The Biosynthetic Monophosphoryl Lipid A Enhances the Therapeutic Outcome of Antibiotic Therapy in Pneumococcal Pneumonia.

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ABSTRACT

Alternative treatment strategies against bacterial infections are required to decrease the use of antibiotics. This study tested the hypothesis that stimulation of the innate immune receptor Toll-like receptor 4 can be combined with antibiotics to improve the treatment of invasive pneumonia. The efficacy of the biosynthetic monophosphoryl lipid A (MPLA), a clinically-approved Toll-like receptor 4 activator, was tested in a mouse model of *Streptococcus pneumoniae* respiratory infection. Interestingly, administration of amoxicillin or MPLA decreased 400 to 11,000-fold the bacterial load in lung and spleen but did not enhance survival compared to mock treatment. The single administration of a combination of MPLA and amoxicillin further reduced 10 to 18-fold the bacterial colonization and invasion and significantly improved protection against lethal disease. The combined administration of MPLA and amoxicillin in a context of infection was associated to transient increase of the serum concentrations of amoxicillin and pro-inflammatory cytokines and chemokines as well as the expression of immune genes in lung tissue. Remarkably, the systemic and lung immune activation extended beyond amoxicillin elimination, suggesting a two-step and cooperative anti-infective effect, *i.e*., rapid antibiotic-mediated alteration of bacteria and a long-lasting impact through mucosal and systemic immunity. Our proof-of-concept study demonstrated for the first time that boosting Toll-like receptor 4 signaling can synergize with antibiotics in order to increase the efficacy of therapy of bacterial pneumonia thereby *in fine* reducing the dose or regimen of antibiotics.

KEYWORDS

Toll-like receptor; amoxicillin; adjunct therapy; bacterial pneumonia; Streptococcus pneumoniae

The development of antibiotics enabled the treatment of infectious diseases. Decades later, the spread in antimicrobial resistance (AMR) promoted the alarming decline of therapeutic effectiveness. Actually, AMR is a major threat to human health because it compromises our ability to treat infections and to carry out medical interventions that rely on antibiotic use, such as chemotherapy, and surgery [1]. The World Health Organization raised concerns about the lack of new antibiotics in development and emphasizes the need of innovative anti-infective approaches for preventing AMR dissemination [2].

The host's recognition of danger is instrumental in mounting innate immune defenses against invading pathogens. Host-directed therapeutic strategies targeting innate immunity has thus gained much attention as an alternative to antibiotics [3]. Stimulation of innate immunity has three main advantages: (i) it makes use of universal, built-in machinery that is ready to activate in most individuals, (ii) it has both anti-infective and pro-recovery effects, and (iii) it mobilizes many antimicrobial effectors, and thus counters the potential development of AMR. *In vivo*, many cell types are equipped with Toll-like receptors (TLRs) that detect pathogenic microorganisms [4]. Thus, TLR engagement triggers the expression of immune genes that regulate antibacterial mechanisms, such as the production of cytokines, chemokines and antimicrobial compounds, the activation of complement pathways, and the mobilization of leukocytes. Accordingly, researchers have sought to design novel therapies against viral and bacterial infections using TLR agonists [3, 5]. Monophosphoryl lipid A (MPLA) is a derivative of the lipid A component of the outer-membrane-expressed lipopolysaccharide (LPS) from the bacterium *Salmonella minnesota* R595 [6]. The LPS synthesized by the rough mutant bacteria R595 lacks the O-specific chain but harbors the keto-3-deoxy-octonate (KDO) component. After specific acid and base treatments, the lipid A is detoxified by hydrolysis of KDO and the selection of attenuated lipid A sugars and fatty acyl chains [6-7]. Lipopolysaccharide (LPS) itself is highly toxic, due to its ability to activate TLR4 signaling at low doses via both of the adaptor proteins, namely myeloid differentiation primary response 88 (MyD88) and Toll/interleukin-1 receptor domain containing adaptor inducing interferon beta (TRIF). In contrast, activation of TLR4 by MPLA is biased towards TRIF-dependent TLR4 signaling, making MPLA safe for use in humans [8]. Monophosphoryl lipid A induces a significant but attenuated innate immune response [9-11]. Years of research have paved the way to MPLA becoming the first TLR agonist to be licensed as an adjuvant in vaccine formulations [12-14]. Unlike the more widely-used aluminum salt adjuvants which work by promoting

antigen deposition and uptake, adjuvant activity of MPLA is achieved by functional activation of TLR4, a receptor which is present in structural cells as well as in antigen presenting cells (APCs), including dendritic cells and monocytes. Thus, in addition to activating innate immune responses, TLR4-dependent activation by MPLA can also directly enhance functional activation of APCs, priming of B and T lymphocytes, thereby promoting adaptive immunity. The continued interest for MPLA led to focus on its use as a prophylactic treatment against bacterial infections. Indeed, MPLA confers protection against infections by (i) *Pseudomonas aeruginosa* in burn-wounds, (ii) *Staphylococcus aureus* under post-hemorrhagic conditions, and (iii) nontypeable *Haemophilus influenzae* in the nasopharynx [15-18]. Whereas prophylaxis activity against infections was demonstrated for many TLR agonists, there are few evidence that such strategy is working for treatment of infections. The most significant therapeutic effect of TLR agonist was demonstrated using LPS and TLR9 agonist in the context of mouse salmonellosis in combination with antibiotic [19]. The treatments were able to reduce the counts of antibiotic tolerant bacteria within dendritic cells and thereby augmenting the therapeutic activity of antibiotics.

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

RESULTS

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

The MPLA is derived from the lipid A moiety of *S. minnesota* R595 lipopolysaccharide and is strongly attenuated for toxic effects in mammals compared to bacterial LPS [6-7]. To determine the appropriate dose to stimulate immunity, 0.5 to 50 µg of MPLA were administered by intraperitoneal injection to naïve C57BL/6JRj mice (**Figure S1**). The magnitude of the systemic immune responses (*i.e.*, in the liver and blood) was strongly dependent on the dose of MPLA. Innate immune responses were also observed in the lungs after the systemic injection of MPLA, albeit only at the highest dose (50 µg). This result suggests that the systemic administration of MPLA promotes both systemic and pulmonary immune responses - a feature that could potentially be exploited in the host-directed therapy of respiratory infectious diseases. We selected the dose of 50 µg per mouse for the next part of the study. As expected, the MPLA effect depends on TLR4-mediated signaling. Incubating *Tlr4*$^{-/-}$ peritoneal cells, mainly macrophages as described previously [22], for 16 h with 50 ng/ml MPLA was unable to trigger any secretion of IL-6 in contrast to TLR4-proficient peritoneal cells from congenic C57BL/6JRj mice (< 10 *vs.* 2540±148 pg/ml, respectively).

