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Streptococcus cristatus modulates the *Fusobacterium nucleatum*-induced epithelial interleukin-8 response through the nuclear factor-kappa B pathway

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Background and Objective: We previously reported that the interleukin-8 (IL-8) response to *Fusobacterum nucleatum* was attenuated in the presence of *Streptococcus cristatus*. Here, we further examined the underlying mechanism(s) involved in the modulating effect of *S. cristatus* by looking specifically at its impact on the nuclear factor-kappa B (NF-κB) pathway under the toll-like receptor (TLR) signaling background.

Material and Methods: OKF6/TERT-2 and KB cells were co-cultured with *F. nucleatum* and *S. cristatus*, either alone or in combination. Secretion of IL-8 protein was measured by ELISA. The nuclear translocation of NF-κB was evaluated by confocal microscopy, while DNA-binding activity was quantified using TransAM™ ELISA kits. Western blot analysis was performed to determine whether the anti-inflammatory effect of *S. cristatus* is related to the modulation of the NF-κB inhibitory protein IκB-α.

Results: Incubation with *F. nucleatum* significantly enhanced the nuclear translocation of NF-κB. Exposure to *S. cristatus* alone did not cause detectable NF-κB translocation and was able to inhibit the *F. nucleatum*-induced NF-κB nuclear translocation. The TransAM assay further confirmed that *S. cristatus* blocked the nuclear translocation of NF-κB in response to *F. nucleatum* stimulation. In contrast to the nearly complete degradation of IκB-α induced by *F. nucleatum* alone, the presence of *S. cristatus* stabilized IκB-α. Pre-incubation with TLR2 and TLR4 antibodies, however, did not affect the epithelial response to either species alone or in combination.

Conclusion: The mechanism by which *S. cristatus* attenuates *F. nucleatum*-induced proinflammatory responses in oral epithelial cells appears to involve blockade of NF-κB nuclear translocation at the level of IκB-α degradation.

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It is estimated that indigenous bacteria, which normally colonize the human body, outnumber eukaryotic cells by 10-fold. A large proportion (approximately 700 species) of the body's normal or commensal bacteria reside in the oral cavity (1), interacting closely with different bacterial species and with host epithelial cells in the form of biofilms (2,3). Although the distribution of normal flora in the oral cavity is highly diverse and site- and subject-specific, most studies have identified streptococcal species, most commonly members of the alpha-hemolytic viridans group, as the predominant components of the normal oral flora (4–6). Conventional and molecular analyses of plaque samples have revealed that colonization of oral streptococci is associated with periodontal health and that a shift in the flora towards an increase in the number of gram-negative anaerobic and proteolytic bacteria accompanies the initiation and progression of periodontal disease (7–10).

Studies, mostly of gastrointestinal bacteria, have shown that endogenous microbial communities are not passive bystanders; instead, they are able to provide significant benefit to the host through blocking colonization by pathogens (11,12), and influencing the development of cell structure and function (13,14) and of the immune system (15,16). Evidence is accumulating to support a similar role for oral commensal bacteria. It has been shown that certain oral streptococci can inhibit the colonization of *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis* on epithelial surfaces (17,18), produce antimicrobial agents against mucosal and periodontal pathogens (19,20) or enhance cellular homeostatic mechanisms (21).

One of the most important characteristics of commensal bacteria is their ability to prevent the host immune system from being activated. A combination of finely tuned processes exists at the mucosal surfaces: permanent surveillance for trespassing microorganisms; and general tolerance to innocent residents to avoid clinical inflammation. The mechanisms that allow commensal microorganisms to be tolerated by host tissues are not well understood, and current evidence indicates that certain properties of the bacteria themselves, of the epithelial surface and of the immune cells present in the lamina propria might be involved (22). Some studies have demonstrated that pathogenic and nonpathogenic bacteria activate different signaling pathways and innate immune responses in epithelial cells (21,23), while others have shown that alteration in host toll-like receptor (TLR) signaling (24–28) or in the production of anti-inflammatory cytokines, such as interleukin (IL)-10 and transforming growth factor-β, were associated with tolerance to mucosal commensal bacteria (29).

Recently, emerging evidence has suggested that certain gut commensal and probiotic bacteria might participate in an active cross-talk with the host epithelium, to limit inflammatory signals triggered by proinflammatory stimuli (30,31). In the oral cavity, two members of the commensal streptococci – *Streptococcus cristatus* (32) and *Streptococcus salivarius* (33) – have been reported to be able to attenuate IL-8 production by *Fusobacterium nucleatum* and *Pseudomonas aeruginosa*, respectively, providing the first evidence for an active role of oral commensals in modulating the host defense process. Although the exact mechanisms for regulation and control of proinflammatory stimuli by the normal flora are not fully understood, inhibition of the nuclear factor-kappa B (NF–κB) pathway at different levels has been demonstrated (30,31,34,35).

We have previously shown that *S. cristatus* downregulates the proinflammatory responses induced by *F. nucleatum* in oral epithelial cells (32). In this study, we further examined the impact of both *S. cristatus* and *F. nucleatum* on NF-κB and on its major negative feedback regulator, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α), to test the hypothesis that *S. cristatus* is able to modulate epithelial proinflammatory responses to *F. nucleatum* through inhibiting the NF-κB pathway. The involvement of the pattern recognition receptors TLR2 and TLR4 was also investigated. We demonstrated that *S. cristatus* inhibited *F. nucleatum*-induced NF-κB activation through stabilizing IκB-α, which seemed to be independent of surface TLR blocking. Our data therefore contribute to understanding the oral commensal bacteria-exerted immunomodulatory effect on epithelial surfaces.

