Salmonella-induced thrombi in mice develop asynchronously in the spleen and liver and are not effective bacterial traps

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Supplemental Materials and Methods

Mice and infection with STm

Wild-type (WT), C57BL/6 mice (6-8 weeks old; Harlan OLAC Ltd.) were used in accordance with local and national ethical approval HO licence numbers 3028/50 and P2E63AE7B. Mice were infected intraperitoneally (i.p.) or intravenously (i.v.) with 1-5x10^5 attenuated STm SL3261 or virulent SL1344[12]. Non-infected (Day 0), vehicle-immunized mice served as controls. Viable bacterial numbers in tissues were calculated by plating out tissue homogenates and counting the numbers of colonies[13].

Immunohistology, fluorescent microscopy and quantification of thrombi and bacteria

Tissues were frozen in liquid nitrogen. Five-six µm acetone-fixed cryosections were stained either for immunohistochemistry (IHC) or immunofluorescence (IF) as described elsewhere[13,14]. Briefly, primary antibodies were incubated for 45min at RT before adding HRP-conjugated or biotin-conjugated secondary antibodies. ABComplex alkaline phosphatase (Vector) was used. Signal was detected using diaminobenzidine for HRP activity and naphthol AS-MX phosphate with Fast Blue salt and levamisole for alkaline phosphatase activity. Antibodies were used to detect CD41, CD31, fibrin/fibrinogen, Ly6G, Ly6C, F4/80, *Salmonella* and nuclei (DAPI; supplemental Table 1)[9]. Images were obtained at x20 using a Zeiss (Jena, Germany) AxioScan.Z1 Scanner. Quantification of frequency and proportion of thrombus per total tissue section and bacteria per thrombus was performed using Zen 2012 blue edition (Jena, Germany) software.

Clodronate treatment

Mice were treated i.p. with either 200 µl (5 mg/ml) of clodronate or PBS liposomes (Liposoma, B.V., Amsterdam, The Netherlands) 24 h before STm infection as described elsewhere[15,16]

Statistical and data analysis

Statistical significance was determined using the 2-tailed Mann-Whitney non-parametric sum of ranks test, 1-wayANOVA with the Kruskal-Wallis test. P values were calculated using GraphPad Prism software (GraphPad, La Jolla, CA) and were considered statistically significant when $p<0.05$. Data presented are medians unless stated

Table S1. Antibodies used for immunohistology, immunofluorescence and flow cytometry staining.

Reactivity	Isotype	Clone	Conjugate	Supplier
CD11b	Rat IgG2b, k	M1/70	eFluor 450	eBioscience
CD31	Rat IgG2a, κ	390	Biotin	eBioscience
CD41	Rat IgG1, κ	eBioMWReg30	Purified	eBioscience
CD41	Rat IgG1, κ	eBioMWReg30	PE	eBioscience
F4/80	Rat IgG2a, k	BM8	APC	eBioscience
Fibrin/Fibrinogen	Goat, IgG	Polyclonal	Purified	Accurate Chemical & Scientific corporation
Goat	Donkey, IgG	Polyclonal	Alexa Fluor 647	Jackson Immunoresearch
Ly6C	Rat IgG2c, k	HK1.4	Biotin	eBioscience
Ly6G	Rat IgG2a, k	1A8	Biotin	BioLegend
Rabbit	Donkey, IgG	Polyclonal	Alexa Fluor 488	Jackson Immunoresearch
Salmonella	Rabbit, IgG	Polyclonal	Purified	Abcam
Myeloperoxidase	Rabbit, IgG	Polyclonal	Purified	Dako
Citrullinated histone H3	Rabbit, IgG	Polyclonal	Purified	Abcam
Streptavidin			Alexa Fluor 555	Invitrogen
Streptavidin			PE-Texas Red	BD Pharmingen

