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1	Anti-thrombotic treatment enhances antibiotic efficiency
2	in a humanized model of meningococcemia
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4	Running title: Antibiotic resistance during Nm sepsis
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23 Abstract

24 Meningococcal infections remain particularly difficult to treat. Despite antibiotic therapy, the 25 state of the patients often rapidly deteriorates. Early clinical studies suggest that meningococci 26 acquire a form of resistance to antibiotic treatments during infections. Taking advantage of a 27 humanized animal model of infection, we confirm that adherent bacteria become highly 28 resistant to antibiotic treatments as early as 3-6 hours post infection, although fully sensitive in 29 vitro. Within this time frame, meningococci adhere to the endothelium via their type IV pili. 30 proliferate and eventually fill the vessel lumen. Using intravital imaging, we show that rapidly 31 upon infection blood flow is dramatically decreased, thus limiting antibiotic access to infected 32 vessels. Concomitantly, fibrin is deposited inside infected vessels in proximity to bacterial 33 aggregates. Pharmacologically impairing thrombin generation by inhibiting Factor X activity not 34 only improves blood flow in infected vessels, but also enhances the efficacy of the antibiotic 35 treatment. Our results indicate that the combined administration of anticoagulants together 36 with antibiotics might represent a therapeutic approach to treat meningococcal sepsis more 37 efficiently.

38 Introduction

39 Despite intensive medical care meningitis and purpura fulminans induced by Neisseria 40 meningitidis remain a major concern worldwide, reflecting our limited understanding of the 41 mechanisms underlying these diseases. Systemic forms of the infection are often the most 42 life-threatening as they account for up to 90% of the mortality associated to invasive 43 meningococcal diseases (IMD) (Brandtzaeg & van Deuren, 2012; van de Beek et al, 2006). 44 Meningococcal sepsis is characterized by specific clinical manifestations such as the 45 appearance of skin purpuric/petechial lesions and most importantly a particularly fast and 46 severe progression (Stephens, 2007; Thompson et al, 2006). Otherwise healthy patients 47 evolve from an absence of symptoms to being critically ill within 12 to 24 hours. Although 48 made difficult by the relatively unspecific clinical features at disease onset, the early 49 recognition and treatment of meningococcal systemic infections by parenteral administration 50 of antibiotics is key in determining patient outcome (Dellinger et al, 2013). Remarkably, 51 despite antibiotic administration, disease often progresses relentlessly towards septic shock 52 making these infections particularly difficult to treat. While it is generally considered that 53 treatments will be inefficient past a certain tipping point, the reasons for these failures remain 54 largely unexplained. A possible explanation would be that bacteria acquire a form of 55 resistance to the treatments at a certain point of infection. Accordingly, biopsies of skin 56 lesions performed on patients suffering from meningococcal septic shock revealed the 57 presence of live bacteria up to 13 hours after the initiation of the antibiotic treatment (de 58 Greeff et al, 2008; van Deuren et al, 2000; van Deuren et al, 1993). The mechanisms 59 underlying bacterial antibiotic resistance at play during meningococcal infections remains 60 elusive. Due to the human-species specificity of the bacterium, the lack of reliable animal 61 models of meningococcal infections has precluded any in-depth experimental studies of this 62 process. Today, this limitation has been overcome by the development of a human skin 63 xenograft mouse model that supports the anastomosis of dermal human blood vessels with 64 the murine circulatory system (Melican et al, 2013). This model recapitulates the hallmark

- 65 features of meningococcal systemic infections, such as the adhesion of bacteria to the
- 66 human endothelial surface and the occurrence of skin purpuric lesions, hence allowing the
- 67 exploration of *Nm* systemic infections in the full complexity of the host tissue *in vivo* (Bonazzi
- 68 *et al*, 2018; Melican *et al.*, 2013). Combining the use of such a model and intravital imaging,
- 69 we show that bacteria become resistant to antibiotic treatments as early as 3-6h post
- 70 infection and that this effect is due to a fast decrease in blood flow induced by the deposition
- 71 of fibrin inside infected vessels.

Results 72 73 Meningococci adhering along the vascular wall rapidly acquire antibiotic resistance 74 We first sought to evaluate whether the humanized model of meningococcal infection could 75 provide new information on the clinical observations pertaining to antibiotic resistance during 76 infection. In this model, intravenously injected Neisseria meningitidis circulate in the blood 77 stream and a fraction of bacteria specifically colonizes dermal human vessels in the grafted 78 skin, forming tight bacterial aggregates that progressively fill the infected human vessels (Fig 79 1A-B) (Manriquez et al, 2021; Melican et al., 2013). Bacterial sensitivity to intravenously 80 injected antibiotics was assessed in the animal model 3h, 6h and 16h post infection by 81 treating mice with Cefotaxime (CTX, Fig 1C), a standard of care antibiotic for meningococcal 82 infections in patients. Mice were treated with doses reflecting the concentration used in 83 patients (200 mg/kg) (Griffiths et al, 2018). As for most clinical meningococcal isolates, the 84 strain used for these experiments is sensitive to low doses of Cefotaxime when cultured in 85 medium containing serum in vitro (Fig EV1). Antibiotic administration led to a strong 86 reduction in the numbers of blood-circulating bacteria regardless the time post infection (Fig 87 1D). Numbers of adherent bacteria also decreased upon antibiotic treatment 3h post 88 infection. In contrast, 6 and 16h post infection, bacteria became strongly resistant to the 89 antibiotic treatment. While bacterial counts were reduced by up to 11 000-fold 3h post 90 infection (55 410 \pm 12 445 versus 5 \pm 3 CFU/mg of skin in non-treated versus CTX-treated 91 animals, respectively), 16h post infection bacterial counts only decreased by 12-fold (90 731 92 \pm 50 772 versus 7 642 \pm 2 200 CFU/mg of skin in non-treated versus CTX-treated animals, 93 respectively) (Fig 1E). Similar results were obtained at 6h post infection (Fig 1E). Taken 94 together, these data show that between 3h and 6h post infection, bacteria adhering to the 95 endothelium experience a change, either intrinsic or in their environment, which leads to 96 antibiotic treatment resistance. Given the ability of meningococci to occlude infected vessels 97 combined with their pro-coagulant activity, a straightforward hypothesis would be that a 98 reduction in microvascular patency could alter the access of antibiotics to bacteria.

