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NOD2 triggers PGE2 synthesis leading to IL-8 activation in *Staphylococcus aureus*-infected human conjunctival epithelial cells

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ABSTRACT

We previously showed that *Staphylococcus aureus* and *Pseudomonas aeruginosa* stimulate IL-8 expression in human conjunctival epithelial cells through different signal transduction pathways. As in some cell types both the bacteria may induce the release of prostaglandin E2 (PGE2) and PGE2 may affect the expression of IL-8, we aimed at investigating whether in human conjunctival cells infected with *S. aureus* or *P. aeruginosa* the activation of IL-8 transcription was mediated by PGE2 and which were the underlying molecular mechanisms. We found that *S. aureus*, but not *P. aeruginosa*, triggered IL-8 activation by increasing COX-2 expression and PGE2 levels in a time-dependent manner. Overexpression of nucleotide-binding oligomerization domain-2 (NOD2) resulted to be essential in the enhancement of IL-8 induced by *S. aureus*. It dramatically activated c-jun NH_2-terminal kinase (JNK) pathway which in turn led to COX2 upregulation and ultimately to *IL-8* transcription. The full understanding of the *S. aureus*-induced biochemical processes in human conjunctival epithelium will bring new insight to the knowledge of the molecular mechanisms involved in conjunctiva bacterial infections and develop novel treatment aiming at phlogosis modulation.

1. Introduction

The epithelium of the ocular surface, and particularly the conjunctival epithelium, is positioned at the interface between the internal milieu and the external environment and helps to prevent the entrance of microbes into the eye. It serves a critical function in the front-line defense of the innate immune system against microbes through non-specific mechanisms, among which the production of a variety of proinflammatory cytokines/chemokines [1]. This feature enables a swift immune response to invaders but may create the danger of over-reaction that may induce the initiation and perpetuation of inflammatory responses [1].

The overproduction of the chemokine IL-8, through transcriptional or epigenetic mechanisms, may lead to exacerbation of tissue injury through the amplification of inflammation [2]. IL-8 was shown to be activated as in *Staphylococcus aureus*- as in *Pseudomonas aeruginosa*-triggered human conjunctivitis albeit through different signal transduction pathways: *S. aureus* via JNK activation and *P. aeruginosa* [3–5] via the cooperative binding of Rel-A and C/EBP-β to IL-8 promoter. As in a variety of cells these two bacteria increase the production of PGE2 [6,7], which at times may induce IL-8 production [8–10], here we investigated whether in conjunctival epithelial cells they activate IL-8 through PGE2. Furthermore, we attempted to identify the signaling components upstream PGE2 secretion. Elucidating these mechanisms may be of interest, as in several instances an increase of PGE2 synthesis may be responsible for exaggerated host defense reactions against bacterial infections [11–13]. In this light, a setting of PGE2 signaling may have potential as a form of adjuvant therapy for bacterial conjunctivitis.

2. Materials and methods

2.1. Cell cultures and treatment

The investigation adhered to the Declaration of Helsinki and it was approved by the Ethics Committee of the University Hospital of Messina. Primary and Chang conjunctival epithelial cells were cultured and challenged with *S. aureus* and *P. aeruginosa*, as previously reported [4,5,14]. Where indicated, cells were transfected with COX-2 or NOD2 siRNAs (0.5 μg; Santa-Cruz Biotechnology, CA), or with NOD2 plasmid (2 μg) [15], or treated with SP600125 (SP; 20 μM; Calbiochem, Milan, Italy); U0126 (1 μM; Calbiochem); SB203580 (SB; 1 μM; Calbiochem); CAPE (10 μM; Calbiochem),

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SC-506 (300 μM, Cayman Chemical, Ann Arbor, MI), NS-398 (300 μM, Cayman Chemical) 1 h before *S. aureus* [3×10^6 CFU; multiplicity of infection (MOI) of 1] or muramyldipeptide (MDP; 10 μg/ml; Sigma–Aldrich Milan, Italy) stimulation.

2.2. IL-8 and PGE2 production

IL-8 and PGE2 were measured by enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical).

2.3. RNA extraction and reverse transcription

1 μg of total RNA extracted with TRIZOL (Invitrogen, Milan, Italy) was reverse-transcribed with IMProm-II™ kit (Promega, Milan, Italy).

2.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Milan, Italy). Primers and probes were as follows: for IL-8, *5′-CTCACTGTGTGTAAACATGACTTCCA-3′* (forward primer), *5′-TTCACACAGAGCTGCAGAAATCA-3′* (reverse primer) and *5′-FAM-CCGTGGCTCTCTTTGGCAGCCTTC-MGBNFQ-3′* (probe); for COX-1, *5′-AGCAGCTTTTCCAGACGACC-3′* (forward primer), *5′-CGGTTGCGGTATTGGAACTG-3′* (reverse primer) and *5′-FAM-CGTGCAGCAGCTGAGTGGCTATTTCC-MGBNFQ-3′* (probe); for COX-2, *5′-CCAGCACTTCACGCATCAGT-3′* (forward primer), *5′-ACGCTGTCTAGCCAGAGTTTCAC-3′* (reverse primer) and *5′-FAM-GGCTGGGCCATGGGGTGGACTTAAAT-MGBNFQ-3′* (probe); for NOD2, *5′-CCGAGGCATCTGCAAGCTCA-3′* (forward primer), *5′-TGCAAGGCTCTGTATTTGC-3′* (reverse primer) and *5′-FAM-CTCGCAGTGAAGAGCACATT-MGBNFQ-3′* (probe). Thermal cycling conditions included activation at 95 °C (10 min) followed by 40 cycles each of denaturation at 95 °C (15 s) and annealing/elongation (1 min) at 60 °C. Each sample was analyzed with beta-actin (Applied Biosystems) as housekeeping gene.

