

Synergistic Induction of Eotaxin and VCAM-1 Expression in Human Corneal Fibroblasts by Staphylococcal Peptidoglycan and Either IL-4 or IL-13

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ABSTRACT

Background: Common features of allergic or atopic ocular and skin diseases are the participation of Th2 lymphocytes and eosinophils and colonization by *Staphylococcus aureus*. To examine the role of interaction between Th2 cells and bacterial infection in tissue eosinophilia, we determined the effects of Th2 cytokines and peptidoglycan derived from the cell wall of *S. aureus* on corneal fibroblasts.

Methods: Chemokine concentrations and the cell surface expression of adhesion molecules were determined by ELISAs, and chemokine and adhesion molecule mRNAs were quantitated by real-time PCR analysis. Signaling by the transcription factor NF- κ B was evaluated by immunoblot and immunofluorescence analyses as well as by assay of DNA binding activity.

Results: Among Th2 cytokines tested, only interleukin (IL)-4 and IL-13 induced a low level of eotaxin release by corneal fibroblasts, as did peptidoglycan. However, the combination of peptidoglycan and either IL-4 or IL-13 induced a marked synergistic increase both in eotaxin release (without affecting that of IL-8) and in the abundance of eotaxin mRNA. The combination of peptidoglycan and IL-4 or IL-13 also synergistically increased the surface expression of VCAM-1, but not that of ICAM-1. Peptidoglycan activated NF- κ B in corneal fibroblasts, and inhibitors of NF- κ B attenuated eotaxin release induced by peptidoglycan alone or in combination with IL-4 or IL-13.

Conclusions: Interaction of innate and adaptive immunity, as manifested by synergistic stimulation of eotaxin and VCAM-1 expression in corneal fibroblasts by peptidoglycan and Th2 cytokines, may play an important role in tissue eosinophilia associated with ocular allergy.

KEY WORDS

adhesion molecule, chemokine, eosinophil, fibroblast, innate immunity

INTRODUCTION

The Th2 immune system, mediated in part by Th2 cytokines such as IL-4 and IL-13, plays a central role in the pathogenesis of allergic diseases such as asthma, atopic dermatitis, and ocular allergic diseases.¹ Another hallmark of these diseases is tissue eosinophilia. The C-C chemokine eotaxin (CCL11) is a potent and selective chemoattractant for eosinophils and contributes to tissue-specific eosinophil recruitment in allergic diseases.² Although the sources and

regulation of eotaxin production remain incompletely characterized, we and others have shown that resident fibroblasts activated by Th2 cytokines are a potential major source of eotaxin in tissue affected by allergic diseases.³⁻⁵

Eosinophils express the adhesion molecule VLA-4, which binds to VCAM-1 expressed on endothelial and other cell types.⁶ We have shown that the inhibition of the VLA-4-VCAM-1 interaction in vivo suppressed allergen-induced infiltration of eosinophils into the conjunctiva in mouse model of allergic conjunctivitis.⁷

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Characterization of the regulation of eotaxin and VCAM-1 expression is therefore likely to provide insight into the mechanisms that underlie the local accumulation of eosinophils in allergic inflammation.

The Gram-positive bacterium *Staphylococcus aureus* colonizes the skin of individuals with atopic eczema, and the skin of patients with atopic dermatitis has been shown to exhibit an increased avidity for *S. aureus* and to be deficient in the ability to produce antimicrobial peptides that contribute to the eradication of infectious agents.⁸ The ocular surface is also commonly affected by infectious diseases and allergic reactions, given that it is continuously exposed to a variety of airborne bacteria and antigens. Several clinical forms are included in the classification of allergic ocular surface diseases including seasonal or perennial allergic conjunctivitis, vernal keratoconjunctivitis, atopic keratoconjunctivitis (AKC), and giant papillary conjunctivitis. AKC is severe type of ocular allergy and is defined as a chronic allergic inflammation of the ocular surface including, conjunctiva, eyelids, and cornea, affecting individuals with a history of atopic dermatitis.⁹ In addition to allergic inflammation, complications of AKC also include *S. aureus* and herpes simplex infection of ocular surface in some patients. Indeed, high rates of bacterial colonization, especially with *S. aureus*, have thus been demonstrated in the conjunctival sacs and eyelid margins as well as on the skin of individuals with atopic dermatitis.¹⁰ Toll-like receptors (TLRs) comprise a family of cell surface proteins in mammals that play an important role in innate immune responses by stimulating the production of proinflammatory cytokines in response to various microbial ligands.¹¹ TLR4 thus recognizes LPS, a component of Gram-negative bacteria, whereas TLR2 mediates responses to lipoteichoic acid, lipopeptides, and peptidoglycan (PGN) of Gram-positive bacteria and mycobacteria. Corneal fibroblasts and epithelial cells express various TLRs for microbial ligands.^{12,13} Cell wall components of *S. aureus* might thus also be expected to activate tissue-resident cells, but the role of PGN in allergic diseases has remained unknown.

