

Pharmaceutical and Histopathological Analyses of the Developing Mechanism of Severe Allergic Conjunctival Diseases Using Experimental Animal Models: Roles of Systemic and Local Cytokines

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Abstract: Eosinophils are major effector cells for the development of severe allergic conjunctival diseases (ACD), such as vernal keratoconjunctivitis. Recruitment of eosinophils into the conjunctiva is mediated by chemokines and cytokines. In this review, using an animal model for ACD (experimental immune-mediated blepharoconjunctivitis, EC), the roles of cytokines for eosinophil infiltration into the conjunctiva are presented. Using cytokine knockout mice, importance of endogenous IL-4 for eosinophil infiltration was confirmed. In contrast, endogenous IFN- γ was identified to be inhibitory for eosinophil infiltration. When EC was induced by transfer of *in vitro*-stimulated antigen (Ag)-primed lymphocytes, then the addition of IL-4 to the culture augmented eosinophil infiltration. Systemic administration of IFN- γ suppressed EC only when IFN- γ was injected during the induction phase. Concerning cytokines in the conjunctiva, expression of Th1 cytokines increased time-dependently, whereas that of Th2 cytokines peaked at 12 hours after Ag challenge. In accord with the data, macrophage infiltration increased in a time-dependent manner, while eosinophil infiltration peaked at 12 hours. Subconjunctival injection of IL-4, as well as eotaxin, induced eosinophil infiltration into the conjunctiva. This IL-4-induced eosinophil infiltration was inhibited by co-injection of IFN- γ . Taken altogether, both systemic and local IL-4 is important for eosinophil infiltration into the conjunctiva and is regulated by IFN- γ .

INTRODUCTION

Definition of Allergic Conjunctival Diseases (ACD) and Involvement of Eosinophils in ACD

Allergic conjunctival diseases (ACD) [1] are defined as conjunctival disorders in which type I allergic reactions are involved. ACD can be classified into 4 types, namely allergic conjunctivitis (seasonal and perennial), atopic keratoconjunctivitis, vernal keratoconjunctivitis and giant papillary conjunctivitis [2,3]. Depending on the severity, ACD can be divided into either mild or severe. The index to distinguish whether ACD is mild or severe is the presence of proliferative changes in the conjunctiva [4]. The typical proliferative changes in the conjunctiva are giant papillae. Histopathologic study demonstrated that giant papillae are composed of many fibroblasts and infiltrating inflammatory cells [5,6]. The pathologic changes are not only restricted in the conjunctiva but also noted in an adjunct tissue, cornea. Corneal damages frequently seen in ACD are ulcer and plaque formation and these changes lead to sight loss in certain circumstances [7]. Thus, proliferative changes in the conjunctiva and corneal damages are the hallmark to distinguish whether ACD is severe or not. How these pathologic changes occur has not been fully elucidated, but accumulating evidences suggest that eosinophils are major causative effector cells [6,8,9]. The importance of eosinophils for the development of severe ACD is strongly

supported by the report that severity of ACD evaluated by corneal damage is in parallel with the number of infiltrating eosinophils into the conjunctiva [10]. In addition, eosinophils, which infiltrate into the conjunctival giant papillae produce growth factors, and are also involved in fibroblast growth [11]. Thus, eosinophils are the key for development of severe ACD.

ACD is an antigen (Ag)-specific disease [12], but eosinophils, which are the key effector cells in severe ACD, do not have Ag-specific receptors. How eosinophils infiltrate into the conjunctiva is still unclear but recent evidences suggest that eotaxin [13,14], a chemokine for eosinophils, is involved in the infiltration of eosinophils into the conjunctiva. It is also unclear which kind of conjunctival cells produce eotaxin to recruit eosinophils into the conjunctiva. One hypothesis [15] is that cytokines produced by T cells activate conjunctival fibroblasts and these activated fibroblasts produce chemokines including eotaxin (Fig. 1), based on the facts that nasal fibroblasts produce eotaxin by stimulation of cytokines [16,17]. Following this hypothesis, T cells are the initiator for the infiltration of eosinophils into the conjunctiva. Because T cells have receptors specific to Ag peptides [18], this hypothesis would be logic.

Using samples from patients, we are able to evaluate the situation only at one time-point and variations of ACD severity among the tested patients can not be ruled out. Furthermore, to verify the hypothesis, it is crucial to detect Ag-specific T cells in the conjunctiva and to demonstrate the interaction between T cells and eosinophils *in vivo*. However, at the present, it is impossible to detect Ag-specific T cells in the conjunctiva using human samples.