99

100 Meningococcal vascular colonization leads to a rapid and complete block of blood 101 flow

102 Using intravital imaging, we next assessed whether meningococcal vascular colonization 103 alters vascular perfusion and consequently the proper access of antibiotics to sites of 104 infection. Blood flow velocities were assessed using 1 µm fluorescent microspheres injected 105 intravenously. High temporal resolution imaging with a spinning disk confocal microscope 106 allowed the visualization of beads freely circulating in human vessels of non-infected animals 107 (Fig 2A and Movie EV1). Beads circulated at average speeds of $1174 \pm 141 \mu m/s$ in the 108 observed arterioles (n = 31, average diameter 35 μ m) and 505 ± 219 μ m/s in venules (n = 109 13, average diameter 54 µm). Similar values were observed for the first 2 hours of infection 110 (Fig 2B). In contrast, a steep reduction was observed at 3 hours post infection in all infected 111 vessels, culminating in a complete absence of flow at 4 hours post infection (Fig 2B and 112 Movie EV1). No variation in the blood vessel diameter was observed during the infection (Fig 113 2C), indicating that blood flow reduction was not due to a global collapse of vessels. We 114 recently showed that meningococci can infect dermal arterioles and venules (Manriquez et 115 al., 2021) and the same dramatic decrease in blood flow was observed in infected venules 116 and arterioles (Fig 2D), suggesting that the effect observed is independent of the vessel type. 117 Taken together, our results indicate that by preventing the perfusion of infected vessels 118 starting at 3 hours post infection, vascular colonization by meningococci limits the 119 bioavailability of antibiotics, thus restraining their ability to eliminate intravascular adherent 120 bacteria. We then explored whether this blood flow reduction was due to the bacteria 121 themselves or secondary to intravascular coagulation or thrombosis. 122

123 Platelets are not involved in the acquisition of antibiotic resistance

124 Meningococcal systemic infections are associated with disturbed coagulation ranging from

125 vessel occlusion and thrombus formation to disseminated intravascular coagulation (DIC), as

126 evidenced by the post-mortem analysis of skin biopsies (Harrison et al, 2002; Pathan et al, 2003; Sotto *et al*, 1976). Similar observations have been recently made using the humanized
mouse model of meningococcal infections (Manriquez *et al.*, 2021). We therefore tested
whether infection-induced microvascular thrombosis might restrict the proper access of
antibiotics to adherent meningococci.

131 We first assessed the role of platelets in the process. Visualization of platelets by intravital 132 imaging showed limited platelet aggregation/accumulation, if any, at sites of bacterial 133 adhesion (Fig EV2A). Platelets could be found in spaces where no bacteria were found, and 134 small volumes of blood expected. Interestingly, no evidence of bacteria-platelet interactions 135 could be found, in contrast to what has been observed with other pathogenic bacteria 136 (Fitzgerald et al, 2006; Sun, 2006). We observed circulating platelets moving past bacterial 137 aggregates without evidence of adhesive contacts (Movie EV2). We then evaluated the 138 impact of platelet depletion on the efficiency of Cefotaxime treatment (Fig EV2B). To this 139 end, grafted mice were administered an antibody targeting the platelet receptor 140 GPIb α /CD42b 6h prior to the infection, as previously described (Bergmeier *et al*, 2000; 141 Nieswandt et al. 2000). This treatment led to the efficient depletion of platelets (Fig EV2C). 142 Antibiotic treatment was then administrated 16h post infection and bacterial counts assessed 143 3 hours later. As expected, low numbers of blood-circulating bacteria were found upon 144 antibiotic treatment when compared to the infected but non-treated control grafted animals 145 (Fig EV2D). Numbers of adherent bacteria following the antibiotic treatment were similar to 146 the numbers observed in non-depleted control animals (6 835 \pm 3 495 versus 7 642 \pm 2 200 147 CFU/mg of skin in platelet-depleted versus non-depleted animals, respectively) (Fig EV2E to 148 be compared to Fig 1E), suggesting that platelets do not limit the efficacy of antibiotic 149 treatment upon vascular colonization by *Neisseria meningitidis*. Taken together, these results 150 argue against a role for platelets in restricting the efficiency of antibiotic treatments upon 151 meningococcal systemic infections.

