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To cite this article: Osamu Sakai, Yoshiyuki Tamada, Thomas R. Shearer & Mitsuyoshi Azuma (2013) Involvement of NF κ B in the Production of Chemokines by Rat and Human Conjunctival Cells Cultured Under Allergenic Conditions, Current Eye Research, 38:8, 825-834, DOI: [10.3109/02713683.2013.780623](https://doi.org/10.3109/02713683.2013.780623)

To link to this article: <http://dx.doi.org/10.3109/02713683.2013.780623>



Published online: 26 Apr 2013.



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ORIGINAL ARTICLE

Involvement of NFκB in the Production of Chemokines by Rat and Human Conjunctival Cells Cultured Under Allergenic Conditions

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ABSTRACT

Purpose: The purpose of present studies was to determine the involvement of NFκB and STAT6 transcription factors in the production of cytokines by the fibroblasts and epithelial cells in conjunctiva.

Methods: An *in vitro* model of allergic conjunctivitis was developed by sensitizing and challenging rat mast cells with anti-dinitrophenyl (DNP) IgE and DNP-BSA, and then using the conditioned medium to stimulate rat conjunctival fibroblasts. Chemokines (eotaxin-1, IL-8, and RANTES – Regulated and Normal T cell Expressed and Secreted) released from cells into the medium was determined by ELISA. Human conjunctival fibroblasts and epithelial cells were also directly stimulated with exogenous cytokines tumor necrosis factor (TNF)-α or IL-4. Degradation of IκB-α and phosphorylation of STAT6 were assessed by immunoblotting. For inhibition of NFκB or STAT6 activation, upstream regulators IκB kinase and Janus protein tyrosine kinases (JAK) were inhibited by use of BMS-345541 and JAK inhibitor 1. An *in vivo* model of conjunctivitis was also produced in rats by intraperitoneal injection of ovalbumin (OA) with aluminum hydroxide and challenge at 21 d with OA eye drops.

Results: Stimulated rat mast cells released TNF-α and IL-4. TNF-α induced NFκB activation in rat and human conjunctival fibroblasts and epithelial cells, and caused production and release of cytokines IL-8 and RANTES. IL-4 activation of STAT6 did not cause release of these cytokines. Only fibroblasts produced the eosinophil-recruiting cytokine, eotaxin-1, after treatment with TNF-α plus IL-4. As observed in the cultured cells, allergic stimulation in the *in vivo* model caused degradation of IκB-α in conjunctiva, and infiltration of eosinophils and other inflammatory cells.

Conclusion: Activated NFκB was found to be a major transcription factor for the release of cytokines from conjunctival cells and intensification of the allergic response. Inhibition of the NFκB pathway by therapeutic drugs may be an important objective for the treatment of human allergic conjunctivitis.

Keywords: Allergic conjunctivitis, chemokine, conjunctival cells, mast cells, NFκB

INTRODUCTION

Allergic conjunctivitis is a common eye disease with symptoms ranging from mild irritation to sight-threatening, acute erosion and scarring of the cornea.^{1–3} The condition commonly develops through the well-defined, type I (immediate) hypersensitivity reaction (Figure 1). Airborne allergens such as pollen, house dust and animal dander are presented by IgE to

mast cell receptors on the conjunctiva (Step 1). Mast cells are present at 5000–6000 cell/mm³ in human conjunctiva⁴ and are increased in the patients with severe allergic conjunctivitis.^{5,6} Stimulated mast cells then release histamine and cytokines – such as tumor necrosis factor (TNF)-α and interleukin (IL)-4.^{7,8} In patients with allergic conjunctivitis, levels of TNF-α and IL-4 in the tears are significantly higher than in healthy subjects.^{9,10}

Received 9 November 2012; revised 31 January 2013; accepted 22 February 2013; published online 26 April 2013

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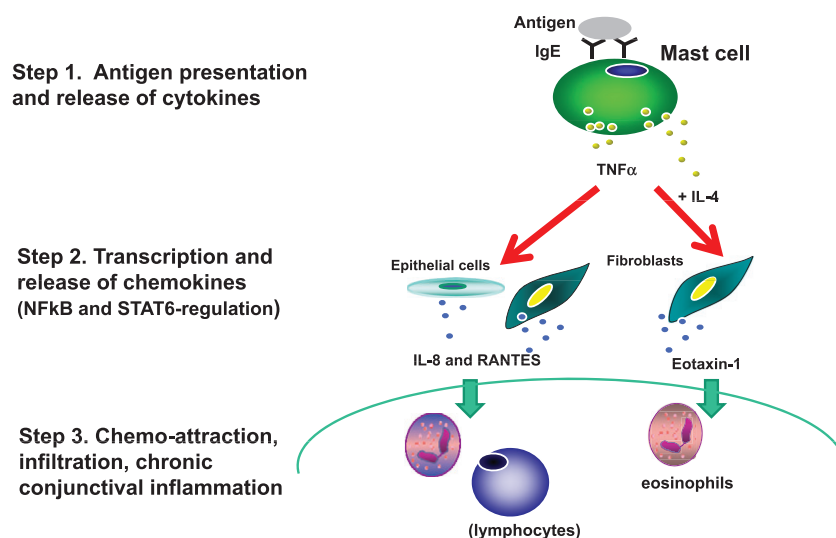


FIGURE 1 Schematic pathway for type I (immediate) hypersensitivity in allergic conjunctivitis, highlighting the central role of activated NFκB.

