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**Control of *Pseudomonas aeruginosa* in the lung requires  
the recognition of either lipopolysaccharide or flagellin <sup>1</sup>**

**Running title : TLRs and *P. aeruginosa***

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## Abstract.

Acute lung infection due to *Pseudomonas aeruginosa* is an increasingly serious problem which results in high mortality especially in the compromised host. In this report, we set out to ascertain what components of the toll-like receptor (TLR) system are most important for innate immunity to this microorganism. We previously demonstrated that TLR2,4<sup>-/-</sup> mice were not hypersusceptible to infection by a wild type *P. aeruginosa* strain. However, we now find that TLR2,4<sup>-/-</sup> mice are hypersusceptible to infection following challenge with a *P. aeruginosa* mutant devoid of flagellin production. We demonstrate that this hypersusceptibility is largely due to a lack of innate defense by the host that fails to control bacterial replication in the lung. Further evidence that a response to flagellin is a key factor in the failure of TLR2,4<sup>-/-</sup> mice to control the infection with the mutant strain was obtained by demonstrating that the intrapulmonary administration of flagellin over a 18 h period following infection, saved 100% of TLR2,4<sup>-/-</sup> mice from death. We conclude that the interactions of either TLR4 with LPS or TLR5 with flagellin can effectively defend the lung from *P. aeruginosa* infection and the absence of a response by both results in hypersusceptibility to this infection.

## Introduction

Infection of the lungs by *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, results in two distinct clinical syndromes. In ventilated patients, it causes an acute pneumonia with a high mortality rate and in the genetic disorder Cystic Fibrosis, it is the prime cause of chronic inflammation which is a key factor in the destructive lung disease that occurs in these patients (1). The different clinical courses of these two lung diseases likely result from the interplay of different host and bacterial factors but inflammation is a common feature of both illnesses with bacterial toxins possibly playing a significant role in acute disease (2,3,4). The inflammatory response in both cases is most likely due to the recognition of the lipid A portion of lipopolysaccharide (LPS) moieties on the cell wall (5) and the flagellin of the microorganism (6,7,8) but there are no data that can definitively exclude other pathogen-associated molecular patterns (PAMPs) of *P. aeruginosa*.

Host recognition of PAMPs may have two entirely different consequences. An appropriate response leads to the eradication of a microorganism (9) but an exaggerated inflammatory response may lead to illnesses such as sepsis and shock (10). The corollary of the former response is that failure to recognize a microorganism results in failure to eradicate it and in susceptibility to disease. Indeed, animal models of infection have demonstrated that the susceptibility to a number of Gram-negative pathogens is linked to the lack of recognition of LPS (11,12) in which case a defective response leads to extreme susceptibility. These experimental findings are supported by clinical observations that polymorphisms in human genes that encode pattern recognition receptors result in greater susceptibility to certain infections (13). However, in the case of *P. aeruginosa*, whose LPS is recognized by toll-like receptor (TLR) 4 (5) and possibly by TLR2 (5,14), mice lacking both TLR2 and 4 (TLR2,4<sup>-/-</sup>) are not hypersusceptible to this bacterium and mount an effective innate response that clears the microorganism (15,16). In contrast MyD88<sup>-/-</sup> mice are extremely susceptible with 100% of

mice dying within 48 h with a low dose of *P. aeruginosa* (15,16), indicating the potential involvement of TLR-ligand interactions in host defense. In *P. aeruginosa*, one other possible TLR ligand is flagellin, the known TLR5 ligand, which has been implicated in a pathogenic role in acute pneumonia (6,15) and which has been demonstrated to cause inflammation when instilled into the lungs (6,7,8). However, in a lung model of acute infection using a *P. aeruginosa* mutant devoid of flagellin production (a  $\Delta fliC$  mutant), we recently demonstrated that the absence of flagellin does not significantly alter the LD<sub>50</sub> but results in slower clearance of this microorganism from the lungs and a delay in the time to death (8). Thus, neither of the Pseudomonas interactions, LPS-TLR2,4 or flagellin-TLR5 by themselves, play an essential role although they both may participate in the innate immune response.