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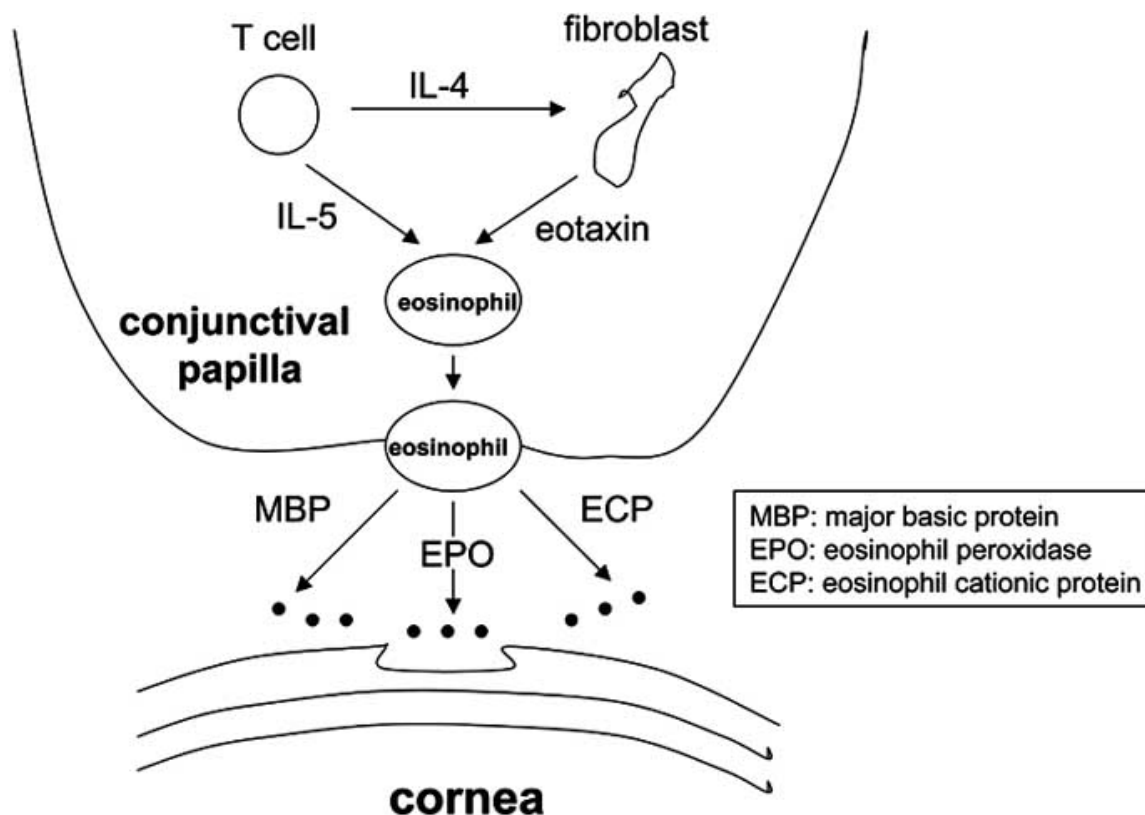


Fig. (1). A hypothetical mechanism of eosinophilic infiltration into the conjunctiva of ACD. T cells infiltrate into the conjunctiva and activate conjunctival fibroblasts by IL-4. These activated fibroblasts produce chemokines including eotaxin. Eotaxin recruits eosinophils into the conjunctiva and the infiltrating eosinophils were activated by IL-5, which is produced by T cells. These activated eosinophils secrete cytotoxic molecules, which cause corneal tissue damage.

Therefore, our group has established experimental model for severe ACD [19,20,21] and investigated how cytokines produced by T cells affect infiltration of inflammatory cells such as eosinophils into the conjunctiva.