Material and methods

Bacterial strains and culture conditions

F. nucleatum (ATCC 10953) and *S. cristatus* CC5A (ATCC 49999) were routinely maintained under anaerobic conditions (85% N_2, 10% H_2 and 5% CO_2) at 37°C. For all experiments, *F. nucleatum* was cultured in Trypticase Soy Broth (BBL; Becton Dickinson, Sparks, MD, USA) supplemented with 1 g/L of yeast extract, 5 mg/L of hemin and 1 mg/L of menadione, while *S. cristatus* was grown in Todd Hewitt broth (BBL; Becton Dickinson).

Epithelial cell cultures

The epithelial carcinoma cell line KB was kindly provided by Dr Mark Herzberg (University of Minnesota). The immortalized normal human oral keratinocytes OKF6/TERT-2 were obtained under a materials transfer agreement from Dr James Rheinwald (Brigham and Women's Hospital, Boston, MA, USA). KB and OKF6/TERT-2 cell lines were maintained in 75-cm^2 flasks (Corning, Corning, NY, USA) in a humidified atmosphere of 5% CO_2 at 37°C. The OKF6/TERT-2 cell line was cultured in keratinocyte serum-free medium (Invitrogen, Grand Island, NY, USA) supplemented with $CaCl_2$ (0.4 mM), bovine pituitary extract (25 μg/mL) and epidermal growth factor (0.2 ng/mL). KB cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Invitrogen). Twenty-four hours before infection, cells were seeded into tissue culture plates or dishes (Corning) in triplicate. For the NF-κB inhibitor assays, KB cells were pretreated with IKK Inhibitor VII (Calbiochem, San Diego, CA, USA) in serum-free MEM for 1 h at

37°C in 5% CO_2. For TLR blocking, KB cells were incubated with 20 μg/mL of rabbit polyclonal anti-human TLR2 (sc-10739) or TLR4 (sc-10741) (Santa Crutz Biotechnology, Santa Crutz, CA, USA) in serum-free MEM for 1 h at 37°C in 5% CO_2. After pretreatment, cells were washed three times with 1× Dulbecco's phosphate-buffered saline (DPBS) before the addition of experimental stimuli.

Bacterial infection procedure

Overnight broth cultures of bacteria were harvested by centrifugation, washed twice with 1× DPBS and then resuspended in serum-free cell culture medium. After adjustment of the resuspended cells to a concentration of 10^8 colony-forming units/mL by alteration of the optical density with reference to a standard, *F. nucleatum* and/or *S. cristatus* were added to cell monolayers at a multiplicity of infection of 100 for each species and incubated for various periods of time at 37°C in 5% CO_2. Wells containing no bacteria served as negative controls. After stimulation, cell supernatants were collected for IL-8 assays in some experiments, and the cell monolayers were either fixed or harvested for various protein assays. All assays were carried out in triplicate and three independent experiments were performed. For all experiments, the viability of infected cells was examined by Trypan Blue exclusion.

Confocal microscopy

To visualize the intracellular localization of NF-κB, OKF6/TERT-2 and KB cells were grown overnight on LAB-TEK chamber slides (Nalge Nunc International, Naperville, IL, USA) at 37°C in 5% CO_2, and then incubated with *F. nucleatum* and *S. cristatus*, either alone or in combination, for various periods of time, at a multiplicity of infection of 100. After stimulation, cell monolayers on chamber slides were washed three times for 5 minutes each with ice-cold DPBS and then fixed with freshly made 4% paraformaldehyde (Sigma, St Louis, MO, USA) for 15 min at room temperature. The monolayers were then washed another three times, permeabilized with 0.2% Triton X-100 (Sigma) for 20 min and then incubated with 5% goat serum (Sigma) for 30 min to suppress the nonspecific binding of immunoglobulin G (IgG). Rabbit anti-human NF-κB p65 (sc-109; 1 μg/mL) and control IgG (sc-2027; 1 μg/mL) (Santa Crutz Biotechnology) were prepared in 1.5% normal blocking goat serum and incubated with cell monolayers for 1 h at room temperature. Cell monolayers were washed three times and then incubated with 2.5 μg/mL of goat anti-rabbit IgG Alexa Fluor 568 conjugate (Invitrogen; Molecular Probes, Carlsbad, CA, USA) for another 1 h. Samples were then mounted with coverslips and observed under a confocal laser scanning microscope (Olympus FluoView FV1000; Olympus Corporation, Tokyo, Japan).

A similar protocol was followed when conducting experiments for TLR analysis, with a few exceptions. For blocking experiments, KB cells were pre-incubated with 2 μg/mL of rabbit anti-human TLR2 (sc-10739) and TLR4 (sc-10741) (Santa Crutz Biotechnology) in serum-free MEM for 1 h before infection to block the surface receptors. In experiments studying the cellular localization of TLRs, 1 μg/mL of TLR2 (sc-10739) and 1 μg/mL of TLR4 (sc-10741) were used as primary antibodies after cell permeabilization, without prior pretreatment. To distinguish nuclear and cytoplasmic localization of TLRs, 200 nM 4′, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes) was utilized to stain the nuclei for 2 min at room temperature.

ELISA

The content of IL-8 in culture supernatants was quantified by ELISA. Briefly, high-binding enzyme immunoassay plates (Corning, Corning, NY, USA) were coated with polyclonal antibodies to IL-8 (Pierce, Rockford, IL, USA) and blocked with phosphate-buffered saline containing 4% bovine serum albumin and 5% sucrose. Samples or a recombinant IL-8 standard (Pierce) were added to the wells for 1.5 h and then the plates were washed three times with PBST (0.5% Tween 20). Biotinylated polyclonal antibodies to IL-8 (Pierce) were added for another 1.5 h. After extensive washing, NeutrAvidin-AP (Pierce) was added and the plates were further washed before the substrate para-nitrophenylphosphate (PNPP) (Pierce) was added. When color developed, the reaction was stopped with 2 N NaOH and the absorbance was read at 405 nm in an automated ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve of optical densities vs. concentrations of IL-8 was generated to determine the concentrations of IL-8 in the samples. The detection limit of the assay was 10 pg/mL.