Two recent studies have shed more light on the role played by these interactions in defense of the lungs but the reported data are not entirely consistent on whether these TLRs are critical for the control of a Pseudomonas lung infection and survival of infected mice. Feuillet et al. (17) demonstrated that TLR4,5<sup>-/-</sup> mice are hypersusceptible to infection by wild type *P. aeruginosa* strain PAK but the innate immune response to the strain and bacterial clearance were not measured. A second study by Skerrett et al. (16) using TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup> or TLR2,4<sup>-/-</sup> mice and a *P. aeruginosa* strain PAK lacking flagellin, in effect abrogating the TLR5 response, concluded that none of the mutant mouse strains were hypersusceptible to this strain that lacked flagellin and that bacterial clearance was not affected in any of these mutant mice but was defective in MyD88<sup>-/-</sup> mice. Thus, the results of these studies have not been consistent in regard to control of bacterial replication and mortality. This may be due to the fact that the outcome of infection by *P. aeruginosa* is much more complex and may involve inflammatory injury in response to the microorganism, the initiation of an effective innate immune response to clear the organism, as well as the pathogenic effects of secreted and injected toxins. As

evidence for this, strains of *P. aeruginosa* defective in the production of various exoenzymes have been shown to be less virulent in the mouse model of pneumonia (2,3,4). With respect to the role of inflammation, the inflammatory response to the inhalation of Pseudomonas LPS leads to lung injury (18) and the inflammatory response to strains of *P. aeruginosa* that produce an excess of flagellin results in rapid death when compared to a wild type strain (8).

To ascertain what factors are involved in mortality and control of bacterial replication, we examined different combinations of wild type, TLR2,4<sup>-/-</sup>, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice infected by *P. aeruginosa* or its  $\Delta$ *fliC* mutant. We demonstrate that TLR4 and TLR5 play major but redundant roles in controlling bacterial replication and in host defense against *P. aeruginosa* pneumonia.

## Materials and Methods.

**Mice.** Males of several mouse strains were used for the experiments. TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice were obtained from S. Akira, Osaka University, Japan and were backcrossed eight times with C57BL/6 to ensure similar genetic backgrounds. TLR2,4<sup>-/-</sup> mice were generated by breeding TLR2<sup>-/-</sup> mice and TLR4<sup>-/-</sup> mice. C57BL/6 mice used as control mice were supplied by the Centre d'Élevage R. Janvier, Le Genest Saint-Isle, France and used at about 8 weeks of age. Mice were fed normal mouse chow and water *ad libitum* and were reared and housed under standard conditions with air filtration. Mice were cared for in accordance with Pasteur Institute guidelines in compliance with the European Animal Welfare regulations.

**Bacterial strains.** The wild type strain, PAK, a commonly studied *P. aeruginosa* strain was obtained from Stephen Lory, originally isolated by D. Bradley. This strain of *P. aeruginosa* is known to contain and express a full complement of virulence factors, including pili, flagella, the type II secreted enzymes, exotoxin A, elastases and phospholipases and the type III secreted exoenzymes S, T and Y. PAK  $\Delta$ *fliC* and PAK-L88 are mutants of the parent strain where the *fliC* gene has been deleted (19), and where there is an L88A substitution in the TLR5 binding site of *Pseudomonas* flagellin (20), respectively. PAO1  $\Delta$ *fliC* is a *fliC* mutant of strain PAO1 (21), another well studied strain of *P. aeruginosa*. Luminescent strains of PAK and its  $\Delta$ *fliC* mutant, were constructed by inserting *luxAB* into the neutral *att* site of the chromosome of strain PAK and its derivative PAK-C using a mini-Tn7-*lux* plasmid provided by Microbiotix, Worcester, Massachusetts, where *luxAB* is driven by the *lac* promoter (22). Bacteria were grown overnight in Luria-Bertani (LB) broth then transferred to fresh medium and grown for 4-5 hours to midlog phase. The cultures were centrifuged at 4,000 g for 15 min

and the cell pellets washed twice with phosphate buffered saline (PBS). The bacterial pellet was diluted in its original volume and the optical density adjusted to give the approximate desired inocula. The inocula were verified by serial 10 fold dilutions of the bacterial suspensions and plating on LB agar.