We next investigated whether MPLA's effects affected bacterial infection when administered together with antibiotic (**Figure 1A**). We used the serotype 1 *S. pneumoniae* strain E1586 that induce a continuing and invasive lethal pneumonia in the outbred Swiss mice and the inbred BALB/cJRj strain [20, 23-24]. Twelve hours after intranasal inoculation with *S. pneumoniae*, Swiss mice received either a sub-curative dose of AMX, a first-line antibiotic (10 µg per animal; 0.4 mg/kg, administered by oral gavage; AMX_{10}), MPLA (50 µg per animal; 2 mg/kg, administered by intraperitoneal injection) or a combination of the two treatments (AMX_{10}+MPLA). Bacterial counts in the lungs and spleen were determined at different times post-treatment as surrogate markers of pneumonia and bacterial dissemination, respectively. At 12 h post-treatment, the bacterial loads in the lungs and spleens were lower in all treated animals than in mock-treated animals; however, the differences between the three treatments were not statistically significant (**Figure 1B-C-D**). In contrast, we observed significant intergroup differences in the bacterial loads at 36 h post-treatment (**Figure 1B-C and 1E**). Bacterial numbers were the lowest in the AMX_{10}+MPLA group, with a median CFU value of 5.1×10^2 in the lungs, and nearly undetectable bacterial levels in the spleen; this can be compared with values of 3.6×10^7 and 1.9×10^6 CFU

recorded in the lungs and spleen of mock-treated infected animals, respectively. The lung bacterial load for AMX_{10}-treated mice and MPLA-treated mice, was 255 and 18 times higher than in the AMX_{10}+MPLA group, respectively. Importantly, AMX_{10} or MPLA monotherapies were unable to prevent dissemination.

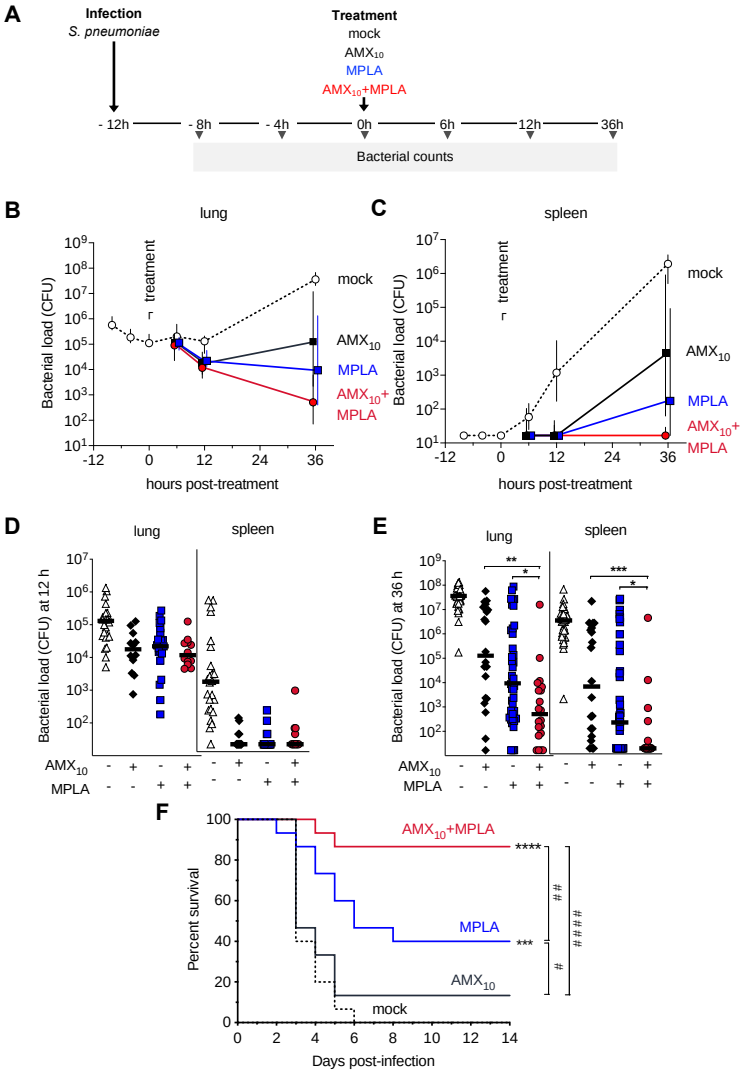

Figure 1. Combination treatment with AMX and MPLA is effective against *S. pneumoniae in vivo*. (A) Swiss mice were infected intranasally with 10^6 *S. pneumoniae* and then given either 10 µg of AMX (AMX_{10}) intragastrically, 50 µg MPLA intraperitoneally, a combination of the two treatments (AMX_{10}+MPLA), or water and saline mock treatments 12 h post-infection. Lungs and spleens were collected at different times for quantification of the bacterial load using standard plate counting methods. (B-C) Bacterial growth over time in infected mice, showing the total bacterial load (CFU). Symbols represent the median value (n≥6/group) and error bars represent the interquartile range. (D-E) Lung and spleen bacterial counts from individual mice (n≥12/group) 12 h (D) and 36 h (E) post-treatment. The solid lines indicate the median value for each group. A one-way ANOVA (the Kruskal-Wallis test with Dunn's post-test for multiple comparisons)

was applied. *=p<0.05, **=p<0.01, ***=p<0.001 vs. the indicated comparator groups. To compare the efficacy of treatments, data from the mock group were excluded from statistical analyses. (F) Survival curves (n=15 mice per group). Log-rank (Mantel-Cox) test was applied as follows: *** = $p<0.001$, **** = $p<0.0001$ *vs.* mock group, # = p<0.05, ## = $p<0.01$, #### = $p<0.0001$ *vs.* the indicated comparator groups.

