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Supplemental Information

Zika Virus Infects Human Placental Macrophages

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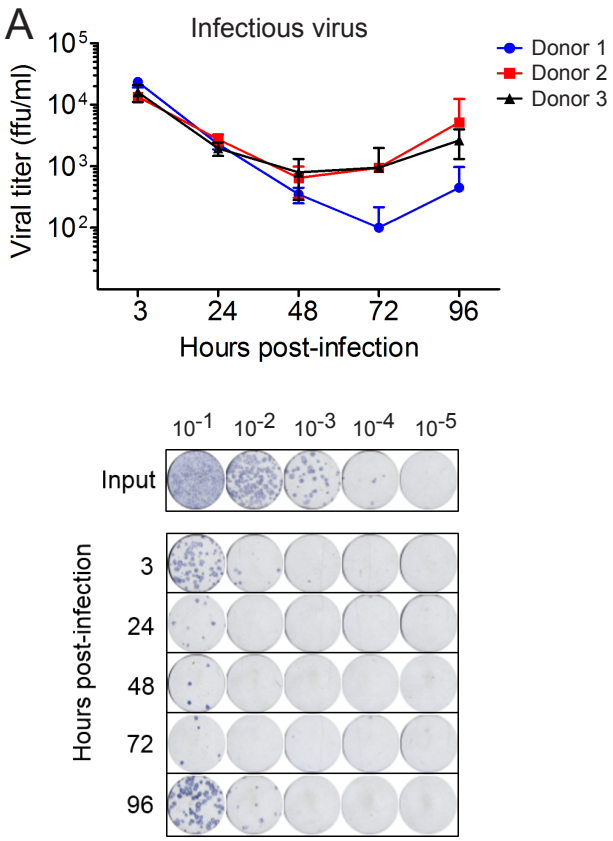
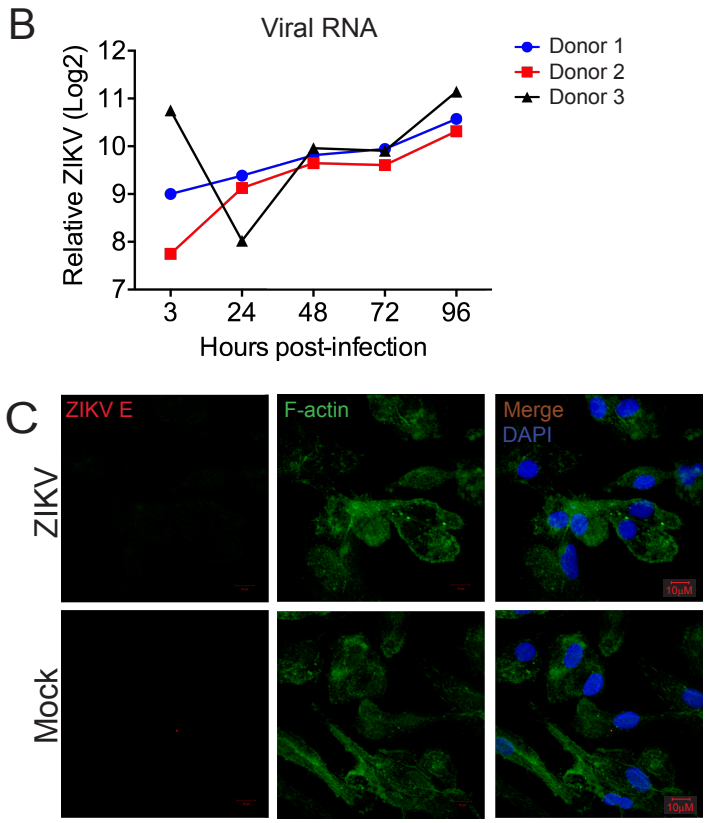
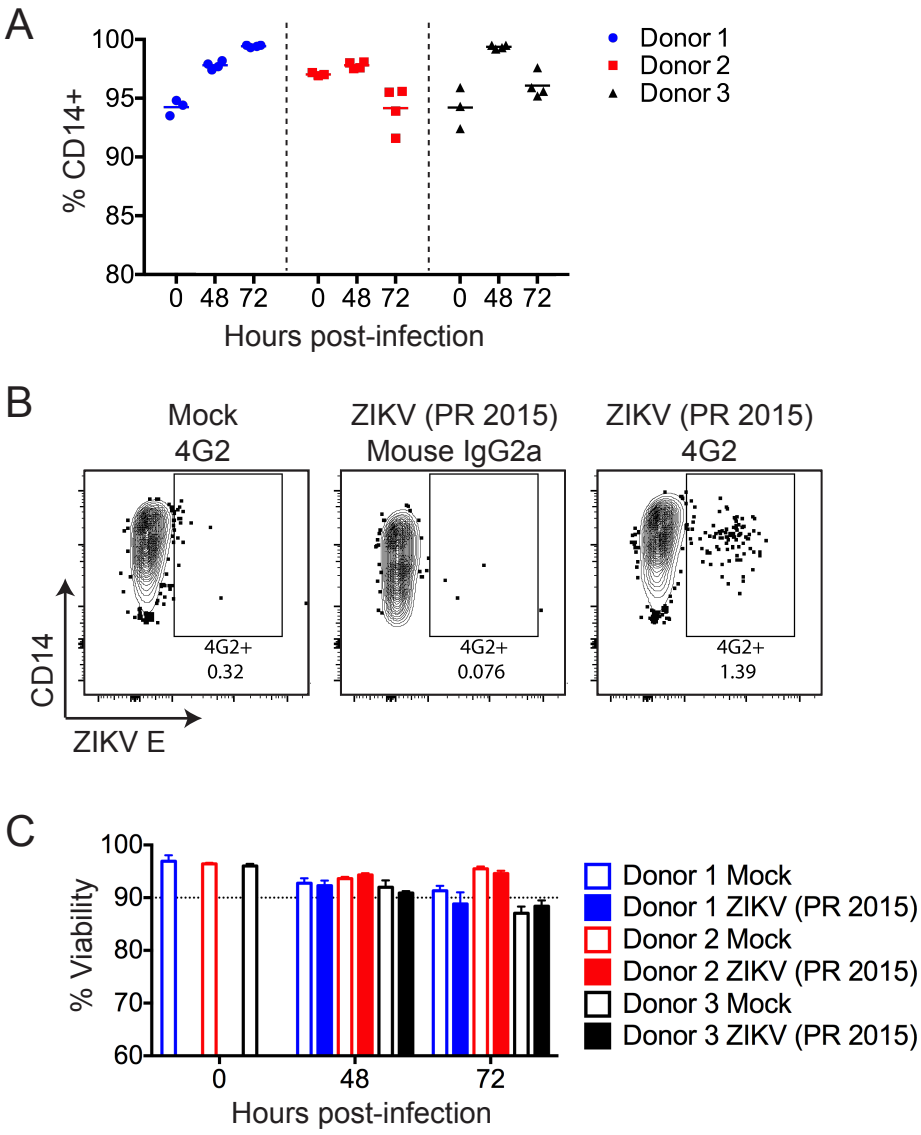

