Cytokine 123 (2019) 154772

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

IL-1RA is part of the inflammasome-regulated immune response in bladder epithelial cells and influences colonization of uropathogenic *E. coli*

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ARTICLE INFO

Keywords:
Inflammasome
IL-1 receptor antagonist
NLRP3
Uropathogenic *Escherichia coli*
Urinary tract infections

ABSTRACT

The NLRP3 inflammasome, IL-1β release and pyroptosis (cell lysis) have recently been proposed to be essential for the progression of urinary tract infection (UTI) and elimination of intracellular bacterial niches. However, the effects of IL-1R antagonist (IL-1RA) on immune responses during UTI, except for its ability to disrupt IL-1β signalling, are not well understood. The aim of this study was to investigate the role of IL-1RA in UPEC colonization of bladder epithelial cells and the subsequent host inflammatory response. Human bladder epithelial cells (5637) and CRISPR/Cas9 generated NLRP3 and caspase-1 knockdown cells and IL-1RA knockout cells were stimulated with the UPEC isolate CFT073. The results showed that the UPEC virulence factor α-hemolysin is essential for IL-1RA release, and that the inflammasome-associated proteins caspase-1 and NLRP3 affect the release of IL-1RA. IL-1RA deficient cells showed a reduced adherence and invasion by CFT073 compared to wild-type cells, suggesting that IL-1RA may oppose mechanisms that protects against bacterial colonization. A targeted protein analysis of inflammation-related proteins showed that the basal expression of 23 proteins and the UPEC-induced expression of 10 proteins were significantly altered in IL-1RA deficient bladder epithelial cells compared to Cas9 control cells. This suggests that IL-1RA has a broad effect on the inflammatory response in bladder epithelial cells.

1. Introduction

The most common cause of urinary tract infection (UTI) is uropathogenic *Escherichia coli* (UPEC) that express several virulence factors like α-hemolysin, lipopolysaccharides (LPS), type 1 and P fimbriae, capsules and iron scavenging systems that facilitate its colonization of the urinary tract [1]. The type 1 fimbriae is one of the most well characterized UPEC adhesins and mediate attachment of UPEC to host receptors localized on the superficial bladder epithelial cells. Following attachment, a subset of the adherent UPEC may invade into the cytosol of superficial bladder epithelial cells [2]and establish intracellular bacterial communities [2,3] or invade into deeper layers of the urothelium and form quiescent intracellular reservoirs that may persist for extended periods [4,5]. Establishment of intracellular bacterial niches, which are protected from the host immune response and many antibiotics, has been suggested to be associated with an increased risk of reinfection and recurrent UTI [3,6,7]. Recurrence of UTI, which occurs in approximately 25% of women [8], results in exaggerated consumption of antibiotics and has due to the rise of multidrug resistant isolates been a challenge to tackle with limited treatment options.

The bladder epithelium is an immunoactive barrier which protects the urinary tract from infections. The interaction of UPEC virulence factors with host recognition receptors triggers a production of various inflammatory mediators of which the pro-inflammatory cytokines and chemokines IL-6 and IL-8 are the most studied [9,10]. Recently, the interest in the role of the pro-inflammatory cytokine IL-1β in regulating host defence mechanism in UTI has increased. IL-1β is one of the most potent mediators of inflammation, but also a major cytokine produced during dysregulated inflammation. Host production of IL-1β involves activation of inflammasomes that are cytosolic multiprotein complexes that sense bacterial virulence factors and danger/damage associated molecules [11]. Formation of the inflammasome triggers activation of caspase-1, which in turn results in release of the active form of IL-1β and an inflammatory form of cell lysis (pyroptotis) [11]. The most studied inflammasome is the nod-like-receptor pyrin domain-containing 3 (NLRP3) inflammasome, and it was recently shown that UPEC strains that express a TLR-signalling inhibitory protein (TcpC) are able to prevent NLRP3 complex formation as a virulence strategy to subvert the host immune system [12]. The role of IL-1β receptor signalling to the local host defence of the bladder mucosa and its contribution to UPEC colonization is unclear and the results from the literature are inconsistent. Some studies report that mice lacking IL-1β are protected

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https://doi.org/10.1016/j.cyto.2019.154772
Received 14 March 2019; Received in revised form 20 June 2019; Accepted 4 July 2019
Available online 09 July 2019
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from UTI [13,14], suggesting that IL-1 β may increase the susceptibility of the bladder mucosa to infection and enhance UPEC colonisation. However, other studies suggest that IL-1 β promotes a protective and beneficial innate immune response during UTI and is of importance for controlling UPEC colonisation [12,15,16].

The production and biological effects of IL-1β are controlled at many levels, one being the inhibition of IL-1 β mediated activities by the secreted form of the IL-1R antagonist (IL-1RA). Thus, the inconsistent results regarding the role of IL-1β in UTI may partly be explained by a variable local production and ratio of IL-1β/IL-1RA under different experimental conditions. The IL-1RA binds with high affinity to the same receptor as IL-1β (the IL1R), and by acting as an antagonist, it disrupts the pro-inflammatory signalling pathways evoked by IL-1β [17,18]. It is currently thought that an imbalance between IL-1β and IL-1RA production contributes to the development of inflammatory responses and concomitant tissue damage [19,20]. This is supported by studies showing excessive inflammation in IL-1RA-deficient mice in various disease models, and that patients with IL-1RA deficiency exhibit severe inflammatory conditions [19,21,22]. Furthermore, many studies demonstrate the efficacy of recombinant IL-1RA (generic anakinra) to treat a large number of systemic inflammatory diseases and rheumatoid arthritis [23].

Clinical data have demonstrated that the urinary levels of IL-1RA are significantly elevated in UTI patients compared with healthy individuals [24,25]. Anakinra, recombinant IL-1RA, was reported to reduce bladder pathology and bacterial and neutrophil counts in a mouse UTI-model characterized by IL-1β overactivity [13,14] and to have a protective effect in a hemorrhagic cystitis mouse model [26]. Thus, although some evidence exists in support of a protective role for IL-1RA during UTI more mechanistic knowledge, e.g., on how the release of IL-1RA is activated and regulated in human uroepithelial cells, is needed. In this study, we constructed IL-1RA deficient bladder epithelial cells and used these cells to reveal the role of IL-1RA in UPEC colonization and invasion, and in the inflammatory host response network.

