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1 **Boosting Toll-like receptor 4 signaling enhances the therapeutic outcome of antibiotic therapy in**
2 **pneumococcal pneumonia**
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18 Running title: TLR4-mediated boosting of antibiotic in pneumonia
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20 **ABSTRACT**

21 The emergence and spread of antibiotic resistance emphasize the need for alternative treatment strategies
22 against bacterial infections. Boosting the host innate immunity is not only readily deployable in most individuals
23 but can also mobilize many different antibacterial defenses. This study tested the hypothesis whereby
24 stimulation of the innate immune receptor Toll-like receptor 4 (TLR4) can be combined with antibiotics in the
25 treatment of invasive pneumonia. In a mouse model of *Streptococcus pneumoniae* infection, a single oral
26 administration of low-dose amoxicillin (AMX) or the systemic delivery of monophosphoryl lipid A (MPLA, a
27 clinically-approved TLR4 activator) decreased the bacterial load in lung and spleen, although this was not
28 sufficient for long-term survival. In contrast, a single treatment with a combination of MPLA and AMX induced
29 significant bacterial clearance with little to no regrowth over time, and was associated with longer survival.
30 Upregulation of genes related to granulocyte infiltration in lung tissue and elevation of blood levels of pro-
31 inflammatory cytokines was immediate and transient in MPLA-treated mice; this indicates activation of the
32 innate immune system in a context of infection. Combination treatment was associated with a well-preserved
33 lung tissue architecture and more rapid recovery from inflammation - suggesting that immune activation by
34 MPLA does not exacerbate pneumonia-induced damage. After AMX administration, plasma AMX
35 concentrations rapidly reached the maximum and declined, whereas the downstream effects of MPLA extended
36 beyond AMX elimination; these findings suggested a two-step effect. Our results demonstrated that leveraging
37 host innate immunity increases the efficacy of antibiotic therapy in bacterial pneumonia.

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39 **INTRODUCTION**

40 The discovery and development of antibiotics in the 20th century was a major turning point in medicine;
41 it enabled the successful treatment and/or prevention of many infectious diseases in humans and in other
42 animals. Decades later, these drugs are back in the headlines but for the wrong reasons: the alarming decline in
43 their therapeutic effectiveness and the spread in antimicrobial resistance (AMR). The latter is a major threat to
44 human health because it compromises our ability to treat bacterial infections and to carry out medical procedures
45 that rely on prophylactic antibiotic use, such as chemotherapy, transplantation, and surgery (1). A single
46 pathogen can express multiple resistance mechanisms, which in turn can often confer protection against several
47 classes of antibiotics. In a clinical setting, this usually necessitates treatment with a "last resort" antibiotic or
48 with combinations of antibiotics. In 2015, a report from the World Health Organization raised concerns about
49 the lack of new antibiotics in development (2). This emphasizes the need to come up with innovative anti-
50 infective approaches for treating resistant pathogens and preventing the further dissemination of AMR.

51 Advances in medicine and technology have provided deeper insights into immunology and thus
52 increased the viability of host-directed therapeutic strategies. For example, the targeted stimulation of innate
53 immunity using immunomodulatory drugs has gained much attention in recent years (3). This approach has
54 three main advantages: (i) it makes use of universal, built-in machinery that is ready to activate in most
55 individuals, (ii) it has both anti-infective and pro-recovery effects, and (iii) the complex innate immune system
56 mobilizes many different effectors through a tightly coordinated string of events, and thus counters the potential
57 development of AMR by invading pathogens. The host's recognition of immediate danger is instrumental in
58 mounting a successful defense against invading pathogens. *In vivo*, many cell types are equipped with pattern-
59 recognition receptors; for example, Toll-like receptors (TLRs) are present on many cell types and are capable
60 of binding to conserved macromolecules expressed by microorganisms (4). Thus, TLR engagement by an
61 agonist triggers signaling cascades that lead to the transcriptional activation of immune genes and the regulation
62 of antibacterial mechanisms for eliminating the threat. Signaling by TLRs triggers the production of various
63 chemokines and antimicrobial compounds, activates complement, and stimulates leukocyte differentiation and
64 mobilization. In view of the TLR-dependent response mechanisms' early involvement in host defense, their
65 robust activation and their highly inducible nature, researchers have sought to design novel or improved current

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66 therapies against viral and bacterial infections (3, 5). Monophosphoryl lipid A (MPLA) is a derivative of the
67 immunostimulatory lipid A component of the outer-membrane-expressed lipopolysaccharide (LPS) from the
68 bacterium *Salmonella minnesota* R595 (6). Lipopolysaccharide itself is highly toxic, due to its ability to strongly
69 activate TLR4 downstream signaling at low doses via both of the receptor's adaptor proteins, namely myeloid
70 differentiation primary response 88 (MyD88) and Toll/interleukin-1 receptor domain-c activation protein
71 inducing interferon beta (TRIF). In contrast, activation of TLR4 by MPLA is biased towards TRIF-dependent
72 TLR4 signaling, making MPLA safe for use in humans (7). Monophosphoryl lipid A induces a significant but
73 attenuated innate immune response (8-10); this property is related to the differences in its molecular structure
74 vs. LPS (11, 12). The combination of immunostimulatory activity and low toxicity make MPLA an attractive
75 candidate for therapeutic use in humans. Years of research have paved the way to MPLA becoming the first
76 TLR agonist to be licensed as an adjuvant in certain vaccine formulations (12-14). Despite the growing body
77 of literature data (indicating continued interest in finding further applications for MPLA), most studies have
78 focused on its use as a prophylactic treatment. Indeed, MPLA has been shown to confer protection against
79 infections by (i) *Pseudomonas aeruginosa* in burn-wounds, (ii) *Staphylococcus aureus* under post-hemorrhagic
80 conditions, and (iii) nontypeable *Haemophilus influenzae* in the nasopharynx (15-18). In view of these
81 observations, we hypothesized that MPLA may be a viable treatment against an ongoing bacterial infection,
82 and so looked into both its applicability and efficacy as an anti-infective therapy. To the best of our knowledge,
83 the only other previous study of this approach was performed in the context of fungal infection (19).

84 In order to tackle the challenges of developing alternative therapeutic strategies against bacterial
85 infections and combating the spread of AMR, we designed and performed the present proof-of-concept study.
86 The prime objective was to establish whether host immune responses can be leveraged to achieve a successful
87 treatment outcome. Using a previously established murine model of invasive pneumococcal disease (20, 21),
88 we determined whether deliberately TLR4-activated innate immune responses can constitute an adjunct to
89 standard antibiotic therapy, improve the latter's efficacy, and/or promote quicker tissue recovery after an
90 infection. To this purpose, the study investigates the MPLA effect on amoxicillin (AMX), a beta-lactam
91 antibiotic used as first-line treatment against *S. pneumoniae*.

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92 **RESULTS**

93 **The combination of MPLA and amoxicillin increases *S. pneumoniae* clearance and extends survival**

94 Intraperitoneal administration of MPLA to naïve animals (0.5 to 50 µg per mouse) increased the mRNA and
95 protein levels of inflammatory mediators - indicating MPLA's ability to induce innate immune responses three
96 hours post-administration (**Figure S1**). The magnitude of the systemic immune responses (i.e. in the liver and
97 blood) was strongly dependent on the dose of MPLA. Innate immune responses were also observed in the lungs
98 after the systemic injection of MPLA, albeit only at the highest dose (50 µg). This result suggests that the
99 systemic administration of MPLA promotes both systemic and pulmonary immune responses - a feature that
100 could potentially be exploited in the host-directed therapy of respiratory infectious diseases.

