

Impact of a Case Series of Corneal Transplant Rejection on the Kinetics of Cytokine Concentrations in Human Tears after Keratoplasty

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ABSTRACT

AIM: Our purpose was to monitor cytokine levels in tears after keratoplasty, and to establish correlations with corneal rejection.

METHODS: This prospective study included 18 healthy subjects (control group) and 30 patients (31 eyes; treated group) who underwent penetrating keratoplasty (PKP; $N=25$) and lamellar keratoplasty (LKP; $N=6$). They were followed for at least six months to detect transplant rejection and evaluate tear fluid composition in cytokines [interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF), interferon (IFN)- γ , and IL-17A].

RESULTS: Transplant rejection occurred in four eyes after PKP, in the first month ($N=1$), third month ($N=2$), and sixth month ($N=1$). The most abundant cytokines in all groups were IL-6 and IL-17A, whereas IFN- γ was >10-fold higher in the nonrejection group than in the control and rejection groups. Kinetics analysis showed that IL-6 response was limited to a transient increase during the first 24 h, whereas the other cytokines accumulated throughout the follow-up

period, starting after Day 7. The concentrations of IL-2, IL-4, IL-10, TNF, IFN- γ , and IL-17A spiked 24 h at least before rejection ($P<0.05$), and were significantly higher than those of the nonrejection group ($P<0.01$), except for IFN- γ .

CONCLUSION: This study should be some of a useful step that corneal transplant rejection is preceded by a massive increase in proinflammatory cytokines that may constitute quantitative predictors of rejection.

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Key words: Interferon gamma; Human tears; Corneal graft rejection; Penetrating keratoplasty; Lamellar keratoplasty

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INTRODUCTION

Corneal graft rejection is one of the most significant complications of corneal transplantation^[1-5]. Despite the immunologically privileged nature of the cornea, immune-mediated graft rejection remains the major cause of unsuccessful human corneal allograft transplantation^[6,7]. The precise mechanisms leading to graft rejection remains poorly understood. Therefore, there is currently no quantitative method to predict surgical outcome or to detect early signs of rejection^[8,9].

During corneal transplant rejection, the inflammatory responses are dominated by T helper cell type 1 (Th₁)-type cytokines, such as interleukin-2 (IL-2), interferon gamma (IFN- γ), or tumor necrosis

factor alpha (TNF- α), and strategies are proposed to restore balance through an upregulation of Th2-type responses from cytokines, such as interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), or interleukin-10 (IL-10)^[10]. Therefore, the Th1/Th2 factors susceptible of inducing corneal graft rejection after penetrating keratoplasty (PKP) are indirectly investigated by the determination of cytokine levels in aqueous humor or tears^[11,12]. The expression of cytokines and chemokines has been monitored after corneal transplant at the mRNA and protein levels in animal models and at the protein level in the aqueous humor of human subjects^[5,11-17]. The importance and the role of various cytokines in different inflammatory diseases are well documented, but the levels and exact contributions of cytokines in human tears in the postkeratoplasty period are not clarified^[13,14]. A recent study reported the impact of corneal transplant rejection on the kinetic of cytokine concentrations in the tears of patients after PKP^[8]. However, this study did not include a control group of healthy subjects to compare baseline cytokine levels before surgery and identify factors predisposing patients to a poor outcome.

The aim of this study was to examine the signs of corneal graft rejection using the safety method of collecting tears in a filter paper. We investigated longitudinal cytokines in human tear fluid with or without rejection and their correlation with the occurrence of rejection. It has been previously reported that tear levels of several cytokines showed increase or decrease before rejection^[6]. We report the following seven different cytokines: IL-2, IL-4, IL-6, IL-10, TNF, IFN- γ , and IL-17A in the human tears of patients with keratoplasty to evaluate their possible roles in predicting corneal rejection.

METHODS AND METHODS

Study subjects

This prospective study was conducted on 30 consecutive patients who underwent keratoplasty between August 2011 and September 2012 at the Fukuoka University Hospital. All subjects were followed for at least six months. This study also included a group of healthy control subjects, including preoperative patients who underwent cataract or vitrectomy but without ocular or systemic infection or allergy. The control subjects did not take any medication that would interfere with tear secretion. None of the subjects suffered from any disease of known immunological origin. All procedures were performed in adherence to the Declaration of Helsinki for research involving human subjects. This study was approved by the local ethics committee and written informed consent was obtained from all participants before sample collection and surgery.

