Supplementary Information for:

PET imaging of TSPO expression in immune cells can assess organ-level pathophysiology in high-consequence viral infections

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SUPPLEMENTARY METHODS:

Flow cytometry

Whole blood samples from eight healthy human subjects (6 male and 2 females with an average age of 52.1±16.4) and five healthy female macaques (4.4±0.5 years) were obtained to determine the baseline distribution of TSPO expression in various immune cells. The human subjects’ samples were obtained from the NIH blood bank and were de-identified prior to use in this study. 8-10 ml of whole blood were collected in an EDTA vacutainer tubes and the entire volume was spun down at 1200rpm/5mins to separate the plasma layer on top and the cells in the bottom. The bottom layer was mixed with RBC lysis buffer and incubated at room temperature (RT) for 10mins on a rocking platform. The mix was centrifuged at 1200rpm/5mins and the supernatant was discarded. This RBC lysis step was repeated. The pelleted cells were then washed and resuspended in 2ml FACS buffer (1% mouse serum + 0.25mM EDTA+ 0.05% BSA in PBS). 200µl aliquots of the cells were taken into individual tubes for staining. Single color controls were made initially to set the compensation parameters on the flow cytometer. To the cell aliquots, 5µl Fc blocking reagent was added for 10 mins. The aliquots for each blood sample were designated as – unstained, all stains without TSPO, all stains with TSPO. Then the cells were incubated with the surface antibodies for 15mins/RT in the dark (CD3, CD19, CD20, CD15, CD66abce, CD45, CD45, HLA-Dr, CD11c and CD14, supplementary table 1). 1µl of the live/dead stain was then added to cell and they were further incubated for another 10mins. After one wash with the FACS buffer, the cells were resuspended in 250µl of Fixation/Permeabilization solution (BD Biosciences) at 4°C for 20mins. After the incubation, the cells were washed twice with 1ml of Perm/Wash buffer (BD Biosciences) and resuspended in 20µl of the wash buffer. The Fc block incubation was repeated and then 50µl of wash buffer containing TSPO antibody (1:300 dilution) was added to the “All stains with TSPO” sample for 30mins at 4°C (the others just contained 50ul of blank wash buffer). All samples were washed with the wash buffer and finally resuspended with 350µl of FACS buffer supplemented with 1% paraformaldehyde. The samples were stored at 4°C in the dark and read on the flow cytometer (BD Biosciences) the next day. The data was analyzed using FlowJo software version 9.9.6.

The following gating strategy was used to separate cell populations from macaque blood (Suppl Fig S2). Forward and side scatter plots (FSC vs SSC) were used to gate out the debris and
doublets. A gate was then drawn on the SSC vs live/dead stain to select the live cells. The SSC/CD45 plots have regularly been used to distinguish granulocytes (high SSC/low CD45), monocytes (mid SSC/high CD45), macrophages (high SSC/high CD45) and lymphocytes (low SSC/high CD45) based on their granularity and level of CD45 expression [1]. Each cell population was gated and further separated using their respective markers to identify neutrophils (CD66abce+/CD14-/HLA-DR-), monocytes (mid SSC/CD45+/CD14+/HLA-DR+), macrophages (high SSC/CD45+/CD14+/HLA-DR+), dendritic cells (CD3-/CD20-/CD11c+/HLA-DR+), T cells (CD3+/HLA-DR+) and B cells (CD20+/HLA-DR+). A similar gating strategy was used for separation of human immune cell populations with the only differences being in the antibodies used for B cells (CD19) and neutrophils (CD15).

**Biomarker testing**

For all animals, whole blood samples were obtained at least twice prior to inoculation and subsequently on each post-infection scan day until euthanasia.

Analyte measures: Complete blood counts with differential, as well as kidney and liver function panels, were performed. Plasma cytokines were assessed using a Milliplex NHP primate kit (Millipore) that has been validated to cross-react with rhesus macaques.

Plasma viral load: Viral RNA was extracted from 70 µL of plasma that was inactivated by Trizol LS, and added to 280 µL of buffer AVL (Qiagen, Germantown, MD Cat No. 19073). Samples were then extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD Cat No 52904) in accordance with the manufacturer’s instructions, eluted in 70 µL of buffer AVE (Qiagen), aliquoted, and frozen. Five microliters of sample was assayed in duplicate reactions using the Ebola Zaire Target 1 LightCycler/Rapid Master Mix (Cat. No. BEI CRP PCR-EBZ-1R-K) and compared to a standard curve of serial tenfold dilutions (Cat no. BEI CRP Ebola Zaire Target 1 Custom Conc. Positive Control 1E+9 copies/rxn). The lower limit of detection was 100 viral RNA copies.

