



Cytokine responses by conjunctival epithelial cells: An in vitro model of ocular inflammation

Amalia Enríquez-de-Salamanca^{a,b,*}, Virginia Calder^c, Jianping Gao^d, Grazyna Galatowicz^c, Carmen García-Vázquez^a, Itziar Fernández^{b,a}, Michael E. Stern^d, Yolanda Diebold^{a,b}, Margarita Calonge^{a,b}

^aIOBA (Institute of Applied Ophthalmobiology)-Ocular Surface Group, University of Valladolid, Campus Miguel Delibes, Valladolid E-47011, Spain

^bCIBER-BBN, Valladolid, Spain

^cUCL, Institute of Ophthalmology, London, UK

^dAllergan Inc., Irvine, CA, USA

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ABSTRACT

Objectives: We examined the differential secretion of cytokines by a conjunctival epithelial cell line in response to proinflammatory cytokines to identify the potential contributions during ocular surface inflammation. **Methods:** A conjunctival epithelial cell line was exposed to IFN- γ , TNF- α , IL-4, or IL-13, and cytokine production was determined in supernatants at different times after exposure. Cell apoptosis was measured by flow cytometry. **Results:** TNF- α induced the greatest effect on cytokine secretion, which was time-dependent. TNF- α -stimulated secretion of IL-12p40 was significantly increased by 30 min; GM-CSF, MCP-1, IL-6, IL-7, IL-8, and RANTES were significantly increased by 2 h, and IFN- γ and IL-1 α by 24 h. After 48 h, TNF- α also induced a significant increase in IL-1 β , IL-3, and IP-10 secretion. IFN- γ significantly enhanced IP-10 and RANTES secretion after 48 h of exposure. Following IL-4 treatment there was a significant increase in eotaxin-1 after 24 h, and IL-12p40 and IL-3 after 48 h. IL-13 significantly increased the secretion of eotaxin-1 after 24 h, and IL-8 after 48 h. **Conclusion:** Our results suggest that conjunctival epithelial cells are an important source of cytokines and chemokines that are regulated by proinflammatory cytokines and may play an important role in ocular surface inflammation.

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1. Introduction

There are several types of inflammatory disorders that affect the ocular surface. Some are acute and mild, such as seasonal allergic conjunctivitis (SAC) and transient infectious conjunctivitis. Others are chronic and/or more severe, such as vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), dry eye syndrome, and cicatrizing autoimmune conjunctivitis [1–3], which can involve the cornea and lead to permanent visual loss. Both acute and chronic forms involve the recruitment of inflammatory cells, and these processes are intricately regulated. Cytokines and chemokines play an integral role in the coordination and persistence of the inflammation in chronic extraocular diseases.

Several cytokines, including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)- α and interferon (IFN)- γ , are increased both on the conjunctival epithelium and in the tear fluid of dry eye

patients. The expression of Th2-type cytokines is increased in tissues affected by allergy, and much of the pathology observed can be attributed to a direct effect of these cytokines on the epithelium. For this reason, allergic conjunctivitis is considered as a classical Th2 ocular surface inflammatory response. Although it is widely assumed that Th1 and Th2 cytokines are mutually inhibitory, there is growing evidence that interactions among these elements may modulate the immune response. Thus, Th1 cells play a role in allergic asthma [4,5], and there is evidence for an important role for Th1-type IFN- γ in a mouse model of chronic allergic conjunctivitis [6] and in the inflammatory phase of chronic allergic diseases [7]. In VKC, both tear TNF- α levels and TNF- α -positive cells are significantly increased, while no differences occur in seasonal allergic conjunctivitis tears [8]. The correlation of TNF- α levels in VKC tears with the severity of the disease suggests an important role of this cytokine in severe forms of allergic conjunctivitis [8].

There is increasing evidence for a role of epithelial cells in inflammatory processes in many tissues, including the ocular surface. Epithelial cells derived from airway, intestinal and ocular mucosal sites actively participate during inflammatory processes. They express adhesion and co-stimulatory molecules in

* Corresponding author. Address: IOBA (Institute of Applied Ophthalmobiology)-Ocular Surface Group, University of Valladolid, Campus Miguel Delibes, Valladolid E-47011, Spain. Fax: +34 983 184762.

E-mail address: amalia@ioba.med.uva.es (A. Enríquez-de-Salamanca).

response to different cytokines and/or chemokines, and they also secrete several cytokines/chemokines that contribute to inflammation [9–17]. Epithelial cells may not only be targets of immune responses, but they can also act as mediators of immune responses. For instance, epithelial cells can play roles as non-professional antigen presenting cells in the recruitment and activation of lymphoid cells [17]. Several authors have suggested that the ocular surface epithelium is a potential therapeutic target during inflammatory processes [10,14,18–20] because it expresses adhesion and co-stimulatory molecules under in vivo or in vitro inflammatory conditions [10,20–25]. In addition, alterations in neuroreceptor expression in conjunctival epithelial cells under inflammatory conditions have been described both in vivo [26] and in vitro [27]. These studies have provided further evidence that neural alterations are implicated in inflammatory ocular surface diseases, like dry eye syndrome or allergic disorders.