Experimental Immune-Mediated Blepharoconjunctivitis as a Model of Severe ACD

Ag-specific IgE is the key molecule for the development of type I allergic reactions. Production of IgE from B cells is mediated by IL-4, which is produced by Th2 cells (Fig. 2) [22]. Therefore, if we wish to establish an experimental ACD model, it is necessary to use animals which are prone to Th2 immune responses. In mice [23] and rats [24], the strains which are prone to Th2 immune responses are known. For example, Brown Norway rats are prone to Th2 immune responses and thus, often used to induce experimental asthma [25] and autoantibody-mediated systemic autoimmune diseases [26]. Therefore, we used BN rats to induce a severe experimental ACD, experimental immune-mediated blepharoconjunctivitis (EC) [19,20,21]. We immunized BN rats with ovalbumin (OVA) or short ragweed pollen (RW) in adjuvants such as complete Freund's adjuvant and aluminum hydroxide. Two or three weeks later, the immunized rats were challenged in eye drops with the same Ag as used for immunization. Twenty-four hours after the challenge, infiltration of inflammatory cells such as mononuclear cells and eosinophils was identified in the conjunctiva and these pathologic changes are compatible with severe ACD in humans. It is of note that passive immunization by transfer

of Ag-specific T cells is able to induce similar pathologic changes seen in EC induced by active immunization [20,21].

Involvement of Cytokines on the Development of EC

From the hypothesis [15], cytokines in the conjunctiva are essential for infiltration of eosinophils into the conjunctiva. As well as these local cytokines, systemic (in lymphoid tissue) cytokines are considered to be important, because they determine the differentiation of T cells either into Th1 or Th2 cells (Fig. 2) [27]. From the accumulating evidences using human samples such as tears [28,29] and conjunctival specimen [30,31], involvement of local cytokines on the development of severe ACD is becoming clearer. However, the roles of systemic cytokines for the development of ACD have not been examined using human specimens. In this review, using animal models, I would like to explain the involvement of systemic and local cytokines, in this order.

ANALYTICAL TECHNIQUES AND QUALITY PARAMETERS

Histological Analysis

The eyes, including the conjunctivas, were fixed with 10% buffered formalin and embedded in paraffin. Four μ m-thick-sections were stained with Hematoxylin-Eosin and May-Giemsa. Concerning immunohistochemistry, the eyes were immediately frozen in a 3% carboxymethyl cellulose (CMC) gel and 4- μ m-thick sections were prepared and fixed

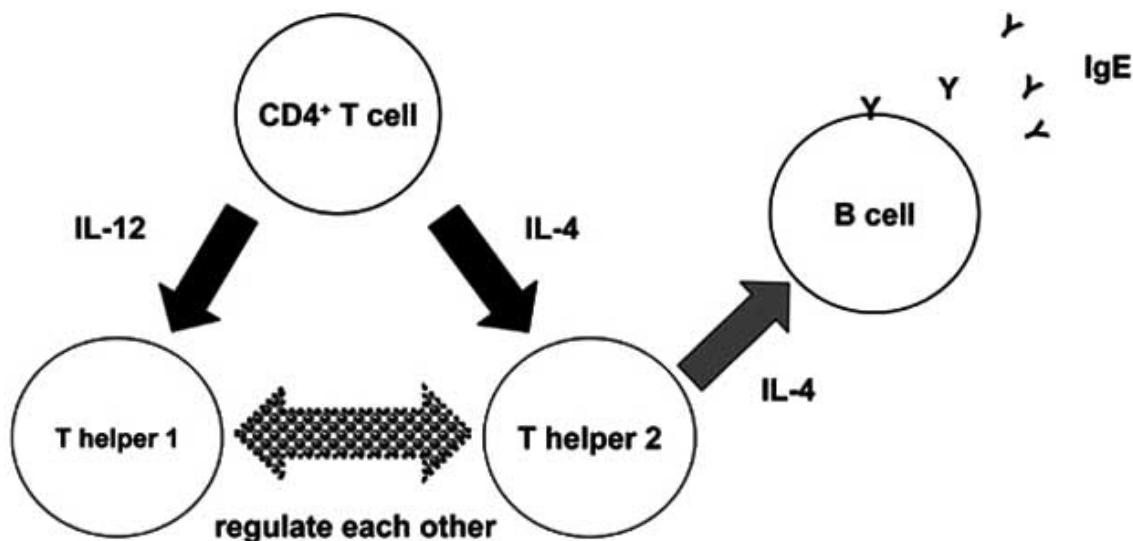


Fig. (2). Th2 development and IgE production are mediated by IL-4 and regulated by Th1 cells. Naive CD4 positive T cells differentiate into either Th1 or Th2 cells, in the presence of IL-12 or IL-4. Th2 cells produce IL-4, which promotes B cells to produce IgE. Th1 and Th2 cells regulate each other by their producing cytokines such as IFN- and IL-10.

in methanol. Endogenous peroxidase activity was inhibited by incubating the sections with 0.1% NaN₃ and 0.3% H₂O₂ in distilled water for 10 minutes at room temperature. The samples were exposed to the primary antibodies for 30 minutes and then to the appropriate biotinylated secondary antibodies for another 30 minutes. The antibody binding was then revealed by using an Avidin-Biotin-Complex kit (Vector laboratories Inc, USA), followed by development with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO).