Subjects Characteristics

This study included a control group (8 males, 10 females) with mean age of 39.4 \pm 10.3 years and a treated group (17 males, 14 females) with mean age of 64.2 \pm 14.6 years. The characteristics of each patient who underwent keratoplasty are presented in Table 1. Among the 30 treated patients, six patients had lamellar keratoplasty (LKP) and 25 patients had penetrating keratoplasty (PKP). Rejection occurred in four PKP eyes, with one case in the first month, two cases in the third month, and one case in the sixth month. At six months after keratoplasty, 16% of the PKP eyes had corneal graft rejection. Therefore, the treated group was separated into a nonrejection group and a rejection group for the cytokine analysis.

Table 1 Patient Characteristics.

Patient	LKP /PKP	Age	Sex	Cause of transplantation	Previous rejection	Months before rejection	Initial visual acuity (log MAR)	Final visual acuity (log MAR)
1	LKP	58	M	Dystrophy	(-)	(-)	0.4	0.16
2	LKP	62	M	"	(-)	(-)	0.4	0
3	LKP	77	M	"	(-)	(-)	0.4	0.7
4	LKP	43	M	Acanthamoeba keratitis	(-)	(-)	2.3	0.4
5	LKP	60	M	Fungal keratitis	(-)	(-)	0.7	-0.08
6	LKP	75	F	Bullous keratopathy	(-)	(-)	-0.08	0.52
7	PKP	65	M	" , retinitis pigmentosa	(-)	(-)	1.4	0.7
8	PKP	71	M	"	(-)	(-)	1.85	0.52
9	PKP	71	F	"	(-)	(-)	0.4	0.52
10	PKP	77	F	"	(-)	(-)	2.3	0.08
11	PKP	80	F	"	(-)	(-)	1.3	0
12	PKP	82	F	"	(-)	(-)	1.9	0.52
13	PKP	33	M	Keratoconus	(-)	(-)	0.7	0
14	PKP	40	M	"	(-)	(-)	1.52	0.04
15	PKP	44	M	" , retinitis pigmentosa	(-)	(-)	1.85	0.52
16	PKP	45	F	"	(-)	(-)	1.3	-0.08
17	PKP	55	M	"	(-)	(-)	0.82	0
18	PKP	62	F	Corneal opacity, post vitrectomy for proliferative diabetic retinopathy	(-)	(-)	2.3	2.3
19	PKP	73	F	"	(-)	(-)	0.7	0.7
20	PKP	79	M	Corneal leukoma	(-)	(-)	1.3	1
21	PKP	81	F	Traumatic corneal opacity	(-)	(-)	2.3	1.3
22	PKP	52	F	Transplant rejection (second PKP), bullous keratopathy, Behcet's disease	(+)	(-)	0.52	0.1
23	PKP	78	F	" , "	(+)	(-)	1.22	0.3
24	PKP	79	F	" , "	(+)	(-)	2.3	1.85
25	PKP	41	M	" , traumatic corneal opacity	(+)	(-)	1.52	1.04
26	PKP	57	M	" , traumatic corneal perforation	(+)	(-)	2.3	1.52
27	PKP	69	F	" , dystrophy	(+)	(-)	1.52	0.08
28	PKP	75	F	Bullous keratopathy	(-)	1M	0.7	0.08
29	PKP	57	M	Transplant rejection (LKP, second PKP), Mooren's ulcer	(+)	3M	1.52	0.22
30	PKP	65	M	Bullous keratopathy, retinitis pigmentosa	(-)	3M	1.16	0.4
31	PKP	85	M	"	(-)	6M	1.9	1.52

Keroplasty Procedure

All surgeries were conducted by the same surgeon. The donor material was preserved in Optisol-GS (Bausch & Lomb, NY) before the surgery. Routine medication (local corticosteroids and antibiotics) was provided for six months after keratoplasty. Patients only received systemic anti-inflammatory therapy (oral corticosteroid or oral immunosuppressant) when an increased risk for immune reactions was identified.