Corneal involvement secondary to severe conjunctival allergic inflammation is the most serious complication of AKC. The expression of eotaxin and adhesion molecules by corneal fibroblasts but not corneal epithelial cells likely contributes to the formation of corneal lesions by promoting the local infiltration, activation, and survival of eosinophils.^{14,15} Corneal epithelial cells thus do not express chemokines in response to Th2 cytokines but rather likely act as a barrier not to penetrate inflammatory mediators into the stroma. Mechanical compression as well as inflammatory mediators may disrupt the barrier function of corneal epithelial cells during allergic inflammation. Indeed, corneal epithelial barrier function is impaired in individuals with atopic blepharoconjunctivitis.¹⁶ In addition, we have recently shown that the loss of the

barrier function of the corneal epithelium exacerbated conjunctival inflammation in a rat model of ocular allergy.¹⁷ We have also reported that restoration of the corneal epithelium resulted in amelioration of conjunctival inflammation in a case of AKC.¹⁸ Taken together, these reports suggest that the vicious cycle between conjunctival inflammation and corneal lesions is mediated by the activation of corneal fibroblasts.

Given the increased rate of ocular infection with *S. aureus* and the role of Th2 cytokines in AKC and atopic dermatitis, we hypothesized that the innate and adaptive immune systems might cooperate to activate resident cells such as fibroblasts at the site of allergic inflammation. To explore this possibility, we examined the effects of exposure of corneal or dermal fibroblasts to the combination of PGN and Th2 cytokines on the expression of eotaxin and VCAM-1 at the mRNA and protein levels.

METHODS

MATERIALS

MEM, FBS, PBS, and trypsin-EDTA were obtained from Gibco-BRL (Grand Island, NY, USA), and cell culture dishes (Falcon) were from Becton Dickinson (Franklin Lakes, NJ, USA). PGN of *S. aureus* was from Fluka (St. Louis, MO, USA). Human recombinant cytokines, paired antibodies to eotaxin or to IL-8, as well as human eotaxin and IL-8 standards for ELISAs were from R&D Systems (Minneapolis, MN, USA). A Reverse Transcription System was from Promega (Madison, WI, USA), and a QuantiTect SYBR Green PCR kit was from Qiagen (Hilden, Germany). Rabbit polyclonal Abs to I κ B- α and to the p65 subunit of NF- κ B, normal rabbit or mouse IgG, and HRP-conjugated goat Abs to rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A mouse mAb to VCAM-1 was from Pharmingen (San Diego, CA, USA), and HRP-conjugated goat Abs to mouse IgG were from Chemicon (Temecula, CA, USA). ECL immunoblot detection reagents and Hyperfilm were from Amersham Pharmacia Biotech (Little Chalfont, UK). Alexa488-conjugated goat Abs to rabbit IgG were from Molecular Probes (Eugene, OR, USA). The pyrrolidine derivative of dithiocarbamate (PDTC) and actinomycin D were from Sigma (St. Louis, MO, USA), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was from Wako Pure Chemical Industries (Osaka, Japan). All reagents used for cell culture were endotoxin minimized.

CELL CULTURE AND INCUBATION FOR MEASUREMENT OF CHEMOKINE RELEASE

Human corneal fibroblasts were isolated as described previously.^{3,19} The human tissue was used in strict accordance with the basic principles of the Declaration of Helsinki. Normal human dermal fibroblasts were obtained from Cascade Biologics (Portland, OR,

USA) or Clonetics (Walkersville, MD, USA). Cells were seeded in 24-well culture plates and cultured for 3 days in MEM supplemented with 10% FBS. They were then washed with PBS, cultured for 24 h in MEM without added serum, and then incubated for the indicated times in the same medium supplemented with PGN or cytokines. The medium was then collected and centrifuged at $120 \times g$ for 5 min, and the resulting supernatant was stored at -80°C for subsequent assay of chemokines. The fibroblasts remaining in each well were exposed to trypsin-EDTA, and their number was determined with a hemocytometer.

DETERMINATION OF CHEMOKINE CONCENTRATION BY ELISA

The concentration of chemokines in culture supernatants was determined in quadruplicate with solid-phase ELISAs. The limit of detection for both eotaxin and IL-8 was 3.9 pg/ml, and data are presented as nanograms of chemokine per 1×10^6 cells.

QUANTITATIVE RT-PCR ANALYSIS OF EOTAXIN AND VCAM-1 mRNAs

The abundance of eotaxin and VCAM-1 mRNAs was determined by RT and quantitative real-time PCR analysis as described previously.^{20,21} In brief, total RNA was extracted from fibroblasts and subjected to RT, and the resulting cDNA was then subjected to real-time PCR with a LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN, USA). Transcripts of the constitutively expressed gene for GAPDH served to normalize the amount of target mRNA in each sample. The sequences of the PCR primers for eotaxin, VCAM-1, and GAPDH were described previously.^{20,21} Real-time PCR data were analyzed with LightCycler software 3.01 (Roche Molecular Biochemicals).

IN SITU WHOLE-CELL ELISA FOR VCAM-1 AND ICAM-1

An in situ whole-cell ELISA for VCAM-1 and ICAM-1 was performed as described.²¹ Cells were seeded in 96-well culture plates and cultured and incubated as for measurement of chemokine release. After incubation with PGN or cytokines for 24 h, the cells were washed twice with PBS, fixed for 15 min at room temperature with PBS containing 1% paraformaldehyde, washed with PBS containing 0.1% BSA, and incubated for 1 h at 37°C with a mAb to VCAM-1 or ICAM-1 (1 : 10,000 dilution) in PBS-BSA (1%). After washing three times with PBS-BSA (0.1%), the cells were incubated for 1 h at 37°C with HRP-conjugated goat Abs to mouse IgG in PBS-BSA (1%), washed three times with PBS-BSA (0.1%), and then incubated for 20 min in the dark with 100 μl of 3,3',5,5'-tetramethylbenzidine solution. The reaction was terminated by the addition of 50 μl of 1 M H_2SO_4 , and the absor-

bance of each well was measured at 450 nm with a microplate reader.