101 We next looked at whether MPLA's immunomodulatory effects impacted the bacterial load during
102 pneumococcal infection in mice when the TLR4 activator was administered together with AMX, a first-line
103 treatment against *S. pneumoniae*. Twelve hours after intranasal inoculation with *S. pneumoniae*, Swiss (CD-1)
104 mice received either a sub-curative dose of AMX (10 µg per animal; 0.4 mg/kg, administered by oral gavage),
105 MPLA (50 µg per animal; 2.0 mg/kg, administered by intraperitoneal injection) or a combination of the two
106 treatments (AMX+MPLA). Bacterial counts in the lungs and spleen were determined at different time points as
107 surrogate markers of pneumonia and bacterial dissemination, respectively (**Figure 1**). At 24 hours post-
108 infection, the bacterial loads in the lungs and spleens were lower in all treated animals than in mock-treated
109 animals; however, the differences between the three treatments were not statistically significant (**Figure 1B-**
110 **D**). In contrast, we observed significant intergroup differences in the bacterial loads at 48 hours post-infection
111 (**Figure 1B-C and 1E**). Bacterial clearance was greatest in the AMX+MPLA group, with a median CFU value
112 of 5.1×10^2 in the lungs, and nearly undetectable bacterial levels in the spleen; this can be compared with values
113 of 3.6×10^7 and 1.9×10^6 CFU recorded in the lungs and spleen of mock-treated infected animals, respectively
114 (corresponding to 7.1×10^4- and 1.1×10^5-fold differences in the bacterial load, respectively). The lung
115 bacterial load was 1.3×10^5 CFU for AMX-treated mice and 9.4×10^3 CFU for MPLA-treated mice, i.e.
116 respectively 255 and 18 times higher than in the AMX+MPLA group. Importantly, AMX and MPLA
117 monotherapies were unable to prevent bacteremia; the respective median splenic bacterial loads were 4.6×10^3
118 and 1.8×10^2 CFU.

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119 Interestingly, the bacterial load in the spleen was a strong predictor of survival (**Figure 1F**). All mock animals
120 succumbed to infection within 3 to 6 days, whereas AMX and MPLA monotherapies were associated with
121 survival rates of 13.3% and 40%, respectively. The AMX+MPLA treatment outperformed the two
122 monotherapies, with a survival rate of 86.7% - more than twice the value for MPLA, and over six times the
123 value for AMX. It is noteworthy that the difference in the survival rate between AMX+MPLA treatment and
124 high-dose AMX monotherapy (30 µg per animal; 1.2 mg/kg) was not significant (**Figure S2A**). This suggests
125 that co-administration of MPLA with low-dose AMX can boost the antibiotic's efficacy to levels comparable
126 with standalone, higher-dose treatment. A similar potentiating effect was observed in the congenic BALB/c
127 mice (**Figure S2B**). Overall, the present results demonstrate that AMX+MPLA combination treatment
128 improves the therapeutic outcome of low-dose AMX and is efficacious against *S. pneumoniae in vivo* by
129 minimizing bacterial lung colonization and dissemination, and promoting long-term survival.
130

131 **Combination treatment with AMX+MPLA mitigates pneumonia-induced lung damage**

132 We next looked at whether or not MPLA-mediated pro-inflammatory signaling exacerbated inflammation due
133 to *S. pneumoniae* infection. To this end, the lung tissue architecture in animals having been treated 12 hours
134 post-infection with AMX, MPLA or AMX+MPLA was analyzed 48 hours post-infection. As a positive control,
135 a group of animals was treated with a single, curative, high dose of AMX (350 µg per animal; 14 mg/kg). The
136 histopathological assessment revealed that treatment with MPLA in the presence or absence of AMX did not
137 exacerbate lung inflammation (**Figure 2**). Notably, all the AMX+MPLA-treated mice did not show any signs
138 of the perivascular inflammatory cell infiltration observed in the other groups (including animals having
139 receiving the curative dose of AMX). The total histopathological scores also showed that AMX+MPLA
140 treatment had the greatest impact on preservation of the lung tissue architecture, with the lowest score of 5; the
141 corresponding values were 5.75, 6.25, 8.75 and 12 in the high-dose AMX, MPLA, low-dose AMX and mock
142 groups, respectively (**Figure 2F)**. These findings suggest that MPLA treatment not only mitigate the effects of
143 infection-induced tissue damage but also (when combined with AMX) promotes tissue recovery and improves
144 the antibiotic's efficacy without exacerbating inflammation.
145

146 **Systemic MPLA treatment boosts the airway's innate immune responses during pneumonia**

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147 It has already been shown that MPLA confers protection against bacterial challenge when administrated
148 prophylactically, i.e. prior to the infectious challenge (15, 17, 18). Our study showed that MPLA also has an
149 immunomodulatory effect after the infection has been established. To further characterize the local immune
150 responses that were elicited and could participate to bacterial clearance, we used microarrays to analyze the
151 transcriptome of lung tissue. We then investigated post-treatment changes in gene expression by initially
152 comparing the AMX- and AMX+MPLA-treated groups 2, 4, and 8 h after treatment (i.e. 14, 16 and 20 h post-
153 infection) (**Figure 3**). The overall response to the AMX+MPLA treatment indicated an enrichment in the
154 granulocyte adhesion and diapedesis pathway and the leukocyte mobilization pathways (**Figure 3B**). Moreover,
155 the difference between the AMX- and AMX+MPLA-treated groups in the number of transcripts that were
156 expressed >2- or <0.5-fold was highest at 2 h post-treatment (n=188 transcripts) and decreased over time, with
157 106 transcripts at 4 h and 13 transcripts at 8 h (**Figure 3C**). There were 173 upregulated transcripts at 2 h, 75
158 at 4 h, and 12 at 8 h (**Figure 3C**). The pattern and time course of expression in lungs suggested that the MPLA-
159 induced transcriptional effects at the infection site were immediate and transient. Some of the lung transcripts
160 strongly expressed within a few hours of treatment were associated with neutrophil function (e.g. *Ngp*, *Itgb2l*,
161 and *Mmp8*) or encoded proteins with known antibacterial properties (e.g. CAMP or S100A8) (**Figure 3D**).
162 In a series of follow-up experiments *in vivo*, we confirmed the above results by using RT-qPCR assays for
163 selected genes. We extended the transcriptional study by including groups of infected and untreated animals,
164 infected and MPLA-treated animals, and uninfected and untreated (naïve) animals (**Figure 4**). In line with the
165 microarray data, MPLA treatment was found to accelerate the onset of potentially antimicrobial-related *Ngp*,
166 *Itgb2l*, *Mmp8*, *Camp*, *S100a9*, *Fkbp5, Ifitm6, Il4i1, Prok2,* and *Zbtb16* transcript expression in infected animals
167 in both the MPLA-only and AMX+MPLA groups (**Figure 4B**). The effect of MPLA on transcript expression
168 was therefore independent of AMX treatment. The lung expression of pro-inflammatory genes coding for
169 cytokines and chemokines (such as *Ccl2, Ccl20*, *Il1b*, or *Il12b*) was increased by infection but was not further
170 impacted by MPLA or AMX+MPLA treatment - indicating that the expression of these genes was primarily
171 regulated in an infection-dependent manner (**Figure 4B**). Given that we compared AMX+MPLA-treated with
172 AMX-treated groups in our initial screening, this might explain why we failed to detect the differential
173 expression of *Ccl2, Ccl20*, *Il1b*, or *Il12b* in our microarray experiments. Lastly, by comparing infected and
174 untreated animals, infected and MPLA-treated animals, and uninfected and MPLA-treated animals (**Figure S3**),

