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# Human corneal epithelial cells respond to ocular-pathogenic, but not to nonpathogenic-flagellin

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**Abstract**

In this study, we investigated the expression of TLR5 in human corneal epithelial cells (CEC), and the functional outcome of TLR5 triggering by flagellins of pathogenic- and nonpathogenic bacteria. Flagellins derived from *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescense* or *Bacillus subtilis* were used. The TLR5 protein and TLR5 specific mRNA expression was evident on human CEC. In human corneal epithelium tissues, TLR5 protein was detected at the basal and wing cells of the tissues. Ocular pathogenic bacteria, namely *P. aeruginosa* and *S. marcescense*, derived flagellin induced the significantly increased level of gene activation and IL-6 and IL-8 production. In contrast, ocular nonpathogenic *S. typhimurium*- and *B. subtilis*-derived flagellin induced neither the gene activation nor the increased production of IL-6 and IL-8 in human CEC. Human CEC would respond only to flagellin derived of ocular pathogenic bacteria, but not to those derived of ocular nonpathogenic bacteria, to generate pro-inflammatory cytokines.


*Keywords:* Human corneal epithelial cells; Flagellin; Inflammation; Toll-like receptor 5 (TLR5); Innate immunity

The surface epithelium serves a critical function as the defensive front line of the innate immune system in mucosal tissues such as intestinal, renal, airway, and urinary tracts [1]. The ability to detect microbes is arguably the most indispensable task undertaken by the immune system, and initiation and perpetuation of the inflammatory mucosal responses may result from an exaggerated host defense reaction of the epithelium to endogenous bacterial flora [2].

In current thinking, it is accepted that the ability of cells to recognize pathogen-associated molecular patterns (PAMPs) [3–5] depends on expression of a family of Toll-like receptors (TLRs) [6–14]. TLR expression is not restricted to macrophages (Mϕ) and dendritic cells (DCs) and the expression is located either in the cell membrane or in the cytosol [7,15–20]. Signaling through TLRs leads to the activation of NF-κB, and NF-κB target genes [3,5,8,21], leading to the coordinate activation of several transcription factors for antimicrobial genes, cytokines, chemokines, and costimulatory molecules [3–5,22].

The eye is relatively impermeable and refractory to microorganisms, partly due to the presence of nonspecific ocular innate defense mechanisms including blinking, tear flow, and mucin [23–25]. However, if corneal integrity is breached by trauma or contact lens wear, a sight-threatening bacterial infection may occur [26,27]. Corneal epithelial cells (CEC) are in the unique position of being in constant contact with bacteria and bacterial products [23] and form a barrier against numerous bacteria, irrespective of their pathogenicity. The refractory nature of CEC to bacteria or bacterial products is intriguing. This is true even more so of the avascular and transparent cornea, where formation of scar tissue, due to a host inflammatory reaction, results in opacification and loss of vision [24,28]. Thus, the ability to discriminate between pathogenic and nonpathogenic bacteria is extremely critical for CEC [29]. We

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reported that cell surface TLR3 of human CEC responds to virus double-stranded RNA-mimic poly(I:C) to generate pro-inflammatory cytokines and IFN-β, and that the innate immune responses in human CEC differ from those in immune-competent cells [30]. Among ocular surface related bacteria only a few of pathogens such as *P. aeruginosa* share flagella, while *Staphylococcus epidermidis*, *S. aureus* or Propionibacterium acnes do not [31,32]. Only one report so far described that flagellin of *P. aeruginosa* contributes to the inflammatory responses of human corneal epithelium in a TLR5-NF-κB signaling pathway-dependent manner [29]. To date, TLR5 expression in humans has mostly been shown for DCs, monocytes, and intestinal epithelial cells [33–39]. Albeit a number of other epithelial responses to flagellin have been examined [40–42], there remains strong controversy on the influence of flagellin to nonimmune competent cells.

In this study, we demonstrated that human CEC would respond only to flagellin derived of pathogenic bacteria, but not to those derived of nonpathogenic bacteria, to generate pro-inflammatory cytokines. The data showed that *P. aeruginosa* and *Serratia marcescense* derived flagellin stimulated the innate immune response, whereas *Salmonella typhimurium* and *Bacillus marcescense* derived flagellin did not trigger the response in TLR5 positive human CEC.

## Materials and methods

*Human corneal epithelial cells*. The human corneal epithelial cell line (HCE-T) transformed with SV40 [43] was maintained in the Department of Ophthalmology, Kyoto Prefectural University of Medicine and cultured in modified SHEM medium consisting of Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F-12) (Gibco-BRL Life Technologies Ltd., Paisley, UK), supplemented with 10% fetal calf serum (FCS) (Gibco), 10 ng/ml murine natural epidermal growth factor (EGF) (Gibco), 5 μg/ml insulin from bovine pancreas (Sigma), and 1% antibiotic–antimycotic solution (100 U penicillin per ml; 100 μg/ml streptomycin per ml; 250 ng amphotericin B per ml) (Gibco), at 37 °C under 95% humidity and 5% $CO_2$. Primary human corneal epithelial cells (PHCE) were obtained from KURABO, Osaka, Japan, and then cultured in a serum-free medium consisting of EpiLife (KURABO), supplemented with human corneal epithelial cell growth supplement (HCGS) containing 1 ng/ml murine epidermal growth factor (mEGF), 5 μg/ml insulin from bovine pancreas, 0.18 μg/ml hydrocortisone, and 0.4% v/v bovine pituitary extract (KURABO), and 1% antibiotic-antimycotic solution consisting of 100 U penicillin per ml, 100 μg/ml streptomycin per ml, and 250 ng amphotericin B per ml (Gibco) at 37 °C under 95% humidity and 5% $CO_2$.