IMMUNOBLOT ANALYSIS OF $\text{I}\kappa\text{B-}\alpha$

The degradation of $\text{I}\kappa\text{B-}\alpha$ in corneal fibroblasts was examined by immunoblot analysis as described previously.²² In brief, cells were incubated for various times at 37°C with MEM containing PGN (30 $\mu\text{g}/\text{ml}$), washed twice, and then lysed with RIPA buffer. Cell lysates (20 μg of protein) were subjected to SDS-PAGE on a 10% gel under reducing conditions, and the separated proteins were then transferred electrophoretically to a polyvinylidene difluoride membrane. After blocking of nonspecific sites, the membrane was incubated with rabbit polyclonal Abs to $\text{I}\kappa\text{B-}\alpha$, and immune complexes were detected with HRP-conjugated secondary Abs and ECL reagents.

IMMUNOFLUORESCENCE ANALYSIS OF $\text{NF-}\kappa\text{B}$

Immunostaining of $\text{NF-}\kappa\text{B}$ in corneal fibroblasts was performed as described previously.²³ In brief, cell monolayers grown on eight-well chamber slides were cultured for 24 h at 37°C in serum-free MEM, incubated for various times at 37°C with MEM in the absence or presence of PGN (30 $\mu\text{g}/\text{ml}$), washed twice with PBS, and fixed with 4% paraformaldehyde in PBS. After three additional washes, the cells were permeabilized with 100% methanol at -20°C for 6 min, and nonspecific adsorption of antibodies was blocked by incubation of the cells for 30 min at room temperature with PBS containing 3% BSA. The cells were then incubated for 1 h at room temperature with Abs to the p65 subunit of $\text{NF-}\kappa\text{B}$ (1:100 dilution in PBS containing 1% BSA), and incubated for 30 min at room temperature with Alexa488-conjugated secondary Abs (1:500 dilution in PBS containing 1% BSA). They were finally washed, mounted in Vectashield mounting medium, and examined with a fluorescence microscope (Axiovert; Carl Zeiss, München-Hallbergmoos, Germany).

ASSAY OF DNA BINDING ACTIVITY OF $\text{NF-}\kappa\text{B}$ p65

Binding of $\text{NF-}\kappa\text{B}$ to κB sites was assessed with the use of a Trans-AM $\text{NF-}\kappa\text{B}$ p65 assay kit (Active Motif, Rixensart, Belgium). In this assay, an oligonucleotide containing the $\text{NF-}\kappa\text{B}$ consensus site is attached to the wells of a 96-well plate, and binding of the active form of $\text{NF-}\kappa\text{B}$ present in nuclear extracts to this oligonucleotide is detected with ELISA technology.²⁴ Cells were stimulated for 60 min with PGN (30 $\mu\text{g}/\text{ml}$), or with $\text{TNF-}\alpha$ (10 ng/ml) as a positive control, and nuclear extracts were then prepared. Portions of the extracts (8 μg of protein) were then analyzed for p65 binding to the κB oligonucleotide. The specificity of the assay was monitored on the basis of competition with free wild-type κB oligonucleotide or a mutated κB oligonucleotide.

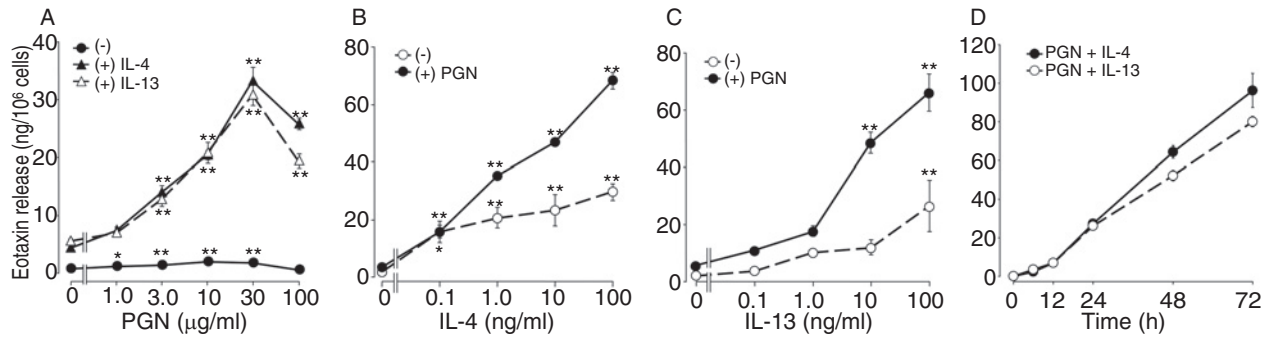


Fig. 1 Effects of PGN, IL-4, and IL-13 on eotaxin release by human corneal fibroblasts. **(A)** Cells were cultured for 24 h with the indicated concentrations of PGN in the absence (circles) or presence of IL-4 (closed triangles) or IL-13 (open triangles) each at a concentration of 10 ng/ml. **(B, C)** Cells were cultured for 24 h with the indicated concentrations of IL-4 **(B)** or IL-13 **(C)** in the absence (open circles) or presence (closed circles) of PGN (30 µg/ml). **(D)** Cells were cultured for the indicated times in the presence of PGN (30 µg/ml) and either IL-4 (closed circles) or IL-13 (open circles), each at a concentration of 10 ng/ml. Culture supernatants from all incubations were assayed for eotaxin by ELISA. Data are means $\pm$ SEM of values from four separate experiments. * p < 0.05, ** p < 0.01 (Dunnett's test) versus the corresponding value for cells incubated in the absence of PGN or cytokine.