2. Material and methods

2.1. Human bladder epithelial cells

The human bladder epithelial cell line 5637 (ATTCC HBT-9) was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle Medium (DMEM) (BioWhittaker, Lonza, Basel, Switzerland) complemented with 2 mM L-glutamin (Hyclone, GE Lifescience, UK), 10% fetal bovine serum (FBS) and 1 mM non-essential amino acids at 37 °C in a 5% CO_2 atmosphere. During experiments, the FBS levels were reduced from 10% to 2%.

2.2. Bacterial strains, mutants and growth conditions

In this study we included the UPEC strain CFT073 and the non-pathogenic K-12 strain MG1655. CFT073 deletion mutants CFT073Δ*hlyA* and CFT073Δ*fimH* were created with the λ red recombinase system [27] using primer sets *hlyA*_Fwd 5′-AAAAACAAGA CAGATTTCAATTTTTCATTAACAGGTTAAGAGATAATTAAGTGTAGGC TGGAGCTGCTTC-′3 and *hlyA*_Rev 5′-AATCTTATGTGGCACAGCCCAGT AAGATTGCTATTATTTAAATTAATAAAATGGGAATTAGCCATGGTCC-′3 and primer sets *fimH*_Fwd 5′- CATTCAGGCAGTGATTAGCATCACCT ATACCTACAGCTGAACCCAAAGAGGTGTAGGCTGGAGCTGCTTC -′3 and *fimH*_Rev 5′- TAGCTTCAGGTAATATTGCGTACCTGCATTAGCAAT GCCCTGTGATTTCTATGGGAATTAGCCATGGTCC -′3, respectively (Thermo Fisher Scientific, Waltham, MA, USA). The CFT073Δ*pap* isolate [28] was kindly provided by Professor Harry Mobley at University of Michigan. The pGNH404 plasmid expressing hlyCABD was kindly provided by Professor Agneta Richter-Dahlfors at Karolinska Institute. The bacteria were grown aerobically overnight in Luria broth (LB) (Lennox; Franklin Lakes, NJ, USA) at 37 °C on a shaker.

2.3. Stimulation of bladder epithelial cells

The bladder epithelial cells (wild-type, Cas9 controls, caspase-1 and NLRP3 knockdown cells and IL-1RA-deficient cells) were infected with CFT073, different CFT073 mutants or with MG1655 for 3 or 6 h at a multiplicity of infection (MOI) of 10. Supernatants, protein and mRNA were collected and stored at −80 °C until analysis.

2.4. Measurement of cytokine release and cell viability

Supernatants were collected after bacterial infection of epithelial cells and centrifuged at 5000g for 5 min and stored at −80 °C. Released IL-1β and IL-1RA from bladder epithelial cells were measured by an enzyme-linked immunosorbent assay (ELISA). The cytokines were measured using a human IL-1β kit (ELISA MAX™ Deluxe Sets, BioLegend, San Diego, CA, USA) and a human IL-1RA kit (Duo set, ELISA, R&D Systems, Minneapolis, USA) following the manufacturer's instructions. A lactate dehydrogenase (LDH) assay (Pierce LDH assay, Thermo fisher, Massachusetts, USA) was performed to measure cell viability according to the manufacturer's instructions. Both assays were analyzed in a spectrophotometer (Multiskan Ascent, Thermo Labsystems, Helsinki, Finland).

2.5. RNA isolation and real time RT-PCR

E.Z.N.A. Total RNA Kit I (Omega Bio-tek, GA, USA) was used for isolating RNA according to manufacturer's instructions. The RNA yield was measured using Nano-Drop ND-1000 (Wilmington, NC, USA). First-strand cDNA synthesis was performed using the High capacity cDNA RT kit (Thermo Fisher Scientific). For the real time-RT-PCR amplification, Maxima SYBR Green qPCR Master Mix (Thermofisher), 250 nM of each primer IL-1RA (Forward: 5′-ATGGAGGGAAGATGTGCCTGTC-3, Revers: 5′-GTCCTGCTTTCTGTTCTCGCTC-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward: 5′-GTCTCCTCTGACTTCAACAGCG-3, Revers: 5′-ACCACCCTGTTGCTGTAGCCAA-3′) (Eurofins MWG Synthesis GmbH, Ebersberg, Munich) and 10 ng cDNA was used. The amplification was performed in a CFX96 Touch™ Real-Time PCR Detection System (Biorad, Hercules, CA, USA) using the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s followed by 60 °C for 60 s. The mRNA expression was analyzed with the comparative Ct (ΔΔCt) method and normalized to GAPDH. Fold change was calculated as $2^{-\Delta\Delta Ct}$.

2.6. CRISPR/Cas9 gene editing

The pSpCas9 (BB)-2A-Puro (PX459, V2.0) (Addgene plasmid #62988) [29] was used for CRISPR/Cas9 gene editing in bladder epithelial cells. The target sites were: GCTAATGATCGACTTCAATG (NLRP3), GACAGTATTCCTAGAAGAAC (caspase-1), and GCTCTGTTC TTGGGAATCCA (IL-1RA). The epithelial cells were transfected with 1.5 μl of Lipofectamine 2000 and 500 ng plasmid in Opti-MEM (Thermo Fisher Scientific). The cells were selected using pyromycin (2.5 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) 24 h after transfection. A polyclonal pool of gene-edited cells was used for all experiments.

