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A Toll-Like Receptor 5 Agonist Improves the Efficacy of Antibiotics in Treatment of Primary and Influenza Virus-Associated Pneumococcal Mouse Infections

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Prophylactic intranasal administration of the Toll-like receptor 5 (TLR5) agonist flagellin protects mice against respiratory pathogenic bacteria. We hypothesized that TLR5-mediated stimulation of lung immunity might improve the therapeutic index of antibiotics for the treatment of *Streptococcus pneumoniae* respiratory infections in mice. Intranasal administration of flagellin was combined with either oral administration of amoxicillin or intraperitoneal injection of trimethoprim-sulfamethoxazole to treat *S. pneumoniae*-infected animals. Compared with standalone treatments, the combination of antibiotic and flagellin resulted in a lower bacterial load in the lungs and greater protection against *S. pneumoniae* dissemination and was associated with an early increase in neutrophil infiltration in the airways. The antibiotic-flagellin combination treatment was, however, not associated with any exacerbation of inflammation. Moreover, combination treatment was more efficacious than standalone antibiotic treatments in the context of post-influenza virus pneumococcal infection. Lastly, TLR5 signaling was shown to be mandatory for the efficacy of the combined antibacterial therapy. This report is the first to show that combining antibiotic treatment with the stimulation of mucosal innate immunity is a potent antibacterial strategy against pneumonia.

Antibiotic treatment is widely acknowledged to be an effective medical intervention against bacterial infections. However, this therapeutic approach is often associated with adverse effects. For example, antibiotics induce the emergence of resistance and promote major changes in the composition of the mucosal microbiota (a phenomenon known as dysbiosis) (1). At the dawn of the postantibiotic era (as defined by the World Health Organization) and faced with the lack of new antibiotics, there is an urgent need for novel antibacterial strategies (2). From this perspective, manipulation of the innate immune system of the host might constitute a therapeutic option in the fight against bacterial pathogens (3). Indeed, the stimulation of innate immunity (i) promotes the production of immune mediators and antimicrobial effectors and (ii) boosts the recruitment and activation of effector immune cells; together, these actions contribute to bacterial clearance.

Pattern recognition receptors (including the Toll-like receptors [TLRs]) are key components of the innate immune system. TLRs recognize conserved microbe-associated molecular patterns and are expressed in all cells (4). The activation of TLRs by microbial components triggers signaling cascades and promotes the archetypal proinflammatory responses involved in antimicrobial defense (4, 5). Primary immunodeficiencies and polymorphisms in TLRs or components of the signaling cascade are strongly associated with susceptibility to infections in both humans and animal models (5). In view of their broad cellular distribution and important role in immunity, the TLRs have emerged as therapeutic targets in the fight against infectious diseases and sepsis (3, 6, 7).

TLR5 is expressed at the surfaces of epithelial cells and dendritic cells. It detects flagellin, the main component of the bacterial flagella (8). There is now strong evidence to suggest that TLR5 signaling induces protective responses to bacterial infections. In humans, the dominant mutation $TLR5_{1174T}$ is thought to be associated with susceptibility to *Legionella pneumophila* (9). In rodents, TLR5 was shown to be essential for the activity of mucosal innate defenses against *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and uropathogenic *Escherichia coli* (10–13). Furthermore, it was shown that the exogenous administration of flagellin can protect mice against mucosal infections, including bacterial pneumonia (14–19). Indeed, intranasal administration of mice with flagellin prior to *P. aeruginosa* inoculation protects against pneumonia via the production of the cathelicidin-related antimicrobial peptides (19). We and others have shown that nasal administration of flagellin also confers prophylactic neutrophil-dependent protection against lung infection with *Streptococcus pneumoniae* (18). Notably, our recent studies

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highlighted the fact that the flagellin-induced innate immune response in the respiratory tract specifically requires TLR5 signaling in the airway epithelium and is independent of the cytosolic Nod-like receptor family caspase activation and recruitment domain-containing protein 4 (NLRC4) (20, 21).

Although TLR targeting represents an interesting strategy for the induction of antimicrobial effectors, TLR agonists have not shown significant therapeutic effects on infections when administered alone (3, 6). Here, we hypothesized that the flagellin might be therapeutically active in the presence of agents with direct antibacterial action (e.g., antibiotics) and that stimulation of mucosal innate immunity might enhance the therapeutic index of antibiotics. We tested this hypothesis with *S. pneumoniae*, the Gram-positive pathogen that is the leading cause of community-acquired pneumonia and invasive infections (22). *S. pneumoniae* is also the main pathogen associated with secondary pneumonia in the context of respiratory infection by influenza A virus (IAV) (23). In the present study, we combined antibiotic treatment (amoxicillin [AMX] or trimethoprim-sulfamethoxazole [SXT]) with the administration of a recombinant flagellin that stimulates innate responses but has low intrinsic immunogenicity (24). Our results show that such combination treatment enhanced the cure rate for both primary *S. pneumoniae* infections and secondary post-influenza virus pneumococcal infections.

MATERIALS AND METHODS

Bacterial preparation. *S. pneumoniae* serotype 1 (clinical isolate E1586) was obtained from the National Reference Laboratory, Ministry of Health, Uruguay, and working stocks were prepared, as previously described (18, 25). Briefly, Todd-Hewitt yeast broth (THYB) (Sigma-Aldrich, St. Louis, MO) was inoculated with fresh colonies grown in blood agar plates and incubated at 37°C until an optical density at 600 nm (OD_{600}) of 0.7 to 0.9 units was reached. Cultures were stored at −80°C in THYB plus 12% (vol/vol) glycerol for up to 3 months. For mouse infection, working stocks were thawed and washed with sterile Dulbecco's phosphate-buffered saline (PBS) (Gibco, Grand Island, NY) and diluted to the appropriate concentration. The number of bacteria (CFU) was confirmed by plating serial dilutions onto 5% sheep blood agar plates.

