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Kingella kingae cytotoxin RtxA induces an innate immune response in oral epithelial cells

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ABSTRACT

The genus *Kingella* comprises four common species: the commensals *K. oralis* and *K. denitrificans*, the emerging paediatric pathogen *K. kingae* and the novel species *K. negevensis*. Improved diagnostic methods have led to the recognition of *K. kingae* as a major cause of septic arthritis in young children. The key virulence factor responsible for the pathogenesis of *K. kingae* is its cytotoxin RtxA, which is thought to facilitate host invasion. After binding to target cells, RtxA inserts into the host cell membrane and forms ion-conducting membrane pores that disrupt normal cell physiology and ultimately lead to cell death. In this study, we analysed the pro-inflammatory response of oral epithelial cells to a clinical isolate of *K. kingae*, its isogenic RtxA-deficient mutant and the commensals *K. oralis* and *K. denitrificans*, which do not produce RtxA. The results show that infection of cells with *K. kingae*, but not with the RtxA-deficient mutant and the commensal species, leads to increased expression and secretion of certain pro-inflammatory cytokines and chemokines. Furthermore, the RtxA-producing *K. kingae*, but not the RtxA-deficient mutant, upregulated the expression of *DEFB4A* and *SLPI* genes encoding antimicrobial peptides. These findings demonstrate that the RtxA toxin induces an innate immune response in oral epithelial cells.

1. Introduction

The Gram-negative genus *Kingella*, belonging to the *Neisseriaceae* family, consists of four main species: *K. kingae* (Henriksen and Bovre, 1976), *K. denitrificans* (Snell and Lapage, 1976), *K. oralis* (Dewhirst et al., 1993) and the novel *K. negevensis* (El Houmami et al., 2017). Three other species assigned to this genus, *K. potus* (Lawson et al., 2005), *K. bonacorsii* (Antezack et al., 2021) and *K. pumchi* (Xiao et al., 2023), have so far been isolated only once. The four main *Kingella* species, which appear in pairs or short chains, have a similar morphology and resemble each other with only small metabolic differences. Although all these four organisms have type 4 pili, which allow twitching motility, they are considered non-motile due to the lack of flagella. They also have a *Kingella* NhhA homolog (Knh) trimeric autotransporter (Morreale et al., 2023). The presence of the type 4 pili and the Knh protein, which are both used to anchor bacteria to cells, indicates that all these organisms are equipped to colonise epithelial surfaces. Indeed, all the four main members of the genus *Kingella* were first isolated from the nasal or oral cavity, and today, it is generally accepted that they are a part of the oral microbiome at some point in life (Dewhirst et al., 1993, 2010; Chen, 1996; Yagupsky, 2015; El Houmami et al., 2017). Although *K. oralis* is associated with periodontitis and a few case studies have identified *K. denitrificans* as a rare cause of infections, they are considered to be non-pathogenic commensal organisms (Swann and Holmes, 1984; Khan et al., 1986; Brown et al., 1987; Chen, 1996). On the other hand, *K. kingae* has recently been recognised as an emerging facultative pathogen and a leading cause of septic arthritis and osteomyelitis in children between 6 and 36 months of age (Williams et al., 2014; Hernandez-Ruperez et al., 2018; Khattak et al., 2021). Other infections caused by *K. kingae* include bacteraemia, endocarditis, pneumonia, meningitis, spondylodiscitis and soft tissue infections (Walterspiel, 1983; Yagupsky et al., 1993; Reekmans et al., 2000; Rolle et al., 2001; Syridou et al., 2020; Samara et al., 2022).

The pathogenicity of *K. kingae* is attributed to its ability to produce a repeats-in-toxin (RTX) cytotoxin called RtxA (Kehl-Fie and St Geme, 2007). Like other RTX toxins, RtxA is a multidomain protein that

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contains several transmembrane α-helices in the N-terminal hydrophobic domain that are responsible for its pore-forming activity (Ruzickova et al., 2024). RtxA is produced as an inactive protoxin (proRtxA) that is activated by fatty acid chains bound to specific lysine residues in the acylated segment (Osickova et al., 2018; Khaliq et al., 2024). The C-terminal part of the molecule consists of an RTX domain, known for the presence of nonapeptide repeat sequences, rich in glycine and aspartate residues, that are important for binding of calcium ions, and a C-terminal secretion signal recognised by the type 1 secretion system (Linhartova et al., 2010; Filipi et al., 2022). After secretion, RtxA binds to host cells via glycosylated cell surface structures such as glycoproteins and gangliosides, inserts into the cell membrane, and forms ion-conducting membrane pores that disrupt normal cell physiology and ultimately lead to cell death (Osickova et al., 2018; Rahman et al., 2020, 2023; Ruzickova et al., 2024). Since genes encoding the inactive RtxA toxin and proteins for its activation and secretion were found in the genomes of all *K. kingae* clinical isolates causing invasive infections, it was hypothesised that it could be a key virulence factor of *K. kingae* (Kehl-Fie and St Geme, 2007; Cherkaoui et al., 2009; Lehours et al., 2011). Indeed, this was later confirmed by observing histopathological changes in an infant rat model infected with a *K. kingae* septic arthritis isolate PYKK081 and its isogenic RtxA-deficient mutant variant KKNB100 (Chang et al., 2014).

K. kingae colonises the oropharynx of young children and is considered as part of the oral microbiota. Longitudinal studies showed that *K. kingae* was not isolated from children under 6 months of age. The colonisation rate increased in 12-month-old children and lasted until 24 months of age, after which it began to decrease until 48 months of age. Children older than 48 months carry *K. kingae* rarely. The age-dependent colonisation rate is often explained by the presence of vertically transmitted immunity in children under 6 months of age and the development of an immune response to polysaccharides after the second year of life (Yagupsky et al., 1993, 2011; Anderson de la Llana et al., 2015; Yagupsky, 2015; Basmaci et al., 2019). Conversely, the colonisation rate of *K. oralis* and *K. denitrificans* was not found to be age-specific, and these organisms were isolated primarily from adults (Hollis et al., 1972; Snell and Lapage, 1976; Dewhirst et al., 1993, 2010; Chen, 1996). Typically, the first contact of bacteria with the host occurs on epithelial surfaces that serve as a protective barrier and provide the first line of defence against pathogens. In order to breach the epithelial surface and invade the host, pathogens must either modulate the epithelial immune response or develop mechanisms to escape the response. Some RTX toxins have been shown to affect the host epithelial immune system by suppressing the immune response and allowing the pathogens to evade the immune defences, but they have also been shown to enhance the immune reaction. For example, the *Bordetella pertussis* adenylate cyclase toxin (CyaA) inhibits the interleukin (IL)-17A-induced secretion of IL-8 and human β-defensin-2 (hBD-2), thus compromising the airway immune response, but also enhances the secretion of IL-6 (Hasan et al., 2018). The mechanism by which *K. kingae* compromises the oral epithelial barrier and modulates the airway immune response remains unknown, but is thought to involve the cell-permeabilising activity of the RtxA toxin.

In this study, we investigated the effect of RtxA on the innate immune response of oral epithelial cells. Using quantitative PCR and flow cytometry, we compared the levels of cytokines, chemokines and antimicrobial peptides (AMPs) produced by cells after infection with the facultative pathogen *K. kingae* and its isogenic RtxA-deficient variant, as well as with the commensal species *K. oralis* and *K. denitrificans*, which do not produce RtxA.

