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Original article

Innate immune responses to *Proteus mirabilis* flagellin in the urinary tract

Ana Umpiérrez [a], Paola Scavone [a], David Romanin [b], Juan Martín Marqués [c], José Alejandro Chabalgoity [c], Martín Rumbo [b], Pablo Zunino [a,*]

[a] *Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, Av. Italia 3318, PC 11600 Montevideo, Uruguay*
[b] *Laboratorio de Investigaciones del Sistema Inmune, Facultad de Ciencias Exactas, Universidad de La Plata, 47 y 15, La Plata, Argentina*
[c] *Departamento de Desarrollo Biotecnológico, Instituto de Higiene, Facultad de Medicina, UDELAR, Av. Alfredo Navarro, 3051 Montevideo, Uruguay*

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Abstract

Flagella are bacterial virulence factors allowing microorganisms to move over surfaces. Flagellin, the structural component of flagella, is sensed by the host via Toll and NOD-like receptors and triggers pro-inflammatory responses. The use of Toll-like receptors agonists to modulate innate immune responses has aroused great interest as an alternative to improve the treatment of diverse infectious diseases. *Proteus mirabilis* is a Gram negative bacterium that causes urinary tract infections in humans. In the present work we used different approaches to study the ability of *P. mirabilis* flagellin to induce an innate immune response. We demonstrated that *P. mirabilis* flagellin has the ability to induce pro-inflammatory chemokines expression in T24 bladder cultures cells and in the mouse bladder after instillation. It was evidenced also that flagellin from different *P. mirabilis* strains differed in their capacity to induce an innate immune response in the CacoCCL20-Luc system. Also, flagellin elicited inflammation, with recruitment of leukocytes to the bladder epithelium. Flagellin instillation before an experimental *P. mirabilis* infection showed that the inflammatory response due to flagellin did not help to clear the infection but favored bacterial colonization. Thus, induction of inflammatory response in the bladder did not contribute to *P. mirabilis* infection neutralization.

Keywords: *Proteus mirabilis*; UTI; Flagellin; Innate immune response

1. Introduction

Urinary tract infections (UTI) are among the most frequent human infection diseases diagnosed in outpatients as well as in hospitalized patients worldwide [1]. Over than 150 million of people are diagnosed with UTI every year [2] making it an illness that needs enormous amounts of money for patient health management. Only in the USA, expenses associated to UTI treatment surpassed the 3.5 billion dollars in 2006 [3]. UTI commonly affect neonates, adults and sexually active women, being this group particularly susceptible to UTI recurrence [3]. Diverse epidemiologic studies revealed that about 50% of women have one episode of UTI during their lives and about 30% of them experience a recurrence episode within 6 months after the first episode [4].

Proteus mirabilis is a common cause of ascending opportunistic and nosocomial UTI in humans. It is frequently associated with complicated UTI, which occur in patients with functional and/or structural abnormalities in the urinary tract (UT) [5,6]. *P. mirabilis* is the fourth more frequent UTI pathogen after *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas aeruginosa* in Latin America [7] and the third more frequent in North America [8].

Flagella are surface organelles that allow bacteria to move over different surfaces and may contribute to virulence through different mechanisms [9–11]. The major structural component of the flagella filament is a small protein called flagellin, which is considered an important bacterial MAMP recognized by the innate immune system [12–14]. The number of flagellin subunits can vary between species and

* Corresponding author. Tel.: +598 24871616; fax: +598 25461.
E-mail addresses: pzunino@iibce.edu.uy, pmzunino@gmail.com, pablo@iibce.edu.uy (P. Zunino).

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strains which determines the length and direction of flagella rotation [9].

P. mirabilis has two tandem flagellin genes denominated *flaA* and *flaB* [15], recently renamed as *fliC1* and *fliC2* (access number in NCBI: P42272 and P42273 respectively). Both genes have domains of high homology, being the amino and carboxyl terminus of the proteins 100% alike [16]. Early studies have demonstrated that *P. mirabilis* commonly express *flaA* gene to produce FlaA flagellin (dominant phenotype), whereas *flaB* gene remains silent. However, under certain circumstances *P. mirabilis* can express hybrid FlaAB proteins of nearly the size of FlaA [15]. Moreover, these phase variations have been associated to antigenic differences between strains and also it results in mechanical changes in the flagellar helicity, which would improve flagellar propulsion efficiency [15].

In terms of innate immune response, it has been evidenced by different in vivo and in vitro studies that recognition of conserved regions of flagellin by TLR5 induces intracellular activation of NF-κB and mitogen-activated protein kinase [17–19]. Furthermore, it has been recently shown that flagellin recognition by cytosolic proteins Naip5 and Ipaf activates signaling pathways which stimulate pro-inflammatory genes expression [9,17–19].