TNF- α and IL-4 stimulated the transcription of chemokines in conjunctival fibroblasts and epithelial cells^{11–14} (Step 2, Figure 1). The small protein chemokines, include eotaxin-1, IL-8 and RANTES (Regulated on Activation Normal T Expressed and Secreted), which are secreted. Eotaxin-1, IL-8 and RANTES have been identified in tears of patients with allergic conjunctivitis.^{15–18}

Chemokines generally function by regulating leukocyte chemo-attraction and infiltration into conjunctiva and cornea (Step 3, Figure 1). For example, eotaxin-1 is a strong and selective chemokine for attracting eosinophil infiltration through the chemokine receptor-3 (CCR3).¹⁹ IL-8 acts through CXCR1 and CXCR2 receptors and causes chemotaxis of neutrophils, eosinophils and lymphocytes.^{20,21} RANTES acts through CCR1, CCR3, CCR4 and CCR5 and is a non-selective chemokine for various inflammatory cells.^{22,23} Inflammatory cells have been identified in conjunctival tissue from patients with severe allergic conjunctivitis,^{24,25} where they induce tissue damage by producing a variety of cytotoxic substances, such as eosinophil cationic protein, eosinophil peroxidase, neutrophil toxic oxygen radical, proteases and Th2 lymphocytic inflammatory cytokines.^{26–29}

As discussed above, a key step in the mechanism of allergic conjunctivitis is the induction of chemokines IL-8, eotaxin-1 and RANTES. However, the regulatory mechanism for chemokine production is not well defined in conjunctiva, and it could be an attractive target for inhibition by drug therapy. In other cell types, TNF- α activates transcription factor NFκB which regulates expression of many pro-inflammatory genes.^{30–33} IL-4 activates transcription factor STAT6 via receptor-associated janus kinases (JAK) 1 and 3,^{34,35} and STAT6 regulates the expression of eotaxin-1.^{31,36} Thus, the purpose of

present study was to determine the involvement of NFκB and STAT6 in production of chemokines in conjunctival fibroblasts and epithelial cells.

METHODS

Experimental Animals

Male Sprague-Dawley rats (Charles River Laboratories, Shizuoka, Japan) at 6–8 weeks of age were used for isolating conjunctival fibroblasts. Male Brown-Norway rats (Charles River) at 6 weeks of age were used for our *in vivo* model of allergic conjunctivitis. Animals were maintained in ordinary animal cages under a constant 12 h light/dark cycles. Food and water were available *ad libitum*. All experimental animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23).

Production of Conditioned Medium from Cultured Mast Cells

Rat mast cells (RBL-2H3, American Type Culture Collection) were cultured under 5% CO₂ at 37°C in MEM (Invitrogen, Carlsbad, CA) containing 10% FBS and 100 U penicillin plus 100 μg streptomycin/mL. Rat mast cells at 50% confluency in 225 cm² flasks were sensitized overnight with 500 ng anti-dinitrophenyl (DNP) IgE/ml (Sigma, St. Louis, MO). After washing with phosphate buffered saline (PBS), mast cells were then challenged with 200 ng DNP-bovine serum albumin/ml (Calbiochem) in 15 ml fresh medium. After 18 h incubation, media were collected

and centrifuged at $2000 \times g$ for 4 min to remove cellular debris. The condition medium was then stored at -80°C . Concentrations of IL-4 and TNF- α in the conditioned medium were measured using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Treatment of Conjunctival Fibroblasts with Conditioned Medium

Conjunctivas excised from euthanized rats were incubated in 2 mg dispase/mL MEM for 1 h at 37°C . The epithelial sheet was mechanically removed from the conjunctivas, and single cell suspensions of conjunctival fibroblasts were obtained by treatment with 2 mg collagenase/mL MEM (Invitrogen) at 37°C . Isolated conjunctival fibroblasts were cultured under 5% CO_2 at 37°C in MEM containing 10% FBS and 100 U penicillin plus 100 μg streptomycin/mL. The cells were subcultured in 3–5 passages, and when confluent, they were used for the experiments below.

