SUPPLEMENTARY INFORMATION FILE

Dissecting pathways to thrombocytopenia in a mouse model of visceral leishmaniasis

Gulab Fatima Rani[1], Olivier Preham[1,*], Helen Ashwin[1], Najmeeyah Brown[1], Ian S. Hitchcock[1], Paul M. Kaye[1]

SUPPLEMENTARY METHODS

Transmission electron microscopy

Femurs were harvested and fixed in 4% PFA + 1% glutaraldehyde in 0.1M Sodium cacodylate buffer (pH 7.4) for 4 hours at RT and then overnight at 4^{o}C followed by post-fixation with Osmium Ferricyanide (1% Osmium Tetroxide and 1.5% Potassium Ferricyanide in 0.1M Sodium cacodylate buffer-pH 7.4) for 1 hour. Femurs washed in 0.1M Sodium cacodylate buffer were decalcified in 1.9% glutaraldehyde and 0.15M EDTA in 0.06M sodium cacodylate buffer for 2 weeks and cut in small fragments (longitudinal and transverse). Femur fragments were then dehydration in a series of ethanol solutions at 25%, 50%, 70%, 90% concentrations for 30-60 minutes at RT and 100% concentration overnight at 4^{o}C. These dehydrated femur fragments were immersed in propylene oxide with continuous shaking for 30 minutes at RT and passed through a series of propylene oxide and epoxy resin (Epon-A) at various ratios (3:1 for 2 hours, 1:1 for 2-3 hours, 1:3 overnight) at RT followed by freshly made 100% epoxy resin for few hours at 37^{o}C. Bone fragments were embedded in polymerised resin at 60^{o}C and semithin sections (0.5-1µm thickness) were cut using Leica ultramicrotome (EM UC7, Leica microsystems, Wetzlar, Germany) with a diamond knife at 6^{o} cutting angle. Femur sections were then stained with saturated Uranyl Acetate (in 50% Acetate) and Reynold's Lead Citrate,

for 10 minutes each. Stained sections were placed on EM mesh grids (200-Grid coated with Carbon support film) and images were collected on Transmission Electron Microscope (TEM), FEI Tecnai 12G^2 (Thermo Fisher Scientific, Oregon, USA) fitted with digital imaging system with SIS MegaView III camera (Ceta™16M, Thermo Scientific™, Waltham, Massachusetts, United States). Segmentation analysis on these TEM images was done using Fiji ImageJ software (version 2.0.0-rc-65/1.51s; Madison, Wisconsin, United States) (**Supplementary Figure 3**).