2. Materials and methods

2.1. Bacterial strains and growth conditions

K. kingae clinical isolate PYKK081 (Kaplan et al., 2012), *K. denitrificans* CCUG12510 and *K. oralis* CCUG30450 (Czech Collection of Microorganisms, Brno, Czech Republic) were grown on solid Columbia agar supplemented with 5 % sheep blood (CBA). *K. kingae* strain KKNB100 (Chang et al., 2014) was grown on solid CBA supplemented with 40 μg/ml of kanamycin. The bacteria were grown in a 5 % CO_2 atmosphere at 37 °C for 48 h, then restreaked and grown for another 24 h. All bacterial strains were stored at −80 °C in brain heart infusion (BHI) broth containing 20 % glycerol. PYKK081 and KKNB100 were a kind gift from N. Balashova, University of Pennsylvania, PA.

2.2. Human oral epithelial cells

Human HSC-3 oral epithelial cells were isolated from human oral squamous carcinoma (Momose et al., 1989) and purchased from the JCRB Cell Bank (Tokyo, Japan). HSC-3 cells were grown in Minimum Essential Medium Eagle (MEME; Sigma-Aldrich, St. Louis, MO) supplemented with 10 % fetal calf serum (FCS; GIBCO Invitrogen, Grand Island, NY) and an antibiotic-antimycotic solution (0.1 mg/ml streptomycin, 1000 U/ml penicillin and 0.25 mg/ml amphotericin; Sigma-Aldrich, St. Louis, MO) in a 5 % CO_2 atmosphere at 37 °C.

2.3. Infection of HSC-3 cells

Grown HSC-3 cells were harvested and seeded at 1.5×10^6 cells/6 cm tissue culture plate in MEME supplemented with 10 % FCS and cultured for 16 h. To set the multiplicity of infection (MOI), the number of HSC-3 cells was counted before each infection experiment and was repeatedly found to be approximately 2.3×10^6. Bacterial cells were taken from solid CBA and resuspended in MEME supplemented with 10 % FCS to an OD_{600} of 1, which corresponded to 2.5×10^8 cells/ml for *K. kingae* and 8×10^8 cells/ml for *K. oralis* and *K. denitrificans*. Growth medium from HSC-3 cell cultures was discarded and replaced with bacterial suspensions to achieve the desired MOI. In some experiments, gentamicin was added to the culture medium at a final concentration of 100 μg/ml to kill the bacteria. Cell culture media were collected at indicated times post-infection to determine cell viability and/or to quantify cytokines and chemokines. The cells were lysed by lysis buffer included in a Monarch Total RNA MiniPrep Kit (New England Biolabs, Ipswich, MA) and total RNA was isolated from the lysed cells.

2.4. Cell viability assay

Cell viability was measured by release of lactate dehydrogenase (LDH) using a CyQUANT LDH Cytotoxicity Assay (Invitrogen, Grand Island, NY) as instructed by the manufacturer.

2.5. RNA isolation and quantitative real-time PCR

Total RNA was isolated from HSC-3 cells infected with PYKK081, KKNB100 and uninfected control cells using a Monarch Total RNA MiniPrep Kit (New England Biolabs, Ipswich, MA). Traces of contaminating DNA were removed using a TURBO DNA-free Kit (Thermo Fisher Scientific, Waltham, MA) and total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The isolated RNA was reverse-transcribed into cDNA using a LunaScript RT SuperMix (New England Biolabs, Ipswich, MA). The cDNA was quantified using a Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA) on a CFX Opus Real-Time PCR System (Bio-Rad, Hercules, CA). All kits were used according to the manufacturer's instructions. The gene for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference in all quantitative real-time polymerase chain reaction (qRT-PCR) experiments. All primers used in the qRT-PCR reactions were purchased from Merck (KiCqStar Primers, Merck, Darmstadt, Germany) and used at a final concentration of 500 nM. All primer pairs yielded a single PCR product, as indicated by melting curves. The cycling conditions were as follows: (i) initial denaturation at 95 °C for 1 min, followed

by 45 cycles of (ii) denaturation at 95 °C for 15 s and (iii) annealing/extension at 60 °C for 30 s. The $2^{(-\Delta\Delta CT)}$ method was used to calculate the fold difference in gene expression in HSC-3 cells infected with PYKK081 or KKNB100 compared to that in uninfected control cells (Livak and Schmittgen, 2001).

2.6. Cytometric bead arrays

Bead arrays were used to measure the concentrations of cytokines and chemokines released into the cell culture media of HSC-3 cells infected with *K. kingae* PYKK081, *K. kingae* KKNB100, *K. oralis, K. denitrificans* and uninfected control cells. The concentrations of released cytokines were measured using a Human Inflammatory Cytokine Cytometric Bead Array (BD Biosciences, San Diego, CA) and the concentrations of released chemokines were measured using a Human Chemokine Cytometric Bead Array (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Data were analysed using FlowJo software (Tree Star, Ashland, OR) and appropriate gatings were used to select individual populations. The concentrations of individual cytokines and chemokines from the cytometric data were calculated using GraphPad Prism 10.2.2 (GraphPad Software, La Jolla, CA).

2.7. Statistical analysis

All results were presented as the arithmetic mean with standard error of the mean. Statistical significance was calculated by two-way ANOVA followed by Dunnett's or Sidak's post-test using GraphPad Prism 10.2.2 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Infection of HSC-3 oral epithelial cells with Kingella species

Kingella spp. colonise human oropharyngeal epithelial cells, which act as an interface between the host organism and the environment and are therefore the first to respond to bacterial colonisation by producing cytokines, chemokines and other effector molecules. To investigate whether *K. kingae* and its cytotoxin RtxA elicit an innate immune response in epithelial cells, we first determined the MOI at which the bacterium secretes RtxA in sufficient quantity to observe its pore-forming activity in target cells, while ensuring that the toxin causes only limited cell lysis. As target cells, we used the human HSC-3 oral epithelial cells, which are frequently used in studies of various oral disease processes, including bacteria-host cell interactions (Momose et al., 1989). The pore-forming activity of RtxA secreted by *K. kingae* was determined by the extent of cell lysis monitored by the amount of LDH released into the medium upon plasma membrane damage using the CyQUANT LDH Cytotoxicity Assay.

HSC-3 cells were infected with either the *K. kingae* clinical isolate PYKK081 (Kaplan et al., 2012) or its isogenic RtxA-deficient strain KKNB100 (Chang et al., 2014). In addition, the cells were also infected with the commensals *K. oralis* or *K. denitrificans*, which lack RtxA and two other virulence factors, a polysaccharide capsule and a lipopolysaccharide-associated exopolysaccharide (Morreale et al., 2023). HSC-3 cells were infected with a range of MOIs (5, 10, 25 and 50) of PYKK081, as this strain is the only one of the four used in this study that causes RtxA-dependent cell lysis. As a control, MOI 50 was used to infect HSC-3 cells with the RtxA non-producing strains (KKNB100, *K. oralis* and *K. denitrificans*). As shown in Fig. 1A, even at the lowest