Conjunctival fibroblasts were stimulated by replacing the culture medium with the conditioned medium from sensitized mast cells in volumes of 5 mL (10 cm^2 dish) or 0.2 mL (96-well plate). For the inhibition studies, cells were pre-treated with inhibitor of I κ B kinase (IKK) BMS-345541 (Merck Biosciences, Bad Soden, Germany), JAK inhibitor 1 (Merck Biosciences) or vehicle 30 min before stimulation with conditioned medium, and each inhibitor was continuously present during culture. Signal-induced phosphorylation of I κ B- α by IKK normally targets I κ B- α inhibitor for proteosomal degradation, allowing NF κ B activation and transcription.³⁷ BMS-345541 inhibits the kinase and was used to confirm that loss of I κ B- α in the present experiments was a valid marker for activation of the NF κ B pathway. JAK inhibitor 1 was used because it functions by preventing phosphorylation of cytokine receptors and STAT6, preventing dimerization to the active STAT6 transcription factor.^{34,35}

Thirty minutes after stimulation, the cells in 10 cm^2 dishes were washed twice with ice-cold PBS, and suspended in 100 μL of the lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.6% Nonidet P-40, and protease inhibitor cocktail (Wako, Tokyo, Japan). The cell suspensions were kept for 15 min on ice to extract proteins, and cell debris were removed by centrifugation at $20,000 \times g$ for 30 min at 4°C . Protein concentrations in the supernates were determined by the Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as standards and then used for immunoblotting.

Twenty-four hours after stimulation, the culture medium in 96-well plates was assayed for rat eotaxin-1 (R&D Systems), rat CINC-1 (IL-8 like chemokine, R&D Systems) and rat RANTES (R&D Systems) using

an ELISA kit according to the manufacturer's instructions.

CINC-1 was assessed instead of IL-8, since (1) IL-8 has not yet been identified in rat,^{38,39} (2) CINC-1 is corresponds to human growth-regulated gene (GRO) in the IL-8 family^{39,40} and (3) The function of CINC-1 as a neutrophil chemo-attractant in the inflammatory process is similar to that of human IL-8.⁴¹

Treatment of Human Conjunctival Fibroblasts and Epithelial Cells with Cytokines

Human conjunctival fibroblasts (GM01123, Coriell Institute for Medical Research, Camden, NJ) were cultured under 5% CO_2 at 37°C in MEM (Invitrogen) containing 15% fetal bovine serum (FBS; Invitrogen), 0.1 mM non-essential amino acids, and 100 U penicillin plus 100 μg streptomycin/mL. Human conjunctival epithelial cells (Wong-Kilbourne derivative of Chang conjunctiva, American Type Culture Collection) were cultured under 5% CO_2 at 37°C in Medium 199 (Invitrogen) containing 10% FBS and 100 U penicillin plus 100 μg /mL streptomycin.

When confluent, media were replaced with 10 mL (10 cm^2 dish) or 0.2 mL (96-well plate) of fresh medium containing 1% FBS. The cells were then stimulated with human recombinant TNF- α (10 ng/mL, R&D Systems), human recombinant IL-4 (10 ng/mL, R&D Systems) or TNF- α plus IL-4. Treatment with inhibitors, preparation of cellular proteins and measurement of chemokines in media were as described above.

Immunoblotting

To test for activation of NF κ B, degradation of I κ B- α protein was determined by immunoblotting. I κ B- α is an endogenous inhibitor of NF κ B; degradation of the inhibitor allows NF κ B to become active.^{42,43} Thus, loss of I κ B- α inhibitor has been widely used in previous studies as a marker for activation of NF κ B.^{44–48} For activation of STAT6, phosphorylated STAT6 was determined by immunoblotting, since STAT6 is activated by phosphorylation via JAK1 and 3.^{34,35}

SDS-PAGE of proteins (20 μg /lane) was performed on 4–12% Bis-Tris gels (Nu-PAGE, Invitrogen) with the MOPS buffer system (Invitrogen). The separated proteins were transferred to PVDF membrane with iBlot Gel Transfer system (Invitrogen). The membranes were probed with antibodies to I κ B- α (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-STAT6 (1:100, Santa Cruz Biotechnology). Binding of secondary antibodies, conjugated to alkaline phosphatase or to horseradish peroxidase, was

visualized with BCIP/NBT substrate (Bio-Rad) or chemiluminescent substrate (Pierce, Rockford, IL).

In Vivo Model of Allergic Conjunctivitis

Rats were sensitized by intraperitoneal injection of 1 mL saline containing 1 mg ovalbumin (OA, Sigma) and 20mg aluminum hydroxide on days 0 and 7. Twenty-one days after the first sensitization, allergic reactions were challenged by topical application of 10 μ L 10% OA to the conjunctiva.

Rats were sacrificed at 0, 3, 6, 12 and 24 h after OA application. Dissected conjunctivas were cut into small pieces, and homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.6% Nonidet P-40 and protease inhibitor cocktail (Wako). The cell lysate were centrifuged at 20,000 $\times$ g for 30 min at 4°C to obtain soluble proteins. After measurement of protein concentrations, immunoblotting for I κ B was performed as described above.

