34+ HSPCs do not support ZIKV propagation.

**Cells of the erythroid and megakaryocytic lineage do not support ZIKV infection**

To explore if cells of erythroid and megakaryocytic origin interact with ZIKV, CD34+ HSPCs were differentiated in the respective lineages by treatment with specific differentiation medium for 12 days (Fig. S3A, available as supporting information in the online version of this paper). Erythropoiesis was confirmed on the morphological level using May-Grünwald/Giema staining (Fig. S3B, available as supporting information in the online version of this paper), by IF staining of GLYA (Fig. S3C, available as supporting information in the online version of this paper) and FACS analysis of CD71/GLYA-positive cells (Fig. 4A). Differentiation of cells from three donors resulted in 30-50% CD71/GLYA double-positive cells indicating sufficient erythroid differentiation (Fig. 4B). Infection with the human parvovirus B19, which is known to infect erythroid progenitor cells, was employed to confirm adequate erythropoiesis. Digital droplet PCR (ddPCR) showed synthesis of the spliced version of the parvovirus B19 capsid protein (VP)—being a surrogate for productive B19 replication—in erythroid differentiated but not in CD34+ HSPC progenitor cells (Fig. S3D, available as supporting information in the online version of this paper). However, ZIKV infection of erythroid differentiated cells neither resulted in replication (Fig. 4C) nor infectious particle production (Fig. 4D) as indicated by unaltered levels of ZIKV RNA and decreasing levels of infectious units in the cellular fraction and supernatant, respectively.

Megaerythroidic differentiation was demonstrated by microscopy of May-Grünwald/Giema-staining showing cells of >50 μm in diameter with multiple nuclei (Fig. S3E, available as supporting information in the online version of this paper). IF staining for von Willebrand factor (vWF) indicated the typical, speckled distribution in the cytoplasm being phenotypic for polyploid, megaerythrocytic cells (Fig. S3F, available as supporting information in the online version of this paper). Polyplody was additionally demonstrated by propidium iodide (PI) incorporation (Fig. S3G and H, available as supporting information in the online version of this paper). Productive megakaryopoiesis was further confirmed...
by FACS analysis determining the number of CD41/CD42b double-positive cells to 23-78% depending on the donor (Fig. 5A and B). Similar to the erythroid cells, infection of the megakaryocytic cells with ZIKV did not result in ZIKV propagation as monitored in cellular fraction and supernatant on the level of vRNA (Fig. 5C) and infectious particle production (Fig. 5D).

Since production of platelets from megakaryocytes in cell culture is challenging, we directly isolated platelets from whole blood of healthy donors for infection studies with ZIKV. As indicated by FACS, platelet preparations generally showed a purity of 98% and activation of CD41-positive platelets was kept down to <30% as indicated by CD62P expression (Fig. S4A, available as supporting information in the online version of this paper). However, in line with the results for megakaryocytes, ZIKV infection did not result in an increase of ZIKV RNA (Fig. S4B, available as supporting information in the online version of this paper).
Fig. 3. CD34+ hematopoietic progenitor cells are non-permissive for ZIKV. (A) Schematic workflow of ZIKV infection studies with CD34+ progenitor cells. CD34+ HSPCs were infected with ZIKV (MOI 2) for 2.5 hours. Inoculum was removed and cells were washed twice with PBS. Cells were resuspended in medium and seeded. Samples of cellular fraction and supernatant were taken directly after seeding (D0) and over a time course of infection at the indicated days (D2, D5, D7). Cellular fraction was washed once with PBS before analysis. (B and C) CD34+ cells were infected with ZIKV (MOI 5). Cellular fraction and supernatant were analyzed at the indicated time points. (B) Total RNA was extracted and quantified by qRT-PCR. Shown are mean absolute genome copies per mL (IU/mL) ± SD for each donor (n = 5). (C) TCID50 assay was performed. Shown are mean TCID50 values per mL ± SD for each donor (n = 5). Dotted lines indicate LOD.

Fig. 4. Erythroid cells do not support ZIKV propagation. (A and B) After 12 days of erythroid differentiation, expression of the erythroid markers CD71 and GLYA was determined by FACS. Living cells were gated and expression of CD71 against GLYA was plotted; one exemplary experiment is shown (n = 3). (B) Percentages of live, CD71/GLYA-double positive cells are demonstrated in the bar graph for all tested donors (n = 3). (C and D) Erythroid cells were infected with ZIKV (MOI 5). Cellular fraction and supernatant were analyzed over a time course of infection at the indicated time points. (C) Total RNA was extracted and quantified by qRT-PCR. Shown are mean absolute genome copies per mL (IU/mL) ± SD for each donor (n = 3). (D) TCID50 assay was performed. Shown are mean TCID50 values per mL ± SD for each donor (n = 3). Dotted lines indicate LOD.
paper) or infectious particles (Fig. S4C, available as supporting information in the online version of this paper) over a 7-day period indicating that platelets also do not support ZIKV propagation.