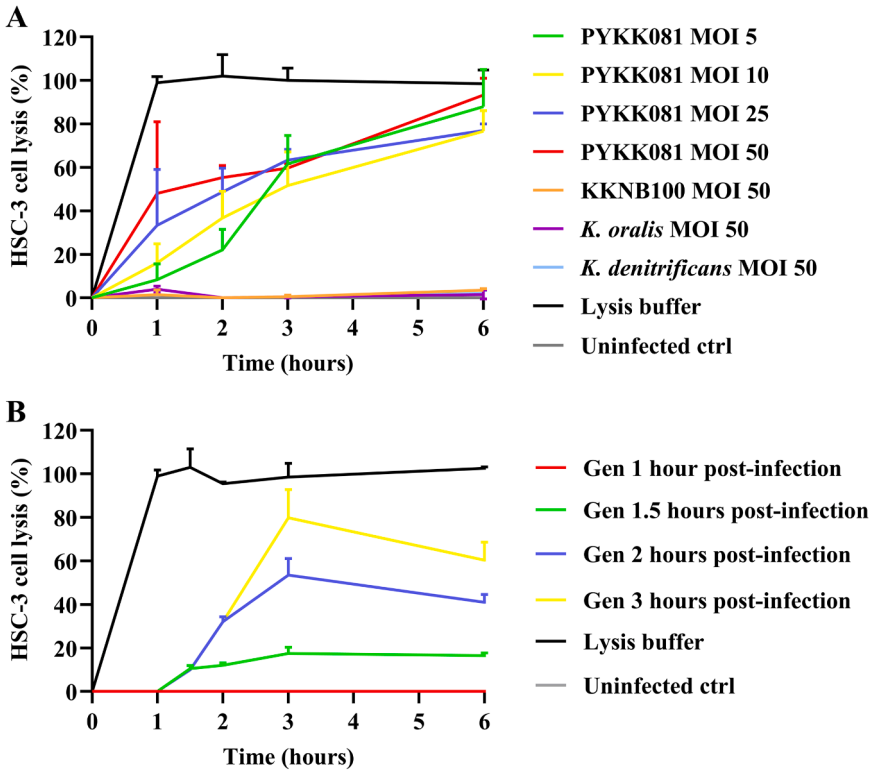

Fig. 1. *K. kingae* PYKK081 added to HSC-3 cells at an MOI of 5 must be killed with gentamycin 1.5 h after infection to ensure the secretion of appropriately low levels of RtxA. (**A**) HSC-3 cells were infected with *K. kingae* PYKK081 at an MOI of 5, 10, 25 or 50 and with *K. kingae* KKNB100, *K. oralis* and *K. denitrificans* at an MOI of 50. Cell lysis was measured 1, 2, 3 and 6 h post-infection by quantifying LDH release into the culture medium. As a negative control (ctrl), cells were incubated without bacteria. Complete lysis (average value was set to 100 %) was achieved by adding lysis buffer to the cells. (**B**) HSC-3 cells were infected with PYKK081 at an MOI of 5 and gentamicin (Gen; 100 μg/ml) was added 1, 1.5, 2 or 3 h after infection to kill the bacteria. Uninfected cells and cells incubated with lysis buffer served as controls. Cell lysis was measured 1, 1.5, 2, 3 and 6 h after infection.

tested MOI of 5, the PYKK081 strain lysed the HSC-3 cells in a time-dependent manner and caused almost complete lysis of the cells 6 h after infection. As expected, no lysis was observed when the cells were infected with the RtxA non-producing strains (Fig. 1A). Since we wanted to investigate the *Kingella*-induced innate immune response in HSC-3 cells at time points 3, 6, 12 and 24 h, it was necessary to use an antibiotic to kill the RtxA-secreting strain PYKK081. To determine the time point for the addition of the antibiotic, HSC-3 cells were infected with PYKK081 at an MOI of 5 and gentamicin (100 μg/ml) was added at different time points after infection. As shown in Fig. 1B, no RtxA-dependent lysis of HSC-3 cells was observed when gentamicin was added 1 h after infection. On the other hand, when gentamicin was added 2 or 3 h after infection, >50 % of the cells were lysed 6 h after infection (Fig. 1B). The optimal result was achieved by adding gentamicin 1.5 h after infection, when the secretion of functional RtxA by *K. kingae* was observed as lysis of <20 % of HSC-3 cells (Fig. 1B).

Based on these results, we performed infection experiments in which HSC-3 cells were infected with either *K. kingae* PYKK081 or its isogenic RtxA-deficient strain KKNB100 with an MOI of 5 and the bacteria were killed with gentamicin 1.5 h after infection. Uninfected HSC-3 cells were used as negative control. Cell culture media were collected from all samples at 3, 6, 12 and 24 h post-infection to determine cytokine and chemokine levels. In parallel, total RNA was isolated from the cells to analyse the changes in gene expression.

3.2. *RtxA stimulates the production of pro-inflammatory cytokines in oral epithelial cells*

The isolated RNA was reverse-transcribed and cDNA was used to analyse changes in expression levels of the genes *CXCL8, IL1B, IL6, IL12B, IL10* and *TNF* by qRT-PCR. In most cases, no significant differences in gene expression were observed between the uninfected control cells and the cells infected with the RtxA-deficient strain KKNB100 (Fig. 2). However, the *CXCL8* (Fig. 2A), *IL1B* (Fig. 2B) and *IL6* (Fig. 2C) genes were significantly upregulated in cells infected with PYKK081 at all time points, with a peak expression at 3 h post-infection. The *CXCL8* gene encodes a protein that belongs to the CXC chemokine family and is commonly known as IL-8. It acts as a chemoattractant for many immune cells. The *IL1B* gene encodes a precursor protein, pro-IL-1B, which, once processed into its active form, serves as an important mediator of the inflammatory response. *IL6* encodes IL-6, an endogenous pyrogen that is involved in the differentiation of T and B cells (Akdis et al., 2011). The *TNF* gene, that encodes a multifunctional pro-inflammatory cytokine called tumour necrosis factor (TNF), was also upregulated at all time points in HSC-3 cells infected with PYKK081. It was also the only cytokine-encoding gene whose expression was slightly but significantly increased in cells infected with KKNB100 at 24 h post-infection (Fig. 2D). Finally, very low or no expression of the *IL10* and *IL12B* genes was observed in HSC-3 cells, as indicated by the predominantly non-specific melting curves of the final DNA products amplified by qRT-PCR. The *IL10* gene encodes the anti-inflammatory IL-10, while the *IL12B* gene encodes a subunit of IL-12 that acts on T cells and natural killer (NK) cells (Akdis et al., 2011).