2.5. Transient transfections

Cells were transiently transfected with IL-8 [8], COX-2 [16] or JNK (PathDetect Kit, Stratagene) promoter/luciferase reporter constructs. Cells were harvested, and protein extracts were prepared for the luciferase activity using luciferine (Promega, Milan, Italy) as substrate.

2.6. Western blotting

The membranes were probed with anti-NOD2 (Santa Cruz Biotechnology, CA), anti-COX-2 (Cell Signaling Technology, Milan, Italy) and phosphorylated JNK (Cell Signaling Technology). Immunoreactive bands were detected by autoradiography (SuperSignal West Pico Chemiluminescent Substrate System, Pierce).

2.7. Statistical analysis

Data are expressed as means ± S.D. from four determinations. Results were analyzed by two-tailed Student's *t* test. *p* values less than 0.05 were considered significant.

3. Results

3.1. S. aureus, but not P. aeruginosa, increases COX-2 expression and PGE2 production in conjunctival epithelial cells

Primary and Chang conjunctival epithelial cells were infected with 3×10^6 CFU live *P. aeruginosa* or *S. aureus* for 2, 3, 4, 6, 8, 12, 18, and 24 h and PGE2 amounts were determined. Fig. 1A shows that PGE2 rose at 2 h after *S. aureus* treatment and backed to basal levels within 12–24 h. On the contrary, *P. aeruginosa* did not affect PGE2 production. To examine whether the *S. aureus*-induced increase of PGE2 was correlated to the activation of the expression of cyclooxygenase-1 and -2 (COX-1 and COX-2), quantitative real-time PCR (qRT-PCR) analysis was performed. The results reported in Fig. 1B and C show that, as compared to untreated cells, COX-1 mRNA expression remained unchanged after *S. aureus* stimulation, while the levels of COX-2 significantly increased by 2 h, peaked at 3 h, and were still elevated at 8 h. In contrast, *P. aeruginosa* challenge had no effect on either COX-1 or COX-2 mRNA abundance. To confirm that the PGE2 produced by *S. aureus* was through COX-2, cells were treated with the specific COX-1 inhibitor SC-506 and the COX-2-selective inhibitor NS-398. As shown in Fig. 1D, NS-398 suppressed PGE2 production induced by *S. aureus* in a dose-dependent manner, whereas SC-506 did not affect the PGE2 synthesis induced by the bacterium. These data show a key role for COX-2 in the PGE2 response to *S. aureus* in conjunctival epithelium and point to any contribution of COX-1.

3.2. S. aureus-induced IL-8 increase is mediated by COX-2 and PGE2

To verify whether the *S. aureus*-induced increase of COX-2 transcript was responsible for the induction of IL-8 upregulation, we investigated the effects of either NS-398, or a synthetic siRNA targeting COX-2, on IL-8 activation in *S. aureus*-treated cells. Both the treatments significantly reduced the upregulation of IL-8 promoter (Fig. 2B), the overexpression of IL8 mRNA (Fig. 2C), and the overproduction of IL-8 protein (Fig. 2D) induced by *S. aureus*. On the contrary, the inhibition of COX-2 expression had no effect on *P. aeruginosa*-triggered IL-8 activation (data not shown). To determine the involvement of PGE2 in *S. aureus*-induced IL-8 production, cultures were treated for 6 h with various concentrations of this prostaglandin in the presence or absence of the bacterium. As seen in Fig. 2E, progressively elevated levels of IL-8 were observed when the cells were treated with PGE2 from 10^{-6} M up to 10^{-4} M, and even more so when the cells were also infected by *S. aureus*. Therefore, it can be assumed that *S. aureus* led to IL-8 release through PGE2 enhancement in conjunctival epithelial cells.

3.3. S. aureus induced enhancement of COX-2 and IL-8 through upregulation of nucleotide-binding oligomerization domain-2 (NOD2)

As previous studies indicated that multiple intracellular pathogens, including *S. aureus*, are sensed by NOD2 [17], we investigated whether NOD2 is involved in *S. aureus*-induced conjunctivitis. Conjunctival epithelial cells treated with the pathogen exhibited a significant increase in NOD2 mRNA and protein levels with a peak at 2 h and 3 h after treatment, respectively (Fig. 3A). Then we investigated whether the overexpression of NOD2 affected COX2 levels. Cells overexpressing NOD2 led to enhanced promoter activation, mRNA levels and protein amount of COX-2 and stimulation of NOD2-transfected cells with *S. aureus* resulted in significantly higher levels of COX-2 expression (Fig. 3B). To confirm the involvement of NOD2 in the *S. aureus*-induced COX-2 activation, we performed gene silencing experiments using a synthetic NOD2 siRNA. Inhibition of NOD2 expression completely suppressed the *S. aureus*-induced COX-2 expression at promoter, mRNA and protein level (Fig. 3C), thus providing definitive evidence of the functional role of NOD2 in *S. aureus*-induced COX-2 gene expression in conjunctival cells. Next we investigated the signaling pathway involved in the NOD2-mediated enhancement of COX-2 expression in response to *S. aureus* challenge. Fig. 3D shows that either *S. aureus* or the NOD2 agonist MDP increased COX-2 mRNA levels through JNK, as their effects were antagonized by treatment

