

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

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

3 **Short title:**

4 ***Salmonella*-induced thrombi do not trap bacteria**

5
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35 **Key points:**

- 36 • Thrombosis develops in the spleen and liver with distinct kinetics following *Salmonella*
37 infection
- 38 • Thrombi in the spleen and liver are not major sites of bacterial localisation

39 **Abstract**

40 Thrombosis is a frequent, life-threatening complication of systemic infection, associated with multiple
41 organ damage. We have previously described a novel mechanism of inflammation-driven thrombosis
42 induced by *Salmonella* Typhimurium infection of mice. Thrombosis in the liver develops 7 days post-
43 infection persisting after the infection resolves, and is monocytic cell-dependent. Unexpectedly,
44 thrombosis was not prominent in the spleen at this time, despite carrying a similar bacterial burden as
45 the liver. In this study, we show that thrombosis does occur in the spleen but with strikingly
46 accelerated kinetics compared to the liver, being evident by 24 h and resolving rapidly thereafter. The
47 distinct kinetics of thrombosis and bacterial burden provide a test of the hypothesis that thrombi form
48 in healthy vessels to trap or remove bacteria from the circulation, often termed immunothrombosis.
49 Remarkably, despite bacteria being detected throughout infected spleens and livers in the early days
50 of infection, immunohistological analysis of tissue sections show that thrombi contain very low
51 numbers of bacteria. In contrast, bacteria are present throughout platelet aggregates induced by
52 *Salmonella in vitro*. Therefore, we show that thrombosis develops with organ-specific kinetics and
53 challenge the universality of immunothrombosis as a mechanism to capture bacteria *in vivo*.

54 **Introduction**

55 The consequences of thrombosis are the leading cause of death worldwide¹. Thrombosis is common
56 after infection and can lead to organ failure and poor outcome²⁻⁵. There are however significant gaps
57 in our understanding of blood-borne infection-associated thrombosis, including whether it occurs at
58 multiple sites through distinct mechanisms and/or kinetics². Immune-driven thrombosis, broadly-
59 termed “immunothrombosis”, can occur in the presence or absence of infection. Nevertheless, when

60 triggered by infection, it is still unclear whether the induced thrombi capture and contain blood-borne
61 pathogens within the vasculature as proposed⁶⁻⁸.

62 We recently reported on a novel pathway of thrombosis in the liver after infection with *Salmonella*
63 Typhimurium (STm), involving inflammation-driven upregulation of podoplanin on monocytic cells
64 and activation of platelets⁹. A striking feature of this thrombosis is that it takes a week to develop and
65 then persists as the bacterial burden declines. Furthermore, thrombi are largely undetectable in the
66 spleen at this time, despite this organ being a major site of bacterial colonisation^{10,11}. In this paper, we
67 show that extensive thrombosis does occur in the spleen but is rapid in onset and transient, with
68 distinct kinetics to liver. Furthermore, we show that thrombi present in either organ contain
69 surprisingly few bacteria, despite the high bacterial burdens in the organs themselves, indicating that
70 bacterial entrapment is not a major consequence of thrombosis after infection with STm.

71 **Study design**

72 **Full details are provided in the Supplemental Material**

73 **Mice and infection with STm**

74 Wild-type (WT), C57BL/6 mice (Home Office Licenses 3028/50 and P2E63AE7B) were infected
75 intraperitoneally (i.p.) or intravenously (i.v.) with $1-5 \times 10^5$ attenuated SL3261 or virulent SL1344
76 STm^{12,13}.

77 **Immunohistology and fluorescent microscopy**

78 Cryosections were stained for immunohistochemistry (IHC) or immunofluorescence (IF)^{13,14} to detect
79 CD41, CD31, fibrin/fibrinogen, Ly6G, Ly6C, F4/80, *Salmonella* and nuclei (DAPI; Supplemental
80 Table 1)⁹.

81 **Clodronate treatment**

82 Mice were treated i.p. with either 200 μ l (5 mg/ml) of clodronate or PBS liposomes 24 h before STm
83 infection^{15,16}