Interestingly, a low number of bacteria in the spleen at 36 h post-treatment was associated to a significant survival (**Figure 1F**). All mock animals succumbed to infection within 3 to 6 days, whereas AMX_{10} or MPLA monotherapies were associated with survival rates of 13.3% and 40%, respectively. The AMX_{10}+MPLA treatment outperformed the monotherapies, with a survival rate of 86.7%. Moreover, the survival rates between AMX_{10}+MPLA treatment and AMX_{30} monotherapy (30 µg per animal; 1.2 mg/kg) were similar (**Figure S2**). This suggests that co-administration of MPLA with low-dose AMX can boost the antibiotic's efficacy to levels comparable with standalone, higher-dose treatment. A similar potentiating effect was observed in the inbred BALB/cJRj mice (**Figure S2**). Overall, these results demonstrate that AMX+MPLA combination treatment improves the therapeutic outcome of AMX and is efficacious against *S. pneumoniae in vivo* by minimizing bacterial lung colonization and dissemination, and promoting long-term survival.

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

We next looked at whether or not MPLA-mediated pro-inflammatory signaling would result in increased inflammation and tissue damage during *S. pneumoniae* infection. The lung tissue architecture in animals having been treated was analyzed 36 h post-treatment. As a control of treatment effectiveness, a group of animals was treated with a single, curative, high dose of AMX (350 µg per animal; 14 mg/kg; AMX_{350}). The histopathological assessment revealed that treatment with MPLA in the presence or absence of AMX_{10} did not increase lung inflammation (**Figure 2**). Notably, the AMX_{10}+MPLA-treated mice did not show any signs of the perivascular inflammatory cell infiltration observed in the other groups (including animals having receiving AMX_{350} or animals that were not infected, i.e., mock, as shown in Figure 2C and 2F respectively). The total histopathological scores also showed that AMX_{10}+MPLA treatment had the greatest impact on preservation of the lung tissue architecture, with the lowest score of 3.3 (**Figure 2G)**. These findings suggest that MPLA

treatment not only mitigate the effects of infection-induced tissue damage but also (when combined with AMX) promotes tissue recovery and improves the antibiotic's efficacy without exacerbating inflammation.

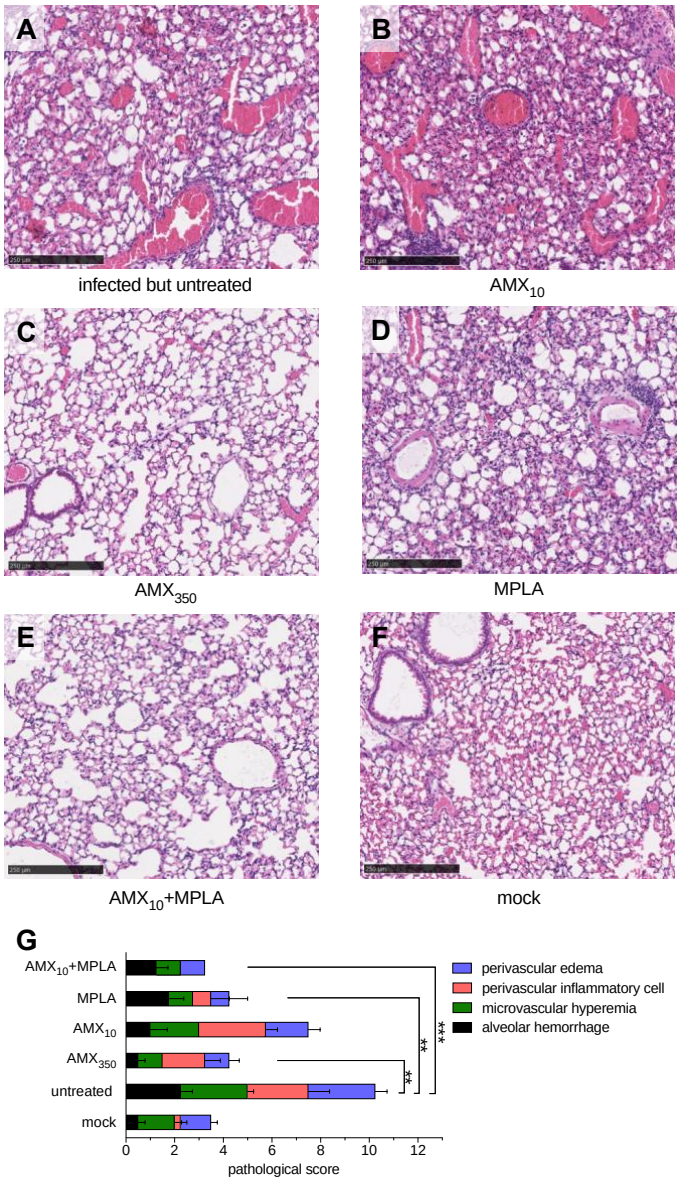

Figure 2. Combination treatment with AMX and MPLA mitigates infection-induced tissue damage. Swiss mice (n=4/group) were infected intranasally with 10^6 *S. pneumoniae*, and then given treatments 12 h later as indicated: (A) No treatment, *i.e.*, untreated, (B) intragastric treatment with 10 µg of amoxicillin (AMX_{10}) or (C) 350 µg amoxicillin (AMX_{350}), (D) intraperitoneal treatment with 50 µg of MPLA, (E) a combination of intragastric treatment with 10 µg AMX and intraperitoneal treatment with 50 µg of MPLA (AMX_{10}+MPLA). The panel F represents section of untreated and uninfected animals, *i.e.*, mock. Hematoxylin- and eosin-stained tissue sections showing the lung architecture 36 h post-treatment (A-F). The images are representative of four biological replicates per group. Scale bar = 150 µm. (G) Histopathological scores were assessed on a 0-5 scale: 0=absence, 1=minimal, 2=slight, 3=moderate, 4=marked, and

5=severe. The bars represent the mean ± SEM. A one-way ANOVA (the Kruskal-Wallis test with Dunn's post-test for multiple comparisons) was applied. ** = p<0.01 and *** = p<0.001.