Mouse model of infection. Female BALB/cJ or male C57BL/6J (6- to 8-week-old) mice (Janvier Laboratories, Saint-Berthevin, France) and male *Tlr5*$^{-/-}$ mice (backcrossed on C57BL/6J) were maintained in individually ventilated cages and handled in a vertical laminar flow cabinet (class II A2; Esco, Hatboro, PA). All experiments complied with current national and institutional regulations and ethical guidelines (B59-350009, Institut Pasteur de Lille). For infection by the intranasal route, each mouse was anesthetized by intraperitoneal injection of 1.25 mg of ketamine plus 0.25 mg of xylazine in 250 μl of PBS. Primary infections were performed with 2×10^6 *S. pneumoniae* organisms in 30 μl of PBS. For superinfection, mice were infected with 50 μl of PBS containing 30 PFU of the highly pathogenic murine-adapted H3N2 influenza A virus strain Scotland/20/74, as previously described (26, 27). Seven days post-viral challenge, each mouse was infected intranasally with 10^3 *S. pneumoniae* organisms. Mouse survival was recorded every 12 h. For a determination of the bacterial numbers in the lungs and spleen, mice were sacrificed at selected times by intraperitoneal injection of 5.47 mg of sodium pentobarbital in 100 μl of PBS. Tissues were collected and homogenized with an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany), and viable counts were determined by plating serial dilutions onto blood agar plates.

Flagellin and antibiotic administration. The recombinant flagellins $FliC_{\Delta 174-400}$, harboring a carboxy-terminal histidine tag, and rFliC, harboring an amino-terminal histidine tag, originated from *S.* Typhimurium FliC, as described previously (24). The constructs were generated by PCR and cloned into the expression vector pET22b$^+$. Site-directed mutagenesis was performed on the plasmid harboring rFliC in order to replace residues 89 to 96 (QRVRELAV) involved in TLR5 detection by the corresponding sequences from a nonsignaling flagellin (DTVKVKAT) (28); the resulting protein was designated $rFliC_{89-96*}$. Flagellins were produced in *E. coli* BL21(DE3). After disruption on a French press, the soluble fraction was depleted of lipopolysaccharide (LPS) using Triton X-114 extraction (29). The proteins were purified successively on nickel affinity, anion exchange, and gel filtration chromatography by Fast protein liquid chromatography (GE Healthcare, Pittsburgh, PA). Using the *Limulus* assay (Associates of Cape Cod, Inc., East Falmouth, MA), the residual LPS concentration was determined to be <20 pg per μg of flagellin. To ensure that flagellins were mostly monomers, they were heated for 10 min at 65°C before use. Flagellins (5 μg of rFliC and $rFliC_{89-96*}$ or 2.5 μg of $FliC_{\Delta 174-400}$ in 30 μl of PBS) or PBS alone were administered intranasally under light anesthesia by inhalation of isoflurane (Axience, Pantin, France). The infected mice were treated either intragastrically with AMX (5 μg in 200 μl of water per animal, 250 μg/kg of body weight; Vertanal; Sigma-Aldrich) or intraperitoneally with SXT (160 μg of trimethoprim and 800 μg of sulfamethoxazole in 200 μl of PBS per animal, 48 mg/kg of body weight; Bactrim Roche, Basel, Switzerland).

Lung histology. Lungs were perfused with PBS and fixed in 4% formaldehyde. 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. A blinded evaluation of the pathology was scored according to a 6-category scale for neutrophil infiltration, perivascular infiltration, edema, and pleuritis, in which 0 is no lesion/change and 1 to 5 correspond to minimal, slight, moderate, marked, and severe lesions, respectively.

Flow cytometry. Bronchoalveolar lavage (BAL) fluid samples were obtained after intratracheal injection of 1 ml of RPMI 1640 medium (Gibco) supplemented with 5% fetal calf serum, as described previously (18). Lung cells were isolated, as described previously (25). Cells were stained with anti-CD45-Brilliant Violet 711 (clone 30F11), anti-CD11b-Brilliant Violet 785 (clone M1.70), anti-SiglecF-allophycocyanin (clone E50-2440), anti-Ly6C-allophycocyanin-cyanine 7 (clone HK1.4), anti-Ly6G-Alexa Fluor 700 (clone 1A8), and anti-CD11c-phycoerythrin-cyanine 7 (clone HL3) antibodies. The antibodies were purchased from BD Biosciences (San Jose, CA) or BioLegend (San Diego, CA). Data were collected on a BD LSR Fortessa and analyzed with the BD FACSDiva software.

Gene expression quantification by real-time RT-PCR. Total lung RNAs were extracted with the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany) and reverse transcribed with the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). The cDNA was amplified using SYBR green-based real-time PCR on a 7300 real-time PCR system (Applied Biosystems). Primers specific for *Actb*, *Ccl20*, and *Cxcl1* were described previously (30). Relative mRNA levels ($2^{-\Delta\Delta CT}$) were determined by comparing first the PCR cycle thresholds (C_T) for the gene of interest and *Actb* (ΔC_T), and second, the ΔC_T values for the treated and untreated (mock) groups ($\Delta\Delta C_T$) (30).

Statistical analysis. The results are expressed as medians or means ± standard deviations (SD), as indicated. Statistical differences were analyzed using the Mann-Whitney test and the log rank test (GraphPad Prism 5.0a) and were considered to be significant for P values of <0.05.