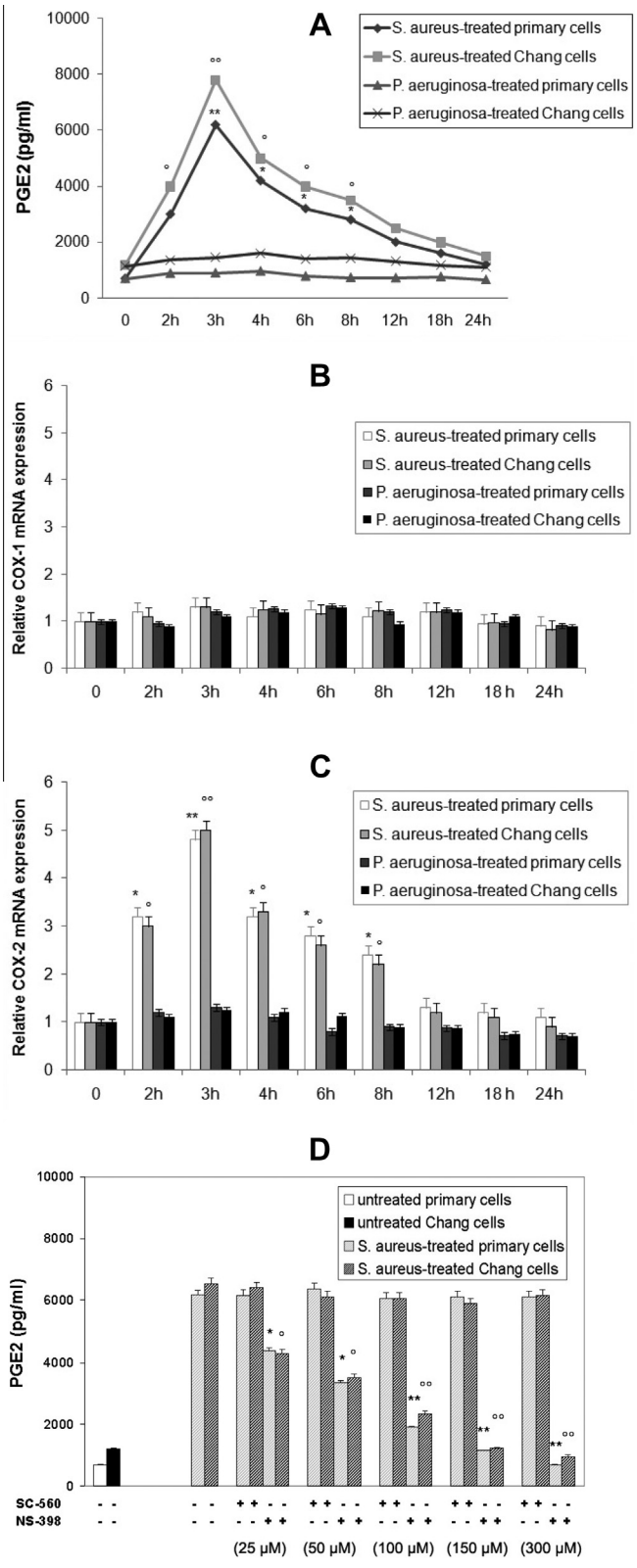

Fig. 1. PGE2, COX-1, and COX-2 expression in conjunctival epithelial cells infected by *S. aureus* or *P. aeruginosa*. Cells were incubated with 3×10^6 CFU live *S. aureus* or *P. aeruginosa* for the indicated times. (A) PGE2 was measured by ELISA. (B) and (C) RNA was extracted, reverse-transcribed, and quantified by real-time PCR as its ratio to β-actin. $^{*}p < 0.05$ and $^{**}p < 0.01$, and $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$, as compared with primary and Chang cells, respectively. (D) Cells were treated with *S. aureus* and incubated with 25, 50, 100, 150, and 300 μM SC-560 or NS-398. PGE2 was measured as in Section A. $^{*}p < 0.05$ and $^{**}p < 0.01$, and $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$, as compared with *S. aureus*-treated primary and Chang cells, respectively. The data shown are means ± S.D. of four determinations.

with the specific inhibitor SP600125, and remained unchanged after treatment with MEK1/2, p38MAPK and NF-kB inhibitors. These data show that JNK pathway is crucial in COX-2 up-regulation induced by *S. aureus* and NOD2. To define their effects on JNK activation, a JNK luciferase reporter assay and Western blot experiments were conducted in conjunctival epithelial cells transfected with NOD2 expression plasmid or specific siRNA in the absence or presence of *S. aureus*. As shown in Fig. 3 (Section E), ectopic expression of NOD2 in untreated cells resulted in JNK promoter up-regulation and phosphorylation that were further enhanced by *S. aureus* treatment. On the contrary, NOD2 silencing repressed JNK activation at transcriptional and post-transductional levels induced by *S. aureus*.

We next aimed at evaluating whether IL-8 activation induced by *S. aureus* was mediated by NOD2. To this end, *S. aureus*-infected conjunctival epithelial cells were transfected with NOD2-expressing plasmid and IL-8 expression was evaluated. Fig. 4A shows that NOD2 overexpression increased IL-8 transcriptional activation as well as mRNA and protein levels and that these effects were significantly enhanced by *S. aureus* treatment. To definitely prove that NOD2-triggered events participate in the *S. aureus*-mediated effects ultimately leading to IL-8 overproduction, we knocked down NOD2 by a siRNA approach. Fig. 4B shows that NOD2 silencing abrogated the *S. aureus*-induced enhancement of IL-8 promoter activation, mRNA expression and protein synthesis. These results show that in conjunctival epithelium NOD2 is required to activate IL-8 in response to *S. aureus* stimulation.

