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HOST RESPONSE AND INFLAMMATION

Flagellin-Mediated Protection against Intestinal *Yersinia pseudotuberculosis* Infection Does Not Require Interleukin-22

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ABSTRACT Signaling through Toll-like receptors (TLRs), the main receptors in innate immunity, is essential for the defense of mucosal surfaces. It was previously shown that systemic TLR5 stimulation by bacterial flagellin induces an immediate, transient interleukin-22 (IL-22)-dependent antimicrobial response to bacterial or viral infections of the mucosa. This process was dependent on the activation of type 3 innate lymphoid cells (ILCs). The objective of the present study was to analyze the effects of flagellin treatment in a murine model of oral infection with *Yersinia pseudotuberculosis* (an invasive, Gram-negative, enteropathogenic bacterium that targets the small intestine). We found that systemic administration of flagellin significantly increased the survival rate after intestinal infection (but not systemic infection) by *Y. pseudotuberculosis*. This protection was associated with a low bacterial count in the gut and the spleen. In contrast, no protection was afforded by administration of the TLR4 agonist lipopolysaccharide, suggesting the presence of a flagellin-specific effect. Lastly, we found that TLR5- and MyD88-mediated signaling was required for the protective effects of flagellin, whereas neither lymphoid cells nor IL-22 was involved.

KEYWORDS interleukin-22, TLR5, *Yersinia pseudotuberculosis*, flagellin, intestine, mouse infection, Toll-like receptors

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The cross talk between mucosal immune cells and intestinal epithelial cells is essential for controlling the bacterial flora in the gut and protecting against pathogenic microbes (1, 2). Many studies have demonstrated that interleukin-22 (IL-22) has a role in the host's immune defense and in tissue-protective and regenerative functions in the gastrointestinal tract (3, 4). IL-22 is a cytokine from the IL-10 family. It is produced by T helper 17 (Th17) lymphocytes, γδ T cells, or NKT cells. Most of the IL-22 in the gut is produced by type 3 innate lymphoid cells (ILC3) (5–8). IL-22 targets epithelial cells and mesenchymal stromal cells but not immune cells, since the IL-22R1 subunit of the IL-22 receptor is not expressed by hematopoietic cells (9). IL-22 induces the signal transducer and activator of transcription-3 (STAT-3) pathway, which in turn leads to the induction of antimicrobial molecules (like C-type lectin RegIIIγ and cal-

granulin S100A9) (10), mucin production (11), and intestinal wound healing (12, 13). Innate IL-22 production by ILC3 requires transactivation by IL-23 (plus IL-1β, in some cases) secreted by dendritic cells (6), and this process has been linked to colonization resistance and protection against *Citrobacter rodentium* via the production of RegIIIγ (7, 14–17). In contrast, it was recently shown that IL-22 can promote *Salmonella* colonization, indicating that the cytokine's role depends on the pathogen in question (18).

Earlier research has shown that immunostimulatory treatments can promote the production of IL-22 in the gut and thus boost antibacterial defenses. Indeed, systemic administration of flagellin induces IL-22 production by ILC3 in the spleen and in the intestinal mucosa (19, 20). Flagellin (the main protein component of the bacterial flagella) binds to Toll-like receptor 5 (TLR5), an innate receptor expressed at the surface of epithelial and dendritic cells (21), and can also be sensed by NAIP5, a cytosolic protein associated with the NLRC4 inflammasome (22, 23). It has been demonstrated that the IL-22 production induced by flagellin was linked to the production of IL-23 by intestinal lamina propria CD103$^+$ CD11b$^+$ dendritic cells and TLR5 signaling (20). Interestingly, flagellin administration induces IL-22-dependent protection against vancomycin-resistant *Enterococcus faecalis* infections and against rotavirus infections (10, 24). Systemic flagellin administration also protects against *Clostridium difficile* colitis or *Salmonella enterica* serovar Typhimurium infection, although it has not yet been established whether this protection requires IL-22 (25, 26). Much as in the intestine, flagellin-mediated protection against *Streptococcus pneumoniae* respiratory infections is associated with IL-22 production by lung ILC3 (27).

In the present study, we hypothesized that flagellin treatment could protect against intestinal infection by *Yersinia pseudotuberculosis*, an invasive, Gram-negative enteropathogen responsible for enteritis and mesenteric lymphadenitis. *Y. pseudotuberculosis* targets the ileum, actively translocates across the intestinal barrier through M cells, infects the Peyer's patches and mesenteric lymph nodes, and disseminates to the spleen and the liver (28). We found that preexposing mice to flagellin protected against intestinal infection (but not systemic infection) by *Y. pseudotuberculosis*, and that this protection was independent of IL-22.

RESULTS

Administration of flagellin protects against intestinal infection (but not systemic infection) by *Y. pseudotuberculosis*. We first looked at whether the TLR5 agonist flagellin could induce resistance against oral infection with *Y. pseudotuberculosis*. To this end, BALB/c mice were injected intraperitoneally with TLR agonists and intragastrically infected 30 min later with a lethal dose of *Y. pseudotuberculosis*. Although all animals that had received PBS alone died within 11 days, only 33% of the animals that received flagellin died (Fig. 1A). Consistent with these findings, the *Y. pseudotuberculosis* count in stools of flagellin-treated animals was six times lower than that in samples from untreated animals (Fig. 1C), and the count in the spleen of animals was seven times lower (Fig. 1D). In contrast, the TLR4 agonist lipopolysaccharide (LPS) did not induce any protection, since LPS-treated animals rapidly died after oral *Y. pseudotuberculosis* infection (only 1 out of the 9 treated animals survived) (Fig. 1A). Production of IL-6 and CXCL2 in the blood and the ileum revealed that both flagellin and LPS significantly induced systemic and local inflammatory responses in mice (Fig. 2). However, LPS triggered a stronger response in blood, whereas flagellin induced a higher response in the ileum. Altogether, these results indicate that the protection is specific for flagellin and is not due to overall stimulation of innate defense mechanisms. When animals were infected intravenously with *Y. pseudotuberculosis* (in order to bypass intestinal tissue invasion), flagellin did not elicit a protective effect (Fig. 1B). These results show that systemic administration of flagellin protects against mucosal (but not systemic) *Y. pseudotuberculosis* infection.