NF-κB transcription factor assay

OKF6/TERT-2 and KB cells (5×10^7) were seeded into tissue culture dishes and grown overnight at 37°C in 5% CO_2. After infection with *F. nucleatum* and/or *S. cristatus* for varying periods of time, nuclear proteins were extracted using the Active Motif Nuclear Extract Protocol (Active Motif, Carlsbad, CA, USA) and the total protein concentration of the lysates was determined using the BCA Protein Assay kit (Pierce). Activation of the NF-κB p65 subunit in 5 μg of nuclear extracts was determined using an NF-κB p65 ELISA-based transcription factor assay kit (TransAM™ assay; Active Motif) according to the manufacturer's instructions. The NF-κB-detecting antibody recognizes an epitope on p65 that is accessible only when NF-κB is activated. The colorimetric reading at 450 nm was determined in a microplate reader (Molecular Devices). The positive-control Jurkat nuclear extract provided with the kit was used to assess assay specificity.

Western blot analysis

OKF6/TERT-2 and KB cells were seeded into tissue culture dishes and grown overnight at 37°C in 5% CO_2. After infection with *F. nucleatum* and/or *S. cristatus* for varying periods of time, cell monolayers were washed three times with ice-cold DPBS and then lysed in radioimmunoprecipitation buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate,

0.1% sodium dodecyl sulfate and 1.0% Nonidet P-40 containing the proteinase inhibitors 1 mM phenylmethanesulfonyl fluoride, 1.0 μg/mL of pepstatin A, 1.0 μg/mL of aprotinin, 1.0 μg/mL of leupeptin, 1 mM Na_3VO_4 and 1 mM NaF; Sigma). The protein concentration of the lysate was determined using the BCA Protein Assay kit (Pierce). Approximately 20 μg of protein extract was separated in 7.5% Tris–HCl Ready Gel Precast Gels (Bio-Rad, Hercules, CA, USA) and transferred to 0.45 μm nitrocellulose membranes (Bio-Rad). The membranes were blocked overnight with 5% nonfat milk in Tris-buffered saline/Tween 20 (TBST) buffer (0.5 M NaCl, 20 mM Tris, pH 7.5, containing 0.1% Tween-20) and then incubated with rabbit anti-human Iκβ-α (sc-371) and rabbit anti-human β-tubulin (sc-9104) (Santa Cruz Biotechnology) for 60 min at room temperature. After washing three times with TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature. After washing, immunoblots were developed for enhanced chemiluminescence using the Pierce SuperSignal West Pico substrate (Pierce) and exposed to X-ray film (Kodak, Rochester, NY, USA). Band intensity was analyzed from scanned images using NIH IMAGE software (NIH, Bethesda, MD, USA). The expression level of Iκβ-α proteins was normalized to that of β-tubulin (loading control) in each condition.

Statistical analysis

Statistical analyses were performed using analysis of variance followed by Duncan's multiple range tests. Values that were statistically different are indicated by asterisks in the figures. Error bars indicate the mean ± standard deviation of three independent experiments performed in triplicate.

Results

NF-κB pathway is involved in IL-8 induction by *F. nucleatum*

It has been reported that *F. nucleatum* upregulates *IL-8* mRNA mainly through activating the NF-κB pathway (36). To verify that the same signal pathway also participated in our model system, we first examined, by immunofluorescence microscopy, the localization of NF-κB following *F. nucleatum* challenge in KB cells. A specific NF-κB inhibitor, IKK Inhibitor VII (Calbiochem) was used to pretreat KB cells for 1 h before infection with *F. nucleatum*. We found that significant NF-κB nuclear translocation was induced by *F. nucleatum* at both 60 and 120 min (Fig. 1A). By contrast, pretreatment with the IKK Inhibitor VII significantly reduced the *F. nucleatum*-stimulated NF-κB nuclear translocation (Fig. 1A). To confirm that NF-κB activation is involved in *F. nucleatum*-induced IL-8 production, we further examined the IL-8 protein levels in cell culture supernatants using ELISA. As expected, IL-8 production induced by *F. nucleatum* was significantly inhibited by NF-κB Inhibitor VII (Fig. 1B). These results indicate that *F. nucleatum* induces IL-8 production in KB cells at least partly through activation of the NF-κB signaling pathway and that modulation of the NF-κB signaling pathway can lead to inhibition of IL-8 production.

Differential impacts on NF-κB by *F. nucleatum* and *S. cristatus*

To determine whether *S. cristatus* acts directly on the NF-κB signaling pathway and also to compare its effects with *F. nucleatum*, we first employed immunofluorescence microscopy to monitor the kinetics of NF-κB localization within 2 h in our dual-infection model. We observed that NF-κB remained exclusively in the cytoplasm of untreated control cells, except at 120 min in OKF6/TERT-2 cells, when subtle signs of NF-κB nuclear translocation with a diffuse pattern were noted (Fig. 2A,B). In KB cells, exposure to *S. cristatus* did not cause detectable NF-κB translocation into the nuclei at any study time-point (Fig. 2A), whereas *F. nucleatum* significantly activated NF-κB at 60 and 120 min (Fig. 2A). Similar NF-κB activation patterns were also found in OKF6/TERT-2 cells, with some variations (Fig. 2B). First, OKF6/TERT-2 cells treated with *S. cristatus* showed a low level of NF-κB nuclear translocation with a diffuse pattern at 120 min, similarly to untreated control cells. In addition, *F. nucleatum*-induced NF-κB activation occurred earlier, at 30 min.