2.7. Western blot analysis

The epithelial cells were lysed in RIPA buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) was used for protein quantification. Equal amounts of Laemmli buffer and protein were mixed and boiled for 5 min at 95 °C. 10 μg of each sample was separated by 4–20% SDS-polyacrylamine gel electrophoresis and later transferred to a polyvinylidene fluoride membrane (Bio-Rad

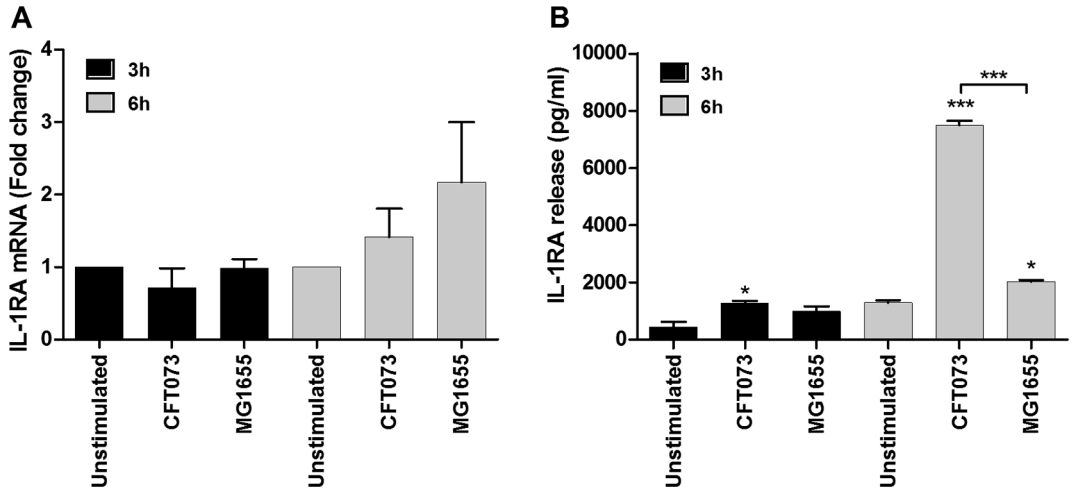

Fig. 1. IL-1RA mRNA expression and release. Bladder epithelial cells were stimulated with CFT073 and MG1655 at MOI 10 for 3 and 6 h followed by gene expression analysis of IL-1RA (A) and IL-1RA release (B). Data are shown as mean ± SEM (n = 3 independent experiments). Asterisks above the bars denote statistical significance compared to unstimulated controls ($^{*}p < 0.05$, $^{***}p < 0.001$).

Laboratories). The polyvinylidene fluoride membrane was blocked with 3% BSA for 1 h. A mouse monoclonal antibody (AdipoGen Life Sciences, Buckingham, UK) was used for detecting human caspase-1, a rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) was used for detecting human NLRP3, a mouse monoclonal antibody (Santa Cruz Biotechnology Inc, Heidelberg, Germany) was used for detecting human IL-1RA and a rabbit polyclonal antibody (Santa Cruz Biotechnology Inc) was used for detection human GAPDH, all overnight. A goat anti rabbit IgG (HRP) (Abcam, Cambridge, UK) and a goat anti mouse IgG (HRP) (Abcam) were used as secondary antibody and incubated for 1 h at room temperature. The blots were developed using Luminata Forte Western HRP Substrate (Merck Millipore, Darmstadt, Germany).

2.8. Colonization and invasion assay

Bladder epithelial cells (300,000 cells) were stimulated with CFT073 (3,000,000 CFU) carrying an eGFP-plasmid (enhanced green fluorescence protein, kindly provided by Professor Philip Poole) at MOI 10 and incubated at 37 °C for 4 h. In some experiments, the cells were pre-incubated with 500 ng/ml recombinant IL-1RA (R&D Systems) for 1 h prior to infection. The plate was then washed with PBS and the adhered/invaded (referred to as colonized) bacteria (eGFP) were imaged and quantified with the Cytation 3 plate reader (BioTek, Winooski, VT, USA). Colonization is reported as mean fluorescence intensity (MFI) after subtraction of background fluorescence. Intracellular invasion of UPEC was assessed by stimulating epithelial cells with CFT073 at MOI 10 for 2 h at 37 °C. The plate was washed after stimulation with PBS and the culturing medium was replaced with DMEM complemented with 2% FBS and 100 μg/ml gentamicin for an additional 2 h. The cells were thereafter washed and lysed with 0.1% Triton-X 100 in PBS. The intracellular UPEC bacteria were plated on TSA plates and grown overnight at 37 °C followed by CFU counting.

2.9. Targeted protein analysis

Bladder epithelial cells (Cas9 controls and IL-1RA deficient cells) were stimulated with CFT073 at MOI 10 for 6 h. Supernatants were collected after infection and centrifuged at 5000*g* for 5 min and stored at −80 °C. Cell supernatants were analyzed using the proximity extension assay (PEA) technology. Briefly, a pair of oligonucleotide-labeled antibodies are allowed to target proteins and when in close proximity, a PCR target sequence is formed, detected and quantified using standard real-time PCR. The Olink inflammation panel (Olink Bioscience AB, Uppsala, Sweden) is based on the PEA technology and enables analysis of 92 inflammation-related proteins (Table S1). The protein values are reported as normalized protein expression levels (NPX). Proteins with signals below the limit of detection (LOD) were excluded from further analysis.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2019.154772.

2.10. Statistical methods

All data are presented as mean values with standard error of the mean (SEM). Differences between individual groups were evaluated statistical by a one-way ANOVA followed by Bonferroni multiple testing correction. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. UPEC-evoked expression and release of IL-1RA and the involvement of different UPEC virulence factors

Bladder epithelial cells were infected with the UPEC strain CFT073 or the K-12 non-pathogenic *E. coli* strain MG1655, and the mRNA expression and release of IL-1RA were analysed. The UPEC strain CFT073 or MG1655 had no significant effects on the mRNA expression of IL-1RA after 3 or 6 h of stimulation (Fig. 1A). However, CFT073 induced a 3-fold and 6-fold increase in IL-1RA release from the bladder cells compared to uninfected cells after 3 and 6 h, respectively (Fig. 1B). MG1655 evoked a small, but significant, increase in IL-1RA release after 6 h (Fig. 1B). These findings show that the UPEC strain CFT073 induces a more pronounced release of IL-1RA from bladder epithelial cells than the non-pathogenic MG1655 strain.

Experiments were performed to investigate the involvement of some established UPEC virulence factors on IL-1RA secretion using α-hemolysin (Δ*hlyA*), P fimbriae (Δ*pap*) and type 1 fimbriae (Δ*fimH*) CFT073 deletion mutants. These experiments showed that the CFT073Δ*hlyA* failed to induce IL-1RA release from bladder epithelial cells (Fig. 2). The ability to stimulate IL-IRA was, however, restored in a α-hemolysin complemented CFT073Δ*hlyA*/pGNH404 strain (Fig. 2). The release of IL-1RA evoked by the Δ*pap* and Δ*fimH* deletion mutants did not differ from the release evoked by the wild-type CFT073 (Fig. 2). These data suggest that α-hemolysin is a crucial virulence factor for UPEC-evoked release of IL-1RA from bladder epithelial cells.