A
Infectious virus
Viral titer (ffu/ml)
10^5
10^4
10^3
10^2
3
24
48
72
96
Hours post-infection
Donor 1
Donor 2
Donor 3
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
Input
Hours post-infection
3
24
48
72
96


B
Viral RNA
Relative ZIKV (Log2)
12
11
10
9
8
7
3
24
48
72
96
Hours post-infection
Donor 1
Donor 2
Donor 3
C
ZIKV E
F-actin
Merge
DAPI
ZIKV
Mock


A
100
95
90
85
80
% CD14+
0 48 72 0 48 72 0 48 72
Hours post-infection
Donor 1
Donor 2
Donor 3
B
Mock
4G2
ZIKV (PR 2015)
Mouse IgG2a
ZIKV (PR 2015)
4G2
4G2+
0.32
4G2+
0.076
4G2+
1.39
CD14
ZIKV E
C
100
90
80
70
60
% Viability
0
48
72
Hours post-infection
Donor 1 Mock
Donor 1 ZIKV (PR 2015)
Donor 2 Mock
Donor 2 ZIKV (PR 2015)
Donor 3 Mock
Donor 3 ZIKV (PR 2015)

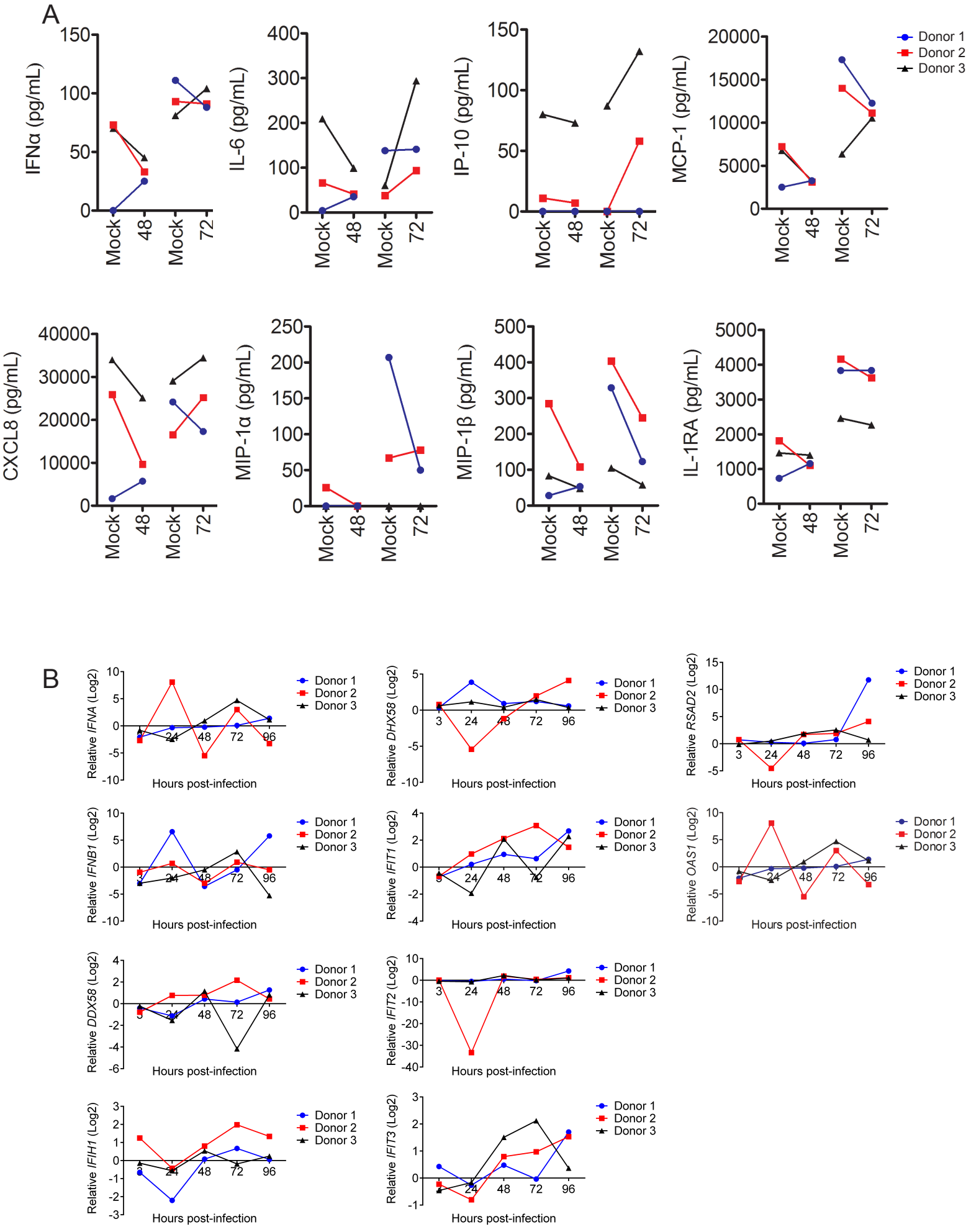

A
IFNα (pg/mL)
IL-6 (pg/mL)
IP-10 (pg/mL)
MCP-1 (pg/mL)
CXCL8 (pg/mL)
MIP-1α (pg/mL)
MIP-1β (pg/mL)
IL-1RA (pg/mL)
Mock
48
72
Donor 1
Donor 2
Donor 3
B
Relative IFNA (Log2)
Relative DHX58 (Log2)
Relative RSAD2 (Log2)
Relative IFNB1 (Log2)
Relative IFIT1 (Log2)
Relative OAS1 (Log2)
Relative DDX58 (Log2)
Relative IFIT2 (Log2)
Relative IFIH1 (Log2)
Relative IFIT3 (Log2)
Hours post-infection
3
24
48
72
96

Figure S1, related to Figure 1. Cytotrophoblasts are permissive to ZIKV infection. (A) CTBs from three donors were infected with ZIKV (PR 2015) at an MOI of 1 and viral titers in supernatants determined by FFA. Data are represented as the mean of four technical replicates +/- SD (top). Representative FFA staining (bottom). ffu, focus forming units. **(B)** Viral RNA detected by qRT-PCR in CTBs infected with ZIKV (PR 2015). Data are relative to GAPDH control and mock-infected cells ($\Delta\Delta C_T$). **(C)** Confocal microscopy of mock- and ZIKV (PR 2015)-infected CTBs at 72hpi.

Figure S2, related to Figures 1-4. Controls for HC flow cytometry analysis. (A) HCs used in these experiments were on average >95% pure by CD14 staining. Horizontal bars indicate the mean. For 0hpi, n=3; for 48 and 72hpi, n=4. **(B)** No ZIKV E protein was detected by mouse 4G2 antibody in mock-infected cells, or by mouse IgG2A isotype control in ZIKV-infected cells. **(C)** Both mock- and ZIKV-infected HCs retained ~90% or better viability over the time course as determined by Ghost Dye Red 780 staining. Data are represented as the mean +/- SD.