4. Discussion

Data presented in this study showed that, following *S. aureus* infection, human conjunctival epithelial cells increase their PGE2 content through upregulation of NOD2, JNK and COX-2 leading to IL-8 overexpression. *S. aureus* had been previously shown to induce PGE2 synthesis in some cell systems [6,18], but this is the first evidence of PGE2 as mediator of *S. aureus*-induced conjunctivitis. Here we also showed that conjunctival epithelium did not exhibit any variation in NOD2-dependent COX-2 expression and PGE2 synthesis after *P. aeruginosa* challenge. It may sound odd since NOD2 is an intracellular sensor that recognizes MDP, a common component of peptidoglycan of both gram positive and gram negative bacteria [19]. But it may be postulated that *P. aeruginosa* did not do the same as *S. aureus* in inducing NOD2, COX2 and PGE2 for the different interplay occurring between toll-like receptors (TLRs) and NOD2 signaling following gram-negative and gram-positive infection. Recent findings showed that RICK (receptor-interacting protein-like interacting caspase-like apoptosis regulatory protein kinase), an adaptor that is required for NOD1 and NOD2 signaling, plays a role in the inflammatory response induced by *P. aeruginosa* only in macrophages prestimulated with LPS, a condition associated with TLR4 tolerization. Therefore, it is well established that NOD2 signaling is secondary to TLRs involvement in gram-negative recognition [20]. A similar mechanism may be postulated in *P. aeruginosa*-infected conjunctival epithelial cells. Human conjunctival epithelium has a pivotal role in the defense of ocular surface for its ability to mount an innate immune response [21] and to modulate the synthesis of PGE2 following a variety of stimuli [22]. PGE2 is one of the most important soluble factors that serves to mediate the phlogosis and the immune response against infections by upregulating the levels of various cytokines [23,24]. Data presented here not only differentiated the role of *S. aureus* and *P. aeruginosa* in inducing PGE2 production by conjunctiva, but also established that this prostaglandin is the key hub in the *S. aureus*-triggered overexpression of IL-8 (Fig. 2). PGE2 has been previously shown to activate IL-8 gene expression in some cell