In the last decade the utilization of TLRs agonists to modulate the innate immune response against microbial infections has raised interest in the immunology community [20]. Flagellin has the capacity to induce strong innate immune responses in the host, characterized by pro-inflammatory cytokines and chemokines production, neutrophils and dendritic cells recruitment to the mucosa and lymphocytes T and B migration to the lymph nodes [18,21]. In this context, diverse studies have used flagellin to modulate the innate immune response in mucosal surfaces and to develop stronger adaptive responses. Thereby, its utilization as an adjuvant has been studied in a broad range of recombinant vaccines [20,22]. For instance, adjuvant effect of recombinant *Salmonella typhimurium* flagellin in combination with F1 antigen of *Yersinia pestis* and with Pneumococcal surface protein A of *Streptococcus pneumoniae* has been evaluated in murine models of infection obtaining very promising results [23,24].

The aim of this work was to evaluate *P. mirabilis* flagellin capacity to induce an innate immune response in the urinary tract through different in vitro and in vivo approaches.

2. Materials and methods

2.1. Bacterial strains

Eight wild type *P. mirabilis* isolates obtained from hospitalized patients with UTI in Montevideo, Uruguay, were utilized in this work. All the strains belong to the collection of the Department of Microbiology, IIBCE (Table 1).

2.2. Media and reagents

P. mirabilis wild type strains were routinely cultured in LB broth. When solid media was required, agar 1.5% was added.

Table 1
Uropathogenic *P. mirabilis* strains used in this work and purified flagellin nomenclature of each protein.

Strain	Origin	Reference	Flagellin nomenclature
P. mirabilis 6515	Clinical UTI	[25]	"A"
P. mirabilis 19287	Clinical UTI	Microbiology Department collection	"B"
P. mirabilis 47	Clinical UTI	Microbiology Department collection	"C"
P. mirabilis 268	Clinical UTI	Microbiology Department collection	"D"
P. mirabilis 2921	Clinical UTI	[26]	"E"
P. mirabilis 289	Clinical UTI	Microbiology Department collection	"F"
P. mirabilis 783	Clinical UTI	[27]	"G"
P. mirabilis 2932	Clinical UTI	[27]	"H"

For bacterial counts, nutrient agar (NA) was used. All media components were from Difco Laboratories (Detroit, MI, USA).

2.3. Animals

Eight-week old female CD-1 mice were utilized in this work. Animals were provided with food-pellets and tap water ad libitum. All animals' procedures had been previously approved by the Bioethics Committee of IIBCE (CEUA), Montevideo, Uruguay.

2.4. Flagellin extraction and purification

Flagellin from the eight wild type *P. mirabilis* strains was obtained by mechanical treatment and centrifugation following the protocol described by Allison and colleagues [28]. For the characterization of the obtained products, aliquots of each flagellin suspension were heated and run in a 10% SDS–PAGE. In addition, MALDI-TOF analysis of *P. mirabilis* 2921 flagellin to corroborate the efficiency of the extraction and purification procedure was performed at the Biochemist and Analytical Proteomics Laboratory of the Institut Pasteur of Montevideo (IPMont).

Pr2921 flagellin sample was evaluated to rule out bacterial LPS contamination [29]. Different animal groups were intraperitoneal injected with flagellin or trypsin-treated flagellin. Groups of mice treated with trypsin and PBS only were used as controls. Two hours after injection, relative mRNA expression of hepatic pro-inflammatory *Ccl20* chemokine was evaluated by quantitative real-time PCR in each animal group. Absence of LPS was corroborated by total abrogation of *Ccl20* induction by trypsin-treated flagellin preparation. Reverse transcription and real-time PCR were performed following the protocols described below (Section 2.6).

Flagellin samples were heated at 80 °C for 5 min before starting the different procedures, to obtain monomer sus pensions.

2.5. Stimulation of CacoCCL20-Luc reporter system with P. mirabilis *flagellin*

CacoCCL20-Luc cells [19] were routinely grown in DMEM base (Gibco) supplemented with 10% FBS, 10 mM HEPES, 1× nonessential amino acids, 100 U/ml penicillin and 100 U/ml streptomycin, at 37 °C and 5% of CO_2. Confluent monolayers of CacoCCL20-Luc cells were stimulated with the different *P. mirabilis* flagellin preparations and with recombinant *S. typhimurium* FliC [30] for 6 h. Luciferase activity was measured using a Luciferase Assay (Promega, Madison, WI, USA) and luminescence was expressed as the fold increase respect to non stimulated cells. Four concentrations of protein were tested: 10 μg/ml, 2 μg/ml, 400 ng/ml and 80 ng/ml.

The presence of factors in *P. mirabilis* flagellins suspensions that could down regulate flagellin activity was evaluated by mixing each preparation with *S. typhimurium* FliC. Protein mixtures (2.0 μg/ml of each *P. mirabilis* flagellin and 0.5 μg/ml of FliC) were evaluated in the CacoCCL20-Luc system for 6 h.

Additionally, the effect of heat on the *P. mirabilis* flagellin suspensions was evaluated in the CacoCCL20-Luc reporter system, following the procedure described above. Before stimulation, different Pr2921 flagellin ("E") suspensions were heated at 70, 75, 80 and 90 °C and then used in the reporter system separately.