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175 we found that the transcription of some genes was somehow dependent on both infection- and MPLA-induced
176 signaling; this suggests the presence of a priming effect in which prior *S. pneumoniae* infection results in a more
177 robust response following the administration of MPLA. We also found that the AMX+MPLA combination did
178 not influence the neutrophil count in the lungs and spleen 12 h post-treatment (**Figure S4**). A similar pattern
179 was observed for alveolar macrophages. In contrast, the lung monocyte count was higher in the AMX+MPLA
180 group than in the mock treatment group (**Figure S4A**). Overall, these results demonstrated that while a bacterial
181 pneumonia insult can prompt airway innate immune responses, the latter are enhanced by post-infection
182 treatment with MPLA; this probably contributes to greater bacterial clearance in the lungs.
183

184 **The systemic response to AMX+MPLA combination treatment during pneumonia**

185 Since combination treatment had significantly outperformed AMX and MPLA monotherapies by limiting
186 bacterial dissemination, we further investigated the effects of treatment on the systemic compartment. In
187 accordance with the gene expression patterns observed in the lungs, we found that the administration of MPLA
188 (whether concomitant with AMX treatment or not) resulted in the sharp release of pro-inflammatory mediators
189 (such as IL-12 p40, IL-6, and CCL2) into the blood (**Figure 5**). The serum concentrations of these cytokines
190 peaked within the first two hours of administration, after which time they fell gradually and returned to baseline
191 levels within six to eight hours. It is noteworthy that during the first 12 hours post-treatment, neither mock- nor
192 AMX-treated infected animals appeared to produce this type of cytokine response - further suggesting that an
193 MPLA-dependent immediate cytokine response is instrumental in better controlling the systemic spreading of
194 bacteria. Although the MPLA- and AMX+MPLA-treated animals displayed similar blood levels of
195 inflammatory mediators, the survival rate was significantly higher in AMX+MPLA group – thus highlighting
196 the importance of the antibiotic's contribution to the therapeutic efficacy of combination treatment. Since our
197 attempts to measure MPLA in serum were not successful, we assumed that systemic as well as lung immune
198 responses were surrogate markers of MPLA's effect and thus a way to quantify the pharmacodynamics (PD).

199 We observed a typical pharmacokinetic (PK) profile after the oral administration of 10 µg AMX per mouse,
200 with a rapid increase and a rapid subsequent decline in the serum concentration of AMX (**Figure 6**).
201 Interestingly, we observed that the antibiotic's maximum serum concentration and rate of decline in the

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202 AMX+MPLA treatment group were slightly but significantly different from those recorded in the AMX group.
203 Taken as a whole, these findings suggest that MPLA and AMX's particular effects and different PK profiles in
204 the systemic compartment may contribute to the observed efficacy of the combination treatment.

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206 **DISCUSSION**

207 The growing incidence of AMR threatens to limit the currently available treatment options for many
208 bacterial infections. In the present study, we described an alternative strategy for combating invasive
209 pneumococcal disease in an experimental model. We used an immunomodulator (the TLR4 agonist MPLA) as
210 an add-on treatment to boost the efficacy of first-line antibiotic therapy. Our findings confirmed that the
211 outcomes of sub-curative antibiotic treatment are significantly improved (i.e. greater bacterial clearance and
212 better tissue recovery) following targeted stimulation of the host's innate immune system.

213 It has already been shown that prophylactic administration of MPLA confers protection against
214 bacterial infection in sepsis, pneumonia, and burn-wound models of disease (15, 17, 18, 22). In our mouse
215 model of progressive pneumococcal pneumonia, we observed that the single-shot, systemic, post-infection
216 administration of MPLA was associated with a significantly lower bacterial load in lung and spleen and higher
217 survival rates. These observations demonstrated that MPLA could potentially function as a therapeutic agent.
218 On the same lines, a recent study evidenced the therapeutic effect of MPLA-containing adjuvants in the context
219 of systemic mycosis (19). Another study reported that administration of LPS, the highly pyrogenic and toxic
220 TLR4 agonist with antibiotic is able to eliminate Salmonella invading the mesenteric lymph nodes, in contrast
221 to stand-alone antibiotic treatment (23). Together with our present findings, these literature data suggest that
222 MPLA could be repurposed as a universal antimicrobial drug whose therapeutic activity is independent of the
223 infection route and the microbial pathogen. Despite MPLA's proven adjuvant potency, a single dose of MPLA
224 alone had a limited therapeutic effect. We therefore hypothesized that MPLA's immunostimulatory properties
225 could be best exploited as a non-specific and safe booster of innate immune responses (thus improving the
226 efficacy of an otherwise suboptimal dose of antibiotic), rather than as a direct antimicrobial treatment.

227 In the present proof-of-concept study with an AMX-susceptible strain of *S. pneumoniae*, we sought to
228 replicate clinical conditions. Firstly, we treated the mice with a low dose of AMX; this mimics the context of
229 AMR in which a poorly administered or incorrectly dosed antibiotic treatment leads to incomplete bacterial
230 clearance or the development of resistance. Secondly, we gave the bacteria time to establish an infection prior
231 to treatment; this simulated both the lag between diagnosis and treatment, and the cascade of immune responses
232 to the initial bacterial insult. The combination of AMX and MPLA in a single administration had a greater

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233 therapeutic effect than each monotherapy alone. It is known that *S. pneumoniae* possesses a large number of
234 virulence factors that facilitate colonization. These include cell wall peptidoglycan components and
235 pneumolysin, both of which appear to elicit MyD88-dependent immune responses via TLR2- and TLR4-
236 specific pathways, respectively (24-27). Furthermore, TLR9 has been shown to have a major role in early host
237 defenses against *S. pneumoniae* infections (28, 29). Given that MPLA immunostimulation is biased towards
238 TRIF-mediated signaling downstream of TLR4, it is tempting to speculate that the introduction of
239 *S. pneumoniae* provides just the right type and right amount of primary stimulation via MyD88, which is then
240 amplified by the introduction of the secondary stimulus (MPLA) - leading to an enhanced immune response
241 through the activation of both MyD88- and TRIF-dependent pathways. Toll-like receptor-specific priming
242 induced by pneumococcal pneumonia may be a major factor in the enhanced immune response observed upon
243 MPLA treatment in our model.

244 It remains to be seen how MPLA is involved in greater bacterial clearance in the context of treatment
245 with sub-curative doses of AMX. The MPLA-associated transcriptional signature in the lungs of infected
246 animals highlighted the upregulation of genes associated with neutrophil function and tissue homing. One can
247 assume that a greater neutrophil count and an enhanced killing capacity may drive MPLA's antibacterial action
248 and synergize them with the antibiotic's effects in the airways. Our preliminary data at 12h post-treatment did
249 not highlighted changes in neutrophil number in lung and spleen; however, monocytes were found in higher
250 number in lung (**Figure S4**). Similarly, the systemic effects of MPLA-mediated signaling (such as the transient
251 production of cytokines and chemokines) may link immune cell activation to bacterial clearance in the
252 peripheral tissues. Our previous research on the TLR5 agonist flagellin identified two contributory mechanisms
253 in bacterial clearance: (i) greater infiltration of myeloid cells into the lungs and airways, and (ii) the activation
254 of IL-17/IL-22 responses by innate lymphoid cells (20, 21, 30, 31). Here, the lung transcriptional signature
255 suggests that MPLA influences the myeloid cell compartment by promoting recruitment and activating
256 antibacterial activity. In contrast to flagellin, MPLA administration was not associated with the rapid production
257 of IL-17/IL-22 by lung innate lymphoid cells; this is also the case for LPS (31). TLR signaling is associated
258 with broad range of antibacterial mechanisms in various cell types, some of which are probably independent of
259 AMR mechanisms (4). A multifactorial action is also a means of developing a therapeutic approach that is less

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260 likely to promote AMR. With a view to defining specific immune targets, it will be important to determine how
261 the MPLA-stimulated immune system cooperates with an antibiotic to improve treatment efficacy.