Figure S3, related to Figures 3 and 4. ZIKV infection of CTBs induces limited type I IFN and proinflammatory cytokine response. (**A**) CTBs isolated from three donors were infected with ZIKV (PR 2015) at an MOI of 1 or mock-infected. Cytokine levels in the supernatants were determined by multiplex bead array. All values are represented in “pg/ml” and shown with a connecting line between ZIKV-infected samples (48 and 72hpi) and their respective donor- and time-matched mock-infected samples. See also Table S2, (**B**) Antiviral gene expression determined by qRT-PCR in CTBs (three donors) infected with ZIKV (PR 2015). Gene expression data are represented as fold change relative to time-matched mock-infected controls (gene expression normalized to GAPDH - $\Delta\Delta C_T$ method).

Table S1, related to Figures 3 and S3 | Cytokine analysis of Hofbauer cells at 48 and 72 hours post infection with ZIKV (PR 2015)[a]

	Mock					48hpi ZIKV (PR 2015)				
	Donor 1	**Donor 2**	**Donor 3**	**Donor 4**	**Donor 5**	**Donor 1**	**Donor 2**	**Donor 3**	**Donor 4**	**Donor 5**
IL-1β	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-13	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-6	17	83	74	25	46	40	119	110	66	41
IL-12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
RANTES	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Eotaxin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-17	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIP-1α	ND	87	473	123	ND	ND	166	349	117	ND
GM-CSF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIP-1β	481	1333	3218	864	490	630	1646	1962	798	278
MCP-1	3005	9691	7852	927	3173	6340	16624	10744	2711	3621
IL-15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IFNγ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-1RA	372	5437	2249	465	1915	666	6029	1268	776	1721
TNFα	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IP-10	ND	ND	36	ND	ND	59	177	71	33	16
IL-2R	ND	ND	56	ND	ND	ND	41	22	ND	ND
MIG	ND	ND	ND	4	ND	ND	ND	ND	4	ND
IL-4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-8	20060	27644	37556	37510	38402	24535	28257	35014	36993	28748
IFNα	42	92	73	ND	13	66	122	101	59	40
IFNβ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IFNλ1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