It has previously been shown that BALB/c and C57BL/6 mice differ in their susceptibility to *Yersinia* infection (29). BALB/c mice have been classified as susceptible, whereas C57BL/6 mice are resistant because of their ability to mount strong IL-12,

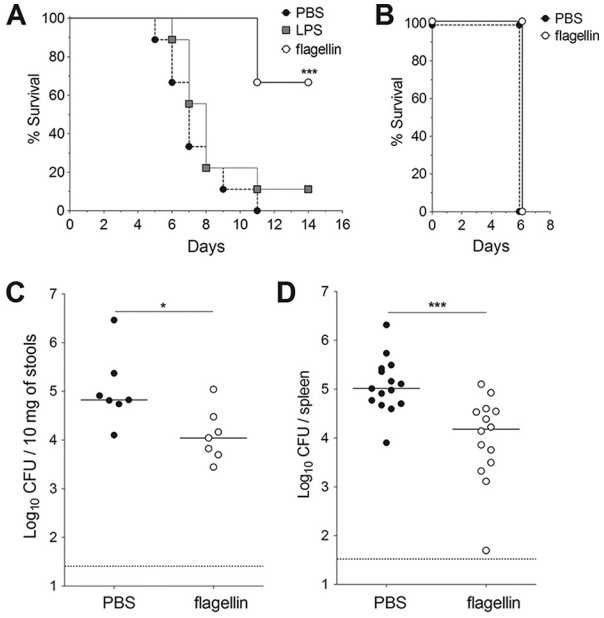

FIG 1 Flagellin protects against intestinal infection (but not systemic infection) by *Y. pseudotuberculosis*. Female BALB/c mice were treated intraperitoneally with flagellin (5 μg) or LPS (5 μg) in phosphate-buffered saline (PBS) or with PBS alone. The animals were challenged 30 min later with an intragastric inoculation of 5×10^8 CFU of *Y. pseudotuberculosis* (A, C, and D) or an intravenous injection of 10^3 CFU of *Y. pseudotuberculosis* (B). (A) The survival of mice ($n = 9$) after an oral challenge was monitored for 14 days. One of three representative survival experiments is shown here. Statistical significance was determined using a log rank test compared to the untreated mice (***, $P < 0.001$). (B) The survival of mice ($n = 10$) after an intravenous challenge was monitored for 7 days. Bacterial counts in the stools (C) and the spleen (D) were determined 72 h after the oral challenge. CFU counts for individual mice ($n = 7$ to 14) are shown. The solid line corresponds to the median value, and the dashed line represents the detection threshold. Data from flagellin-treated and untreated mice were compared in a Mann-Whitney test (*, $P < 0.05$; ***, $P < 0.001$).

gamma interferon (IFN-γ), and Th1 responses. We therefore analyzed the ability of flagellin treatment to promote protection against oral infection by *Y. pseudotuberculosis* in C57BL/6 animals (Fig. 3). Consistent with the trend of improved survival, we observed a significant increase of bacterial clearance upon flagellin treatment. Indeed, the

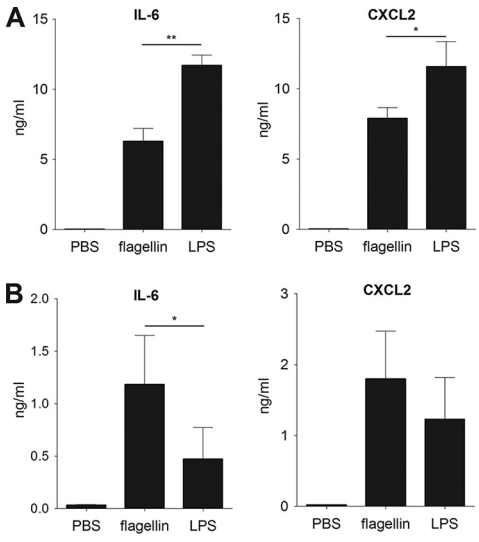

FIG 2 Flagellin and LPS activate systemic and local inflammation. BALB/c mice ($n = 5$) were treated intraperitoneally with flagellin (5 μg) or LPS (5 μg) in PBS or with PBS alone. Blood and ileum were sampled 2 h posttreatment. IL-6 and CXCL2 levels were measured by ELISA in serum (A) and ileum homogenates (B). The data are shown as the means $\pm$ SD. Data from flagellin-treated and LPS-treated mice were compared in a Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$).