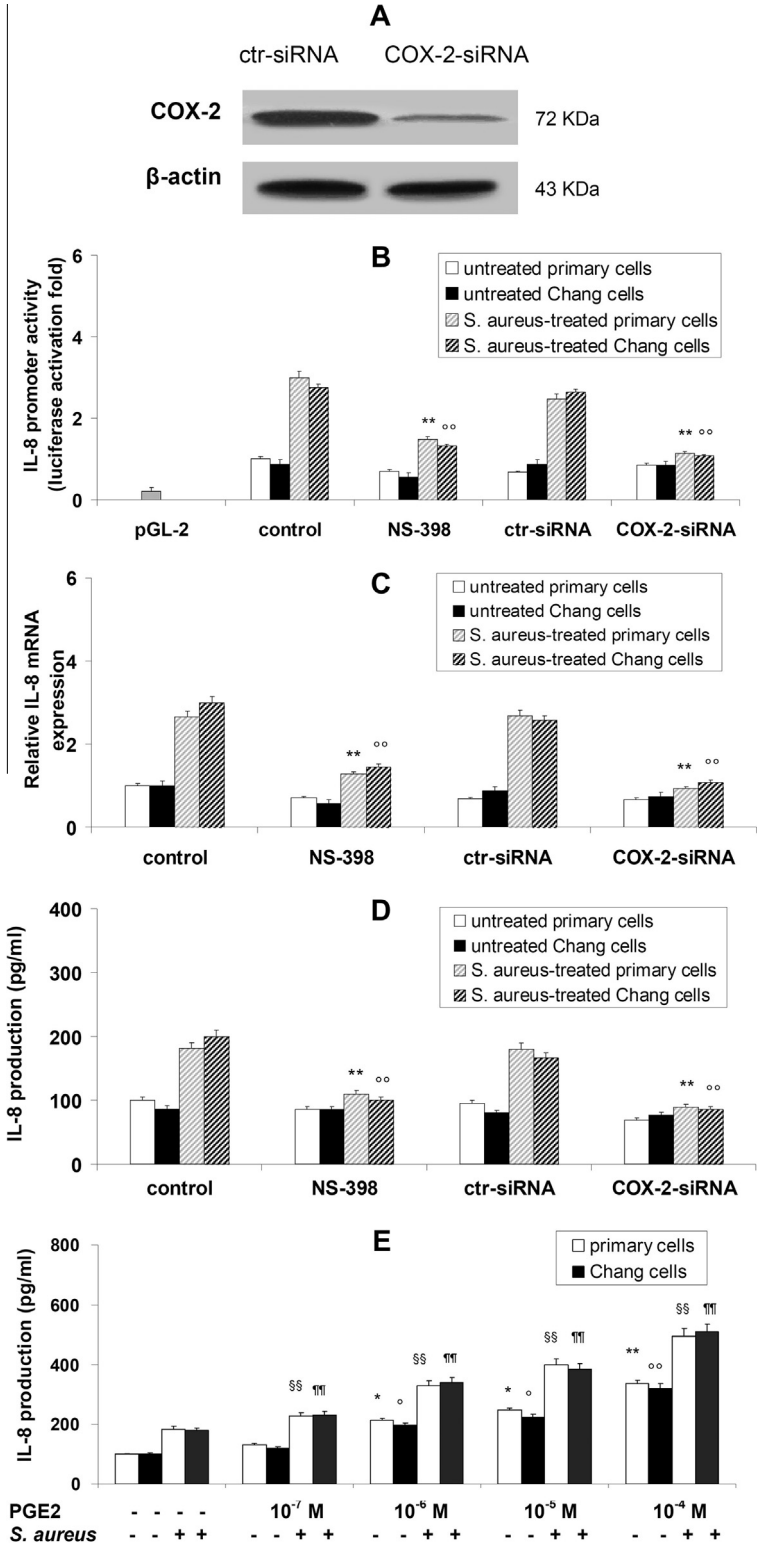

Fig. 2. Effects of COX-2 inhibition and PGE2 treatment on *S. aureus*-induced IL-8 expression. (A) Representative immunoblot of COX-2 protein in Chang conjunctival cells transfected with negative control (ctr) and COX-2-specific siRNA and stimulated with *S. aureus*. (B) The promoter, (C) mRNA, and (D) protein expression levels of IL-8 were examined by luciferase assay, qRT-PCR and ELISA, respectively, in unstimulated or *S. aureus*-stimulated primary and Chang cells treated with NS-398 or transfected with a COX-2 siRNA. $^{**}p < 0.01$, NS-398-treated, or COX-2 siRNA-transfected and *S. aureus*-stimulated primary cells *versus* *S. aureus*-stimulated cells, $^{\circ\circ}p < 0.01$, NS-398-treated, or COX-2 siRNA-transfected and *S. aureus*-stimulated Chang cells *versus* *S. aureus*-stimulated cells. (E) Cells were treated with 10^{-7}–10^{-4} M PGE2 for 6 h and where indicated with *S. aureus*. IL-8 protein was measured by ELISA. $^{*}p < 0.05$ and $^{**}p < 0.01$, and $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$ PGE2-treated primary and Chang cells, respectively, *versus* untreated cells; $^{\S\S}p < 0.01$ and $^{\P\P}p < 0.01$, PGE2-treated and *S. aureus*-stimulated primary and Chang cells, respectively, *versus* PGE2-treated cells. Data are depicted as the mean ± S.D. (*error bars*) of four independent experiments.