Systemic pharmacokinetics and response to AMX+MPLA combination treatment during pneumonia

We next investigated the effects of the treatments on the systemic circulation. We observed in mouse serum a characteristic pharmacokinetic (PK) profile of AMX after oral administration of 10 µg of antibiotic per mouse (**Figure 3**). The AMX serum concentrations increased during 10 min after administration of AMX alone and then declined to levels below the minimal inhibitory concentration (MIC) of AMX for *S. pneumoniae* within 2 h. Interestingly, we observed that the AMX's maximum serum concentration was achieved at 30 min in the AMX_{10}+MPLA treatment group and the rate of decline was different from those recorded in the AMX group. These finding suggests that the particular PK profile of AMX in the presence of MPLA may contribute to the observed efficacy of the combination treatment.

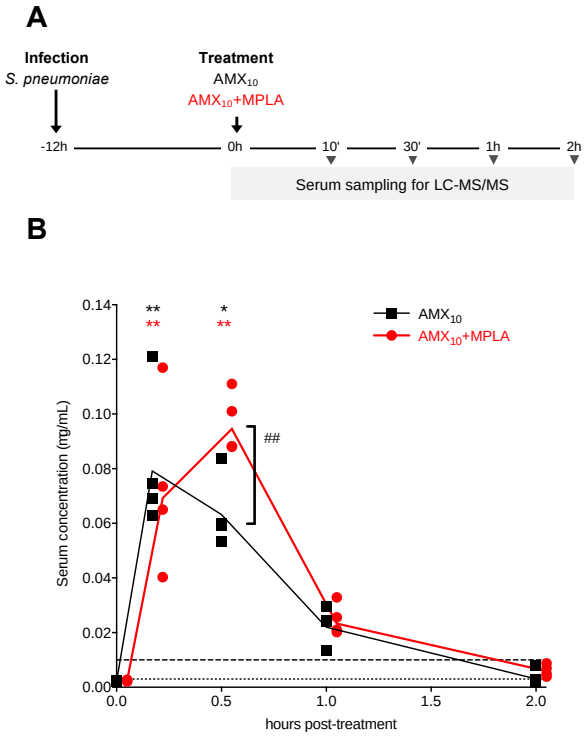

Figure 3. Pharmacokinetics of orally administered AMX in mice. (A) Swiss mice (n=4/group) infected with 10^6 *S. pneumoniae* and treated with either 10 µg intragastrically administered of amoxicillin (AMX_{10}) or a combination of AMX and a 50 µg intraperitoneally administered MPLA (AMX_{10}+MPLA). Blood samples were collected at different times post-

treatment. (B) Change in the serum AMX concentration over time, as determined using LC-MS/MS. A one-way ANOVA (the Kruskal-Wallis test with Dunn's post-test for multiple comparisons) was applied to analyze kinetic changes regarding time 0 in AMX_{10} or AMX_{10}+MPLA groups. The lines indicate the median value and the symbols represent individual values; Groups were compared using a Kruskal-Wallis test: *=p<0.05 and **=p<0.01 *vs.* mock, or a two-way ANOVA with Bonferroni's post-test for multiple comparisons for AMX_{10} vs. AMX_{10}+MPLA comparison at indicated times: ## = $p<0.01$. The dashed and dotted lines along the x-axis indicate the lower limit of quantification (0.01 μg/mL) and the limit of detection (0.003 μg/mL), respectively.

Since we were unable to directly detect MPLA in the blood using Limulus Amebocyte assays or TLR4 reporter cells, we analyzed the systemic immune responses, i.e. the pharmacodynamics (PD) of MPLA during pneumococcal respiratory infection. The administration of MPLA (whether concomitant with AMX_{10} treatment or not) resulted in the release of pro-inflammatory mediators into the blood of infected and treated animals (**Figure 4 and Figure S3**). The serum concentrations of CCL2, IL-12 p40, IL-6, and TNF peaked within the two first hours of administration, after which time they fell gradually and returned to baseline levels. Moreover, the immune mediators IL-1α, IL-1β, IL-12p70, IL-17A, IL-23, IL-27, IFNγ, or GM-CSF were neither produced by infection nor induced by the various treatments. These data indicate that the therapeutic efficacy of AMX+MPLA against bacteria correlates with the MPLA-dependent systemic immune response.