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis of Cytokine and Chemokine Expression

RNA extracted from conjunctivas by a commercially available homogenizer (Mixer Mill MM 300, Qiagen KK, Tokyo, Japan) was transcribed into cDNA. PCR was performed on a DNA thermal cycler (Perkin Elmer, Foster City, CA) using one 10 minute cycle at 94°C followed by 30-50 cycles consisting of denaturation at 94°C for 30 seconds, annealing at the optimal temperature of the primer pairs employed for 30 seconds, and extension at 72°C for 90 seconds. The molecular size marker used was X174/Hae III digest (Wako, Osaka, Japan). The PCR products were subjected to electrophoresis in a 2% agarose gel and then stained with ethidium bromide. Signal intensity was measured by an Image analyzer (Aishincosmos, Tokyo, Japan) and quantified by NIH Image. The intensity of a given cytokine was standardized by calculation (intensity of cytokine divided by the intensity of β -actin) to compare the expression of cytokines among tested groups.

ROLES OF SYSTEMIC CYTOKINES FOR THE DEVELOPMENT OF EXPERIMENTAL ACD (TABLE 1)

Systemic Cytokines: Evidence Obtained from Cytokine Knockout Mice

To clearly demonstrate the involvement of a cytokine on the development of experimental ACD, it is necessary to

compare the pathology of experimental ACD induced in WT animals, animals with excess of a cytokine and animals with the absence of a cytokine. Recently, cytokine transgenic and knockout (KO) mice become commonly available, and these advantages allow us to investigate the roles of cytokines for the development of experimental ACD. IL-12 [32] and IFN- [33] are important for induction and maintenance of Th1 immune responses, respectively, while IL-4 [34] is important for both of Th2 immune responses. Therefore, we induced experimental ACD by active immunization in WT, IL-4 KO, IL-12 KO and IFN- KO mice, and compared the severity of inflammation in the conjunctiva [35]. Compared with WT mice, negligible infiltration of inflammatory cells into the conjunctiva was noted in IL-4 KO mice [35]. In contrast, IFN- KO mice exhibited more severe infiltration of inflammatory cells than WT mice [35]. From these results, it could be suggested that IL-4 is necessary to induce experimental ACD whereas IFN- is inhibitory for its development. Surprisingly, IL-12, which is considered to be necessary for the production of IFN- , is less likely to be inhibitory [35]. The discrepancy on the data between IFN- KO and IL-12 KO mice could be partially explained by a recent finding using experimental asthma, demonstrating that IL-12 is important for the expression of adhesion molecules at the inflammatory sites and therefore crucial for the infiltration of inflammatory cells [36]. It should be kept in mind that the above findings were obtained by active immunization and therefore, the involvement of cytokines was evaluated in a situation combined of both the induction and effector phases [37].

Systemic Cytokines: Evidence Obtained from Passive Immunization

To better understand when the function of cytokines was exerted, we applied passive immunization for the induction of experimental ACD. For this purpose, we induced EC in Brown Norway rats by transfer of Ag-primed lymphocytes or Ag-specific T cells. I would like to describe the involvement of IL-4 and IFN- , in this order.

Table 1. Effects of systemic cytokines on infiltration of inflammatory cells into the conjunctiva

Procedure	Animal	Control	IL-4	IFN-g	IL-12
active immunization ^a	knockout mice (C57BL/6 background)	+ (WT)	-	++	+/-
active immunization (<i>in vivo</i> injection of a cytokine) ^b	BN rats	+ (PBS)	ND	+/-	+
passive immunization (<i>in vitro</i> treatment with a cytokine) ^c	BN rats	+ (PBS)	++	ND	+/-
passive immunization (<i>in vivo</i> injection of a cytokine) ^d	BN rats	+ (PBS)	ND	+	ND

-: no infiltration, +/-: mild infiltration, +: moderate infiltration, ++: severe infiltration.

^aSeverity was evaluated by total infiltrating inflammatory cells into the conjunctiva.