Eye globes with lid margins from rats sacrificed at 24 h were also enucleated, fixed in 10% buffered

formalin and embedded in paraffin. Horizontal 3 μ m thick sections were cut and stained with Giemsa. Infiltrating eosinophils in conjunctiva were identified with light microscopy (X 20).

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed with the Dunnett's multiple comparison tests. $p < 0.05$ was considered statistically significant.

RESULTS

Rat Conjunctival Fibroblasts: Activation of NF κ B and STAT6 by Mast Cell Conditioned Medium

Conditioned medium from mast cells stimulated with anti-DNP IgE contained large amounts of TNF- α and IL-4, which were negligible in the medium from untreated mast cells (Figure 2A). The amount of

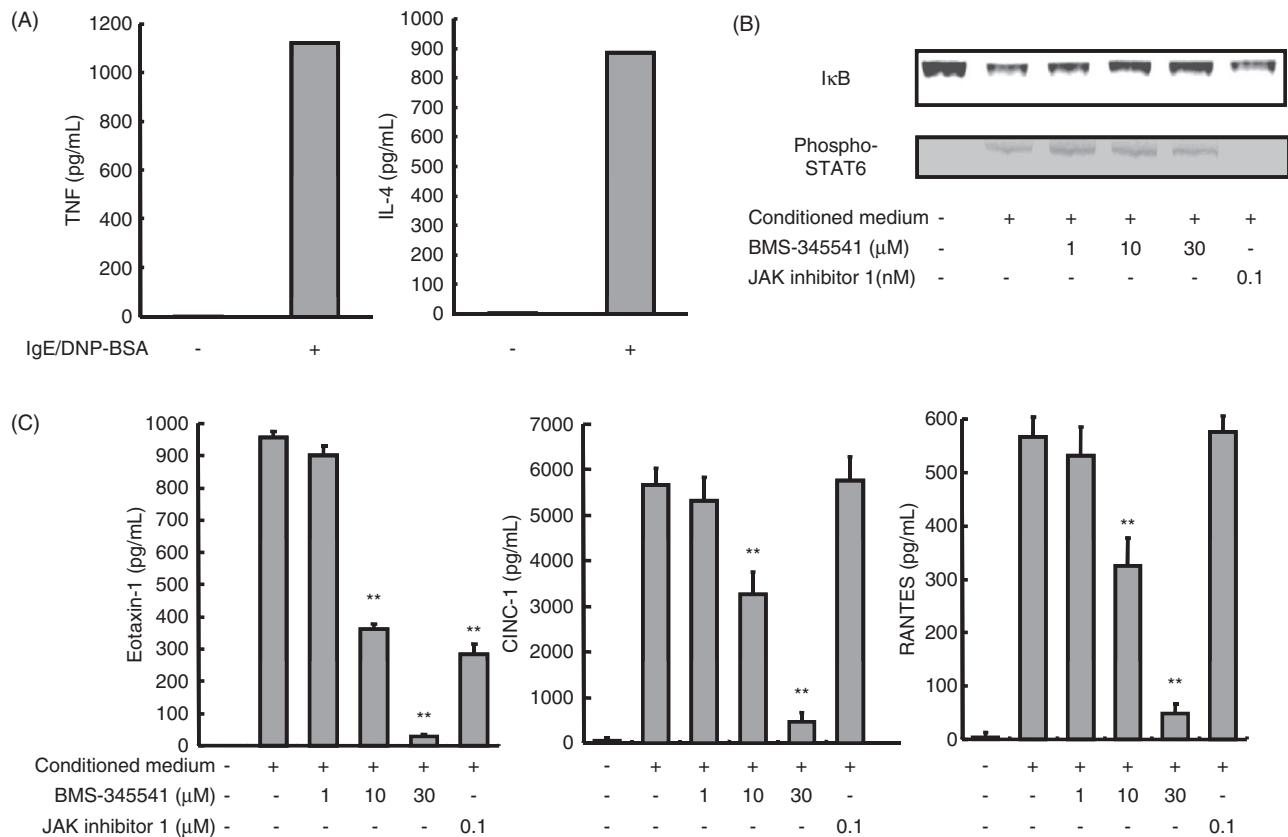


FIGURE 2 Activation of NF κ B and STAT6 signaling pathways in conjunctival rat fibroblasts stimulated by mast cell-conditioned medium. (A) ELISA showing release of cytokines TNF- α and IL-4 by cultured rat mast cells activated with DNP-BSA antigen and producing “conditioned” medium. (B) Immunoblots of rat conjunctival fibroblasts after 30 min culture in the conditioned medium, showing activation of signaling pathways by a decrease in I κ B inhibitory protein (upper) and an increase in active phosphorylated STAT6 (lower). Pre-treatment with BMS-345541 (IKK inhibitor) and JAK inhibitor 1 inhibited the signaling pathways. (C) After 24 h, ELISA showing release of cytokines eotaxin-1, CINC-1 and RANTES from conjunctival fibroblasts and inhibition by pretreatment with BMS-345541 and JAK inhibitor 1. Data are means $\pm$ S.E. ($n = 4$). ** $p < 0.01$ relative to vehicle-treated group.