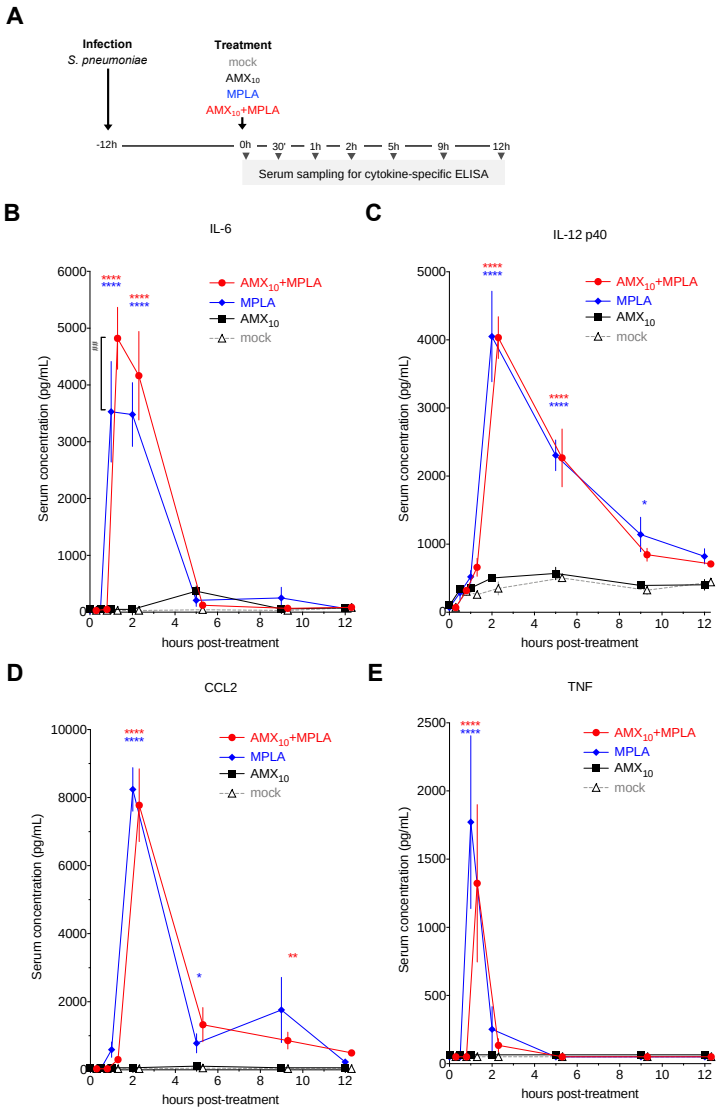

Figure 4. MPLA treatment induces an immediate, transient cytokine response. (A) Swiss mice (n=4/group) were infected intranasally with 10^6 *S. pneumoniae* and then treated 12 h later with either AMX_{10} (10 µg, intragastric administration), MPLA (50 µg intraperitoneal administration), a combination of AMX_{10} and MPLA, or left untreated. Blood samples were collected at different times post-treatment. Serum levels of pro-inflammatory mediators were determined using ELISAs. (B) IL-6, (C) IL-12 p40 subunit, (D) CCL2, and (E) TNF. The mean ± SEM values are shown. A two-way ANOVA with Bonferroni's post-test for multiple comparisons was applied. At indicated times: *=p<0.05, **=p<0.01 and ***=p<0.001 vs. the untreated group; ##=p<0.01 vs. the indicated comparator groups.

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

We hypothesized that, beyond the systemic response, the respiratory immune responses to MPLA participate in bacterial clearance. To challenge this idea, we analyzed the transcriptome of lung tissue of infected animals. We investigated changes in gene expression by comparing the AMX_{10}- and AMX_{10}+MPLA-treated groups 2 h, 4 h, and 8 h after administration (**Figure 5A**).

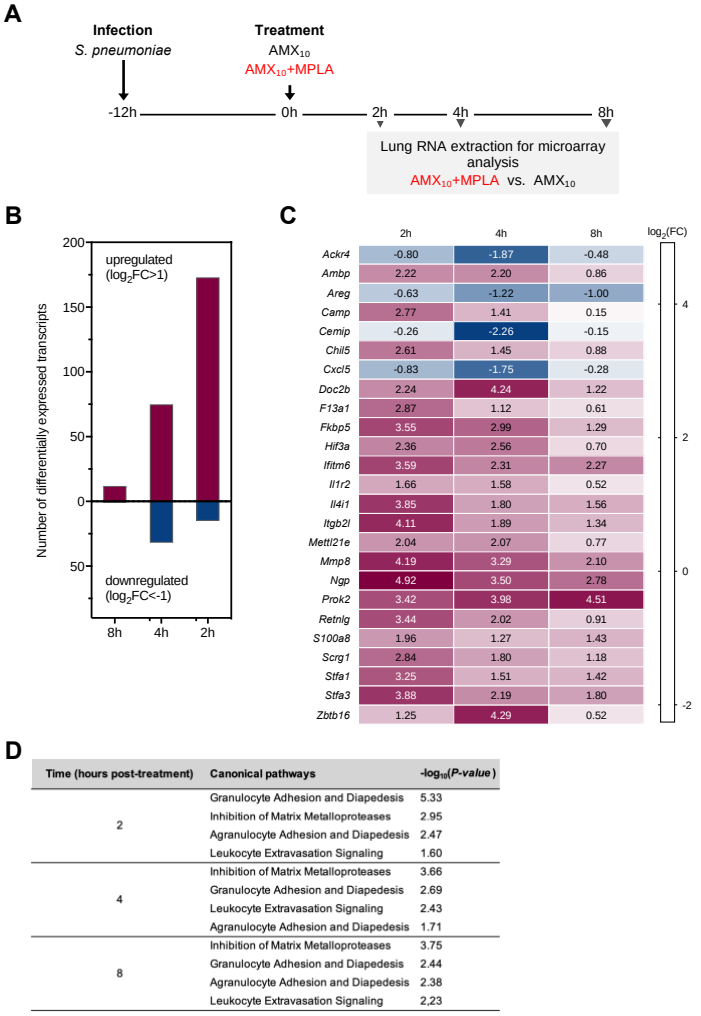

Figure 5. Characterization of lung immune response signatures of MPLA treatment. (A) BALB/cJRj mice (n=3/group) were infected with 10^6 *S. pneumoniae* and then treated with either AMX_{10} (10 µg, intragastric administration) or a combination of AMX_{10} and 50 µg of intraperitoneally administered MPLA (AMX_{10}+MPLA). Total RNA was extracted from lungs collected at different times, and mRNA transcripts were compared in a microarray analysis. (B) Number of transcripts with significantly greater expression (upregulation, log_2(fold change [FC] >1) or significantly lower expression (downregulation, $log_2FC < -1$) in (AMX_{10}+MPLA)-treated vs. AMX_{10}-treated animals. (C) The 25 genes with the highest or lowest differential expression levels in the microarray analysis (AMX_{10}+MPLA vs. AMX_{10}). (D) Enrichment of canonical pathways, according to an Ingenuity Pathway Analysis of the microarray datasets.

We found that 188 transcripts were differentially expressed at 2h post-treatment, among which 173 were upregulated >2-fold ($log_2FC>1$) and 15 were downregulated <0.5-fold ($log_2FC<-1$) in the AMX_{10}+MPLA-treated group compared to AMX_{10}. The number then decreased over time, with 106 transcripts at 4 h and 13 transcripts at 8 h (**Figure 5B**). The pattern and time course of expression in lungs suggested that the MPLA-induced transcriptional effects at the infection site were immediate and transient. Some of the lung transcripts strongly expressed within a few hours of treatment were associated with neutrophil function (e.g. *Ngp*, *Itgb2l*, and *Mmp8*) or antibacterial molecules (e.g. *Camp* or *S100a8*) (**Figure 5C**). Moreover, the overall response to the AMX_{10}+MPLA treatment indicated an enrichment in the granulocyte adhesion and diapedesis pathway and the leukocyte mobilization pathways (**Figure 5D**).