152

153 Coagulation inhibition restores antibiotic efficiency

154 Clinical and experimental evidence point to the presence of fibrin clots in the vicinity of 155 infected vessels (Faust et al, 2001; Guarner et al, 2004; Melican et al., 2013). Using intravital imaging, we therefore monitored the deposition of fibrin inside infected vessels during 156 157 vascular colonization by Neisseria meningitidis. In the first 3 hours following infection, no 158 evidence of fibrin deposition could be found. In contrast, starting 3 hours post infection we 159 observed the progressive accumulation of fibrin close to sites of bacterial adhesion (Fig 3A-160 B, Fig EV3A and Movie EV3). In absence of infection, fibrin deposition was not observed (n = 161 17 vessels). Thrombus formation thus coincides with the time of blood flow decrease and the 162 emergence of the resistance to the antibiotic treatment (Fig 1E and Fig 2B). Importantly, 163 fibrin deposition occurs in most infected vessels, reaching 64% coverage at 4h30 post 164 infection (Fig 3B). Interestingly, in patients, circulating monocytes have been shown to 165 express Tissue Factor (TF) during meningococcemia (Osterud & Flaegstad, 1983), 166 suggesting a role for TF in triggering disseminated intravascular coagulation (DIC). 167 Accordingly, TF expression on the surface of monocytes (CD115⁺ Gr-1⁺) from infected mice 168 increased with kinetics similar to microvascular fibrin deposition (Fig EV4A). In contrast, 169 endothelial cells did not show any increase in TF expression, even though E-Selectin 170 expression strongly increased, as shown previously (Manriquez et al., 2021) (Fig EV4B). 171 We next sought to determine whether infection-induced fibrin deposition negatively impacts 172 the efficacy of antibiotic therapy by inhibiting the thrombin-dependent cleavage of fibrinogen 173 into fibrin strands. Fondaparinux prevents the formation of fibrin clots by inhibiting Factor Xa. 174 and thus the conversion of prothrombin into active thrombin (Bauer et al, 2002). To ensure 175 the constant delivery of the anticoagulant treatment to grafted mice. Fondaparinux was 176 infused subcutaneously using osmotic minipumps. Grafted mice were then infected, and the 177 antibiotic treatment (Cefotaxime, CTX) was administrated 16h post infection (Fig 3C). As 178 expected. Fondaparinux strongly reduced plasma Factor Xa (Fig 3D). Interestingly, 179 Fondaparinux treatment per se did not alter, nor promote meningococci survival either in the 180 blood or adhering to the vessel walls (Fig EV3B-C). The antibiotic treatment also efficiently 181 decreased the numbers of circulating bacteria in conditions of Factor X inhibition (Fig 3E).

- 182 Strikingly, upon inhibition of coagulation and antibiotic treatment, numbers of adherent
- 183 bacteria in the human grafts were decreased by more than 500-fold when compared to mice
- 184 that did not received antibiotics (29 591 \pm 23 065 versus 52 \pm 45 CFU/mg of skin in
- 185 Fondaparinux- versus Fondaparinux+CTX-treated animals, respectively) (Fig 3F).
- 186 Accordingly, the perfusion of infected vessels was partially restored upon inhibition of
- 187 coagulation by Fondaparinux (Fig 3G-H and Movie EV4). Taken together these results
- 188 strongly suggest that meningococcal-induced fibrin deposition reduces microvascular
- 189 patency and thereby the tissue distribution of antibiotics, thus preventing their efficacy.

190 **Discussion**

191 Our results show that within a few hours of infection, meningococcal vascular colonization 192 leads to a dramatic reduction in the perfusion of infected vessels. While bacterial colonization 193 alone is sufficient to partially occlude vessels and reduce blood flow, ensuing thrombus 194 formation leads to the complete cessation of blood flow in affected vessels. This, in turn, has 195 important implications for disease progression. Complete absence of perfusion will result in 196 severe ischemia and irreversible tissue injury, driving the rapid progression of the disease 197 towards multiorgan failure. Another consequence of reduced microvascular perfusion, 198 detailed in this study, is its impact on the access of antibiotics to target tissues. This is 199 observed in the animal model described here but also in human cases where bacteria have 200 been demonstrated to resist intensive antibiotic treatment (van Deuren et al., 1993). 201 202 The molecular process driving thrombin generation during meningococcal infection has been 203 extensively studied clinically and in vitro but remains to be fully established (Lecuyer et al. 204 2017). Our results using a humanized model of Neisseria meningitidis systemic infection 205 show extensive fibrin deposition in close proximity to bacterial aggregates early during 206 infection and provide new insight into meningococci-induced thrombus formation. Our results 207 suggest that the process is largely independent of platelets, which do not stably interact with 208 meningococci, as opposed to many other bacterial infections (Fitzgerald et al., 2006; Sun, 209 2006). Rather, thrombus formation appears to be triggered by the extrinsic pathway of blood 210 coagulation through the rapid expression of TF/CD142 by monocytes. Accordingly, in 211 meningococcemia cases (Osterud & Flaegstad, 1983) and the humanized model used here, 212 monocytes express TF at early stages of infection. In contrast and despite massive local 213 bacterial accumulation, human endothelial cells did not display TF expression in vivo. 214 Interestingly, the contact (intrinsic) pathway of blood coagulation was also shown to be 215 activated during meningococcal infections (Wuillemin et al, 1995). The negative charges 216 present on the bacterial LOS or capsule could be a trigger of the contact pathway (Oehmcke

217 & Herwald, 2010). This could provide an alternative explanation for fibrin deposition in 218 proximity to bacterial colonies. In any case, vascular colonization by Neisseria meningitidis 219 leads to the rapid formation of thrombi that restrict the efficacy of antibiotic treatment. Many 220 pathogenic bacteria have evolved specific strategies to inhibit coagulation, a process that is 221 thought to favor bacterial dissemination (Popov et al, 1991). Meningococci do not seem to be 222 equipped to efficiently block thrombus formation, hence permitting the thrombotic occlusion 223 of infected vessels and the sealing of the infected regions by the host. This likely reflects the 224 main lifestyle of *Neisseria meningitidis* at the surface of the nasopharyngeal epithelium rather 225 than in the vasculature.