^{b-d}Severity was evaluated by infiltrating eosinophils into the conjunctiva.

^aConjunctivas from WT or a cytokine knockout mice immunized and challenged with RW were collected 24 hours after challenge detailed in reference [35].

^{b,d}After immunization, BN rats were injected with a cytokine or PBS detailed in reference [38, 39].

^cBefore transfer, lymphocytes or T cells were cultured with a cytokine or PBS detailed in reference [38].

1) When transferred lymphocytes produce excessive IL-4

IL-4 is important for the differentiation of Th0 cells into Th2 cells [34]. To examine the effects of IL-4 on EC by transfer of Ag-primed lymphocytes, we stimulated Ag-primed lymphocytes with the Ag in the presence or absence of IL-4. The presence of IL-4 induced higher expression of IL-4 mRNA in lymphocytes, compared with those stimulated in the absence of IL-4 [38]. These lymphocytes were transferred into syngeneic BN rats and EC was induced by Ag challenge in the conjunctiva. The rats received with lymphocytes stimulated in the presence of IL-4 had more infiltration of eosinophils in the conjunctiva [38]. Thus, it could be suggested that IL-4 in donor cells is important for the infiltration of eosinophils into the conjunctiva. This notion is in good agreement with the hypothesis [15] that IL-4 produced by T cells is important for the infiltration of eosinophils into the conjunctiva.

2) Mechanism on suppression of eosinophil infiltration by IFN-

From the analysis using IFN- KO mice [35], IFN- is identified to be suppressive for the infiltration of eosinophils into the conjunctiva. However, it had been still unclear when and how the suppressive effect of IFN- was exerted, because experimental ACD in IFN- KO mice was induced by active immunization. Therefore, to investigate the suppressive mechanism of IFN-, we induced EC in BN rats either by active or passive immunization. Depending on the phases, EC could be divided into either induction or effector phase [37]. As previously reported, induction phase could be evaluated by active immunization, while effector phase by passive immunization of Ag-primed lymphocytes or Ag-specific T cells [21,37]. Applying these two different methods, we examined the suppressive effects of IFN- on EC during both induction and effector phases. Intraperitoneal injection of IFN- after active immunization until Ag challenge significantly suppressed the infiltration of eosinophils into the conjunctiva [39]. On the other hand, in EC by transfer of Ag-specific T cells, injection of IFN- did not affect the infiltration of inflammatory cells into the conjunctiva [39]. In addition, administration of IFN-

neutralizing antibodies did not affect EC by transfer of Ag-specific T cells [39]. Taken together, suppressive effects of IFN- on EC are exerted during the induction phase but not the effector phase.

ROLES OF LOCAL CYTOKINES FOR THE DEVELOPMENT OF EXPERIMENTAL ACD

Action of Infiltrating Inflammatory Cells in the Conjunctiva (Table 2)

Mononuclear cells and eosinophils are the major infiltrating cells into the conjunctiva of EC [19,20,21]. At first, we examined the phenotypes of infiltrating inflammatory cells into the conjunctiva and the kinetic changes after Ag challenge. Harvested conjunctivas after Ag challenge were processed to immunohistochemical analyses. CD3+ T cells became detectable 6 hours after Ag challenge and peaked and plateaued at 12 hours [40]. Notably, CD4+ cells existed in the conjunctiva before EC induction [40]. Using mouse EC, we confirmed that these residential CD4+ cells are a subset of macrophages [41]. CD68+ cells, which also belong to a macrophage subset, existed before challenge and their numbers increased from 6 hours onwards in a time-dependent manner [40]. Kinetic changes of RT1.B+ (a rat

Table 2. Expression pattern of cytokines in the conjunctiva and phenotypes of infiltrating cells into the conjunctiva

	IL-4	IFN-g
^a kinetics of expression	transient	persistent
^b phenotypes of infiltrating cells concordant with expression pattern of a cytokine	eosinophil	macrophage

EC was induced in BN rats by transfer of Ag-specific T cells followed by Ag challenge. Then, the conjunctivas were harvested at different time-points, detailed in reference [40].

^aExpression of a cytokine was evaluated by RT-PCR.

^bPhenotypes of infiltrating cells into the conjunctiva was evaluated by immunohistochemistry.