### **Animal infections.**

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine-xylazine and were placed supine. A plastic catheter (diameter of 0.86 mm) was inserted into the trachea *via* the oropharynx. The proper insertion was verified by checking the formation of mist due to expiration on a mirror placed in front of the external end. A 50  $\mu$ l bacterial suspension was laid down at the internal end of the catheter with a micropipette using a sterile disposable tip for gel loading, introduced into the catheter. Mice were then immediately held upright in order to facilitate bacterial inhalation and until normal breathing resumed. A series of experiments were done to ascertain the effects of the TLR mutations in bacterial clearance of the *fliC* mutant of *P. aeruginosa*. Groups of mice were infected by the intratracheal route using inocula of bacteria that were known to be less the LD<sub>50</sub> for wild type mice from previous experiments (8,15). In one series of experiments recombinant *P. aeruginosa* flagellin prepared as described (20) was mixed with the bacterial inoculum prior to intratracheal instillation and at several times after infection.

Broncho-alveolar lavages (BAL) were performed on some mice at 6 hours after infection following pentobarbital euthanasia. The lavage fluids (4 ml) were diluted and plated on LB agar plates, to obtain viable bacterial counts in the lavage fluid. Total cell counts were measured in the BAL fluids with a Coulter Counter (Coulter Electronics, Margency, France) and cell differential counts were determined after cytopsin centrifugation and staining with



Diff-Quick products. Murine cytokine concentrations in BAL fluid were determined using DuoSet ELISA kits obtained from R&D systems (Minneapolis, MN).

**Luminescence measurements.** Photon emission of the luminescent bacteria in the lungs of infected mice was measured using the IVIS system (Xenogen Biosciences, Cranbury, NJ). In preliminary experiments we ascertained that the system could detect  $10^6$  cfu of luminescent *P. aeruginosa* given intratracheally. To achieve detection in black C57BL/6 mice, it was necessary to remove hair from their chests with a depilatory agent used for this purpose in humans. After infection, analysis of photons was done in mice under isoflurane inhalation anesthesia in an IVIS CCD (charge-coupled device) camera coupled to the LivingImage software package (Xenogen). A digital false-color photon emission image of the mouse was generated and photons were counted within a constant defined area corresponding to the surface of the chest encompassing the whole lung region. Photon emission was measured as photons/second emitted.

**Statistical calculations.** Cytokine levels, myeloperoxidase (MPO) concentrations, polymorphonuclear neutrophil (PMN) counts, and pathogen counts were expressed as mean  $\pm$  SEM. Differences between groups were assessed for statistical significance using the analysis of variance test followed by Fischer's test. A value of  $p < 0.05$  was considered statistically significant.

## Results

### **Effect of TLR2,4 mutations on survival of mice following infection by a $\Delta$ *fliC* mutant of *P. aeruginosa*.**

Survival of C57BL/6 mice and TLR2,4<sup>-/-</sup> was examined using wild type strain PAK, and its flagellin deficient mutant PAK $\Delta$ *fliC*. The dose of bacteria ( $5 \times 10^6$  cfu/mouse) was chosen after preliminary experiments showed that the mutant mice were dying rapidly when given  $\frac{1}{2}$  the LD<sub>50</sub> of the  $\Delta$ *fliC* mutant for wild type mice as measured in a previous study (8). The dose chosen was expected to cause little or no mortality among wild type mice, but should uncover any hypersusceptibility among knockout mice.

As previously demonstrated with wild type strain PAK (15), there were no differences in the survival of the control and the TLR2,4<sup>-/-</sup> mice (Fig. 1). In contrast, the survival studies with the  $\Delta$ *fliC* mutant demonstrated extreme susceptibility of the TLR2,4<sup>-/-</sup> mice, with all mice dying within 2 days of challenge with a dose of this strain that caused no mortality among the control mice. These results suggested that the extreme susceptibility was likely due to the absence of a response to both LPS and flagellin consequent to the mutations in TLR2,4 and the concomitant absence of flagellin from the bacterium.