*Bacterial flagellins used in this study*. Flagellins derived of *P. aeruginosa* and *S. marcescense* were obtained from Inotek Pharmaceuticals (Beverly, Massachusetts, USA), and those of *Salmonella typhimurium* and *Bacillus subtilis* were from InvivoGen (San Diego, USA). *S. typhimurium* flagellin represents strong pathogen that gains entrance to intestinal mucosa by penetrating enterocytes [39,44–46], but not to ocular surface mucosa. *S. marcescense* is another pathogen that causes approximately 5–10% of Gram-negative corneal ulcers related to contact lens wear [47–51]. *B. subtilis* has been reported that its pathogenic potential is generally low or absent [52]. *P. aeruginosa* was chosen as a Gram-negative bacterium being an opportunistic ocular pathogen that can initiate a highly destructive corneal infection in humans [28]. *P. aeruginosa* is the pathogen most commonly involved in contact lens-related bacterial keratitis and accounts for up to 70% of all cases of contact lens-related bacterial keratitis [53].

*Purification of mononuclear cells from peripheral blood*. After the purpose of the research and the experimental protocol had been explained to and informed consent, human venous blood samples were obtained from volunteers. Mononuclear cells were isolated from peripheral blood by lymphoprep tube (Daiichi Pure chemicals, Tokyo, Japan). Mononuclear cells were gently aspirated from the interface and washed with PBS(−). For stimulation with LPS or flagellin, isolated human peripheral mononuclear cells were cultured in RPMI medium (Gibco-BRL Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (Gibco) and 1% antibiotic-antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B) (Gibco).

*Reverse transcription polymerase chain reaction (RT-PCR) analysis*. Total RNA was purified from HCE-T and PHCE using TRIZOL Reagent (InvivoGen) according to the manufacturer's instructions. For the RT reaction, we used the SuperScript™ Preamplification kit (Invitrogen). PCR amplication was performed using DNA polymerase (γTaq; TOYOBO, Japan) on the GeneAmp® PCR system (PE Applied Biosystems) for 35 cycles using the following cycling conditions; 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and then a final extension of 72 °C for 2 min. The specific primers for TLR5 and GAPDH we used were described in previous report [40,54]. The primer for TLR5 was 5′-CCTCATGACCATCCTCACAG-3′ and 5′-GGCTTCAAGGCA CCAGCCAT-3. The primer for GAPDH was 5′-CCATCACCATCT TCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3. To assess mRNA expression, RT-PCR products were run on a 1% ethidium bromide-stained agarose gel.

*Flow cytometric analysis*. HCE-T and PHCE were treated with 0.02% EDTA. For TLR5 expression, mouse anti-human TLR5 monoclonal antibody was derived from Abcam (Cambridge, UK). Because this mouse anti-human TLR5 monoclonal antibody recognizes an intracellular epitope in the cytoplasmic domain of TLR5, we performed intracellular FACS using the cell fixation/permeabilization kit (BD Pharmingen, San Diego, CA). Cells were fixed with Cytofix/Cytoperm and then stained with mouse anti-human TLR5 monoclonal antibody or isotype control mouse IgG2a Ab in Perm/Wash solution at 4 °C for 30 min. They were then reacted with Alexa Fluor 488 goat anti-mouse IgG (H+L) Ab (Molecular Probes, Eugene, OR). Stained cells were analyzed with a FACSCalibur (Becton–Dickinson, San Jose, CA), and data were analyzed using Cellquest software (Becton–Dickinson). Gating of lymphocytes or monocytes was made from a distribution pattern in forward and side scatter.

*Immunohistochemistry of TLR5 in human cornea sections*. Serial sections (6 μm) of human cornea were prepared from human corneal graft after corneal transplantations for one pre-bullous keratopathy due to Fuch's dystrophy. All experimental procedures have been conducted in accordance with the principles set forth in the Helsinki declaration. Slides were fixed with methanol for 30 min. And then, slides were incubated with mouse anti-human TLR5 monoclonal antibody (mAb; Abcam, Cambridge, UK) or isotype control mouse IgG2a (DakoCytomation, Kyoto, Japan) at 4 °C in a moist chamber overnight. After the slides were washed in PBS-, Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR) was applied for 1 h at room temperature. After the slides were washed, they were mounted with antifade mounting medium with PI (Vectashield; Vector Laboratories, Burlingame, CA).