MHC class II equivalent to mouse I-A) and RT1.D+ (a rat MHC class II equivalent to mouse I-E) cells paralleled with those of the CD68-positive cells [40]. CD45RA+ B cells were not detectable up to 48 hours after Ag challenge [40]. A small number of eosinophils, confirmed by MBP staining, was detectable before EC induction [40]. Eosinophil infiltration was first detected at 6 hours. It peaked at 12 hours and then decreased [40].

Action of Cytokines and Chemokines in the Conjunctiva (Table 2)

Following the hypothesis [15], cytokines and chemokines should be involved in the infiltration of these inflammatory cells into the conjunctiva. To understand whether the expressions of cytokines and chemokines in the conjunctiva are related to the infiltration of the inflammatory cells, we examined naive, passively immunized but unchallenged, and EC-developing conjunctivas at different timepoints after Ag challenge for the mRNA expression of 11 cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, IL-13, IFN- γ , transforming growth factor (TGF)- β and tumor necrosis factor (TNF)- α), 7 chemokines (CCL2 (macrophage chemoattractant protein (MCP)-1), CCL3 (macrophage inflammatory protein (MIP)-1), CCL4 (MIP-1), CCL5 (regulated on activation, normal T-cell expressed and secreted (RANTES)), CCL10 (eotaxin), CXCL4 (platelet factor (PF)-4) and CXCL10 (interferon-induced protein of 10 kDa (IP-10)) and 3 chemokine receptors (CCR3, CCR5 and CXCR3). mRNA expression was evaluated by RT-PCR. Expression was evaluated by following three points. 1) Expression is either constitutive (detectable in naive rats) or inducible. 2) Upregulation of expression does or does not occur after challenge. 3) Expression is either transient or persistent (expression continues up to 48 hrs after it is initiated). Constitutive expression in the conjunctiva was noted in TGF- β , TNF- α , eotaxin, RANTES, MIP-1, MIP-1, IP-10, CCR3, CCR5 and CXCR3, whereas others were inducible [41]. In most of tested molecules except for eotaxin and CCR5, expression was upregulated [41]. Expression of IL-4, IL-6, IL-13 and MIP-1 was transient but the expression of the other tested molecules persisted [41]. IL-5 and IL-12p35 were not detectable at any time-point.

Regulation of Infiltrating Inflammatory Cells into the Conjunctiva by Local Cytokines and Chemokines

As previously reported, major infiltrating cells into the conjunctiva of EC are mononuclear cells and eosinophils [19,20,21]. From the above study, a major component of mononuclear cells is identified to be CD68+ macrophages [41]. These two different subsets of infiltrating cells exhibited different kinetic change. Infiltration of eosinophils peaked at 12 hours while that of macrophages gradually increased. These changes are just in accord with the kinetic change of Th1/Th2 cytokines; a Th2 cytokine IL-4 peaked at 6-12 hours, whereas a Th1 cytokine IFN- γ persisted up to 48 hours after Ag challenge. Expression of IP-10, which is upregulated by IFN- γ , changed similar to IFN- γ . Expressions of RANTES and MIP-1, which are related to eosinophil infiltration, are in agreement with the kinetic changes of eosinophils. Although eotaxin expression was not

upregulated in the conjunctiva of EC in BN rats by T cell transfer, these data support the hypothesis [15] that cytokines and chemokines mediate infiltration of inflammatory cells into the conjunctiva during the development of ACD.

Effects of Exogenous Eotaxin and IL-4 by Subconjunctival Injection (Table 3)