Diagnosis of Corneal Transplant Rejection

Corneal endothelial rejection was diagnosed by the onset of an acute inflammatory episode, combined with endothelial precipitates and/or stromal edema, which was depicted by increased central corneal thickness.

Collection of Tear Samples

The anterior ocular status of each subject was carefully assessed before tear collection. Tear samples were collected at least 15 min after the eye drop instillation. We used antibiotic and steroid drops, having dwindled it in the order of betamethasone sodium phosphate, dexamethasone sodium phosphate, and fluorometholone for every 3 months. Corneal endothelial rejection was diagnosed by the onset of an acute inflammatory episode combined with endothelial precipitates and/or stromal edema with increased central corneal thickness. Tear fluid was extracted by the Schirmer method, as previously described^[18]. Tears were sampled on preoperative day 0 and postoperative day 1, day 7, month 1, month 3, and month 6. All samples were rapidly frozen at -20°C and maintained at -80°C until cytokine analysis. Tear fluid extraction was performed with 0.5 M NaCl and 0.5% Tween 20 in 0.01 M phosphate buffer (pH 7.2).

Measurements of Cytokine Concentrations

The cytokine composition of the tears was analyzed using the BDTM (Becton, Dickinson and Company, Franklin Lakes, NJ) Cytometric Bead Array system and a flow cytometer (BD™ FACS Canto II), according to the manufacturer's instructions. Data were acquired and analyzed using the FCAP Array™ software (version 1.0.1, BD Biosciences). Standard curves were generated using the reference cytokine concentrations supplied by the manufacturer. The following seven inflammatory cytokines were analyzed: IL-2, IL-4, IL-6, IL-10, TNF, IFN- γ , and IL-17A.

Statistical Analysis

All data were expressed as mean \pm standard deviations (SD). The treated group was separated into a nonrejection group and a rejection group for the cytokine analysis. The cytokine concentrations in tear samples of the control, treated, nonrejection, and rejection groups were compared by Wilcoxon's rank-sum test or Mann-Whitney's test, as deemed appropriate. Statistical significance was set at $p < 0.05$.

RESULTS

Impact of Transplant Rejection on the Preoperative Cytokine Profile
The baseline cytokine concentration profiles of tears were determined for the control group and before keratoplasty for the nonrejection and rejection groups (Figure 1). In the control group, all cytokine concentrations were low (<5 pg/mL), except for IL-6 (26.16 pg/mL) and IL-17A (21.97 pg/mL). LKP, PKP without rejection and PKP with rejection were compared. There was no

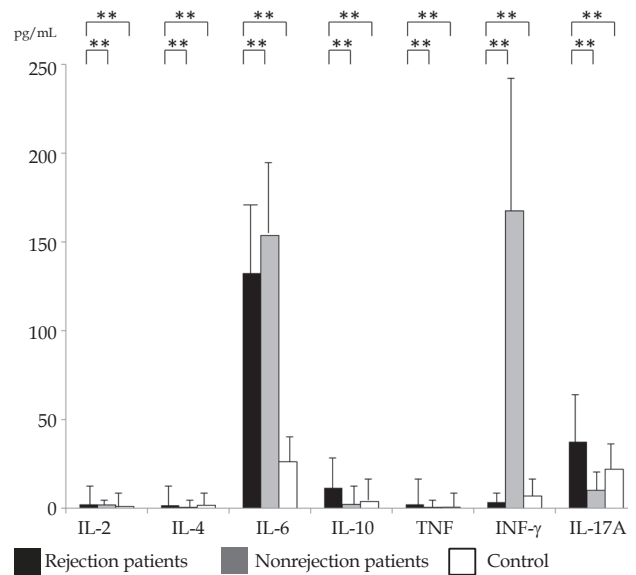


Figure 1 Baseline cytokine concentrations in the tears of control subjects and treated (nonrejection and rejection) patients before surgery. Concentrations of IL-2, IL-10, TNF, and IL-17A were higher in the rejection group than those in the nonrejection and control groups (** $P < 0.01$).