Cytokine/chemokine production by stimulated conjunctival epithelial cells has been demonstrated in several studies [24,25,28,29], although only the effects of TNF- α , IFN- γ , and/or IL-1 β were investigated and a limited range of cytokines assayed. Based on these findings, we hypothesize that conjunctival epithelial cells play an important role in the pathogenesis of ocular surface inflammatory diseases. The purpose of our study was to determine the profile of cytokines secreted from conjunctival epithelial cells in vitro under the effect of proinflammatory (TNF- α), Th1-type (INF- γ) and Th2-type (IL-4 and IL-13) cytokines to further understand the contribution of conjunctival epithelial cells to ocular surface inflammation.

2. Materials and methods

2.1. Human conjunctival epithelial cells

The IOBA-NHC cell line derived from normal human conjunctival epithelium was used [22]. The culture medium was DMEM/F12 (Invitrogen-Gibco, Inchinnan, UK) supplemented with 2 ng/ml human epidermal growth factor, 0.1 μ g/ml cholera toxin, 1 μ g/ml bovine pancreatic insulin, 10% fetal bovine serum, 5 μ g/ml hydrocortisone, and antibiotics (50 U/ml penicillin, 50 mg/ml streptomycin, and 2.5 μ g/ml amphotericin B), all from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cytokine treatment

Cells were plated in either 25 cm² flasks or 24-well plates and grown until confluent (2 days). They were then maintained for 24 h in serum-free, non-supplemented medium. Prior to cytokine exposure, the medium was replaced with serum-free, non-supplemented medium before treating the cells with 25 ng/ml TNF- α , 500 U/ml INF- γ , 20 ng/ml IL-4, or 20 ng/ml IL-13 (all from Pepro-Tech, London, UK) for a further 24–72 h. Control cells received no cytokines. Doses of the stimulatory cytokines used here were chosen based on previous work where dose and time-response curves were performed by us [7,20,22,24,27,30] and also by other groups [13,31–33]. In those studies, agonist concentrations of cytokines were identified by the induction of epithelial cell responses, and the same concentrations were used here. After treatment, the culture medium was collected, centrifuged, and cell-free supernatants were stored at –80 °C until assayed. Three independent experiments were performed for each time point. For the time course study of TNF- α exposure, cells were treated as described, and the culture medium was collected at 30 min, 2 h and 24 h. Four independent experiments were done at each time point. For each experiment, all cytokine treatments and unstimulated controls were performed simultaneously.

2.3. Cytokine responses

2.3.1. Protein content in cell lysates and cell apoptosis

To ensure that cytokine production was not affected by differences in cell density and/or viability due to cytokine treatment, total protein in cell lysates and cell apoptosis were determined in cytokine-exposed cells. After treatments, cells were lysed in ice-cold RIPA buffer (10 mM Tris-HCl (pH 7.4) 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) supplemented with protease inhibitors (0.57 mM phenylmethylsulfonyl fluoride, 1 U/ml aprotinin and 1 mM sodium orthovanadate), centrifuged at 14,000g, and stored at –80 °C until assayed. Total protein content was determined spectrophotometrically in cell lysates by the BCA assay (Pierce, Rockford, IL, USA). Absorbance at 590 nm was measured in a SpectraMax® M5 spectrophotometer (Molecular Devices Corporation, CA, USA), and protein content was calculated by interpolation of absorbance values in a standard curve. Each sample was assayed in duplicate.

To analyze early and late apoptosis following cytokine treatment for up to 72 h (as described in Section 2.2), cells from confluent monolayers were enzymatically dissociated, pooled with non-adherent cells in the culture supernatants, and pelleted. Resuspended single cells were stained with markers for early apoptosis (0.5 μ M YoPro, Molecular Probes, Cambridge Bioscience, Cambridge, UK) and late apoptosis/necrosis (4 μ g/ml propidium iodide, PI, Sigma), and acquired for flow cytometry (Facsan, Becton Dickinson, B-D, Oxford, UK). At least 10,000 events were acquired per sample and all flow cytometry was analyzed using CellQuest (B-D).