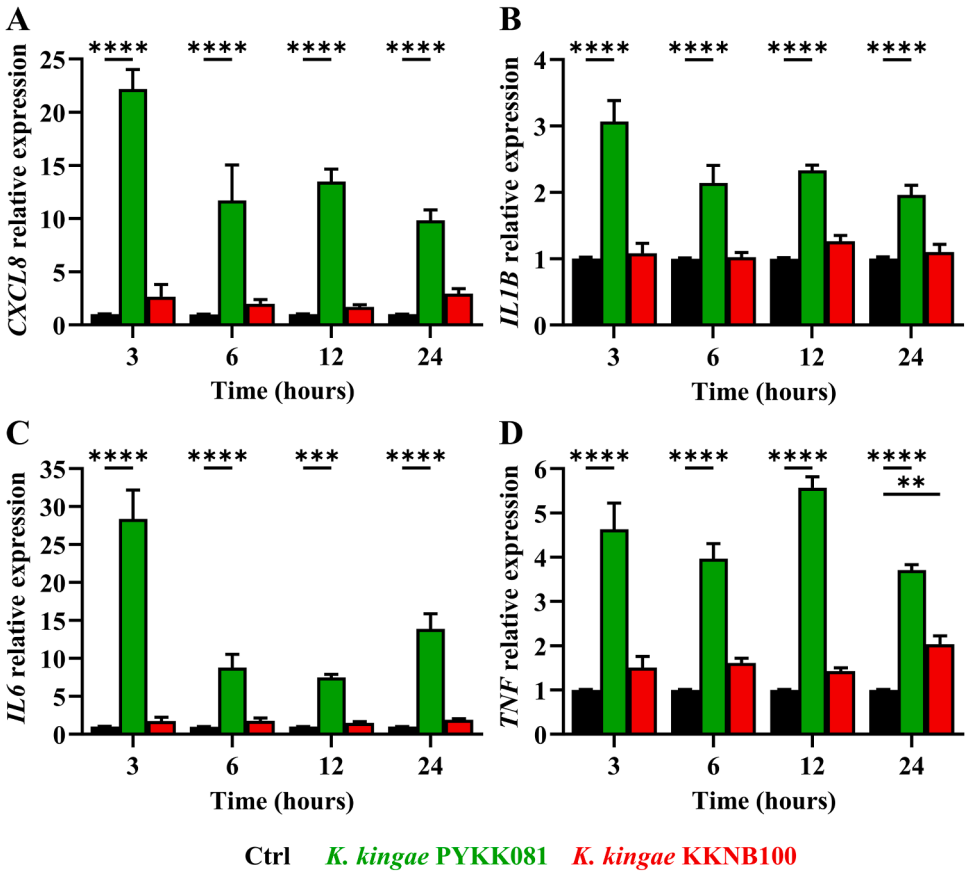

Fig. 2. RtxA secreted by *K. kingae* PYKK081 increases the expression of cytokine-encoding genes in oral epithelial cells. HSC-3 cells were infected with either *K. kingae* PYKK081 or its isogenic RtxA-deficient mutant KKNB100 at an MOI of 5 and the bacteria were killed with gentamicin 1.5 h post-infection. Uninfected HSC-3 cells were used as negative control (Ctrl). At 3, 6, 12 and 24 h post-infection, total RNA was isolated, reverse-transcribed and the resulting cDNA was used for qRT-PCR analysis to determine the relative expression of *CXCL8* (**A**), *IL1B* (**B**), *IL6* (**C**) and *TNF* (**D**). Each bar represents the mean value of four independent biological replicates (ANOVA; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; statistically insignificant differences are not shown).

To confirm the qRT-PCR data, we analysed the levels of cytokines released into the culture media by HSC-3 cells infected with *K. kingae* PYKK081 and its isogenic RtxA-deficient strain KKNB100. In addition, we performed an infection experiment with *K. oralis* and *K. denitrificans* to analyse whether these commensals contain components capable of stimulating the production of pro-inflammatory cytokines, similar to the RtxA toxin secreted by *K. kingae*. The concentrations of cytokines released into the culture media were quantified by flow cytometry using the BD Human Inflammatory Cytokine Cytometric Bead Array.

Compared to uninfected control cells, neither *K. oralis, K. denitrificans* nor the *K. kingae* strain KKNB100 significantly increased the release of IL-8 (Fig. 3A) or IL-1B (Fig. 3B) into the culture media of the infected cells. However, in HSC-3 cells infected with the RtxA-producing strain PYKK081, a significant increase in the production of these cytokines was observed 6, 12 and 24 h after infection (Fig. 3A and B).

Compared to control cells, IL-6 levels were significantly increased in the media of cells infected with PYKK081 at all time points (Fig. 3C). Nevertheless, a slightly increased production of IL-6 was also observed 12 and 24 h after infection of HSC-3 cells with *K. denitrificans* and 24 h after infection of the cells with *K. oralis* (Fig. 3C). TNF production was significantly increased by PYKK081 at 3, 6, 12 and 24 h and slightly increased by KKNB100 at 6 h (Fig. 3D). In agreement with the qRT-PCR data, only very low concentrations of IL-10 and IL-12, barely above the detection limit, were observed in both control and infected cells (Fig. S1A and S1B). All these data show that RtxA secreted by *K. kingae* PYKK081 is the main factor responsible for the increased production of the pro-inflammatory cytokines IL-8, IL-1B, IL-6 and TNF, although the production of IL-6 and TNF may also be slightly increased by some other *Kingella* components.

3.3. RtxA stimulates the production of pro-inflammatory chemokines in oral epithelial cells

To further investigate the role of *K. kingae* and its cytotoxin RtxA on the innate immune response of epithelial cells, we analysed the production of several pro-inflammatory chemokines. These molecules are responsible for the migration and residence of immune cells and therefore represent an important part of the immune system (Sokol and Luster, 2015). First, we analysed the expression of the genes *CCL2, CCL5, CCL20* and *CXCL10* in HSC-3 cells infected with either PYKK081 or KKNB100, as well as in uninfected control cells.

The results showed that PYKK081, but not its RtxA-deficient mutant KKNB100, significantly upregulated the expression of *CCL2* in infected HSC-3 cells at 3 and 6 h post-infection when compared to uninfected cells (Fig. 4A). After 12 and 24 h, we observed a significant upregulation of *CCL2* in cells infected with PYKK081 and a slight but statistically significant upregulation in cells infected with KKNB100 (Fig. 4A). The *CCL2* gene encodes the chemokine C-C motif ligand (CCL) 2, which is responsible for the recruitment of monocytes and basophils to the site of infection (Sokol and Luster, 2015). The expression of *CCL5*, which encodes a chemokine responsible for migration of macrophages and NK cells (Sokol and Luster, 2015), was strongly upregulated in cells infected with PYKK081 but not with KKNB100 at all time points (Fig. 4B). In addition, we observed an upregulated expression of *CCL20* only in cells infected with PYKK081, with the highest level 3 h post-infection and a decrease thereafter (Fig. 4C). The *CCL20* gene encodes the CCL20