226

227 From the clinical point of view, our observations suggest that anticoagulation therapy could 228 potentially provide a synergistic effect in conjunction with antibiotic treatment for 229 meningococcal infections. A number of clinical studies have tested the benefits of 230 anticoagulants such as activated protein C in combination with antibiotics. Activated protein 231 C acts as a negative regulator of coagulation by proteolytically inactivating coagulation 232 cofactors Factor Va and Factor VIIIa. Unfortunately, the use of recombinant Protein C has 233 had variable success (Silva et al, 2010). Our study suggests that anticoagulation would 234 mainly be effective in the very early stages of infection and that direct targeting of Factor Xa 235 or thrombin may be more effective than Protein C. The efficacy of anticoagulation described 236 here is likely to be linked to the pathogenesis of meningococcal infections and more 237 specifically to the ability of this bacterium to adhere to the endothelium and colonize vessels. 238 The utility of anticoagulation is thus likely to depend on the pathogen. Although the process 239 described here is local, in close association with infected vessels, it is a host-driven response 240 and a likely precursor of DIC. The induction of local coagulation appears to represent a 241 tipping point in the progression of the disease after which antibiotic therapy is no longer as 242 efficient.

While our study suggests the potential of anticoagulant therapy for increased efficacy ofantibiotic treatment, it also argues that such therapy would have to be administered early in

the progression of the disease and that thrombolytic therapy is likely to be more efficacious 245 246 once the bacteria have taken hold and thrombi are established. Importantly, the use of 247 anticoagulant and fibrinolytic therapy is always associated with a potential risk of bleeding 248 that will need to be carefully assessed. Although anticoagulation has been used in the 249 context of sepsis in the past (Levi et al, 2001; Silva et al., 2010), the strong implication of the 250 cerebral vasculature render safety considerations particularly important for meningococcal 251 infections. That said, experience from ischemic stroke shows that such risks can be 252 managed by the use of strict inclusion criteria based on timing and individual risk/benefit

assessment.

254 Materials and Methods

Mice. SCID/Beige (CB17.Cg-*Prkdc^{scid}Lyst^{bg-J}*/Crl) mice were purchased from Charles Rivers 255 256 (France) and housed under specific pathogen-free condition at Institut Pasteur. Mice were 257 kept under standard conditions (light 07.00-19.00h; temperature 22±1°C; humidity 50±10%) 258 and received sterilized rodent feed and water ad libitum. All experiments were performed in 259 agreement with guidelines established by the French and European regulations for the care 260 and use of laboratory animals and approved by the Institut Pasteur committee on Animal 261 Welfare (CETEA) under the protocol code DAP 180022. For all experiments, male and 262 female mice between 9 and 16-weeks of age were used. Littermates were randomly 263 assigned to experimental groups. 264 Human skin. Normal human skin was obtained from adult patients (20-60 years old), both 265 males and females, undergoing plastic surgery in the service de chirurgie reconstructrice et 266 plastique of Groupe Hospitalier Saint Joseph (Paris, France). In accordance with the French 267 legislation, patients were informed and did not refuse to participate in the study. All 268 procedures were approved by the local ethical committee Comité d'Evaluation Ethique de 269 I'INSERM IRB 00003888 FWA 00005881, Paris, France Opinion: 11-048. 270 Xenograft model of infection. 6-13 weeks old mice, both males and females, were grafted 271 with human skin as previously described (Melican et al., 2013). Briefly, mice were 272 anesthetized with a mixture of ketamine hydrochloride (100 mg/kg, Boehringer Ingelheim) 273 and xylazine hydrochloride (8.5 mg/kg, Bayer) and a graft bed of approximately 1-2 cm² was 274 prepared on their flank by removing the mouse epithelium and the upper dermis layer. A 275 human skin graft (200 µm thick) comprising the human epidermis and the papillary dermis 276 was immediately placed over the graft bed. Grafts were fixed in place with surgical glue 277 (Vetbond, 3M, USA) and dressings were applied for 2 weeks. Grafted mice were used for 278 experimentation 3-6 weeks post-surgery when the human dermal microvasculature is 279 anastomosed to the mouse circulation without evidence of local inflammation, as previously 280 described (Melican et al., 2013). All efforts were made to minimize suffering.

281 Neisseria meningitidis strains and mouse infection. Experiments were performed using *N. meningitidis* 8013 serogroup C strain (http://www.genoscope.cns.fr/agc/nemesys) 282 283 (Rusniok et al, 2009). Strains were streaked from -80°C freezer stock onto GCB agar plates 284 and grown overnight in a moist atmosphere containing 5% CO₂ at 37°C. For all experiments, 285 bacteria were transferred to liquid cultures in pre-warmed RPMI-1640 medium (Gibco) 286 supplemented with 10% FBS at adjusted OD_{600nm} = 0.05 and incubated with gentle agitation 287 for 2 hours at 37°C in the presence of 5% CO₂. Bacteria were washed twice in PBS and 288 resuspended to 10⁸ CFU/ml in 1x PBS. Prior to infection, mice were injected intraperitoneally 289 with 8 mg of human transferrin (Sigma Aldrich) to promote bacterial growth in vivo as 290 previously described (Melican et al., 2013). Mice were infected by retro-orbital injection of 291 100 µl of the bacterial inoculum (10⁷ CFU total). When indicated, mice received a retro-orbital 292 injection of 4 mg Cefotaxime (CTX, #C7912, Sigma-Aldrich) at 3h, 6h or 16h post infection 293 and 3h prior to mouse sacrifice. No influence of mice sex on bacterial colonization have been 294 observed. 295 Wholemount immunofluorescent staining. Following mouse infection with mCherry-