significant difference between LKP and PKP without rejection in concentrations of IL-2, IL-4, IL-6, IL-10, TNF, IFN- γ , and IL-17A ($P=0.63, 0.43, 0.63, 0.42, 0.63, 0.43,$ and 0.63 , respectively). The preoperative concentrations of IL-2, IL-6, IL-10, TNF, and IL-17A in the rejection group were significantly higher than those in the control group ($P=0.0023, 0.0025, 0.0025, 0.0015,$ and 0.0025 , respectively). In contrast, IL-4 and IFN- γ concentrations were significantly lower in the rejection group than those in the control group ($P=0.0025$ and 0.0025 , respectively). The preoperative concentrations of IL-2, IL-4, IL-10, TNF, and IL-17A in the rejection group were significantly higher than those in the nonrejection group ($P=0.0014, 0.0014, 0.0016, 0.0011,$ and 0.0015 , respectively). In contrast, IL-6 and IFN- γ concentrations were significantly lower in the rejection group than those in the nonrejection group ($P=0.0016$ and 0.0016 , respectively). The most remarkable difference between the two treated groups was a five-fold higher IL-10 concentration in the rejection group than that in the nonrejection group.

Kinetics of Cytokine Responses after Keratoplasty

Time-course analysis was conducted to compare the cytokine responses of the nonrejection group and rejection group on keratoplasty (Figure 2). In the nonrejection group, the concentrations of proinflammatory cytokines (IL-2, IL-4, IL-10, TNF, and IL-17A) gradually increased from day 7 until the end of the follow-up (6 months), whereas IL-6 concentration was only elevated during the first week. In contrast, anti-inflammatory IFN- γ responded to the surgery by an initial decrease in concentration during the first 24 h at least. Subsequently, the levels gradual returned to normal over the first month and then drastically decreased during the remaining five months. The initial increase in IL-6 was the only significant direct response to surgery in both groups ($P < 0.01$). Transplant rejection did not significantly affect the kinetics, except for IFN- γ ($P=0.03$). In the rejection group, IFN- γ concentration was initially very low, and remained significantly lower than that in the nonrejection group during at least the first 30 days postoperation.