*ELISA*. To quantify cytokine secretion, HCE-T and PHCE were plated in 25-cm dishes and, after reaching subconfluence, were either left untreated or incubated with each flagellin at a final concentration of 100 ng/ml or 10 ng/ml of human IL-1α (R&D Systems, Inc., Minneapolis, MN, USA) for 24 h. As the secreted amounts of IL-6 and IL-8 after exposure to flagellin rise in proportion to a rise in the concentration of flagellin, the concentration of flagellin was used 100 ng/ml in this study. IL-6 and IL-8 secretion in HCE-T culture supernatant examined during the time-course experiment showed a high secretion of IL-6 and IL-8, starting as early as 24 h. The concentration of IL-1α was optimal for the maximum induction of inflammatory cytokines [54]. The culture supernatants were harvested and then were cleared of cellular debris by centrifugation for 1 min at 10,000*g*, quick frozen, and stored at −80 °C prior to measurement. IL-6 and IL-8 release into the cell supernatants was quantitated using the OPTEIATM IL-6 and IL-8 set (BD Pharmingen,

San Diego, CA, USA) according to the manufacturer's instructions. Each assay was performed in triplicate. Means and SE (standard error) of three independently performed coincubations were calculated.

*Real-time quantitative PCR*. Real-time quantitative PCR was performed on an ABI-prism 7700 (Applied Biosystems, Foster City, CA) according to a previously described protocol [30] and the manufacturer's instructions. Total cellular RNA extraction and the first cDNA synthesis were as described above. The primers and probes for human IL-6, IL-8, and human GAPDH were from Perkin-Elmer Applied Biosystems. To amplify human IL-6, IL-8, TLR5, and GAPDH cDNA, PCR was performed in a 25-μl total volume that contained a 1μl cDNA template and 2× TaqMan universal PCR master mix (Applied Biosystems) at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. These quantification data were analyzed with sequence detection software (Applied Biosystems) and the expression level of each mRNA was normalized to the expression of the housekeeping gene GAPDH.

*Data analysis*. Data were expressed as means ± SE and were evaluated by Student's *t*-test using the Excel program.

## Results

### *HCE-T and PHEC express TLR5*

To determine whether TLR5 was expressed in human corneal epithelium, we first investigated the expression of TLR5 on HCE-T and PHCE. Flow cytometric analysis showed that TLR5 was expressed in HCE-T comparable to the expression levels on human peripheral lymphocytes and monocytes (Fig. 1A). This finding is quite consistent with the previous findings by Zhang et al. [29]. It is quite conclusive that not only a HCE-T, but also PHCE displays the expression of TLR5. To further confirm the TLR5 expression in stratified corneal epithelium tissues, immunohistochemical studies were performed on corneal tissues from human corneal grafts after corneal tansplantations