296 expressing Neisseria meningitidis for 9 hours, the human grafts were harvested and fixed in 297 4% PFA overnight at 4°C with gentle agitation. Skin samples were then washed and 298 permeabilized/blocked overnight at 4°C with gentle agitation using a solution of 0.3% Triton-299 X100, 1% BSA, 1% normal goat serum in 1X PBS. The basal lamina of human blood vessels 300 was stained using an AlexaFluor647-conjugated anti-human Collagen IV antibody (#51-301 9871-82, eBioscience, 1/100) diluted in permeabilization/blocking solution and incubated 3 302 days at 4°C with gentle agitation. Samples were then extensively washed and tissue 303 transparization and mounting were performed using RapidClear 1.47 (SunJin Lab) according 304 to manufacturer's recommendations.

305 *Neisseria meningitidis* growth curves. Bacterial liquid culture was diluted to an OD_{600nm} of
306 0.05 and 150 µl of the bacterial suspension was transferred into wells of a 96-well plate.
307 Bacteria were then treated or not with the indicated final concentration of Cefotaxime, each
308 performed in quintuplicates. Plates were placed in a Cytation 5 multimode reader (Biotek) at

309 37°C with 5% CO₂ and continuous agitation and optical densities at 600 nm were recorded
310 every 30 minutes for 20 hours.

311 Colony-forming units (CFU) enumeration. To assess bacteraemia (blood-circulating 312 bacteria) in infected animals, 10 µl of blood was sampled at the time of sacrifice. Serial 313 dilutions of blood were plated on GCB agar plates and incubated overnight at 37°C and in a 314 moist atmosphere containing 5% CO₂. Bacterial counts were expressed in colony-forming 315 units (CFU) per ml of blood. To assess the extent of vascular colonization by meningococci 316 (adherent bacteria) following mouse sacrifice at indicated times post infection, tissue biopsies 317 were collected using a sterile dermatological biopsy puncher (approximately 4 mm²), 318 weighted and placed in 500 µl 1x PBS. Skin biopsies were dissociated and homogenized 319 using a BeadMill4 homogenizer (Fisher Brand) and serial dilutions of skin homogenates were 320 plated on GCB plates incubated overnight at 37°C and in a moist atmosphere containing 5% 321 CO₂. Bacterial counts were expressed in colony-forming units (CFU) per mg of skin. 322 **Platelet depletion.** Platelets were depleted in grafted mice by retro-orbital injection of 40 µg 323 of anti-mouse GPIba (CD42b) (#R300, Emfret Analytics) monoclonal antibodies diluted in 324 100 µl 1x PBS 6 hours prior to infection, as previously described (Bergmeier et al., 2000; 325 Nieswandt et al., 2000). The efficiency of platelet depletion was assessed by counting 326 platelets on citrated blood using an automated hemocytometer (SCIL Vet ABC plus) 327 according to manufacturer's instructions.

328 Spinning disk confocal intravital imaging. Intravital imaging of the human xenograft was 329 adapted from (Ho et al, 2000) and previously described (Manriquez et al., 2021). Briefly, 30 330 min prior to surgery, mice were injected subcutaneously with buprenorphine (0.05 mg/kg, 331 CEVA) and anesthetized by spontaneous inhalation of isoflurane in 100% oxygen (induction: 332 4%; maintenance: 1.5% at 0.3 L/min). A flap of skin supporting the human graft was 333 immobilized onto a custom-made heated deck (36°C) and continuously moistened with 334 warmed 1x PBS (36°C). Mouse body temperature was maintained at 37°C using a heating 335 pad and oxygen saturation and heart rate were monitored using the pulse oximetry

336 Physiosuit apparatus (Kent Scientific). Image acquisition was performed using a Leica DM6

337 FS upright microscope equipped with a motorized stage, a Fluotar 25x/0.95 objective (Leica), 338 and coupled to a Yokogawa CSU-W1 confocal head modified with Borealis technology 339 (Andor). Four laser excitation wavelengths (488, 561, 642, and 730 nm) were used in fast 340 succession and visualized with the appropriate long-pass filters. Fluorescence signals were 341 detected using a sCMOS 2048x2048 pixel camera (Orca Flash v2+, Hamamatsu). 342 Metamorph acquisition software (Molecular devices) was used to drive the confocal 343 microscope. 344 The human vessels were labelled using Dylight755-conjugated UEA-1 lectin (100 µg, Vector 345 Laboratories), platelets using DvLight488-anti-mouse GPIb_B antibody (0.1 µg, #X488, Emfret 346 Analytics) and fibrin deposits using DyLight488-anti-human/mouse fibrin antibody (8 µg, 347 clone 59D8, Sigma-Aldrich). All antibodies and dyes were injected intravenously 15 min prior 348 to intravital imaging. Five to ten fields of view of interest were selected per animal and time-349 lapse z-stack series (2-2.5 µm z-step, 50-80 µm range) were captured every 30-60 min for 4-350 5 hours following the intravenous injection of iRFP-expressing bacteria (10^7 CFU/100 µl 1x 351 PBS). In mice treated with anticoagulant, Fondaparinux was injected subcutaneously 5 min 352 prior to imaging (80 µl, 1 mg). 353 The blood flow during vascular colonization was monitored by perfusing 1 µm diameter 354 fluorescent microspheres (Yellow/Green Fluoresbrite carboxylate, Polysciences, 10⁷ 355 microspheres/ml 1x PBS) at a rate of 15 µl/min before each z-stack series and images 356 acquired on a single plane at high speed (50 frames per second, 300 frames). Blood flow 357 was calculated from the vessel diameter and the centerline microsphere mean velocity, 358 according to (Hidalgo et al, 2009), and normalized with respect to the basal blood flow. 359 Platelet dynamics during vascular colonization was assessed by fast (50 frames/sec) 360 simultaneous dual-color imaging of platelets and bacteria. 361 Anticoagulant treatment. The inhibition of coagulation was achieved by treating grafted