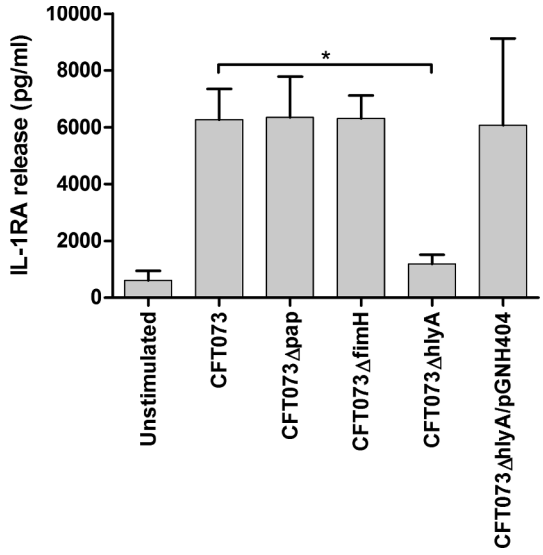

Fig. 2. UPEC virulence factors and IL-1RA release. Bladder epithelial cells were infected with wild-type CFT073, CFT073Δ*pap*, CFT073Δ*fimH*, CFT073Δ*hlyA* and CFT073ΔhlyA/pGNH404 (hemolysin complemented) strain at MOI 10 for 6 h followed by analysis of IL-1RA release. Data are shown as mean ± SEM (n = 3 independent experiments). Asterisks show statistical significance ($^{*}p < 0.05$).

3.2. The involvement of inflammasome-associated proteins in IL-1RA release induced by UPEC

We next investigated if inflammasome-associated proteins are involved in UPEC-evoked release of IL-1RA. Caspase-1 and NLRP3-knockdown bladder epithelial cell lines were created using the CRISPR/Cas9 system. Decreased protein expression of caspase-1 (72%) and NLRP3 (> 90%) compared to bladder epithelial cells transfected with the control Cas9 plasmid was confirmed by western blot (Fig. 3A). UPEC-stimulated Cas9 control cells showed a significant increase in IL-1RA release, in line with the findings in UPEC-stimulated wild-type cells (Fig. 3B). The CFT073-induced release of IL-1RA was significantly higher ($p < 0.001$) in caspase-1 knockdown cells than in Cas9 controls (Fig. 3B). In contrast, the CFT073-induced release of IL-1RA from NLRP3 knockdown cells was negligibly and significantly lower ($p < 0.001$) than Cas9 controls (Fig. 3B). These data show that inflammasome-associated proteins have pronounced effects on the release of IL-1RA.

3.3. Colonization and invasion of IL-1RA deficient bladder epithelial cells

To investigate the involvement of IL-1RA on UPEC colonization and invasion of bladder epithelial cells we created an IL-1RA deficient cell line using the CRISPR/Cas9 system

(Fig. 4A). The IL-1RA deficient cells did not release IL-1RA at basal level or following stimulation with the UPEC strain CFT073 (Fig. 4B), confirming that the knockdown had been successful. Basal or CFT073-induced release of IL-1β was not significantly changed in IL-1RA deficient cells (Fig. 4C). However, the ratio of IL-1β/IL-1RA release was significantly increased in IL-1RA-deficent cells compared to Cas9 controls (Fig. 4D), suggesting that more IL-1β may be accessible for activation of the IL-1 receptor. The cell viability (LDH release) of CFT073-infected IL-1RA-deficient cells did not differ compared to the viability of infected Cas9 control cells (Fig. 4E).

Bacterial colonization of IL-1RA deficient bladder epithelial cells was imaged using GFP-labelled CFT073 (Fig. 5A) and quantified by measurement of mean fluorescence intensity (MFI) (Fig. 5B). In this model, bacterial colonization reflects a combination of attached extracellular bacteria and intracellular bacteria. The bacterial colonization of IL-1RA deficient cells was significantly reduced compared to wild-type cells (Fig. 5B). To specifically assess whether IL-1RA deficiency affects the ability of CFT073 to invade bladder epithelial cells, the number of intracellular bacteria was quantified after lysis of cells. The bacterial invasion of IL-1RA deficient cells was significantly reduced compared to the invasion of wild-type cells (Fig. 5C). We next investigated whether administration of recombinant IL-1RA would be able to restore the reduced colonization and invasion noted in IL-1RA deficient cells. However, pre-incubating the IL-1RA deficient cells with recombinant IL-1RA did not restore bacterial colonization or invasion (Fig. 5B and C). Taken together, these data show that IL-1RA deficient cells are less prone to be colonized and invaded by UPEC.

3.4. Differentially regulated proteins in IL-1RA deficient bladder epithelial cells

To gain more knowledge on co-regulation of IL-1RA with other host response factors, we performed a targeted protein analysis of inflammation-related proteins (a total of 92 proteins, Table S1) in IL-1RA deficient cells. The basal expression of 23 proteins was identified as being significantly altered in IL-1RA deficient cells. Eight proteins showed an increased basal expression compared to Cas9 control cells and 15 showed a decreased expression (Fig. 6).