TNF-α and IL-4 were several times higher than those in previous report.⁴⁹ This may be due to higher amounts of IgE and antigen used in the present study.

In rat conjunctival fibroblasts, conditioned medium from rat mast cells caused a significant decrease in IκB-α and increases in phosphorylation of STAT6 (Figure 2B). IKK inhibitor BMS-345541 inhibited degradation of IκB-α in a dose dependent manner (Figure 2B). JAK inhibitor 1 inhibited phosphorylation of STAT6. Inhibitors were specific without cross inhibitions.

Conditioned medium increased the release of eotaxin-1, CINC-1 and RANTES from rat conjunctival fibroblasts (Figure 2C). BMS-345541 inhibited release of all three chemokines in a dose-dependent manner. On the other hand, JAK inhibitor 1 only inhibited release of eotaxin-1, and did not inhibit release of CINC-1 and RANTES (Figure 2C). These data suggested activation of the NFκB pathway during production of all three chemokines, but simultaneous activation of STAT6 only occurred during the production of eotaxin-1. Data also suggested that TNF-α and IL-4 in conditioned medium might be inducers for signal transduction in conjunctival fibroblasts, which were next tested by direct stimulation with recombinant TNF-α and IL-4 proteins.

1 (0.1 μM) suppressed the degradation of IκB-α and phosphorylation of STAT6 (Figure 3B). These results suggested that TNF-α and IL-4 specifically activated NFκB and STAT6, respectively, without cross activation.

In human conjunctival fibroblasts, TNF-α by itself induced release of IL-8 and RANTES, but IL-4 did not induce either (Figure 4A). An additive effect was not observed by combined treatment with TNF-α and IL-4. BMS-345541 (≥10 μM) inhibited release of IL-8 and RANTES, but JAK inhibitor 1 (0.1 μM) did not inhibit their release (Figure 4B), suggesting involvement of NFκB, but not STAT6. In contrast, production of eotaxin-1 required treatment with both TNF-α and IL-4, and TNF-α or IL-4 alone did not induce either (Figure 4A). In support of this, BMS-345541 (≥10 μM) as well as JAK inhibitor 1 (0.1 μM) inhibited release of eotaxin-1 (Figure 4B), suggesting that both NFκB and STAT6 activation were essential for eotaxin-1 induction.

In human conjunctival epithelial cells, the release of IL-8 and RANTES showed similar results as those observed in fibroblasts (Figure 5A and B). However, eotaxin-1 was not released even when epithelial cells received combined treatment with TNF-α plus IL-4 (Figure 5A).

Human Conjunctival Fibroblasts and Epithelial Cells: Activation of NFκB and STAT6 by TNF-α and IL-4

In human conjunctival fibroblasts and epithelial cells, exogenous TNF-α and IL-4 induced loss of IκB-α and promoted phosphorylation of STAT6, respectively (Figure 3A). TNF-α plus IL-4 showed an additive effect. BMS-345541 (10 μM) and JAK inhibitor

Activation of NFκB and Infiltration by Inflammatory Cells

In our *in vivo* rat model of allergic conjunctivitis, degradation of IκB-α was first observed 6 h after OA application; degradation was even more severe at 12 h (Figure 6A). This may be caused by TNF-α from mast cells, since several hours are needed for production of TNF-α in mast cells.^{50–52} After 24 h,

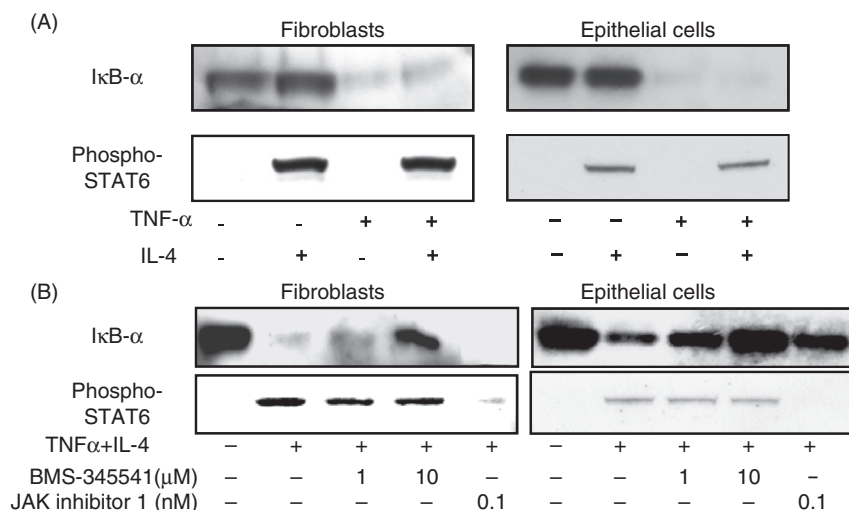


FIGURE 3 Exogenous TNF-α and IL-4-activated NFκB and STAT6 pathways in human conjunctival fibroblasts and epithelial cells. (A) Immunoblots showing decreased IκB (upper) and increased phosphorylated STAT6 (lower) in human conjunctival fibroblasts (left) and epithelial cells (right) 30 min after induction by TNF-α, IL-4 or TNF-α. (B) Immunoblots showing inhibition by BMS-345541 (upper) and by JAK inhibitor 1 (lower).