mice with the factor Xa inhibitor Fondaparinux (ARIXTRA[®], ASPEN) prior to and/or during
the course of the infection. For short-term infection (6h and 9h), grafted mice received a
subcutaneous injection of 80 µl Fondaparinux (corresponding to 1 mg) immediately after the

365 intravenous injection of bacteria and 3h and 6h post infection. For long-term infection (19h), 366 grafted mice received a subcutaneous injection of 80 µl Fondaparinux (corresponding to 1 367 mg) immediately after the infection and the constant delivery (infusion) of Fondaparinux was 368 then ensured by ALZET[®] osmotic pumps (200 µl, 8 µl/h during 24h, #2001D) filled with 200 369 µl Fondaparinux and placed subcutaneously, according to manufacturer's instructions. The 370 efficiency of the anticoagulant treatment was assessed using a chromogenic enzymatic 371 method (Biophen Heparin 6 kit, #221006, HYPHEN Biomed), according to manufacturer's 372 instructions.

373 Flow cytometry. To monitor the surface levels of Tissue Factor and E-Selectin on human 374 endothelial cells, biopsies of human xenograft harvested at the indicated time post infection 375 were digested with 0.4 mg/ml Liberase TL (Sigma Aldrich) in CO₂-independent medium for 376 60 min at 37°C with gentle agitation (100 rpm). The resulting single-cell suspension was passed through a 70-µm cell strainer (BD Bioscience), washed once with FACS buffer (1× 377 378 PBS supplemented with 0.5% BSA and 2 mM EDTA) and stained in FACS buffer according 379 to standard protocols. Briefly, human and murine Fc receptors were blocked using Human 380 TruStain FcX (Biolegend, #422302, 1/20) and anti-mouse CD16/CD32 (FcBlock clone 2.4G2, 381 BD Biosciences, #553141, 1/200), respectively, and cells were stained for 30 min at 4°C with 382 the following anti-human antibodies: PerCP-Vio700-conjugated REAfinity recombinant anti-383 CD31 (Miltenyi Biotec, clone REA730, #130-110-673, 1/20), BV421-conjugated anti-Tissue 384 Factor/CD142 (BD Biosciences, clone HTF-1, #744003, 1/20), and PE-conjugated anti-385 CD62E/E-Selectin (eBioscience, clone P2H3, #12-0627-42, 1/20). 386 To monitor the surface levels of Tissue Factor on circulating murine monocytes, blood was 387 harvested at the indicated time post infection and treated with 1x RBC lysis solution 388 (BioLegend). Murine Fc receptors were blocked using anti-mouse CD16/CD32 (FcBlock 389 clone 2.4G2, BD Biosciences, #553141, 1/200) and cells were stained in FACS buffer for 390 30 min at 4°C with the following anti-mouse antibodies: PacificBlue-conjugated anti-CD45 391 (Biolegend, clone 30-F11, #103126, 1/100), PE-Cy7-conjugated anti-CD11b (BD 392 Biosciences, clone M1/70, #552850, 1/200), APC-conjugated anti-CD115 (Invitrogen, clone

- 393 AFS98, #17-1152-82, 1/100), BV650-conjugated anti-Gr-1 (Biolegend, clone RB6-8C5,
- #108441, 1/50), and PE-conjugated anti-Tissue Factor/CD142 (R&D Systems, polyclonal,
 #FAB3178P, 1/50).
- 396 After staining, cells were washed twice in 1× PBS and fixed for 15 min at 4 °C with 1%
- 397 paraformaldehyde in 1× PBS. Data were acquired using a CytoFLEX S flow cytometer
- 398 controlled with the CytExpert software (Beckman Coulter). Data analysis was carried out
- 399 using FlowJo software v10 (Tree Star).
- 400 **Statistics.** All graphs and statistical analyses were performed with GraphPad Prism 9
- 401 (GraphPad Software). No statistical method was used to predetermine sample size.
- 402 Kolmogorov–Smirnov test was used to assess the normality of all data sets. Scatter dot
- 403 plots, bar graphs and values are provided as the mean ± sem. P-values were considered as
- 404 statistically significant when inferior at 0.05. Statistical details of experiments (sample size,
- 405 replicate number, statistical significance) can be found in the figures, figure legends and
- 406 source data file.
- 407

408 Data availability

409 This study includes no data deposited in external repositories.

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415

416 **Author contributions**

- 417 G.D. designed research and supervised overall data analysis; J.-P.C., P.N., A.Y. and D.O.
- 418 performed and analyzed experiments; T.S. provided human skin samples; D.O. assembled
- 419 figures; D.O. and G.D. wrote the manuscript.