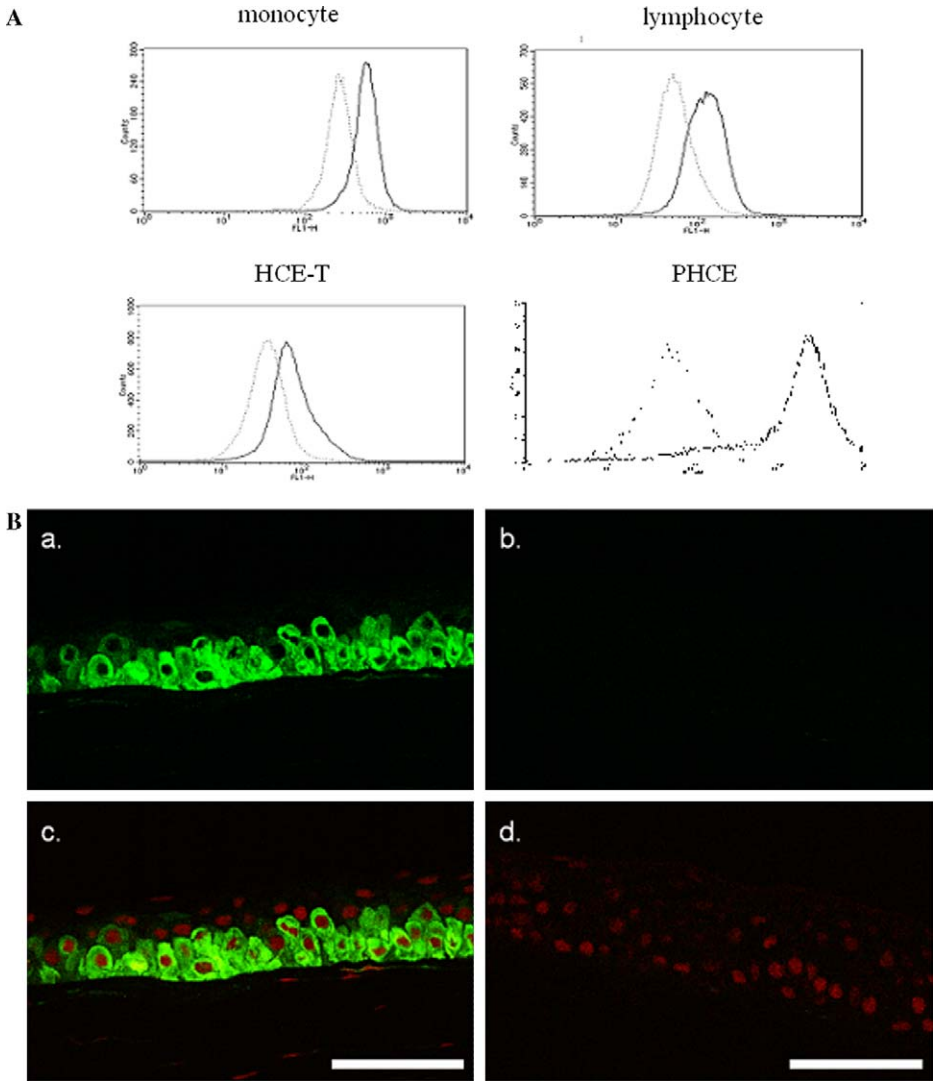



Fig. 1. Human corneal epithelial cells express TLR5 protein. (A) Intracellular FACS analysis of TLR5 showed that TLR5 is expressed in human corneal epithelial cells. Monocytes and lymphocytes served as positive controls. Histogram data are representative of three separate experiments. (B) Immunolocalization of TLR5 in a human cornea. TLR5 was detected by immunofluorescence staining. Frozen cryostat sections of a human cornea were incubated with anti-TLR5 antibody (a,c) or isotype control incubation as a negative control (b,d). The bound antibodies were visualized after incubation with Alexa Fluor 488 goat anti-mouse IgG and nuclei by PI staining. (c,d) Double staining with TLR5 labeling in green and PI indicate no TLR5 staining associated with the apical layer of the epithelium.

for one pre-bullous keratopathy due to Fuch's dystrophy. TLR5 protein was consistently and abundantly expressed in human corneal epithelium tissues, and was detected at the basal and wing, but not in superficial, cells of the tissues (Fig. 1B).

*Expression of TLR5-specific mRNA in human CEC*

As we identified TLR5 protein expression in human corneal epithelial cells and tissues, we prepared mRNA from PHCE and HCE-T cell line, and performed RT-PCR to determine whether TLR5-mRNA was constitutively expressed in human CEC. The specificity of the PCR product for TLR5 was confirmed using human mononuclear cells as a positive control. RT-PCR showed that TLR5-specific mRNA was present both in PHCE and HCE-T at a comparable level to peripheral mononuclear cells (Fig. 2). This result demonstrates that TLR5 gene is constitutively expressed in human CEC also at the level of mRNA.

*Induction of pro-inflammatory cytokines by flagellin in human peripheral mononuclear cells*

In order to identify whether flagellin used in this study would induce an inflammatory response, fractionated human peripheral mononuclear cells were stimulated with *P. aeruginosa*-, *S. typhimurium*-, *B. subtilis*- or *S. marcescense*-derived flagellin at a concentration of 100 ng/ml for 24 h. All of these flagellins induced considerably high levels of inflammatory cytokine, IL-6 and IL-8, production (Fig. 3). The levels of the IL-6 production were well paralleled with those of IL-8 production stimulated with flagellin derived of four distinct bacteria, and they were almost comparable with those of IL-6 and IL-8 production by

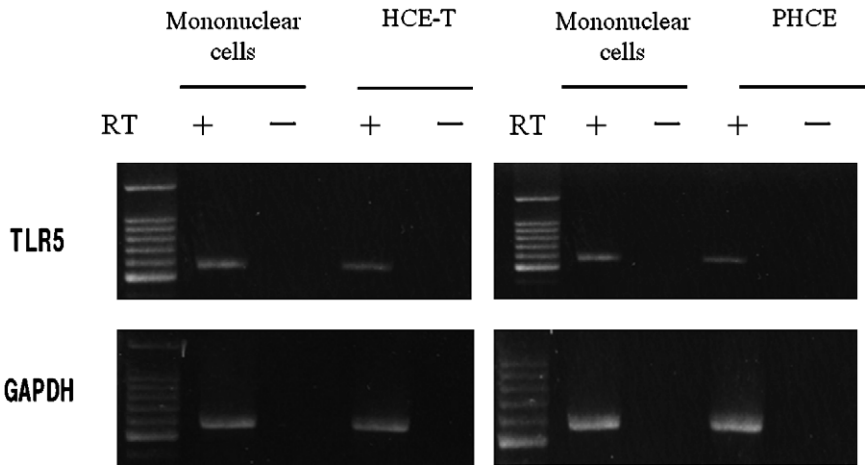



Fig. 2. Human corneal epithelial cells express TLR5-specific mRNA. Total RNA was isolated from human corneal cell lines (HCE-T) and primary human corneal epithelial cells (PHCE). For RT reaction, the SuperScript preamplification system was applied. PCR amplification was performed with DNA polymerase. Human mononuclear cells served as a positive control.