To confirm the microscopic observations and to further quantify the level of NF-κB activation, we then assayed the DNA-binding capacity of NF-κB p65 in nuclear extracts, using the TransAM™ method. We observed that within 2 h, *F. nucleatum* induced an oscillatory NF-κB activation profile in OKF6/TERT-2 cells (Fig. 2D). Specifically, a significant amount of NF-κB translocated into nuclei between 15 and 30 min, and achieved its maximum levels probably between 60 and 120 min. After 120 min, the NF-κB-binding activity was reduced. In KB cells (Fig. 2C), however, *F. nucleatum* stimulation did not induce significant NF-κB translocation until 60 min, and the 120-min observation period was not long enough to see the reduction of NF-κB. In contrast to *F. nucleatum*, *S. cristatus* stimulation did not lead to a significant peak of NF-κB response, even after 2 h, although an insignificant increase in the nuclear NF-κB levels was observed at 120 min in OKF6/TERT-2 cells.

S. cristatus attenuates *F. nucleatum*-induced NF-κB nuclear translocation

We previously reported that *S. cristatus* attenuates the expression of a number of *F. nucleatum*-induced proinflammatory cytokines, including IL-1, IL-6, IL-8 and tumor necrosis factor-α (32). Here, the impact of *S. cristatus* on *F. nucleatum*-induced NF-κB activation was examined. Immunofluorescence microscopy demonstrated that, when compared with *F. nucleatum* alone, the addition of *S. cristatus* dramatically repressed the *F. nucleatum*-induced NF-κB nuclear translocation in KB cells (Fig. 2A) and to a lesser degree in OKF6/TERT-2 cells (Fig. 2B). The TransAM™ DNA-binding assay further confirmed that, in KB cells, inhibition of NF-κB translocation was evident in the presence of

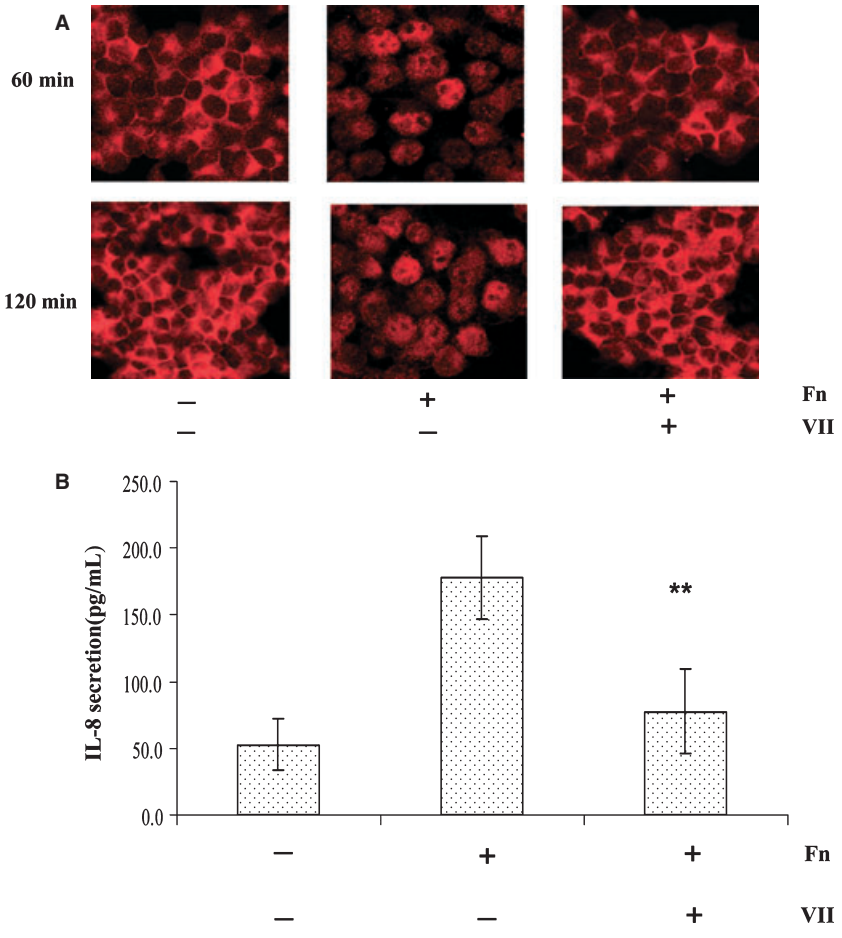

Fig. 1. The effects of IKK Inhibitor VII on nuclear factor-kappa B (NF-κB) activation and interleukin-8 (IL-8) induction in *Fusobacterium nucleatum*-infected KB cells. KB cells were pretreated with or without IKK Inhibitor VII for 1 h before bacterial challenge. (A) After incubation with *F. nucleatum* for 60 and 120 min, cell monolayers were fixed and permeabilized. NF-κB subunit p65 was labeled with Alexa Fluor 568 (red) and then examined under a confocal microscope. (B) At 120 min, the culture supernatants were collected and the concentration of IL-8 was measured by ELISA. Values represent the mean ± standard deviation of three independent assays. $^{**}p < 0.01$ compared with the corresponding stimulus alone. Fn, *F. nucleatum*; VII, IKK Inhibitor VII.

S. cristatus at both 60 and 120 min (Fig. 2C). However, in OKF6/TERT-2 cells, the inhibition of NF-κB translocation became nonsignificant at 120 min, probably as a result of both the reduction of nuclear NF-κB in *F. nucleatum*-challenged cells and the insignificant NF-κB nuclear translocation induced by *S. cristatus* (Fig. 2D).

S. cristatus inhibits the *F. nucleatum*-induced degradation of IκB-α

In mammalian cells, nuclear translocation of NF-κB is controlled by IκBs. To determine, in greater detail, whether the blockage of NF-κB activation by *S. cristatus* was occurring at the level of IκB, we performed kinetic western blot assays to monitor the degradation of IκB-α in our model cells. In KB cells, we found that IκB-α was relatively stable at 30 min in all experimental conditions (Fig. 3A,C), corresponding to the cytoplasmic sequestration of NF-κB observed previously (Fig. 2A,C). A progressive degradation of IκB-α was induced by *F. nucleatum* from 60 to 120 min, concomitant with the increased nuclear localization of NF-κB (Fig. 3A,C). The coincubation with *S. cristatus* inhibited the IκB-α degradation induced by *F. nucleatum* at 60 and 120 min (Fig. 3A,C).