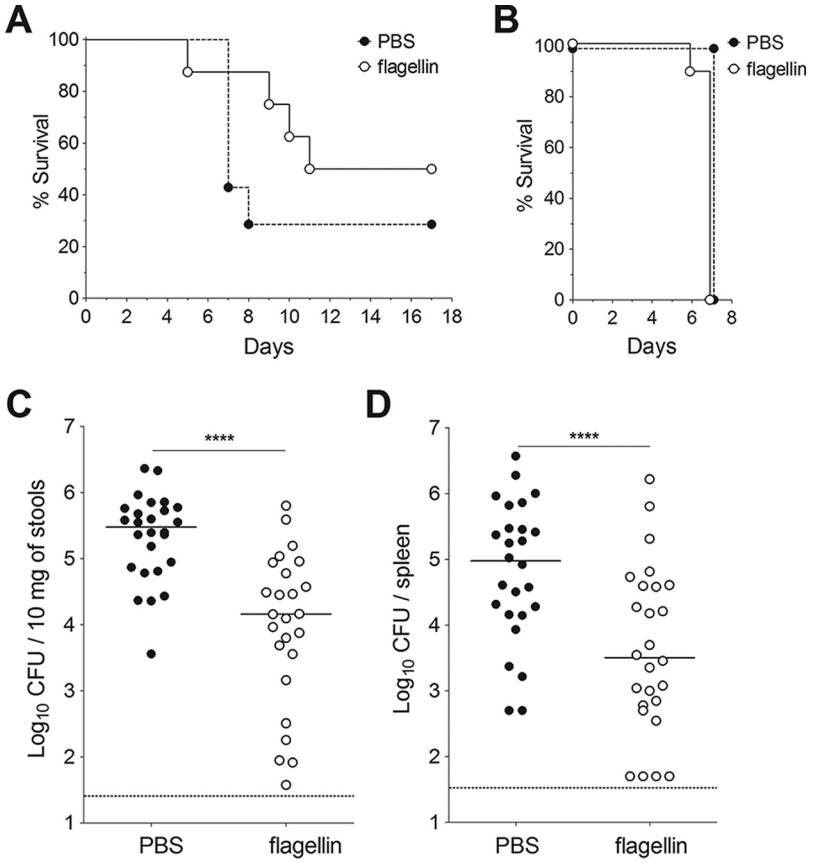

FIG 3 Flagellin protects against *Y. pseudotuberculosis* intestinal infection in *Yersinia*-resistant C57BL/6 mice. Female C57BL/6 mice were treated intraperitoneally with flagellin (5 μg) in PBS or with PBS alone 30 min before an intragastric challenge with 5×10^8 CFU of *Y. pseudotuberculosis* (A, C, and D) or an intravenous challenge with 10^3 CFU of *Y. pseudotuberculosis* (B). Survival was monitored daily for 17 days ($n = 7$ to 8) (A) or 7 days ($n = 10$) (B). Survival data from flagellin-treated and untreated mice were compared in a log rank test. Bacterial counts were determined in the stools (C) and the spleen (D). CFU counts for individual mice ($n = 25$ to 26) at 72 h postinfection are shown. The solid bar corresponds to the median value, and the dashed line represents the detection threshold. Data from flagellin-treated and untreated mice were compared in a Mann-Whitney test (****, $P < 0.0001$).

bacterial counts in the stools and spleen of flagellin-treated mice were 20- and 65-fold lower, respectively, than in untreated animals (Fig. 3A, C, and D). Analysis of the time course of *Y. pseudotuberculosis* colonization of the stools and spleen of C57BL/6 mice showed that flagellin-associated protection was observed from 72 h following infection (see Fig. S1 in the supplemental material). As seen in BALB/c mice, flagellin provided protection in C57BL/6 mice when *Y. pseudotuberculosis* was administered by the oral but not the systemic route (Fig. 3B). In conclusion, flagellin-mediated protection against *Y. pseudotuberculosis* is independent of the animal's intrinsic resistance to oral infection.

TLR5 signaling is specifically required for flagellin-mediated protection against *Y. pseudotuberculosis*. Two innate receptors are able to recognize flagellin: TLR5 detects extracellular flagellin, whereas the cytoplasmic NAIP5/NLRC4 complex detects intracellular flagellin. To define the receptors' respective contributions to the flagellin-induced protection, we compared the activity of recombinant flagellins impaired in their ability to stimulate TLR5 ($rFliC_{89-96*}$) (30) or the NAIP5/NLRC4 complex ($rFliC_{492stop}$) to that of unmutated recombinant flagellin (rFliC) (Fig. S2). As expected, $rFliC_{89-96*}$ was unable to trigger TLR5-specific responses (e.g., the systemic production of CXCL2 and CCL20 after intravenous injection), in contrast to rFliC or $rFliC_{492stop}$ (Fig. S2A and B). $rFliC_{492stop}$ was unable to induce the NLRC4-dependent processing of procaspase-1 seen with rFliC (Fig. S2C and D). Treatment with rFliC protected mice against *Y. pseudotuberculosis* infection, since the bacterial counts in stools and spleen were 20

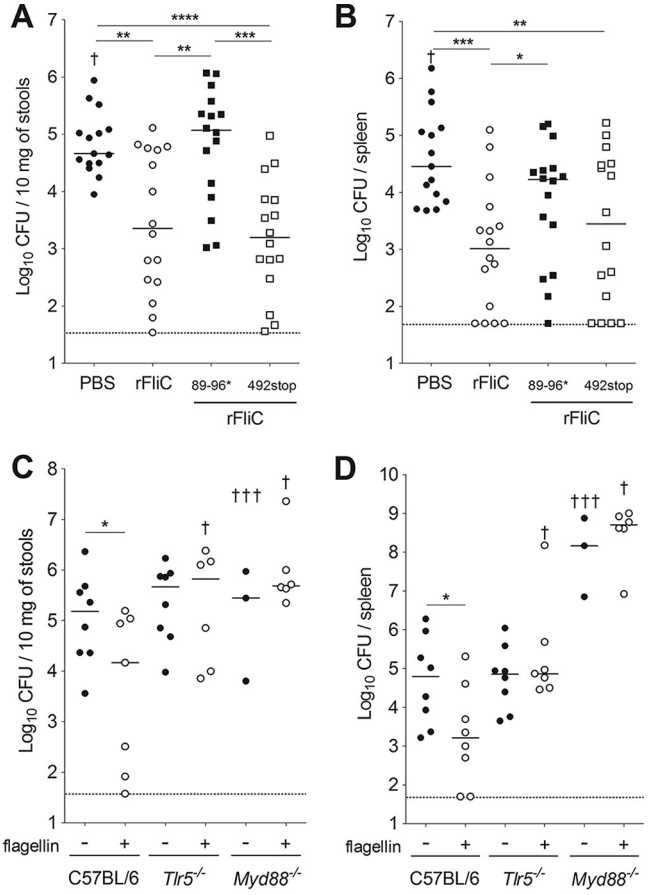

FIG 4 Flagellin-mediated antibacterial defenses require TLR5 signaling. (A and B) C57BL/6 mice were treated intraperitoneally with 5 μg of the histidine-tagged flagellin rFliC, histidine-tagged mutant flagellins (rFliC$_{89/96*}$ or rFliC$_{492stop}$), or PBS alone 30 min before an intragastric challenge with 5×10^8 CFU of *Y. pseudotuberculosis*. (C and D) *Tlr5*$^{-/-}$ or *Myd88*$^{-/-}$ mice were treated with 5 μg of native flagellin in PBS or with PBS alone 30 min before an intragastric challenge with 5×10^8 CFU of *Y. pseudotuberculosis*. Bacterial counts were determined in the stools (A and C) and the spleen (B and D). CFU counts for individual mice ($n = 7$ to 16) at 72 h postinfection are shown. The solid bar corresponds to the median value, and the dashed line represents the detection threshold. The dagger symbol indicates animals that died. Bacterial counts were not determined in these animals. Intergroup differences were analyzed in a Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