RESULTS

Combined administration of flagellin and AMX improves the treatment of respiratory pneumococcal infection. The murine model of respiratory bacterial infection used here consisted of intranasal administration of a lethal dose of *S. pneumoniae*. This usually causes pneumococcal invasive disease 24 h postinfection and results in death within 3 to 4 days (see Fig. S1A and B in the supplemental material). We hypothesized that flagellin administration might boost the activity of antibiotics against *S. pneumoniae*. Hence, we first established that intranasal treatment with the recombinant immunostimulatory nonimmunogenic flagellin

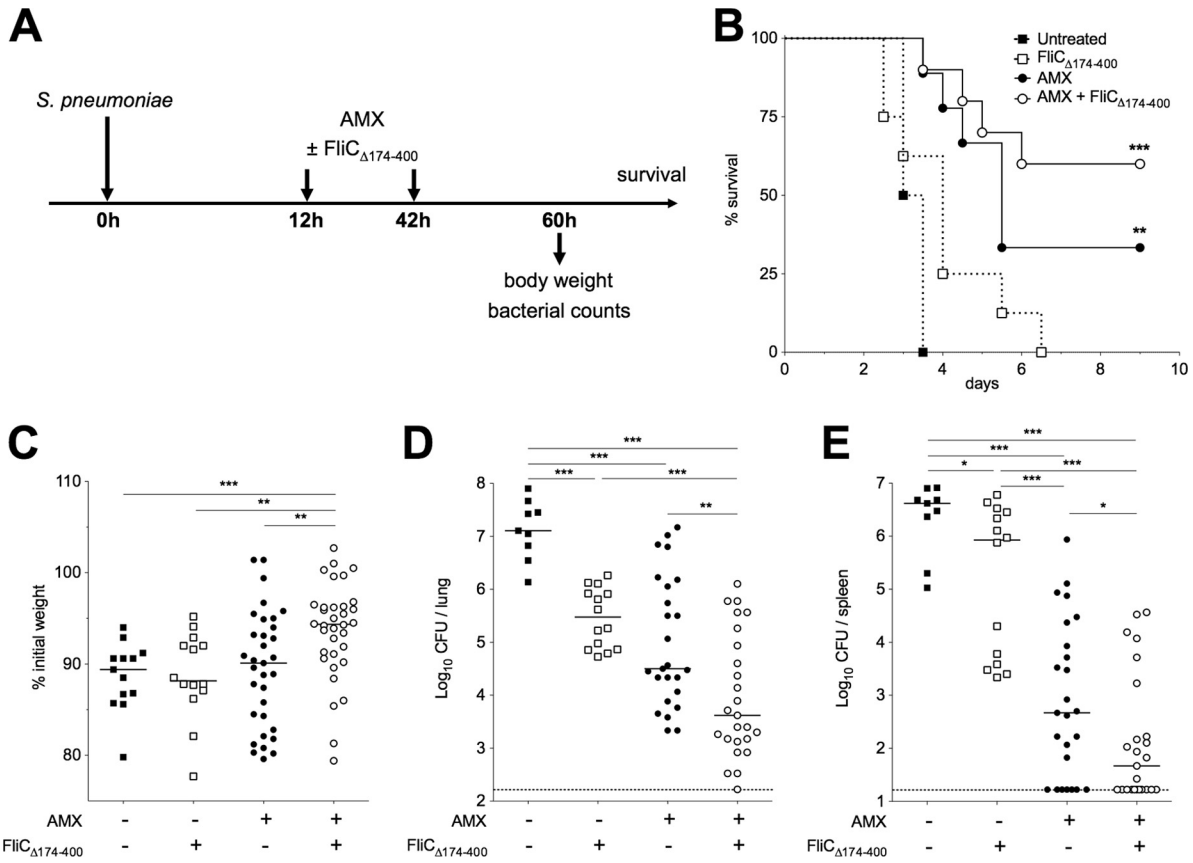

FIG 1 Flagellin improves the therapeutic efficacy of amoxicillin during *S. pneumoniae* respiratory infection. (A) Female BALB/c mice were infected intranasally with 2×10^6 *S. pneumoniae* organisms. The animals were then either left untreated or were treated at 12 and 42 h (i) intranasally with 2.5 μg of flagellin FliC$_{\Delta174-400}$ in phosphate-buffered saline (PBS), (ii) intragastrically with 5 μg of amoxicillin (AMX) and intranasally with PBS, or (iii) with a combination of AMX and FliC$_{\Delta174-400}$. (B) Survival of mice ($n = 9$ to 10) was monitored for 9 days. Statistical significance compared to the untreated mice was determined by a log rank test. (C) The body weights of the infected animals ($n = 13$ to 34) were measured at 60 h and expressed relative to the initial weights. Bacterial counts were determined in the lungs (D) and spleen (E). CFU counts for individual mice ($n = 9$ to 25) at 60 h are shown. The solid lines correspond to the median values, and the dashed lines represent the detection threshold. Statistical significance was assessed by a Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

FliC$_{\Delta174-400}$ activates lung innate immune signaling during the course of infection (see Fig. S1C to E in the supplemental material). Indeed, flagellin enhanced the transcription of *Ccl20* and *Cxcl1* (both surrogate markers of TLR5-mediated lung stimulation [21]) in animals from 0 to 24 h postinfection. These results indicated that flagellin is able to boost innate signaling even in the context of ongoing pneumococcal infection.

AMX impairs bacterial wall synthesis and is a clinically relevant antibiotic for the treatment of pneumonia. We therefore assessed the ability of a combination of flagellin and AMX to cure infections. BALB/c mice were infected with *S. pneumoniae* and treated 12 and 42 h postinfection with intragastric low-dose AMX and/or intranasal flagellin FliC$_{\Delta174-400}$ (Fig. 1A). None of the infected animals survived when left untreated or treated with flagellin alone (Fig. 1B). The survival rates were 33% for standalone antibiotic therapy and 60% for the AMX-flagellin combination treatment. The combination treatment was also associated with a lower degree of weight loss, relative to standalone antibiotic, standalone flagellin, or untreated conditions (Fig. 1C). Consistent with the results for survival, the combination treatment decreased the lung bacterial burden by factors of 72 and 8 compared with standalone treatment with flagellin FliC$_{\Delta174-400}$ and AMX, respectively (Fig. 1D). Moreover, the dissemination of *S. pneumoniae* into the spleen of animals treated with the combination treatment was 18,000- or 10-fold lower than that in animals treated with flagellin or AMX alone, respectively (Fig. 1E).

In a histologic assessment, the lungs of *S. pneumoniae*-infected and untreated mice were characterized by acute perivascular inflammation and by fibrinonecrotizing pleuropneumonia of various intensities. The airways and alveoli were not greatly altered (Fig. 2 and Table 1). In contrast, treatment with AMX alone or AMX plus FliC$_{\Delta174-400}$ appeared to protect the lung architecture to a similar extent, with less-severe signs of perivascular and pleural necrosis or inflammation.