A similar trend was also observed in OKF6/TERT-2 cells, but there were some differences in timing. *F. nucleatum*-induced IκB-α degradation became evident at 30 min, which was significantly prevented by the presence of *S. cristatus* (Fig. 3B,D). The IκB-α stabilization by *S. cristatus* continued to 60 min, but became insignificant compared with *F. nucleatum* alone (Fig. 3D). At 120 min, the total amount of IκB-α protein was enhanced in *F. nucleatum*-infected cells, indicative of negative feedback (Fig. 3B,D). By contrast, untreated cells and *S. cristatus*-infected cells demonstrated gradual degradation of IκB-α from 60 to 120 min (Fig. 3B,D).

Blocking TLR2 and TLR4 does not affect IL-8 production and attenuation

TLR2 and TLR4 have been identified as the principal signaling receptors for bacterial cell-wall components, and their expression on oral epithelial cells has been reported (37–39). To determine whether TLR2 and/or TLR4 were involved in modulating oral epithelial cell responses to *S. cristatus* and *F. nucleatum*, we first studied the localization of TLRs on KB cells by immunofluorescence confocal microscopy. In agreement with the previous results, both TLR2 and TLR4 were present and localized to the cell surface and cytosolic compartments but not to the nucleus (Fig. 4A). To further establish whether surface TLR2 and/or TLR4 are involved in IL-8 production and NF-κB activation, antibodies to TLR2 and TLR4 were used to block the surface receptors on KB cells before infection. Interestingly, immunofluorescence microscopy demonstrated that blocking of neither TLR2 nor TLR4 changed the NF-κB activation patterns in our dual-infection model (Fig. 4B). In support of the microscopic findings, ELISA further revealed that pretreatment with antibodies to TLR2 and TLR4 did not affect IL-8 production induced by

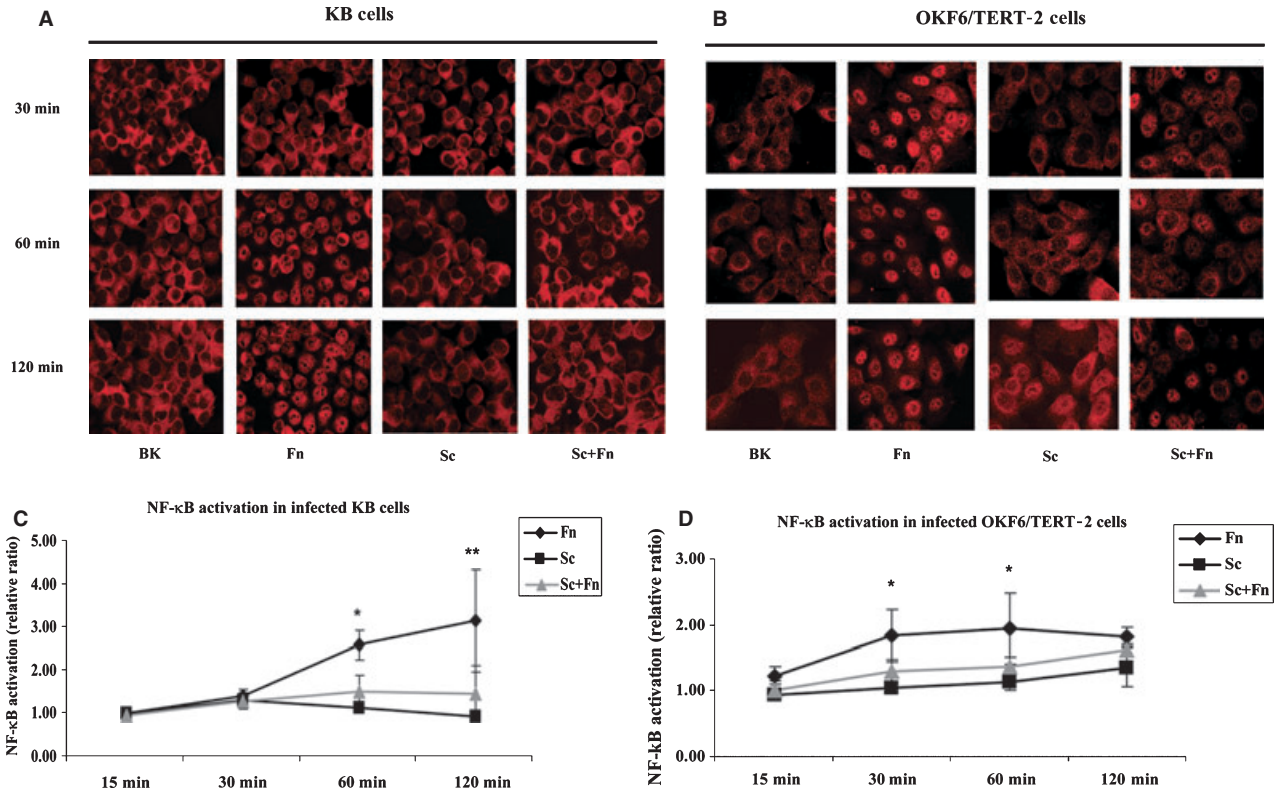

Fig. 2. Time course analysis of nuclear factor-kappa B (NF-κB) activation in KB and OKF6/TERT-2 cells following stimulation with or without bacteria. For immunofluorescence microscopy, KB (A) and OKF6/TERT-2 (B) cell monolayers were fixed at the indicated time-points and permeabilized. NF-κB subunit p65 was labeled with Alexa Fluor 568 (red), and then examined under a confocal microscope. For TransAM™ assays, nuclear extracts of KB (C) and OKF6/TERT-2 (D) were prepared at the indicated time-points and the DNA-binding activity of p65 was measured using an ELISA-based TransAM kit. The results are presented as a ratio of the unstimulated control and graphed with trend lines. Values are the mean ± standard deviation of three independent experiments performed in duplicate. $^{**}p < 0.01$; $^{*}p < 0.05$, compared between treatments. BK, medium control; Fn, *Fusobacterium nucleatum*; Sc, *Streptococcus cristatus*; Sc + Fn, *S. cristatus* plus *F. nucleatum*. As ratio data may not be normally distributed, the statistical analysis was run on both the original and log-transformed ratios. The results were the same in both cases, with regard to statistical significance.