### **Effect of the TLR2,4 mutations on bacterial proliferation in the lungs.**

We have previously shown that proliferation of wild type strain PAK was controlled in TLR2,4<sup>-/-</sup> mice (15). Skerrett et al (16) recently demonstrated that proliferation of a  $\Delta$ *fliC* mutant of this same strain of *P. aeruginosa* is also controlled in TLR2,4<sup>-/-</sup> mice even though host innate immune responses are severely blunted. These recently published data did not however explain the hypersusceptibility that we observed (Fig.1). We therefore examined

bacterial proliferation to ascertain whether hypersusceptibility was accompanied by failure to control bacterial proliferation as occurs in MyD88 deficient mice (15,16). When examined at 6 h post infection (Fig. 2A), TLR2,4<sup>-/-</sup> mice failed to control replication of the  $\Delta$ *fliC* mutant, with bacterial counts reaching 10 times the challenge dose in these mice whereas it was down to less than 20% of the inoculum in the control mice. We also measured proliferation *in vivo* at 6 and 20 h by detecting the growth of luminescent bacteria directly in the lungs of living anesthetized mice. Representative pictures of bacterial luminescence are shown in Fig. 2B. Measured in photons/second, luminescence increased at least 10 fold in a 24 h period in the TLR2,4<sup>-/-</sup> mice infected with the  $\Delta$ *fliC* mutant whereas it decreased in wild type mice (Fig. 2C). In agreement with our previous report (15), luminescence decreased as a function of time in wild type and TLR2,4<sup>-/-</sup> mice challenged with the wild type strain PAK (data not shown).

#### **Evaluation of TNF- $\alpha$ , IL-6, KC and G-CSF production in response to infection of TLR2,4<sup>-/-</sup> mice by a $\Delta$ *fliC* mutant of *P. aeruginosa*.**

We have previously noted that when TLR2,4<sup>-/-</sup> mice are infected with the wild type *P. aeruginosa* strain, the TNF- $\alpha$  response is severely attenuated but the IL-6 response is intact (15) suggesting that a PAMP other than LPS may also stimulate the IL-6 response. We thus examined the production of these two and other inflammatory mediators under the different experimental combinations used in the present study. Studies were conducted at 6 h post infection, as mutant mice were often dead at 18-24 h following challenge with the  $\Delta$ *fliC* mutant. We confirmed the earlier data that the TNF- $\alpha$  response is lost in the TLR2,4<sup>-/-</sup> mice (Fig. 3A) and that the IL-6 (Fig. 3B) response is preserved. The most striking observation was that infection of TLR2,4<sup>-/-</sup> mice with the  $\Delta$ *fliC* mutant led to a complete suppression of host response in terms of both TNF- $\alpha$  and IL-6 formation. Given the complete loss of TNF- $\alpha$

synthesis in the TLR2,4<sup>-/-</sup> mice infected with the flagellated strain and the additional loss of IL-6 in these mice only when flagellin is absent, it is possible that the TNF- $\alpha$  response is mainly due to a PAMP recognized by TLR2,4, and that the IL-6 response is a sum of the effects of a PAMP recognized by TLR2/4 and the activity of flagellin. The KC and G-CSF responses (Fig. 3C and D) to the absence of flagellin were similar to those of IL-6 in these mice. Thus absence of IL-6, G-CSF and KC responses, was correlated with hypersusceptibility but the absence of a TNF- $\alpha$  response was not.

### **Evaluation of PMN recruitment and activation.**

In agreement with the above data, PMN recruitment in the airspaces and their activation as measured by the release of free MPO, were similar under the different experimental combinations, except in the case of TLR2,4<sup>-/-</sup> mice infected with the  $\Delta$ *fliC* mutant (Fig. 4). Under the latter, the values for both parameters were not different from those of uninfected control mice suggesting a failure of recruitment and activation by PMN in these mice.

### **Role of flagellin in the innate immune response.**

To examine whether an innate immune response to flagellin as opposed to LPS (TLR2 or 4) was critical to the survival of these mice, one group of TLR2,4 mutant mice was given 0.1  $\mu$ g flagellin mixed with the challenge bacteria, followed by another dose of flagellin 6 h later by the intranasal route. A second group was given the same dose at the time of infection then at 6, 12 and 18 h post infection by the intranasal route. A control group of TLR2,4<sup>-/-</sup> mice received PBS instead of flagellin but did not survive beyond 24 h. Survival of the mice that received two doses of flagellin was prolonged by about a day but all were dead by 48 h post

bacterial challenge, whereas the administration of four doses of flagellin resulted in the survival of 100% of the mice even as long as 2 weeks post bacterial challenge (Fig 5).