2.3.2. Cytokine secretion

A 22-multiplex (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IP-10, eotaxin-1, IFN- γ , GM-CSF, MCP-1, MIP-1 α , RANTES, and TNF- α) cytokine/chemokine immunobead-based assay (22-plex Beadlyte®, Upstate-Millipore, UK) was first performed on cell supernatants after 48 h of cytokine stimulation as a “screening” detection. This was followed by a second experiment in which an earlier (24 h) and later (72 h) cytokine exposure time points were included. In this experiment, the cytokine secretion was evaluated with a 9-multiplex (IL-1 β , IL-5, IL-6, IL-10, IL-15, eotaxin-1, MCP-1, IL-8, RANTES) immunobead-based assay (9-plex Beadlyte®, Upstate-Millipore, UK). A third cytokine stimulation experiment was performed at earlier time points (30 min, 2 h, and 24 h), with the most potent stimulator, TNF- α . In this case, cytokine secretion was measured with the 22-multiplex assay. Cytokine multiplex bead assays were done following the manufacturer's protocol. Briefly, 50 μ l of each sample were incubated for 2 h on a plate shaker in the dark at room temperature with Beadlyte® anti-human multicytokine beads (Upstate-Millipore, UK). Beads were washed and incubated with Beadlyte® Multi-cytokine Biotin for 1.5 h, followed by 30 min incubation with streptavidin-phycoerythrin solution. Beads were washed and read on a Luminex™100-IS instrument (Luminex Corporation, Austin, Tx, USA). The minimum detectable levels for cytokine/chemokines were 1.5 pg/ml for RANTES; 2.4 pg/ml for GM-CSF, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IP-10, and TNF- α ; and 4.4 pg/ml for eotaxin-1, IFN- γ , IL-12p40, MCP-1, and MIP-1 α . Samples were analyzed in duplicate and data analyzed with the BeadView™ Software (Upstate, UK). Eotaxin-2 and -3 were assayed by commercial Quantikine® (R&D Systems, UK) ELISA kits. Undiluted samples were analyzed in triplicate. The minimum detection levels were 8.5 and 7.8 pg/ml for eotaxin-2 and -3, respectively. Optical densities were measured at 450 nm with correction at 540 nm in a Tecan Safire spectrophotometer (Tecan, West Berkshire, UK).

2.4. Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Fold increase values were calculated as the mean of individual fold increase values for each of the experiments. Statistics were analyzed using the SPSS software package (SPSS 13.0 for Windows, Chicago, IL, USA) by a statistician (coauthor IF). Statistical significance of the differences between cytokine secretion stimulated by each cytokine treatment and basal secretion of unstimulated control cells was analyzed by one-way analysis of variance (ANOVA) with Student–Newman–Keuls post hoc test. If there was significant heterogeneity of variance among groups, the Games–Howell post hoc test was employed. When Box–Cox transformation failed to normalize the data, multiple comparisons between groups were performed by the non-parametric Kruskal–Wallis one-way ANOVA on ranks followed by Dunnett's test. Time-dependent changes were analyzed using repeated-measures ANOVA. Pairwise multiple comparisons were done using the Bonferroni correction. A p -value < 0.05 was considered the limit for statistical significance.

3. Results

3.1. Protein content and apoptosis measured in cytokine-treated cells

Protein concentrations in cell lysates were consistent (2600 ± 86 ng/ml) and did not vary significantly among control and treatment cultures (data not shown). Exposure to any of the cytokines did not induce early apoptosis in comparison with untreated cells (Fig. 1). However, at 24 h there was a 15% increase in PI staining after exposure to TNF- α and a 10% increase after IFN- γ , but there was no effect with IL-4 or IL-13 (data not shown). This increase in PI staining was reduced to $<5\%$ at 48–72 h cytokine treatment, presumably due to the breakdown of dead cells into debris, which was not included in the flow cytometry gated region. Following 48 h treatment with IL-13, 5% of the cells stained with PI, and this returned to basal levels by 72 h.

3.2. Cytokine secretion by conjunctival epithelial cells stimulated with TNF- α , IFN- γ , IL-4, or IL-13

3.2.1. Cytokine secretion after 48 h treatment

At 48 h of culture, unstimulated cells showed basal secretion of many cytokines (Table 1), with the highest levels for IL-8. In contrast, IL-2, IL-7, IL-12p70, IL-15, eotaxin-1, eotaxin-3, GM-CSF, MCP-1, and MIP-1 α levels were only minimally detected (data not shown). Levels of IL-4, IL-5, IL-10, IL-13, TNF- α , IFN- γ , and eotaxin-2 were undetectable.

TNF- α induced the highest levels of cytokine secretion (Table 1). Cells secreted >100 pg/ml of IL-1 α , IL-6, IL-8, IL-12p40, RANTES, and IP-10. Eotaxin-1, eotaxin-3, IL-3, IL-7 MCP-1, and MIP-1 α were present at concentrations between 100 and 10 pg/ml, while GM-CSF, IL-1 β , IL-2, IL-15, and IL-12p70 were at less than 10 pg/ml. Upon IFN- γ treatment, IL-6, IL-12p40, RANTES, IL-1 α , IL-8, and IP-10 were secreted at concentrations exceeding 100 pg/ml. After exposure to IL-4 and IL-13, the highest levels secreted were for IL-8, IP-10, IL-12p40, and RANTES. IL-2, IL-7, IL-12p70, IL-15, eotaxin-1, GM-CSF, MCP-1, and MIP-1 α levels were unaffected by cytokine treatments. IL-5, IL-10, and eotaxin-2 remained undetectable with any of the cytokine treatments.