F. nucleatum or the IL-8-attenuating effect of *S. cristatus* (Fig. 4C).

Discussion

Immune and inflammatory responses in immunocompetent tissues often involve the transcription factor NF-κB. Proinflammatory stimuli activate NF-κB through tightly regulated phosphorylation, ubiquitination and proteolysis of a physically associated class of inhibitor molecules, IκBs (40). In the present study, we demonstrated that *S. cristatus*, a commensal oral viridans streptococcus, is able to downregulate the nuclear translocation of NF-κB induced by *F. nucleatum*, an oral proinflammatory species, through stabilizing IκB-α, which as a result might dampen the epithelial proinflammatory responses.

The oral epithelium is constantly exposed to commensal and pathogenic microorganisms. Currently, the role of *F. nucleatum* in periodontal disease remains controversial. Some authors consider it a commensal because it is present in healthy sites in large numbers. In contrast, the prevalence of *F. nucleatum* is significantly increased at diseased sites (41), and it possess putative virulence factors, such as the ability to invade oral epithelial cells (36,42). Regardless of whether one defines it as a putative pathogen or as a commensal species, it has been shown that, in contrast to most oral streptococci, *F. nucleatum* is able to induce a wide range of proinflammatory responses in various types of host cells, including marked IL-8 induction in oral epithelial cells (36,42). Expression of IL-8 is largely regulated by NF-κB, ERK, JNK and p38 MAPK pathways (43). Up-regulation of *IL-8* mRNA by *F. nucleatum* has been found to mainly involve NF-κB pathways and to some extent MAPK p38 and MAPK/ERK pathways (36). In the current study, we observed that exposure to *F. nucleatum* induced significant NF-κB activation in KB and OKF6/TERT-2 oral epithelial cells, which was accompanied by marked production of IL-8. On the other hand, when the IKK complex, the master regulator of the NF-κB pathway, was inhibited by IKK Inhibitor VII, the production of IL-8 in *F. nucleatum*-infected KB cells was strongly impaired. These data confirmed that induction of IL-8 by *F. nucleatum* in our cell models depends, at least in part, on the NF-κB pathway.

Both commensal and pathogenic species have molecular motifs that can

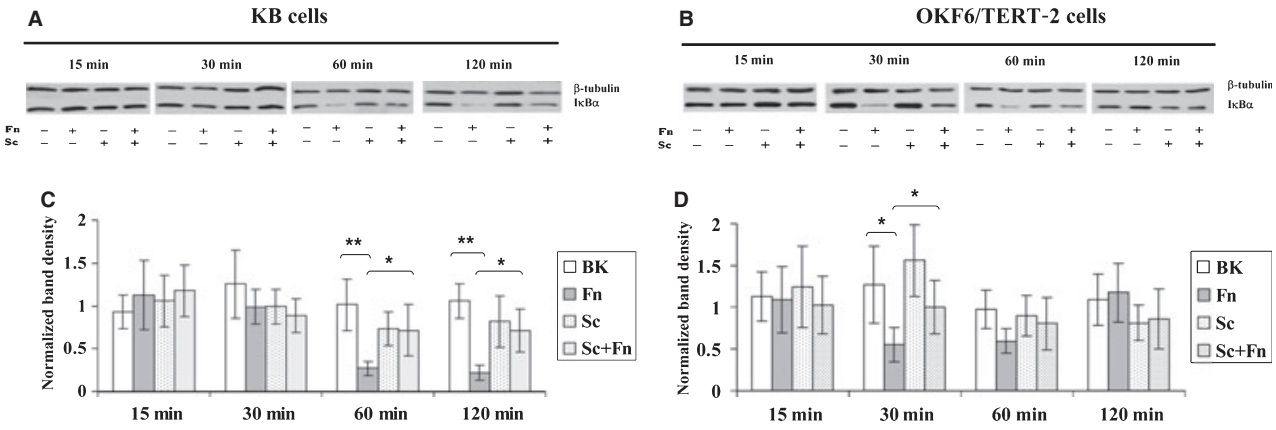

Fig. 3. Time course analysis of IκB-α degradation in KB and OKF6/TERT-2 cells following stimulation with or without bacteria. For the western blot assay, whole-cell lysates of KB (A) and OKF6/TERT-2 (B) cells were prepared at the indicated time-points, separated by electrophoresis through a 7.5% sodium dodecyl sulfate polyacrylamide gel and then subjected to western blot analysis with rabbit anti-human-IκB-α immunoglobulin as a probe. The loading control was β-tubulin. Images are representative of three independent experiments. For semiquantitative analysis, bands in the KB (C) and OKF6/TERT-2 (D) western blot were scanned and densitometric signals of IκB-α were normalized to those of β-tubulin. The results are expressed as the mean ± standard deviation of three independent experiments. $^{**}p < 0.01$; $^{*}p < 0.05$, compared between indicated treatments. BK, medium control; Fn, *Fusobacterium nucleatum*; Sc, *Streptococcus cristatus*; Sc + Fn, *S. cristatus* plus *F. nucleatum*.