In conclusion, mucosal administration of flagellin enhances the antibacterial activity of AMX in pneumonia and associated sepsis and does not exacerbate respiratory inflammation.

Combination therapy with flagellin requires TLR5. We next looked at whether TLR5 signaling is necessary for the protection elicited by the combination treatment. To this end, we used two recombinant flagellins: (i) rFliC, which has the same ability to promote mucosal TLR5 signaling as native flagellin, and (ii) rFliC$_{89-96*}$, in which substitution of amino acids 89 to 96 prevents TLR5 signaling (24, 28). Combination therapy with rFliC and

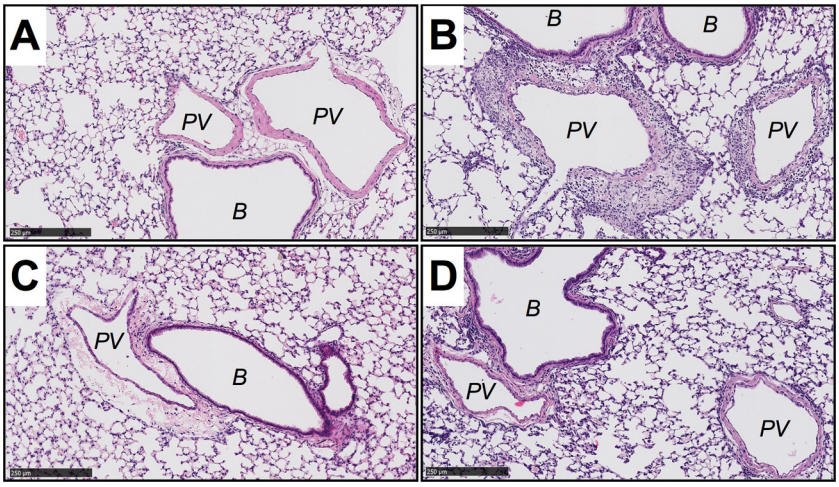

FIG 2 Combination therapy does not exacerbate inflammation or damage lung architecture. Female BALB/c mice ($n = 5$) were treated intranasally with phosphate-buffered saline (PBS) as a reference (A) or infected intranasally with 2×10^6 *S. pneumoniae* organisms (B to D). Infected mice were either left untreated (B) or treated at 12 and 42 h intragastrically with 5 μg of amoxicillin (AMX) and intranasally with PBS (C) or treated with a combination of amoxicillin and 2.5 μg of intranasally administered flagellin $FliC_{\Delta 174-400}$ (D). The lungs were sampled at 48 h (A and B) or 60 h (C and D), and the sections were stained with hematoxylin and eosin reagent and examined at a magnification of ×100. The scale bar on each panel represents 250 μm. *B*, bronchiole; *PV*, pulmonary venule.

AMX was efficacious against *S. pneumoniae* infection in female BALB/c mice, whereas the therapeutic effect was lost when the $rFliC_{89-96^*}$ and AMX were administered (Fig. 3A and B). The contribution of TLR5 signaling was further confirmed by studying TLR5-deficient animals. The bacterial counts in the lungs and spleen of $Tlr5^{-/-}$ animals that received AMX alone or the AMX-$FliC_{\Delta 174-400}$ combination therapy were essentially the same (Fig. 3C and D). Interestingly, the combination therapy was efficacious in male C57BL/6 mice and female BALB/c mice. These results demonstrate that TLR5 signaling is required for the protection and efficacy afforded by AMX-flagellin combination therapy. These experiments also highlighted the minor impact of the genetic background and gender of the host on TLR5-dependent activity.

Flagellin enhances efficacy of SXT in the treatment of respiratory pneumococcal infection. We next looked at whether flagellin improves the therapeutic index of antibiotics other than AMX. We selected SXT, a drug formulation containing two antimicrobial agents impairing folic acid synthesis. This antibiotic is not commonly used as a first-line treatment for pneumococcal infections but could be repurposed for these diseases. Starting 12 h after *S. pneumoniae* infection, each mouse was treated intraperitoneally with SXT every 12 h for 3 days and intranasally with $FliC_{\Delta 174-400}$ every 24 h (Fig. 4A). The survival rates were 41% for standalone SXT therapy and 67% for the SXT-flagellin combination treatment (Fig. 4B). As was seen with AMX, flagellin was able to enhance the local and systemic clearance of bacteria and attenuate weight loss (Fig. 4C to E). Interestingly, 68% of the animals treated with SXT and flagellin were completely free of the bacteria in the spleen, whereas this was true for only 22% of the mice treated with standalone SXT (Fig. 4E). Compared with infected untreated animals, both SXT and the combination therapy protected against histological changes in the lung (data not shown). Our data suggest that combination therapy with flagellin can repurpose disused or second-line antibiotics for the treatment of bacterial pneumonia.

Combination therapy increases neutrophil recruitment in the lungs. It was previously shown that intranasal administration of flagellin induces neutrophil infiltration into airways (18, 31). In the present study, we observed that flagellin stimulated the expression of CXCL1, a chemokine that promotes recruitment of neutrophils (see Fig. S1E in the supplemental material). We then wondered whether the combination therapy increases the neutrophil recruitment to the lung tissue. Therefore, neutrophils were detected in lung extracts and BAL fluid by flow cytometry 60 and 88 h postinfection in the AMX-flagellin and SXT-flagellin models, respectively. We found that both combination treatments increased the neutrophil counts in the BAL fluid compared to those seen with standalone antibiotic treatments (Fig. 5A and C). A significant increase in neutrophils was also observed in the lungs

TABLE 1 Lung histopathological score of mice infected with *S. pneumoniae* and treated with AMX standalone or combinatory treatment[a]