To further implicate the requirement for TLR5 recognition of flagellin in TLR2,4<sup>-/-</sup> mice, we challenged these mice with *P. aeruginosa* strain PAK-L88 (8) which is a PAK mutant with an L88A amino acid change in the putative TLR5 binding site of *Pseudomonas* flagellin (20). This single amino acid change results in a significant reduction in the inflammatory response of the mouse lung when recombinant L88 flagellin is instilled into the lungs (8). Using different concentrations of the L88 mutant (between 1-2 X 10<sup>7</sup>) we observed greater mortality than that seen with the use of the wild type strain but not as great as that seen with the  $\Delta$ *fliC* mutant. The differences in survival observed within the two first days of infection of the TLR2,4<sup>-/-</sup> mice challenged with wild type strain PAK or PAK-L88 were statistically significant ( $p < 0.05$ ;  $n = 3$ ), with mean survivals at 48 h of 78% with the wild type strain and 35% with the L88 mutant. Under the same experimental conditions, the survival of the mice was 0% for the  $\Delta$ *fliC* mutant (Fig 6). Some possible reasons for the lack of total susceptibility of TLR2,4<sup>-/-</sup> mice to infection with the PAK-L88 mutant are discussed below.

### **Role of TLR2 versus TLR4 in innate immunity to a $\Delta$ *fliC* mutant of *P. aeruginosa*.**

Since TLR2,4<sup>-/-</sup> were hypersusceptible to a  $\Delta$ *fliC* mutant of strain PAK, we sought to examine which of these two TLRs was instrumental in defense against this organism in the absence of a response to flagellin. TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice were infected with the  $\Delta$ *fliC* mutant of strain PAK at a dose of 5x10<sup>6</sup> cfu which does not kill wild type C57BL/6 mice. All TLR4<sup>-/-</sup> mice died within 48 h of infection whereas all TLR2<sup>-/-</sup> mice survived (Fig. 7). To confirm that this hypersusceptibility was more general to *P. aeruginosa* strains, a  $\Delta$ *fliC* mutant of strain PAO1 was used to infect wild type and TLR4<sup>-/-</sup> mice. In preliminary experiments, an inoculum of

$10^6$  cfu failed to kill wild type mice. However all TLR4<sup>-/-</sup> rapidly succumbed to infection by this mutant (Fig. 7).

## Discussion

The outcome of an acute pulmonary infection due to *P. aeruginosa* is a balance between an appropriate innate immune response and bacterial virulence. The pathway to an appropriate response is through the recognition of PAMPs by TLRs and possibly other nonTLR systems such as the NOD-like receptor system and the inflammasome (23,24). However, although it has been shown that *P. aeruginosa* triggers the NOD system (25) and its flagellin is recognized by the inflammasome (26), a role for host defense by these systems *in vivo* has not been demonstrated to date. The data from this study and that of Feuillet et al. (17) suggests that the most important systems may be the TLR system and that TLR4 and TLR5 are redundant for the recognition of *P. aeruginosa* and possibly other flagellated bacteria. However, there have been reported instances where defense against Gram-negative bacteria required only TLR4 (11,12,27,28). On closer examination of those studies, some of the microorganisms studied do not possess a flagellum or do not express flagellin *in vivo*. For example *Klebsiella pneumoniae* and *Hemophilus influenzae* are not flagellated bacteria and Bordetella down regulates flagellin production *in vivo* (29). One study of *P. aeruginosa* that showed TLR4 alone to be essential (30), used strain PA103, which is known to be a nonflagellated strain (31). Other studies have alternatively shown TLR4 mutant mice not to be hypersusceptible to *Escherichia coli* strains (32,33,34), however whether these strains or clones of a given strain expressed flagellin and made a flagellum, is unknown. It is possible that the lack of flagellin expression in the strains or clones of a given strain that was used in the studies that showed hypersusceptibility to *E. coli* was responsible for this observation, as TLR4 mutant mice would be able to not mount an effective response if flagellin were absent.