activate proinflammatory gene expression, but commensal bacteria appear to have a different impact on host cell responses and therefore are able to maintain an immune homeostasis at mucosal surfaces (22). We previously demonstrated that the gram-positive oral commensal *S. cristatus* alone is a poor IL-8 inducer and capable of suppressing the IL-8 production induced by *F. nucleatum* (32). Indeed, emerging evidence has shown that certain commensal species and probiotics, including *S. salivarius* K12, *Lactobacillus salivarius*, *Lactobacillus reuteri*, *Bacteroides thetaiotaomicron* and *Samonella pullorum*, have similar effects on pathogen-induced IL-8 secretion in epithelial cells and do not stimulate IL-8 production per se (30,31,33,44,45). The molecular basis of commensal-exerted IL-8 attenuation has recently been investigated and studies have pinpointed the IκB/NF-κB signaling module as the target (30,34,35,45). In support of those previous results, we observed that *S. cristatus* alone did not significantly affect NF-κB activation in KB and OKF6/TERT-2 cells, and its presence significantly inhibited *F. nucleatum*-induced NF-κB translocation to the nucleus.

The investigation of molecular mechanisms involved in IκB/NF-κB signaling modulation has mainly focused on studying the downstream events. Several distinct modes of action by which commensal bacteria impinge on NF-κB signaling to limit inflammation have been elucidated. These include blockage of IκB-α polyubiquitination (30), inhibition of epithelial proteasome function (34,35) or promoting nuclear export of the transcriptionally active NF-κB subunit p65 (31). Kinetic analysis of the NF-κB activation profile by the TransAM assay and of IκB-α degradation by western blotting revealed that the IκB/NF-κB signaling module in the two oral epithelial cells used in this study was activated more efficiently by a longer time-period of stimulation (≥ 1 h), especially in KB cells. Moreover, OKF6/TERT-2 cells were more responsive than KB cells, represented by gradual spontaneous activation of IκB/NF-κB with time. Despite the subtle difference in IκB/NF-κB responses existing in two cell lines, our results confirmed that *S. cristatus* did not signal the proinflammatory pathway in oral epithelial cells, as opposed to *F. nucleatum*. The observation that *S. cristatus* was able to stabilize the IκB-α levels in *F. nucleatum*-infected cells is considered as a significant finding of the present study, which adds to evidence supporting the imunoregulatory role of certain commensal species at mucosal surfaces and the potential of using NF-κB as a drug target for chronic inflammatory diseases.

As we have previously shown that the inhibitory effect of *S. cristatus* appeared to require bacterial contact with epithelial cells (32), we speculated that it might interfere with the recognition of *F. nucleatum* by the host, thereby resulting in the modulation of downstream IκB/NF-κB signaling. TLRs have been recognized as key sensors of microbial infections in mammalian cells through activating the major transcription factors NF-κB and/or MAPK to result in the expression of proinflammatory cytokines (46). In line with the previous reports (47), we found that TLR2 and TLR4 were clearly expressed both on the KB cell surface and intracellularly. The expression of TLR2 and TLR4 in the cytoplasmic compartment has been recently demonstrated in different types of epithelial cells (28,48,49), and it is speculated that the subcellular localization of TLRs could imply an immunoregulatory mechanism to avoid proinflammatory stimulation by