2.6. In vitro Ccl20, Cxcl2, Ccl2 mRNA levels

Uroepithelial bladder T24 cells [31] were routinely grown in high Glucose DMEM (Gibco) supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin, at 37 °C and 5% of CO_2. Confluent monolayers were stimulated with either Pr2921 flagellin ("E") or recombinant *S. typhimurium* FliC [30] for 2 and 8 h. Two concentrations of above mentioned flagellins were tested: 10 μg/ml and 50 μg/ml. After stimulation, cells were homogenized in RA1 lysis buffer (GE, Healthcare, Germany) and total RNA was extracted with Nucleo Spin RNA II kit (Macherey-Nagel, Germany). Reverse transcription was performed using 200 U M-MLV reverse transcriptase (Invitrogen, USA), 0.5 mM dNTPs, 1 nM random primers, 20 U RNAseOUT, 7.5 mM DTT, 1× reaction buffer and 400 ng of RNA in a final volume of 20 μl. The RT reaction was performed at 25 °C for 10 min followed by 37 °C for 60 min. For real-time PCR, MyiQ Single color Real-time PCR Detection System (BioRad, USA) and SYBR® Green Master Mix (Invitrogen) were utilized. The thermocycler programme consisted in an initial activation step at 95 °C for 10 min, then 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min followed by an extension at 72 °C for 30 s) and chilling to 4 °C. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product. Each reaction mix consisted in 0.9 μM of primers, 12 μl of master mix and 10 μl of cDNA in a final volume of 24 μl. Primers to evaluate mRNA levels of pro-inflammatory chemokines: CCL20, CCL2, CXCL2 (MIP-2α) and human β-actin were previously designed and described [30]. Relative mRNA expression of each chemokine was evaluated through the $2^{-\Delta\Delta Ct}$ method and by using the β-actin gene as reporter gene [32].

2.7. Flagellin instillation in mice

To evaluate the innate immune response induced by the administration of *P. mirabilis* flagellin in the UT, a mouse bladder instillation model was developed, based on the work of Saban and colleagues [33]. Two groups of animals were transurethrally instilled with 5 and 25 μg of Pr2921 flagellin respectively, suspended in 50 μl of PBS. The third group was instilled only with 50 μl of PBS. All animals were subcutaneous anesthetized with xylazine and ketamine (1:5 mg/kg) and their bladders were emptied with gentle abdominal massages before starting the procedure.

2.8. Urine collection and immune cells counts

Urine samples were obtained at zero, 6 and 24 h post-instillation of mice by gentle abdominal massages. The numbers of leukocytes in each sample of urine was determined using a Neubauer chamber. For the enumeration of leukocytes under light microscopy, cells size and shape were taken into account.

2.9. Bladder histological modifications

Animals treated with 5 μg of flagellin ($n = 10$) and with PBS ($n = 7$) were sacrificed 24 h after instillation. Then, bladders were extracted and embedded in Tissue Freezing Medium (Leica Biosystems). After that, all samples were sectioned, stained with hematoxylin and eosin and examined microscopically. Bladder sections (12 μm) were analyzed using a light microscope OLYMPUS DP71 and CellF program (OLYMPUS). The severity of histological modifications of each bladder was expressed as a semiquantitative score previously described by Alamuri and colleagues [34]. The histological modification score used was: zero, absence of histological modifications; 1, occasional submucosal inflammatory cell infiltrates; 2, widespread submucosal inflammatory cell infiltration with minimal spread to the muscularis or epithelium; and 3, widespread inflammation with dense perivascular cuffs, transmural distribution, and intraepithelial inflammatory cells. Two researchers observed all samples separately, in a double-blind manner.

2.10. In vivo IL6, Cxcl1, Cxcl10 mRNA levels

In this experiment, two groups of mice were transurethrally instilled with 5 μg of Pr2921 flagellin per mouse and after 2 and 24 h, bladders were extracted, homogenized in 500 μl TRIzol (Invitrogen, USA), mechanically disaggregated with an Ultra Turrax (IKA-Werke, Germany) and kept at −80 °C until their utilization. Total RNA of each sample was extracted with chloroform, suspended in RNAse free H_2O and treated

with 0.35 U of DNAse I (Invitrogen, USA). cDNA synthesis was performed using 1.0 μg of total RNA, 200 U M-MLV reverse transcriptase (Invitrogen, USA), 1.0 mM dNTPs, 200 ng of random primers, 400U RNaseOUT, 20 mM DTT, 1× reaction buffer and Gradient Palm-Cycler™ High Performance Thermal Cycler (Corbett Life Science). Real-time PCR was performed using the Rotor-Gene 6000 (Corbett Life Science) and PCR QuantiTect® SYBR® Green Qiagen (Hilden-Germany), following the next protocol: an initial activation step at 95 °C for 15 min, then 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, extension at 72 °C for 30 s) and chilling to 4 °C. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product. Each reaction mix consisted in 0.9 μM of primers, 5.0 μl of master mix (Qiagen) and 2.0 μl of DNAc in a final volume of 10 μl. Primers to evaluate mRNA levels of pro-inflammatory chemokines: IL6, CXCL1, CXCL10, and mouse β-actin were previously designed and described [35,36]. Relative mRNA expression of each chemokine was evaluated through the $2^{-\Delta\Delta Ct}$ method and by using the β-actin gene as reporter gene [32].