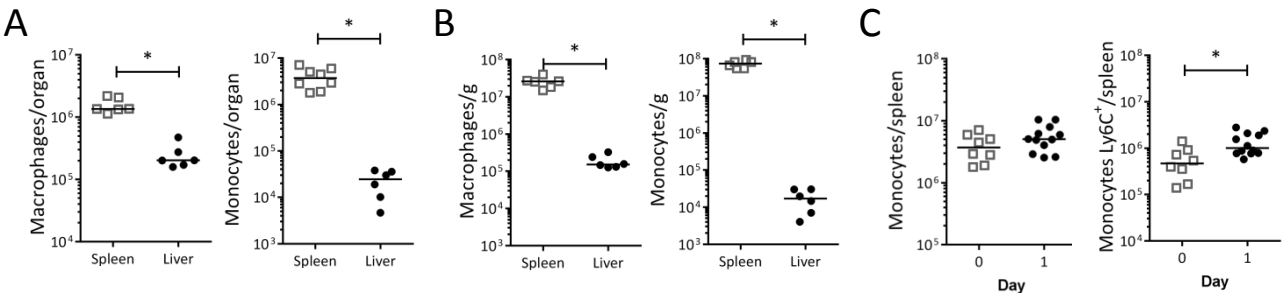


Figure S1. Numbers of monocytic cells before and after infection. (A) Graphs showing the numbers of macrophages (left, F4/80^{hi}CD11b^{lo}Ly6G^{-} cells) and total monocytes (right, F4/80^{lo}CD11b^{hi}Ly6G^{-} cells) (gating strategy from Rose, Misharin et al. 2012, Cytom; Tam et. al. 2014, Infect. & Immun.) present in the spleen and liver in non-infected mice. (B) Shows equivalent cell numbers per 1 g of tissue to adjust for organ size. (C) Graphs display number of total monocytes (left, F4/80^{lo}CD11b^{hi}Ly6G^{-} cells) and inflammatory monocytes (right, F4/80^{lo}CD11b^{hi}Ly6G^{-}Ly6C^{+} cells) present in the spleen before and 1 day after infection with 5x10^{5} STm SL3261.