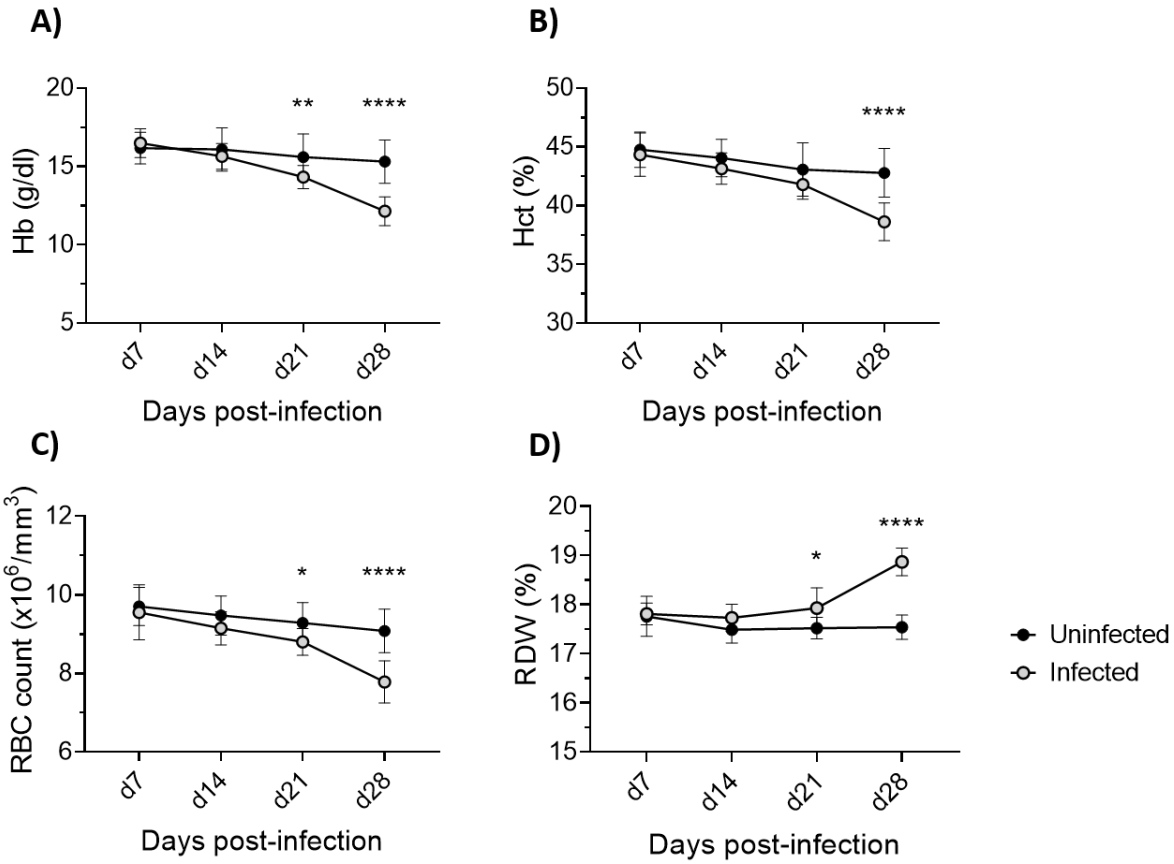
Immunohistochemistry on livers

Post-mortem liver fragments were embedded and snap-frozen on dry ice as described above without prior processing and cut at a thickness of 8-10 µm using CM1900 cryostat (Leica Microsystems, Wetzlar, Germany) onto Polysines® slides (Thermo Fisher). Liver cryosections were fixed in ice-cold acetone for five minutes followed by blocking with a dilution buffer containing 5% serum in wash buffer (0.05% w/v bovine serum albumin; BSA (Sigma-Aldrich, USA) in sterile 1x PBS) for 30 minutes at RT. For TPO staining, tissue sections were incubated with primary antibodies; unconjugated anti-TPO antibody (1:100; Cat no: ab196026 Abcam) and F4/80 AF647 (1:200; Cat no: 123122 BioLegend) for 45 minutes at RT. Slides were washed three times in wash buffer while shaking continuously and then incubated with AF488 goat anti-rabbit secondary antibody (1:200; Cat no: A-11034 Invitrogen) for 30 minutes at RT. All tissue sections after antibody staining were washed thoroughly with wash buffer and 1x PBS and counter-stained with a nuclear stain i.e. 4′,6-diamidino-2-phenylindole (DAPI; 1µg/ml in 1x sterile PBS). Slides were washed twice with 1x PBS and mounted with coverslips using ProLong® gold antifade mountant (Thermo Fisher Scientific). Images were captured using Zeiss LSM 710 upright confocal microscope (Zeiss, Oberkochen, Germany) using Zen software (Zeiss, Oberkochen, Germany) at 63x resolution with oil immersion lens.

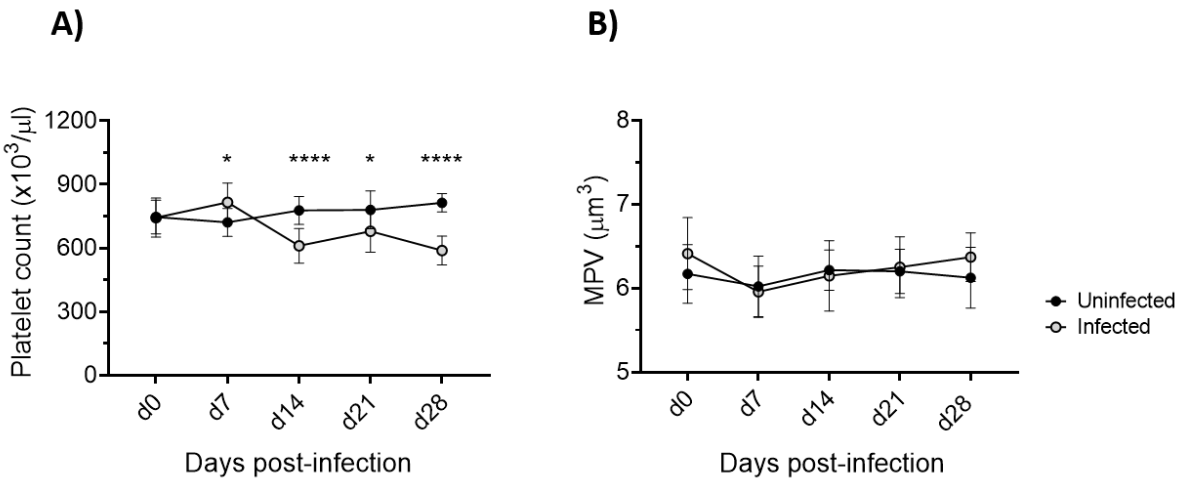
Segmentation image analysis

Segmentation analysis was done on the immunohistochemistry images using StrataQuest TissueGnostics image analysis software (TissueGnostics, Vienna, Austria). Images were analysed either with the standard image analysis applications provided in the software or advanced mode by setting up a specific profile with the desired parameters.