420

421 **Conflict of interest**

422 The authors declare no conflict interests.



424 425

426 Figure 1 - Limited antibiotic efficiency during Neisseria meningitidis vascular

427 colonization

- 428 A Schematic representation of the human skin xenograft mouse model.
- 429 B Representative whole-mount immunofluorescence pictures (maximum intensity z-
- 430 projection) of the colonization of human vessels (grey, anti-human Collagen IV) by mCherry-
- expressing Neisseria meningitidis (green) in grafted animals 9 hours post infection (p.i.). 431
- 432 Scale bar: 40 μ m. (n = 3 mice).
- 433 **C** Schematic representation of the experimental approach used to assess bacterial sensitivity
- 434 to Cefotaxime (CTX) treatment at 3h, 6h and 16h post infection (p.i.).
- 435 D, E Bacterial colony forming unit (CFU) counts from (D) blood (circulating bacteria) and (E)
- 436 dissociated human xenografts (adherent bacteria) collected from mice infected for the
- 437 indicated time points and treated (+) or not (-) with Cefotaxime (CTX). (n = 12, 6 and 10 mice
- 438 per group at 3h, 6h and 16h p.i., respectively, pooled from N = 3 independent experiments 439 per time point).
- 440 Data information: In (D-E), data are presented as the mean ± SEM. Kruskal-Wallis test with
- 441 Dunn's correction for multiple comparisons.



442 443

444 Figure 2 – Vascular colonization by *Neisseria meningitidis* blocks the blood flow

445 **A** Representative image of fluorescent microsphere tracking as a read-out of blood flow

446 during the early phase of the infection (0h and 4h p.i.). Tracks were color-coded according to

447 the mean velocity of the corresponding microsphere. Inset shows the extent of vascular

colonization by iRFP-expressing *Nm* (green) 4h post infection. Blue arrowheads indicate
 immobilized microspheres. Scale bar: 50 μm.

- 450 **B, C** Normalized blood flow (**B**) and vessel diameter (**C**) as a function of the time post
- infection. (n = 18 and 19 vessels for non-infected and infected animals, respectively, pooled
 from N = 2 mice per condition and imaged independently).
- 453 **D** Data in (B) were classified according to the vessel type: venules (left) and arterioles (right).
- 454 (n = 13 and 14 arterioles, and 5 and 5 venules in non-infected and infected mice,
- 455 respectively).
- 456 Data information: In (B-D), data are presented as the mean ± SEM. Two-way ANOVA test.



- 457
- 458

459 Figure 3 - The efficiency of antibiotic treatments is restored upon inhibition of

460 coagulation

- 461 A Representative image (maximum intensity z-projection) and orthogonal view of fibrin
- 462 deposition (red, anti-Fibrin) in human vessels (grey, UEA1 lectin) infected by iRFP-
- 463 expressing *Neisseria meningitidis* (green). Scale bar: 50 μm. (n = 28 infected vessels, pooled
- 464 from N = 3 mice imaged independently).

- 465 **B** Percentage of vessels with fibrin deposition in control and infected animals (n = 28 infected
- 466 vessels and 17 non-infected control vessels, pooled from N = 3 and 2 mice, respectively,
- 467 imaged independently).
- 468 **C** Schematic representation of the experimental approach used to assess the impact of
- 469 coagulation inhibition on bacterial sensitivity to Cefotaxime (CTX) treatment.
- 470 **D** Chromogenic-based quantification of the coagulation Factor Xa in the plasma of control
- 471 (no Fondaparinux, no Cefotaxime), Fondaparinux-treated (no Cefotaxime) and
- 472 Fondaparinux+CTX-treated mice. (n = 8, 10 and 10 mice for control, Fondaparinux-treated,
- and Fondaparinux+CTX-treated mice, respectively, pooled from N = 3 independentexperiments).
- 475 **E**, **F** Bacterial colony forming unit (CFU) counts from (E) blood (circulating bacteria) and (F)
- 476 dissociated human xenografts (adherent bacteria) collected from mice treated or not with the
- 477 Fondaparinux anticoagulant and infected for 19h and treated or not with Cefotaxime (CTX).
- 478 (n = 9 mice per group, pooled from N = 3 independent experiments).
- 479 **G** Representative images (maximum intensity z-projection) of fluorescent microsphere
- 480 tracking in human vessels (white dashed line) infected by iRFP-expressing bacteria (green,
- 481 inset) in mice treated with Fondaparinux. Tracks were color-coded according to the mean
- 482 $\,$ velocity of the corresponding microsphere. Scale bar: 50 $\mu m.$ (n = 3 mice).
- 483 **H** Violin plot showing the distribution of the blood flow in vessels from non-infected, infected,
- 484 and infected+Fondaparinux-treated animal 4h post infection. (n = 16, 19 and 13 vessels for
- 485 non-infected, infected, and infected+Fondaparinux, respectively, pooled from N = 2, 2 and 3
- 486 mice for control, infected, and infected+Fondaparinux-treated animals, respectively).
- 487 Data information: In (D), data are presented as the mean ± SEM. Kruskal-Wallis test with
 488 Dunn's correction for multiple comparisons.
- 489 In (E-F), data are presented as the mean ± SEM. Two-tailed Mann-Whitney test.
- 490 In (H), data are presented as violin plot showing the distribution of the dataset. Kruskal-Wallis
- 491 test with Dunn's correction for multiple comparisons.



- 494 Figure EV1 *Neisseria meningitidis* is sensitive to low doses of Cefotaxime *in vitro*
- 495 Growth curves of *N. meningitidis* in the presence of the indicated final concentrations of
- 496 Cefotaxime. (N = 1 experiment). Data are presented as the mean ± SEM of quintuplicates.