and 28 times lower, respectively, than in untreated, infected animals (Fig. 4A and B). Similar effects were obtained after treatment with rFliC$_{492stop}$, the flagellin unable to bind to NLRC4: the bacterial counts in the stools and spleen of treated mice were 29 and 10 times lower, respectively, than those for untreated animals (Fig. 4A and B). In contrast, protection was abrogated when mice were treated with rFliC$_{89-96*}$ prior to infection (Fig. 4A and B).

We also found that flagellin treatment was not associated with low bacterial counts in *Tlr5*$^{-/-}$ mice compared with those in C57BL/6 animals; this finding demonstrates that TLR5 signaling is required for the protection (Fig. 4C and D). Flagellin-mediated protection was also abolished in mice lacking myeloid differentiation factor 88 (MyD88), which is required for TLR5 signaling (Fig. 4C and D). It is noteworthy that high bacterial counts were found in the spleen of *Myd88*$^{-/-}$ mice infected with *Y. pseudotuberculosis*, confirming that MyD88 is critical for protection against *Yersinia* infection (31). Taken as a whole, these results showed that flagellin exerts its protective effect through the activation of TLR5.

Flagellin induces a transient inflammatory response independently of *Y. pseudotuberculosis* infection. To characterize the flagellin-mediated response, we analyzed gene expression in the ileum of C57BL/6 mice treated with flagellin and infected (or