Plaque assay: Viral titers in tissue were determined through plaque. The tissue samples were frozen and then processed to a 10% homogenate by bead-beating. These samples were serially diluted in DMEM with 10% fetal calf serum and added to confluent monolayers of VeroE6 and incubated for 1 hour at 37°C in 5% CO₂. The monolayers were then overlaid with a 1:1 mix of
2.5% Avicel and 2X Plaque Assay media and incubated at 37°C for 8 days. After the overlay was removed the cells were fixed and stained for 30 minutes with 0.2% mixture of Crystal Violet in 10% NBF. After 30 minutes, the fixative/stain was removed, plates washed in water, and plaques counted.

**PET/CT Imaging**

CT and PET scans were performed with a Gemini TF PET/CT scanner (Philips). The Gemini PET/CT scanner has 16-slice CT component that shares a common imaging bed with the PET scanner. Prior to the scan, subjects were anesthetized with ketamine (15 mg/kg, intramuscular), then intubated, immobilized using isoflurane (2–2.5%) inhalation, positioned supine on the scanner bed and monitored during anesthesia. CT images were acquired in helical scan mode with the following parameter settings: 140 kVp, 250 mAs/slice, 3 mm thickness, 1.5 mm increment, 0.688 mm pitch, collimation 16x0.75 and 0.5 s rotation. Two CT images were reconstructed from the raw data. An initial CT image was reconstructed into a 600 mm diameter field-of-view (FOV), resulting in a pixel size of 1.17 mm and a slice spacing of 3 mm. This CT image was then used to correct the PET images for photon attenuation. The raw CT data were reconstructed a second time into a diagnostic quality CT image by reducing the FOV size to 250 mm, which resulted in a pixel size of 0.352 mm while leaving the slice spacing to 1 mm. No contrast was given and the animals were allowed to breathe freely during the scan. The diagnostic quality CT scans were evaluated carefully by a board-certified radiologist to rule out major structural changes such as organomegaly or lung infiltrates.

Following the CT scans, 40 minute dynamic PET imaging was performed using DPA-714 (intravenous injection, dose: 1.95 ± 0.092 mCi) followed by a full body static scan at 60 minutes. Dynamic PET data were acquired in list mode and binned into a total of 42 time-frames with the following durations: 15 x 2 sec, 6 x 5 sec, 4 x 10 sec, 4 x 20 sec, 3 x 1 min, 3 x 2 min and 7 x 4 min. The sinograms were reconstructed using Philips’ iterative, maximum-likelihood reconstruction algorithm (3D-RAMLA) into images consisting of 90 slices with a 128x128 matrix size and 2 mm wide, cubic voxels. To ensure quantitative accuracy, all reconstructed PET images were corrected for radioactive decay, uniformity, random coincidences, attenuation, and scattering. Both PET and CT images were then sent for radiologic interpretation and analysis.
Necropsy and histopathology
Tissue samples were obtained from animals sacrificed at various time points post-infection. The organ sample collected at necropsy were fixed for 72 hours in 10% neutral buffered formalin. After fixation and removal from the BSL-4 laboratory, tissue samples were processed in a Tissue-Tek VIP-6 automated vacuum infiltration processor (Sakura Finetek USA), followed by paraffin embedding with a Tissue-Tek model TEC unit (Sakura Finetek USA). Using a standard semiautomated rotary microtome and lighted water flotation bath (Leica Biosystems), tissue sections were cut to a thickness of 4 µm and mounted on positively charged uncoated glass slides (Thermo Fisher), air dried at room temperature, stained with hematoxylin-eosin, and covered slipped for microscopic evaluation by the pathologist.