After 48 h of TNF- α stimulation, secretion of IL-1 β , IL-6, IL-12p40, RANTES, and IP-10 were significantly increased over basal levels (Table 1). IL-8 and IL-1 α were detected at high levels, but the values were highly variable and failed to reach significance. When these values were expressed as fold increases relative to unstimulated cells (Fig. 2), the variability was reduced and significant

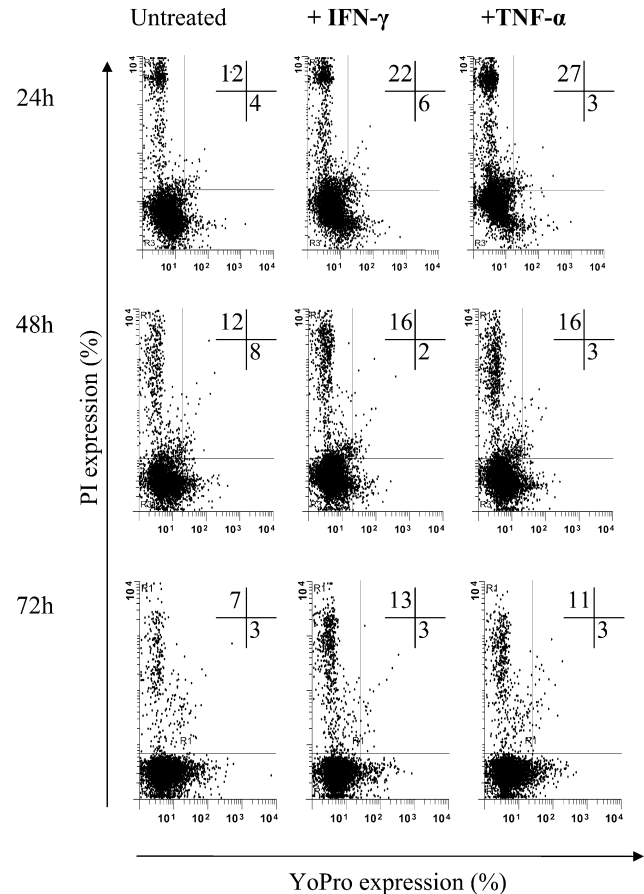


Fig. 1. Flow cytometric analysis YoPro-PI staining in conjunctival epithelial cells after 24, 48, and 72 h of cytokine stimulation. Dot plots of propidium iodide and YoPro were generated from untreated, IFN- γ -treated, and TNF- α -treated IOBA-NHC cells at 24, 48, and 72 h. The percentage staining values are shown for both markers.

increases were detected. IFN- γ treatment induced significant increase in the secretion of IP-10 and RANTES (Table 1), and IL-4 similarly increased the secretion of IL-12p40 and IL-3. No significant changes were found in secreted cytokine levels from cells treated with IL-13 when compared with basal levels.

When expressed as fold increases over basal secretion (Fig. 2), TNF- α provoked the highest increase in secretion for almost all of the cytokines. IP-10 was the most increased (~ 45 -fold), followed by IL-1 α , IL-6, RANTES, and IL-8. Upon IFN- γ exposure, the highest increase in secreted cytokine was IP-10 (~ 20 -fold), followed by IL-1 α (5-fold). Upon IL-4 exposure, IP-10 and IL-13 were the most increased (~ 6 -fold).

3.2.2. Cytokine secretion: 24, 48, and 72 h time course

In this study IL-1 β , IL-5, IL-6, IL-10, IL-15, eotaxin-1, eotaxin-2, eotaxin-3, MCP-1, IL-8, and RANTES, were further studied temporally. These molecules were chosen because of their suggested involvement in inflammation and inflammatory cell recruitment. Only six out of the 11 cytokines were detected (Fig. 3). TNF- α treatment induced significantly higher levels of IL-6 and IL-8 at all three time points. RANTES production was also significantly higher at 48 h, but not at 24 or 72 h. Eotaxin-1 secretion following TNF- α treatment was significantly higher than control at 24 h but not afterwards. IL-8 production was significantly increased by IL-13 treatment at all three time points. IL-4- and IL-13-stimulated eotaxin-1 production was significantly higher than basal only at 24 h. A significant increase with time in the production of IL-6, IL-8, and RANTES occurred even in the absence of cytokine treatment.

Table 1Cytokines secreted by conjunctival epithelial cells either basally or in response to TNF- α , IFN- γ , IL-4, or IL-13 by 48 h

Cytokine/chemokine	Basal	IL-4 (20 ng/ml)	IL-13 (20 ng/ml)	TNF- α (25 ng/ml)	IFN- γ (500 U/ml)
IL-1 α	34.2 $\pm$ 23.0	50.9 $\pm$ 20.4	45.4 $\pm$ 23.1	396.4 $\pm$ 230.9	337.6 $\pm$ 283.0
IL-1 β	1.3 $\pm$ 0.1	1.45 $\pm$ 1.5	1.42 $\pm$ 0.6	2.2 $\pm$ 0.3*	1.5 $\pm$ 0.2
IL-3	6.3 $\pm$ 0.6	28.4 $\pm$ 7.9*	12.4 $\pm$ 3.6	17.4 $\pm$ 7.2	10.8 $\pm$ 2.9
IL-6	57.2 $\pm$ 12.1	81.4 $\pm$ 16.9	75.1 $\pm$ 15.9	691.2 $\pm$ 257.5*	81.8 $\pm$ 18.7
IL-8	787.0 $\pm$ 405.6	1619.9 $\pm$ 888.0	1354.0 $\pm$ 725.3	3660.3 $\pm$ 1674.5	1144.3 $\pm$ 596.4
IL-12p40	85.4 $\pm$ 9.4	204.0 $\pm$ 42.1*	122.1 $\pm$ 25.4	257.0 $\pm$ 9.0**	146.4 $\pm$ 20.6
IP-10	121.6 $\pm$ 41.8	506.5 $\pm$ 308.6	176.2 $\pm$ 26.1	5158.8 $\pm$ 2076.0**	3898.9 $\pm$ 3434.0*
RANTES	92.8 $\pm$ 16.5	194.9 $\pm$ 66.0	162.9 $\pm$ 43.6	809.3 $\pm$ 110.0**	267.9 $\pm$ 33.9*