84 **Results and Discussion**

85 **Thrombosis develops with distinct kinetics in the spleen and liver**

86 Thrombosis in the liver becomes established 7 days after infection, whereas few thrombi are
87 detectable in the spleen at this time⁹. In the liver, thrombosis is driven by the up-regulation of
88 podoplanin on monocytes/macrophages, triggering activation of CLEC-2 on platelets⁹. The spleen is
89 a reservoir of monocytic cells, with high numbers of these cells present pre-infection compared to the
90 liver, and 24 hours post-infection there were increased numbers of inflammatory splenic monocytes
91 (Supplemental Figure S1), suggesting that thrombosis may occur at a much earlier stage in the
92 infection. Consistent with this, we found numerous, large platelet-rich thrombi within the spleen at 24
93 h post-infection (Figure 1A), independent of whether mice were infected i.v. or i.p. or of the virulence
94 of the infecting strain (Figure 1B-C). Thrombi typically stained positive for citrullinated-histone H3,
95 Ly6G⁺ cells and myeloperoxidase (Supplemental Figure S2). Ly6C⁺ and F4/80⁺ cells were located at
96 the periphery of thrombi (Supplemental Figure S3). Splenic thrombosis resolved rapidly after day 1,
97 with few thrombi detected thereafter (Figure 1D,E), often leaving the remnants of a fibrin core (Figure
98 1 and 2A). In contrast, at these early times, thrombosis was undetectable in the liver (Figure
99 1B,C,D,F). Moreover, thrombosis was absent in the spleens of clodronate-liposome treated mice
100 (Figure 1G) suggesting that, like in the liver, monocytic cells are important in this process⁹. Therefore,
101 systemic infection with STm can induce thrombosis in distinct sites and with distinct kinetics, likely
102 due to the levels of tissue-resident macrophages present at the time of infection.

103 **Most thrombi induced in the spleen and liver contain limited numbers of bacteria**

104 It has been proposed that thrombus formation can trap and remove bacteria, a process sometimes
105 known as immunothrombosis⁶. After platelet activation induced by STm *in vitro*, bacteria are present
106 throughout the aggregate as shown in Supplemental Figure S4 and video 1. This demonstrates that
107 bacteria can closely associate with platelets in aggregates formed *in vitro*. We used IHC and IF
108 microscopy to identify the relationship between bacterial localisation and thrombi *in vivo* at the peak
109 times of thrombosis in the spleen (day 1) and liver (day 7) (Figure 2A-B and Supplemental Figure

110 S5). A 3-dimensional reconstruction of a thrombus and proximal bacteria is shown in Supplemental
111 Figure S6 and Supplemental Video 2. Collectively, these approaches all showed that thrombi
112 contained a surprisingly low number of bacteria, despite their relative abundance in the surrounding
113 tissues. Quantification of the bacteria within sections of splenic thrombi (>200 thrombi from 37 mice,
114 3 time points evaluated) showed that no bacteria were detected in 38% of thrombus sections at day 1,
115 and that 33% of sectioned thrombi contained 1-2 bacteria (Figure 2C). At later times, bacteria were
116 detected at an even lower frequency within sections of splenic thrombi, with >90% of thrombi
117 containing 0-2 bacteria at day 7 and day 21 (Figure 2C). In the liver, only ~20% of thrombi sections
118 (>400 thrombi counted from 23 mice) contained bacteria at day 7 and this proportion was even lower
119 (<5%) at day 21 (Figure 2D). Analysis of serial sections from the same thrombi confirmed a paucity
120 of bacteria within individual thrombi (Supplemental Figure S7). When the bacterial burdens per organ
121 were compared with the levels of thrombosis at days 1 to 21 post-infection, no direct relationship was
122 found between the two, other than the necessity for infection to induce thrombosis. In the spleen,
123 thrombosis peaks before bacterial numbers peak (Figure 2E) and falls whilst bacterial numbers are
124 still rising, whereas in the liver thrombosis develops later and peaks when the bacterial levels are
125 beginning to decline (Figure 2F)^{9,13,17}. Therefore, thrombi induced during this infection do not trap
126 significant numbers of bacteria, regardless of the bacterial loads in the organs. This contrasts with
127 other models of infection^{18,19}, which used 1000-fold higher numbers of bacteria compared to here⁹.
128 Thus, although STm infection can drive thrombosis, thrombi do not necessarily contribute to bacterial
129 containment and moreover they form in different organs with distinct kinetics. These findings are
130 important for our understanding of the consequences of infection on the haematological system since
131 they show that the presence of equivalent levels of bacteria is not enough to induce thrombosis in an
132 organ.

133 Although these data show that during a single infection thrombosis can occur sequentially in multiple
134 tissues, further work is needed to evaluate whether other systemic bacterial infections induce
135 thrombosis with similar kinetics. Thrombi are induced by many different pathogens and although the
136 role of thrombosis after infection remains unclear, the presumption must be that they are pathological

137 in some circumstances, particularly if they are large and/or embolise. Since we have shown that
138 thrombi develop in the venous system, they may form due to differences in local infection-associated
139 changes in blood flow and as part of altered vessel homeostasis. Perhaps the bigger clinical question is
140 about what controls their ultimate size and what triggers thrombus resolution as this may influence
141 whether thrombosis becomes clinically problematic. Therefore, for a known infection it may be
142 possible to target therapeutically those organs at greatest risk of developing thrombosis at particular
143 stages of infection. These findings deepen our understanding of the concept of immunothrombosis and
144 shows thrombi can form as a non-canonical haemostatic response to infection-driven inflammation
145 but not to capture bacteria.