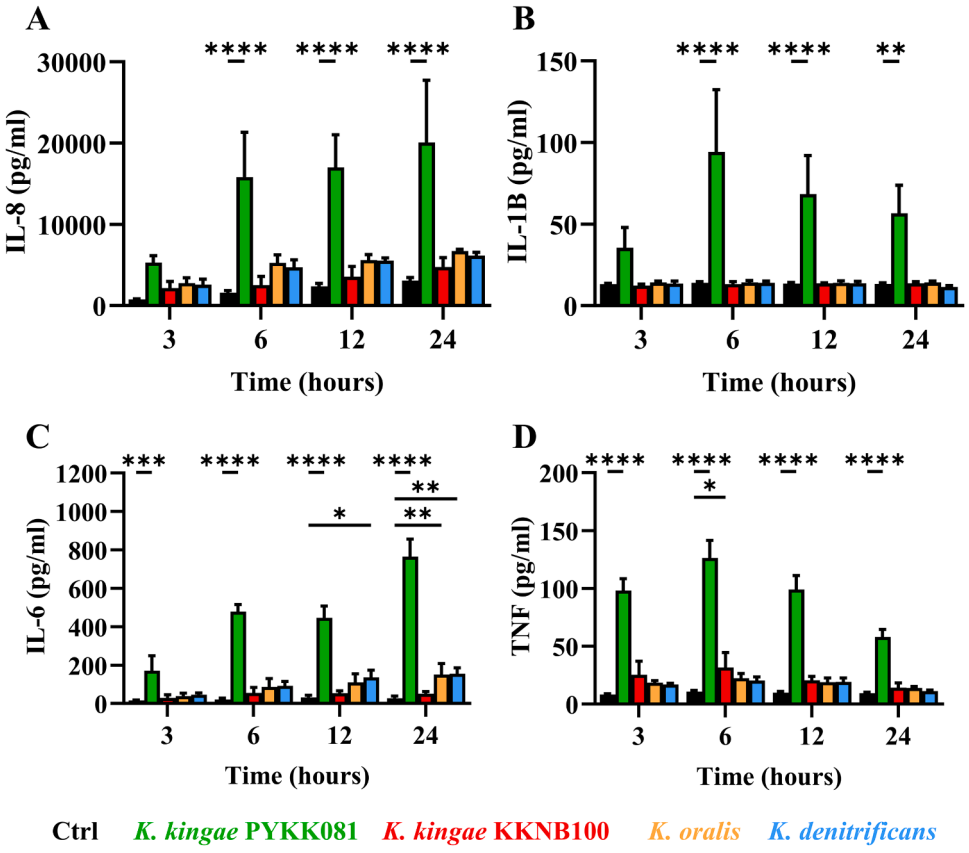

Fig. 3. RtxA secreted by *K. kingae* PYKK081 is the major factor of *Kingella* species that increases cytokine levels in the culture media of oral epithelial cells. HSC-3 cells were infected with *K. kingae* PYKK081, *K. kingae* KKNB100, *K. oralis* or *K. denitrificans* at an MOI of 5 and the bacteria were killed with gentamicin 1.5 h after infection. Uninfected HSC-3 cells served as negative control (Ctrl). At 3, 6, 12 and 24 h post-infection, the concentrations (pg/ml) of the cytokines IL-8 (**A**), IL-1B (**B**), IL-6 (**C**) and TNF (**D**) released into the culture media were quantified by flow cytometry using the BD Human Inflammatory Cytokine Cytometric Bead Array. Each bar represents the mean value of four independent biological replicates (ANOVA; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; statistically insignificant differences are not shown).

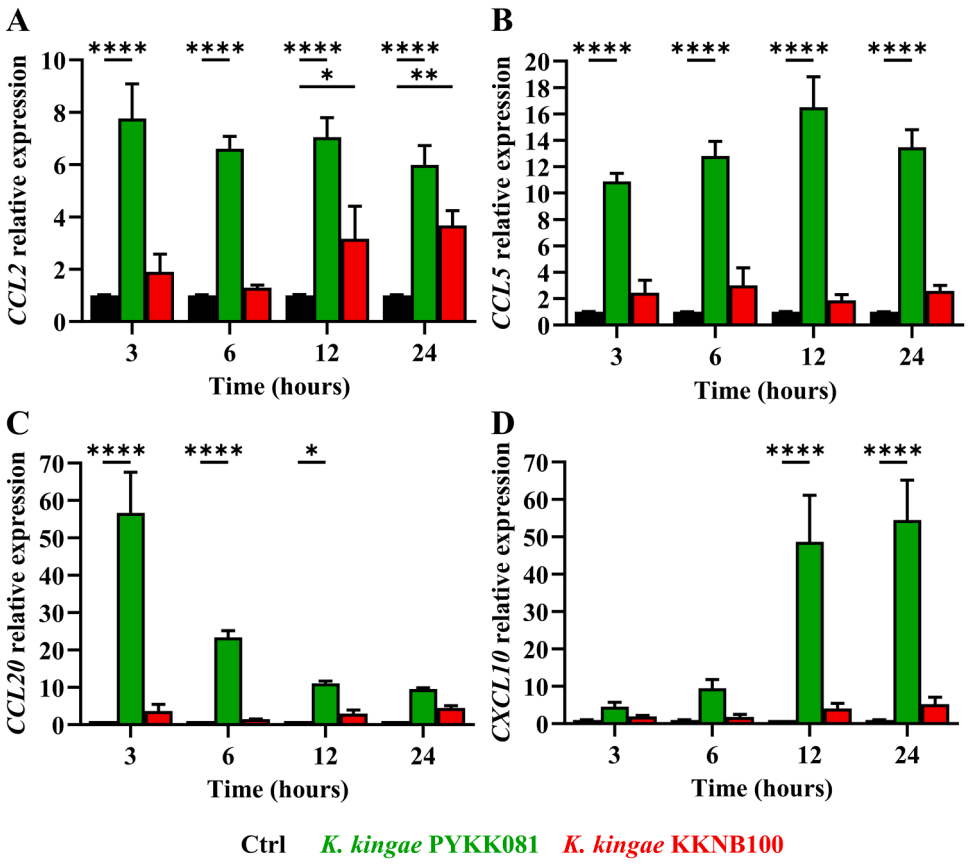

Fig. 4. RtxA secreted by *K. kingae* PYKK081 increases the expression of chemokine-encoding genes in HSC-3 cells. The infection experiment and qRT-PCR were performed as described in the legend to Fig 2. The relative expression of the genes *CCL2* (A), *CCL5* (B), *CCL20* (C) and *CXCL10* (D) is shown. Each bar represents the mean value of four independent biological replicates (ANOVA; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; statistically insignificant differences are not shown).

chemokine that acts on dendritic cells, T cells and B cells (Sokol and Luster, 2015). The *CXCL10* gene, which encodes an antimicrobial chemokine C-X-C motif ligand (CXCL) 10, was strongly upregulated by PYKK081 at 12 and 24 h after infection, but not by KKNB100 (Fig. 4D).

To confirm the qRT-PCR results, we quantified the concentrations of chemokines released by HSC-3 cells infected with *K. kingae* PYKK081 and its isogenic mutant KKNB100. In addition, we investigated the effect of *K. oralis* and *K. denitrificans* on the induction of chemokine production. The levels of released chemokines were quantified by flow cytometry using the BD Human Chemokine Cytometric Bead Array.

Although the expression of the *CCL2* gene was increased by *K. kingae* PYKK081 (Fig. 4A), no significant increase in the concentration of the secreted CCL2 chemokine was observed at any time point (Fig. S1C). In agreement with the qRT-PCR data, CCL5 production was increased by *K. kingae* PYKK081 at 12 and 24 h post-infection when compared to uninfected control cells (Fig. 5A). Furthermore, PYKK081 also significantly increased the production of CXCL10 at 24 h post-infection. Slightly increased production of CXCL10 was also observed in the

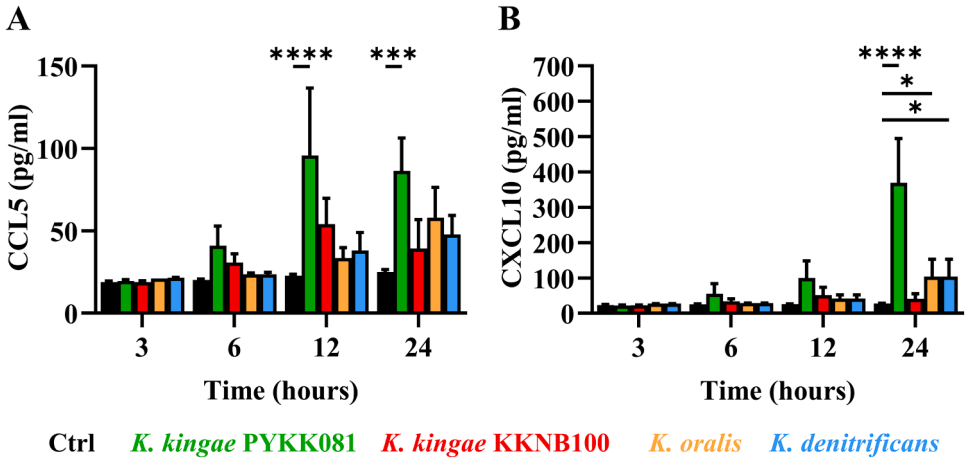

Fig. 5. RtxA is the major factor of the genus *Kingella* that increases the production of pro-inflammatory chemokines in oral epithelial cells. The infection experiment and the quantification of the CCL5 (**A**) and CXCL10 (**B**) chemokines by flow cytometry were performed as described in the legend to Fig. 3. Each bar represents the mean value of four independent biological replicates (ANOVA; *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$; statistically insignificant differences are not shown).

media collected from cells infected with *K. oralis* or *K. denitrificans* at 24 h post-infection (Fig. 5B). All these data indicate that RtxA is the major inducer of pro-inflammatory chemokines in oral epithelial cells.