2.11. Flagellin instillation and experimental UTI in mice

Two groups of animals were transurethrally instilled with 5 μg ($n = 9$) and 25 μg ($n = 6$) of Pr2921 flagellin per mouse. Also a group of 7 animals was instilled with PBS. After 24 h, all animals were challenged with 50 μl of a bacterial suspension in PBS containing 10^8 colony-forming units (CFU) of *P. mirabilis* 2921, using the ascending UTI model [26]. All animals were subcutaneously anesthetized with xylazine and ketamine (1:5 mg/kg) and their bladders were emptied with gentle abdominal massages before starting the procedure. Animals were examined daily and then scarified 7 days after the challenge by cervical dislocation. Each bladder and kidney was aseptically removed and separately homogenized in 10 ml of PBS using a stomacher 80 Lab Blender (Seward). Homogenized tissues were quantitatively cultured on NA plates and the numbers of CFU per bladder and kidney were determined after 24 h of incubation at 37 °C.

2.12. Statistical analysis

To analyze bacterial colonization levels in kidneys and bladders and chemokine and cytokine transcriptional levels in bladders the non-parametric two-tailed Mann–Whitney *U*-test was used. Stimulation CacoCCL20-Luc reporter system experiment and in vitro chemokine expression in T24 culture cells were analyzed with the Student-*t* test and the non-parametric two-tailed Mann–Whitney *U*-test respectively. Urine leukocytes counts between animal groups were analyzed with the Kruskal Wallis test.

3. Results

3.1. Flagellin stimulation of CacoCCL20-Luc cells

Flagellin from 8 different clinical *P. mirabilis* UTI was correctly purified, as a single band of approximately 41 kDa was observed by SDS–PAGE (Fig. 1a). Subsequently MALDI-TOF analysis confirmed that Pr2921 flagellin suspension corresponded to *P. mirabilis* flagellin (Figure S1). Furthermore, LPS contamination of Pr2921 flagellin preparation was evaluated by quantitative real-time PCR.

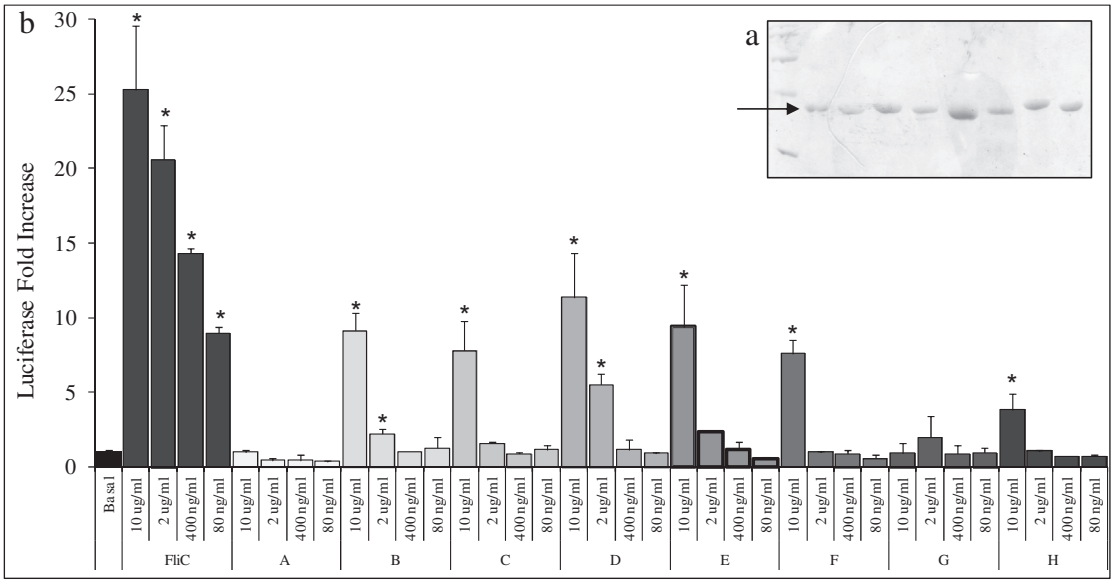

Fig. 1. (a) Purified *P. mirabilis* flagellins run in 10% SDS–PAGE. From left to right, Lane 1: Broad range molecular weight marker (Bio-Rad) (bands correspond to 21.5, 45 and 61 kDa proteins), Lanes 2–9: *P. mirabilis* purified flagellin "A", "B", "C", "D", "E", "F", "G" and "H" respectively. Arrow shows purified protein. (b) Flagellin stimulation of CacoCCL20-Luc cells. Luciferase fold increase in CacoCCL20-Luc culture cells stimulated with *S. typhimurium* FliC and 8 *P. mirabilis* purified flagellins for 6 h. Four concentrations of protein were tested: 10 μg/ml, 2 μg/ml, 400 ng/ml and 80 ng/ml. Asterisks (*) show significant differences of each flagellin respect to non-stimulated (basal) cells (Student *t*-test, $p < 0.05$).