262 Drug PK and PD studies provide a quantitative basis for dosing regimens in humans and other animals.
263 Orally administered AMX is absorbed rapidly in humans, and has a relatively short period of serum availability
264 (32-34). The absorption in mice is reportedly even faster (35, 36). The PK characteristics of AMX indicate that
265 this antibiotic is efficacious very soon after administration. Hence, AMX's bactericidal activity depends greatly
266 on the time during which the serum concentration exceeds the minimum inhibitory concentration for its target;
267 accordingly, multiple, regular administrations are required to ensure efficacy (37, 38). Although these treatment
268 regimens are used widely, they are always associated with the risk of encouraging the development of AMR.
269 Amoxicillin's rapid PK maximum and minimum concentrations were in contrast with the PD characteristics of
270 MPLA, i.e. longer-term activation of the immune system. Our present findings suggest that the different time
271 scales of AMX's and MPLA's respective activities increased the overall efficacy of treatment when the two are
272 administered together. On a PK level, we also observed a slight increase in AMX retention when latter was
273 combined with MPLA. Future research must focus on whether and how the two substances interact specifically
274 *in vivo* in an infectious context.

275 Our present results highlighted on the potential of targeting innate immunity with a TLR4 agonist as a
276 viable strategy for improving antibiotic therapy against a bacterial infection. This pragmatic approach uses the
277 host's immune system to strengthen the attack against invading microorganisms, and may also help to repurpose
278 currently available drugs with known characteristics. Our experimental evidence suggests that the enhanced
279 therapeutic effect of the MPLA-AMX combination is achieved through a combination of TLR4 priming and
280 the time scales of MPLA's and AMX's respective peak biological activities. In the future, it will be important
281 to investigating possible synergistic relationships between individual treatment components by using (i) PK/PD
282 analyses and comprehensive mathematical modelling, and (ii) further *in vivo* simulations in multidrug resistant
283 strains treated with high-dose and/or multiple-dose antibiotic regimens. Ultimately, this promising approach
284 may open up new avenues for the design of host-directed therapeutics for infectious diseases.
285

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286 **MATERIALS AND METHODS**

287 **Ethics statement.** Animals were maintained in individually ventilated cages and were handled in a vertical
288 laminar flow cabinet (biosafety level 2). All experiments complied with current national, institutional and
289 European regulations and ethical guidelines, were approved by our Institutional Animal Care and Use
290 Committee (animal facility agreement C59-350009, Institut Pasteur de Lille; reference: APAFIS#5164,
291 protocol 2015121722429127_v4) and were conducted by qualified, accredited personnel.

292

293 **Bacterial strains and cell cultures.** *Streptococcus pneumoniae* serotype 1 (clinical isolate E1586) was obtained
294 from the Uruguayan Ministry of Health's National Reference Laboratory (Montevideo, Uruguay). Working
295 stocks were prepared as described previously (20, 21). Briefly, Todd Hewitt Yeast Broth (THYB) (Sigma-
296 Aldrich, St. Louis, MO, USA) was inoculated with fresh colonies grown in trypcase soy agar plates
297 supplemented with 5% sheep blood (BioMérieux, Marcy-l'Étoile, France), and incubated at 37°C until the
298 OD_{600nm} reached 0.7-0.9 units. Cultures were stored at -80ºC in THYB + glycerol 12% (vol/vol) for up to 3
299 months. For mouse infections, working stocks were thawed and washed with sterile Dulbecco's phosphate-
300 buffered saline (PBS; Gibco, Grand Island, NY, USA) and diluted to the appropriate concentration. The number
301 of bacteria (expressed in colony forming units [nezs]) was confirmed by plating serial dilutions onto blood agar
302 plates.

303

304 **The mouse model of infection.** Six- to eight-week-old female BALB/cJRj (BALB/c), C57BL/6JRj (C57BL/6)
305 or RjOrl:SWISS (CD-1) mice (Janvier Laboratories, Saint Berthevin, France) were used for all *in vivo*
306 experiments. Infection was carried out as described previously (30, 39). Briefly, mice were first anesthetized
307 by intraperitoneal injection with a solution of 1.25 mg ketamine plus 0.25 mg xylazine in 250 µL of PBS, after
308 which they were infected intranasally with a 30 µL PBS suspension containing 1 to 4×10^6 CFU of *S.*
309 *pneumoniae*. All the treatments described below were administered once 12h post-infection. Mice were
310 sacrificed at selected times via the intraperitoneal injection of 5.47 mg of sodium pentobarbital in 100 µl PBS
311 (Euthasol, Virbac, Carros, France). Blood was sampled by retro-orbital puncture into Z-Gel micro tubes to
312 prepare serum for downstream applications (Sarstedt, Nümbrecht, Germany). Lungs and spleens were collected,
313 homogenized, and plated onto blood agar to determine the endpoint bacterial load. For survival assays, both

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314 mortality and changes in body weight were monitored; mice were individually weighed prior to infection and
315 then every 24h for a period of up to two weeks.
316
317 **Reagents and administration of treatments *in vivo*.** Amoxicillin trihydrate (Sigma-Aldrich) was prepared in
318 a stock solution of 1.75 mg/mL in sterile water and then adjusted to a final dose of 5, 30, or 350 µg/mouse (i.e.,
319 0.2, 1.2, or 14 mg/kg) before intragastric administration of a volume of 200 µL by oral gavage.
320 Lipopolysaccharide from *E. coli* O111:B4 (S-form) and MPLA from *S. minnesota* R595 (Re) (TLR*pure*™,
321 Innaxon Therapeutics, Bristol, United Kingdom) were obtained as sterile solutions or prepared from powder
322 with sterile distilled water to a concentration of 1 mg/L (according to the manufacturer's recommendations)
323 and then adjusted to different final concentrations in sterile PBS and administered by intraperitoneal injection
324 (200 µL).
325
326 **Quantification of gene expression and microarrays.** Lungs were perfused with PBS prior to sampling. Lung
327 or liver total RNA was extracted with the Nucleospin RNA II kit (Macherey Nagel, Düren, Germany) and
328 reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA).
329 The cDNA was amplified using SYBR-Green-based real-time PCR on a Quantstudio™ 12K Real-Time PCR
330 System Thermo Fisher Scientific, Carlsbad, CA, USA). Specific primers used are listed in **Table S1**. Relative
331 mRNA levels were determined by comparing the PCR cycle thresholds (Ct) for the gene of interest vs. *Actb*
332 (ΔCt) and then the ΔCt values for treated vs. untreated (mock) groups (ΔΔCt). For the microarray analysis, total
333 RNA yield and quality were further assessed on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa
334 Clara, CA, USA). One-color whole mouse (084809_D_F_20150624 slides) 60-mer oligonucleotide 8x60k v2
335 microarrays (Agilent Technologies) were used to analyze gene expression. The cRNA labelling, hybridization
336 and detection steps were carried out according to the supplier's instructions (Agilent Technologies). For each
337 microarray, cyanine-3-labeled cRNA was synthesized from 50 ng of total RNA using the low-input QuickAmp
338 labeling kit. RNA Spike-In was added to all tubes and used as a positive control in the labelling and
339 amplification steps. Next, 600 ng of each purified labelled cRNA were then hybridized and washed following
340 manufacturer's instructions. Microarrays were scanned on an Agilent G2505C scanner, and the data were
341 extracted using Agilent Feature Extraction Software (version 10.7.3.1, Agilent Technologies). Microarray data