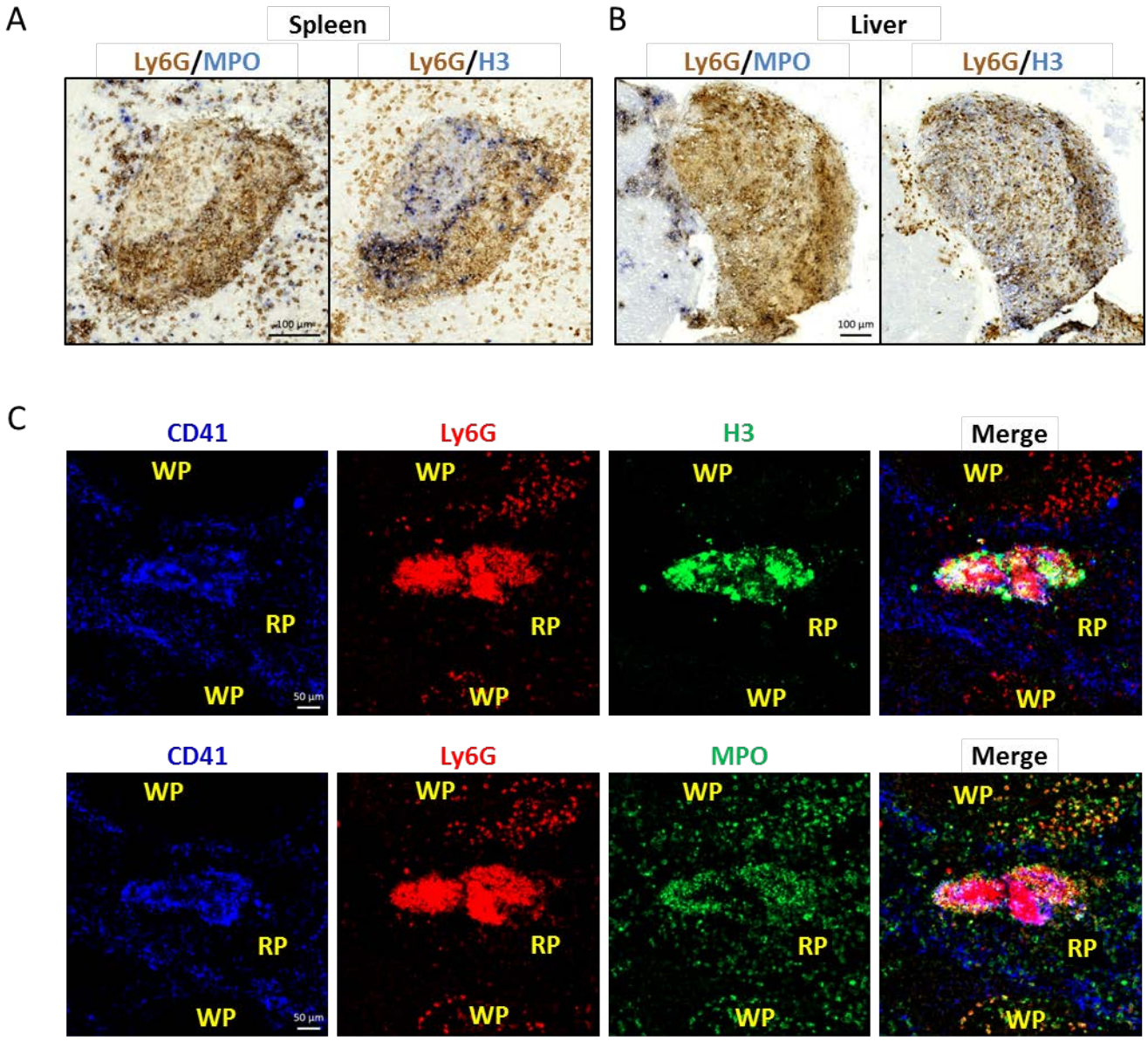


Figure S2. MPO and citrullinated histone H3 are detected within thrombi after STm infection. (A) Representative photomicrographs of a thrombus from the spleen of a mouse infected with $5x10^5$ STm SL3261 for 24h. (B) Representative photomicrographs of a thrombus from the liver of a mouse infected with $5x10^5$ STm SL3261 for 7 days. For A and B serial sections were stained to identify neutrophils (Ly6G; brown) together with either Myeloperoxidase (MPO; blue) or citrullinated histone H3 (H3; blue). (C) Immunofluoresence of a thrombus in the spleen from a 1 day-infected ($5x10^5$ STm SL3261) mouse co-incidentally stained for H3, Ly6G and CD41 (top row) or MPO, Ly6G and CD41 (bottom row).

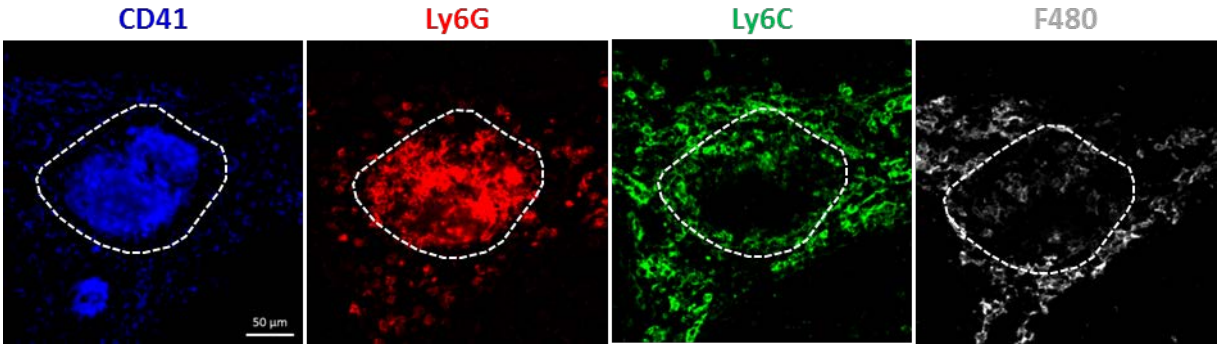


Figure S3. Association of different myeloid cells with thrombi. Immunofluorescence photomicrographs of the same thrombus from a spleen taken from a mouse infected for 24h with $5x10^5$ STm SL3261. Sections were stained to identify platelets (CD41, blue), neutrophils (Ly6G, red), $Ly6C^+$ cells (green) and $F4/80^+$ cells (grey). Dotted line outlines the thrombus.