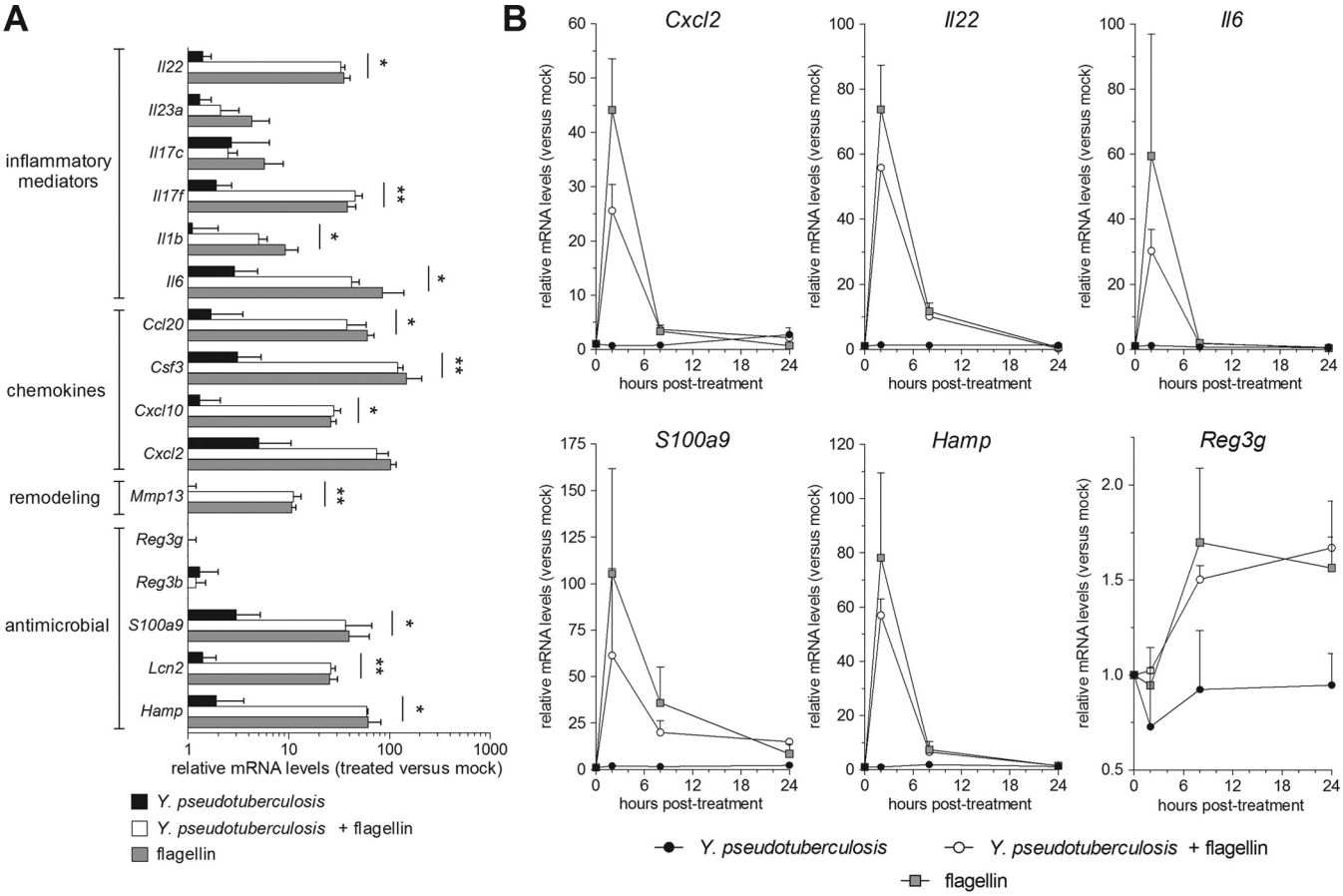

FIG 5 *Y. pseudotuberculosis* infection does not influence the flagellin-specific gene expression signature. (A and B) Female C57BL/6 mice ($n = 4$) were treated intraperitoneally with flagellin (5 μg) in PBS or with PBS alone (mock). Thirty minutes later, the animals were challenged intragastrically with 5×10^8 CFU of *Y. pseudotuberculosis*. One group of mice was treated with flagellin but not *Y. pseudotuberculosis*. Ilea were sampled postinfection and mRNA was quantified by quantitative reverse transcription-PCR. mRNA levels are expressed relative to those of uninfected and PBS-treated animals (the mock group; arbitrarily set to a value of 1) and are shown as the means ± SD. (A) Analysis of gene expression at 2 h posttreatment. Intergroup differences compared to flagellin-treated animals were analyzed in a Limma test with Benjamini-Hochberg FDR correction (*, $P < 0.05$; **, $P < 0.01$). Significant differences in mRNA expression levels were not found between flagellin and *Y. pseudotuberculosis* plus flagellin groups. (B) A time course analysis of gene expression.

not) with *Y. pseudotuberculosis*. Two hours after flagellin treatment, we observed strong expression of genes coding for inflammatory cytokines (e.g., IL-6, IL-1β, and IL-17F), chemokines (e.g., CCL20 and CXCL10), remodeling protein (MMP13), and antimicrobial molecules (S100A9, lipocalin-2, and hepcidin) (Fig. 5A). As expected, flagellin administration triggered a 100-fold relative increase in *Il22* mRNA levels within 2 h. Interestingly, flagellin-dependent gene expression occurred in both infected and noninfected animals, indicating that *Y. pseudotuberculosis* infection does not modify flagellin's biological activity *per se*. We next analyzed the time course of the flagellin-mediated transcriptional response (Fig. 5B). In both infected and uninfected mice, flagellin rapidly upregulated the transcription of *Cxcl2*, *Il22*, *Il6*, *Hamp*, and *S100a9* genes. This was followed by a strong decrease in mRNA levels 8 h posttreatment and a return to baseline after 24 h (Fig. 5B). However, the gene coding for RegIIIγ displayed a delayed expression profile; expression started 8 h posttreatment and was maintained until 24 h, thus confirming a previous report (19). In summary, the flagellin-induced gene expression in the ileum was not changed by *Y. pseudotuberculosis* infection.

We found that unlike flagellin, LPS was not able to induce protection against oral infection with *Y. pseudotuberculosis* (Fig. 1A). We compared the transcriptional response to LPS and flagellin for a set of 10 genes known to be activated by TLR stimulation (19). Whereas both flagellin and LPS were associated with similar expression levels in the spleen, flagellin was 10- to 100-fold more potent than LPS in the ileum (Fig. S3). These