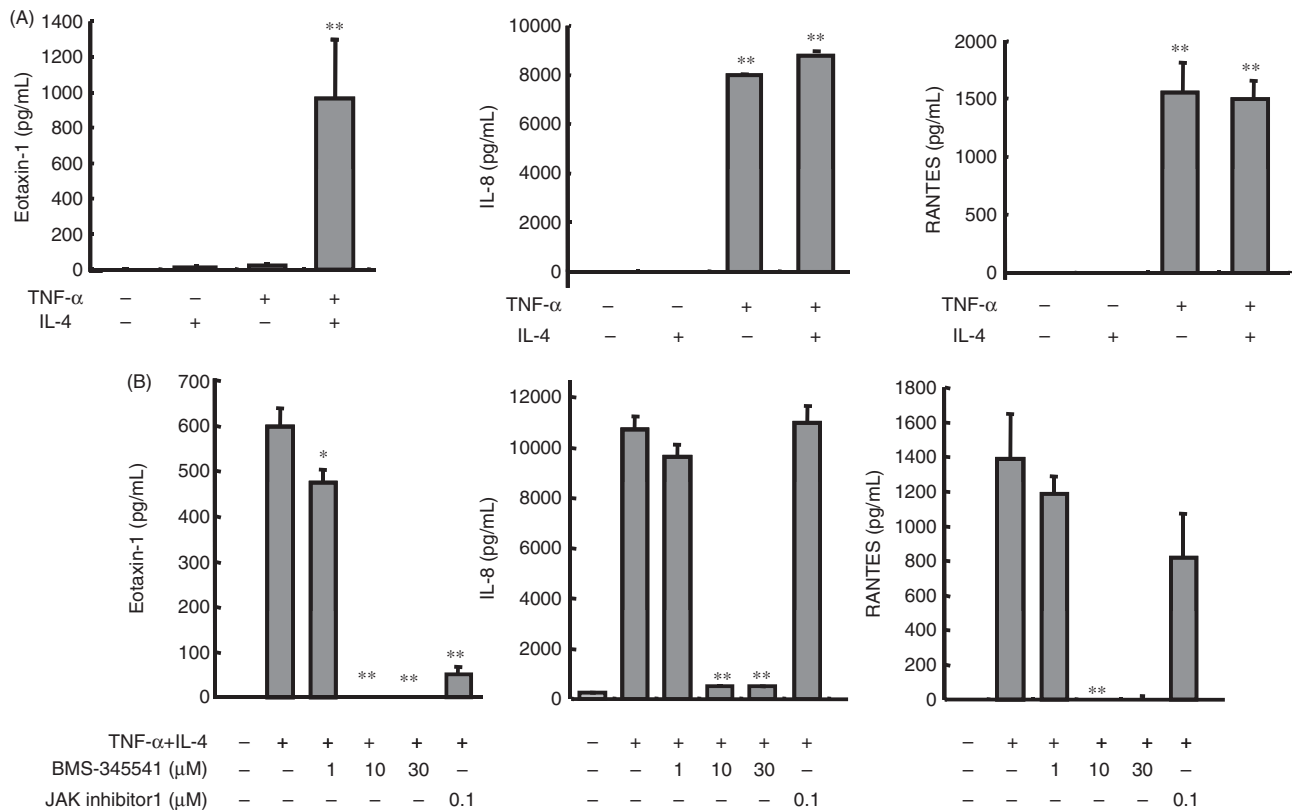


FIGURE 4 Fibroblasts: stimulated release of chemokines. (A) ELISA of culture medium showing that TNF- α alone, but not IL-4, released IL-8 and RANTES from human conjunctival fibroblasts. TNF- α required IL-4 to cause release of eotaxin-1. Data are means $\pm$ S.E. ($n = 3$). ** $p < 0.01$ relative to the untreated normal group. (B) IKK inhibitor BMS-345541, but not JAK inhibitor 1, inhibited release of IL-8 and RANTES after stimulation of fibroblasts by TNF- α plus IL-4. BMS-345541 or JAK inhibitor 1 alone inhibited release of eotaxin-1. Data are means $\pm$ S.E. ($n = 4$). * $p < 0.05$ and ** $p < 0.01$ relative to the vehicle-treated group.

cells, including eosinophils, infiltrated and accumulated in conjunctiva (Figure 6B) even though the level of I κ B- α had somewhat returned to normal (Figure 6A).