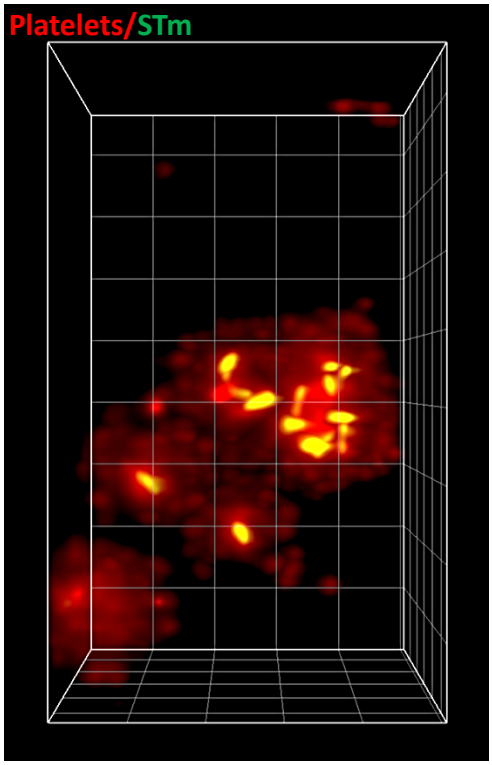


Figure S4. *Salmonella* are found throughout platelet aggregates formed *in vitro*. Platelet-Rich Plasma (PRP) was obtained from healthy donors and mixed with DiOC6 (3,3'-dihexyloxacarbocyanine iodide) to stain platelets. The PRP was mixed, with agitation, for 30 min with mCherry-expressing *Salmonella* Typhimurium (SL1344) at a ratio 5 platelets to 3 bacteria. At the end of the incubation, aggregates were fixed in 4% (w/v) paraformaldehyde. Whole aggregates were placed on a coverslip and imaged using Dual Inverted Selective Plane Illumination Microscopy (diSPIM). The image shows a representative z-stack, the grid size is 10 µm. A 3D reconstruction can be observed in supplemental video 1.