497 498

499 Figure EV2 - Platelets do not prevent the efficiency of the antibiotic treatment

- 500 A Representative image (maximum intensity z-projection) and orthogonal view of platelets
- 501 (purple, anti-GPIb β) in bacteria-free spaces in human vessels (grey, UEA1 lectin) infected by
- 502 iRFP-expressing *Neisseria meningitidis* (green) for 4 hours. Scale bar: 25 μm. (n = 3 mice).
- 503 **B** Schematic representation of the experimental approach used to assess the impact of
- 504 platelet depletion on bacterial sensitivity to Cefotaxime (CTX) treatment.
- 505 **C** Quantification of platelet numbers in the blood of mice treated (+) or not (-) with the
- 506 platelet-depleting antibody directed against GPIb α . (n = 6 and 12 mice for control and anti-
- 507 GPIb α -treated mice, respectively, pooled from N = 2 independent experiments).
- 508 **D**, **E** Bacterial colony forming unit (CFU) counts from (**D**) blood (circulating bacteria) and (**E**)
- 509 dissociated human xenografts (adherent bacteria) collected from platelet-depleted mice (anti-
- 510 GPIb α) infected for 19h and treated (+) or not (-) with Cefotaxime (CTX). (n = 6 mice per
- 511 group, pooled from N = 2 independent experiments).
- 512 Data information: In (C), data are presented as the mean \pm SEM. Unpaired t test.
- 513 In (D-E), data are presented as the mean \pm SEM. Two-tailed Mann-Whitney test.



514 515

516 Figure EV3 - The efficiency of the antibiotic treatment is restored upon coagulation 517 inhibition

- 518 A Representative time sequence (maximum intensity z-projection) of fibrin deposition (red,
- 519 anti-Fibrin) in human vessels (dashed line) infected by iRFP-expressing *Neisseria*
- 520 *meningitidis* (green). Scale bar: 50 μ m. (n = 28 infected vessels, pooled from N = 3 mice 521 imaged independently).
- 522 **B**, **C** Bacterial colony forming unit (CFU) counts from (B) blood (circulating bacteria) and (C)
- 523 dissociated human xenografts (adherent bacteria) collected from control (-) and
- 524 Fondaparinux-treated (+) mice infected for 5 minutes or 6 hours. (n = 7 mice per group,
- 525 pooled from N = 2 independent experiments).
- 526 Data information: In (B-C), data are presented as the mean ± SEM. (B) Kruskal-Wallis test
- 527 with Dunn's correction for multiple comparisons. (C) Two-tailed Mann-Whitney test.



528 529

530 Figure EV4 - Early upregulation of Tissue Factor at the surface of circulating 531 monocytes upon meningococcal vascular colonization

- 532 A Representative flow cytometry analysis (left) and quantification (right) of the cell surface
- 533 expression of murine Tissue Factor (CD142) on blood-circulating monocytes (gated as
- 534 $CD45^+ CD11b^+ CD115^+ Gr-1^+$) in non-infected control mice (black) and mice infected for 6h 535 (red). (n = 3 mice).
- 536 **B** Representative flow cytometry analysis (left) and quantification (right) of the cell surface
- 537 expression of human Tissue Factor (CD142, Top) and human E-Selectin (CD62E, bottom)
- 538 on CD31⁺ human endothelial cells isolated from human skin xenografts in non-infected
- 539 control mice (black) and mice infected for 6h (red). (n = 3 mice).
- 540 Data information: In (A-B), data are presented as the mean \pm SEM and were normalized with
- 541 respect to the mean fluorescence intensity (MFI) of the non-infected controls.

542 Movie legends

543

544 Movie EV1. Altered blood flow during *Neisseria meningitidis* infection

545 High-speed (50 frames/sec) intravital imaging (single focal plane) of 1 µm fluorescent

546 microspheres (white) intravenously perfused allowing the visualization and quantification of

547 the blood flow in human blood vessels (UEA1 lectin, gray) at the indicated time points in

infected and non-infected control mice. The extend of vascular colonization by iRFP-

549 expressing *Neisseria meningitidis* (green) is shown as a maximum intensity z-projection.

550 Scale bar: 50 μm. 551

552 Movie EV2. Dynamics of platelets during *Neisseria meningitidis* infection

- 553 Simultaneous dual color intravital imaging (single focal plane) of iRFP-expressing *Neisseria* 554 *meningitidis* (green) and platelets (anti-GPIbβ, magenta) in a human vessel (white dashed 555 line) 2h post infection. Scale bar: 50 μm.
- 556

Movie EV3. Intravascular fibrin deposits in *Neisseria meningitidis* infected human vessels

3D volume rendering of a fibrin deposit (anti-Fibrin, red) in a human vessel (UEA-1 lectin,

gray) infected with iRFP-expressing *Neisseria meningitidis* (green) for 5 hours. Scale bar: 30
 μm.

562 µ

563 Movie EV4. Coagulation inhibition restores the blood flow in *Neisseria meningitidis*

564 infected vessels

565 High-speed (50 frames/sec) intravital imaging (single focal plane) of 1 µm fluorescent

566 microspheres (white) intravenously perfused allowing the visualization and quantification of

the blood flow in human blood vessels (UEA1 lectin, gray) before (t0h00) and after (t4h00)

568 infection upon anticoagulant (Fondaparinux) treatment. The extend of vascular colonization

- 569 by iRFP-expressing *Neisseria meningitidis* (green) is shown as a maximum intensity z-
- 570 projection. Scale bar: 50 µm.

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