Histological change	Group change score (individual scores) in[b]:		
	Untreated[c]	AMX	AMX + $FliC_{\Delta 174-400}$
Alveolar neutrophil infiltration	1.6 (1, 1, 2, 2, 2)	1.0 (1, 1, 1, 1, 1)	1.0 (1, 1, 1, 1, 1)
Perivascular mixed inflammatory cells	2.8 (4, 1, 4, 4, 1)	1.0 (0, 1, 2, 1, 1)	0.6 (0, 0, 1, 1, 1)
Perivenous/perivenular edema	3.4 (4, 1, 4, 4, 4)	1.2 (3, 0, 1, 2, 0)	1.6 (0, 0, 2, 4, 2)
Fibrinoleukocytic/suppurative pleuritis	3.4 (4, 1, 4, 4, 4)	1.2 (3, 0, 1, 2, 0)	1.6 (0, 0, 2, 4, 2)

[a] See Fig. 1A and Fig. 2 for infection and treatment protocol.
[b] The scores for the indicated changes in individual infected animals ranged from 0 (no lesions) to 6 (severe lesions).
[c] Mice were infected and left untreated, and the lungs were sampled at 48 h postchallenge.

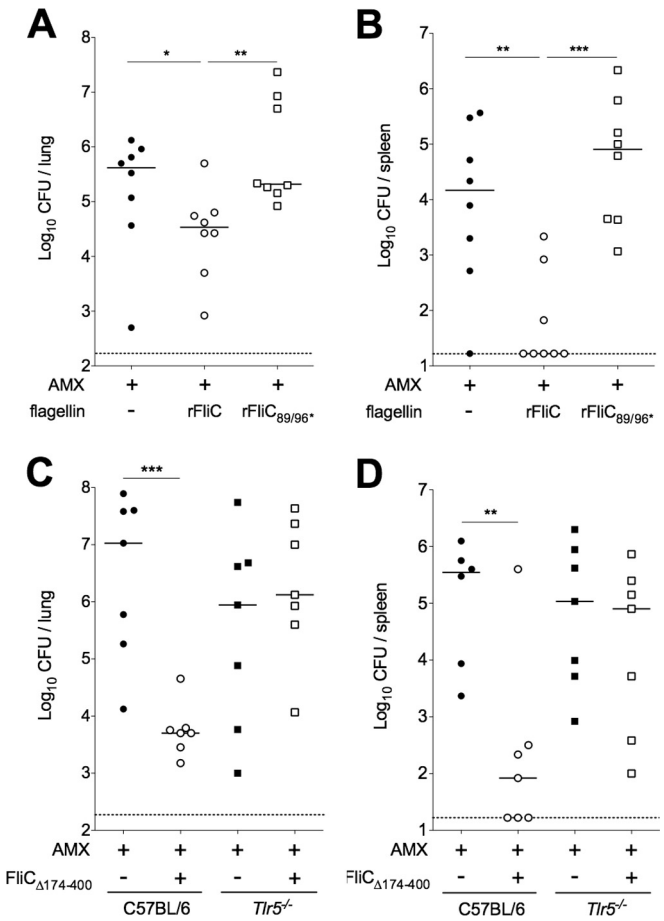

FIG 3 Antibacterial activity associated with combination treatment with flagellin and amoxicillin requires TLR5. (A and B) Female BALB/c mice were infected intranasally with 2×10^6 *S. pneumoniae* organisms and were treated at 12 and 42 h intragastrically with 5 μg of amoxicillin (AMX) and intranasally with phosphate-buffered saline (PBS) or a combination of AMX and intranasally administered flagellin (5 μg of either histidine-tagged flagellin [rFliC] or histidine-tagged mutant flagellin [$rFliC_{89-96*}$]). (C and D) C57BL/6 and *Tlr5*$^{-/-}$ male mice were infected intranasally with 2×10^6 *S. pneumoniae* organisms and were treated at 12 and 42 h intragastrically with 5 μg of AMX and intranasally with PBS or 2.5 μg of flagellin $FliC_{\Delta 174-400}$. Bacterial counts were determined after 60 h in the lungs (A and C) and spleen (B and D). The CFU counts for individual mice ($n = 6$ to 8) are shown, and the solid lines represent the median values. The dashed lines represent the detection threshold. (A and B) Statistical significance was assessed in a Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C and D) Statistically significant differences with respect to the antibiotic-treated mice were assessed by a Mann-Whitney test (**, $P < 0.01$; ***, $P < 0.001$).

of animals treated with the AMX-flagellin combination compared to AMX alone but not in the SXT model (Fig. 5B and D). All together, these data showed that the protective effect of the combination treatments is associated with the infiltration of neutrophils into the airways.

Combination therapy is efficacious against post-influenza virus superinfection. Severe pneumococcal infections are often associated with IAV, and this lethal synergy significantly contributes to the excess morbidity and mortality rates for influenza virus infection (23). We therefore investigated the efficacy of combination therapy in the context of IAV infection. Data in the literature indicate that after the resolution of infection, IAV induces the sustained desensitization of TLR-mediated lung innate responses (32). We found that *Tlr5* mRNA levels were decreased 40% ± 10% 7 days after IAV infection. We thus investigated the functionality of TLR5 signaling in the context of acute IAV infection. As shown in Fig. 6A and B, intranasal administration of $FliC_{\Delta 174-400}$ 7 days post-IAV infection specifically triggered the transcription of *Ccl20* and *Cxcl1* in the lung tissue. A similar pattern of flagellin-mediated responses was observed 14 days post-IAV infection (data not shown). These data demonstrate that flagellin can promote innate immune signaling during acute viral infection. The therapeutic activity of the combination treatment was then tested in the context of post-influenza virus superinfection with *S. pneumoniae*. Seven days after IAV infection, animals were infected with *S. pneumoniae* and treated with AMX or the combination of AMX and flagellin $FliC_{\Delta 174-400}$ (Fig. 6C). Compared with animals receiving AMX alone, bacterial counts in the lungs of mice treated with AMX and flagellin were 217-fold lower (Fig. 6D). Moreover, the dissemination of bacteria into the spleen was 1,700-fold lower (Fig. 6E). Interestingly, 57% of the mice that had received the combination treatment did not harbor any bacteria in the spleen, whereas this was true for only 5% of the AMX-treated animals. Therefore, the present data demonstrate that the combination therapy was highly efficacious in increasing the therapeutic index of AMX in the context of post-influenza virus superinfection.