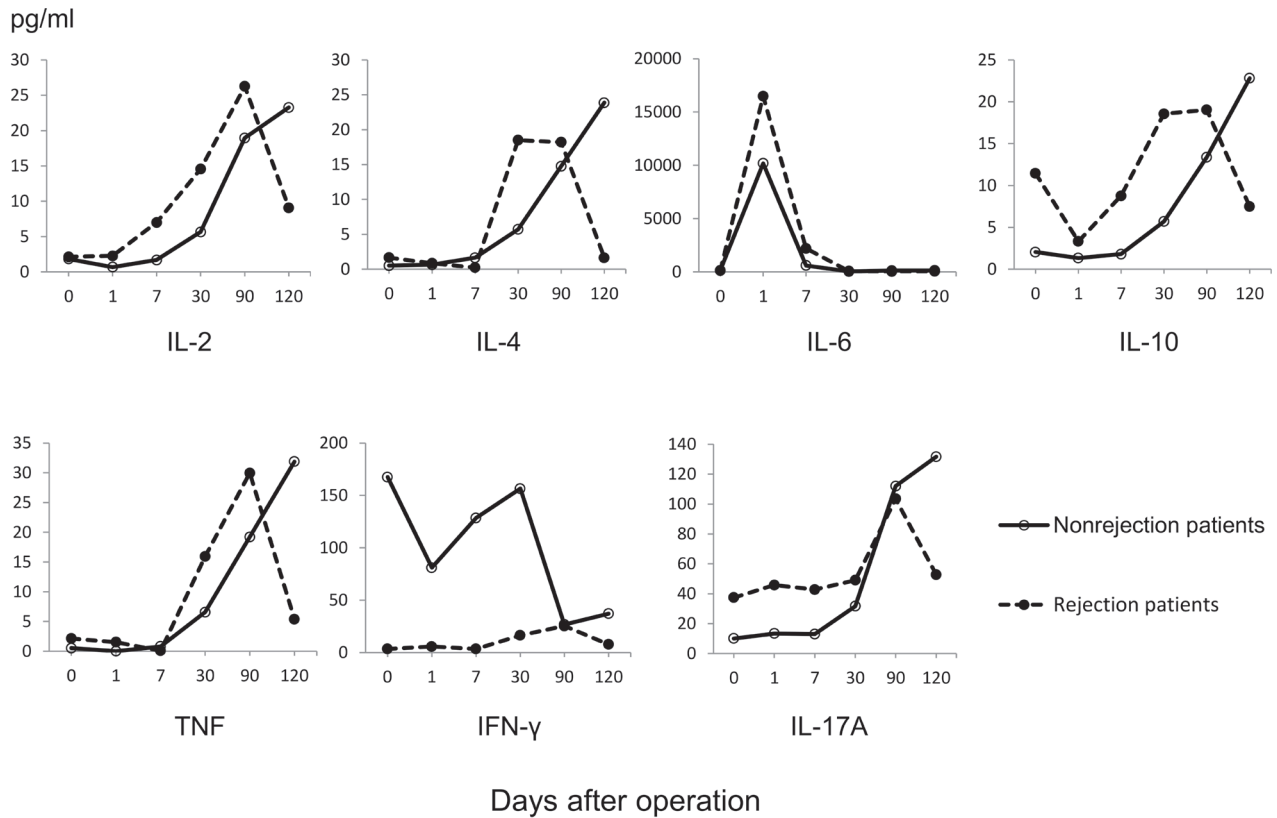


Figure 2 Long-term kinetics of cytokine concentrations in the tears of patients after keratoplasty. The only significant differences between the nonrejection and rejection groups were found for IFN- γ . Only IL-6 showed significant difference before and after the operation with or without rejection.