The expression of 32 proteins was significantly altered following stimulation with the UPEC strains CFT073; 25 proteins showed an increased expression (Fig. 7A) and 7 proteins showed a decreased

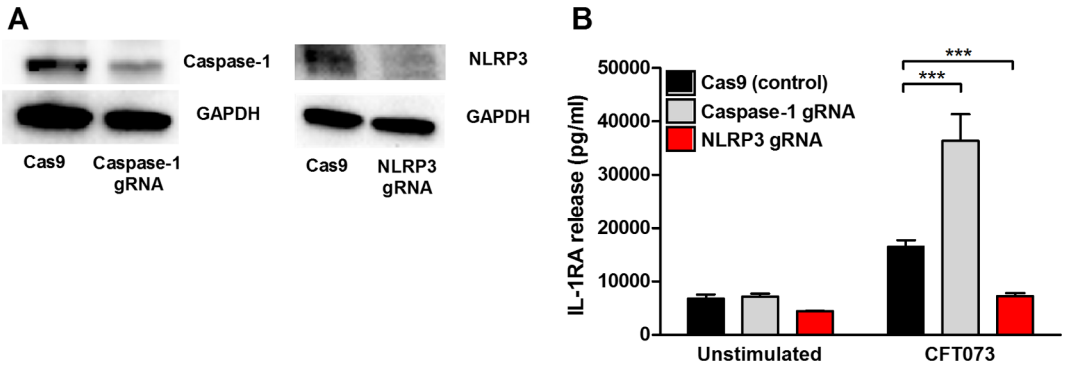

Fig. 3. Inflammasome-associated proteins and IL-1RA release. Western blot analysis showing knockdown of caspase-1 and of NLRP3 in bladder epithelial cells using the CRISPR/Cas9 system. GAPDH was used as a loading control (A). Release of IL-1RA from Cas9 controls and caspase-1 and NLRP3-deficient bladder epithelial cells after stimulation with CFT073 at MOI 10 for 6 h (B). Data is shown as mean ± SEM (n = 3 independent experiments). Asterisks show statistical significance ($^{***}p < 0.001$).

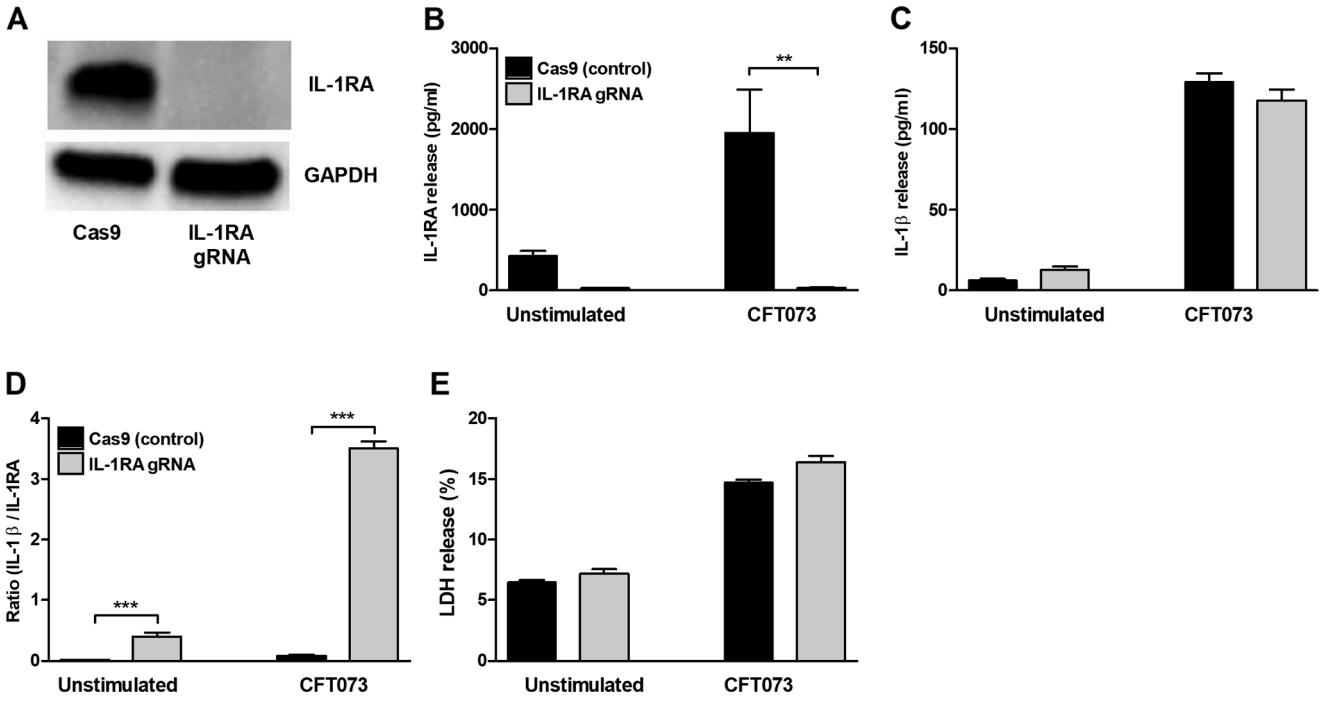

Fig. 4. Characterization of IL-1RA-deficient bladder epithelial cells. Western blot analysis showing knockout of IL-1RA in bladder epithelial cells using the CRISPR/Cas9 system (A). IL-1RA-deficient bladder epithelial cells and Cas9 controls were infected with UPEC strain CFT073 for 6 h followed by analysis of IL-1RA release (B), IL-1 β release (C), the ratio of IL-1β/IL-1RA release (D) and cell viability (LDH release) (E). Cell viability is presented as % of total LDH. Data are shown as mean ± SEM (n = 3 independent experiments. Asterisks show statistical significance ($^{**}p < 0.01$, $^{***}p < 0.001$).

expression (Fig. 7B) compared to unstimulated cells. A total of 10 proteins (CDCP-1, IL-18, IL-18R1, CXCL10, 4E-BP1, STAMPB, ADA, SIRT2, CCL20, MMP-10) demonstrated a significantly different expression in UPEC-stimulated IL-1RA deficient cells compared to UPEC-stimulated Cas9 controls. All of these proteins, except IL-18R1, showed a lower expression in IL-1RA deficient cells.