We confirmed the results by using RT-qPCR assays and extended the transcriptional study by including groups of infected and mock-treated animals, and infected and MPLA-treated animals (**Figure 6**). The effect of MPLA on lung transcript expression was found to be independent of AMX treatment. The expression of pro-inflammatory genes coding for cytokines and chemokines (such as *Cxcl2, Ccl20*, or *Il1b*) that are usually increased by infection was not further impacted by MPLA or AMX+MPLA treatment [23-24]. In line with the microarray data, MPLA treatment was found to accelerate the onset of potentially antimicrobial-related *Ngp*, *Itgb2l*, *Mmp8*, *Camp*, *S100a9*, *Fkbp5*, *Ifitm6*, and *Zbtb16* transcript expression in infected animals in both the MPLA-only and AMX_{10}+MPLA groups. Lastly, we found that the AMX+MPLA combination did not influence the neutrophil count in the lungs 12 h post-treatment (**Figure S4**). A similar pattern was observed for alveolar macrophages. In contrast, the lung monocyte count was higher in the AMX+MPLA group than in the AMX treatment group. Moreover, treatments did not influence monocyte and neutrophils counts in the same condition. Overall, the prompt airway innate immune responses induced by the AMX_{10}+MPLA treatment suggest that lung phagocytes, including monocytes may be involved in the mucosal bacterial clearance and the prevention of invasion/dissemination.

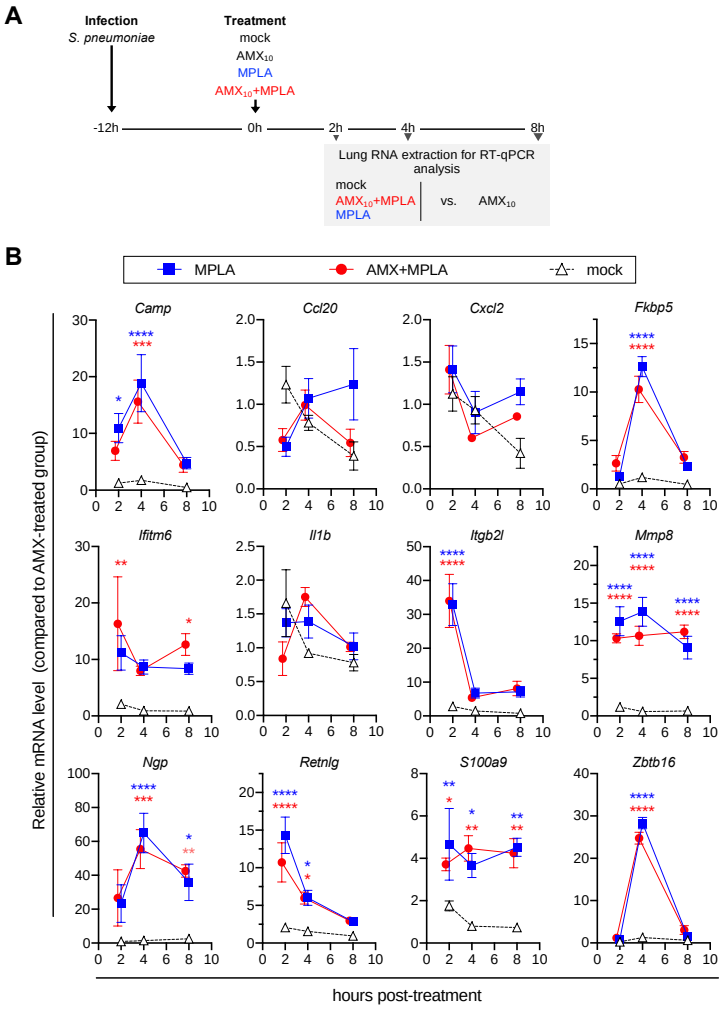

Figure 6. MPLA treatment boosts immune responses at the infection site. (A) BALB/cJRj mice (n=3-8/group) were infected with *S. pneumoniae* and treated 12 h post-infection with either AMX_{10} (10 µg, intragastric administration), 50 µg MPLA intraperitoneally, a combination of the two treatments (AMX_{10}+MPLA), or mock-treated; total RNA was extracted from lungs collected at different times, and mRNA transcripts were compared using RT-qPCR assays. Expression levels were normalized against infected, and AMX-treated controls. (B) Change in relative mRNA levels over time. The mean ± SEM values are shown. A two-way ANOVA with Bonferroni's post-test for multiple comparisons was applied. *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001*vs.* AMX-treated group.

DISCUSSION

This study described an alternative strategy for combating invasive pneumococcal disease and reduce the dose and regimen of antibiotics. We used a biosynthetic immunomodulator (the TLR4 agonist MPLA) as an add-on treatment to boost the efficacy of first-line antibiotic therapy. Our findings showed that the outcomes of sub-curative antibiotic treatment are significantly improved (i.e. greater bacterial clearance or inhibition and better survival) following targeted stimulation of the host's innate immune system in inbred and outbred animals. According to this study, the use of MPLA with antibiotics can be considered as a leverage to decrease the dose of antibiotics for treatment and, consequently, the selective pressure on the emergence of resistant bacteria.

The prophylactic administration of MPLA confers protection against bacterial sepsis, pneumonia, and burn-wound models of disease [15, 17-18, 25]. The therapeutic effect of MPLA-containing adjuvants in the context of systemic mycosis was evidenced [26]. The administration of LPS, the highly pyrogenic and toxic TLR4 agonist with antibiotic is able to eliminate *Salmonella* invading the mesenteric lymph nodes, in contrast to stand-alone antibiotic treatment [19]. All these data suggested that MPLA could be repurposed as a universal antimicrobial booster. Despite MPLA's proven potency, a single dose of MPLA alone had a limited therapeutic effect in pneumonia. The present proof-of-concept study with *S. pneumoniae* replicated clinical conditions since the respiratory infection was established prior to treatment, thereby simulating the lag between diagnosis and treatment in clinical context. Moreover, mice were administered a low dose of AMX that mimics ineffective treatment and incomplete bacterial clearance; a clinical context that will require extended duration or increased dose of antibiotics and promote emergence of resistance. Here, we demonstrated that the combination of AMX and MPLA had a greater therapeutic effect than AMX monotherapy alone.