3.4. The increased production of inflammatory cytokines and chemokines in HSC-3 cells is not caused by damage-associated molecular patterns released from cells lysed by RtxA

Since the RtxA toxin secreted by *K. kingae* PYK0081 caused damage

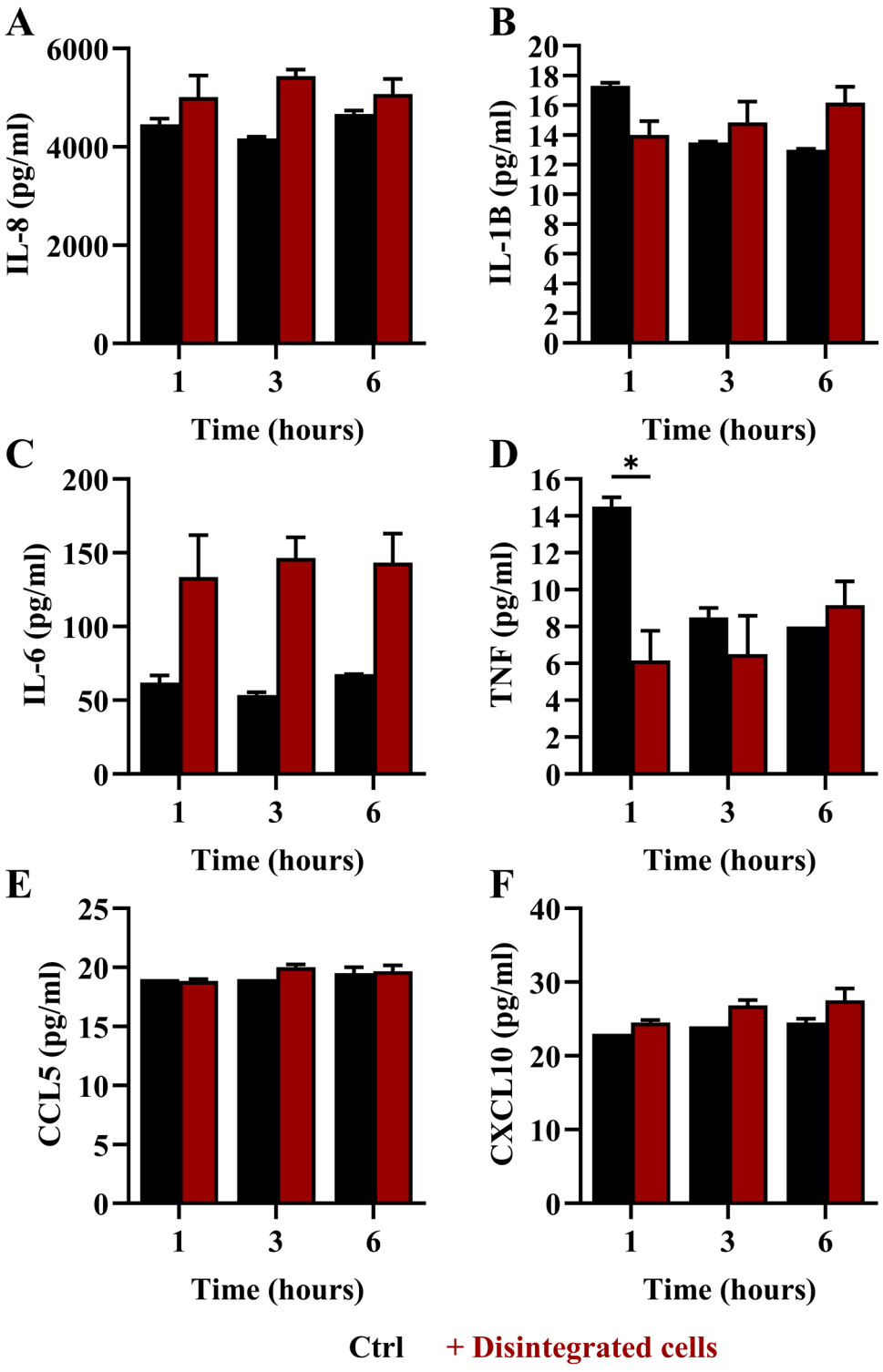

Fig. 6. The increased production of pro-inflammatory cytokines and chemokines is not induced by DAMPs. HSC-3 cells were incubated with disintegrated cells and the concentrations (pg/ml) of the cytokines IL-8 (**A**), IL-1B (**B**), IL-6 (**C**) and TNF (**D**) and the chemokines CCL5 (**E**) and CXCL10 (**F**) released into the culture media were quantified 1, 3 and 6 h post-infection using flow cytometry. Untreated HSC-3 cells served as negative control (Ctrl). Each bar represents the mean value of two independent biological replicates, performed in monoplicate (Ctrl) or triplicate (+ Disintegrated cells) (ANOVA; *, $p < 0.05$; statistically insignificant differences are not shown).

to a small proportion of the HSC-3 cells in our infection experiment (Fig. 1B), we sought to determine whether the increased production of pro-inflammatory cytokines and chemokines could be triggered by damage-associated molecular patterns (DAMPs) released from the lysed cells. To address this, a similar number of HSC-3 cells as in the infection experiment were disintegrated by sonication and 25 % of the disintegrated cells were added to the culture of HSC-3 cells grown as in the infection experiment. This was designed to simulate the RtxA-mediated cell damage that occurred during the infection experiment. After 1, 3 and 6 h of incubation, the culture media were collected, and the concentrations of released cytokines and chemokines were measured by flow cytometry. A comparison of the concentrations of cytokines and chemokines released by control cells and cells incubated with the disintegrated cells showed no statistically significant difference in the concentrations of the analysed proteins (Fig. 6). It can therefore be concluded that the increased production of cytokines and chemokines in HSC-3 cells was not caused by signalling through DAMPs, but by the activity of the RtxA toxin secreted by *K. kingae*.

3.5. *RtxA upregulates the expression of β-defensin-2 and secretory leukocyte peptidase inhibitor*

Finally, we analysed the effect of *K. kingae* PYKK081 and its isogenic mutant KKNB100 on the expression of genes encoding various AMPs in HSC3-cells. AMPs are small peptides that play a crucial role in the innate immune response against a broad range of microorganisms, including bacteria.