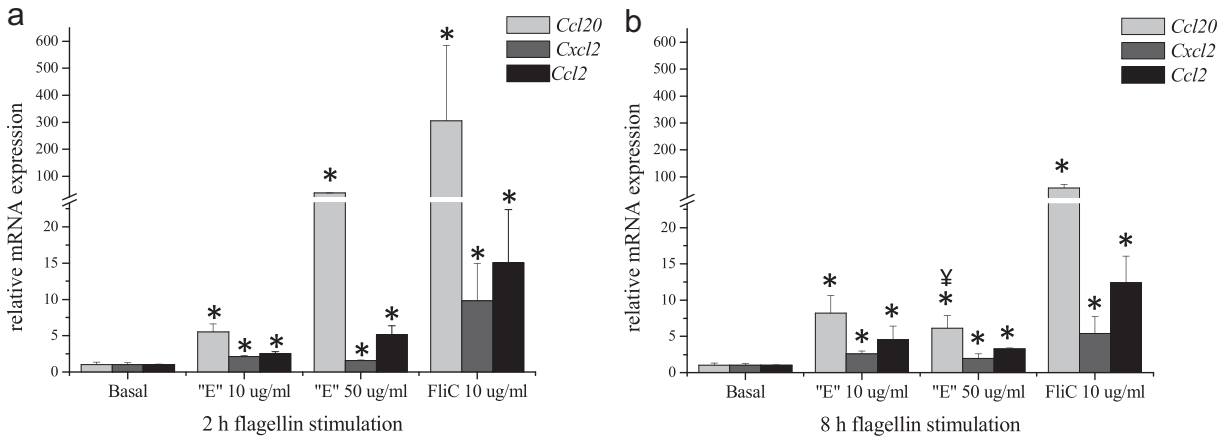

Fig. 2. Chemokine production in culture cells. *Ccl20*, *Cxcl2* and *Ccl2* relative mRNA expression in T24 culture cells stimulated with Pr2921 flagellin ("E") and *S. typhimurium* FliC flagellin. β-actin gene was employed as the reference gene. Asterisks (*) indicate significant differences of relative expression at 2 (a) and 8 h (b) of all chemokines respect to non-stimulated (Basal) cells. (¥) indicates significant decrease of *Ccl20* mRNA relative expression, using 50 μg/ml of Pr2921 flagellin at 8 h respect to 2 h (Student *t*-test, $p < 0.05$).

Intraperitoneal-injected Pr2921 flagellin induced a 271 fold increase of mRNA expression of pro-inflammatory CCL20 chemokine ($p < 0.05$, compared to basal cells) in mice livers, whereas the treatment of flagellin preparation with trypsin before mice administration abrogated the *Ccl20* induction (0.8 fold increase, respect to basal cells). Thus, we could show that bioactivity was due to the protein extract.

Capacity of *P. mirabilis* flagellin to induce *Ccl20* promoter in the CacoCCL20-Luc reporter system was evaluated. All *P. mirabilis* flagellins showed different capacities to activate the CacoCCL20-Luc reporter system, being in almost all the cases, stimulating proteins (Fig. 1b). We identified two flagellins (obtained from Pr6515 and Pr783 strains) that did not induce the *Ccl20* promoter expression whereas the rest of the proteins induced a significant fold increase in luciferase light emission compared to basal cells when 10 and 2 μg/ml of protein were utilized (Fig. 1b). Pr2921 flagellin, purified from the most thoroughly characterized uropathogenic strain of our laboratory, induced a significant 8-fold increase in the reporter activity compared to the basal state when 10 μg/ml of protein was employed. This strain produced one of the highest values of the reporter system activation among all *P. mirabilis* flagellins tested (Fig. 1b).

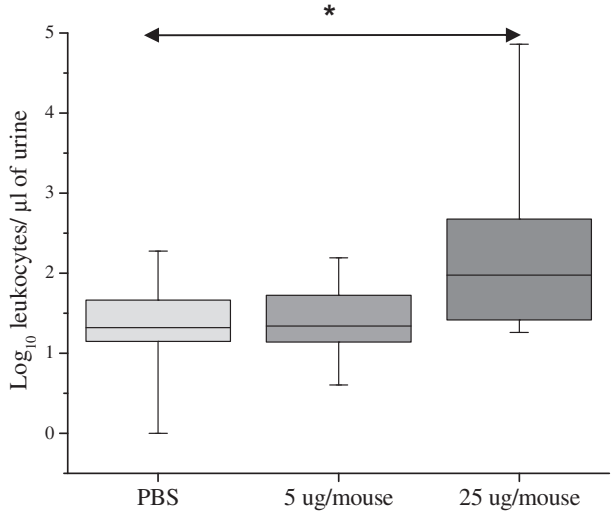

Fig. 3. Quantification of leukocytes in urine. BoxPlot of Log10 of leukocytes/ μl urine (t24/tzero h). Leukocytes counts were obtained by Neubauer Chamber urine counts at zero and 24 h post-transurethral instillation of mice with PBS ($n = 7$) and 25 μg ($n = 10$) of Pr2921 flagellin ("E") per mouse. Asterisks (*) indicate significant differences respect to PBS group (Kruskal Wallis test, $p < 0.05$).