Immunostaining
Multiplex fluorescence immunohistochemistry (MF-IHC) staining was performed on spleen tissue sections for the following targets: TSPO, CD3, CD20, CD68, CD31/PECAM-1, CD144/VE-Cadherin, Cleaved Caspase-3/Cleaved PARP1 (CC3/PARP) and VP40 (supplementary table 2). Lung tissue sections were stained for the following targets: TSPO, CD68, CD66abce and VP40 (supplementary table 3). The cell nuclei in the spleen and lung sections were counterstained using 1 ug/ml DAPI (diamidino-2-phenylindole) to facilitate cell counting. All fluorescence signals were imaged using an Axio Imager.Z2 upright scanning wide-field fluorescence microscope (Zeiss) equipped with an Orca Flash 4.0 high-resolution sCMOS camera (Hamamatsu), 200W X-cite 200DC broadband light source (Lumen Dynamics), and standard DAPI and Alexa Fluor filter sets (Semrock). After imaging, the image datasets were processed for image stitching and illumination correction and the images were imported into Adobe Photoshop CS6 to produce pseudo-colored composites. Quantification of the immunofluorescent staining was performed using FIJI image processing package, based on ImageJ (NIH, Bethesda, MD). The RGB bitmap images were first converted to 8-bit grayscale, and the threshold was adjusted to include only cells of interest and eliminate the background. This was followed by calculating the percent area of positive staining within the ROIs. Four small regions of interest (ROI) of identical size were drawn across the splenic section.
and two small ROIs were drawn in the lung section. The data for all stains is expressed as %area of positive cell stain.

**SI REFERENCES**

1. Nicholson, J.K., M. Hubbard and B.M. Jones, *Use of CD45 fluorescence and side-scatter characteristics for gating lymphocytes when using the whole blood lysis procedure and flow cytometry*. Cytometry, 1996. **26**:p.16-21.
SUPPLEMENTARY FIGURES

Figure S1: Infection and sampling timeline:
The infected macaques were split into 3 groups depending on the PET imaging and sampling time-points. All animals had two baseline scans between days -12 and -6 prior to EBOV inoculation on day 0 (indicated by black dotted line). Blood collection for biomarker sampling was done after each scan session. The animals were euthanized after the final PET scan- group 1 on day 4, group 2 on day 6 while group 3 animals were euthanized at the terminal time-point on day 7.
Figure S2: Flow gating strategy

(A) Forward and side scatter plots (FSC vs SSC) were used to gate out the debris and doublets. A gate was then drawn on the SSC vs live/dead stain to select the live cells. The SSC/CD45 plots were used to distinguish granulocytes (high SSC/low CD45), monocytes (mid SSC/high CD45), macrophages (high SSC/high CD45) and lymphocytes (low SSC/high CD45). Each cell
population was gated and further separated using their respective markers to identify neutrophils (CD66abce+/CD14+/HLA-DR−), monocytes (mid SSC/CD45+/CD14+/HLA-DR+), macrophages (high SSC/CD45+/CD14+/HLA-DR+), dendritic cells (CD3+/CD20−/CD11c+/HLA-DR+), T cells (CD3+/HLA-DR+) and B cells (CD20+/HLA-DR+). (B) A similar gating strategy was used for separation of human immune cell populations with the only differences being in the antibodies used for B cells (CD19) and neutrophils (CD15).
Figure S3: Peripheral biomarker measures during the disease course

The changes in (A) total WBC and platelet counts (x10^3/μl), (B) % lymphocytes, monocytes and neutrophils, (C) plasma VL and (D) organ viral titers during the course of disease at indicated time-points are shown.
**Figure S4: Plasma cytokine measures during the disease course**

Plasma cytokines were measured at baseline and the indicated time-points post EBOV inoculation. The blue bars correspond to the left y-axis while the red bars correspond to the right y-axis.
Figure S5: Spleen volume changes

Total spleen volumes of the macaques were obtained from the CT scans at baseline and the last available time-point (ranging from day 2-7 based on animal group and/or exclusions due to motion). The scans were manually segmented and splenic volumes were quantified using MIM. Statistical analysis was performed using Wilcoxon paired signed rank test.
Figure S6: Post-mortem immunostaining of B cells in the spleen

Representative MF-IHC images of a single spleen section from a control (left panels), day 4 (middle panels) and a terminally infected EBOV macaque (right panels) are shown. The top panel shows brightfield images of the splenic region comprising of both the red pulp (RP) and the white pulp (WP) while the bottom panel shows B cells (stained with CD20) concentrated mostly in the WP area. The scale bar is 50 microns. The quantification data measuring % area positive staining is shown on the right and was obtained by drawing multiple ROIs across the entire splenic tissue section using ImageJ.
**Figure S7: Co-staining of neutrophils with TSPO and macrophages in the lung sections:**
Representative MF-IHC image of a lung section from an infected macaque showing staining with (A) CD68 (macrophages) alone (B) TSPO alone (C) merge of CD68 and CD66abce (neutrophils) and the (D) merged image with all three stains. The scale bar is 50 microns. The white square in (C) indicates instances of co-stained neutrophils and macrophages. The circles in (D) are examples of TSPO co-localization with an individual macrophage (green) or neutrophil (white).
Supplementary Table 1. List of antibodies used for flow cytometry:

| Marker   | Fluor conjugate   | Target            | Species | Company    | Cat no.   |
|----------|-------------------|-------------------|---------|------------|-----------|
| CD3      | APC Cy7           | T Cells           | Rh/H    | BD         | 557757    |
| CD19     | PE                | B cells           | H       | Abcam      | ab1168    |
| CD20     | PE                | B cells           | Rh      | BD         | 556633    |
| CD15     | perCP efluor710   | Neutrophils       | H       | Fisher     | 46-0159-42|
| CD66abce | perCp vio700      | Neutrophils       | Rh      | Miltenyi   | 130-103-864|
| CD45     | BV421             | APCs, T&B cells   | H       | BD         | 563879    |
| CD45     | BV421             | APCs, T&B cells   | Rh      | BD         | 740084    |
| HLA-Dr   | PE Cy7            | APCs, T&B cells   | Rh/H    | BD         | 560651    |
| CD11c    | APC               | Dendritic cells   | Rh/H    | BD         | 340544    |
| CD14     | BV786             | Monocytes         | Rh/H    | BD         | 563698    |
| TSPO     | AF488             | TSPO              | Rh/H    | Abcam      | ab199779  |
| Fc Block | N/A               | Fc receptor       | H       | ebioscience| 14-9161-73|
| Fc Block | N/A               | Fc receptor       | Rh      | ebioscience| 14-9165-42|
Supplementary Table 2. List of antibodies used for spleen immunofluorescence staining:

| Primary antibody | Target/Function         | Source            | Catalog       | Conjugate          |
|------------------|-------------------------|-------------------|---------------|--------------------|
| TSPO             | Various immune cells    | Abcam             | ab199836      | Alexa Fluor 647    |
| CD3              | T-cells                 | Abcam             | ab11089       | Unconjugated       |
| CD20             | B-cells                 | Novus Biologicals| NBP2-44743    | Unconjugated       |
| CD68             | Macrophages             | Novus Biologicals| NB600-985AF647| DyLight 594        |
| Cleaved Caspase-3| Actively Apoptotic Cells| Cell Signaling Tech| 96695        | Alexa Fluor 488    |
| Cleaved PARP1    | Actively Apoptotic Cells| Cell Signaling Tech| 91485        | Alexa Fluor 488    |
| VP40             | Ebola Matrix Protein    | IBT Bioservices   | 0201-17       | Unconjugated       |

| Secondary antibody | Target | Source            | Catalog       | Conjugate          |
|--------------------|--------|-------------------|---------------|--------------------|
| Goat anti-Rat IgG  | CD3    | Li-Cor Biosciences| 926-32219     | IRDye 800CW        |
| Goat anti-Mouse IgG2a | CD20 | Li-Cor Biosciences | 926-68051     | IRDye 680LT        |
| Goat anti-Mouse IgG2b | VP40 | Jackson ImmunoRes | 115-475-207   | DyLight 405        |

Supplementary Table 3. List of antibodies used for lung immunofluorescence staining:

| Primary antibody | Target       | Source                | Catalog          | Conjugate          |
|------------------|--------------|-----------------------|------------------|--------------------|
| CD68             | Macrophages  | Novus Biologicals     | NB600-985DL594   | DyLight 594        |
| TSPO             | Macrophages  | Abcam                 | ab199836         | Alexa Fluor 647    |
| CD66abce         | Neutrophils  | Miltenyi Biotech      | 130-119-848      | Biotin             |
| VP40             | Ebola Matrix Protein | IBT Bioservices | 0201-18         | Unconjugated       |