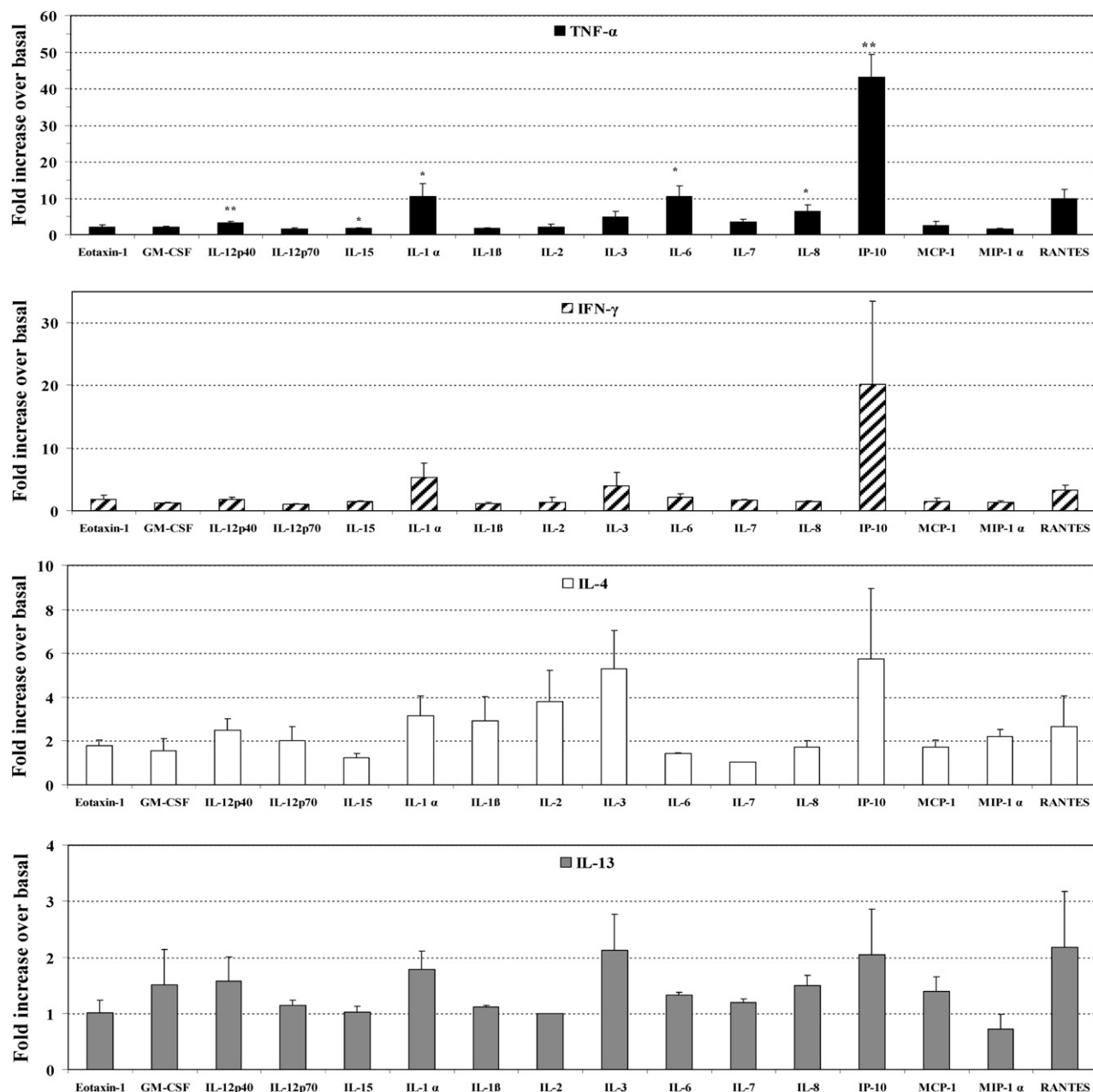
Values are expressed as mean (pg/ml) $\pm$ SEM. *Significantly different from corresponding basal secretion.* $p < 0.05$.** $p < 0.01$.