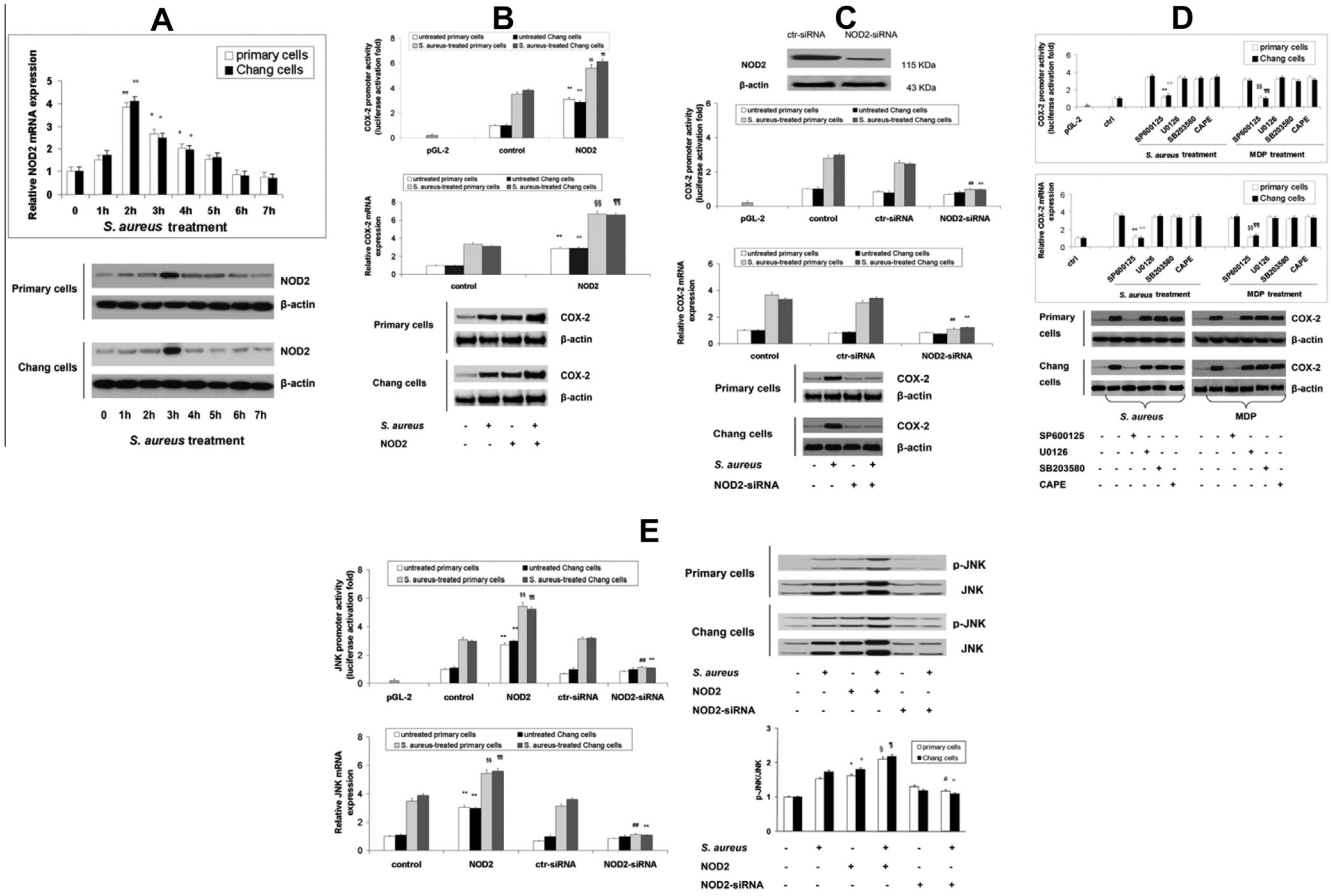

Fig. 3. Role of NOD2 in *S. aureus*-induced COX-2 overexpression. (A) NOD2 mRNA and protein were examined in *S. aureus*-treated cells for the indicated times by qRT-PCR and Western blot, respectively, as its ratio to β-actin. $^{*}p < 0.05$ and $^{**}p < 0.01$, and $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$, as compared with primary and Chang cells, respectively. (B) The promoter, mRNA and protein expression of COX-2 was examined by luciferase assay, qRT-PCR and Western blot, respectively, in unstimulated or *S. aureus*-stimulated cells transfected with a NOD2-expressing plasmid. $^{**}p < 0.01$ and $^{\circ\circ}p < 0.01$, NOD2-transfected primary and Chang cells, respectively, *versus* untransfected cells; $^{\S\S}p < 0.01$ and $^{\P\P}p < 0.01$ NOD2-transfected and *S. aureus*-treated primary and Chang cells, respectively, *versus* NOD2-transfected cells. (C) Representative immunoblot of NOD2 protein in Chang conjunctival cells transfected with negative control (ctr) and NOD2-specific siRNA and stimulated with *S. aureus*. The promoter, mRNA and protein expression of COX-2 was examined in unstimulated or *S. aureus*-stimulated cells transfected with a NOD2 siRNA as in Section B. $^{\#\#}p < 0.01$ and $^{\wedge\wedge}p < 0.01$, NOD2 siRNA-transfected and *S. aureus*-treated primary and Chang cells, respectively, *versus S. aureus*-treated cells. (D) The promoter, mRNA and protein expression of COX-2 was examined in *S. aureus*- or MDP-stimulated cells pretreated with SP600125 (SP; 20 μM), U0126 (1 μM); SB203580 (SB; 1 μM), CAPE (10 μM) by luciferase assay, qRT-PCR and Western blot, respectively. $^{**}p < 0.01$ and $^{\circ\circ}p < 0.01$, SP600125-treated/*S. aureus*-stimulated primary and Chang cells, respectively, as compared with *S. aureus*-stimulated cells. $^{\S\S}p < 0.01$ and $^{\P\P}p < 0.01$, SP600125-treated/MDP-stimulated primary and Chang cells, respectively, as compared with MDP-stimulated cells. (E) JNK luciferase assay, JNK mRNA levels and immunoblot of phosphorylated and total JNK in untreated or *S. aureus*-treated cells transfected with a NOD2 plasmid or a NOD2 siRNA. $^{*}p < 0.05$ and $^{**}p < 0.01$, and $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$, NOD2-transfected primary and Chang cells, respectively, *versus* untransfected cells; $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$, and $^{\P}p < 0.05$ and $^{\P\P}p < 0.01$, NOD2-transfected and *S. aureus*-treated primary and Chang cells, respectively, *versus* NOD2-transfected cells; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, and $^{\wedge}p < 0.05$ and $^{\wedge\wedge}p < 0.01$, NOD2 siRNA-transfected and *S. aureus*-treated primary and Chang cells, respectively, *versus S. aureus*-treated cells. Results are expressed as the mean ± S.D. of four independent experiments performed in triplicate.

types [8,10,13,25–28], but until now no data has been available for a similar effect in conjunctival epithelium. Moreover, despite NOD2-dependent COX2 expression and PGE2-induced IL-8 production have been reported, this is the first study that shows such sequence of correlated events following *S. aureus* infection. The present research filled the gap between *S. aureus* stimulus and IL-8 production in conjunctiva, previously shown by others and us [3–5], and established that PGE2 synthesis is the missing link in conjunctivitis triggered by *S. aureus*. Elucidating this mechanism could lead to a better understanding of pathogenesis, progression, and treatment of the most frequent bacterial conjunctivitis [29]. In this work we also showed that *S. aureus* infection promoted the expression of the inducible form COX-2, but not the constitutive form COX-1, and that COX-2 inhibition suppressed the production of PGE2 following *S. aureus* infection (Fig. 1). These data are in agreement with the research literature on this issue reporting that COX-2 is markedly expressed during several inflammatory processes and this event generally precedes the overproduction of PGE2 [11,30,31]. The present data highlight the crucial role of COX-2 and PGE2 in the production of IL-8 by the *S. aureus*-infected human conjunctiva (Fig. 2) and assess their strategic importance in external eye infections. Moreover, experiments reported here elucidated as well the mechanisms by which *S. aureus* increased PGE2 amounts showing that NOD2 is essential in activating COX2 expression. Our findings support the note that NOD2 upregulation accounted for the sensitization effects upon *S. aureus* treatment of conjunctival epithelial cells as *S. aureus* increased COX2 and IL-8 up-regulation through NOD2 (Figs. 3 and 4). Indeed, several reports have suggested a critical relationship between NOD2 and COX2 production [32–34], but, to our knowledge, none of them concerns the conjunctival epithelium. In the search of molecular mechanisms underlying the induction of PGE2 synthesis by *S. aureus* in conjunctiva, we found that JNK pathway is implicated in the activation of COX-2 mediated by NOD2. It is worth to note that JNK signaling, but not NF-kB and p38 MAPK, was shown by our previous data to be the transduction pathway responsible for *IL-8*