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342 have been deposited in the Gene Expression Omnibus database (accession number: GSE118860). Statistical
343 comparisons and filtering were performed with the Limma R package with 75-percentile normalization.
344 Differentially expressed genes were considered to be those with an adjusted p-value below 0.05 after the false
345 discovery rate had been checked with the Benjamini-Hochberg procedure (40). Pathways were investigated
346 using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA).
347
348 **Histology.** Lungs were fixed by intratracheal perfusion with 4% formaldehyde prior to sampling. The left lobe
349 and the upper right lobe were included in paraffin, and 3- to 5-µm tissue sections were stained with hematoxylin
350 and eosin reagent. The slides were blindly evaluated for neutrophil infiltration, perivascular infiltration, edema,
351 and pleuritis on a 6-level scale, where 0 corresponded to the absence of lesions, and 1 to 5 corresponded to
352 minimal, slight, moderate, marked, and severe lesions, respectively (Althisia, Troyes, France).
353
354 **Quantification of serum cytokine levels.** Serum levels of CCL2, IL-6 and IL-12 p40 were measured using an
355 ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MO, USA).
356
357 **Determination of serum amoxicillin concentrations.** Serum AMX concentrations were assayed using
358 previously developed and validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method
359 (41). In brief, the proteins in 10 µL of serum were precipitated with 40 µL of ice-cold methanol. After diluting
360 the supernatant with water, the sample was injected into the LC system (Agilent Technologies) by using a
361 gradient elution at a flow rate of 0.3 mL/min with acetonitrile and water with formic acid. The AMX ion product
362 (*m/z* 114) was quantified using electrospray ionization MS in positive ion mode over a calibration range from
363 0.01 to 10 µg/mL. In-study validation was performed according to the European Medicines Agency guidelines
364 on bioanalytical method development (42)
365
366 **Statistical analysis.** Results were expressed as the median and individual values, median [interquartile range]
367 or mean ± standard error of the mean (SEM), as appropriate. Groups were compared using a Mann-Whitney
368 test (for two independent groups) or a Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's
369 post-test (for three or more groups). The log rank test was used for survival analyses. Statistical analyses were

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370 performed using GraphPad Prism software (version 8.2, GraphPad Software Inc., San Diego, CA, USA), and

371 the threshold for statistical significance was set to $p<0.05$.

372

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373 **ACKNOWLEDGMENTS**

374 We thank Shéhérazade Sebda and Frédéric Leprêtre from University of Lille's genomics facility for preparing
375 microarrays and depositing the data in the Gene Expression Omnibus database, respectively.
376

377 **FUNDING**

378 The study was funded by INSERM, Institut Pasteur de Lille, Université de Lille, and the Era-Net Joint Program
379 Initiative on Antimicrobial Resistance (ANR-15-JAMR-0001-01 to FC, LM, CC and JCS) and by the German
380 Federal Ministry of Education and Research (031L0097 to SF, RM, and CK).
381

382 **AUTHOR CONTRIBUTIONS**

383 FC performed all animal, RT-qPCR, ELISA, and flow cytometry experiments. SF and RM analyzed antibiotic
384 PK data. LM provided FC with technical assistance. MF performed microarray experiments and bioinformatics
385 analyses. CK, CC, and JCS designed the experiments. FC, JCS, and CC wrote the manuscript. JCS and CC
386 supervised the experimental work as a whole.

387

388 **COMPETING INTERESTS**

389 The authors declare that the research was conducted in the absence of any commercial or financial relationships
390 that could be construed as a potential conflict of interest.

391

392 **DATA AND MATERIALS AVAILABILITY**

393 Microarray data are available in the Gene Expression Omnibus database (accession number: GSE118860).
394 The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue
395 reservation, to any qualified researcher.
396

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507 **FIGURE LEGENDS**

508 **Figure 1. Combination treatment with AMX and MPLA is effective against *S. pneumoniae in vivo*.** (A)
509 CD-1 mice were infected intranasally with 1×10^6 *S. pneumoniae* and then given either 10 µg of AMX
510 intragastrically, 50 µg MPLA intraperitoneally, a combination of the two treatments (AMX+MPLA), or water
511 and saline mock treatments 12 hours post-infection. Lungs and spleens were collected at different time points
512 for quantification of the bacterial load using standard plate counting methods. (B-C) Bacterial growth over time
513 in infected mice, showing the total bacterial load in the indicated tissues (as CFUs). Symbols represent the
514 median value (n≥6/group) and error bars represent the interquartile range; the gray shaded area along the x-axis
515 indicates the assay's limit of detection. (D-E) Lung and spleen bacterial counts from individual mice
516 (n≥12/group) 24 h (D) and 48 h (E) post-infection. The solid lines indicate the median value for each group,
517 and the gray shaded area along the x-axis indicates the assay's limit of detection. A one-way ANOVA (the
518 Kruskal-Wallis test with Dunn's post-test for multiple comparisons) was applied. *=p<0.05, **=p<0.01,
519 ***=p<0.001 vs. the indicated comparator groups. Data from the mock control group (shown in white) were
520 excluded from statistical analyses of treatment groups. (F) Survival curves (n=15 mice per group). Gehan-
521 Breslow-Wilcoxon test. *** = *p*<0.001 vs. infected mock treated control, # = p<0.05, # # = *p*<0.01, # # # =
522 *p*<0.001 vs. the indicated comparator groups.

523

524 **Figure 2. Combination treatment with AMX and MPLA mitigates infection-induced tissue damage.** CD-
525 1 mice (n=4/group) were infected intranasally with 1×10^6 *S. pneumoniae,* and then given treatments 12 hours
526 later as indicated: (A) No treatment, i.e. mock, (B) intragastric treatment with 10 µg of amoxicillin (AMX_{10}) or
527 (C) 350 µg amoxicillin (AMX_{350}), (D) intraperitoneal treatment with 50 µg of MPLA (MPLA), or (E) a
528 combination of intragastric treatment with 10 µg AMX and intraperitoneal treatment with 50 µg of MPLA
529 (AMX+MPLA). Hematoxylin- and eosin-stained tissue sections showing the lung architecture 48 hours post-
530 infection (A-E). The images are representative of four biological replicates per group. Scale bar = 150 µm. (F)
531 Histopathological scores were assessed on a 0-5 scale: 0=absence, 1=minimal, 2=slight, 3=moderate,

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532 4=marked, and 5=severe. The bars represent the mean ± SEM. A one-way ANOVA (the Kruskal-Wallis test
533 with Dunn's post-test for multiple comparisons) was applied. *=p<0.05 and **=p<0.01.