Activity of *S. typhimurium* FliC was not affected by the presence of any of the *P. mirabilis* flagellins suspensions, as we did not observe a significant Luciferase fold decreases in the reporter system in any case. Thus, we demonstrated that *P. mirabilis* suspensions did not contain inhibitors of flagellin activity (Figure S2).

Furthermore, thermal treatment of Pr2921 flagellin did not induce significant changes in the activity on the CacoCCL20-Luc cells, compared to the non-treated protein. Consequently, 80 °C treatment was used to insure the absence of partial flagellin monomers aggregates (Figure S3).

3.2. In vitro chemokine expression

The capacity of *P. mirabilis* flagellin to induce chemokine expression by T24 bladder epithelial cells was first evaluated in vitro. After 2 h stimulation, Pr2921 flagellin significantly induced a 8.2, 2.6 and 4.5 fold increase of *Ccl20*, *Cxcl2* and *Ccl2* mRNA levels respectively ($p < 0.05$, compared to basal cells), when 10 μg/ml of protein was used. Moreover, Pr2921 flagellin induced a 37.4, 1.6 and 5.2 fold increase of the same chemokines, when 50 μg/ml was employed (Fig. 2a). On the other hand, 10 μg/ml per well of *S. typhimurium* FliC flagellin induced a 305, 9.8 and 15 fold increase of mRNA expression of the same analyzed chemokines ($p < 0.05$, respect to basal cells). Also, we observed that after 8 h of incubation with 50 μg/ml of Pr2921 flagellin, CCL20 mRNA level expression decreased respect to mRNA levels at 2 h ($p = 0.003$) (Fig. 2b, ¥). Therefore, these results verified transient in vitro innate immune response elicited by *P. mirabilis* flagellin in bladder epithelium cells (Fig. 2b).

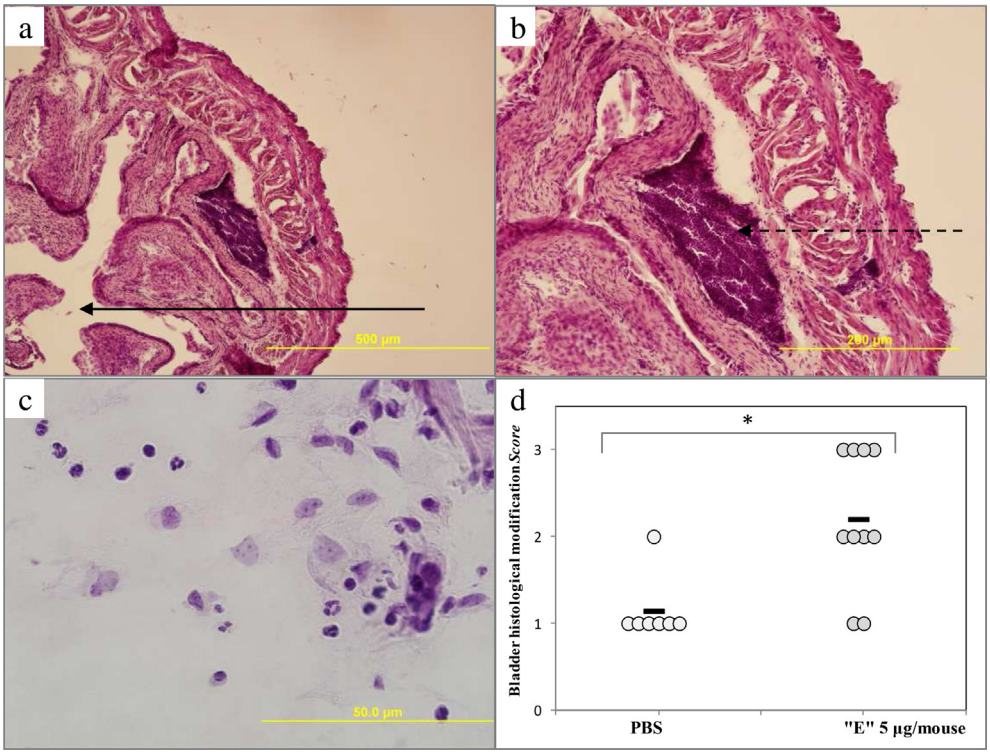

Fig. 4. Bladder histological modifications. Murine bladder transects stained with hematoxylin and eosin and analyzed with Cell^F image analysis programme. Panels a, b and c: Pr2921 flagellin ("E") instilled bladders (5 µg/mouse). Continuous arrow shows bladder lumen and dotted arrow shows a focus of inflammation with leukocytes recruitment to the submucosa. Panel d: Bladder histological modification Score of PBS and Pr2921 flagellin treated animals. Each circle corresponds to a single bladder. Bars represent median of the values and asterisks (*) represent significant differences between groups (non-parametric two-tailed Mann–Whitney *U*-test, $p < 0.05$).