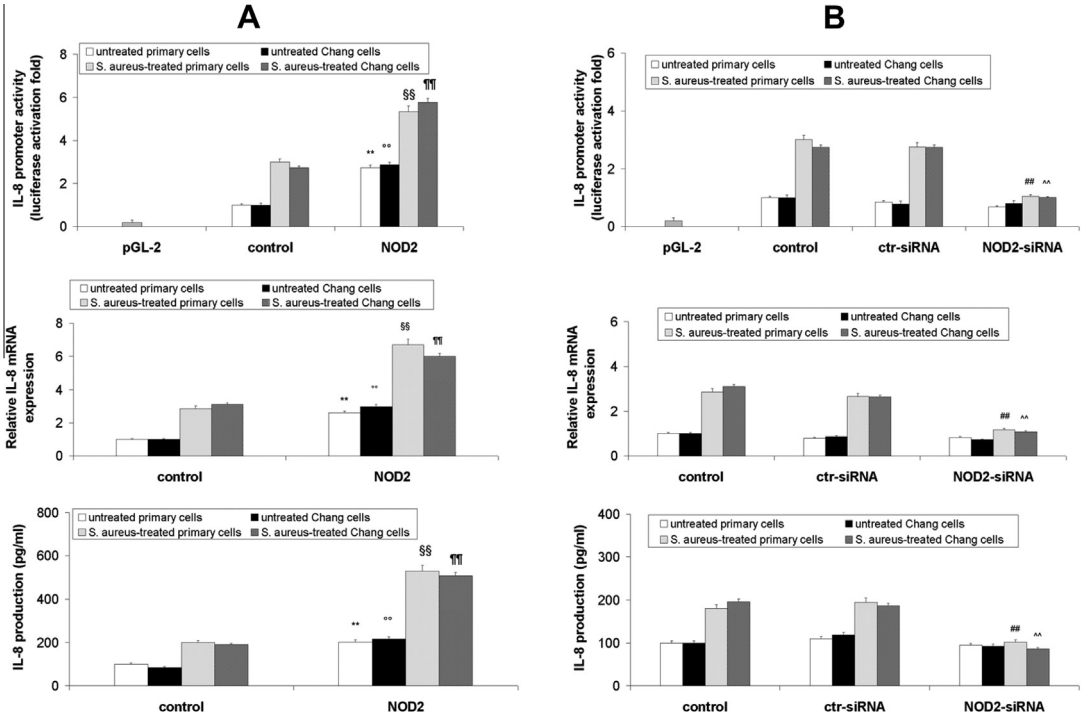

Fig. 4. Effects of NOD2 on IL-8 expression. (A) The promoter, mRNA and protein expression of IL-8 was examined as in Fig. 2 in unstimulated or *S. aureus*-stimulated cells transfected with a NOD2 plasmid. $^{**}p < 0.01$ and $^{\circ\circ}p < 0.01$, NOD2 overexpressing primary and Chang cells, respectively, *versus* untransfected cells; $^{\S\S}p < 0.01$ and $^{\P\P}p < 0.01$, NOD2-transfected and *S. aureus*-stimulated primary and Chang cells, respectively, *versus* NOD2-transfected cells. (B) The promoter, mRNA and protein expression levels of IL-8 were examined as in Fig. 2 in unstimulated or *S. aureus*-stimulated cells transfected with a NOD2 siRNA. $^{\#\#}p < 0.01$ and $^{\wedge\wedge}p < 0.01$, NOD2 siRNA-transfected and *S. aureus*-treated primary and Chang cells, respectively, *versus S. aureus*-treated cells. Data are depicted as the mean ± S.D. (*error bars*) of four independent experiments.

expression by *S. aureus*-infected human conjunctival epithelium [4]. Taken together these data allow to speculate that in this cell system *S. aureus* operates by upregulating the expression of NOD2 which activates JNK transcription and phosphorylation resulting in COX2 activation and PGE2 production responsible for IL-8 overexpression. Our results are in line with reports showing that NOD2 is an important regulatory point for the coordination of signaling pathways activated downstream Gram-positive bacteria infection by inducing activation of MAPKs, including JNK, which usually initiates the production of pro-inflammatory cytokines [35]. But, to the best of our knowledge, the requirement for NOD2 in specific signaling pathways leading to IL-8 production in response to *S. aureus* has never been investigated in human cell systems. Results reported here open up intriguing new scenarios where the conjunctival immune surveillance against *S. aureus* challenge goes through JNK-mediated stimulatory effects on COX2 and PGE2 expression leading to IL-8 upregulation, and highlight that NOD2 was essential for an efficient activation program triggered by *S. aureus*. Findings reported here may serve as a paradigm to understand the pathogenesis of *S. aureus*-associated conjunctivitis and clearly pave a way toward development of novel therapeutics.

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References

[1] M. Ueta, S. Kinoshita, Innate immunity of the ocular surface, Brain Res. Bull. 81 (2–3) (2010) 219–228.
[2] I. Venza, M. Visalli, R. Oteri, et al., Class II-specific histone deacetylase inhibitors MC1568 and MC1575 suppress IL-8 expression in human melanoma cells, Pigment Cell Melanoma Res. 26 (2013) 193–204.
[3] E.B. Cook, J.L. Stahl, S. Esnault, et al., Toll-like receptor 2 expression on human conjunctival epithelial cells: a pathway for *Staphylococcus aureus* involvement in chronic ocular proinflammatory responses, Ann. Allergy Asthma Immunol. 94 (2005) 486–497.
[4] I. Venza, M. Cucinotta, S. Caristi, et al., Transcriptional regulation of IL-8 by *Staphylococcus aureus* in human conjunctival cells involves activation of AP-1, Invest. Ophthalmol. Vis. Sci. 48 (2007) 270–276.
[5] I. Venza, M. Cucinotta, M. Visalli, et al., *Pseudomonas aeruginosa* induces interleukin-8 (IL-8) gene expression in human conjunctiva through the recruitment of both RelA and CCAAT/enhancer-binding protein beta to the IL-8 promoter, J. Biol. Chem. 284 (2009) 4191–4199.
[6] C.A. Pérez-Novo, A. Waeytens, C. Claeys, et al., *Staphylococcus aureus* enterotoxin B regulates prostaglandin E2 synthesis, growth, and migration in nasal tissue fibroblasts, J. Infect. Dis. 197 (2008) 1036–1043.
[7] K.A. Kernacki, R.S. Berk, Characterization of arachidonic acid metabolism and the polymorphonuclear leukocyte response in mice infected intracorneally with *Pseudomonas aeruginosa*, Invest. Ophthalmol. Vis. Sci. 36 (1995) 16–23.
[8] S. Caristi, G. Piraino, M. Cucinotta, et al., Prostaglandin E2 induces interleukin-8 gene transcription by activating C/EBP homologous protein in human T lymphocytes, J. Biol. Chem. 280 (2005) 14433–14442.
[9] W.W. Zhong, P.A. Burke, M.E. Drotar, et al., Effects of prostaglandin E2, cholera toxin and 8-bromo-cyclic AMP on lipopolysaccharide-induced gene expression of cytokines in human macrophages, Immunology 84 (1995) 446–452.
[10] V. Srivastava, I. Dey, P. Leung, et al., Prostaglandin E2 modulates IL-8 expression through formation of a multiprotein enhanceosome in human colonic epithelial cells, Eur. J. Immunol. 42 (2012) 912–923.
[11] M.D. Díaz-Muñoz, I.C. Osma-García, C. Cacheiro-Llaguno, et al., Coordinated up-regulation of cyclooxygenase-2 and microsomal prostaglandin E synthase 1 transcription by nuclear factor kappa B and early growth response-1 in macrophages, Cell. Signal. 22 (2010) 1427–1436.
[12] L. Ulmann, H. Hirbec, F. Rassendren, P2X4 receptors mediate PGE2 release by tissue-resident macrophages and initiate inflammatory pain, EMBO J. 29 (2010) 2290–2300.
[13] M. Venza, M. Visalli, C. Alafaci, et al., Interleukin-8 overexpression in astrocytomas is induced by prostaglandin E2 and is associated with the