SUPPLEMENTARY FIGURES

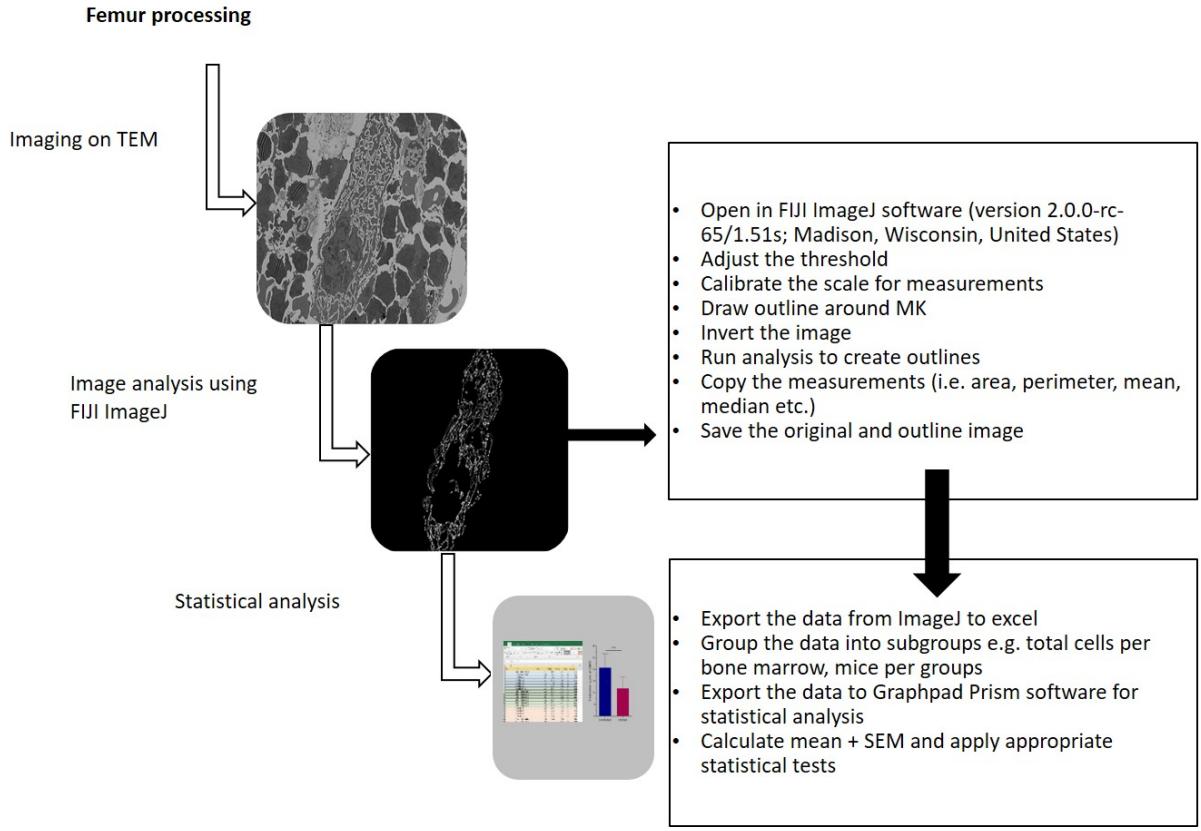


Supplementary Figure 1: Anemia associated with experimental visceral leishmaniasis. A) Hemoglobin (Hb), B) Hematocrit (Hct), C) RBC count and D) Red cell distribution width (RDW) were monitored weekly in C57BL/6 mice infected with *L. donovani*. All data are pooled from three different independent experiments and analysed using unpaired t-test comparing mean ± SD of uninfected (n = 15) vs infected (n = 12) at each time-point, *; $p < 0.05$, **; $p < 0.01$, ****; $p < 0.0001$.