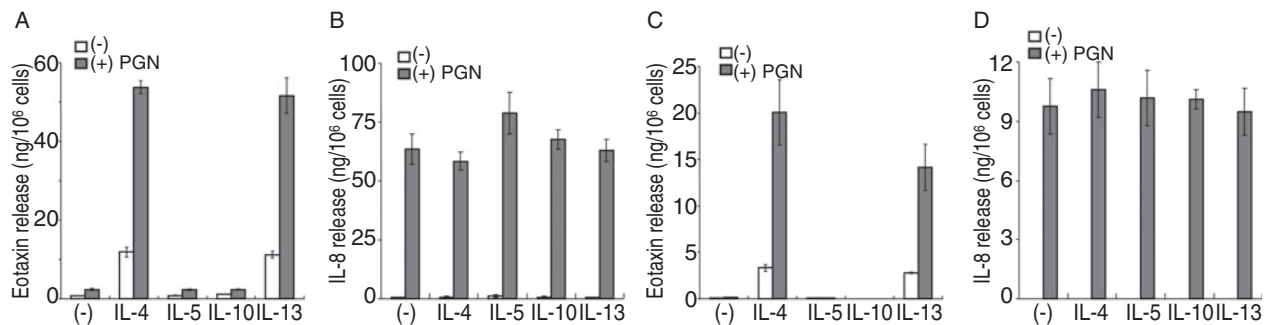


Fig. 2 Effects of PGN and Th2 cytokines on eotaxin and IL-8 release by human corneal and skin fibroblasts. Fibroblasts derived from the cornea **(A, B)** or skin **(C, D)** were incubated for 24 h with the indicated Th2 cytokines (10 ng/ml) in the absence (open bars) or presence (closed bars) of PGN (30 µg/ml), after which the concentration of eotaxin **(A, C)** or IL-8 **(B, D)** in the culture supernatants was measured by ELISA. Data are means $\pm$ SEM of values from four separate experiments. * p < 0.0001 (Dunnett's test) versus the corresponding value for cells incubated in the absence of cytokine.

STATISTICAL ANALYSIS

Data are presented as means $\pm$ SEM. Results were compared between two groups with the unpaired Student's t test and among three or more groups by Dunnett's test. Statistical analyses were performed with StatView for Windows software (version 5.0; SAS Institute, Cary, NC, USA). A p value of <0.05 was considered statistically significant.

RESULTS

EFFECTS OF PGN AND Th2 CYTOKINES ON EOTAXIN RELEASE IN HUMAN CORNEAL AND DERMAL FIBROBLASTS

We first examined the effects of PGN and the Th2 cytokines IL-4 and IL-13 on the release of eotaxin from human corneal fibroblasts. The cells were cultured for 24 h either with various concentrations of PGN in the absence or presence of IL-4 or IL-13 (each at 10

ng/ml) (Fig. 1A) or with various concentrations of IL-4 (Fig. 1B) or IL-13 (Fig. 1C) in the absence or presence of PGN (30 µg/ml). In the absence of IL-4 or IL-13, PGN induced only a small increase in eotaxin release, which was statistically significant at concentrations of 1.0 to 30 µg/ml (Fig. 1A). However, in the presence of IL-4 or IL-13, PGN exhibited a marked stimulatory effect on eotaxin release that was concentration dependent and maximal at 30 µg/ml. Similarly, in the absence of PGN, IL-4 (Fig. 1B) or IL-13 (Fig. 1C) induced relatively small increases in eotaxin release, consistent with our previous observations.³ However, in the presence of PGN, the stimulatory effects of IL-4 or IL-13 on eotaxin release were markedly potentiated. The time course of eotaxin release by corneal fibroblasts incubated with the combination of PGN and either IL-4 or IL-13 was characterized by a delay of -12 h and a subsequent linear phase for up

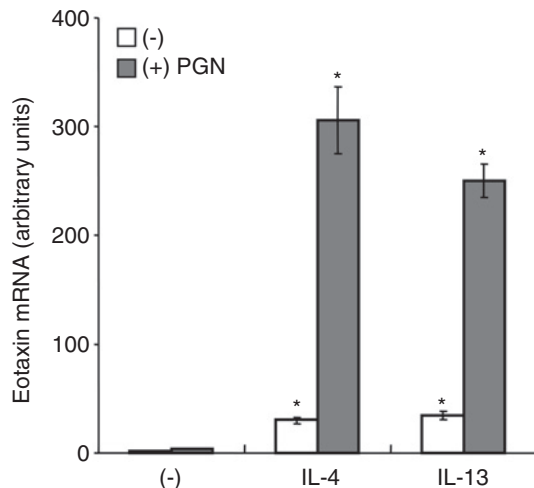


Fig. 3 Effects of PGN, IL-4, and IL-13 on the abundance of eotaxin mRNA in corneal fibroblasts. Cells were cultured for 6 h with IL-4 or IL-13 (each at 10 ng/ml) in the absence (open bars) or presence (closed bars) of PGN (30 μ g/ml), after which total RNA was isolated from the cells and the amount of eotaxin mRNA was determined by quantitative RT-PCR analysis. Data were normalized on the basis of the abundance of GAPDH mRNA, are expressed in arbitrary units, and are means $\pm$ SEM of values from four separate experiments. * p < 0.0001 (Dunnett's test) versus the corresponding value for cells incubated in the absence of cytokine.