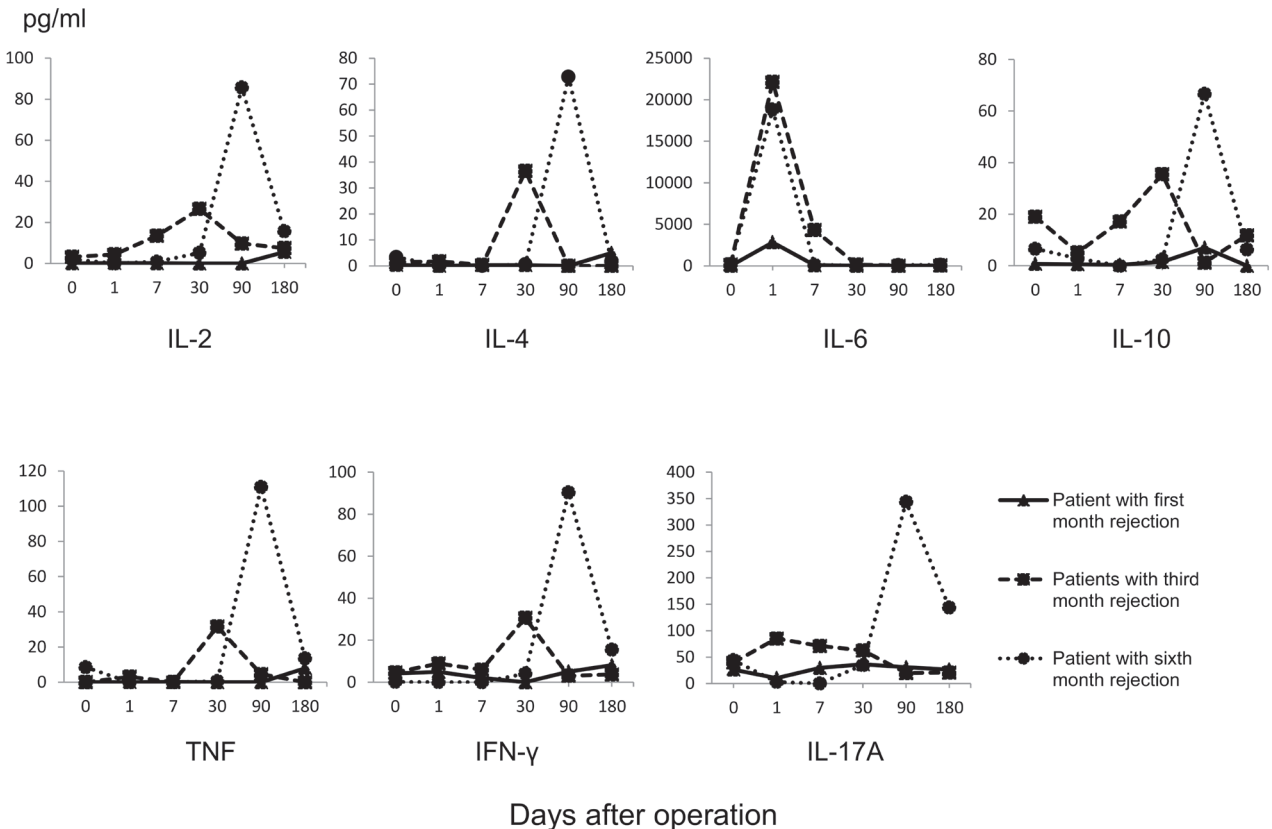


Figure 3 Impact of the timing of rejection on the kinetic of cytokine concentrations in the tears of patients before and after penetrating keratoplasty. There was a spike in IL-2, IL-4, IL-10, TNF, IFN- γ , and IL-17A concentrations 24 h at least before rejection onset.