The qRT-PCR results showed a significant upregulation of *DEFB4A* in HSC-3 cells infected with PYKK081 compared to uninfected control cells at all time points, whereas no increase in the *DEFB4A* expression was observed in cells infected with KKNB100 (Fig. 7A). The *DEFB4A* gene encodes human β-defensin-2 (hBD-2), which exhibits antimicrobial activity against Gram-negative bacteria through direct interaction with their plasma membrane (Schroder and Harder, 1999). In addition to *DEFB4A*, the *SLPI* gene expression was slightly increased in HSC-3 cells infected with PYKK081, but not with KKNB100, at 6, 12 and 24 h post-infection (Fig. 7B). The *SLPI* gene encodes the secretory leukocyte peptidase inhibitor (SLPI), which protects local tissues from inflammatory damage by inhibiting specific proteases (Doumas et al., 2005). Finally, the expression of other genes encoding AMPs, including cathelicidin antimicrobial peptide (CAMP), collectin subfamily member 10 (COLEC10), cystatin 3 (CST3), human β-defensin-1 (hBD-1), lactoferrin (LTF) and lysozyme (LYZ), was only slightly increased in HSC-3 cells infected with PYKK081 at some time points. However, this increase was not statistically significant in most of the analysed samples (Fig. S2). These results indicate that RtxA secreted by PYKK081 is the sole factor in *K. kingae* responsible for inducing the expression of *DEFB4A* and *SLPI*, without significantly affecting the expression of genes encoding other AMPs.

4. Discussion

The oral mucosa, which lines the oral cavity, is comprised of stratified squamous epithelium that has adapted to the requirements of different regions within the oral cavity. The epithelial cells are connected by tight junctions, gap junctions and anchoring junctions, which collectively create a physical barrier. In the stratified oral epithelium, tight junctions are present in the superficial layers and are known to constitute the primary barrier of the epithelium. Cells of the immune system are located at the host-microbiome interface and have the ability to sense microorganisms through their pattern recognition receptors (PRRs). These receptors, which identify pathogen-associated molecular patterns (PAMPs) or DAMPs released by damaged cells, are predominantly expressed by immune cells but can also be found on the surface of epithelial cells. Upon recognition of PAMPs, the signal induces the activation of epithelial cells, which is followed by the production of chemokines, cytokines and antimicrobial molecules (Lebedev and Poniakina, 2006; Senel, 2021).

Another crucial part of oral immunity is microbiota. The microbial communities in the oral cavity are among the most diverse in terms of composition. The most prevalent phyla detected in the oral microbiome include Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes, and over 600 prevalent taxa have been described (Dewhirst et al., 2010; Proctor and Relman, 2017). The oral mucosal immune system must therefore maintain a delicate balance between tolerating commensal microorganisms and harmless antigens while responding effectively to harmful pathogens. Given that the oral mucosa is often the site of the first encounter of the body with viral, microbial and airborne antigens, it is conceivable that immunological responses to microbes and antigens are primed at this site and may influence not only local immunity but also subsequent responses at distant sites. The concept of priming local and systemic immunity at mucosal sites is based on the observation that sublingual delivery of antigens for vaccination produces efficient local and systemic protection in experimental models (Shim et al., 2013).

K. kingae is carried on the tonsillar and oropharyngeal surfaces of young children, with the majority of cases exhibiting no symptoms (Yagupsky et al., 2011; Yagupsky, 2015). It is believed that colonisation is the initial step in the pathogenesis of invasive diseases, although the

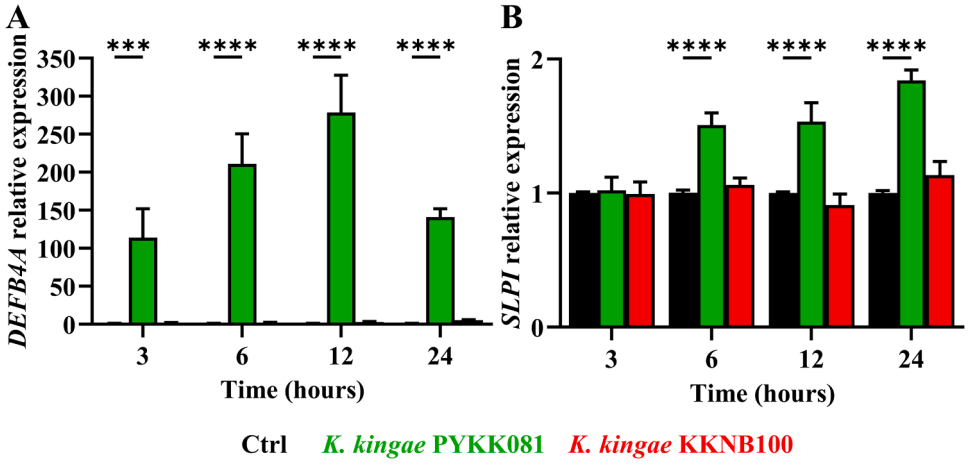

Fig. 7. RtxA secreted by *K. kingae* PYKK081 increases the expression of genes encoding hBD-2 and SLPI in HSC-3 cells. The infection experiment and qRT-PCR were performed as described in the legend to Fig. 2. The relative expression of the *DEFB4A* (**A**) and *SLPI* (**B**) genes is shown. Each bar represents the mean value of four independent biological replicates (ANOVA; ***, $p < 0.001$; ****, $p < 0.0001$; statistically insignificant differences are not shown).

mechanism by which the bacteria penetrate the oral barrier and enter the body remains unknown. However, it most likely involves the key virulence factor of *K. kingae*, the RtxA cytotoxin. Unlike *K. oralis* and *K. denitrificans*, which were isolated from children as well as adults, the *K. kingae* colonisation rate rapidly declines after the age of three years (Dewhirst et al., 1993, 2010; Chen, 1996; Yagupsky et al., 2011; Yagupsky, 2015). As the age of the host is the most significant factor influencing the carriage of *K. kingae*, it has been proposed that clearance of this bacterium from the oral epithelial surface is due to the development of an immune response to polysaccharides, which occurs around the second year of life (Klein Klouwenberg and Bont, 2008; Yagupsky, 2015). The data presented here suggest that the immune response may also result from a priming effect of the cytotoxic activity of RtxA on the immune system. Indeed, the results demonstrate that if oral epithelial cells are infected with *K. oralis* or *K. denitrificans*, which have the type 4 pili and the Knh protein, but do not produce RtxA, or with the *K. kingae* RtxA-deficient mutant, the immune response is either weak or does not occur at all. However, when the cells were infected with the RtxA-producing *K. kingae* strain PYKK081, we observed an immune response in the form of increased production of pro-inflammatory cytokines and chemokines. It has been shown that the secretion of cytokines and chemokines, such as IL-8, by stratified squamous epithelium can lead to the accumulation of neutrophils in junctional areas, which in turn results in the disruption of the epithelial barrier (Schroeder and Listgarten, 1997; Fujita et al., 2018). Therefore, it can be hypothesised that the cytokines and chemokines produced by infected epithelial cells during the innate immune response against *K. kingae* may act on immune cells, whose attraction to the site of infection could, together with the pore-forming activity of RtxA, disrupt the epithelial barrier and thus facilitate bacterial invasion.

RtxA belongs to a broad family of pore-forming RTX toxins produced by numerous Gram-negative bacteria, including those belonging to the genera *Actinobacillus, Aggregatibacter, Bordetella, Escherichia, Kingella, Mannheimia, Moraxella, Morganella, Pasteurella* and *Proteus* (Linhartova et al., 2010; Filipi et al., 2022). Most of the characterised RTX toxins exhibit a pore-forming activity, which, after the permeabilisation of the cellular membrane, leads to an efflux of cytosolic potassium ions (Linhartova et al., 2010). This efflux can modulate the immune response and mobilise host protective responses against bacterial pathogens (Porta et al., 2011; Dikshit et al., 2018; Verma et al., 2020). The objective of this study was to analyse the effect of the pore-forming RtxA toxin and other virulence factors or components of the *Kingella* spp. on the innate immune response of oral epithelial cells by analysing gene expression and the release of various pro-inflammatory cytokines, chemokines and AMPs.