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152 **Authorship**

153 Contribution: N.B.C. and M.P.T. designed and performed the experiments, analysed the data and
154 wrote the manuscript; A.F.L., M.Z., J.R.H., L.D.W.K., W.M.C. performed the experiments and
155 analysed the data; M.R.T., J.R. contributed vital reagents, experimental design and proofread the
156 manuscript; I.R.H. experimental design, novel reagents and proofread the manuscript; A.F.C. and
157 S.P.W. supervised the research, analysed the data, and wrote the manuscript.

158 **Conflict-of-interest disclosure:**

159 The authors declare no competing financial interests.

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198

199 **Figure legends**

200 **Figure 1. Thrombosis in the spleen and liver follows different kinetics after STm infection.** (A)

201 Frozen spleens from WT mice infected with 5×10^5 STm i.p were sectioned longitudinally to the hilum

202 ($\geq 1200 \mu\text{m}$ deep) and $5\text{-}\mu\text{m}$ sections were stained by IHC. Scans of stained spleen sections from day 0,

203 1, 7 and 21-infected mice show blood vessels identified with anti-CD31 in blue and thrombi with

204 anti-fibrin/fibrinogen in brown. Arrows identify individual thrombi. (B) Representative low power

205 images of spleen and liver sections stained for CD41 (platelets; blue) and fibrin/fibrinogen (brown)
206 from mice infected for 24 hours via the i.v. route with 5×10^5 STm SL3261. The left hand graph shows
207 the number of thrombi per spleen section for mice infected i.p. or i.v. The right hand graph shows the
208 number of thrombi per section in the spleen and liver from these i.v. infected mice. (C) Representative
209 low power images of spleen and liver sections stained for CD41 (platelets; blue) and fibrin/fibrinogen
210 (brown) from mice infected for 24 hours with the virulent 10^5 STm SL1344 strain. The left hand
211 graph shows the number of thrombi per spleen section for mice infected with SL3261 (attenuated) or
212 SL1344 (virulent). The right hand graph shows the number of thrombi per section in the spleen and
213 liver from mice infected with SL1344. (D) Representative scans at higher magnification of spleen
214 sections (upper panels) and liver sections (lower panels) from WT mice at day 0, 1, 7 and 21 post-
215 infection with 5×10^5 STm SL3261. Sections are stained for fibrin/fibrinogen (brown) and CD41
216 (blue). V=Vein, RP=Red Pulp, WP=White Pulp, T=Thrombus. (E) Quantification of numbers of
217 thrombi per spleen section (left graph) and the proportion of section area covered by thrombi (right
218 graph) at days 0, 1, 2, 7 and 21 after infection with 5×10^5 STm SL3261. Each point represents a single
219 mouse (Data are combined from 3 independent experiments); * $p < 0.05$, 1 way ANOVA. (F) Line
220 graph showing the level of thrombosis in the spleen (black line) and the liver (grey line) over the first
221 3 weeks of infection with 5×10^5 STm SL3261. The data are expressed as mean \pm S.E.M. from at least
222 4 mice per group combined from 3 independent experiments. (G) Quantification of thrombi in spleen
223 sections from PBS liposomes or clodronate liposome pre-treated mice, infected for 24 hours with
224 5×10^5 STm SL3261. Combined data from 2 experiments with a total of 8 mice in each group.
225 * $p < 0.05$. 2-tailed non-parametric t test. N.D.=Not detected.

226 **Figure 2. Detection of bacteria within thrombi.** Representative immunofluorescence
227 photomicrographs of (A) spleens and (B) livers from WT mice infected with 5×10^5 STm SL3261 for
228 0, 1, 7 and 21 days. (V=Vein, RP=Red Pulp, WP=White Pulp, T=Thrombus, MK=Megakaryocyte).
229 Fibrin, blue; CD31, white; CD41, red and STm, green indicated with white arrows. For both (A) and
230 (B) the second row shows a higher magnification image of the area identified by the white box. (C)
231 and (D) Frequency of detecting 0, 1, 2 or ≥ 3 bacteria in thrombi in sections from spleens and livers

232 respectively, infected with 5×10^5 STm SL3261 for 0, 1, 7 or 21 days. (E) and (F) Line graphs showing
233 the kinetics of thrombosis (black) and bacterial colonisation (gray) in spleens and livers respectively,
234 from mice infected with 5×10^5 STm SL3261 for 0, 1, 2, 7 or 21 days. Data are expressed as mean \pm
235 S.E.M. from 152 thrombi counted in spleens from day 1; 40 from day 7; and 18 from day 21-infected
236 mice. In liver, 411 thrombi were counted for day 7 and 23 for day 21 after infection. In each case
237 thrombi were counted from at least 4 mice per group and are combined from 3 independent
238 experiments. CFU= Colony-forming unit.