All data were confirmed by IF analysis of ZIKV NS4B and ENV protein showing no occurrence of either protein (data not shown) in all tested primary cells. Comparable to the CD34+ HSPCs, vRNA remained stable after infection in all fractions (Fig. 4C, 5C, and S4B, available as supporting information in the online version of this paper) and low amounts of infectious particles were detected in the supernatant at Day 0 only (Fig. 4D and 5D, and S4C, available as supporting information in the online version of this paper).

Thus, the data reveal that erythroid or megakaryocytic lineage decision does not render CD34+ HSPCs permissive for ZIKV infection.

**ZIKV infectious particles do not compartmentalize with erythrocytes or platelets in leukocyte-reduced (LR) packed RBC units and platelet concentrates (PCs)**

To investigate association of ZIKV RNA and infectious particles with erythroid and megakaryocytic cells, leukocyte-reduced packed RBC units (LR-pRBCs) or PCs were spiked with ZIKV, respectively. According to the prescribed storage conditions, three LR-pRBC units from three independent donors were stored at 4°C and two PCs each containing platelets pooled from four whole blood donations were incubated at 22°C under agitation. After incubation for the indicated time points, cells were separated from plasma and additives by centrifugation and ZIKV RNA and infectious particles content were determined in all fractions. High amounts of vRNA were detected in all fractions for 7 days (Fig. 6A and C) apparently having a higher affinity for RBCs (Fig. 6A, cells) compared to platelets (Fig. 6C, cells). Remarkably, evaluation of the supernatant fractions for infectious ZIKV demonstrated infectious particles in the plasma fraction of PCs only on day zero (Fig. 6D, supernatant) which is in contrast to the additive fraction of LR-pRBCs units where virus was still detectable after 7 days (Fig. 6B, supernatant) with titers of 100 TCID$_{50}$/mL. However, no infectious virus particles were found associated with RBCs and platelets (Fig. 6B and D; cells).

The data imply that even if vRNA remains detectable in all fractions, infectious ZIKV particles do not compartmentalize with RBCs or platelets, but appear to be stable for 7 days in the additive solution of LR-pRBC units when stored at 4°C.
This study is, to our knowledge, the first to investigate the infection of in vitro cultured hematopoietic progenitors and cells of the erythroid and megakaryocytic lineage with ZIKV in order to evaluate the risk for ZIKV transmission via purified CD34+ HSPC products, RBC and platelet transfusion units.

Previous studies reported comparable tissue tropism for Asian and African ZIKV strains showing variances only in the extent of the subsequent antiviral and immune responses in neuronal cells.63–66 Due to its close relation to ZIKV strains currently spreading in the Pacific region, the Americas and the Caribbean and because of its association with significant clinical manifestations such as microcephaly and the Guillain-Barré Syndrome we performed our experiments with a low-passaged ZIKV isolate from a French Polynesian patient to evaluate the ZIKV tropism in the context of hematopoietic cells.

We demonstrate that ZIKV is able to conduct RNA replication, translation and infectious particle production in subsets of myeloid-like K562—which is in agreement with previous reports67,68—as well as “erythroid-differentiated” KU812Ep6EPO cells. Surprisingly and in contrast to the supposedly representative cell culture models, neither primary CD34+ HSPCs nor differentiated erythroid cells supported ZIKV propagation. However, K562 and KU812Ep6 cell populations are rather heterogeneous, have multipotent potential and can spontaneously differentiate into cells with basophilic and macrophage-like properties.69,70 Since ZIKV is known to infect cells of the GMP lineage (Fig. S2A, available as supporting information in the online version of this paper44–47), propagation in a small subset of either cell line might explain our observations. In line with this argument, EPO deprivation did not impact ZIKV propagation in the KU812Ep6 subclone suggesting that cells other than those exhibiting erythroid-like features might support infection. Nevertheless, determination of the subsets of K562 and KU812Ep6 cells which are permissive for ZIKV might help to elucidate the mechanism of ZIKV cell tropism and should be further explored. In contrast to K562 and KU812Ep6EPO cells, HEL cells, that are closely related to MEP cells, and the megakaryoblastic cell line Meg01 did not support ZIKV propagation. This suggests that cells of erythroid-megakaryocytic origin are indeed not prone to ZIKV infection which is in agreement with the results obtained using primary cells.