3.3. Transurethral administration of P. mirabilis *flagellin*

In vivo experiments were carried out in order to evaluate capacity of Pr2921 flagellin to induce an innate immune response in the mouse bladder. Analysis of PBS and flagellin instilled bladders showed a transient recruitment of leukocytes in urine (Fig. 3). At 24 h post-instillation we observed a significant increase of leukocytes in the 25 µg of flagellin treated group compared to PBS group ($p = 0.036$) (Fig. 3).

Light microscopy evaluation of mice bladders sections revealed the presence of histological modifications, which were significantly more severe in flagellin-treated mice bladders. Flagellin induced modifications characterized by widespread submucosal inflammatory cell infiltration and dense perivascular cuffs with spread to the muscularis and epithelium and transmural distribution (Fig. 4). All animal groups showed some extent of modification as the semiquantitative score was higher than zero in all cases. These observations are concordant to those observed in urine cell counts and may be due to the fact that mechanical catheter manipulation induces bladder modifications. Despite this, 24 h post-instillation, all flagellin treated bladders ($n = 10$) scored significantly higher than control ones ($n = 7$) ($p = 0.01$) (Fig. 4d). Thus, it was demonstrated that Pr2921 flagellin instillation induces bladder histological modifications in the mice.

Then it was analyzed whether Pr2921 flagellin instillation modified cytokine and chemokine transcriptional response in the mice bladders. After 2 h instillation, Pr2921 flagellin significantly induced a 17.9, 3.1 and 89 fold increase of *Cxcl1*, *Cxcl10* and *Il6* mRNA levels respectively ($p < 0.05$, compared to control group), when 5 µg of Pr2921 of flagellin per mouse was used. Then, 24 h after instillation of Pr2921 flagellin, mRNA levels diminished to initial values (Figure S4).

3.4. Instillation of flagellin and UTI challenge in mice

The role of transurethral bladder instillation of *P. mirabilis* flagellin in the protection of mice against a subsequent experimental UTI was assessed using an experimental UTI model. Bacteria were recovered from bladder and kidneys 7 days after experimental UTI and were counted on NA plates. The number of viable bacteria in kidneys of animals instilled with 25 µg of flagellin per mouse was significantly higher compared to PBS treated animals ($p = 0.007$) and to the group treated with 5 µg of flagellin ($p = 0.019$) (Fig. 5). On the other hand, no statistical differences were observed in the number of viable bacteria in bladders between the two flagellin-treated animal groups and between PBS treated animals (Fig. 5).

4. Discussion

In this work, we characterized the capacity of flagellin from different uropathogenic *P. mirabilis* strains to elicit an innate

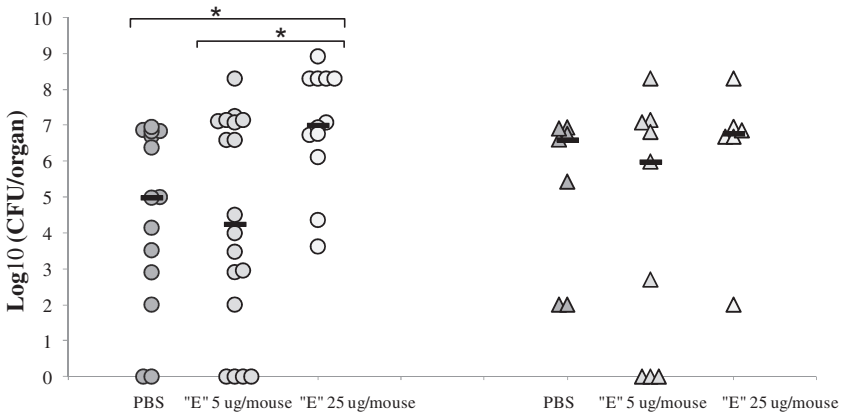

Fig. 5. Instillation of flagellin and *P. mirabilis* challenge in mice. Bacterial counts in bladder and kidneys after 24 h of transurethral Pr2921 flagellin administration and 7 days of UTI experimental challenge. Flagellin concentrations employed: 5 ($n = 9$) and 25 μg ($n = 6$) of Pr2921 flagellin ("E") per mouse in each animal group. Control group ($n = 7$) was instilled with PBS. Circles correspond to kidney Log10 CFU and triangles to bladder Log10 CFU. The medians are represented as the black lines. Asterisks (*) show significant differences between groups (non-parametric two-tailed Mann–Whitney *U*-test, $p < 0.05$).

response using in vitro and in vivo models. Furthermore, we have analyzed the effect of triggering an innate response in the bladder through the recognition of *P. mirabilis* flagellin in the 24 h previous to an experimental UTI in mice.