transcription factors CCAAT/enhancer-binding protein-β and CCAAT/enhancer-binding homologous protein, Neurosurgery 69 (2011) 713–721.
[14] S. Narayanan, W.L. Miller, A.M. McDermott, Expression of human β-defensins in conjunctival epithelium: relevance to dry eye disease, Invest. Ophthalmol. Vis. Sci. 44 (2003) 3795–3801.
[15] P. Hruz, A.S. Zinkernagel, G. Jenikova, et al., NOD2 contributes to cutaneous defense against *Staphylococcus aureus* through alpha-toxin-dependent innate immune activation, Proc. Natl. Acad. Sci. USA 106 (2009) 12873–12878.
[16] Y.M. Chiang, C.P. Lo, Y.P. Chen, et al., Ethyl caffeate suppresses NF-κB activation and its downstream inflammatory mediators, iNOS, COX-2, and PGE2 in vitro or in mouse skin, Br. J. Pharmacol. 146 (2005) 352–363.
[17] X. Xie, L. Wang, F. Gong, et al., Intracellular *Staphylococcus aureus*-induced NF-κB activation and proinflammatory responses of P815 cells are mediated by NOD2, J. Huazhong Univ. Sci. Technol. Med. Sci. 32 (2012) 317–323.
[18] S.N. Somayaji, S. Ritchie, M. Sahraei, et al., *Staphylococcus aureus* induces expression of receptor activator of NF-kappaB ligand and prostaglandin E2 in infected murine osteoblasts, Infect. Immun. 76 (2008) 5120–5126.
[19] S.E. Girardin, I.G. Boneca, J. Viala, et al., Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection, J. Biol. Chem. 278 (2003) 8869–8872.
[20] J.H. Park, Y.G. Kim, G. Núñez, RICK promotes inflammation and lethality after gram-negative bacterial infection in mice stimulated with lipopolysaccharide, Infect. Immun. 77 (2009) 1569–1578.
[21] M. Hingorani, V.L. Calder, R.J. Buckley, et al., The role of conjunctival epithelial cells in chronic ocular allergic disease, Exp. Eye Res. 67 (1998) 491–500.
[22] S. Viau, L. Leclère, B. Buteau, et al., Polyunsaturated fatty acids induce modification in the lipid composition and the prostaglandin production of the conjunctival epithelium cells, Graefes Arch. Clin. Exp. Ophthalmol. 250 (2012) 211–222.
[23] I.M. Toller, I. Hitzler, A. Sayi, et al., Prostaglandin E2 prevents *Helicobacter*-induced gastric preneoplasia and facilitates persistent infection in a mouse model, Gastroenterology 138 (2010) 1455–1467 (1467.e1-4).
[24] M. Takano, H. Nishimura, Y. Kimura, et al., Prostaglandin E2 protects against liver injury after *Escherichia coli* infection but hampers the resolution of the infection in mice, J. Immunol. 161 (1998) 3019–3025.
[25] H. Aso, S. Ito, A. Mori, et al., Prostaglandin E2 enhances interleukin-8 production via EP4 receptor in human pulmonary microvascular endothelial cells, Am. J. Physiol. Lung Cell. Mol. Physiol. 302 (2012) L266–L273.
[26] J.A. Maybin, N. Hirani, H.N. Jabbour, et al., Novel roles for hypoxia and prostaglandin E2 in the regulation of IL-8 during endometrial repair, Am. J. Pathol. 178 (2011) 1245–1256.
[27] T. Li, J. Qi, E.A. Cowley, Activation of the EP4 prostanoid receptor induces prostaglandin E2 and pro-inflammatory cytokine production in human airway epithelial cells, Pulm. Pharmacol. Ther. 24 (2011) 42–48.
[28] I. Venza, M. Visalli, C. Fortunato, et al., PGE2 induces interleukin-8 derepression in human astrocytoma through coordinated DNA demethylation and histone hyperacetylation, Epigenetics 7 (2012) 1315–1330.
[29] K. Cavuoto, D. Zutshi, C.L. Karp, et al., Update on bacterial conjunctivitis in South Florida, Ophthalmology 115 (2008) 51–56.
[30] M. Hämäläinen, R. Nieminen, M.Z. Asmawi, et al., Effects of flavonoids on prostaglandin E2 production and on COX-2 and mPGES-1 expressions in activated macrophages, Planta Med. 77 (2011) 1504–1511.
[31] S. Pugh, G.A. Thomas, Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E2, Gut 35 (1994) 675–678.
[32] M.J. Kang, S.K. Heo, E.J. Song, et al., Activation of Nod1 and Nod2 induces innate immune responses of prostate epithelial cells, Prostate 72 (2012) 1351–1358.
[33] W.H. Tsai, D.Y. Huang, Y.H. Yu, et al., Dual roles of NOD2 in TLR4-mediated signal transduction and -induced inflammatory gene expression in macrophages, Cell. Microbiol. 13 (2011) 717–730.
[34] K. Bansal, K.N. Balaji, Intracellular pathogen sensor NOD2 programs macrophages to trigger Notch1 activation, J. Biol. Chem. 286 (2011) 5823–5835.
[35] S. Akira, K. Takeda, T. Kaisho, Toll-like receptors: critical proteins linking innate and acquired immunity, Nat. Immunol. 2 (2001) 675–680.