DISCUSSION

Central Role of the NF κ B Pathway

The main contribution of the experiments above was that NF κ B was a central and major transcription factor for producing pro-inflammatory chemokines in conjunctival cells (Figure 1). This finding was facilitated by the development and use of our *in vitro* rat model of allergic conjunctivitis, and was confirmed for relevance using human cells. While steroid treatment can be used for symptom relief in patients with allergic conjunctivitis, increased IOP and cataract are potential risks. It is currently well accepted that the therapeutic anti-inflammatory effects of steroids are usually mediated via inhibition of transcription factors such as NF κ B,^{53,54} while the side effects of steroids are usually mediated via DNA-dependent transactivation.^{55,56} The present finding that the NF κ B pathway is a central mediator during inflammation

of the conjunctiva suggests that inhibition of the NF κ B pathway, by IKK inhibitors such as BMS-345541, may present new opportunities for developing and evaluating anti-allergic drugs. These inhibitors may also provide fewer side effects than steroids.

Mast Cell TNF- α Activates NF κ B to Cause Production of Chemokines IL-8 and RANTES from Conjunctival Fibroblasts and Epithelial Cells

Our studies found that activated conjunctival mast cells produce cytokines TNF- α and IL-4. This supports previous reports of TNF- α and IL-4 in the tears of patients with allergic conjunctivitis,^{13,17,29} and mirrors their well-known roles in many other types of inflammatory reactions.^{57–60} TNF- α is known to activate transcription factors NF κ B, NF-IL-6 and AP-1.^{61,62} Note that in our human conjunctival fibroblasts and epithelial cells, TNF- α only activated NF κ B. Further, only TNF- α was involved in release of chemokines IL-8 and RANTES from human conjunctival fibroblast and epithelial cells (Figures 4 and 5). The release of both chemokines was inhibited only by BMS-345541, again suggesting involvement of NF κ B.