534

535 **Figure 3. Characterization of local immune response signatures of MPLA treatment.** (A) BALB/c mice
536 (n=3/group) were infected with 1×10^6 *S. pneumoniae* and then treated with either 10 µg of intragastrically
537 administered AMX or a combination of AMX and 50 µg of intraperitoneally administered MPLA
538 (AMX+MPLA). Total RNA was extracted from lungs collected at different time points, and mRNA transcripts
539 were compared in a microarray analysis. (B) Enrichment of canonical pathways, according to an Ingenuity
540 Pathway Analysis of the microarray datasets. (C) The number of transcripts with significantly greater
541 expression (upregulation, Log_2(fold change [FC] >1) or significantly lower expression (downregulation,
542 $Log_2FC < -1$) in (AMX+MPLA)-treated vs. AMX-treated animals. (D) The 25 genes with the highest or lowest
543 differential expression levels in the microarray analysis (AMX+MPLA vs. AMX);

544

545 **Figure 4. MPLA treatment can accelerate infection-dependent immune responses at the infection site.**
546 (A) BALB/c mice (n=3-8/group) were infected with *S. pneumoniae* and treated 12 hours post-infection with
547 either 10 µg of AMX intragastrically, 50 µg MPLA intraperitoneally, a combination of the two treatments
548 (AMX+MPLA), or left untreated; total RNA was extracted from lungs collected at different time points, and
549 mRNA transcripts were compared using RT-qPCR assays. Expression levels were normalized against
550 uninfected, untreated (naïve) controls. (B) Change in relative mRNA expression levels over time for selected
551 genes under different treatment conditions. The mean ± SEM values are shown. A two-way ANOVA with
552 Bonferroni's post-test for multiple comparisons was applied. *=p<0.05, **=p<0.01 and ***=p<0.001 vs. the
553 mock control group; +=p<0.05, ++=p<0.01, and +++=p<0.001 vs. the indicated comparator groups.

554

555 **Figure 5. MPLA treatment induces an immediate, transient cytokine response.** CD-1 mice (n=4/group)
556 were infected intranasally with 1×10^6 *S. pneumoniae* and then treated 12 hours later with either AMX (10 µg,

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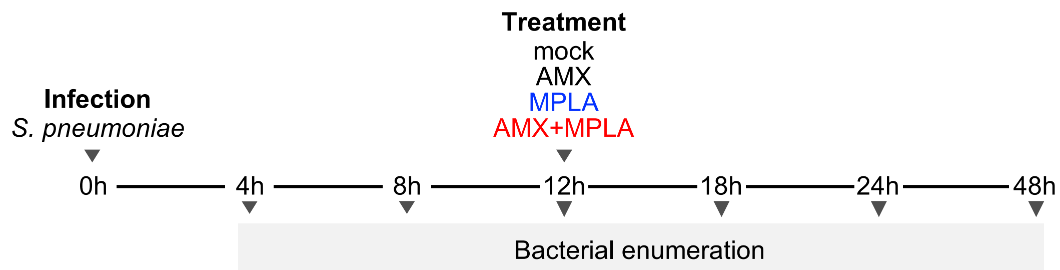
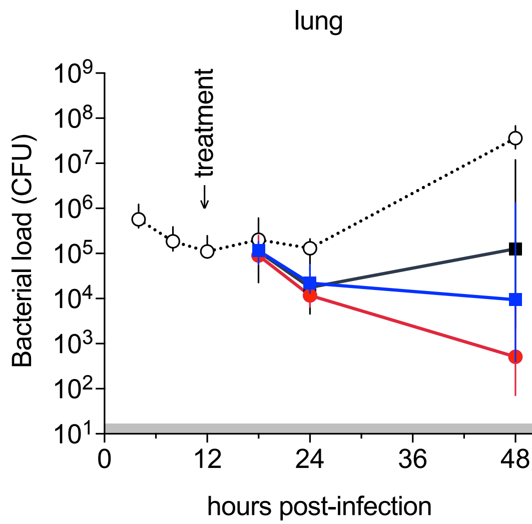
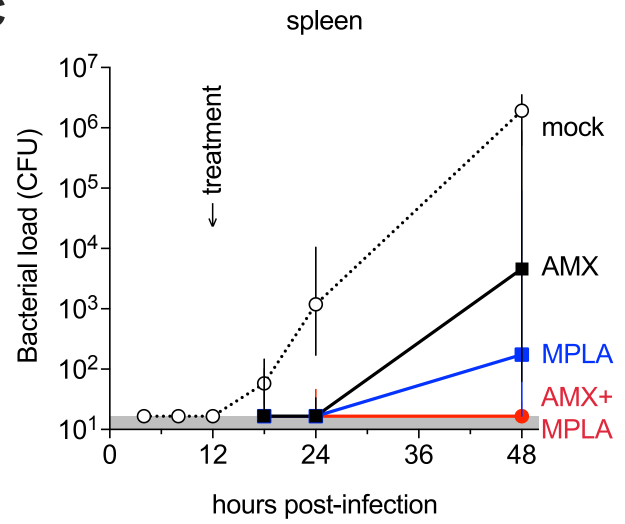
557 intragastric administration), MPLA (50 μg intraperitoneal administration), a combination of AMX and MPLA,
558 or left untreated. Blood samples were collected at different time points post-treatment. Serum levels of pro-
559 inflammatory mediators were determined using ELISAs. (A) IL-12 p40 subunit, (B) IL-6, and (C) CCL2. The
560 mean ± SEM values are shown. A two-way ANOVA with Bonferroni's post-test for multiple comparisons was
561 applied. At indicated time points: *=p<0.05, **=p<0.01 and ***=p<0.001 vs. the untreated group; ++=p<0.01
562 vs. the indicated comparator groups.

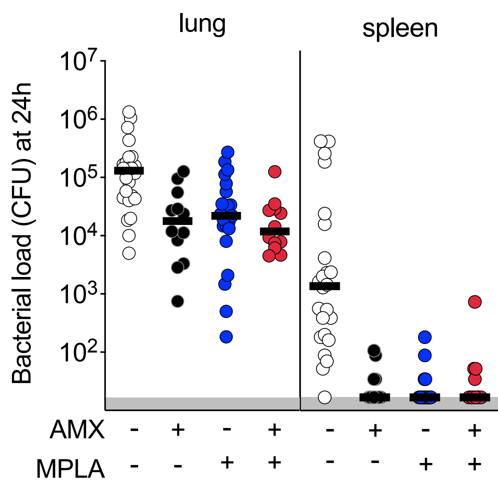
563

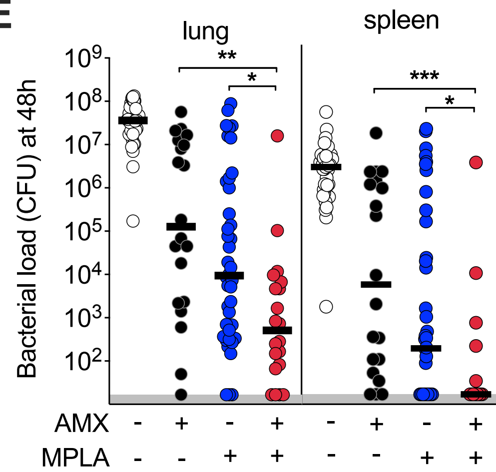
564 **Figure 6. Pharmacokinetics of orally administered AMX in mice.** (A) CD-1 mice (n=4/group) infected with
565 1×10^6 *S. pneumoniae* and treated with either 10 μg intragastrically administered of amoxicillin (AMX) or a
566 combination of AMX and a 50 μg intraperitoneally administered MPLA (AMX+MPLA). Blood samples were
567 collected at different time points post-treatment. (B) Change in the serum AMX concentration over time, as
568 determined using LC-MS/MS. A two-way ANOVA with Bonferroni's post-test for multiple comparisons was
569 applied. The values shown are geometric means + range; *=p<0.05 vs. the indicated comparator groups at
570 indicated time points. The dashed and dotted lines along the x-axis indicate the lower limit of quantification
571 (0.01 μg/mL) and the limit of detection (0.003 μg/mL), respectively.