A role for TLR2 in defense against *P. aeruginosa* infections is more difficult to substantiate, although interactions of TLR2 with both LPS and flagellin have been described (5,14,35). It has been reported that Pseudomonas LPS may be recognized by either TLR2 or TLR4 depending on its structure (5), thus one cannot entirely rule out a role for TLR2. However, Feuillet et al. (17) as well as the current study, could not however measure a contribution of TLR2 in lung defense against *P. aeruginosa*. Since TLR2 does recognize *P. aeruginosa*, the suggestion by Skerrett et al. (16) that it may play a counter-regulatory role seems feasible but it does not appear to have a direct role in defense. The response of strains of *P. aeruginosa* that have been adapted to the airways as well as most common laboratory strains examined appear to be TLR4 dependent rather than to TLR2 (5). Strain PAK used by Feuillet et al. (17), Skerrett et al. (16) as well as this study fall into this group. Moreover, we see the same TLR4 dependency in PAO1, another *P. aeruginosa* strain.

Other features of this study that are worthy of commentary are the differences in deaths and bacterial clearance that was noted between us and Skerrett et al. (16) for TLR4<sup>-/-</sup> and TLR2,4<sup>-/-</sup> mice, when a  $\Delta$ *fliC* mutant of strain PAK was used. Skerrett et al. (16) reported one death in TLR2,4 mutant mice and none in TLR2 or TLR4 mutant mice (data in text, page L317) while we noted hypersusceptibility. Bacterial clearance in TLR2,4<sup>-/-</sup> mice was observed by Skerrett et al. (16) but was markedly reduced in our studies with actual proliferation noted at both 6 and 24 h following lung challenge. The use of different doses of bacteria could be an explanation since mortality appears to be dose dependent (17). Indeed, it can be deduced from the data reported by Skerrett et al. (16) that the bacterial load of their mice fluctuates around 10<sup>5</sup> cfu/mouse while we challenged mice with 5x10<sup>6</sup> cfu. In agreement, Feuillet et al (17) observed a dramatic death rate of the mice with a challenge of 6x10<sup>6</sup> cfu/mouse.



Due to the unavailability of TLR2,4,5<sup>-/-</sup> mice, we used a  $\Delta$ *fliC* mutant of *P. aeruginosa* in most of these studies. However, having a mutant in the flagellin binding site for TLR5 (strain PAK-L88) allowed us to examine the role of TLR5 in our studies. TLR2,4<sup>-/-</sup> mice proved not to be as hypersusceptible to this strain as the  $\Delta$ *fliC* mutant, where we saw 100% mortality after 2 days. However, infection of these mice with PAK-L88 did result in higher mortality than infection with the wild type strain.

We offer several explanations for this reduced hypersusceptibility. The L88 flagellin on the bacterium is glycosylated and the *Pseudomonas* flagellin glycosyl moiety stimulates an inflammatory response (20). The  $\Delta$ *fliC* mutant has no glycosyl groups, as it is a flagellin deletion mutant. *Pseudomonas* flagellin activates caspase-1 through a mechanism that is TLR5 independent (26), thus the L88 mutation in the TLR5 binding site still stimulates IL1 $\beta$  secretion which may offer some protection (26). Our own previous experiments (20) also demonstrate a low level of IL-8 release (10% of wild type flagellin), which may be enough to prevent the hypersusceptibility seen with a  $\Delta$ *fliC* mutant. Thus there may be three possible explanations for the lack of extreme hypersusceptibility as seen with the  $\Delta$ *fliC* mutant.

The current study also points to important roles for flagellin in the generation of IL-6 and G-CSF, since loss of these responses occurred only in TLR2,4<sup>-/-</sup> mice infected with the flagellin mutant. We have previously reported the loss of the IL-6 response in MyD88 deficient mice and its preservation in TLR2,4<sup>-/-</sup> mutant mice, when these mice are infected with a wild type *P. aeruginosa* strain (15). Their loss when a flagellin mutant was used, suggests the existence of a specific pathway that is flagellin dependent. Similar observations, that an IL-6 response was lost in response to an intranasal challenge of TLR5<sup>-/-</sup> mice with flagellin protein were made by Feuillet et al (17) suggesting that the IL-6 response may be TLR5 and flagellin

dependent. Our study expands this by showing the lack of response is solely due to the loss of flagellin. How this response to flagellin is mediated remains to be determined.