Fig. 2. Cytokine secretion by conjunctival epithelial cells after 48 h of cytokine treatment. The response is expressed as fold increase over basal secretion. After 24 h in serum-free culture medium, cells were exposed 25 ng/ml TNF- α , 500 U/ml IFN- γ , 20 ng/ml IL-4, or 20 ng/ml IL-13 for 48 h. Cytokine secretion was measured in the recovered supernatants. Unstimulated cells were used as controls for basal secretion. Production was expressed as fold increase over basal secretion. * $p < 0.05$ and ** $p < 0.01$ compared with basal secretion at the same time point.

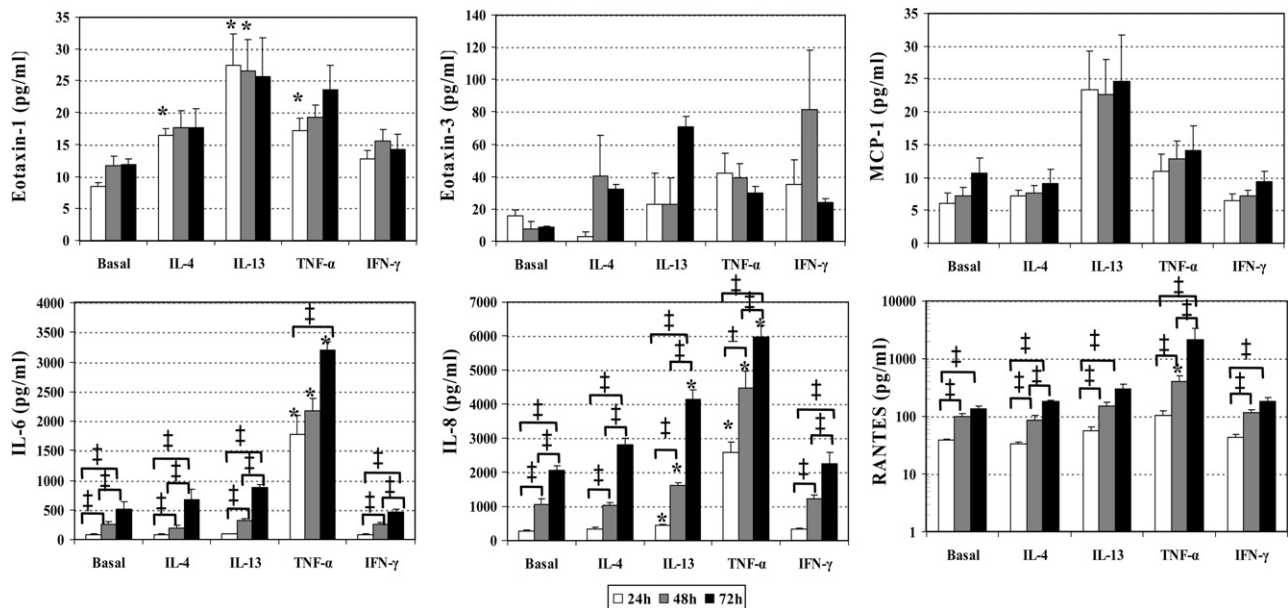


Fig. 3. Time-dependent cytokine secretion by conjunctival epithelial cells. After 24 h in serum-free culture medium, cells were exposed to cytokine treatment (20 ng/ml IL-4, 20 ng/ml IL-13, 25 ng/ml TNF- α , and 500 U/ml IFN- γ for 24, 48 and 72 h. Cytokine secretion was measured in the recovered supernatants. Unstimulated cells were used as control for basal secretion. * $p < 0.05$ compared with basal secretion at the same time point; † $p < 0.05$ compared with secretion at the other time points under the same stimulator.

3.2.3. TNF- α treatment: 30 min, 2 h, and 24 h time course

As TNF- α induced the highest levels of cytokine secretion, we studied additional early time points of exposure at 30 min, 2 h, and 24 h. In general, basal (Fig. 4A) and TNF- α -stimulated (Fig. 4B) cytokines were secreted in a time-dependent manner, with highest levels accumulating by 24 h. IL-8, RANTES, IP-10, and IL-6 were secreted at higher levels following TNF- α treatment. After 30 min of TNF- α treatment, IL-12p40 was the preferentially increased cytokine (5.7-fold) followed by MCP-1, IP-10, and IFN- γ (Fig. 5). After 2 h of stimulation, IL-6, IL-8, and IP-10 were preferen-

tially increased, followed by IFN- γ and IL-12p40. The relative levels of IL-6 obtained after 2 and 24 h in these experiments were higher than those obtained at 48 h (Fig. 2), suggesting that upon TNF- α treatment, IL-6 was mainly released at the 2–24 h period, then decreasing thereafter.