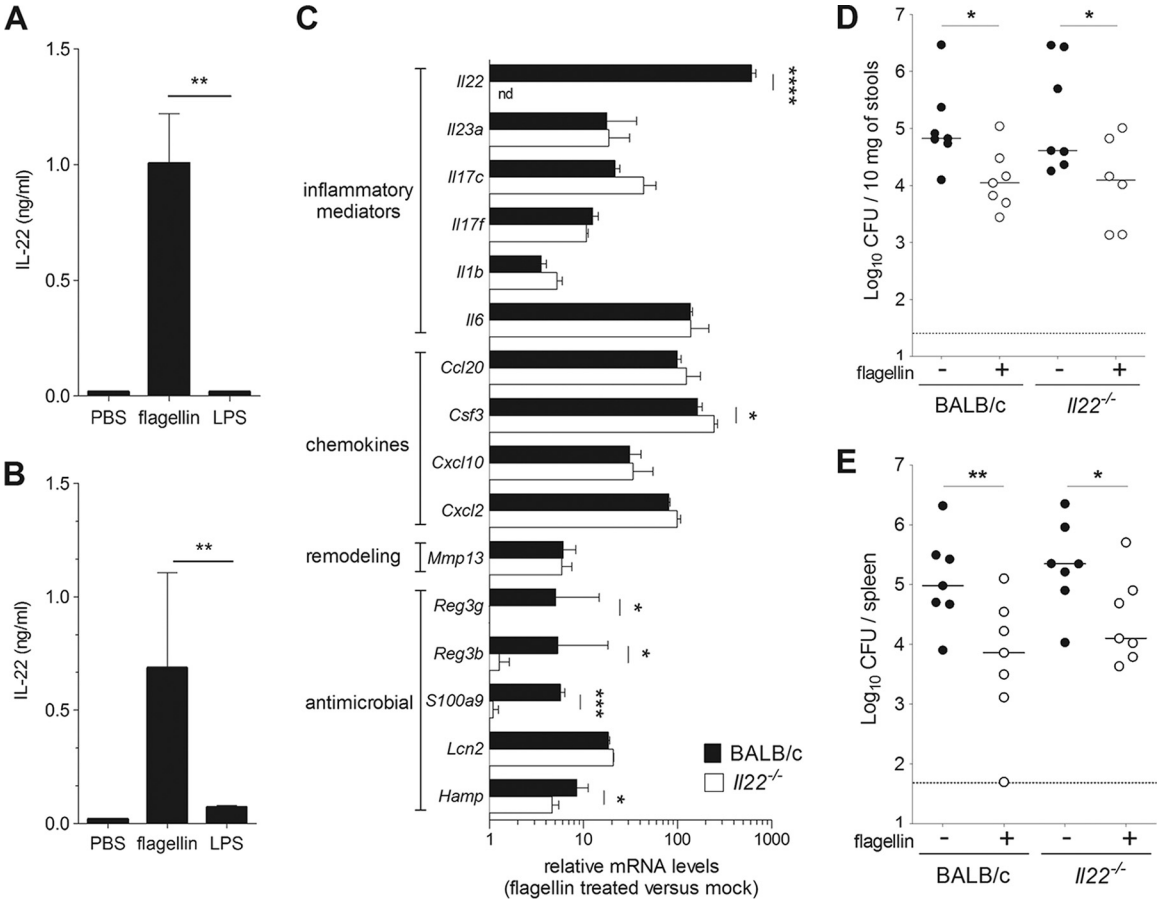

FIG 6 Flagellin-mediated protection does not require IL-22. (A and B) Female BALB/c mice ($n = 5$) were treated intraperitoneally with flagellin (5 μg) or LPS (5 μg) in PBS or with PBS alone. Blood and ileum were sampled 2 h posttreatment. IL-22 levels were measured by ELISA in serum (A) and ileum homogenates (B). The data are presented as means ± SD. Data from flagellin-treated and LPS-treated mice were compared in a Mann-Whitney test (**, $P < 0.01$). (C) *Il22*$^{-/-}$ mice and wild-type BALB/c littermates ($n = 3$) were treated intraperitoneally with PBS alone or flagellin (5 μg) in PBS. The ileum was sampled at 2 h for quantification of mRNA levels using quantitative reverse transcription-PCR. mRNA levels are expressed relative to the PBS group (mock). nd, not detected. The data are presented as means ± SD. Intergroup differences were analyzed in a Limma test with Benjamini-Hochberg FDR correction (*, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$). (D and E) BALB/c and *Il22*$^{-/-}$ mice were treated intraperitoneally with flagellin (5 μg) in PBS or with PBS alone 30 min before an intragastric challenge with 5×10^8 CFU of *Y. pseudotuberculosis*. Bacterial counts were determined in the stools (D) and the spleen (E). CFU counts for individual mice ($n = 7$) at 72 h postinfection are shown. The solid line corresponds to the median value, and the dashed line represents the detection threshold. Statistical significance was assessed in a Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$).

data corroborate our previous observations (Fig. 2) (19) and suggest that flagellin-mediated protection correlated with a specific, intestinal innate response.

IL-22 is not required for flagellin-mediated protection. Given that TLR5 stimulation strongly enhanced intestinal production of IL-22, a cytokine that is critical for mucosal defense (Fig. 5) (10, 19, 20, 24, 25), we further investigated the role of IL-22 in flagellin-mediated protection against *Y. pseudotuberculosis*. First, we found that flagellin administration induced a strong production of IL-22 in blood and ileum (Fig. 6A and B). Interestingly, treatment with LPS was unable to stimulate IL-22 production in the systemic (blood) and local (ileum) compartments (Fig. 6A and B). A transcriptional analysis of ileum samples 2 h after flagellin injection indicated that the overall signatures in *Il22*$^{-/-}$ mice (BALB/c background) and in BALB/c littermates were similar, except, as expected, for the absence of expression of *Il22* and the reduced expression of three well-defined IL-22 target genes (*Reg3b*, *Reg3g*, and *S100a9*) in the knockout animal (Fig. 6C) (20). Finally, flagellin-treated BALB/c and *Il22*$^{-/-}$ mice were inoculated with *Y. pseudotuberculosis*, and bacterial counts in the spleen and stools were measured. As in littermate BALB/c (IL-22 proficient) mice, bacterial counts were significantly

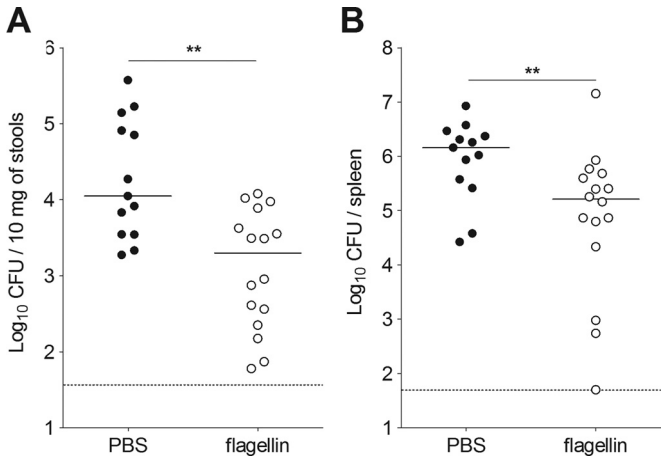

FIG 7 Flagellin protection does not require B, T, NK, and innate lymphoid cells. *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice were treated intraperitoneally with flagellin (5 μg) in PBS or with PBS alone 30 min before an intragastric challenge with 5×10^8 CFU of *Y. pseudotuberculosis*. Bacterial counts were determined in the stools (A) and the spleen (B). CFU counts for individual mice ($n = 13$ to 16) at 72 h postinfection are shown. The solid bar corresponds to the median value, and the dashed line represents the detection threshold. Intergroup differences were analyzed in a Mann-Whitney test (**, $P < 0.01$).

lower in flagellin-treated *Il22*$^{-/-}$ mice than in untreated *Il22*$^{-/-}$ mice (Fig. 6D and E). Similar protection was observed in *Il22*$^{-/-}$ mice with the C57BL/6 background (data not shown). Taken as a whole, these results indicate that IL-22 is not required for flagellin-mediated protection and suggest that the antimicrobial molecules RegIIIβ, RegIIIγ, and S100A9 are not involved in flagellin's mode of action.

Lastly, we also studied flagellin-mediated protection in *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice (Fig. 7). These mice lack T and B lymphocytes and cells that depend on the common γ-chain (natural killer cells, natural killer T cells, and all subsets of ILCs). Innate production of IL-22 is known to be impaired in *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice due to the specific absence of ILC3 (7, 19). First, we observed that the bacterial counts after *Y. pseudotuberculosis* infection were 15-fold higher in the spleen and 27-fold lower in the stools of *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice than in wild-type C57BL/6 mice (Fig. 3C and D; Fig. 7). This indicates that *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice can control the mucosal colonization but not the dissemination of *Y. pseudotuberculosis*. Despite this difference of susceptibility, flagellin was still able to increase the bacterial clearance in *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice. Indeed, the bacterial counts in stools and spleen were 5.6- and 9-fold lower, respectively, in *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ animals treated systemically with flagellin than in mock-treated, infected animals (Fig. 7). These data indicate that lymphoid cells (including ILC3) are not required for flagellin-mediated protection.