DISCUSSION

The present report is the first to show that combined treatment with an antibiotic and a TLR agonist can improve the course of respiratory bacterial infections and prevent bacteremia. In addition to being more efficacious than the standalone antibiotic or flagellin therapy, the antibiotic-flagellin combination treatment was not associated with any exacerbation of inflammation. The therapeutic effect was dependent on TLR5 signaling, associated with neutrophil infiltration in the bronchoalveolar compartment, and independent of the genetic background and gender of the host and the nature of the antibiotic. Furthermore, the combination treatment was highly efficacious in the context of post-influenza bacterial superinfection. In summary, our data indicate that combining antibiotics with a TLR agonist may constitute a promising potentially universal strategy for improving the efficacy of antibiotic therapy.

The activation of innate immunity as a weapon against bacterial infections is an alternative to the use of direct antimicrobial agents, such as antibiotics (3, 6, 33). Several studies have highlighted the ability of TLR2/6, TLR4, TLR5, and TLR9 agonists and microbial extracts (delivered by the respiratory route) to prevent bacterial pneumonia (18, 19, 33–36). However, the effective use of this type of treatment is restricted to prophylactic regimens or is limited by a narrow therapeutic window. These limitations correspond to the function of innate immunity, i.e., the prevention or eradication of pathogenic microorganisms in the early course of an infection. When TLR agonists are administered during pneumonia or invasive disease (as in the present study), their activity may be limited by the high bacterial load present in the respiratory tract or circulation (6). Here, we hypothesized that the combination of a TLR agonist with an antibiotic would decrease the bacterial load in the local and systemic compartments and thus increase the efficacy of the TLR-induced host defenses. Recently, a similar strategy was found to be efficacious in the context of intestinal infections with *S.* Typhimurium (37). Indeed, the combina-

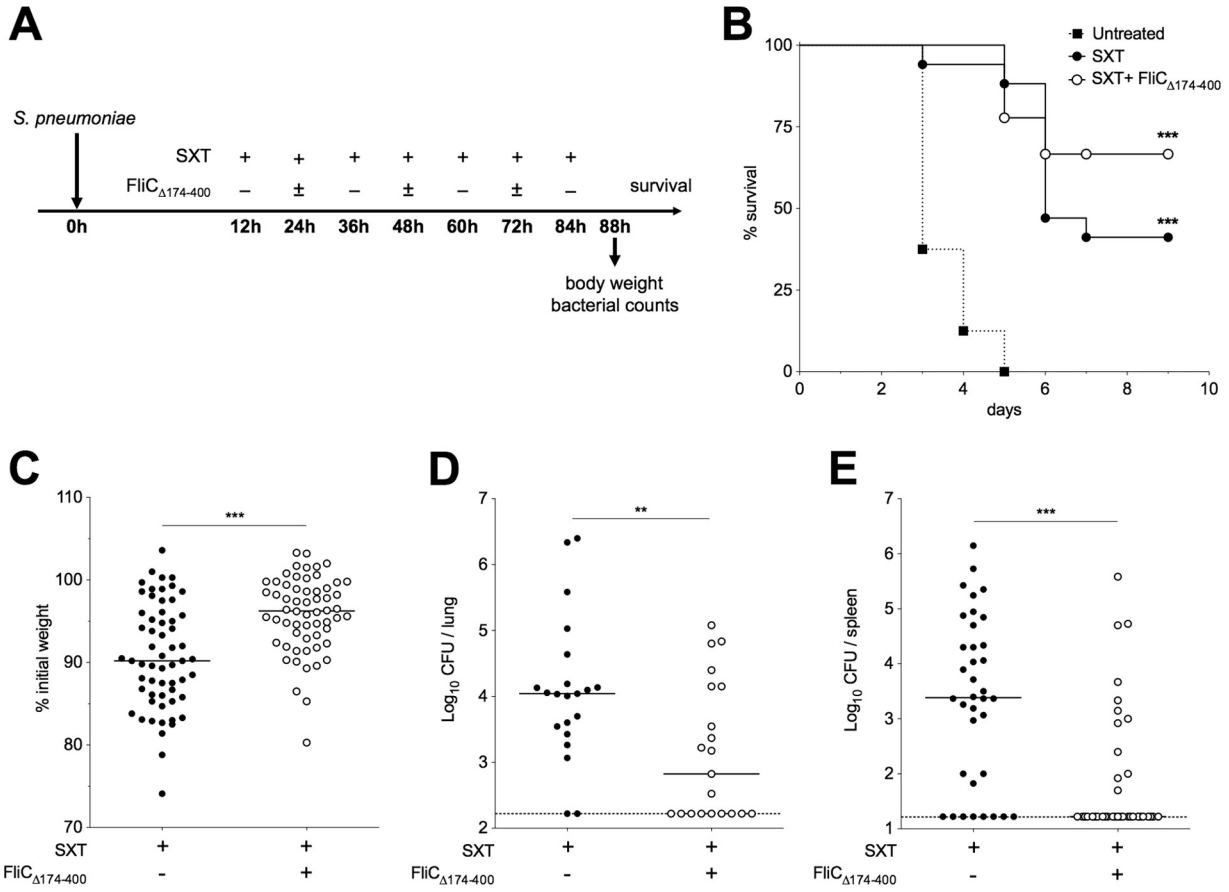

FIG 4 Therapeutic efficacy of trimethoprim-sulfamethoxazole in *S. pneumoniae* infection is improved by combination with flagellin. (A) Female BALB/c mice were infected intranasally with 2×10^6 *S. pneumoniae* organisms. The animals were then treated at 12, 24, 36, 48, 60, 72, and 84 h with 4.8 mg of intraperitoneally administered trimethoprim-sulfamethoxazole (SXT) (i.e., every 12 h postinfection) and treated at 24, 48, and 72 h with intranasally administered flagellin FliC$_{\Delta 174-400}$ (2.5 μg) or phosphate-buffered saline (PBS) (i.e., every 24 h postinfection). (B) Survival of mice ($n = 8$ to 18) was monitored for 9 days. Statistical significance was determined using a log rank test compared to the untreated mice. (C) The body weights of the infected animals ($n = 59$ or 60) were measured at 88 h and expressed relative to the initial weights. Bacterial counts were determined in the lungs (D) and spleen (E). The CFU counts for individual mice ($n = 21$ to 36) at 88 h are shown, and the solid lines represent the median values. The dashed lines represent the detection threshold. Statistically significant differences with respect to antibiotic-treated mice were assessed by a Mann-Whitney test (**, $P < 0.01$; ***, $P < 0.001$).