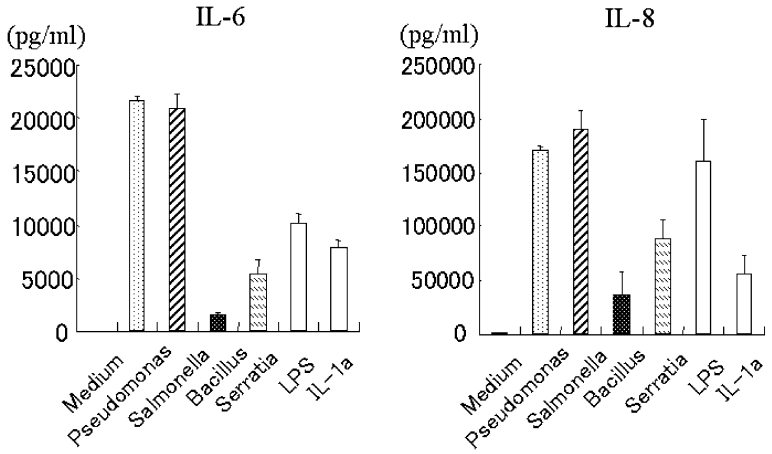



Fig. 3. Production of IL-6 and IL-8 of human peripheral mononuclear cells by various flagellins. To quantify inflammatory cytokine secretion, human peripheral mononuclear cells were isolated and cultured, then were left untreated or were exposed to LPS (1 μg/ml) or IL-1α (10 ng/ml) or flagellin derived from *P. aeruginosa* or *S. typhimurium* or *B. subtilis* or *S. marcescense* (100 ng/ml) for 24 h. The supernatants were harvested for measurement of IL-6 and IL-8. Data represent means ± SEM from an experiment with triplicate dishes.