It remains to be seen how MPLA is involved in greater bacterial clearance in the treatment with AMX. The MPLA-associated transcriptional signature in the lungs of infected animals highlighted the upregulation of genes associated with neutrophil/monocyte function and tissue homing. One can assume that a greater phagocyte count and an enhanced killing capacity may drive MPLA's antibacterial action and synergize them with the antibiotic's effects in the airways. Our preliminary data did not highlighted changes in neutrophil number in lung and spleen; however, monocytes were found in higher number in lung (**Figure S4**). The direct

activation of cells via TLR4 may also enhance the antibacterial potency of monocytes or neutrophils, as suggested with LPS in *Salmonella*-infected dendritic cells [19]. Such immune process may also explain the kinetic of bacterial numbers that decrease over time and was maximal at 36 h in our experimental conditions. We also observed that MPLA induces systemic innate response such as production of circulating cytokines that may contribute to effectiveness of combination treatment after their levels have reached the baseline. The cytokines may modulate downstream immune processes that mobilize a second wave of antibacterial effectors. For instance, the innate immune response due to MPLA may change the activation levels of specific immune cells; the activation may last for days. Remarkably, TLR4 signaling induced innate immune responses are associated with the enhancement of the adaptive response in the context of adjuvant of vaccination [8-9, 11-12, 14]. Regarding this study, it remains to be defined whether the combination of AMX with MPLA can also improve the adaptive immunity specific for pneumococcus, for instance by activation of dendritic cells or other hematopoietic cells that can present pneumococcal antigens. With a view to defining specific immune targets, it will be important to determine how the MPLA-stimulated immune system cooperates with an antibiotic to improve treatment efficacy in the lungs and upon dissemination.

The AMX PK analysis indicated that serum concentrations are above MIC within minutes after administration but rapidly decreases. Hence, AMX's bactericidal activity depends on the time during which the concentration exceeds the MIC for its target; accordingly, several administrations are required to ensure complete efficacy. Amoxicillin's rapid PK maximum and minimum concentrations were in contrast with the PD characteristics of MPLA, i.e. longer-term activation of the immune system. Our present findings suggest that the different time scales of AMX's and MPLA's respective activities increased the overall efficacy of treatment when the two are administered together. On a PK level, we also observed a slight increase in AMX retention in serum when being was combined with MPLA. We recently developed a pharmacometric PK/PD disease-treatment-survival model that revealed a pharmacokinetic interaction and enhanced activity of amoxicillin and MPLA in combination [27]. Future research must focus on how the two substances interact specifically *in vivo* in an infectious context into the airways and upon dissemination of bacteria and how the findings can be translated to human.

Our present results highlighted on the potential of targeting innate immunity with a TLR4 agonist as a viable strategy for improving antibiotic therapy against a bacterial infection. This pragmatic approach uses the

host's immune system to strengthen the attack against invading microorganisms, and may also help to repurpose currently available drugs with known characteristics. Our experimental evidence suggests that the enhanced therapeutic effect of the MPLA and AMX combination is achieved through a combination of TLR4 priming and the time scales of MPLA's and AMX's respective peak biological activities. Many novel agonists of TLR4 either biosynthetic or chemically engineered are currently in development and may represent fascinating molecules with peculiar properties of immunostimulation, PK, or targeted effects that may improve the proof-of-concept shown with MPLA [28-30]. Whereas the improved immunity due to the combined treatment depends on innate immune processes, investigations on the deployment of adaptive immunity will be required to fully understand the mechanisms of action. In the future, it will be important to investigating possible synergistic relationships between individual treatment components by using (i) further *in vivo* simulations in multiple-dose regimens, (ii) infections associated with other tissues, (ii) different classes of antibiotics either bactericidal or bacteriostatic, and (iv) translational mathematical modelling of PK and PD. Ultimately, this promising approach may open up new avenues for the design of host-directed therapeutics for infectious diseases.

METHODS

Ethics statement. All experiments complied with current national, institutional and European regulations and ethical guidelines, were approved by our Institutional Animal Care and Use Committee (animal facility agreement C59-350009, Institut Pasteur de Lille; reference: APAFIS#5164, protocol 2015121722429127_v4) and were conducted by qualified, accredited personnel.

Bacterial strains and cell cultures. *Streptococcus pneumoniae* serotype 1 (clinical isolate E1586) was used [20]. The minimum inhibitory concentration (MIC) of AMX for this strain is about 0.016 µg/ml. Working stocks were prepared as described previously [20-21]. Briefly, Todd Hewitt Yeast Broth (THYB) (Sigma-Aldrich, St. Louis, MO, USA) was inoculated with fresh colonies grown in trypcase soy agar plates supplemented with 5% sheep blood (BioMérieux, Marcy-l'Étoile, France), and incubated at 37°C until the OD_{600nm} reached 0.7-0.9 units. Cultures were stored at -80ºC in THYB + glycerol 12% (vol/vol) for up to 3 months. For mouse infections, working stocks were thawed and washed with sterile Dulbecco's phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) and diluted to the appropriate concentration. The number of bacteria (expressed in colony forming units [CFU]) was confirmed by plating serial dilutions onto blood agar plates.

The mouse model of infection. Six- to eight-week-old female BALB/cJRj, C57BL/6JRj or Swiss (RjOrl:SWISS CD-1) mice (Janvier Laboratories, Saint Berthevin, France) or *Tlr4*$^{-/-}$ (on C57BL/6J genetic background) mice bred in house were used. Infection was carried out as described previously [23-24]. Mice were first anesthetized by intraperitoneal injection with a solution of 1.25 mg ketamine plus 0.25 mg xylazine in 250 µL of PBS, after which they were infected intranasally with a 30 µL PBS suspension containing 1 to 4×10^6 CFU of *S. pneumoniae*. The treatments described below were administered once 12h post-infection. Mice were sacrificed via the intraperitoneal injection of 5.47 mg of sodium pentobarbital in 100 µl PBS (Euthasol, Virbac, Carros, France). Blood was sampled by retro-orbital puncture into Z-Gel micro tubes (Sarstedt, Nümbrecht, Germany) to prepare serum. Lungs and spleens were homogenized, and plated onto blood agar to determine the bacterial load. The lower limit of quantification was set to 17 CFU. For survival assays, both mortality and changes in body weight were monitored; mice were individually weighed prior to infection and then every 24h for two weeks.