to 72 h (Fig. 1D).

We next examined the specificity of this synergistic effect of IL-4 or IL-13 with PGN on eotaxin release by corneal fibroblasts. Among the Th2 cytokines tested, only IL-4 and IL-13, not IL-5 or IL-10, stimulated eotaxin release either alone or synergistically with PGN (Fig. 2A). To determine whether the synergistic action of PGN and either IL-4 or IL-13 was specific to eotaxin, we also investigated the effects of these mediators on the release of another chemokine for neutrophils, IL-8 (CXCL8). None of the Th2 cytokines tested alone was found to affect IL-8 production, whereas PGN stimulated IL-8 release by corneal fibroblasts (Fig. 2B); this effect of PGN was not potentiated by IL-4, IL-13, or any of the other Th2 cytokines.

We also examined the effects of PGN and Th2 cytokines on eotaxin and IL-8 release by human dermal fibroblasts. Whereas IL-4, IL-13, or PGN alone induced small increases in the extent of eotaxin release, the combination of PGN with either IL-4 or IL-13 resulted in a synergistic increase in eotaxin release by these cells (Fig. 2C). Again, as for corneal fibroblasts, the production of IL-8 by skin fibroblasts was stimulated only by PGN, not by any of the Th2 cytokines tested (Fig. 2D).

EFFECTS OF PGN, IL-4, AND IL-13 ON THE ABUNDANCE OF EOTAXIN mRNA IN FIBROBLASTS

We next investigated the effects of PGN, IL-4, and IL-13 on the abundance of eotaxin mRNA in corneal fibroblasts. Cells were cultured for 6 h in the absence or presence of cytokine (10 ng/ml) and PGN (30 μ g/ml), after which the amount of eotaxin mRNA in cell lysates was determined by RT and real-time PCR analysis. Whereas PGN, IL-4, and IL-13 each induced small increases in the amount of eotaxin mRNA, the abundance of this transcript was synergistically increased by simultaneous exposure of the cells to the combination of PGN and either IL-4 or IL-13 (Fig. 3). Similar results were obtained for the effects of these agents on the amount of eotaxin mRNA in skin fibroblasts (data not shown).

EFFECTS OF PGN AND Th2 CYTOKINES ON THE EXPRESSION OF ADHESION MOLECULES IN CORNEAL FIBROBLASTS

We examined the effects of PGN, IL-4, and IL-13 on the expression of adhesion molecules in corneal fibroblasts. The cells were cultured for 24 h with PGN or Th2 cytokines, after which the expression of VCAM-1 and ICAM-1 was measured by in situ whole-cell ELISA (Fig. 4). In the absence of IL-4 or IL-13, PGN induced only a small increase in VCAM-1 expression that was statistically significant at concentrations of 1.0 to 100 μ g/ml. In the presence of IL-4 or IL-13, however, PGN markedly potentiated the stimulatory effects of IL-4 or IL-13 on VCAM-1 expression in a concentration-dependent manner, with the maximal potentiation apparent at 1.0 μ g/ml (Fig. 4A). Similarly, IL-4 and IL-13 each potentiated the effect of PGN on VCAM-1 expression in a concentration-dependent manner (data not shown). Whereas PGN, IL-4, or IL-13 induced only small increases in the amount of VCAM-1 mRNA in corneal fibroblasts, the combination of PGN and either IL-4 or IL-13 elicited a synergistic up-regulation of this transcript (Fig. 4B). Among Th2 cytokines examined, only IL-4 and IL-13, not IL-5 or IL-10, exhibited a stimulatory effect on VCAM-1 expression (Fig. 4C). ICAM-1 was constitutively expressed on cell surface of corneal fibroblasts in unstimulated condition. PGN by itself induced an approximately two-fold increase in the expression of ICAM-1. However, none of these four Th2 cytokines affected ICAM-1 expression in corneal fibroblasts regardless of the stimulation with PGN (Fig. 4D).