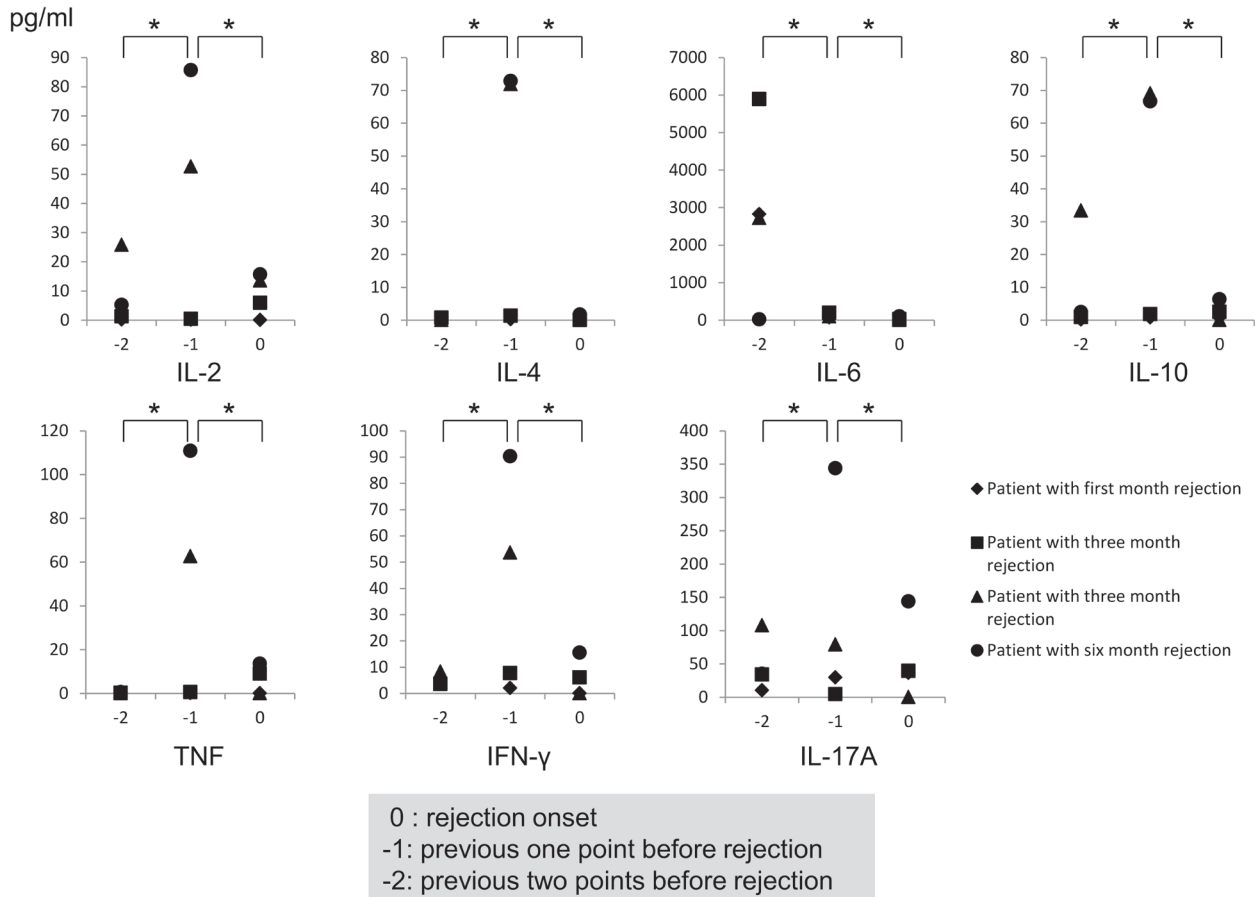


Figure 4 Impact of corneal transplant rejection on cytokine concentrations in the tears of patients before and after penetrating keratoplasty. The concentration of IL-2, IL-4, IL-10, TNF, IFN- γ , and IL-17A spiked 24 h at least before rejection onset (* $P < 0.05$).

Impact of Rejection Delay on Cytokine Kinetics

The patients who developed transplant rejection 1, 3 or 6 months after PKP were compared in terms of cytokine responses during the six months follow-up period (Figure 3). For the patient diagnosed with transplant rejection after 1 month, the cytokine levels remained very low during the entire follow-up period. In contrast, the patient diagnosed with transplant rejection at the three-month follow-up examination showed a transient elevation of proinflammatory cytokines (IL-2, IL-4, IL-10, TNF, IFN- γ) that was detected at the one-month follow-up. In contrast, the patient diagnosed with transplant rejection at the six-month follow-up exam, showed a delayed peak accumulation of the same proinflammatory cytokines (IL-2, IL-4, IL-10, TNF, IFN- γ) and a peak for IL-17A at the three-month follow-up visit. In contrast, the timing of transplant rejection did not affect the IL-6 response, which showed a consistent peak concentration on day 1 postoperation.

The cytokines associated with corneal transplant rejection were further assessed by comparing the concentrations measured in these three patients on day -2, -1, and 0 (onset) of rejection (Figure 4). The large differences detected on Day -1 are consistent with the gradual accumulation of these cytokines during the six-month period and delay before rejection onset. The IL-2, IL-4, IL-10, TNF, IFN- γ , and IL-17A levels increased significantly from Day -2 to Day -1 ($P = 0.028, 0.030, 0.028, 0.028, 0.028, \text{ and } 0.028$, respectively) and decreased significantly from Day -1 to Day 0 ($P=0.028, 0.029, 0.028, 0.030, 0.030, \text{ and } 0.028$, respectively). In contrast, IL-6 levels kept

decreasing after postoperative day 1 and remained very low on Day -1 and Day 0 ($P=0.028, \text{ and } 0.028$, respectively). These data suggest that a combination of cytokines is involved in corneal transplant rejection after PKP and the cytokine responses consistently occur within 24 h at least before the onset of rejection.

As the balance of pro- and anti-inflammatory cytokines determines the inflammatory status of the eye, we calculated the ratio of the average of each cytokine concentration to that of IL-10. The IL-4/IL-10 and IFN- γ /IL-10 ratios of these four patients, measured on Day -1 of rejection onset, were significantly lower than those of the non-rejection group throughout the follow-up period ($P<0.01$). We determined the most pertinent ratios. The ratio of IL-10/TNF- α expression, a measure of cytokine polarization, may be a better indicator of regulatory function than IL-10 expression alone^[19]. We calculated the ratio of IL-10/TNF; however, there was no significant difference between the rejection and non-rejection groups. We measured these ratios to determine any significant difference, which was observed in previous reports^[8]. Plasma levels of IFN- γ , IL-4, and their ratios did not correlate with the rejection or immunosuppressive therapy, Th1/Th2 cytokine monitoring during the first week post-transplant does not predict early rejection^[20]. We consider that tear levels of IFN- γ , IL-4 reflect the latest change of rejection rather than the plasma levels. The tendency observed in the graph was the same as that in the group without rejection if these ratios are similar on days 29, 89, and 119 of the follow-up, because these days correspond to Day -1 of rejection onset for the four PKP patients.