DISCUSSION

In the present study, we showed that flagellin administration protects against intestinal (but not systemic) infection with *Y. pseudotuberculosis*. These data strongly suggest that TLR5 signaling has a mucosal protective effect. We and others have already shown that TLR5 signaling induces the rapid production of IL-22 by stimulating ILC3 in a dendritic cell-dependent manner (19, 20). Since IL-22 reportedly has a protective role against intestinal and intra-abdominal pathogens (14, 24, 32), we hypothesized that this cytokine was also involved in flagellin-mediated mucosal protection of mice against *Y. pseudotuberculosis*. Surprisingly, we found that IL-22 is not required for this protection. Moreover, our present results suggest that neither ILC3 nor the IL-22-dependent antimicrobial agents S100A9, RegIIIβ, and RegIIIγ are required for protection.

These observations contrast with animal studies of infections with the enteric bacterium *C. rodentium*, in which IL-22 and RegIIIγ proteins are essential for intestinal protection (14). The differential involvement of IL-22 in models of experimental infec-

tions with *Citrobacter* and *Yersinia* might be related to differences in the pathogenicity of these two Gram-negative microorganisms. *C. rodentium* is an extracellular attaching/effacing pathogen that is related to enteropathogenic *Escherichia coli* and damages the colonic epithelium (14). In contrast, *Y. pseudotuberculosis* does not colonize the epithelial surface; in fact, it invades intestinal tissues and displays a pronounced tropism for ileal Peyer's patches, mesenteric lymph nodes, and the spleen (28). This invasive feature might make *Y. pseudotuberculosis* resistant to an IL-22-mediated, epithelial, antibacterial response. The infection site (colon versus ileum) might also contribute to specific expression of IL-22-dependent protection. For example, IL-22 might profoundly influence resistance to colonization of colon, where the microbial density is significantly higher than in the ileum.

Our present results also showed that the protection against *Y. pseudotuberculosis* infection requires TLR5 and thus agree with a previous study in which impaired TLR5 signaling was associated with chronic yersiniosis in humans (33). This protection was also specific for flagellin, since a TLR4 agonist did not induce protection. This disparity can be attributed to the differences in cellular expression levels of TLR4 and TLR5; TLR4 is mainly expressed by hematopoietic cells, whereas TLR5 is mainly expressed in the epithelium (34). Moreover, the responsiveness of hematopoietic cells and epithelial cells to TLR4 and TLR5 agonists is associated with gut-specific features. For instance, the gut epithelium's tolerance to LPS is a hallmark feature related to the absence (or lack of function) of signaling molecules like IRAK-1 or coreceptors like CD14 (35). Furthermore, it has been shown that although the intestinal *lamina propria* contains dendritic cells expressing TLR5 and TLR4, the response to TLR5 stimulation is significantly stronger than the response to TLR4 stimulation (36). We confirmed this scenario because flagellin, but not LPS, induced a strong innate response in the ileum (see Fig. S3 in the supplemental material).

Our results strongly suggest that nonlymphoid cells have a major role in flagellin-mediated protection against *Y. pseudotuberculosis*. Recently published data showed that TLR5 signaling deficiency in gut epithelium is involved in spontaneous, low-grade, intestinal inflammation in a process modulated by the microbiota (37). Accordingly, the systemic administration of flagellin is known to activate gut epithelial cells (38). Flagellin's protective activity is not restricted to the gut, since intranasal administration is effective against respiratory infections and depends on the TLR5-mediated activation of epithelial cells and neutrophil recruitment (39, 40). Taken as a whole, these findings suggest that epithelial cells have a role in the flagellin-mediated protection against *Y. pseudotuberculosis*.

Neutrophils constitute the first line of inducible defense against infection. In the present study, we confirmed that flagellin administration induces the rapid, transient transcriptional activation of genes encoding chemokines involved in chemoattraction of neutrophils in the intestine, as described previously (19). Instillation of flagellin into the respiratory tract in both naive and infected animals is associated with the recruitment of neutrophils into lungs (30, 39–41). It remains to be seen whether, in the intestine, neutrophils contribute to flagellin-mediated protection against *Y. pseudotuberculosis*. However, *in vivo* and *in vitro* studies have shown that *Y. pseudotuberculosis* is partially resistant to killing by human neutrophils (42–44); this suggests that flagellin-mediated protection involves additional or unrelated mechanisms. Along with neutrophils, other myeloid cells may also contribute to this protection, because macrophages, monocytes, and dendritic cells are all targets of flagellin-mediated signaling. Interestingly, stimulation of TLR signaling is known to shift the anti-inflammatory effects of *Y. pseudotuberculosis* on macrophages toward inflammatory pyroptosis, a process that contributes to bacterial clearance (45).

In agreement with previous studies, we found that flagellin stimulates the transcription of genes encoding antimicrobial molecules (19, 20, 46, 47). Although RegIIIβ was found to be associated with intestinal resistance to *Y. pseudotuberculosis* (31), the present study suggests that this C-type lectin and other IL-22-induced antimicrobial molecules (such as S100A9 and RegIIIγ) are not essential for flagellin-mediated protec-

tion. We identified other antimicrobial compounds (unrelated to IL-22 activation) that might have a role in the clearance of *Y. pseudotuberculosis*; these include lipocalin-2 (which binds to bacterial siderophores) and hepcidin (which influences the levels of iron available for bacterial growth). Other antimicrobial candidates include the cathelicidin-related antimicrobial peptide or the β-defensins, which also have been linked to TLR5 signaling (47, 48). Understanding the anti-*Yersinia* mechanisms that are primed by TLR5 signaling and contribute to mucosal protection remains an important topic for future research.