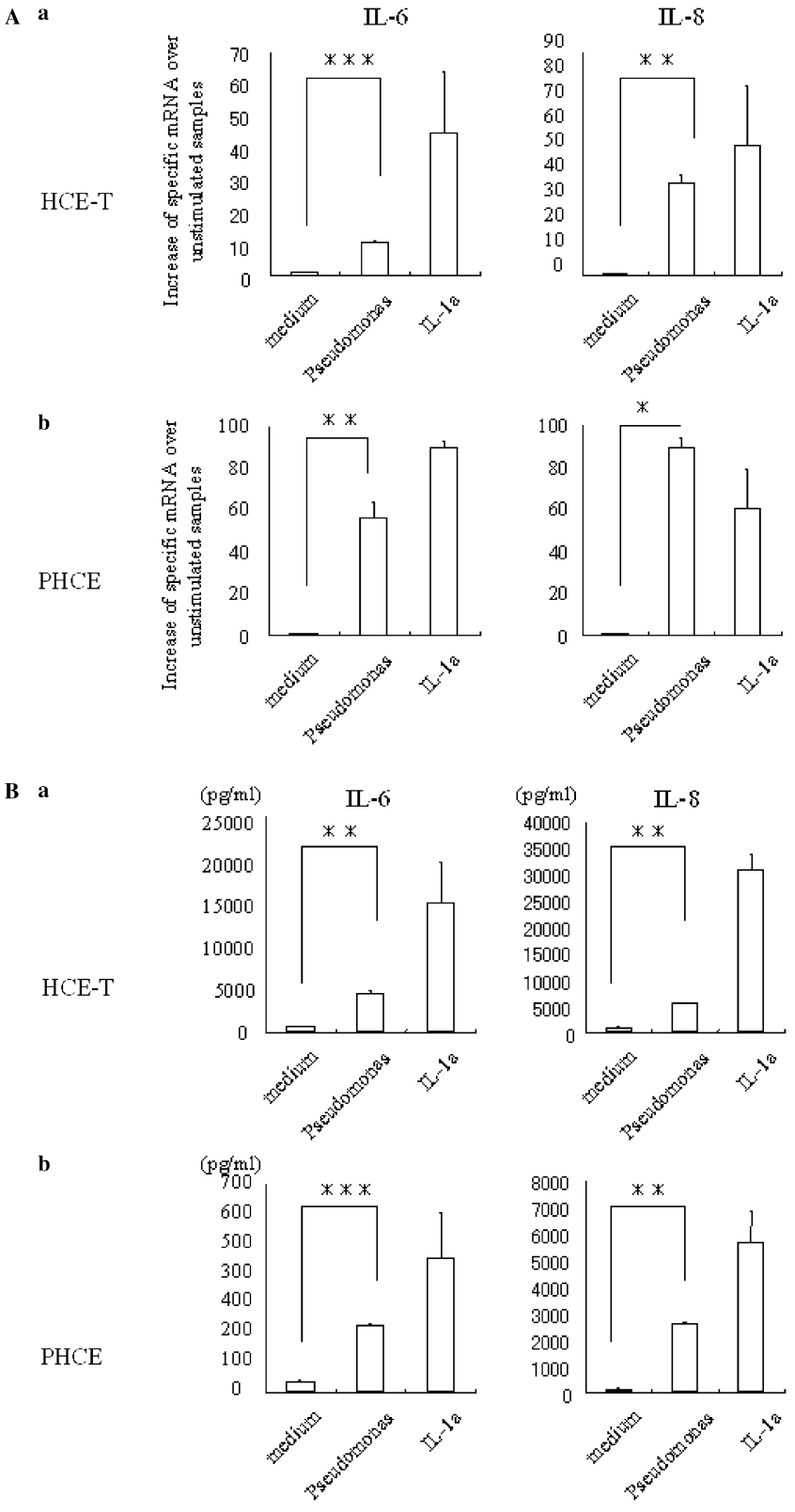



Fig. 4. mRNA expression and production of IL-6 and IL-8 of human corneal epithelial cells by *P. aeruginosa* flagellin. (A) To measure the expression of IL-6, IL-8 mRNA in HCE-T and PHCE after treated with 100 ng/ml *P. aeruginosa* flagellin or human IL-α for 1 h, quantitative RT-PCR was performed. Data represent means ± SEM from an experiment with triplicate dishes. (B) To quantify inflammatory cytokine secretion, HCE-T and PHCE were plated in 25-cm dishes and, upon reaching subconfluence, were left untreated or were exposed to 100 ng/ml *P. aeruginosa* flagellin or 10 ng/ml human IL-1α for 24 h. The supernatants were harvested for measurement of IL-6 and IL-8. Data represent means ± SEM from an experiment with triplicate dishes. $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

LPS. This indicated that all flagellins derived from four distinct microbes used in this study share the similar potency to induce pro-inflammatory cytokine production by human mononuclear cells.

*CEC responded to P. aeruginosa flagellin*

As HCE-T and PHCE were confirmed to express TLR5 at both levels of protein and mRNA, whether HCE-T and PHCE would respond to ocular surface related bacterium *P. aeruginosa* derived flagellin was first examined. The IL-6- and IL-8-specific mRNA detection by real-time quantitative PCR revealed that *P. aeruginosa* derived flagellin induced the elevated levels of IL-6- and IL-8-specific mRNA in both HCE-T and PHCE, comparable to those by IL-1α (Fig. 4A). This finding was further confirmed at the cytokine protein level in culture supernatants. As shown in Fig. 4B, *P. aeruginosa* derived flagellin elicited significantly elevated secretion of IL-6 and IL-8 proteins by both HCE-T and PHCE, although the elevation was relatively lower than that by IL-1α.

*CEC did respond to ocular-pathogenic, but not to nonpathogenic, bacteria derived flagellin*

It is an absolutely intriguing issue to clarify the intrinsic potency of distinct flagellin derived of different, ocular pathogenic versus nonpathogenic, microbial bacteria to induce inflammatory responses in TLR-5 positive human CEC. Aiming to resolve the issue, human CEC were stimulated with different types of flagellin, either of ocular pathogenic or ocular nonpathogenic bacteria and that of bacterium devoid of pathogenicity in human. The mRNA expression levels specific for IL-6 and IL-8 were considerably elevated in HCE-T and PHCE stimulated with *P. aeruginosa*- and *S. marcescense*-derived flagellin. In contrast, neither IL-6- nor IL-8-specific mRNA levels were found to be significantly elevated in HCE-T and PHCE stimulated with *S. typhimurium*- and *B. subtilis*-derived flagellin (Fig. 5A). This finding was further confirmed at the protein level. As shown in Fig. 5B, *P. aeruginosa*- and *S. marcescense*-derived flagellin elicited significantly increased secretion level of IL-6 and IL-8 proteins in HCE-T and PHCE, whereas *S. typhimurium*- and *B. subtilis*-derived flagellin did not induce the increased production of IL-6 and IL-8 proteins (Fig. 5B). It is of note that *P. aeruginosa*- and *S. typhimurium*-derived flagellin share with the identical potency, different from the action on human CEC, to induce IL-8 protein production by human intestinal epithelial cell (IEC) line HT29 (Fig. 5C). These results may suggest the possibility that both HCE-T and PHCE respond to ocular-pathogenic flagellin, but they poorly respond to ocular-nonpathogenic flagellin.