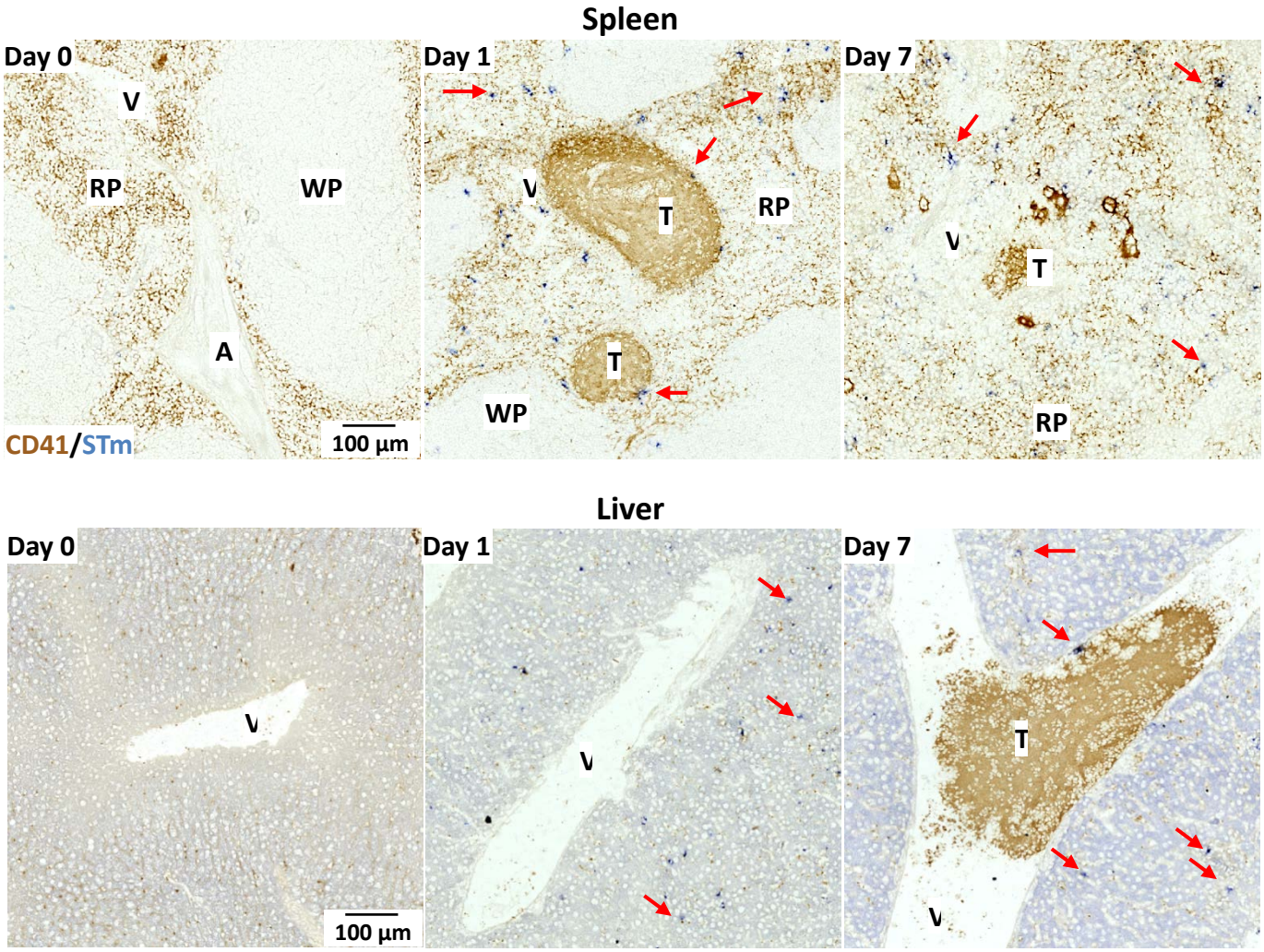


Figure S5. Detection of bacteria within the spleen and liver after infection with STm. Frozen tissue sections (spleen and liver) from WT mice infected with $5x10^5$ STm SL3261 were stained by IHC with anti-*Salmonella* antibody (blue) to identify bacteria and anti-CD41 (brown) to identify platelets, megakaryocytes and thrombi. Representative photomicrographs of spleen and liver sections from WT mice at day 0, 1, and 7 post-infection. Red arrows highlight some STm bacteria, V=Vein, RP=Red Pulp, WP=White Pulp, T=Thrombus.