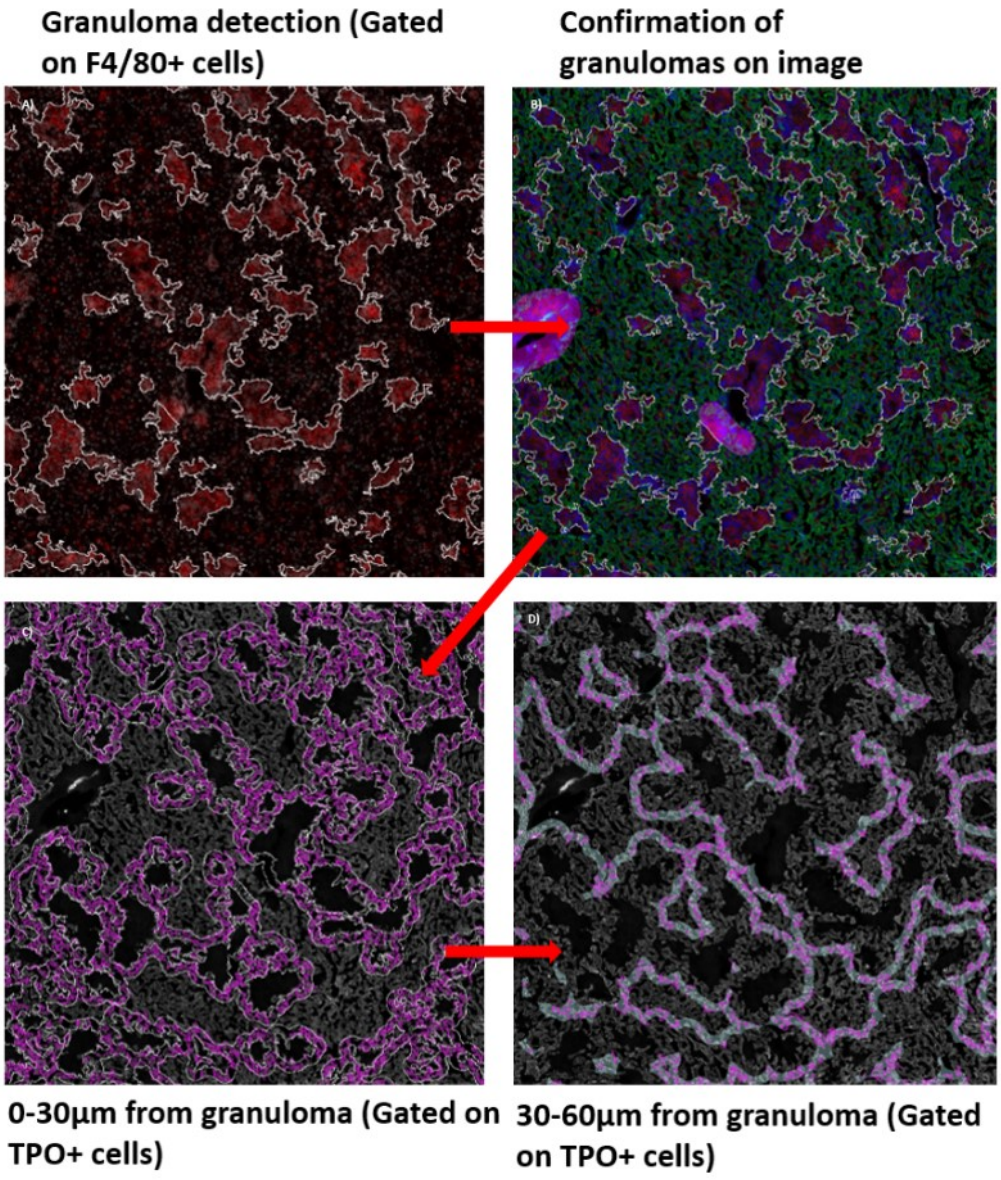


Supplementary Figure 2: Thrombocytopenia associated with experimental visceral leishmaniasis in BALB/c mice.

BALB/c mice were infected with 3 x 10^7 *L. donovani* amastigotes intravenously for 28 days and A) Platelet counts and B) MPV were monitored weekly in infected (n = 17) and uninfected (n = 8) mice. Data are representative of a single independent experiment and analysed using unpaired t-test comparing mean ± SD of uninfected vs infected at each time-point, *; $p < 0.05$, ****; $p < 0.0001$.



Supplementary Figure 3: Segmentation analysis strategy on TEM images. Segmentation analysis was done on the TEM images of BM MKs of infected and uninfected mice using Fiji ImageJ software. Outlines of the demarcation membranes were created after adjusting the image threshold and calibrations for measurements. All measurements were exported to Microsoft Excel after the segmentation analysis and statistical analysis was done using GraphPad Prism 8.0 software.



Supplementary Figure 4: Gating strategy for the segmentation analysis of anti-TPO$^+$ hepatocytes around the hepatic granulomas in infected mice livers. A granuloma mask was generated on F4/80$^+$ Kupffer cells in infected liver sections to identify the granulomas and create the outlines around them. Two regions of interest (ROIs) were drawn around the granuloma margin at a distance of 0-30µm and 30-60µm. Only the cells (TPO$^+$ hepatocytes) with > 50% of their nucleus and cytoplasm inside the ROIs were used for final analysis. Cells close to the margin of any granuloma were excluded. Fluorescent intensity of cells in each ROI was exported to Microsoft Excel for every tissue section (tissue sections; 3/mouse for n=5 infected mice). Further analysis was done using Microsoft Excel and GraphPad Prism 8.0 software for statistical analysis.