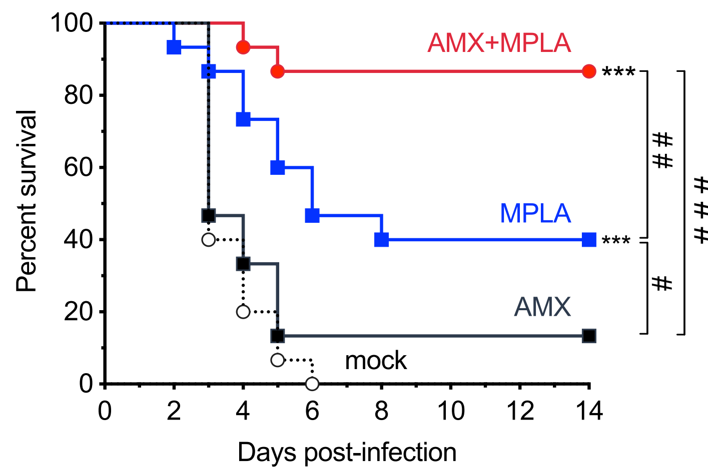


Figure 1

A

250 µm

B

250 µm

C

250 µm

D

250 µm

E

250 µm

F

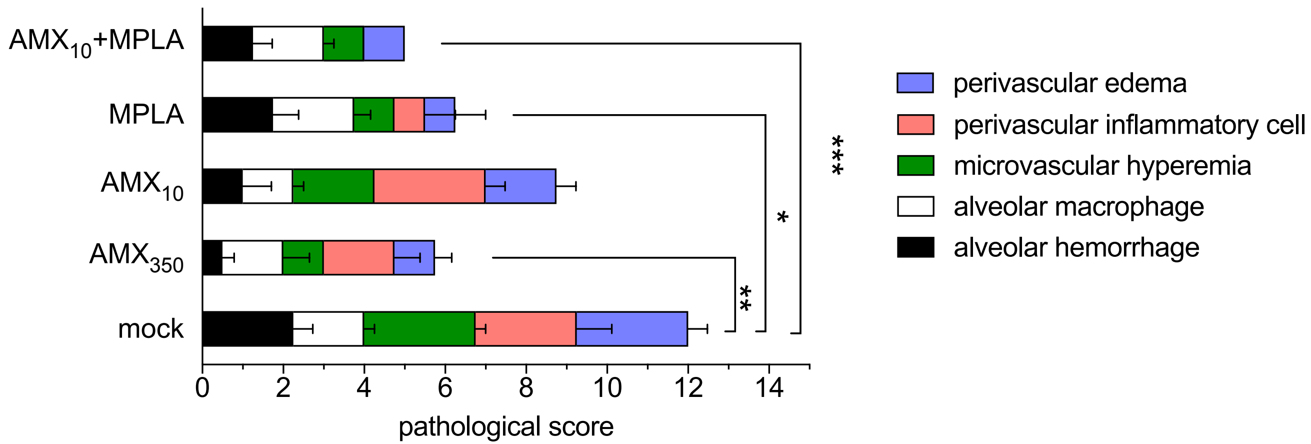
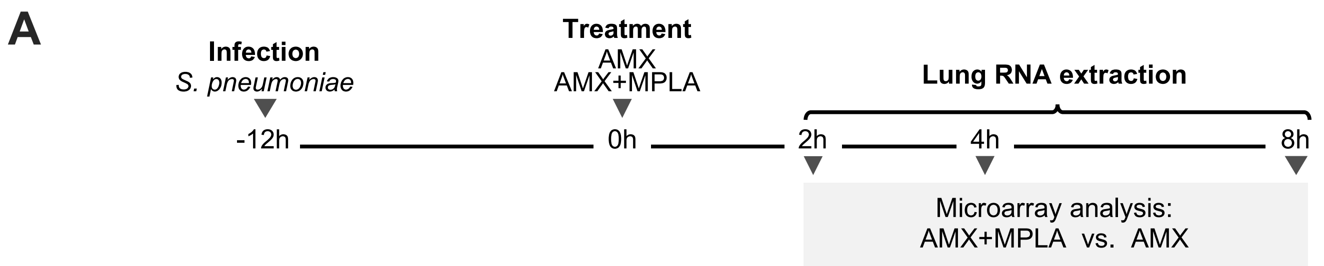


Figure 2

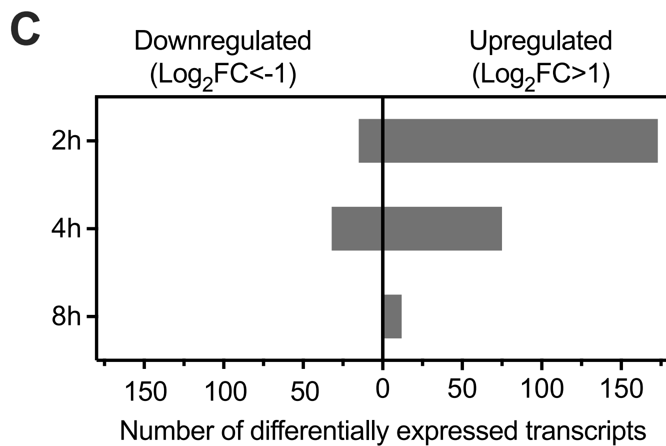
A



B

Time (hours post-treatment)	Canonical Pathways	-Log₁₀ (p value)	Molecules
2	Granulocyte Adhesion and Diapedesis	5.33	C5AR1, FPR1, FPR2, IL1R2, MMP25, MMP8, MMP9, SELL
	Inhibition of Matrix Metalloproteases	2.76	MMP8, MMP9, MMP25
	Leukocyte Extravasation Signalling	1.45	MMP8, MMP9, MMP25, RAPGEF4
4	Inhibition of Matrix Metalloproteases	3.43	MMP8, MMP25, TIMP4
	Granulocyte Adhesion and Diapedesis	2.55	CXCL6, IL1R2, MMP8, MMP25
	Leukocyte Extravasation Signalling	2.22	MMP8, , MMP25, RAPGEF4, TIMP4
8	Inhibition of Matrix Metalloproteases	3.65	MMP8, MMP25
	Granulocyte Adhesion and Diapedesis	2.42	MMP8, MMP9
	Leukocyte Extravasation Signalling	2.23	MMP8, MMP9

C



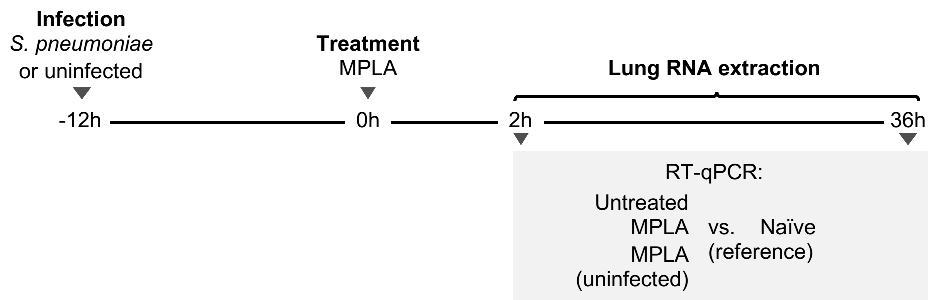
D

	2h	4h	8h
Ngp	4.919	3.497	2.778
Mmp8	4.188	3.292	2.104
Ifitm6	3.590	2.308	2.269
Prok2	3.423	3.983	4.505
Stfa3	3.876	2.191	1.798
Fkbp5	3.552	2.994	1.294
Zbtb16	1.252	4.291	0.519
Doc2b	2.236	4.238	1.224
Itgb2l	4.113	1.891	1.343
Il4i1	3.854	1.796	1.555
Retnlg	3.444	2.019	0.911
Stfa1	3.245	1.508	1.421
Chil5	2.612	1.447	0.878
Scrg1	2.837	1.798	1.181
Camp	2.766	1.409	0.147
F13a1	2.871	1.124	0.605
Hif3a	2.362	2.561	0.702
S100a8	1.956	1.265	1.430
Mettl21e	2.043	2.071	0.769
Ambp	2.220	2.198	0.859
Il1r2	1.663	1.583	0.520
Areg	-0.631	-1.219	-0.996
Cxcl5	-0.827	-1.753	-0.277
Ackr4	-0.802	-1.868	-0.484
Cemip	-0.263	-2.259	-0.147