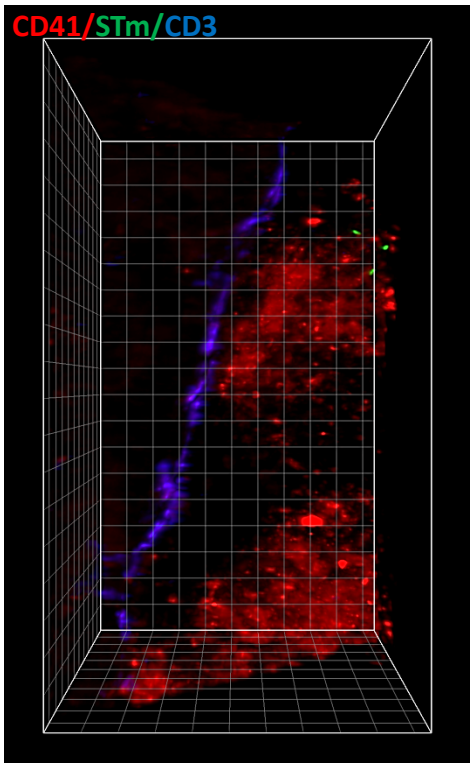
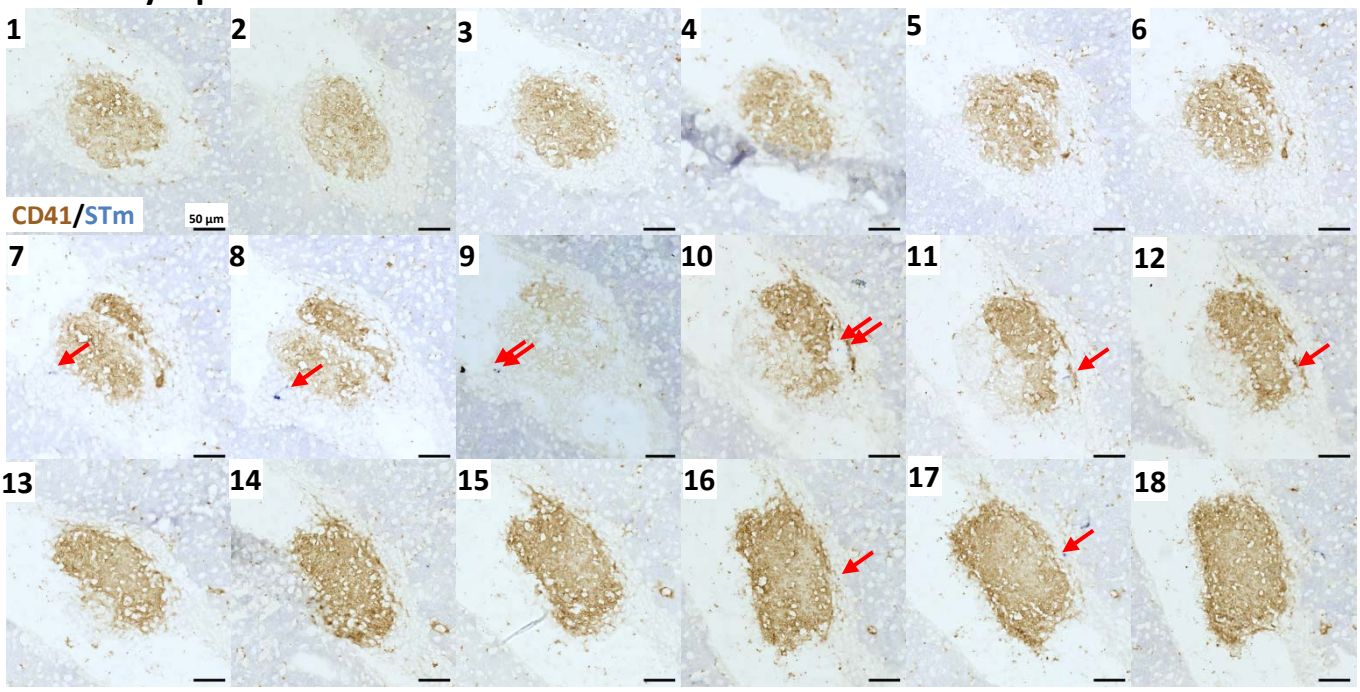