4. Discussion

Although typically secreted by monocytes and neutrophils, IL-1RA could also be released from epithelial cells and may represent a mechanism by which the inflammatory effects of IL-1β are locally modulated. In the present study we found that CFT073 induced IL-1RA release from human bladder epithelial cells and that α-hemolysin was a crucial virulence factor for the IL-1RA release. The pore-forming α-hemolysin provides UPEC with the capacity to cause cell damage and α-hemolysin plays a significant role in UTI pathogenesis [30]. Commensal *E. coli* usually lack α-hemolysin encoding genes and our study showed accordingly that MG1655 was a weak inducer of IL-1RA release from bladder epithelial cells. UPEC α-hemolysin has been shown to be essential for activation of caspase-1/caspase-4-dependent cell death and IL-1β release in human bladder epithelial cell [31]. We have confirmed these results and also presented data showing that activation of caspase-1 and release of IL-1β in human bladder epithelial cells are not dependent on the UPEC virulence factors FimH and PapG [32]. Results from the present study show that IL-1RA release is unaffected by deletion of *fimH* and *papG* in CFT073, suggesting that type 1 or P fimbriae are not necessary for triggering UPEC-evoked IL-1RA release in bladder epithelial cells. Thus, these results show that the release of IL-1RA in bladder epithelial cells is triggered by the same UPEC virulence factors that trigger IL-1β release. This is an expected finding since expression of IL-1RA is known to be induced by signals that also trigger IL-1β production, and also by IL-1β itself [33].

The inflammasome-associated proteins caspase-1 and NLRP3 have recently been emphasised by several researchers to be associated with IL-1β production, pyroptosis and regulation of the host response during UTI [12,13,16,31,32]. The inflammasome is responsible for the maturation and release of IL-1β, while IL-1RA appears to be processed in an inflammasome/caspase-1-independent pathway [34]. The release of IL-1β involves a caspase-1 mediated activation of the pore-forming substrate gasdermin D [35] that initiates pyroptosis, while IL-1RA contains a secretion sequence at the N-terminus for effective secretion [17]. UPEC infection of NLRP3 knockdown bladder epithelial cells demonstrated a decrease in IL-1RA release compared to Cas9-infected cells. This suggests that the release of IL-1RA is induced in an NLRP3-dependent manner in bladder epithelial cells. It has previously been shown that NLRC4, but not the NLRP3 inflammasome, contributes to the production and release of IL-1RA in mice [36]. It has also been shown that NLRP3 activity can be negatively regulated by IL-1RA during mucosal infections caused by *Candida albicans* [37]. However, the precise mechanism by which NLRP3 activity influences the release of IL-1RA in bladder epithelial cells remains to be determined. The finding that the release of IL-1RA was higher in caspase-1 knockdown bladder cells suggests a possible suppressive role of caspase-1 on IL-1RA release. In agreement with our findings, caspase-1 knockout mice were found to exhibit significantly increased mRNA and protein levels for IL-1RA after *Pseudomonas aeruginosa* corneal infection [38]. Furthermore, the release of IL-1β is markedly reduced in both NLRP3 and caspase-1 knockdown cells [32]. It is therefore unlikely that the lack of IL-1β release *per se* in these cells explain the altered release of IL-1RA. Taken together, we found that the inflammasome-associated proteins NLRP3 and caspase-1 regulate UPEC-induced IL-1RA release from bladder epithelial cells in the opposite way.

The ability of UPEC to colonize and persist in the urinary tract depend on a complex interplay between the host responses and the bacteria. The contribution of host production of IL-1RA for UPEC colonization and intracellular invasion of bladder epithelial cells has not previously been investigated. In order to study the involvement of IL-1RA on UPEC colonization and invasion of bladder epithelial cells we created an IL-1RA-deficient cell line. IL-1RA exists as a secreted soluble

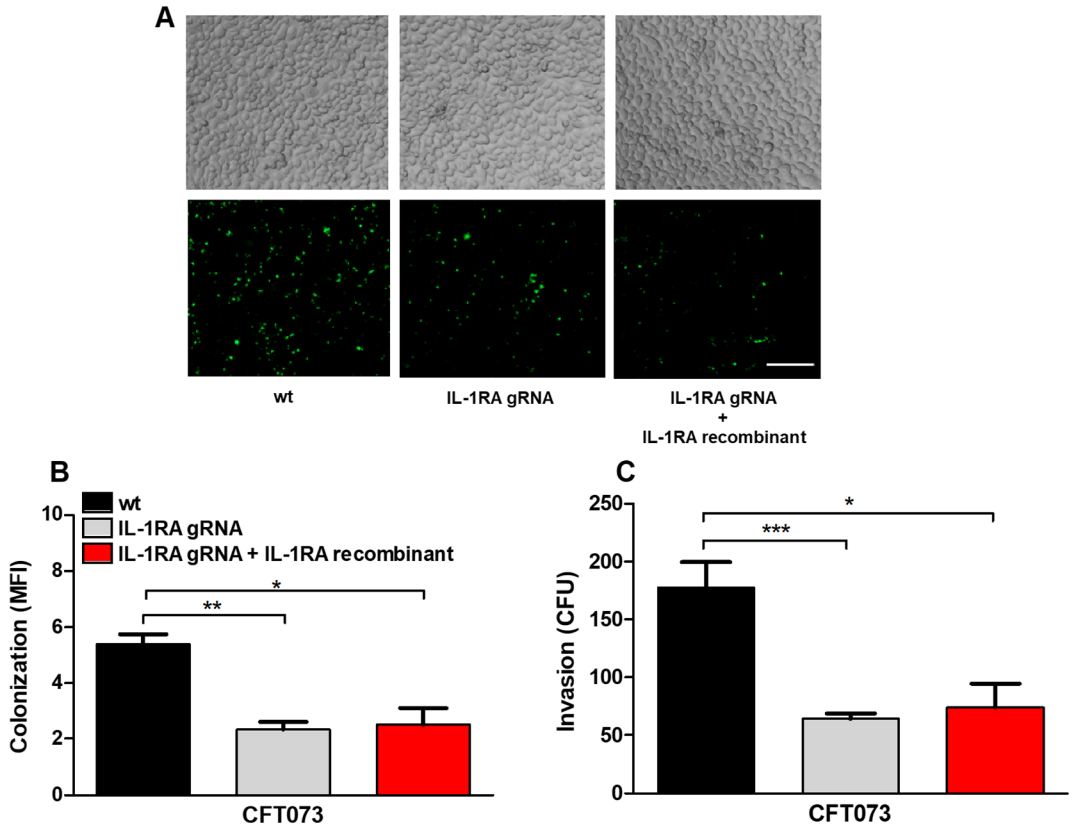

Fig. 5. Bacterial colonisation and invasion of IL-1RA deficient bladder epithelial cells. IL-1RA-deficient bladder epithelial cells and wild-type cells were infected with UPEC strain CFT073 and bacterial colonization and invasion were evaluated. In some experiments, IL-1RA-deficient cells were pre-incubated with 500 ng/ml recombinant IL-1RA for 1 h prior to infection. Bacterial colonization with CFT073 (harbouring a GFP-expressing plasmid) was imaged (A) and the colonization quantified as mean fluorescence intensity (MFI) (B). Bacterial invasion was evaluated using the gentamycin protection assay and the invasion quantified as the number of intracellular bacteria (CFU) (C). Representative images of colonized GFP-expressing bacteria, from 3 independent experiments, are shown in panel A with their representative phase-contrast-image. Scale bar represents 125 μm. Data are shown as mean ± SEM (n = 3 independent experiments). Asterisks show statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