Following the hypothesis [15], chemokines such as eotaxin directly recruit eosinophils into the conjunctiva. However, no evidence has been available whether eotaxin alone is able to induce eosinophil infiltration into the conjunctiva. To clarify this issue, we subconjunctivally injected recombinant human eotaxin and conjunctivas were harvested for histological examination [42]. Subconjunctival injection of eotaxin recruited eosinophils into the conjunctiva in a dose-dependent manner [42]. Thus, involvement of eotaxin in the infiltration of eosinophils into the conjunctiva was confirmed. Next, to investigate the roles of IL-4 on the infiltration of eosinophils into the conjunctiva, we injected recombinant rat IL-4 subconjunctivally. Similar to eotaxin, subconjunctival injection of IL-4 dose-dependently induced eosinophil infiltration into the conjunctiva [42]. Following the hypothesis, IL-4 activates conjunctival fibroblasts to produce eotaxin. Therefore, we examined chemokine (eotaxin, MIP-1, RANTES) expression in the conjunctiva by RT-PCR after IL-4 injection. Surprisingly, upregulation of chemokine mRNA was not noted up to 24 hours after IL-4 injection [42]. In addition, expression of VCAM-1 evaluated by immunohistochemistry was not different compared with PBS-injected conjunctivas. Thus, although the mechanisms are not still unsolved, the data confirmed that eosinophil infiltration into the conjunctiva is mediated by IL-4. There is a possibility that subconjunctival injection of IL-4 induces infiltration of inflammatory cells other than eosinophils. To examine this possibility, immunohisto-chemical analysis using antibodies against T cells, macrophages, MHC class II and major basic protein (MBP) was applied to study the phenotypes of infiltrating cells into the conjunctiva. Six hours after IL-4 injection, there were twice as many macrophages and MHC class II+ cells compared to the naive rats [42]. Their numbers decreased slightly after later time-points. Increase of T cell compartment was minimal. Strikingly, the number of MBP-positive cells increased by 30-fold compared to their numbers before the injection [42]. Thus, recruitment of inflammatory cells into the conjunctiva by subconjunctival injection of IL-4 is relatively specific to eosinophils. Taken together, exogenous local IL-4 induces eosinophil infiltration into the conjunctiva by an unknown mechanism.

Table 3. Effects of subconjunctival injection of cytokines.

Subconjunctival Injection	Eotaxin	IL-4	IL-4+IFN-
Infiltration of Eosinophils into the Conjunctiva	++	++	+

BN rats were subconjunctivally injected with eotaxin, IL-4 or IL-4+IFN- γ . Twenty-four hours later, infiltrating eosinophils into the conjunctiva were evaluated by histology, detailed in reference 42.

+: Moderate infiltration, ++: severe infiltration.

Inhibition of IL-4-Induced Eosinophil Infiltration by IFN- (Table 3)

IFN- inhibits IL-4 function such as Th2 cell development and activation [43]. To examine whether IFN- suppresses IL-4 function in the conjunctiva, IFN- was subconjunctivally injected together with IL-4. Co-administration of IFN- significantly suppressed the infiltration of eosinophils into the conjunctiva induced by IL-4 [42]. From this result, it could be suggested that the regulatory interaction between IL-4 and IFN- exists in the conjunctiva.

Treatment with FK506 in Eye Drops Inhibited the Infiltration of Eosinophils into the Conjunctiva by Suppression of IL-4 mRNA Expression in the Conjunctiva

Importance of local IL-4 for the infiltration of eosinophils into the conjunctiva is further supported by the experiments examining the effects of FK506 in eye drops. When EC-developing BN rats were treated with FK506 in eye drops, infiltration of inflammatory cells was significantly inhibited compared with those treated with vehicle [44]. Mechanistically, activation of T cells in the conjunctiva should be suppressed by FK506 treatment. We evaluated T cell activation in the conjunctiva by examining cytokine mRNA upregulation in the conjunctiva. Compared to the vehicle group, upregulation of IL-4 mRNA expression by Ag challenge was not observed in FK506-treated group [44]. These data further support the notion that IL-4 is important for the infiltration of inflammatory cells, especially eosinophils, into the conjunctiva.

CONCLUDING REMARKS

From the information as above, it becomes clear that both systemic and local cytokines are involved in the development of experimental ACD. However, there are still questions to be solved to confirm whether the hypothesis is correct. Although data obtained using *in vitro* culture of conjunctival fibroblasts suggest that IL-4 activates conjunctival fibroblasts [45], it remains unclear whether IL-4 activates fibroblasts *in vivo*. It is not yet known whether conjunctival fibroblasts are the source for chemokines *in vivo*. Because the initial hypothesis is based on only Th2 cells and obviously Th1 cells are identified in the conjunctiva of both in human ACD [31] and experimental ACD [19,20,21,39,40], the interaction between Th1 and Th2 should be clarified, especially focusing on IFN- and IL-4. Because T cell transfer data demonstrated massive infiltration of macrophages in the conjunctiva [40] and macrophages were identified in the conjunctiva of patients with vernal keratoconjunctivitis [46,47], the roles of macrophages on the development of ACD *in vivo* should be investigated. Involvement of mast cells, another important effector cell compartment for ACD, has not been investigated in our series of experiments. Because mast cells themselves produce cytokines [48] and functions of mast cells are regulated by cytokines [49], investigation of the relationship between cytokines and mast cells is crucial to understand the developing mechanism of ACD. If we are able to clarify these issues, we will be able to better understand the developing mechanism of ACD.

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