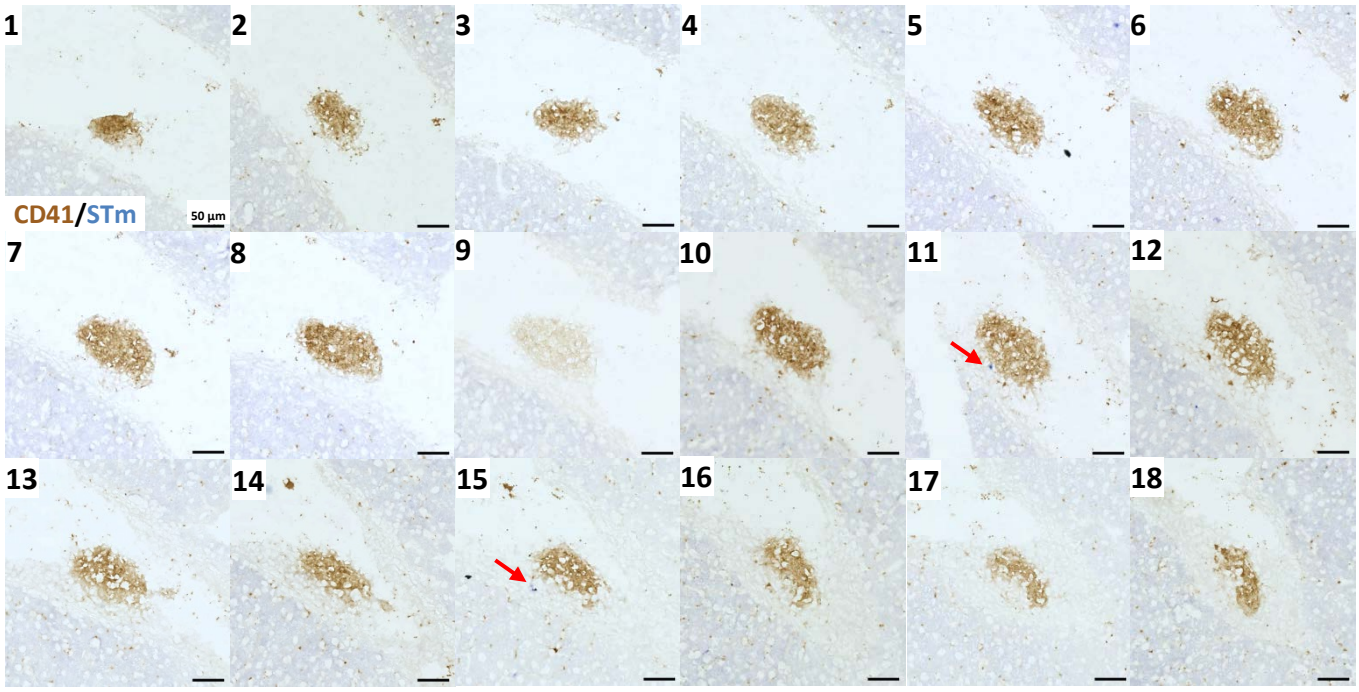
Figure S6. Detection of STm proximal to a thrombus. 30-µm liver sections from mice infected with $5x10^5$ STm SL3261 for 7 days were stained with anti-*Salmonella* antibody to detect bacteria (green), anti-CD41 (red) to detect platelets and anti-CD31 (blue) to delineate the blood vessel. Imaging was performed using Dual Inverted Selective Plane Illumination Microscopy (diSPIM). The image shows a representative z-stack , the grid size is 10 µm. A 3D reconstruction can be observed in supplemental video 2.

A

Liver -Day 7 post infection



B



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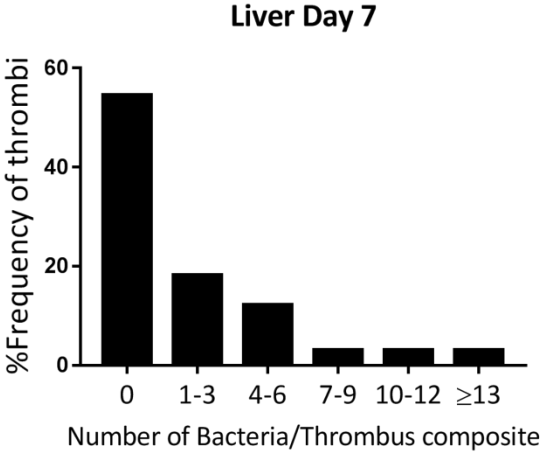

Figure S7. Serial sections of thrombi show that most contain no or few bacteria. A) and B) 6-µm serial sections of two individual thrombi in livers from two different mice infected with $5x10^5$ STm SL3261 for 7 days were stained with anti-*Salmonella* antibody to detect bacteria (blue) and anti-CD41 (brown) to detect platelet-rich thrombi. Red arrows=STm. C) Graph showing numbers of bacteria counted in the serial sections from up to 19 sections from 34 thrombi from two different mice. Data are. Scale bar= 50µm