Our observations on TNF- $\alpha$  are also of interest. We have noted in the past (15) and find once more that animals incapable of mounting a TNF- $\alpha$  response are not susceptible to *Pseudomonas* lung infection and do not demonstrate a defect in *Pseudomonas* clearance. However, most studies, including one that used a TNF- $\alpha$  knockout mouse (36,37) show a defect in protection against such an infection. Thus, the role of TNF- $\alpha$  is not as clear as described since it has also been shown that mice deficient in TNF- $\alpha$  receptors do not have diminished resistance to a *Pseudomonas* lung infection (18), a result which is consistent with our findings.

There also exists the possibility that other systems may play a role in defense against this organism but we have not been able to substantiate this. It has recently been demonstrated that flagellin from two intracellular bacterial species *Salmonella* and *Legionella* (38,39) is recognized by Ipaf which activates caspase-1 (40) leading to the formation of IL-1 $\beta$  and IL-18 which are proinflammatory cytokines. This system also recognizes *Pseudomonas* flagellin (26), however, its role in infection by an extracellular pathogen such as *P. aeruginosa* may not be as critical and be supplemental. Alternatively, this system may be functional but is overwhelmed by high inocula of bacteria such as those used in experimental *in vivo* studies ~~or since the action of the inflammasome requires the formation of pro-IL-1 $\beta$  which in turn requires TLR activation (40), the inflammasome may not be functional.~~ Thus, we conclude that the activation of inflammatory pathways through TLR4 or TLR5 is sufficient to control an acute *P. aeruginosa* lung infection. While the studies of TLR-*Pseudomonas* interactions

have been limited to acute infections, one may conclude that these are equally important in chronic infection in Cystic Fibrosis, however in this case these interactions may fail to control the infection because of host and microbial factors such as microcolony formation (41), defective phagocytosis in the presence of mucins (42) and instead, result in cyclical bouts of uncontrolled inflammation.

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## Figure legends.

### Figure 1.

TLR2,4<sup>-/-</sup> mice are hypersusceptible to lung infection by a *P. aeruginosa* mutant devoid of flagellin production. Survival of wild type mice (WT) (n = 10) and TLR2,4<sup>-/-</sup> mice (n = 10) infected intratracheally with *P. aeruginosa* strain PAK or its  $\Delta$ *fliC* mutant. Inocula used were 5x10<sup>6</sup> cfu in a volume of 50  $\mu$ l of PBS. Survival was monitored up to 7 days. All TLR2,4<sup>-/-</sup> mice infected with the  $\Delta$ *fliC* mutant died by 48 h after bacterial challenge.

### Figure 2.

TLR2,4<sup>-/-</sup> mice are unable to control replication of a flagellin mutant of *P. aeruginosa* in the lungs. **A.** Bacterial replication is shown as a percentage of the initial inoculum (2x10<sup>6</sup> cfu) administered intratracheally. TLR2,4<sup>-/-</sup> mice were unable to control replication of the flagellin mutant of strain PAK (n = 3) when compared to the flagellin positive strain (n = 6) when measured a 6 h post infection ( $p < 0.05$ ). Clearance of the flagellin mutant by the wild type mice (n = 4) was less than that of the flagellin positive bacterium (n = 6), but did not reach statistical significance. A minimum of three mice were used for each assay. **B.** Bacterial luminescence in the lungs of wild type and TLR2,4<sup>-/-</sup> mice measured by injecting luminescent *P. aeruginosa* intratracheally (5x10<sup>6</sup> cfu/mouse) and capturing photon emission from the chest at different times post infection, using the IVIS system. TLR2,4<sup>-/-</sup> mice are unable to control the replication of the  $\Delta$ *fliC* mutant of strain PAK as opposed to wild type mice. **C.** Plots of the photon emission as a function of time. Clearance of the same bacterial strains by wild type and TLR2,4<sup>-/-</sup> mice was recorded at 2, 6 and 20 h post challenge. TLR2,4<sup>-/-</sup> mice show a significant increase in the luminescent  $\Delta$ *fliC* mutant whereas the wild type mice control replication of this strain ( $p < 0.05$ ; n=7 and 9, respectively).