EFFECTS OF PGN ON NF- κ B ACTIVITY IN CORNEAL FIBROBLASTS

The I κ B- α -NF- κ B pathway plays a key role in induction of eotaxin and VCAM-1 gene expression in several cell types.^{25,26} We therefore examined the possible effects of PGN on this signaling pathway in corneal fibroblasts by monitoring the degradation of I κ B-

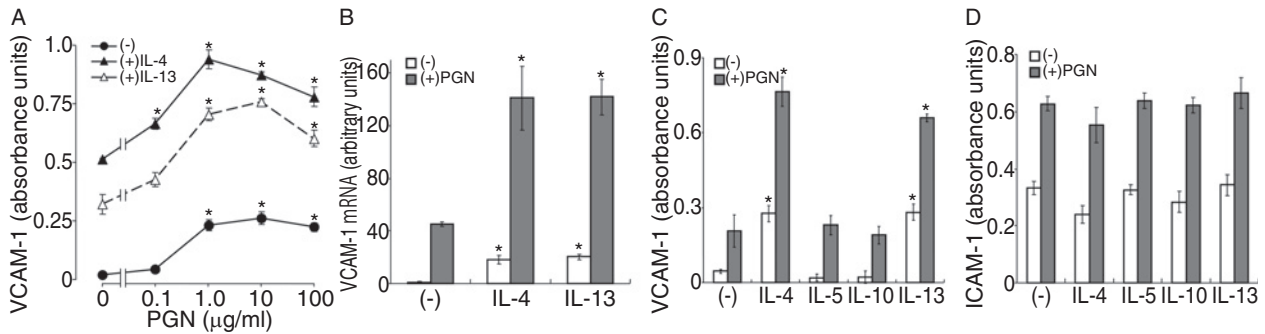


Fig. 4 Effects of PGN, IL-4, and IL-13 on the expression of adhesion molecules in corneal fibroblasts. **(A)** Cells were cultured for 24 h with the indicated concentrations of PGN in the absence (circles) or presence of IL-4 (closed triangles) or IL-13 (open triangles), each at a concentration of 10 ng/ml, after which the expression of VCAM-1 was examined by in situ whole-cell ELISA. **(B)** Cells were cultured for 6 h with IL-4 or IL-13 (each at 10 ng/ml) in the absence (open bars) or presence (closed bars) of PGN (30 μg/ml). Total RNA was then isolated and the amount of VCAM-1 mRNA was determined by quantitative RT-PCR analysis. Data were normalized on the basis of the abundance of GAPDH mRNA and are expressed in arbitrary units. **(C, D)** Cells were incubated for 24 h with the indicated Th2 cytokines (10 ng/ml) in the absence (open bars) or presence (closed bars) of PGN (30 μg/ml), after which the expression of VCAM-1 **(C)** or ICAM-1 **(D)** was examined by in situ whole-cell ELISA. All data are means $\pm$ SEM of values from four separate experiments. * $p < 0.01$ (Dunnett's test) versus the corresponding value for cells incubated in the absence of cytokine or PGN.

α as well as the nuclear translocation and DNA binding activity of NF- κ B. Exposure of cells to PGN (30 μg/ml) induced degradation of I κ B- α in a time-dependent manner (Fig. 5A); the decrease in the amount of I κ B- α was first apparent at 10 min and was maximal at 60 min, after which the abundance of this protein gradually increased up to 4 h after the onset of stimulation. Immunofluorescence analysis revealed that the p65 subunit of NF- κ B was located predominantly in the cytoplasm of corneal fibroblasts under basal conditions (Fig. 5B), consistent with our previous observations.²² No immunofluorescence was apparent in cells stained with normal rabbit IgG as a negative control (data not shown). Treatment of cells with PGN (30 μg/ml) resulted in translocation of NF- κ B to the nucleus in a time-dependent manner; translocation was first detected at 10 min and was maximal at 60 min (Fig. 5C) after the onset of stimulation. Measurement of the binding of the p65 subunit of NF- κ B to an oligonucleotide containing the consensus binding site revealed that exposure of corneal fibroblasts to PGN (30 μg/ml) for 60 min resulted in a pronounced increase in the DNA binding activity of p65 present in nuclear extracts (Fig. 5D). This effect was similar to that observed with TNF- α (10 ng/ml, data not shown), a classical activator of NF- κ B signaling. The specificity of p65 binding was demonstrated by its inhibition with free wild-type oligonucleotide but not with an oligonucleotide in which the binding site is mutated (Fig. 5D). These various observations thus showed that PGN activates the NF- κ B signaling pathway in human corneal fibroblasts.

To confirm the role of NF- κ B activation in the stimulation of eotaxin release by PGN, we examined

the effects of two inhibitors of NF- κ B signaling, PDTC and TPCK, on PGN-induced eotaxin release in corneal fibroblasts. Oxygen radicals have been implicated in the mechanism of NF- κ B activation, and antioxidants such as PDTC have been shown to block this process in several cell lines.²⁷ TPCK blocks NF- κ B activation by preventing the degradation of I κ B and the translocation of NF- κ B to the nucleus.²⁸ PDTC (30 μM) and TPCK (3 μM) each inhibited eotaxin release induced by PGN but not that elicited by IL-4 or IL-13 (Fig. 6). Furthermore, the synergistic increase in eotaxin release induced by PGN and either IL-4 or IL-13 was inhibited by PDTC or TPCK. These findings thus suggest that the activation of NF- κ B plays a major role in the induction of eotaxin release by PGN in corneal fibroblasts.

DISCUSSION

We have shown that PGN of *S. aureus* induces eotaxin production in a synergistic manner with the Th2 cytokines IL-4 or IL-13 in human corneal and dermal fibroblasts. Given that both the up-regulation of Th2 cytokines and a high rate of *S. aureus* colonization of the skin and ocular surface are associated with AKC and atopic dermatitis,²⁹ our data suggest that the innate immune response to bacterial infection and the adaptive immune response mediated by IL-4 or IL-13 may together promote tissue eosinophilia through the intermediary of tissue-resident fibroblasts.