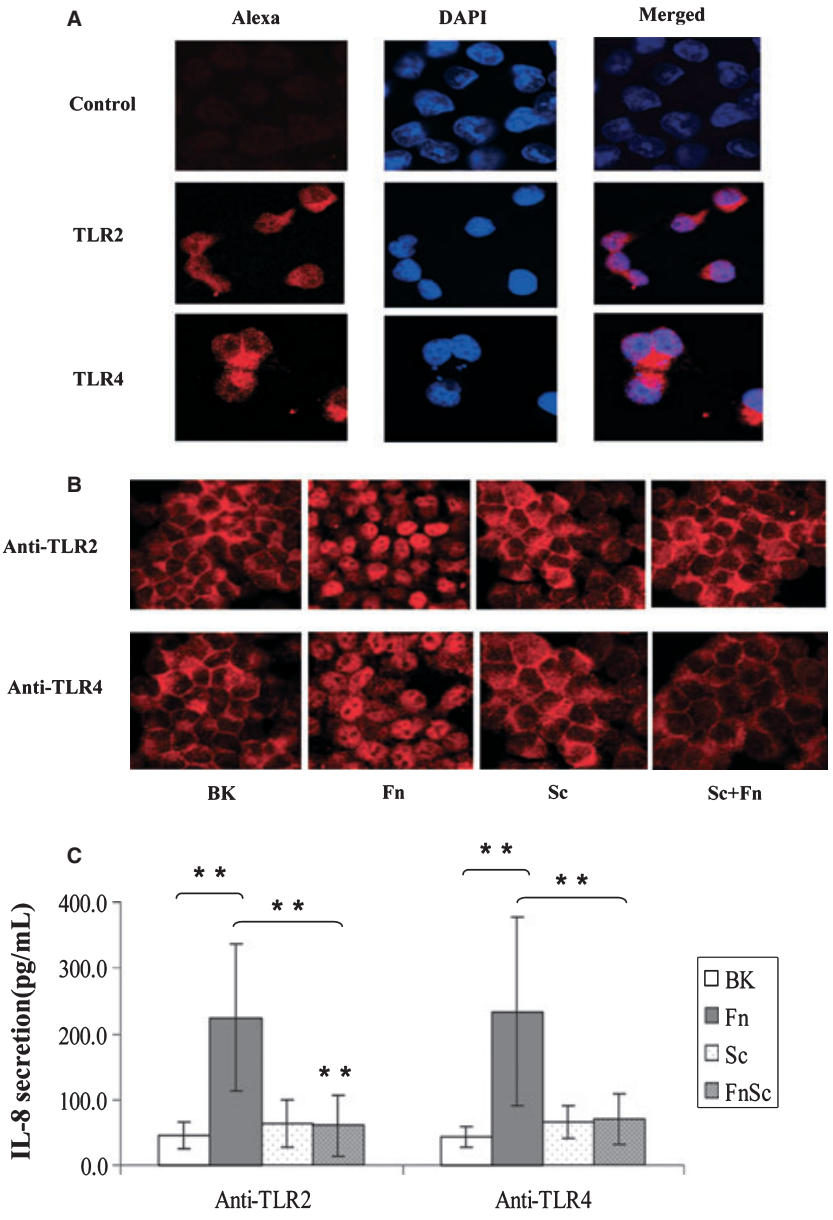

Fig. 4. Effects of antibodies to toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) on nuclear factor-kappa B (NF-κB) activation and interleukin-8 (IL-8) induction in KB cells. KB cells were pretreated with or without antibodies to TLR2 and TLR4 for 1 h before bacterial challenge. (A) Localization of TLR2 and TLR4 in unstimulated cells was examined by immunofluorescence. TLR2 and TLR4 were labeled with Alexa Fluor 568 (red). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (blue). Images were captured under a confocal microscope. (B) NF-κB activation was examined by immunofluorescence following bacterial challenge. NF-κB subunit p65 was labeled with Alexa Fluor 568 (red) and the images were captured under a confocal microscope. (C) IL-8 production under various conditions was measured by ELISA. Values represent the mean ± standard deviation of three independent assays. $^{**}p < 0.01$ compared between indicated treatments. BK, medium control; Fn, *Fusobacterium nucleatum*; Sc, *Streptococcus cristatus*; Sc + Fn, *S. cristatus* plus *F. nucleatum*.

the normal microflora, which, once overcome by invasive species, could turn these cells into active players of innate immunity that are capable of initiating an inflammatory response.

It is known that TLR2 and TLR4 expressed in oral epithelial cells are functional (37). *F. nucleatum* might activate IL-8 production in gingival epithelial cells through both surface TLR2 and TLR4 because its lipopolysaccharide (LPS), a ligand to TLR4, was able to induce *IL-8* mRNA (50), while its cell wall extracts were reported to exclusively interact with TLR2 to trigger IL-8 expression (51,52). However, in the present study, blocking both surface TLR2 and TLR4 with antibodies failed to affect IL-8 production and NF-κB activation induced by live *F. nucleatum* in KB cells. This discrepancy might be partly explained by variation present in the different types of epithelial cells used. In addition, host cells might react very differently to live intact bacteria compared with dead bacteria or bacterial components. Indeed, a recent study reported that knockdown of TLR2 RNA did not affect IL-8 induction by live intact *F. nucleatum* in gingival epithelial cells, suggesting that receptors other than TLR2 or different mechanisms may be involved (53). Given the abundant intracellular expression of TLR4 in KB cells, one possibility might be that the recognition of *F. nucleatum* LPS occurs mainly in the cytoplasmic compartment rather than at the cell surface. There is emerging evidence that in contrast to the situation in cells of the myeloid lineage, recognition of LPS by non-professional immune cells, including epithelial cells, may occur intracellularly and require LPS internalization (28,54). This appears to be relevant to the biological behavior of *F. nucleatum*, because this species is a common member of oral resident flora and its invasiveness is associated with potent IL-8 induction (36).

The TLR-stimulating property of oral streptococci has been studied in less detail. Recently, cell wall extracts of *Streptococcus sanguinis*, a commensal streptococcal species, were reported to limit *F. nucleatum* extracts-induced IL-8 expression through TLR2 (51). In our study, however, the lack of impact of TLR on blocking the production of IL-8 in response to either *S. cristatus* alone or *S. cristatus* in combination with *F. nucleatum* suggested the involvement of some other receptors. More specific studies, such as complete knockout of TLRs from model cells or

animals, would be helpful to clarify this point.

Distinct regulatory mechanisms other than TLR signaling might exist. It has been reported that α5β1 integrins act as cell receptors for antigen I/II (AgI/II), an adhesin expressed by most indigenous species of oral streptococci, and this interaction led to an IL-8 response to *Streptococcus mutans* in endothelial cells by activating MAPK signaling pathways (55). As activation by different receptors at some point converges upon the common signaling pathways, most notably those leading to the activation of NF-κB, it will be interesting to determine whether epithelial integrins are involved in signaling the IL-8 attenuation induced by *S. cristatus*.

In conclusion, in this study we demonstrated that *S. cristatus* was able to inhibit NF-κB activation induced by *F. nucleatum* through stabilizing IκB-α, which might represent a mechanism to regulate the host response and maintain epithelial homeostasis. Interactions between oral commensal streptococci and *F. nucleatum* appear to be complex and interdependent. They co-aggregate avidly, and we have observed streptococci and fusobacteria together within buccal epithelial cells from human subjects, as members of a polymicrobial intracellular flora (56). Subsequently, we established that co-aggregation may provide a mechanism whereby noninvasive *S. cristatus* could be transported inside epithelial cells by invasive *F. nucleatum* (57). Although we later showed that the anti-inflammatory effect of *S. cristatus* does not depend on co-aggregation with *F. nucleatum* (32), co-aggregation is likely to be the normal state of affairs in the mouth. It is possible that one benefit which *F. nucleatum* obtains from its relationship with oral streptococci is suppression of the proinflammatory response that is seen with exposure to *F. nucleatum* alone. That may be one reason why large numbers of *F. nucleatum* can be observed in sites that are periodontally healthy. The development of an imbalance in that relationship might contribute to the relative overgrowth of *F. nucleatum* and the emergence of periodontal disease in susceptible individuals. This may help to explain why *F. nucleatum* can appear to be commensal in some persons but pathogenic in others.

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