DISCUSSION

In the corneal tissue, IL-6 plays critical roles, including the maintenance of clear corneal button and stimulation of collagen synthesis and wound healing^[21]. These functions are consistent with the rapid and robust increase in IL-6 concentration we detected during the first 24 h at least after keratoplasty, the most intensive phase of wound healing. These data are consistent with the acute increase in IL-6 level reported in the tears of patients after PKP^[8]. As the healing process continued, we observed a gradual decrease in IL-6 back to preoperative levels within one month after surgery. Then IL-6 concentrations remained low during the remaining five months, even in the rejection patients. In contrast, previous studies reported an increase in IL-6 concentration in aqueous humor at the time of corneal transplant rejection^[5,14]. These data suggest different kinetics of cytokine levels in the tears and aqueous humor and that IL-6 plays different roles in these two distinct environments.

Other cytokines, i.e., IL-1 β , IL-8, and TNF- α , are involved in the neovascularization process^[22]. Therefore, the enhanced expression of TNF- α may promote the tissue infiltration observed after high-risk corneal transplant in patients performed with inflamed vascular beds^[23]. In the cornea, TNF- α expression was found elevated at the mRNA^[13] and protein^[24] levels in the anterior chamber and serum of hosts with rejected corneal allografts. Likewise, we found a significant increase of TNF levels in the tears of patients sampled 24 h at least before the onset of corneal rejection. It has been suggested that TNF- α increases the susceptibility of corneal endothelial and epithelial cells to apoptosis^[8]. Therefore, these data are consistent with the role for TNF in corneal transplant rejection.

IL-17A is a proinflammatory cytokine that has recently received attention for its role in the pathogenesis of several autoimmune diseases. IL-17A has also been implicated in cardiac and renal allograft rejections^[25-28]. These reports propose that a depletion of IL-17A enhances corneal allograft survival. However, Cunnusamy *et al.* suggested that IL-17A is essential for the maintenance of corneal immune privilege and establishes a new paradigm, whereby interactions between IL-17A and CD4⁺CD25⁺ Tregs is necessary for the survival of corneal allografts^[29]. In our study, IL-17A levels decreased before rejection onset, suggesting a loss of the protective effect of the cytokine.

The most remarkable cytokine responses were observed from the anti-inflammatory cytokines. First, the nonrejection groups had >10-fold higher IFN- γ concentrations than those in the control and rejection groups. The injection of IFN- γ was able to heal corneal transplant rejection in patients after PKP^[30]. Therefore, the preoperative analysis of tear samples showing high IFN- γ levels would support a favorable PKP outcome. Second, IFN- γ and IL-10 concentrations increased >10-fold 24 h before rejection and then decreased back to baseline level at rejection onset. The reduced level of IL-10 in tears of patients with endothelial rejection could be an important promoter of transplant rejection. In animal models, IL-10 had the potential to delay or reduce corneal transplant rejection^[31-33]. IL-10 has been shown to decrease the expression of MHC class II in monocytes/macrophages, and therefore interfering with their antigen-presenting function. In addition, IL-10 modulates monocytes by suppressing the production of their proinflammatory cytokines, such as TNF- α , IL-1 β and IL-8^[34]. In our study, the fact that IL-10 concentration suddenly decreased at the onset of corneal rejection suggests a loss of protection by this cytokine. Therefore, corneal transplant rejection may be initiated by a sudden increase in proinflammatory/anti-inflammatory cytokine ratio.

The main limitations of this study are the short follow-up period and the irregular sampling intervals. First, longer follow-up periods are necessary to confirm the differences in cytokine levels between the rejection and nonrejection group. Second, the irregular sampling intervals (days to months) may have missed important fluctuations in cytokine levels. The number of patients of this study was small. Further investigations are needed.

Therefore, monitoring cytokines in tear fluid is a simple and noninvasive method to identify high-risk patients for corneal transplant rejection before PKP (IFN- γ) and to monitor the first signs of rejection after PKP.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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