	Mock					**72hpi ZIKV (PR 2015)**				
	Donor 1	**Donor 2**	**Donor 3**	**Donor 4**	**Donor 5**	**Donor 1**	**Donor 2**	**Donor 3**	**Donor 4**	**Donor 5**
IL-1β	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-13	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-6	25	192	117	90	46	121	287	286	215	126
IL-12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
RANTES	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Eotaxin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-17	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIP-1α	ND	52	206	129	ND	ND	161	314	132	ND
GM-CSF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIP-1β	302	1267	3613	3269	334	421	2028	3076	1297	182
MCP-1	3826	32675	16101	5078	4644	11758	38672	23022	9512	7399
IL-15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IFNγ	ND	4	ND	ND	ND	ND	4	ND	ND	ND
IL-1RA	1105	11027	3983	2745	3563	1565	17634	5120	1818	3575
TNFα	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

IP-10	4	ND	12	ND	ND	95	394	215	137	72
IL-2R	ND	ND	ND	22	22	ND	56	ND	ND	ND
MIG	ND	ND	ND	23	ND	ND	4	ND	4	ND
IL-4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-8	22801	35633	38583	39257	36938	28784	33427	33837	31804	29587
IFNα	40	111	101	66	70	93	169	131	91	85
IFNβ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IFNλ1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]Cytokine levels in the supernatants of mock or ZIKV (PR 2015) infected HCs at 48 and 72 hours post infection as determined by multiplex bead array. All values are represented in “pg/mL”. Cytokine levels that were below the lower limit of detection are indicated as not detected or “ND”. hpi, hours post-infection.

Table S2, related to Figures 3 and S3 | Cytokine analysis of cytotrophoblasts at 48 and 72 hours post infection with ZIKV (PR 2015)[a]

	Mock			48hpi ZIKV (PR 2015)		
	Donor 1	**Donor 2**	**Donor 3**	**Donor 1**	**Donor 2**	**Donor 3**
IL-1β	ND	ND	ND	ND	ND	ND
IL-10	ND	ND	2	ND	ND	ND
IL-13	ND	ND	ND	ND	ND	ND
IL-6	4	66	209	35	41	99
IL-12	ND	ND	ND	ND	ND	ND
RANTES	ND	ND	ND	ND	ND	ND
Eotaxin	ND	ND	ND	ND	ND	ND
IL-17	ND	ND	ND	ND	ND	ND
MIP-1α	ND	26	ND	ND	ND	ND
GM-CSF	ND	ND	ND	ND	ND	ND
MIP-1β	28	285	83	53	108	48
MCP-1	2505	7234	6766	3256	3103	3273
IL-15	ND	ND	ND	ND	ND	ND
IL-5	ND	ND	ND	ND	ND	ND
IFNγ	ND	ND	ND	ND	ND	ND
IL-1RA	733	1815	1459	1157	1102	1397
TNFα	ND	ND	ND	ND	ND	ND
IL-2	ND	ND	ND	ND	ND	ND
IL-7	ND	ND	ND	ND	ND	ND
IP-10	ND	11	80	ND	7	73
IL-2R	ND	ND	ND	ND	ND	ND
MIG	ND	ND	56	ND	ND	37
IL-4	ND	ND	ND	ND	ND	ND
IL-8	1663	25899	33972	5729	9672	25122
IFNα	ND	73	70	25	33	45
IFNβ	ND	ND	ND	ND	ND	ND
IFNλ1	ND	ND	ND	ND	ND	ND