tion of systemic administration of ciprofloxacin and a TLR4 or TLR9 agonist was able to further reduce the number of antibiotic-tolerant *Salmonella* in the gut and the associated draining lymph nodes compared to that seen with standalone treatments.

Previous studies show that the respiratory delivery of flagellin induces TLR5-dependent neutrophil recruitment into the lungs, together with local production of bactericidal compounds (18–20, 31, 32). Thus, neutrophils and cathelicidin-related antimicrobial peptides have been linked to the prophylactic protective effects of flagellins against respiratory infections with *S. pneumoniae* and *P. aeruginosa*, respectively. Here we showed that the administration of flagellin in association with antibiotics increased neutrophil recruitment in the airways. The role of the neutrophils in the therapeutic effect of the combination treatments and the influence of antimicrobial molecules remain to be elucidated. Furthermore, there is strong evidence to suggest that the primary targets of intranasally administered flagellin are the airway epithelial cells (20, 21, 38). The epithelial cells express TLR5 strongly and respond both *in vitro* and *in vivo* to flagellin by upregulating the transcription of genes encoding host antibacterials and phagocyte-specific chemokines (21, 38–40). Airway epithelial cells were also found to be the driving force behind the inducible resistance to pneumonia produced in response to TLR2/6 and TLR9 agonists (41). Recently, systemic administration of flagellins or intranasal infection with bacteria was found to activate lung type 3 innate lymphoid cells (ILC3) (25, 30). These stimulated ILC3 secrete abundant amounts of interleukin 17 and interleukin 22, which are potent effectors against respiratory infections, particularly pneumococcal infections (25, 42, 43). Whether combination treatments activate ILC3 and play a role in the therapeutic effects remains to be studied. In conclusion, characterization of the mode of action of combination therapy and the contribution of these multiple mechanisms are major issues in the future development of this strategy.

The therapeutic activity of an antibiotic-flagellin combination might conceivably result from the respective additive effects of the TLR5-induced antibacterial effector and the antibiotic. Alternatively, the TLR5 agonist and the antibiotics may have synergistic effects on innate defenses. Indeed, the direct effects of antibiotics on bacterial viability and integrity are known to trigger the release of microbe-associated molecular patterns or endogenous compounds that act as danger signals (44). The resulting integration of

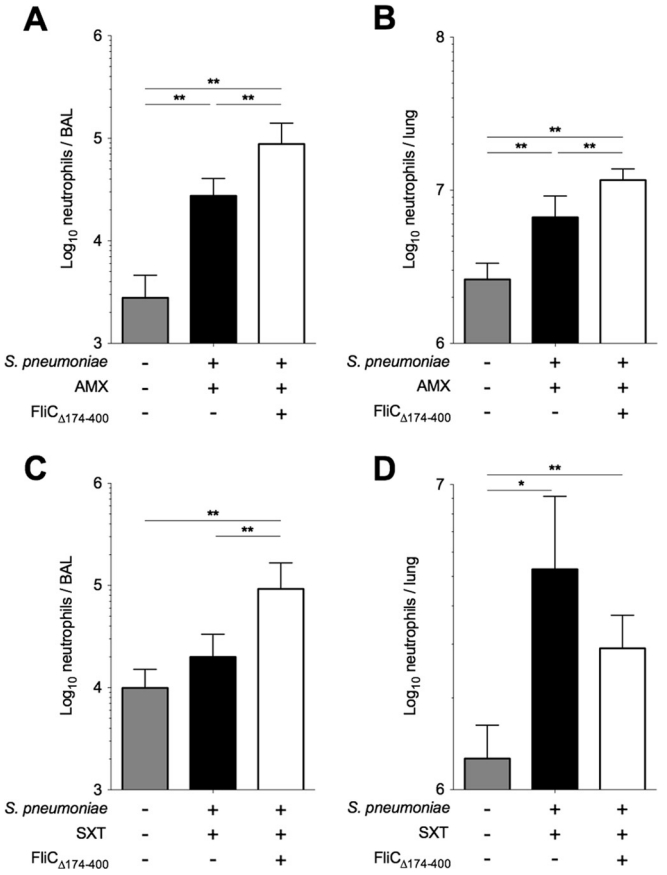

FIG 5 Combination therapies increase neutrophil recruitment in airways during pneumococcal infection. Female BALB/c mice ($n = 4$ to 5) were infected intranasally with 2×10^6 *S. pneumoniae* organisms or left uninfected as a control. (A and B) Infected mice were treated at 12 and 42 h intragastrically with 5 μg of amoxicillin (AMX) and intranasally with phosphate-buffered saline (PBS) or a combination of AMX and 2.5 μg of intranasally administered flagellin $FliC_{\Delta 174-400}$. (C and D) Infected mice were treated at 12, 24, 36, 48, 60, 72, and 84 h with 4.8 mg of intraperitoneally administered trimethoprim-sulfamethoxazole (SXT) (i.e., every 12 h postinfection) and treated at 24, 48, and 72 h with intranasally administered flagellin $FliC_{\Delta 174-400}$ (2.5 μg) or PBS (i.e., every 24 h postinfection). Bronchoalveolar lavage (BAL) fluid and lungs were sampled at 60 h (A and B) and 88 h (C and D), and cell composition was analyzed by flow cytometry and cell counts. The numbers of neutrophils ($CD45^+$ $CD11c^{neg}$ $SiglecF^{neg}$ $CD11b^+$ $Ly6G^+$ $Ly6C^+$) in BAL fluid (A and C) and the lungs (B and D) are shown as the means ± SD. Statistical significance was assessed by a Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$).