IL-1RA isoform and three intracellular (icIL-1RA) isoforms that are not known to exit the cells, at least not under basal conditions [18]. There are data proposing an intracellular role of icIL-1RA in regulating cell signalling, and some cell types appear to possess the mechanisms required to induce extracellular release of icIL-1RA [39]. It is unclear if the Crisp/Cas9 gene editing in our study affects the intracellular forms of IL-1RA, but we demonstrated that the release of IL-1RA from IL-1RA-deficient cells was strongly diminished relative to Cas9 controls. Release of IL-1β was unchanged in IL-1RA-deficent cells, but the ratio of IL-1β/IL-1RA was markedly increased both at basal level and following UPEC-infection, suggesting that IL-1β receptor activation may be exaggerated in IL-1RA-deficent bladder epithelial cells.

Intracellular UPEC undergo a defined differentiation program involving replication and aggregation into biofilm-like communities, and following dispersal of the biofilm the bacteria will eventually efflux from their intracellular niche into the bladder lumen to colonize and invade neighbouring bladder epithelial cells [2,5]. It has been speculated that intracellular UPEC may efflux from their cytosolic niche into the bladder lumen by inflammasome-associated pyroptosis [31]. Thus, inflammasome activation may be a possible regulator for release and elimination of intracellular bacterial niches. Our study showed that UPEC strain CFT073 was less able to adhere to and invade IL-1RA-deficient bladder epithelial cells compared to control cells. This suggests that IL-1RA may counteract mechanisms that protect against bacterial colonization. Part of the effects observed in IL-1RA deficient cells may represent IL-1β-mediated effects, i.e., effects that are normally reduced by IL-1RA. However, pre-incubating the IL-1RA-deficient cells with recombinant IL-1RA did not restore UPECs ability to adhere or invade. This suggests that the protective effect on colonization and invasion are

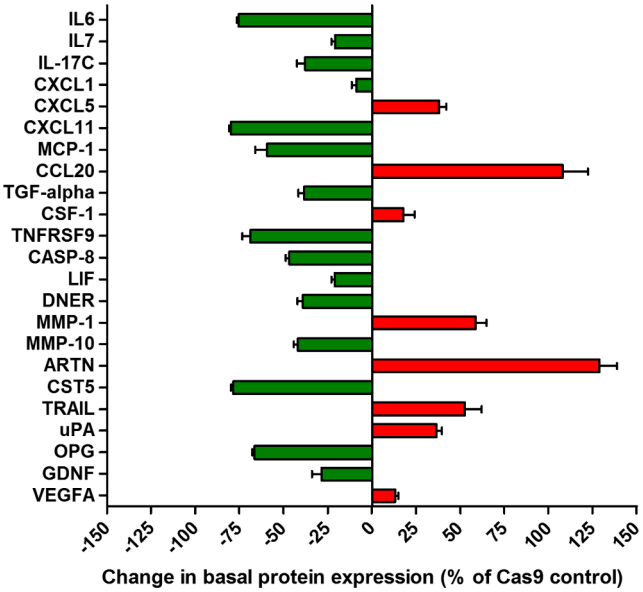

Fig. 6. Targeted protein analysis of IL-1RA deficient cells, basal expression. Analysis of the basal protein expression in IL-1RA-deficient bladder epithelial cells and Cas9 control cells using a panel of inflammation-related proteins. The proteins where the basal expression in IL-1RA deficient cells was either significantly increased (red bars) or decreased (green bars) are shown. The data are presented as the percentage change in basal protein expression in IL-1RA deficient cells compared to the basal expression in Cas9 controls. Data are shown as mean ± SEM (n = 3 independent experiments). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