	Mock			72hpi ZIKV (PR 2015)		
	Donor 1	**Donor 2**	**Donor 3**	**Donor 1**	**Donor 2**	**Donor 3**
IL-1β	ND	ND	ND	ND	ND	ND
IL-10	ND	2	5	ND	ND	14
IL-13	ND	ND	ND	ND	ND	ND
IL-6	138	38	60	141	94	294
IL-12	ND	ND	ND	ND	ND	ND
RANTES	ND	ND	ND	ND	ND	ND
Eotaxin	ND	ND	ND	ND	ND	ND
IL-17	ND	ND	ND	ND	ND	ND
MIP-1α	207	67	ND	50	78	ND
GM-CSF	ND	ND	ND	ND	ND	ND
MIP-1β	329	404	105	123	245	58
MCP-1	17312	14016	6359	12257	11117	10534
IL-15	ND	ND	ND	ND	ND	ND
IL-5	ND	ND	ND	ND	ND	ND
IFNγ	ND	ND	ND	ND	ND	ND
IL-1RA	3833	4162	2461	3837	3623	2264
TNFα	ND	ND	ND	ND	ND	ND
IL-2	ND	ND	ND	ND	ND	ND
IL-7	ND	ND	ND	ND	ND	ND

IP-10	ND	ND	87	ND	58	132
IL-2R	ND	ND	22	ND	ND	ND
MIG	ND	ND	82	ND	ND	94
IL-4	ND	ND	ND	ND	ND	ND
IL-8	24157	16535	29035	17273	25173	34423
IFNα	111	93	81	88	91	104
IFNβ	ND	ND	ND	ND	ND	ND
IFNλ1	ND	ND	ND	ND	ND	ND

[a]Cytokine levels in the supernatants of mock or ZIKV (PR 2015) infected CTBs at 48 and 72 hours post infection as determined by multiplex bead array. All values are represented in “pg/mL”. Cytokine levels that were below the lower limit of detection are indicated as not detected or “ND”. hpi, hours post-infection.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Isolation of primary placental cells. To isolate Hofbauer cells (HCs) and cytotrophoblasts (CTBs), membrane-free villous was dissected from the placenta, as previously described (Johnson and Chakraborty, 2012; Tang et al., 2011). The tissue was washed and mechanically dispersed in Hank's balanced salt solution (HBSS) to minimize peripheral blood contamination. For HC isolation, minced tissue was re-suspended in complete medium containing 10% Trypsin/EDTA (Sigma-Aldrich) for 1hr, followed by resuspension in media containing 1mg/ml collagenase A (Sigma-Aldrich), 10U/ml dispase (Worthington Biochemical Corp.), and 0.2mg/ml of DNase I (Sigma-Aldrich) and incubated in a shaking water bath at 37°C for 1hr. The digested tissue was washed with PBS and passed through gauze and a 70μm cell strainer (BD-Falcon Biosciences). The mononuclear cell population was isolated by density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich). CD14+ Magnetic Cell separation was performed using anti-CD14 magnetic beads (Miltenyi Biotech) as recommended by the manufacturer. The purity of the HC population was assessed by CD14 staining and was on average greater than 97% (Figure S2A). For CTB isolation, minced tissue was subjected to three sequential enzymatic digestions in a solution containing 0.25% trypsin (Mediatech Inc.), 0.2% DNase I (Sigma-Aldrich), 25mM HEPES, 2mM CaCl2, and 0.8mM MgSO4 in HBSS at 37°C. Following each digestion, undigested tissue was removed by passage through gauze and 100μm cell strainer (BD) and washed with PBS. Supernatants from the second and third digestions were collected and the resulting cell pellets resuspended in 1:1 DMEM/F12 supplemented with 10% FBS, 1mM L-glutamine, and 1% pen/strep (Sigma-Aldrich). The CTBs were isolated on a discontinuous gradient of Percoll (GE Healthcare) (50%/45%/35%/30%) by centrifugation. Cells migrating to the 35%/45% Percoll interface were recovered and immunopurified by negative selection with simultaneous treatment with anti-CD9 (to exclude EnC, FB, platelets, smooth muscle, extravillous trophoblast cells, B cells and monocytes) and anti-CD45RA (to exclude leucocytes) antibodies and magnetic beads (Miltenyi Biotech) (Manoussaka et al., 2005). The purity of the CTB population was assessed by cytokeratin-7 staining and was on average greater than 97% (Chiuppesi et al., 2015). HCs were maintained in supplemented RPMI medium and CTBs were maintained in supplemented DMEM medium as described below.