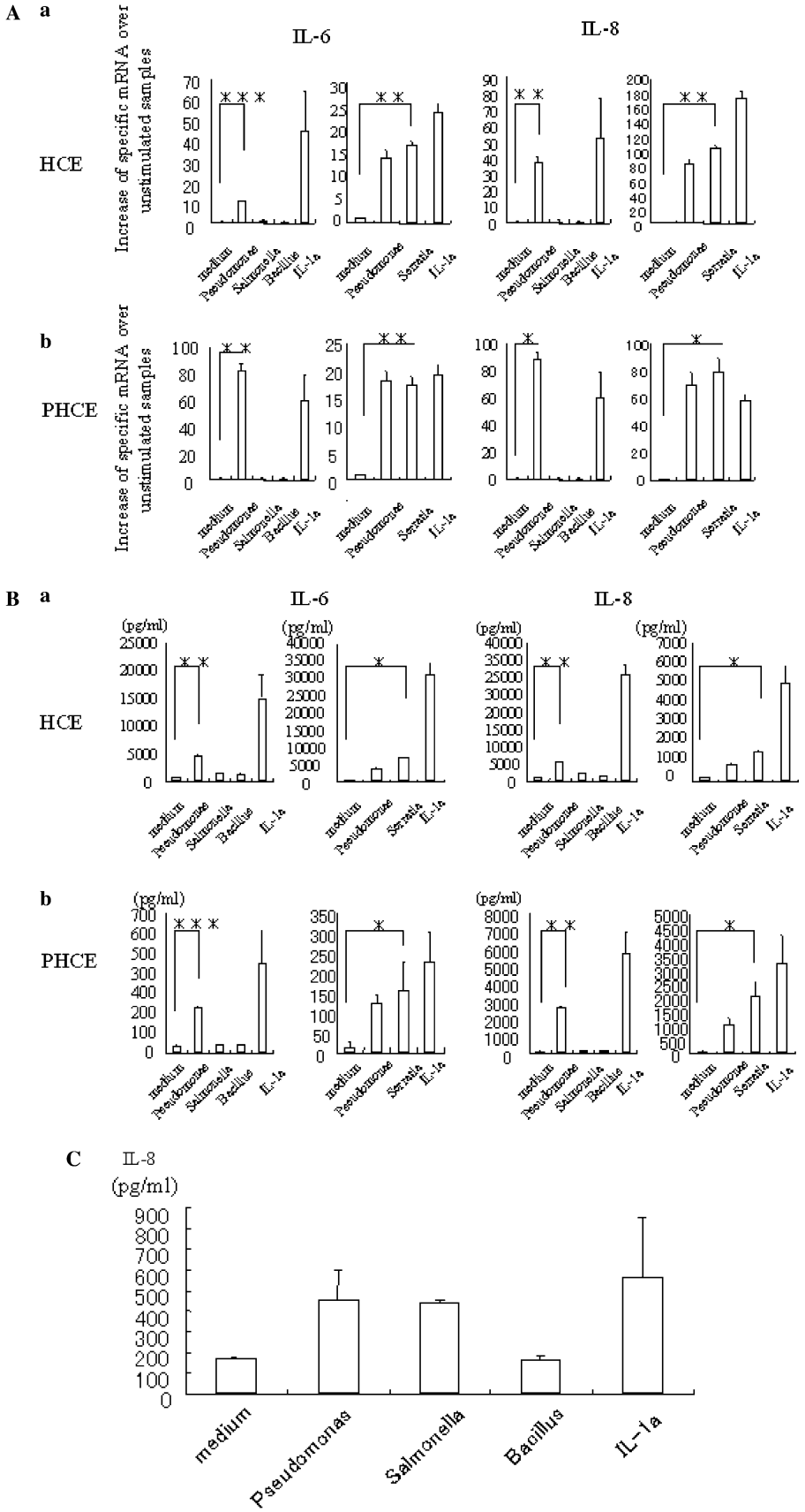
## Discussion

This is the first paper describing the selective reactivity of TLR5 on human CEC to flagellin derived of ocular-pathogenic bacteria, but not to those derived of nonpathogenic species. Furthermore, it is relevant to note that entero-pathogenic, but ocular-nonpathogenic, *Salmonella* strain derived flagellin did not induce pro-inflammatory responses to human CEC, whereas it did to human IEC, indicating the possibility of discriminating pathogenic bacteria from nonpathogenic species through the specified innate immune system on ocular surface.

Induction of the host epithelial cell pro-inflammatory gene program is essential for activating the host mucous inflammatory response and is important for bacterial clearance and host survival following infection [55,56]. On ocular surface, eyelids, tears, epithelium, and stroma help protect the outer eye against the environment [57]. The human corneal epithelium is continually exposed to external circumstance with diverse bacteria and bacterial products. Commensal bacteria in conjunctival sacs or along eyelids commonly cause no symptoms. This is partially dependent on nonspecific ocular innate immune defense mechanisms including blinking, tear flow, and mucin. Once physical or chemical injury to the corneal surface allows entry of live organisms and microbial products to the inner part, they can induce an inflammatory response that may lead to corneal destruction and visual loss.

Flagella are highly conserved among diverse bacterial species. Among ocular surface related bacteria, only a few of common causative ocular pathogens such as *P. aeruginosa*, *S. marcescense* have flagella, whereas *S. epidermidis* or Propionibacterium acnes known as commensal bacteria is devoid of flagella [31,32]. The main virulence protein in flagella is flagellin encoded by the *fliC* gene. Most if not all of the responses to bacterial flagellin are believed to be mediated by TLR5 [38,58,59]. TLR5 recognizes flagellin purified from both Gram-positive and -negative bacteria [60]. The impact of flagellin on human CEC has not been reported except for only one by Zhang et al. describing