The lack of permissiveness of the tested primary hematopoietic cells was surprising considering that the closely
related Dengue virus (DENV) was reported to infect CD34+ HSPCs, GLYA-positive MEPs, megakaryocytes and supposedly also platelets. In addition, ZIKV as well as DENV propagate in cells of the GMP lineage. However, whereas DENV NS1 protein was found to activate platelets, no such effect was observed for ZIKV. Also, since ZIKV, but not DENV, exhibits a distinct neurotropism, we can assume that cell tropism of viruses from the same family indeed overlaps but ultimately differs between even closely related species.

Notably, preliminary experiments indicated that ZIKV infection of CD34+ HSPCs did neither inhibit cell growth nor prevented differentiation of erythroid and megakaryocytic cells (data not shown) as it is reported for other viruses such as DENV. Thus, we speculate that thrombocytopenia and anemia in ZIKV-infected patients might be explained by cell damage due to an overshooting immune response or ZIKV-induced impairment of blood vessel integrity or development rather than by a direct interaction of ZIKV with erythroid and megakaryocytic cells. However, further investigations are crucial to unravel the connection of ZIKV pathogenicity and symptoms manifestation towards the prevention and cure of ZIKV infections.

In our experiments, high amounts of vRNA were found associated with CD34+ HSPCs, erythroid and megakaryocytic cells as well as in the supernatant fractions. In contrast, only low levels of infectious particles were detected at the earliest time points and exclusively in the supernatant fraction, demonstrating low affinity of infectious viral particles for the hematopoietic cells tested. Consequently, the data suggest that ZIKV transmission via purified CD34+ is unlikely. Purified CD34+ HSPCs are often used as starting material for advanced therapy medicinal products (ATMPs). However, purified CD34+ HSPCs are rarely used for hematopoietic reconstitution, whereas unprocessed grafts, containing residual RBCs, high amounts of leukocytes and plasma, are preferentially transplanted. Thus, for unprocessed hematopoietic stem cell transplants the risk of ZIKV transmission via residual ZIKV-susceptible cells or plasma still remains.

Along the same lines, we showed in our spiking experiments of LR-pRBC units and PCs, that despite the detection of stable vRNA levels in all fractions, infectious ZIKV particles were only found in the supernatant of the tested preparations and did not compartmentalize with RBCs and platelets. This is somewhat surprising since the cellular surface of RBCs and platelets is littered with sialic-acid-modified glycans which are known to serve as an attachment receptor for many viruses on other cell types and was indicated to support ZIKV internalization. In addition, a large screening study testing the blood type distribution of over 4000 ZIKV-infected patients revealed that detected viremia is not associated with ABO, Rhesus, and Kell blood groups. Strikingly, despite the consequently low risk for ZIKV transmission due to attachment of infectious particles to RBCs and platelets, we demonstrated high stability of ZIKV particles in the supernatant of 4°C-stored LR-pRBC units and detection of only a small number of infectious particles in the plasma fraction of PCs stored at 22°C shortly after spiking which is most likely due to the temperature-sensitive properties of ZIKV particles. Considering that 250 mL of concentrate are administered with one transfusion, even small numbers of infectious particles remaining in the plasma and additive component of LR-pRBC units and PCs could indeed lead to ZIKV transmission. This is in agreement with three cases, reporting probable ZIKV transfusion-transmissions via PCs. Of note, since our data showed stability of infectious particles in the plasma fraction, the use of fresh frozen plasma as well as platelet lysates containing plasma must be considered as a potential source of ZIKV transmission. Importantly, our data also suggest that vRNA detection does not always mean the presence of infectious particles and does not necessarily imply a risk for ZIKV transmission. This is in line with a previous study, stating that a platelet transfusion received from a donor with detectable levels of ZIKV RNA in plasma and RBCs fraction did not result in infection of the recipient. Further, as implied by our analysis and suggested by a report about West Nile virus RNA in RBC samples, erythrocytes appear to be rather “sticky” for the very stable flavivirus RNA providing an explanation for prolonged ZIKV RNA detection in RBC and whole blood samples as compared to plasma—especially when considering the about 115 days erythrocyte lifespan.