Log2(FC): 4, 2, 0, -2

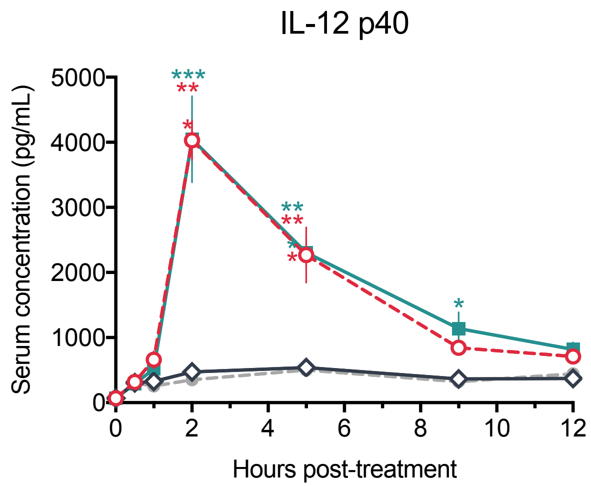
Figure 3

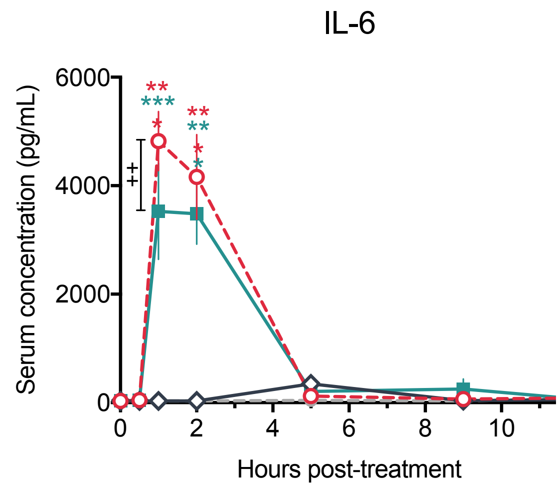
A



B

Figure 4





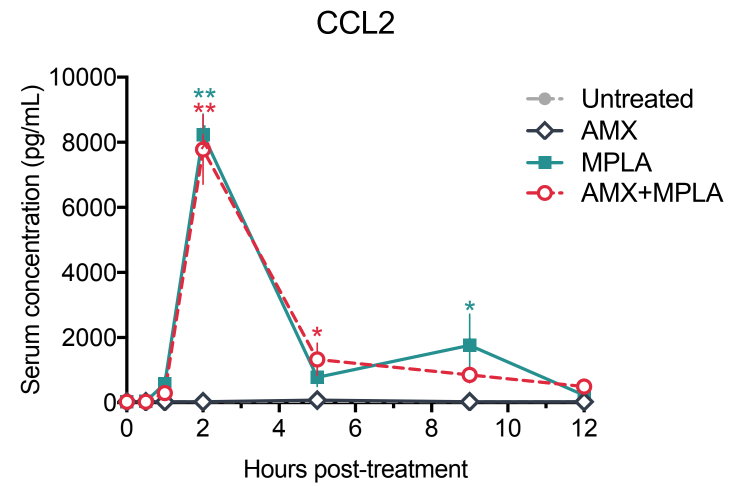
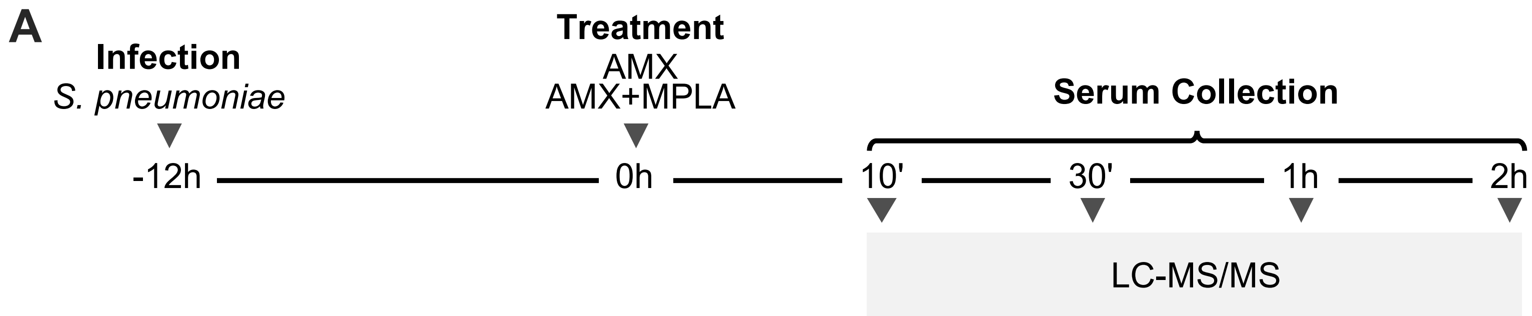


Figure 5



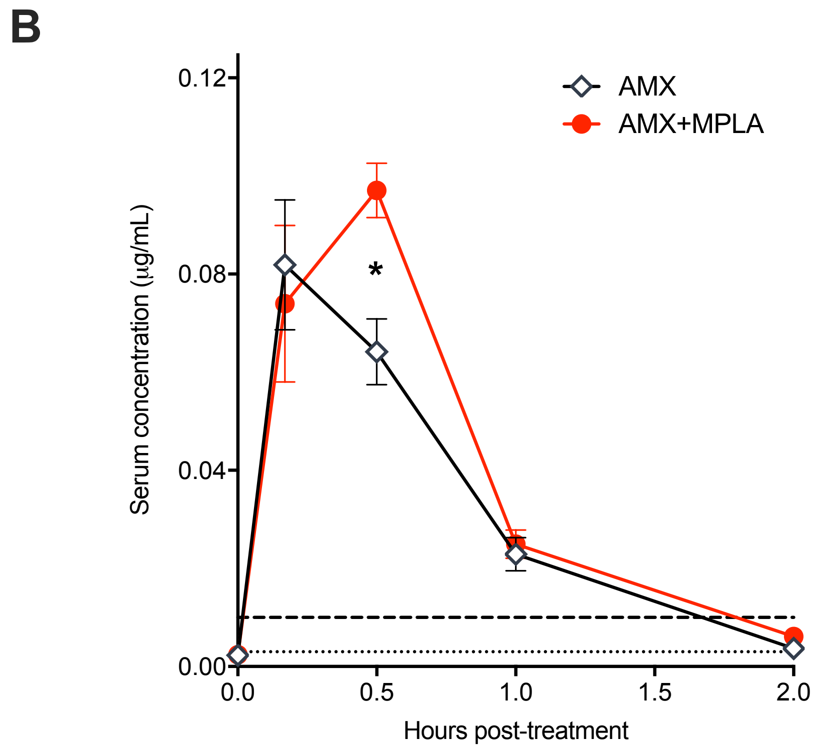


Figure 6