Fig. 5. mRNA expression and production of IL-6 and IL-8 of human corneal epithelial cells by various flagellin. (A) To measure the expression of IL-6, IL-8 mRNA in HCE-T and PHCE after treated with each flagellin derived from *P. aeruginosa* or *S. typhimurium* or *B. subtilis* or *S. marcescense* (100 ng/ml) or human IL-α for 1 h, quantitative RT-PCR was performed. Data represent means ± SEM from an experiment with triplicate dishes. (B) To quantify inflammatory cytokine secretion, HCE-T and PHCE were plated in 25-cm dishes and, upon reaching subconfluence, were left untreated or were exposed to each flagellin derived from four different microbial bacteria (100 ng/ml) or 10 ng/ml human IL-1α for 24 h. The supernatants were harvested for measurement of IL-6 and IL-8. Data represent means ± SEM from an experiment with triplicate dishes. (C) To quantify inflammatory cytokine secretion, human colonic carcinoma cell line HT29 was cultured, and then was left untreated or was exposed to each flagellin derived from four different microbial bacteria (100 ng/ml) or 10 ng/ml human IL-1α for 24 h The supernatants were harvested for measurement of IL-8. Data represent means ± SEM from an experiment with triplicate dishes. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.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the response to *P. aeruginosa* derived flagellin via TLR5 [29]. There are conflicting reports demonstrating that the distinct epithelium evokes a distinct response even to the same flagellin [33,40–42]. We previously reported that human CEC failed to respond functionally to peptideglycan and LPS because of the lack of TLR2 and TLR4 on the surface [54], and that HCEC expressing TLR3 on the cell surface responded to poly(I:C) to generate pro-inflammatory cytokines and IFN-β [30]. In this context, innate immune responses of human CEC would be distinct from those in immune-competent cells, and a better understanding of the symbiotic relationship between CEC and commensal bacteria inhabiting the ocular mucous surface needs further intensive analysis.

Human IEC expressing TLR5 are hypo- or nonresponsive to flagellated bacteria in commensal bacterial flora. As TLR5 was selectively expressed spatially on the basolateral side of the intestinal epithelium, TLR5 triggering to flagellated bacteria might be efficient only when flagellated bacteria cross intestinal epithelia to contact the basolateral membrane [61]. Considering the spatially selective localization of TLR5 protein on the basolateral side of human corneal epithelium (Fig. 1B), it is possible that flagellin of *P. aeruginosa* or *S. marcescense* can induce inflammatory reaction, only when they cross corneal epithelium to contact the basolateral site.

TLRs recognize microbial ligands/flagellin produced by either pathogenic or commensal microbes and trigger inflammatory responses. However, the pathophysiologic relationship between bacterial flagellin and ocular surface inflammation has yet to be determined. It is indispensable to take into a consideration the new findings in regard to the pathology of TLR mediated noxious or beneficial mucosal innate immunity triggered by either pathogenic or commensal bacterial flora [62–64]. It is of special relevance that *S. typhimurium* flagellin, while it is strongly pathogenic and pro-inflammatory to IEC, had little effect on human CEC (Fig. 5). Furthermore, although *B. subtilis* is one of related species of *Bacillus cereus* that is a major cause of severe keratitis, endophthalmitis, and panophthalmitis [65], *B. subtilis* flagellin had no immune influence on human CEC, either. This may suggest the possibility that TLR5 in human CEC discriminates between ocular-pathogenic and ocular-nonpathogenic bacteria, but does not merely discriminate bacterial pathogenicity.

The sequences of the flagellins of *P. aeruginosa* [66], *S. enterica* serovar typhimurium [67], and *Listeria monocytogenes* [68] have similar regions that may be the binding sites of TLR-5 [69]. To the contrary, *P. aeruginosa* flagellin has evolved additional flagellar signaling mechanisms over that described for *Salmonella* flagellin [61]. There is also the possibility that TLR5 signaling activity is consistent with either the aid of another flagellin-recognizing co-receptor or the use of another adapter protein, that is absent or present at low levels in flagellin non- or low-responding cells [33]. A recent study described that gangliosides serve or do not serve as co-receptors for flagellin signaling via TLR5 [35,70]. We previously reported that human CEC expresses specific mRNA for all TLRs 1–10 but TLR8 [30], as well as intracellular TLR2 and TLR4 proteins. Human CEC failed to respond functionally to PAMPs, such as peptidoglycan from *S. aureus* and LPS from *P. aeruginosa* in signaling for the induction of IL-6 and IL-8 and the experimental translocation of LPS to the cytoplasm did not elicit an immune-response despite the existence of TLR2 and TLR4 in the cytoplasm of human CEC [54]. This rules out the possibility of direct participation of contaminated endotoxin, if any, in flagellin preparations in the presented experiments. This rationalization is also supported by the same result confirmed for the induction by human CEC of IL-6 and IL-8 in the presence of polymixyn B (data not shown). It is also quite intriguing in future to show whether it would be direct TLR5-flagellin interactions, either extra- or intra-cellular, that lead to the cytokine response. The use of whole bacteria with many contaminations, as opposed to purified bacterial products, to measure cytokine responses including bacteria strains with mutated flagella that do not bind TLR5 should be complemented to make our conclusion decisive.

It is a critical issue to be resolved to find out the molecular mechanism to explain the distinction in reactivity to human CEC observed among four flagellins derived of either ocular-pathogenic, ocular-nonpathogenic but entero-pathogenic or flagellin with almost null pathogenicity.

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