| Secondary antibody | Target | Source            | Catalog       | Conjugate          |
|--------------------|--------|-------------------|---------------|--------------------|
| Goat anti-Mouse IgG2a | VP40 | Li-Cor Biosciences | 926-68051     | IRDye 680LT        |
| Animal ID | Organ | Avg BL | D1   | D2   | D3   | D4   | D5   | D6   | D7   | D7/Terminal |
|-----------|-------|--------|------|------|------|------|------|------|------|-------------|
| **Group 1 (n=3)** |       |        |      |      |      |      |      |      |      |             |
| DGJF      | Spleen| 7.7988 | 7.0540| 5.9849|      |      |      |      |      |             |
|           | Lungs | 1.5766 | 1.3592| 1.0460|      |      |      |      |      |             |
|           | Liver | 6.3046 | 5.8259| 5.2439|      |      |      |      |      |             |
|           | Bone Marrow | 1.5173 | 1.0436| 1.7789|      |      |      |      |      |             |
| DGTH      | Spleen| 7.2365 | 5.2008| 7.8075|      |      |      |      |      |             |
|           | Lungs | 1.7131 | 1.3248| 1.6724|      |      |      |      |      |             |
|           | Liver | 7.1431 | 5.5585| 8.8349|      |      |      |      |      |             |
|           | Bone Marrow | 1.5379 | 1.1127| 1.7337|      |      |      |      |      |             |
| DGVB      | Spleen| 6.7552 | 7.9230| 5.8017|      |      |      |      |      |             |
|           | Lungs | 2.1702 | 1.9471| 1.4741|      |      |      |      |      |             |
|           | Liver | 5.8971 | 6.2224| 5.7049|      |      |      |      |      |             |
|           | Bone Marrow | 1.5439 | 1.8967| 1.6032|      |      |      |      |      |             |
| **Group 2 (n=3)** |       |        |      |      |      |      |      |      |      |             |
| DG0C      | Spleen| 6.3786 | 6.4076| 5.3597|      |      |      |      |      |             |
|           | Lungs | 1.5953 | 1.7241| 1.1477|      |      |      |      |      |             |
|           | Liver | 6.5546 | 7.0398| 7.6960|      |      |      |      |      |             |
|           | Bone Marrow | 1.9716 | 1.9246| 2.3270|      |      |      |      |      |             |
| DGBT      | Spleen| 5.4704 | 4.0004| 4.9364|      |      |      |      |      |             |
|           | Lungs | 1.7303 | 1.2381| 1.3585|      |      |      |      |      |             |
|           | Liver | 5.6117 | 6.1920| 6.4740|      |      |      |      |      |             |
|           | Bone Marrow | 1.4908 | 1.3161| 2.3665|      |      |      |      |      |             |
| DGRM      | Spleen| 5.3796 | 4.6580| 3.0892|      |      |      |      |      |             |
|           | Lungs | 1.5029 | 1.5011| 1.0922|      |      |      |      |      |             |
|           | Liver | 4.9356 | 5.0854| 5.1064|      |      |      |      |      |             |
|           | Bone Marrow | 1.0508 | 1.0172| 1.4041|      |      |      |      |      |             |
| **Group 3 (n=4)** |       |        |      |      |      |      |      |      |      |             |
| DF3Z      | Spleen| 5.2079 | 4.5408| 2.3938| 1.8528|      |      |      |      |             |
|           | Lungs | 1.4259 | 1.1936| 0.8035| 0.6433|      |      |      |      |             |
|           | Liver | 4.7561 | 4.8171| 4.4876| 4.4895|      |      |      |      |             |
|           | Bone Marrow | 1.1431 | 1.1938| 1.0619| 1.0638|      |      |      |      |             |
| DFSA      | Spleen| 8.5530 | 9.8175| n/a*  | 3.4043|      |      |      |      |             |
|           | Lungs | 2.5110 | 2.6234| n/a*  | 1.503|      |      |      |      |             |
|           | Liver | 7.2781 | 9.2999| n/a*  | 8.3529|      |      |      |      |             |
|           | Bone Marrow | 1.1548 | 1.0126| n/a*  | 2.4079|      |      |      |      |             |
| DFWF      | Spleen| 4.9573 | 4.8211| 4.3442| 3.3588|      |      |      |      |             |
|           | Lungs | 2.0399 | 2.9030| 1.4401| 1.1186|      |      |      |      |             |
|           | Liver | 4.8243 | 5.3598| 5.5257| 6.5907|      |      |      |      |             |
|           | Bone Marrow | 0.8527 | 1.1754| 1.6605| 1.9187|      |      |      |      |             |
| DFWM      | Spleen| 5.9729 | 6.1395| 2.8435| n/a** |      |      |      |      |             |
|           | Lungs | 3.6576 | 3.8770| 1.2551| n/a** |      |      |      |      |             |
|           | Liver | 5.1972 | 6.6928| 4.9501| n/a** |      |      |      |      |             |
|           | Bone Marrow | 0.8519 | 0.5914| 1.0246| n/a** |      |      |      |      |             |

* No data collected due to technical complications on day 5 scan.
** Animal died during the scan and no data was collected.