MATERIALS AND METHODS

Bacterial preparation. *Yersinia pseudotuberculosis* (serogroup O:1, strain IP32777) (49) was grown as described previously (50). Briefly, Luria-Bertani Lennox (LB) (AthenaES, Baltimore, MD) plates were streaked from a −80°C culture stock. Bacterial inocula were prepared from overnight cultures in LB broth at 28°C. This culture was monitored spectrometrically (optical density at 600 nm [OD_{600}]) and diluted to the appropriate concentration. The number of bacteria (in CFU) was confirmed by plating serial dilutions on LB agar plates.

The murine model of infection. Six- to 20-week-old BALB/cJ or C57BL/6J mice (Janvier Laboratories, Saint-Berthevin, France), *Tlr5*$^{-/-}$, *Myd88*$^{-/-}$, *Il22*$^{-/-}$, and *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice (all backcrossed with C57BL/6J), and *Il22*$^{-/-}$ mice (backcrossed with BALB/cJ) 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, Lille, France). Mice were infected with *Y. pseudotuberculosis* via the intravenous or intragastric route, as described previously (50). Briefly, animals were orally challenged with 5×10^8 bacteria in 200 μl of water or intravenously infected with 10^3 bacteria in 100 μl of phosphate-buffered saline (PBS). These bacterial inocula correspond to lethal doses established according to the 50% lethal dose (LD_{50}) values defined previously as $10^{7.3}$ by the oral route and $<10^2$ by the intravenous route (51, 52). PBS alone was administered in control experiments. The mice were observed daily for signs of illness. For the determination of bacterial counts in stools and spleen, mice were sacrificed at selected time points by the intraperitoneal injection of 5.47 mg of sodium pentobarbital. Stools and spleens were weighed and collected in PBS. Samples were then homogenized with an UltraTurrax homogenizer (IKA-Werke, Staufen, Germany), and viable counts were determined by plating serial dilutions onto LB agar plates (for spleens) or LB agar plates containing vancomycin (50 μg/ml) and irgasan (1 μg/ml) (for stools) in order to specifically enable *Yersinia* growth. CFU were counted after 48 h of culture at 28°C.

Administration of flagellin and LPS. Native flagellin FliC from *Salmonella enterica* serovar Typhimurium and the recombinant flagellins rFliC and $rFliC_{89-96*}$ (both harboring an amino-terminal histidine tag) were produced as described previously (30, 53). rFliC is equivalent to the native protein, whereas $rFliC_{89-96*}$ contains TLR5-nonsignaling residues at positions 89 to 96. The recombinant flagellin $FliC_{492stop}$ was generated by site-directed mutagenesis of a plasmid harboring rFliC cloned into the expression vector pET22b$^+$. A premature stop codon was introduced in the plasmid coding for $FliC_{492stop}$ in order to truncate the last two C-terminal residues involved in NAIP5/NLRC4 complex recognition (54). Recombinant flagellins were produced in *E. coli* BL21(DE3), purified using fast protein liquid chromatography (GE Healthcare, Pittsburgh, PA), and depleted of LPS using Triton X-114 extraction and polymyxin B columns (Thermo Fisher Scientific, Waltham, MA). In a *Limulus* assay (Associates of Cape Cod Inc., East Falmouth, MA), the residual LPS concentration was determined to be less than 20 pg per μg of flagellin. To ensure that the flagellins were mostly monomers, the corresponding samples were heated for 10 min at 65°C before use. Flagellins (5 μg in 200 μl PBS), ultrapure LPS from *E. coli* (5 μg in 200 μl PBS; serotype 0111:B4; InvivoGen, Toulouse, France), or PBS alone was administered intraperitoneally 30 min prior to the bacterial challenge.

Gene expression. Total RNA was extracted with the NucleoSpin RNA II kit (Macherey-Nagel, Durel, 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 used in the study are listed in Table S1 in the supplemental material. For high-throughput analysis, TaqMan low-density arrays (Applied Biosystems) were used (references of the assays/probes are listed in Table S2). The analysis was carried out using real-time StatMiner software (Integromics) or ThermoCloud RQ software (Thermo Fisher Scientific). Relative mRNA levels ($2^{-\Delta\Delta CT}$) were determined by comparing (i) the PCR cycle thresholds (C_T) for the gene of interest and the housekeeping genes (*Actb* and/or *B2m*) (ΔC_T) and (ii) the ΔC_T values for flagellin-treated and untreated groups ($\Delta\Delta C_T$). The C_T threshold was set to 33 cycles.

Caspase 1 activation assay. The procaspase-1 processing assay was performed as previously described (55). Briefly, bone marrow-derived macrophages isolated from wild-type C57BL/6 and *Nlrc4*$^{-/-}$ mice were incubated with streptolysin O (SLO) (Sigma-Aldrich, St. Louis, MO) in the presence or absence of recombinant flagellins for 2 h. Cell lysates were prepared in Laemmli buffer and transferred to nitrocellulose membranes. Processing of procaspase-1 was followed by immunoblotting with caspase-1-specific antibody (Adipogen, San Diego, CA).

Cytokine assays. CXCL2, CCL20, IL-6, and IL-22 levels were measured by enzyme-linked immunosorbent assay (ELISA) in serum or ileum tissue homogenates prepared with T-PER reagent (Pierce; Rockford,

IL) supplemented with protease inhibitor cocktail (Roche Diagnostics, Switzerland) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis. Results were expressed as the means ± standard deviations (SD) or the median, as indicated. Intergroup differences were analyzed using the Mann-Whitney test and the log rank test (GraphPad Prism 5.0a). The Limma test with Benjamini-Hochberg false discovery rate (FDR) correction was used for high-throughput PCR with TaqMan low-density arrays (Applied Biosystems) as described previously (19). The threshold for statistical significance was set to a P value of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00806-16.

TEXT S1, PDF file, 0.3 MB.

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