In a mouse model of allergic conjunctivitis, inoculation of *S. aureus* markedly accelerated Th2-type immune responses and accelerates experimental allergic conjunctivitis including tissue eosinophilia, higher levels of IgE in serum.³⁰ However, we have also dem-

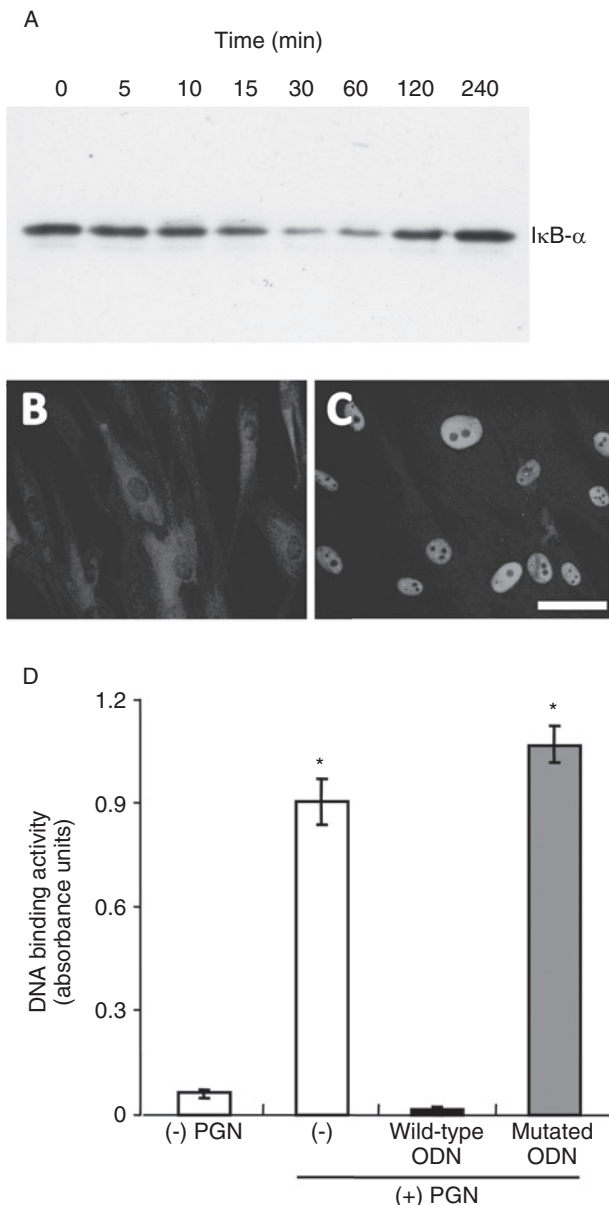


Fig. 5 Effect of PGN on NF- κ B signaling in corneal fibroblasts. **(A)** Cells were incubated for the indicated times with PGN (30 μ g/ml), lysed, and subjected to immunoblot analysis with Abs to I κ B- α . **(B, C)** Cells were incubated in the absence **(B)** or presence **(C)** of PGN (30 μ g/ml) for 60 min, fixed, and subjected to immunofluorescence analysis with Abs to the p65 subunit of NF- κ B. Scale bar, 50 μ m. Data in **(A)** through **(C)** are representative of three independent experiments. **(D)** Cells were incubated in the absence or presence of PGN (30 μ g/ml) for 1 h, after which nuclear extracts were prepared and assayed for the DNA binding activity of the p65 subunit of NF- κ B with a colorimetric assay. The specificity of binding was assessed on the basis of competition with excess wild-type or mutated oligonucleotide (ODN). Data are means $\pm$ SEM of values from four separate experiments. * p < 0.0001 (Dunnett's test) versus control (no PGN).

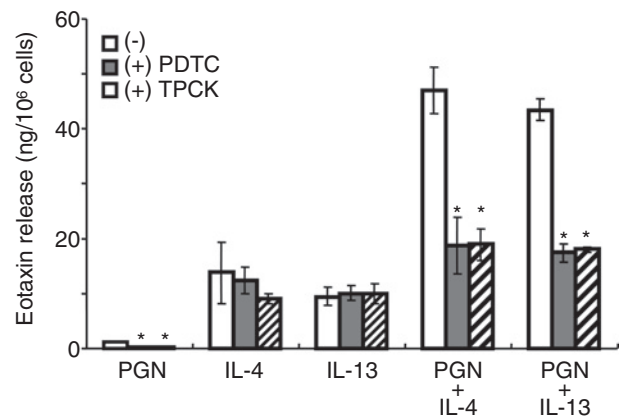


Fig. 6 Effects of NF- κ B inhibitors on eotaxin release induced by PGN and Th2 cytokines in corneal fibroblasts. Cells were preincubated for 1 h in the absence or presence of PDTC (30 μ M) or TPCK (3 μ M) and were then incubated for 24 h in the additional absence or presence of PGN (30 μ g/ml), IL-4 (10 ng/ml), or IL-13 (10 ng/ml), as indicated. The concentration of eotaxin in culture supernatants was then measured by ELISA. Data are means $\pm$ SEM of values from four separate experiments. * p < 0.01 (Dunnett's test) versus the corresponding value for cells incubated without inhibitor.