Studies on the *Escherichia coli* α-hemolysin (HlyA) have shown that the pore-forming properties of the toxin lead to the production of the pro-inflammatory cytokines IL-6 and IL-8 in epithelial cells (Uhlen et al., 2000). The elevated production of IL-6, due to *B. pertussis* CyaA, has been observed in tracheal epithelial and bronchial cells. However, in the case of CyaA, it is unclear whether the elevation of IL-6 is caused by its pore-forming or adenylate cyclase activity (Bassinet et al., 2004; Hasan et al., 2018). Interestingly, when the impact of the *K. kingae* RtxA on the production of IL-6 and IL-8 was investigated in THP-1 macrophages, the results showed that the RtxA toxin suppressed the production of these cytokines (Srikhanta et al., 2017). Similarly, the RTX toxin GtxA, expressed by *Gallibacterium anatis*, appears to suppress the expression of certain pro-inflammatory cytokines in chicken macrophage-like cells (Tang and Bojesen, 2020). Here, we show that only the cytotoxic *K. kingae* strain PYKK081, which is capable of RtxA-mediated cell lysis, as observed by the release of LDH, was able to elevate the expression and release of IL-6 and IL-8 in epithelial cells. Thus, these results indicate the opposite effect of the RtxA toxin on epithelial and immune cells.

It was shown that only active acylated *E. coli* HlyA, in contrast to unacylated proHlyA, triggers the activation of the NLRP3 inflammasome and caspase-1, leading to IL-1B maturation and release *in vitro* (Gleason et al., 1998; Demirel et al., 2018; Murthy et al., 2018; Verma et al., 2020). Furthermore, the pore-forming activity of *B. pertussis* CyaA induces the activation of the NALP3 inflammasome and the release of IL-1B (Dunne et al., 2010). These data demonstrate that the elevated release of IL-1B by RTX toxins from epithelial and immune cells is dependent on the pore-forming activity of the toxin. Although we did not directly demonstrate the activation of the NLRP3 inflammasome and caspase-1, it is reasonable to assume that the *K. kingae* RtxA functions in a similar manner. This assumption is supported by our data showing an increased concentration of IL-1B in the culture medium of HSC-3 cells. IL-1B is produced as a precursor protein, pro-IL-1B, which is cleaved by the pro-inflammatory protease caspase-1 (Thornberry et al., 1992). *IL1B* expression is generally referred to as a priming step, which is not sufficient for the processing and secretion of IL-1B. The primed cell must receive another stimulus for the processing to occur, and therefore, the increased expression of *IL1B* does not necessarily correlate with increased secretion of mature IL-1B. Here, we have shown that RtxA triggers the upregulation of *IL1B* expression, as well as the cleavage and secretion of mature IL-1B.

At subtoxic doses, purified HlyA failed to induce higher levels of TNF in human monocytes (Bhakdi et al., 1990). Similarly, HlyA-producing *E. coli* did not increase TNF levels in infected monocytes *in vitro* (Bhakdi et al., 1990) or in the sera of infected mice (May et al., 1996). Exposure of THP-1 macrophages to a non-cytotoxic *K. kingae* strain led to upregulated expression and production of TNF, but infection with the wild-type strain, on the contrary, led to the suppression of several pro-inflammatory cytokines, including TNF (Srikhanta et al., 2017). Here, we show that only the RtxA-producing *K. kingae* strain PYKK081 was able to increase the expression and release of TNF in epithelial cells. Thus, these results further emphasise the opposing effects of the RtxA toxin on epithelial and immune cells.

Chemokines promote the directional migration of leukocytes and play a vital role in the host defence mechanism. Based on their function, these small proteins can be categorised as homeostatic, which are constitutively expressed, or inflammatory, whose expression is induced by inflammatory stimuli (Raman et al., 2011). Studies on uropathogenic *E. coli* have demonstrated that HlyA upregulates granulocyte-macrophage colony-stimulating factor (GM-CSF) in renal epithelial cells (Wang et al., 2020). This suggests that some RTX toxins may stimulate chemokine production in epithelial cells. Our study demonstrated that RtxA induces the expression of the chemokine-encoding genes *CCL2, CCL5, CCL20* and *CXCL10*, however significant differences in protein levels were only evident in the secretion of CCL5 and CXCL10. While the level of CCL20 was not measured, the absence of CCL2 in the culture medium could be attributed to the degradation of *CCL2* mRNA, which may occur to maintain cellular homeostasis (Fan et al., 2005).

As AMPs represent another crucial component of the innate immune system, we also investigated the effect of *K. kingae* and its RtxA on the expression of certain AMPs. It has been shown that *B. pertussis* CyaA inhibits the secretion of hBD-2 in bronchial cells by increasing intracellular cAMP levels (Hasan et al., 2018). In contrast to CyaA, RtxA stimulates an upregulation of the gene encoding hBD-2. Additionally, we observed an upregulation of the *SLPI* gene, which encodes a peptidase inhibitor responsible for protecting local tissues against inflammation (Doumas et al., 2005).

K. kingae isolates are known to exhibit noteworthy genetic diversity, as demonstrated by multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and whole-genome sequencing, and to express different types of *rtxA* alleles. While certain PFGE clones are associated with distinct types of infections, such as osteoarticular infections, bacteraemia, or endocarditis, others are more frequently detected in asymptomatic carriers (Amit et al., 2012; Basmaci et al., 2012; Murik et al., 2023). These findings suggest that both *rtxA* polymorphism and broader genetic differences among *K. kingae* isolates may influence their pathogenic potential. In our study, we employed the septic arthritis

isolate PYKK081, which carries *rtxA* allele type 1 and belongs to PFGE clone B and MLST sequence type 22 (Amit et al., 2012; Kaplan et al., 2012). Future studies should aim to extend our findings to genetically distinct isolates with diverse *rtxA* alleles to further elucidate their role in the pathophysiology of *K. kingae* infections.

In conclusion, our results show that infection of oral epithelial cells with *K. kingae*, but not with the RtxA-deficient mutant or commensal *Kingella* species, leads to a significant, RtxA-dependent increase in the gene expression and production of certain pro-inflammatory cytokines, chemokines and AMPs. A comparison with previous studies on *K. kingae* infection in THP-1 macrophages revealed that RtxA exerts opposing effects on epithelial and immune cells. Since *K. kingae* is not typically present on oral epithelial surfaces after the third year of life, whereas *K. oralis* and *K. denitrificans* are also isolated from adults, we hypothesise that the cytotoxic effect of RtxA, which triggers increased gene expression and production of pro-inflammatory cytokines, chemokines and AMPs, may, together with other virulence determinants, contribute to the elimination of *K. kingae* once a functional immune response is developed. These insights may provide a novel perspective on the study of the innate immune response to RTX toxins.

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CRediT authorship contribution statement

Eliska Ruzickova: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Karyna Zhuk:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization. **Kevin Munoz Navarrete:** Investigation, Methodology. **Adriana Osickova:** Investigation, Methodology, Supervision. **Radim Osicka:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2025.100393.

Data availability

Data will be made available on request.

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