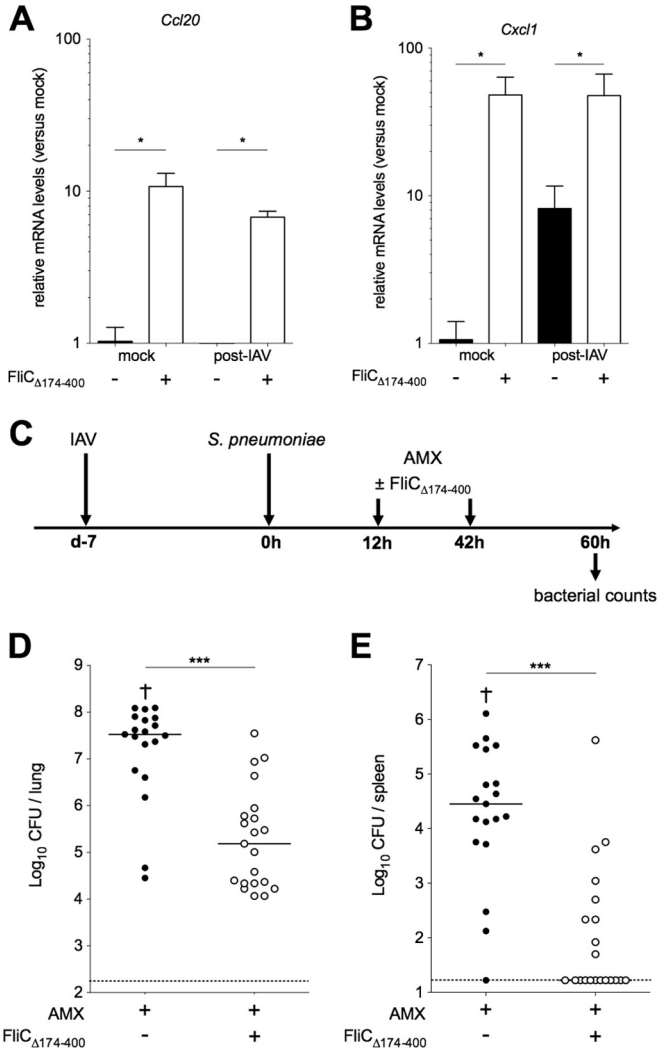

FIG 6 Combination treatment is efficacious in post-influenza virus secondary pneumococcal infection. (A and B) C57BL/6 male mice were infected intranasally with influenza A virus H3N2 (30 PFU). Seven days later, animals were treated intranasally with 2.5 μg of flagellin $FliC_{\Delta 174-400}$. Two hours after flagellin treatment, lungs were collected for analysis of transcript levels by quantitative RT-PCR. The mRNA levels for individual mice ($n = 4$) are expressed relative to those in the uninfected and untreated mock group (arbitrarily set to a value of 1) and are shown as the means ± SD. (C to E) Seven days after H3N2 challenge, animals were infected intranasally with 10^3 *S. pneumoniae* organisms. Mice were then treated at 12 and 42 h intragastrically with 5 μg of amoxicillin (AMX) and intranasally with phosphate-buffered saline (PBS) or a combination of AMX and 2.5 μg of intranasally administered flagellin $FliC_{\Delta 174-400}$ (C). Bacterial counts were determined at 60 h in the lungs (D) and spleen (E). CFU counts for individual mice ($n = 19$ to 21) are shown, and the solid lines represent the median values. The dashed lines represent the detection threshold. †, dead animals. Statistical significance was assessed by a Mann-Whitney test (*, $P < 0.05$; ***, $P < 0.001$).

these danger signals (via TLR5 and innate receptors) may drastically change the degree of stimulation of the immune system relative to each signal alone. Measuring the combination index of flagellin and selected antibiotics (as has been described for a drug combination [45]) and characterizing the innate immune signature in response to individual or combined treatments will be essential for accurately defining any cross talk and synergy between the TLR- and antibiotic-mediated signaling pathways.

Secondary bacterial infections with *S. pneumoniae* are frequently observed in the aftermath of IAV infection and contribute to the severity of the observed post-influenza virus infection manifestations (23, 26). Our present results show that the combination treatment was highly efficacious in controlling the bacterial burden and thus indicate that the immunomodulatory activity of flagellin is not inhibited by IAV infection. It is known that several weeks and months after resolution of influenza, IAV infection induces a partial but sustained desensitization of innate immunity, especially in response to TLR5 stimulation (32). However, we found that the transcriptional response to flagellin was not affected during the first 2 weeks following IAV challenge, indicating that TLR5 signaling is unaffected. Respiratory administration of a TLR3 agonist or a combination of TLR2/6 and TLR9 agonists was

shown to trigger protective antiviral responses (41, 46). Therefore, combining antibiotics with one or more TLR agonists may constitute a unique strategy for combatting viral and bacterial respiratory coinfections.

Antibiotic therapy compromises the mucosal microbiota. This dysbiosis is associated with diminished innate immune defenses (47, 48). Remarkably, exogenous stimulation of mucosal TLR signaling following antibiotic therapy restores immune defenses and prevents infections by opportunistic microorganisms (14, 15, 47, 49). Therefore, combining flagellins with antibiotics might not only represent a potent anti-infectious treatment but also may prevent secondary infections induced by antibiotic-induced dysbiosis.

In conclusion, combination treatment with a TLR5 agonist and an antibiotic might represent a new therapeutic opportunity in a worldwide context of antibiotic resistance and excessive antibiotic consumption. Indeed, this type of strategy might decrease the treatment duration and the doses used and thus the overall consumption of antibiotics. It might also reduce antibiotic-associated adverse effects, such as dysbiosis and the emergence of antibiotic resistance. Last, this combination strategy could be considered for repositioning old or discontinued antibiotics.

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We declare no conflicts of interest.

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