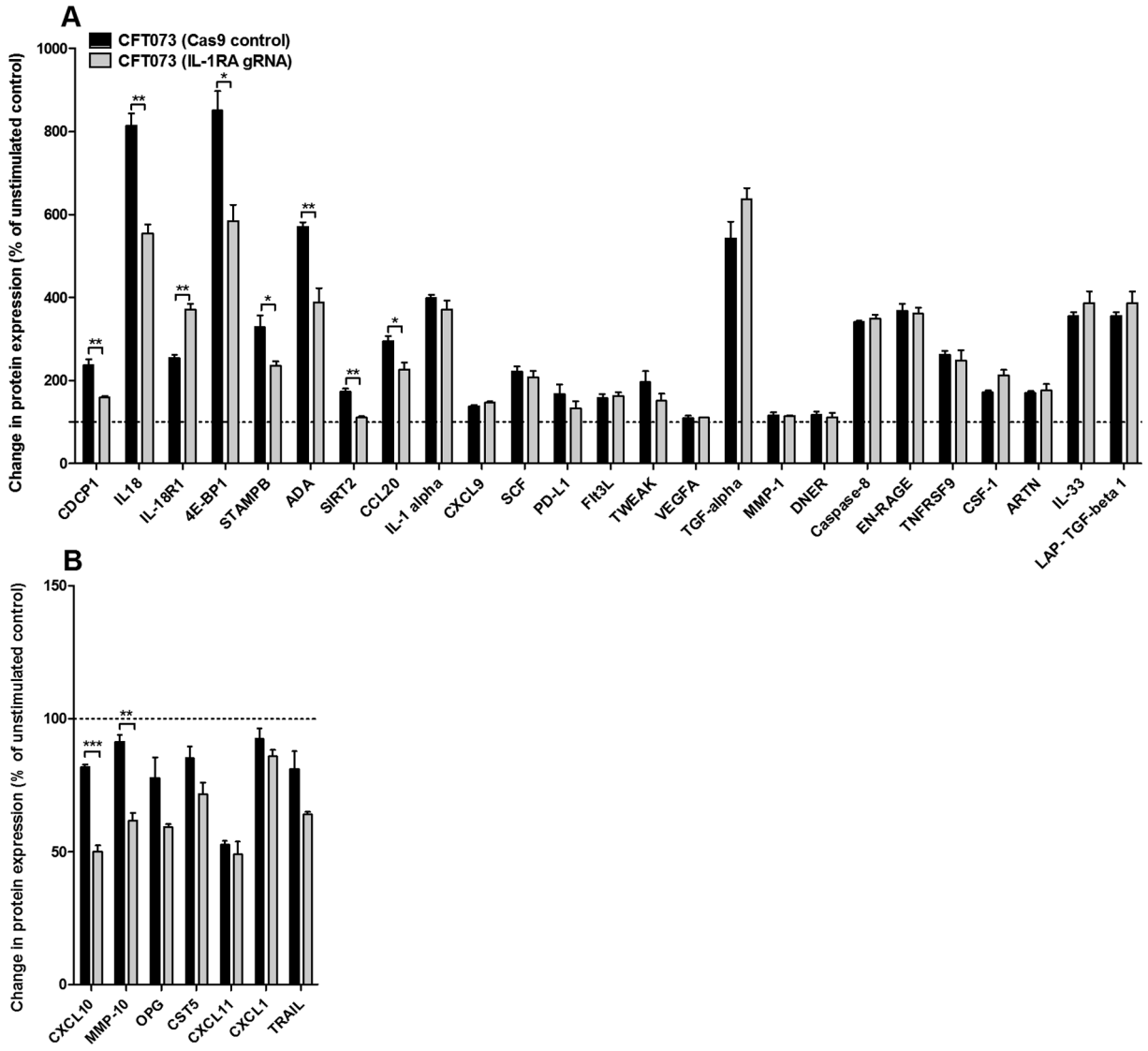

Fig. 7. Targeted protein analysis of IL-1RA-deficient cells, UPEC-induced expression. IL-1RA-deficient bladder epithelial cells and Cas9 controls were infected with UPEC strain CFT073 followed by analysis of protein expression using a panel of inflammation-related proteins. All proteins that responded to stimulation with the UPEC strain CFT073 with either a significant increase (A) or decrease (B) in protein expression are shown and compared in IL-1RA-deficient cells and Cas9 control cells. The data in panel A and B are presented as the percentage change in CFT073-induced protein expression compared to the expression in unstimulated cells (dotted line). Data are shown as mean ± SEM (n = 3 independent experiments). Asterisks show statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

unlikely to primarily be explained by excessive IL-1β receptor stimulation. Furthermore, it is also unlikely that pyroptosis could explain the reduced colonization and invasion of IL-1RA-deficient cells, since the cell death (LDH release) did not differ between IL-1RA-deficent cells and control cells. Hence, we hypothesize that the reduced adhesion/invasion may involve the intracellular isoforms of IL-1RA, which are known to be involved in cell signalling [39]. icIL-1RA deficiency may result in interference with several cellular signalling cascades known to regulate adhesion, invasion and expelling (e.g., focal adhesion kinases, Rho family GTPases and actin rearrangement [7,40]), which subsequently may reduce bacterial adhesion and invasion.

The activation of inflammation-related proteins in IL-1RA-deficient cells was analysed using a multiplex assay including 92 key proteins. Results obtained from the protein analysis disclosed co-regulation of IL-1RA with several inflammatory proteins in bladder epithelial cells. Both the basal and UPEC-induced expression of inflammatory proteins (23 and 10, respectively) was significantly altered in IL-1RA deficient bladder epithelial cells. Several of the differentially regulated proteins in UPEC-infected cells were cytokines or chemokines (IL-18, CXCL10, CCL20), of which IL-18 is processed together with IL-1β following activation of the inflammasome [11]. Unfortunately, IL-1β was not included in the immunoassay panel. The expression of IL-18 was lower in IL-1RA deficient cells while the expression of the IL-18 receptor was higher, consistent with the common pharmacodynamic phenomenon that the receptor population usually upregulates as an adaption to lower availability of agonists. Metallopeptidase-10 (MMP-10), CUB-domain-containing protein 1 (CDCP1), adenosine deaminase (ADA), eukaryotic translation initiation 4E-binding protein 1 (4E-BP1), STAM-binding protein (STAMBP) and SIR2-like protein 2 (SIRT2) were other proteins that demonstrated a lower expression in UPEC-stimulated IL-1RA deficient cells, but these proteins currently lack a known association to the inflammatory response in UTI. Thus, besides the well-known inhibitory effect on IL-1β-mediated receptor signalling, IL-1RA appears to affect the expression of several inflammation-related proteins in bladder epithelial cells. The underlying mechanisms are currently unknown but one hypothesis is that the altered expression of the identified inflammatory proteins is an indirect, secondary phenomena i.e., a result of the unopposed and exaggerated action of IL-1β. However, it has been

proposed that IL-1RA itself may control the expression of matrix metalloproteinase-13 in periodontal tissue, without interference with the IL-1β signalling cascade [41] and a direct regulatory effect by the different isoforms of IL-1RA cannot be excluded. Taken together, the regulatory role of IL-1RA on the inflammatory response in bladder epithelial cells appears to be broad with consequences more far-reaching than inhibition of IL-1β-mediated signalling.

5. Conclusion

Our results showed that IL-1RA is part of the inflammasome-regulated immune response in bladder epithelial cells after UPEC infection and that IL-1RA influences the expression of many inflammatory proteins. IL-1RA deficiency impaired UPEC colonization and invasion, suggesting a role for IL-1RA in controlling intracellular invasion and bacterial persistence. The contribution and impact of IL-1RA release from bladder epithelial cells need to be taken into consideration when evaluating the normal and dysregulated mucosal immune responses during UPEC infection.

Acknowledgement

The authors acknowledge support from the Research Committee of Örebro County Council, the Faculty of Medicine and Health at Örebro University, Capio Research Foundation and the Swedish Society of Medicine.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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