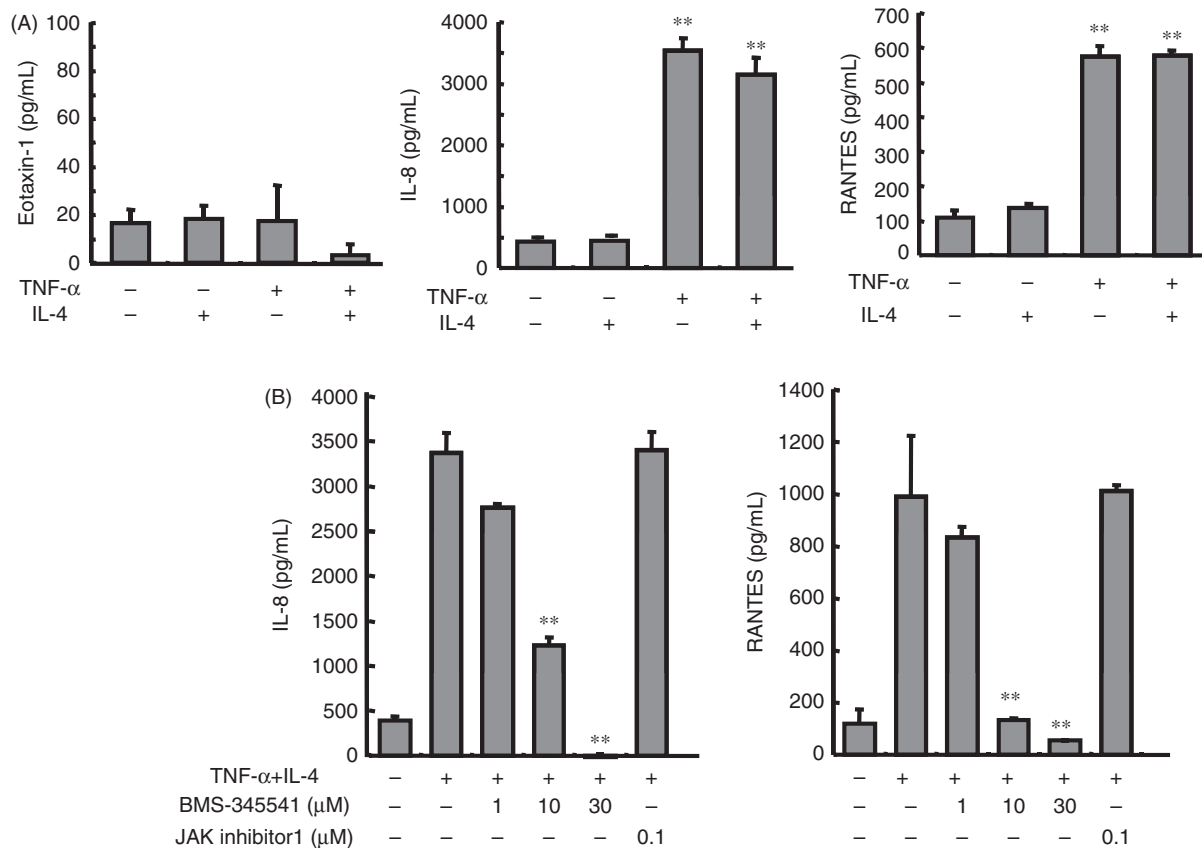


FIGURE 5 Epithelial cells: stimulated release of specific chemokines. (A) ELISA showing that TNF-α alone, but not IL-4, released IL-8 and RANTES from human conjunctival epithelial cells. Eotaxin-1 was not released. ** $p < 0.01$ relative to the untreated normal group. (B) BMS-345541 inhibited release of IL-8 and RANTES induced by TNF-α plus IL-4, but JAK inhibitor 1 did not inhibit. Data are means $\pm$ S.E. ($n = 4$). ** $p < 0.01$ relative to the vehicle-treated group.

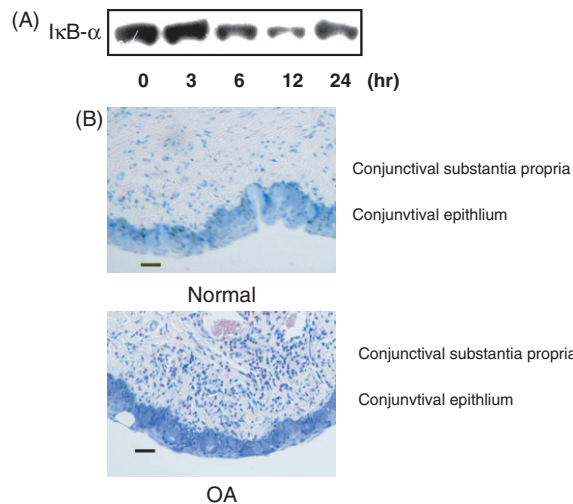


FIGURE 6 Activation of NFκB induced infiltration of cells. (A) Immunoblot showing decrease in IκB in rat conjunctivas after OA challenge, suggesting activation of NFκB pathway. (B) Giemsa-stained histological sections showing eosinophil (red) infiltration into conjunctiva 24 h after OA. Bars, 40 μm.

Similar results were found in previous reports,^{12,13} and indicate the central role of the activated NFκB pathway in release of chemokines IL-8 and RANTES.

Concerted Activation of TNF-α and IL-4 was Required to Cause Chemokine Eotaxin-1 Production from Fibroblasts

In contrast to the above, chemokine eotaxin-1 was only released from fibroblasts, and only when IL-4 was present along with TNF-α (Figures 2 and 4A). The release of eotaxin-1 was inhibited by either BMS-345541 or JAK inhibitor 1, and both inhibitors did not have to be present in combination. Transcription factor STAT6 is essential for most responses to IL-4, and is known to regulate chemokines, CD23 and MHC class II.^{31,36,55–57,63} In other types of cells, TNF-α was found to amplify the activation of STAT6, and IL-4 could also cause activation of NFκB.^{64–66} However, in our cultured cells, TNF-α and IL-4 specifically activated either NFκB or STAT6, respectively, without cross activation. The eotaxin-1 promoter is reported to contain overlapping consensus binding sites for NFκB and STAT6.⁶⁷ All these data suggest that synergistic activation of both NFκB and STAT6 are essential for production of eotaxin-1, although further investigation is required to fully understand the mechanism of interaction of NFκB and STAT6.

Chemokines Recruit Inflammatory Cells to Aggravate Allergic Conjunctivitis

Eotaxin-1, RANTES and IL-8 play important roles in recruiting inflammatory cells,^{19–23} and these chemokines have also been found in tears from patients with severe allergic conjunctivitis.^{15–18} In our studies, these chemokines caused marked accumulation of cells in conjunctiva (Figure 6B). Our tissues were only stained for eosinophils, but they undoubtedly accumulated other inflammatory cells such as neutrophils and lymphocytes, all of which release cytotoxic agents to aggravate allergic conjunctivitis. NFκB was a crucial factor not only for production of chemokines for chemoattraction/infiltration of these inflammatory cells, but also for cytokine production by mast cells. This also suggests the possibility of inhibiting the NFκB pathway to treat of cytotoxic inflammation in human allergic conjunctivitis.

DECLARATION OF INTEREST

Dr Shearer has significant financial interests (research contract and consulting fee) in Senju Pharmaceutical Co., Ltd., and Dr Azuma is an employee of Senju Pharmaceutical Co., Ltd., a company that may have a commercial interest in the results of this research and technology. These potential conflicts of interest have been reviewed and managed by the OHSU Conflict of Interest in Research Committee.

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