The first experimental approach performed to evaluate innate immune activation by flagellin was based in the CacoCCL20-Luc reporter system. Stimulation of these cells allowed us to establish that *P. mirabilis* flagellins were able to induce an innate immune response in the reporter system as we observed statistical fold increases of luciferase light emission in CacoCCL20-Luc cells. Similar results have been obtained by others authors using *S. typhimurium* FliC flagellin in the same reporter system [19] as well as in the HEK293-Luc system, which expresses the reporter gene associated to NF-κB intracellular pathway [37] and using *Listeria monocytogenes* flagellin in CHO-Luc culture cells [17]. Also, we observed that *S. typhimurium* FliC flagellin induced a higher response in the reporter system than any of the *P. mirabilis* flagellins. Probably intrinsic differences between proteins are the cause of such appreciation, as McDermott and colleagues [38] observed with *S. typhimurium*, *E. coli*, *Yersinia enterocolitica* and *P. aeruginosa*. In that work *S. typhimurium* flagellin showed the maximum capacity to elicit an innate response. In the present work, flagellin of the different *P. mirabilis* strains showed different capacities to induce an innate immune response in the CacoCCL20-Luc reporter system. *P. mirabilis* has the ability to express hybrid FlaAB proteins with different aminoacidic sequences and different antigenic properties [15,39]. Moreover, taking into account the CacoCCL20-Luc experiments results, we propose that these rearrangements would lead to the generation of flagellin with different capacities to induce an innate immune response. This property would allow different strains of *P. mirabilis* to avoid innate immune receptors that may provide an advantage to colonize the host UT.

UT epithelium is an important mechanic barrier to infection where potent and organized innate and adaptive immune responses are induced upon recognition of selected microbial ligands [40]. Neutrophils are among the most abundant leukocytes recruited to the site of infection in a UTI episode [3]. During UTI, their recruitment begins when UT epithelial cells secrete chemokines and cytokines [41]. It has been reported that *S. typhimurium* FliC induces the production of TNF-α, IL6, IL-10, IL-12p70, MIP-1α and IFN-γ [13] and recruitment of neutrophils to the mucosa, when is locally and systemically administered in mice [18,35]. We demonstrated for the first time that Pr2921 flagellin has the capacity to induce a transient pro-inflammatory *Ccl20*, *Cxcl2* and *Ccl2* mRNA expression in bladder T24 culture cells. Therefore, flagellin recognition could be involved in the inflammatory response in the bladder in the context of a *P. mirabilis* infection.

In addition, in vivo experiments demonstrated that Pr2921 flagellin bladder instillation induced a local transient inflammatory response, as we observed histological modifications, detected the presence of leukocytes in mice urine and transient pro-inflammatory *Cxcl1*, *Cxcl10* and *Il6* mRNA expression in murine bladders. Similar results had been observed by Saban and colleagues [33] when transurethrally instilled *E. coli* LPS in mice. However, severity of bladder histological modifications and edema formation seen in the present work were lower than the observed by Saban and colleagues [33] and by Alamuri and colleagues when performing an experimental ascendant UTI with *P. mirabilis* [34].

Different authors have proposed that the induction of an innate response could prevent the progress of infection. For example, intranasal administration of *S. typhimurium* FliC flagellin increased the resistance to *S. pneumoniae* infection in mice [35] whereas intranasal pretreatment of *P. aeruginosa* flagellin abrogates *P. aeruginosa* infection in mice lungs [42]. Also, triggering TLR4 previously to *Bordetella pertussis* infection blocks completely the progression of the disease [43]. Furthermore, *S. typhimurium* FliC flagellin administration impaired *Clostridium difficile* or *Enterococcus* intestinal infection [44,45]. In these different scenarios, activation of the innate response may prevent concomitant infection by either enhancing neutrophil recruitment to the infection site,

inducing antimicrobial peptide production and enhancing the capacity of resident cells to respond to other microbial stimuli or a combination of these factors. Consequently, we speculated that administration of *P. mirabilis* flagellin could have a preventive blocking effect in the course of an experimental UTI. However, results presented here demonstrated that bladder instillation of Pr2921 flagellin did not contribute to diminish bacterial colonization in the UT. Moreover, the number of viable bacteria in kidneys was exacerbated when high concentrations of the protein were used. Our observation suggests that inflammation and leukocyte recruitment associated to flagellin transurethral instillation were not favorable for experimental UTI resolution, and even an increase in kidney colonization was observed in mice previously treated with flagellin.

Altogether, our results indicate that different strains of *P. mirabilis* may have different capacities to elicit an innate immune response. Although the urinary tract may respond to triggering of immune receptors in the host, bladder stimulation with Pr2921 flagellin is not beneficial to the neutralization of the infectious process in the host, probably by the modulation of the inflammation and immune response in the bladder.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micinf.2013.06.007.

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