4. Discussion

Among the clinically distinct inflammatory disorders affecting the ocular surface, the exact mechanisms leading to a sight-threat-

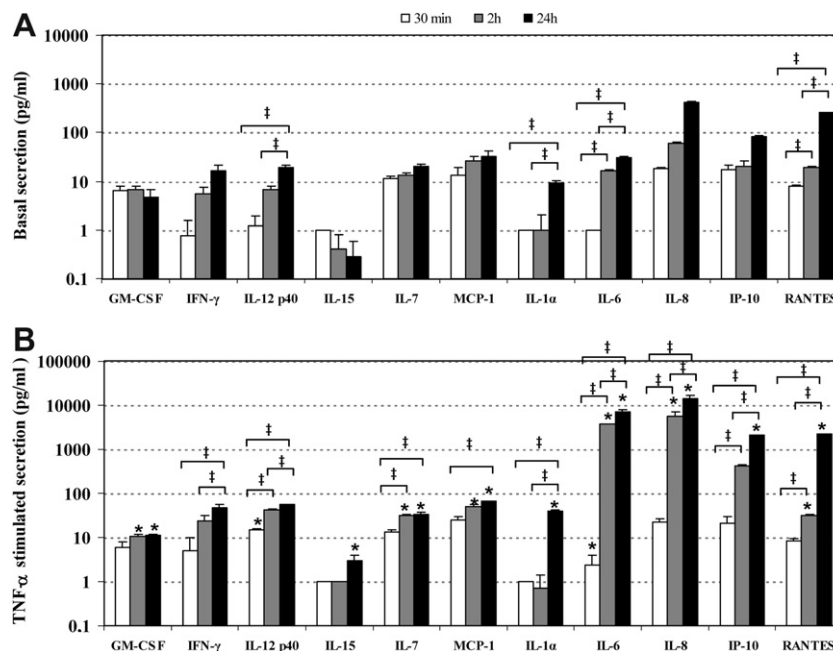


Fig. 4. Early response time-dependent cytokine secretion by TNF- α -treated conjunctival epithelial cells. After 24 h in serum-free culture medium, cells were exposed to 25 ng/ml TNF- α for 30 min, 2 h and 24 h. Cytokine secretion was measured in recovered supernatants. Unstimulated cells were used as control for basal secretion. (A) Basal production. (B) TNF- α -stimulated secretion. * $p < 0.05$ compared with basal secretion at the same time point; † $p < 0.05$ compared with secretion at the other time points under the same stimulator.

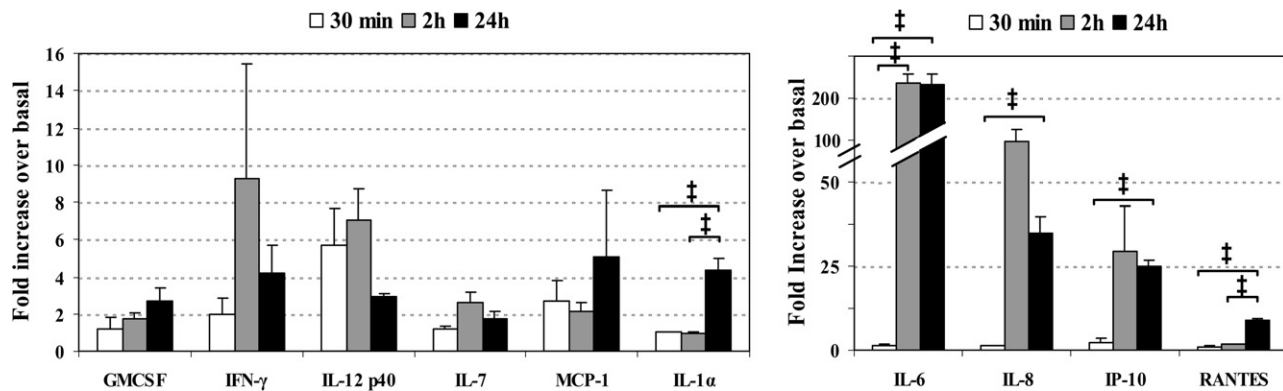


Fig. 5. Early response time-dependent cytokine secretion by TNF- α -treated conjunctival epithelial cells expressed as a fold increase. Cytokine production of conjunctival epithelial cells under 25 ng/ml TNF- α treatment for 30 min, 2 h, and 24 h was expressed as fold increase over corresponding basal secretion. * $p < 0.05$ compared with basal secretion at the same time point; † $p < 0.05$ compared with secretion at the other time points under the same stimulator.

ening outcome are not completely understood. There is a growing body of evidence suggesting that epithelial cells actively participate in inflammatory processes, expressing adhesion and co-stimulatory molecules in response to cytokines and/or chemokines. Epithelial cells themselves can secrete several cytokines and/or chemokines that play integral roles in the coordination and persistence of epithelial inflammatory diseases. Different ocular allergies express specific patterns of cytokines that may be related to the severity of the allergic response [8,10,30,34,35]. The response of conjunctival epithelial cells to cytokines secreted by inflammatory cells could be one of the factors involved in specific inflammatory cell recruitment and the perpetuation of inflammatory processes.