In summary, we demonstrate that ZIKV neither propagates in nor compartmentalizes with CD34+ HSPCs, erythroid and megakaryocytic cells as well as RBCs and platelets. Viral particles from leukocytes or plasma, which are present in unprocessed hematopoietic stem cell transplants and blood products, present a risk for ZIKV transmission rather than purified CD34+ HSPCs or the cellular component of LR-pRBC units or PCs.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.
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TCID50 assay was performed. Shown are mean TCID50/C6 copies per mL (IU/mL) infected with ZIKV (MOI 0.01; C24 (n = 4). Scale bars, 25 μm. (A) Characterization of surfacemarkers on hematopoietic cell lines. The indicated cell lines were FACS analyzed for surface expression of GLYA (GLYA) and megakaryocytic (CD41) markers. Living cells were gated and expression of CD41 against GLYA was plotted; one exemplary experiment is shown (n = 2). (C) Meg01 and HEL were infected with ZIKV (MOI 1), fixed at 96 h p.i. and immunostained using a ZIKV envelope (ENV) and quantified at the indicated time points. (B) Total RNA was extracted and quantified by qRT-PCR. Shown are mean absolute genome copies per mL (IU/mL) ± SD for each donor (n = 2). (D) TCID\textsubscript{50} assay was performed. Shown are mean TCID\textsubscript{50} values per mL ± SD for each donor (n = 2).

**Fig. S2.** ZIKV infection studies in CD34\textsuperscript{+} HSPCs. (A) Schematic representation of human hematopoiesis. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythroid progenitor; EPO, erythropoietin; TPO, thrombopoietin; NK cells, natural killer cell. (B) FACS analysis of unpurified peripheral blood mononuclear cells (PBMCs) and MACS-purified CD34\textsuperscript{+} HSPCs. Total cells were gated and cell size (FSC) was plotted against granularity (SSC). For FACS analysis of hematopoietic markers in the MACS purified population, living CD34\textsuperscript{+} cells (circled area) were gated and expression of CD45 against CD34 was plotted; one exemplary experiment is shown (n = 5).

**Fig. S3.** Characterization of erythroid and megakaryocytic differentiated cells. (A) Schematic representation of erythroid and megakaryocytic differentiation. Characteristic surface markers are indicated in filled areas. HSC, human stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; EPO, erythropoietin; TPO, thrombopoietin. (B and C) May-Grünwald/Giemsa staining (B) and IF analysis of GLYA expression (C; red) of erythroid cells after 12 days of differentiation. Nuclei are visualized by DAPI staining (C; blue). Scale bars, 25 μm. (D) ddPCR analysis of parvovirus B19 replication in erythroid cells. Erythroid cells were infected with 10\textsuperscript{4} B19 genomes per cell. Graph shows absolute numbers of copies of spliced VP mRNA ± SD for each of two differentiated (red) and undifferentiated (gray) donors as determined by ddPCR. (E and F) May-Grünwald/Giemsa staining (E) and IF analysis of vWB factor expression (F; green) of megakaryocytic cells after 12 days of differentiation. Nuclei are visualized by DAPI staining (F; blue). Scale bars, 25 μM (E) and 50 μM (F). (G-H) Polyploidy analysis of megakaryocytic cells. (G) FACS analysis of propidium iodide (PI) incorporation profile of living megakaryocytic cells with a DNA content from 2n (diploid) to 32n (polyploid) is shown. One exemplary result is demonstrated (n = 4). (H) Bar graph showing the percentage of 2n (diploid) to 32n (polyploid) cells for all tested donors (n = 4).

**Fig. S4.** Human platelets isolated from whole blood are non-permissive for ZIKV. (A) Human platelets were isolated from platelet-rich plasma by density centrifugation. Purity of platelets preparation was tested by FACS plotting SSC against FSC. Results are shown in linear and log scale graphs. Living cells were gated (circled area) and platelet surface marker CD41 was plotted against activation marker CD62P. (B and C) Platelets were infected with ZIKV (MOI 5) for the indicated time period and cellular fraction and supernatant were analyzed. (B and C) Platelets were infected with ZIKV (MOI 5) for the indicated time period and cellular fraction and supernatant were analyzed. (B) Total RNA was extracted and quantified by qRT-PCR. Shown are mean absolute genome copies per mL (IU/mL) ± SD for each donor (n = 3). (C) End point dilution assay (TCID\textsubscript{50}) was performed. Shown are mean TCID\textsubscript{50} values per mL ± SD for each donor (n = 3). Dotted lines indicate LOD.