To minimize maternal blood and decidual cell contamination, membrane-free villous tissue was macroscopically dissected from the fetal-facing surface of the placenta. The tissue was washed thoroughly with HBSS until the supernatant ran clear. Following the initial digest with trypsin, the remaining tissue is washed again thoroughly with PBS to further ensure removal of maternal blood. Similar to Tang et. al., our method of isolation (positive selection for CD14), ensures a pure population of placental macrophages, with negligible contamination of fibroblast or trophoblast. The placental macrophages isolated through this method express high levels of DC-SIGN and have a distinct morphology (Johnson and Chakraborty, 2012; Johnson et al., 2015), unlike other peripheral blood and tissue macrophages.

Cells. HCs were maintained in RPMI medium (Corning Cellgro) supplemented with 10% FBS, 2mM L-Glutamine (Corning Cellgro), 1mM sodium pyruvate (Corning Cellgro), 1x MEM Non-essential Amino Acids (Corning Cellgro), and 1x Antibiotics/Antimycotics (Corning Cellgro). CTBs and Vero cells were maintained in DMEM medium (Corning Cellgro) supplemented with 10% FBS, 2mM L-Glutamine (Corning Cellgro), 1mM HEPES (Corning Cellgro), 1mM sodium pyruvate (Corning Cellgro), 1x MEM Non-essential Amino Acids (Corning Cellgro), and 1x Antibiotics/Antimycotics (Corning Cellgro).

Focus forming assay. Supernatants collected from mock- and ZIKV-infected HCs and CTBs ($2x10^5$ cells per condition) were diluted in DMEM supplemented with 1% FBS and used to infect Vero cells for 1hr at 37°C. Cells and inoculum were overlaid with methylcellulose (OptiMEM [Corning Cellgro], 1% Antibiotic/Antimicotic [Corning Cellgro], 2% FBS, 2% methylcellulose [Sigma Aldrich]) and incubated for 72hr at 37°C. Cells were washed with PBS and fixed with a 1:1 methanol/acetone mixture for 30min. Cells were blocked with 5% milk/PBS at RT for 20min and incubated with primary antibody (mouse 4G2 (Hamel et al., 2015)) at 1μg/ml in 5% milk for 2hr at RT. Cells were incubated with secondary antibody (HRP-conjugated goat anti-mouse IgG) diluted 1:3000 in 5% milk for 1hr at RT. Foci were developed with TrueBlue Peroxidase Substrate (KPL). Plates were read on a CTL-ImmunoSpot S6 Micro Analyzer.

Quantitative reverse transcription-PCR (qRT-PCR). ZIKV E gene probe sequences: ZIKV1107 (FAM-AGCCTACCTTGACAAGCAATCAGACACTCAA-TAMRA), ZIKV 1086 (5- CCGCTGCCCAACACAAG-3') and ZIKV 1162c (5'-CCACTAACGTTCTTTTGCAGACAT-3'). All primers were purchased from Integrated DNA Technologies (IDT). Primer sequences used to identify expression of host genes (forward and reverse, 5'---3'): GAPDH: GGAGCGAGATCCCTCCAAAAT and GGCTGTTGTCATACTTCTCATGG; DDX58:

TGTGCTCCTACAGGTTGTGGA and ACGGGTGTATGCGCTTCAC; IFIH1: TCGAATGGGTATTCCACAGACG and GTGGCGACTGTCCTCTGAA; DHX58: ACGGGTGTATGCGCTTCAC and TTGCGGTCATCGAACAGGG; IFNB1: GCTTGGATTCCTACAAAGAAGCA and ATAGATGGTCAATGCGGCGTC; IFIT1: AGAAGCAGGCAATCACAGAAAA and CTGAAACCGACCATAGTGGAAAT; IFIT2: GACACGGTTAAAGTGTGGAGG and TCCAGACGGTAGCTTGCTATT; IFIT3: AAAAGCCCAACAACCCAGAAT and CGTATTGGTTATCAGGACTCAGC; RSAD2: CAGCGTCAACTATCACTTCACT and AACTCTACTTTGCAGAACCTCAC; and OAS1: TGTCCAAGGTGGTAAAGGGTG and CCGGCGATTTAACTGATCCTG. Primer sets were designed using the PrimerBank database and purchased through IDT. All qRT-PCR was performed in 384-well plates on an Applied Biosystems 7500 Real-Time PCR System.

Immunofluorescence and confocal microscopy. HCs and CTBs were grown on glass chamber slides (Thermo Fisher Scientific) and infected with ZIKV (PR 2015) at an MOI of 1. At 72hpi, cells were fixed with 3.7% paraformaldehyde/PBS for 1hr, and permeabilized in 0.3% Triton X-100 for 30min at RT. Cells were blocked in 10% fetal bovine serum/PBS, and stained with primary (mouse 4G2 mAb) and secondary (donkey anti-mouse Alexa-488, Thermo Fisher) antibodies in blocking buffer for 1hr, respectively. Cells were washed with PBS, stained with F-actin probe (BODIPY® 558/568 phalloidin, Thermo Fisher) for 20min, and mounted with ProLong®Gold with DAPI (Thermo Fisher). Fluorescence microscopy was performed with a Zeiss LSM 510 META confocal microscope at the Integrated Cellular Imaging Core of Emory University. Images were processed with ZEN imaging software (Zeiss). 3D images were acquired with Leica SP8 confocal microscopy at 100x magnification. Images were processed with ZEN (Zeiss) and Imaris version 8.1.2 (Bitplane) software for 3D reconstruction.

Flow cytometry. Cells were blocked for 10min on ice in 25µl FACS buffer (PBS, 1mM EDTA, 0.5% BSA) with 0.25µl Human TruStain FcX (BioLegend) and then stained with 25µl surface staining mix (1:200 dilution of each antibody in FACS buffer) for 20min on ice. Cells were washed with PBS and resuspended in 50µl PBS with 0.1µl Ghost Dye Red 780 viability dye (Tonbo Biosciences) and incubated on ice for 20min. Cells were washed with PBS and resuspended in 100µl 1x Foxp3/Transcription Factor Fix/Perm buffer (Tonbo Biosciences) for 20min on ice. To perform intracellular staining of ZIKV E protein, fixed cells were washed twice with 1x Flow Cytometry Perm Buffer (Tonbo Biosciences) and resuspended in 25µl intracellular Fc block (25µl Perm Buffer, 0.25µl Human TruStain FcX, 10% normal mouse serum) for 10min at RT. 25µl E protein staining mixture (25µl Perm Buffer, 0.25µg APC-conjugated 4G2 antibody) was added to cells for 20min at RT. Cells were washed twice in Perm Buffer and resuspended in PBS prior to acquisition on a BD LSR II. All analysis was performed using FlowJo version 10.

Multiplex bead array. Cytokines analyzed included: GM-CSF, TNF-α, IL-4, IL-6, MIP-1α, IL-8, IL-15, IL-2R, IP-10, MIP-1β, Eotaxin, RANTES, MIG, IL-1RA, IL-12 (p40/p70) IL-13, IFN-γ, MCP-1, IL-7, IL-17, IL-10, IL-5, IL-2, IL-1β, IFNα, IFNβ, and IFNλ1.

SUPPLEMENTAL REFERENCES

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