In this study, we examined a wide panel of cytokines/chemokines secreted by human conjunctival epithelial cells in vitro, under both basal and cytokine-stimulated conditions with the purpose of further clarifying the role of epithelial cells in ocular surface inflammation in terms of secretion profiles. We chose the IOBA-NHC cell line for this study because it retains most of the morphologic and functional characteristics of the normal human conjunctival epithelium [22,27]. We found that the cells of this line secreted basal levels of several cytokines and chemokines, and upon cytokine treatment this secretion was highly upregulated. The most effective stimulatory cytokines were TNF- α followed by IFN- γ , a Th1-type cytokine. IL-4 and IL-13, both Th2-type cytokines that are preferentially involved in the disruption of the epithelial barrier of ocular surface [36], did not elicit much epithelial cytokine secretion. Additionally, the differential responses to the stimulatory cytokines were independent of variability in cell density and apoptosis. Only TNF- α and IFN- γ induced a transient, low increase in apoptosis. This transient effect might have been related to the cell-turnover, which could have been upregulated by these cytokines. The late apoptosis values following cytokine treatment were remarkably low compared to reports for other conjunctival epithelial cell lines [37]. This fact could be due to the use of different cell lines in each study. Whether the effects of chronic exposure to these proinflammatory cytokines in vivo might lead to more marked effects on the epithelium is unclear.

Treatment of the IOBA-NHC cells with TNF- α -stimulated secretion of IP-10, RANTES, and IL-8 the most of all the tested cytokines/chemokines. These chemokines are involved in selective lymphocyte/leukocyte recruitment. IP-10 is a potent chemoattractant for activated Th1 and natural killer cells. In an experimental model of dry eye, desiccating stress significantly increases IP-10 expression in both corneal and conjunctival epithelium [38]. Increased expression of this molecule also occurs in the airways of subjects with chronic obstructive pulmonary disease [39] and in patients

with asthma [40,41], where the level is correlated with lymphocyte number. IP-10 binds to cells through the CXCR3 receptor, which is preferentially expressed in Th1 cells as well as in eosinophils. Interestingly, those chemokines that preferentially attract Th1 cells via the CXCR3 receptors can concomitantly block the migration of Th2 cells in response to CCR3 ligands, thus enhancing the polarization of T cell recruitment [42].

The expression of IL-8 and RANTES is increased in the conjunctival epithelium of VKC and AKC patients [10,43,44]. IL-8 levels in the conjunctival epithelium from VKC patients are significantly correlated with the presence of neutrophils and eosinophils in tears and with corneal damage [43]. However, no correlation of RANTES levels with corneal involvement in VKC patients has been found [43]. IL-6 expression is increased in tear of SAC, VKC, and AKC patients [30] and in tears and conjunctival epithelium of dry eye patients [45,46]. Hingorani et al. [10] showed that its expression was raised in giant papillary conjunctivitis, VKC, and AKC epithelial cells, although it did not reach statistical significance. In our study, IL-6 secretion was also significantly increased in TNF- α -stimulated cells as early as 2 h after stimulation, and this effect was maintained until 72 h.

One characteristic of allergic conjunctival diseases is the infiltration of eosinophils. Activated eosinophils release many cytotoxic proteins that have been implicated in the development of corneal lesion in VKC patients. We found that after 24 h, eotaxin-1 secretion was significantly increased in IL-4, IL-13 and TNF- α exposed cells, although the levels of secretion were low. Eotaxin-1 levels in tears in VKC patients are correlated with disease severity [47]. High levels of eotaxin-1 and -2 have been found in mucus of these patients, but only eotaxin-1 was detected in tears and in the primary conjunctival fibroblasts after cytokine treatment [48]. Corneal fibroblasts, but not corneal epithelial cells or conjunctival fibroblasts, are a significant source of eotaxin-1 when stimulated with a combination of IL-1 α , IL-1 β or TNF- α and either IL-4 or IL-13 [49]. We found that eotaxin-3 was secreted by conjunctival epithelial cells both basally and under stimulation. Although its secretion was not significantly increased by any of cytokines tested, some cytokine-specific temporal differences in the pattern of secretion occurred. Differential release of the eotaxins in response to cytokine combinations has also been described in alveolar epithelial cells, in which eotaxin-1 was released during the first 24 h of stimulation, while eotaxin-2 and -3 secretion were increased after 24–48 h [15].

Our findings also demonstrated that, as early as 30 min after exposure to TNF- α , epithelial cells significantly increased IL-12p40 secretion. This early secretion could be an important factor involved

in ocular surface T cell differentiation and/or modulation during inflammatory diseases. The presence of free p40 in the absence of p75 occurs in various pathological conditions, including autoimmunity [50,51], infection [52], sepsis [53], and during intestinal allergic disease [54]. Free p40 may have a physiological role independent of IL-12p70 and IL-23. Additionally, in a mouse intestinal allergic disease, the overexpression of IL-12p40 by intestinal macrophages, dendritic cells, and epithelial cells may play a role in contributing to the generation of a Th2 dominant environment [54].

In summary, we have found that under inflammatory-like conditions, cultured conjunctival epithelial cells secreted cytokines and chemokines that may have a role in initiating, amplifying, and/or modulating ocular surface inflammation. This could occur in part by contributing to the selection of the inflammatory cells recruited to the ocular surface. Therefore, therapeutic interventions may also need to target epithelial cells.

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