Reagents and administration of treatments *in vivo*. Amoxicillin trihydrate (Sigma-Aldrich) was prepared in a stock solution of 1.75 mg/mL in water and then adjusted to a final dose of 5, 30, or 350 µg/mouse in 200 µL water (i.e., 0.2, 1.2, or 14 mg/kg) before intragastric administration. Lipopolysaccharide from *E. coli* O111:B4 (S-form) and MPLA from *S. minnesota* R595 (Re) (TLR*pure*™, Innaxon Therapeutics, Bristol, United Kingdom) were obtained as sterile solutions or prepared from powder with sterile distilled water to a concentration of 1 mg/L (according to the manufacturer's recommendations) and then adjusted to final concentrations in PBS and administered by intraperitoneal injection (200 µL). MPLA is a mixture of congeners containing disaccharide of 2-deoxy-2 aminoglucose, once phosphorylated and substituted with four to seven fatty hydroxyacyl or acyloxyacyl chains [6-7]. This MPLA is used as an adjuvant in prophylactic and therapeutic vaccines.

Quantification of gene expression and microarrays. Lungs were perfused with PBS prior to sampling. Total RNA was extracted with the Nucleospin RNA II kit (Macherey Nagel, Düren, Germany) and reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The cDNA was amplified using SYBR-Green-based real-time PCR on a Quantstudio™ 12K Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA, USA). Specific primers used are listed in **Table S1**. Relative mRNA levels were determined by comparing the PCR cycle thresholds (Ct) for the gene of interest vs. *Actb* (ΔCt) and then the ΔCt values for treated vs. untreated (mock) groups (ΔΔCt). For the microarray analysis, total RNA yield and quality were further assessed on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). One-color whole mouse (084809_D_F_20150624 slides) 60-mer oligonucleotide 8x60k v2 microarrays (Agilent Technologies) were used to analyze gene expression. The cRNA labelling, hybridization and detection steps were carried out according to the supplier's instructions (Agilent Technologies). For each microarray, cyanine-3-labeled cRNA was synthesized from 50 ng of total RNA using the low-input QuickAmp labeling kit. RNA Spike-In was added to all tubes and used as a positive control in the labelling and amplification steps. Next, 600 ng of each purified labelled cRNA were then hybridized and washed following manufacturer's instructions. Microarrays were scanned on an Agilent G2505C scanner, and the data were extracted using Agilent Feature Extraction Software (version 10.7.3.1, Agilent Technologies). Microarray data

have been deposited in the Gene Expression Omnibus database (accession number: GSE118860; token: adsdkgaodnkjrcd). Statistical comparisons and filtering were performed with the Limma R package with 75-percentile normalization. Differentially expressed genes were considered to be those with an adjusted p-value below 0.05 after the false discovery rate had been checked with the Benjamini-Hochberg procedure [31]. Pathways were investigated using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA).

Histology. Lungs were fixed by intratracheal perfusion with 4% formaldehyde prior to sampling. The left lobe and the upper right lobe were included in paraffin, and 3- to 5-µm tissue sections were stained with hematoxylin and eosin reagent. The slides were blindly evaluated for neutrophil infiltration, perivascular infiltration, edema, and pleuritis on a 6-level scale, where 0 corresponded to the absence of lesions, and 1 to 5 corresponded to minimal, slight, moderate, marked, and severe lesions, respectively (Althisia, Troyes, France).

Quantification of serum cytokine levels. Serum levels of CCL2, IL-6, IL-12 p40 and TNF were measured using an ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MO, USA).

Determination of serum amoxicillin concentrations. Serum AMX concentrations were assayed using validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method [32]. In brief, the proteins in 10 µL of serum were precipitated with 40 µL of ice-cold methanol. After diluting the supernatant with water, the sample was injected into the LC system (Agilent Technologies) by using a gradient elution at a flow rate of 0.3 mL/min with acetonitrile and water with formic acid. The AMX ion product (*m/z* 114) was quantified using electrospray ionization MS in positive ion mode over a calibration range from 0.01 to 10 µg/mL. In-study validation was performed according to the European Medicines Agency guidelines on bioanalytical method development (https://www.ema.europa.eu/en/bioanalytical-method-validation).

Peritoneal macrophage preparation. Mice were sacrificed by cervical dislocation. After intraperitoneal injection of 5 ml of cold PBS, macrophages were isolated by aspiration with a syringe. Cells were counted in order to plate 10^6 cells per well (48 well plates). After incubation at 37°C for 4 h, peritoneal macrophages are adherent to the plastic and are stimulated for 24 h using 50 ng/ml of MPLA or PBS alone.

Statistical analysis. Results were expressed as the median and individual values, median [interquartile range] or mean ± standard error of the mean (SEM), as appropriate. Groups were compared using a Mann-Whitney test (for two independent groups) or a Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's post-test (for three or more groups) or a two-way ANOVA with Bonferroni's post-test for multiple comparisons for two variables. The log rank test was used for survival analyses. Statistical analyses were performed using GraphPad Prism software (version 8.2, GraphPad Software Inc., San Diego, CA, USA), and the threshold for statistical significance was set to $p<0.05$.

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SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publication Website.

Systemic MPLA administration leads to dose-dependent immune activation (Figure S1); MPLA treatment augments the therapeutic effect of low-dose amoxicillin (Figure S2); MPLA treatment induces a cytokine response independently of mouse genotype (Figure S3); MPLA treatment influences the number of monocytes but not the number of neutrophils in lung (Figure S4). List of primers used in this study (Table S1)

FUNDING

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

MEETING PRESENTATION

Part of this work was presented at the 29th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).

ABBREVIATIONS

Amoxicillin: AMX; CFU: colony forming unit; monophosphoryl lipid A: interleukin: IL-; keto-3-deoxy-octonate: KDO; MPLA; reverse transcription quantitative PCR: RT-qPCR; Todd Hewitt Yeast Broth: THYB; Toll-like receptor: TLR

AUTHOR CONTRIBUTIONS

FC and LM performed animal, RT-qPCR, ELISA, and flow cytometry experiments. SF and RM analyzed antibiotic PK data. MF performed microarray experiments and bioinformatics analyses. CK, CC, and JCS designed the experiments. FC, LM, JCS, and CC wrote the manuscript. JCS and CC supervised the experimental work as a whole. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

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

DATA AND MATERIALS AVAILABILITY

Microarray data are available in the Gene Expression Omnibus database (accession number: GSE118860).

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ACKNOWLEDGMENTS

We thank Shéhérazade Sebda and Frédéric Leprêtre from University of Lille's genomics facility for preparing microarrays and depositing the data in the Gene Expression Omnibus database, respectively. We also thank Charlotte Costa for analysis of RT-qPCR data and the UMS2014-US41-PLBS, BioImaging Center Lille, Flow Cytometry Core Facility.

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