onstrated that systemic treatment with Pam3CSK4, a TLR2 agonist, suppressed eosinophil infiltration into the conjunctiva in a mouse model of allergic conjunctivitis.³¹ In a mouse model of allergic asthma, the humoral and cellular immune responses, including eosinophilia, were more marked and Th2 biased in mice immunized intranasally with both ovalbumin and PGN than in those immunized with ovalbumin alone.³² Similarly, exposure to a synthetic ligand of TLR2 promoted the Th2 response and eosinophil infiltration induced by allergen in this same model.³³ However, activation of TLR2 immediately before intranasal allergen challenge was found to reduce allergic airway inflammation in the mouse model of asthma,³⁴ and a TLR2 agonist decreased established allergic airway inflammation by promoting a Th1 response.³⁵ Intradermal injection of cell wall components of *S. aureus* in mice induced localized inflammation that was reminiscent of atopic dermatitis and characterized by the infiltration of mast cells, eosinophils, and mononuclear cells. PGN from *S. aureus* also stimulated the production of Th2 cytokines by and degranulation of mast cells in a TLR2-dependent manner, and intradermal injection of PGN induced vasodilation and inflammation in mouse skin.³⁶ Whether TLR2 signaling activates a Th1 or Th2 response thus likely depends on the dose, timing of administration, and nature of TLR2 agonists and the genetic background of the host. Our results showing that the combination of PGN and the Th2 cytokines IL-4 or IL-13 synergistically induces eotaxin release

by tissue-resident fibroblasts suggest that the concomitant local presence of these factors might trigger tissue eosinophilia without the participation of other immune cells.

The development of sight-threatening corneal lesions secondary to severe allergic inflammation of the conjunctiva is the most serious complication of ocular allergy.³⁷ The expression of bioactive molecules, such as eotaxin and adhesion molecules, by corneal fibroblasts likely contributes to the formation of corneal lesions by promoting the local infiltration, activation, and survival of eosinophils.^{14,15} Knowledge of the regulation of eotaxin synthesis by corneal fibroblasts is thus important for an understanding of the pathogenesis of corneal lesions and for the development of potential new treatments for severe ocular allergy. We have previously shown that concomitant stimulation with proinflammatory cytokines (TNF- α or IL-1) and Th2 cytokines (IL-4 or IL-13) synergistically increases eotaxin production by corneal fibroblasts.^{3,15} In addition, TGF- β or oncostatin-M synergizes with IL-4 or IL-13 to increase eotaxin synthesis by airway fibroblasts.^{38,39} We have now shown that the bacterial component PGN also synergistically increases eotaxin synthesis with the Th2 cytokines IL-4 or IL-13 in corneal fibroblasts, and that this effect is mediated by activation of NF- κ B. Putative binding sites for NF- κ B and STAT6 overlap in the eotaxin gene promoter, and these two transcription factors are thought to mediate the synergistic effect of IL-13 or IL-4 with IL-1 β on eotaxin gene expression in epithelial cells and fibroblasts.^{25,40} In addition to the direct effects of PGN on NF- κ B activation, it may be possible that PGN also have indirect effects on the increase of eotaxin synthesis thorough the production of TNF- α or IL-1 in an autocrine or paracrine manner.

Adhesion molecules are another important determinant of eosinophil infiltration into tissue. The role of VCAM-1 in the infiltration of inflammatory cells appears to be selective for eosinophils, given that its ligand VLA-4 is expressed on the surface of eosinophils but not on neutrophils, whereas LFA-1, the ligand for ICAM-1, is expressed on the surface of both eosinophils and neutrophils.⁶ In addition, VCAM-1 augments the function and survival of eosinophils.⁴¹ IL-4 selectively regulates the expression of VCAM-1 on human endothelial cells.⁶ We have also previously shown that IL-4 and IL-13 each act synergistically with TNF- α to increase the expression of VCAM-1, but not that of ICAM-1, in corneal fibroblasts.^{21,42} We have now shown that PGN acts synergistically with IL-4 or IL-13 to increase the expression of VCAM-1 in corneal fibroblasts, suggesting that PGN may play an important role in eosinophil infiltration by up-regulating the expression of both a chemokine (eotaxin) and an adhesion molecule (VCAM-1).

In conclusion, our results provide evidence for a

specific and substantial interaction between the innate immune system and the Th2 immune system. Eotaxin and VCAM-1 play important roles as a chemokine and adhesion molecule, respectively, in the induction and maintenance of allergic inflammation by promoting the infiltration of eosinophils. Although increased tissue eosinophilia by the infection of *S. aureus* in a murine allergic conjunctivitis has been attributed to accelerated Th2-type immune responses,³⁰ our report provides the possibility, for the first time, that the expression of chemokines and adhesion molecules by *S. aureus*-stimulated fibroblasts contributes to eosinophilic inflammation in AKC and atopic dermatitis. Given that *S. aureus* colonizes the skin and ocular surface in most atopic patients, our data suggest that infection with this bacterium in the atopic Th2 background might markedly enhance tissue eosinophilia through up-regulation of the expression of eotaxin and VCAM-1 by tissue-resident fibroblasts. Knowledge of the mechanisms of interaction between infection and allergy may eventually lead to an increased understanding of allergic diseases such as AKC and atopic dermatitis.

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