### Figure 3.

TLR2,4<sup>-/-</sup> mice infected with a *P. aeruginosa* mutant devoid of flagellin production are defective in their TNF- $\alpha$ , IL-6, G-CSF and KC responses. **A.** TNF- $\alpha$  responses are blunted in the TLR2,4<sup>-/-</sup> mice in response to infection by both wild type and  $\Delta$ *fliC* mutant. **B.** By contrast, the IL-6 response is only reduced and by approximately 50% when the  $\Delta$ *fliC* mutant is used to infect wild type mice ( $p < 0.05$  compared to infection by wild type bacteria) but is drastically reduced when this mutant is used to infect TLR2,4<sup>-/-</sup> ( $p < 0.01$  compared to infection of these same mice by the wild type strain). Similarly, the KC (**C**) and G-CSF (**D**) responses are dramatically lost only when the  $\Delta$ *fliC* mutant is used to infect TLR2,4<sup>-/-</sup> mice, ( $p < 0.05$  and  $p < 0.01$ , respectively when compared to infection of these same mice with the flagellin positive strain). The basal values for non infected mice were at maximum 0.7, 7, 16 and 0 pg/ml for TNF- $\alpha$ , IL-6, KC and G-CSF, respectively. Each histogram is the mean  $\pm$  sem obtained with a minimum of 4 mice.

### Figure 4.

PMN influx and activation are defective when TLR2,4<sup>-/-</sup> mice are infected with a *P. aeruginosa* mutant devoid of flagellin production. **A.** PMN influx is significantly reduced only in TLR2,4<sup>-/-</sup> mice infected with the  $\Delta$ *fliC* mutant ( $p < 0.05$ ; n = 3-4). Similarly, **B.** MPO as a measure of PMN activation is reduced only in these same mice infected with the  $\Delta$ *fliC* mutant ( $p < 0.05$ ; n = 3-4)

### Figure 5.

An innate immune response to flagellin alone is sufficient to defend the lungs against *P. aeruginosa*. TLR2,4<sup>-/-</sup> mice were infected with the  $\Delta$ *fliC* mutant of strain PAK and treated

with different regimens of recombinant *P. aeruginosa* flagellin. One group (black squares, n = 6) was given 0.1 µg flagellin mixed with the challenge bacteria, followed by another dose of flagellin 6 h later by the intranasal route (2 doses). A second group (white triangles, n = 7) was given the same dose at the time of infection then at 6, 12 and 18 h post infection (4 doses). A control group of TLR2,4<sup>-/-</sup> mice received PBS (black circles, n = 10) instead of flagellin.

### Figure 6.

Survival of TLR2,4<sup>-/-</sup> mice challenged with *P. aeruginosa* wild type strain PAK, PAK-L88 or PAK  $\Delta$ *fliC*. The different strains were administered (1-2x10<sup>7</sup> cfu/mouse) and survival was recorded two days post infection. Differences in survival following challenged with wild type strain PAK and the L88 or  $\Delta$ *fliC* mutants were statistically significant ( $p < 0.05$ ; n = 3).

### Figure 7.

Susceptibility of TLR2,4<sup>-/-</sup> mice to infection by a *P. aeruginosa* mutant devoid of flagellin production is due to the TLR4 mutation and is not specific to one strain of *P. aeruginosa*. TLR2<sup>-/-</sup> mice (white triangles, n = 10) or TLR4<sup>-/-</sup> mice (black squares, n = 10) were infected with the  $\Delta$ *fliC* mutant of strain PAK (5x10<sup>6</sup> cfu). All TLR2<sup>-/-</sup> mice survived the infection, 90% TLR4<sup>-/-</sup> mice died rapidly. Similarly all TLR4<sup>-/-</sup> mice infected with a flagellin mutant of strain PAO1 (black circles, n = 5) died when infected with an inoculum of 1x10<sup>6</sup> cfu.















