Subnormal Cytokine Profile in the Tear Fluid of Keratoconus Patients

Albert S. Jun1, Leslie Cope2, Caroline Speck3, Xiaojun Feng4, Seakwoo Lee4, Huan Meng1, Abdel Hamad5, Shukti Chakravarti1,4,6*

1 Department of Ophthalmology, Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America, 2 Department of Oncology, Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America, 3 Department of Psychological and Brain Sciences, Johns Hopkins School of Arts and Sciences, Baltimore, Maryland, United States of America, 4 Department of Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America, 5 Department of Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America, 6 Department of Cell Biology, Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America

Abstract

Keratoconus, a historically viewed as a non-inflammatory disease, is an ectatic corneal disorder associated with progressive thinning of the corneal stroma. Recently, a few inflammatory mediators have been reported to be elevated in the tear fluid of keratoconus patients. Consequently, we investigated a wide range of inflammation regulating cytokines in the tears and sera of keratoconus and control subjects. Interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17, interferon (IFN)-γ, chemokine C-C motif ligand 5 (CCL5) and tumor necrosis factor (TNF)-α were tested in tear samples and sera of keratoconus and control individuals by multiplex immuno-bead assays. Selected cytokines were further tested by standard ELISA on pooled tear samples. All cytokines in the sera were generally low, with no significant changes between keratoconus and control subjects. However, in tear fluids, clear differences were detected between the two groups. These differences include increased IL-6, and decreased IL-12, TNF-α, IFN-γ, IL-4, IL-13 and CCL5 in keratoconus compared to control tear fluids. The decreases in IL-12, TNF-α and CCL5 were statistically significant, while the IL-13 decrease was statistically significant in the severe keratoconus group only. IL-17 could not be detected by multiplex immuno-bead assay, but showed an increase in keratoconus by conventional ELISA on a limited number of pooled tear samples. Our findings confirm increased IL-6, but dispute earlier reports of increased TNF-α, and suggest a cytokine imbalance in keratoconus disrupting corneal homeostasis. Moreover, an increase in IL-17 suggests tissue degenerative processes at work, contributing to the thinning and weakening of the corneal connective tissue in keratoconus.

Introduction

Keratoconus is an ectatic corneal disease associated with progressive thinning of the corneal stroma, and a protruding, cone-shaped cornea that produces astigmatism and myopia. Keratoconus is a leading cause of corneal transplantation, affecting 1 in 2000 individuals with a mean age of onset at 15.4 years [1,2,3,4]. The disorder typically progresses until the third to fourth decade of life [3], and the factors that determine the progression or stabilization of the disease are not well characterized or understood.

Although the etiology of keratoconus is poorly understood, it is traditionally viewed as a non-inflammatory corneal thinning disease [1]. Accordingly, cellular infiltration and vascularization are not clinically apparent in keratoconus. However, keratoconus has been linked to atopy since the 1950s [5], with further support coming from later studies that reported elevated levels of immunoglobulin (Ig) E in the sera of keratoconus patients [6,7]. Recent studies have suggested pro-inflammatory factors as key to keratoconus pathogenesis based on their findings of elevated interleukin (IL)-6, tumor necrosis factor (TNF)-α and matrix metalloproteinase (MMP)-9 in the tear fluid of keratoconus patients [8,9]. Moreover, increased binding of IL-1 by keratoconus corneal fibroblasts has led another group to suggest a role for inflammation in the onset or progression of keratoconus as well [10].

Despite these initial findings of specific changes in inflammatory cytokines, there are no studies that have examined a range of cytokines to determine whether keratoconus is associated with an imbalance in the repertoire of cytokines that regulate inflammatory and immune responses driven by subsets of T-helper cells, Th1, Th2, and Th17 in the corneal environment. To begin addressing this question, we quantified Th1 cytokines (IL-12, IFN-γ and TNF-α), Th2 cytokines (IL-4, IL-10 and IL-13), the Th17 representative cytokine IL-17, and other inflammatory cytokines/chemokines (IL-1β, IL-6, and RANTES or CCL5) in tear fluids and serum samples of keratoconus patients and control subjects. Although this initial study is based on a relatively small sample size, decreases in specific cytokines suggest down regulations of both pro-inflammatory and immunoprotective responses to play a role in this disease.
Results

Demographics

A set of 18 keratoconus (KC) and 11 controls were used for the multiplex cytokine analyses on tear fluids and sera (Table 1). Two (11%) of the KC patients were graded as mild (steepest K < 45D), 6 (33%) as moderate (45D ≤ steepest K ≤ 52D), and 10 (56%) as severe (steepest K > 52D). Using the Mann-Whitney test, there was a statistically significant (p = 0.02), 10 year difference in the mean age between the controls and the keratoconus subjects, who were on average 33 and 43 years old, respectively. Nine of the KC and four of the control subjects wore contact lenses. Due to their disease status, keratoconus patients normally wear hard contact lenses, while unaffected subjects wear soft contact lenses. Although matching by contact lens use would not per se remove this difference, to determine if contact lens use had an effect, irrespective of disease status, we fit a multivariate linear model to determine the effects of contact lens wear on tear fluid cytokine levels after adjusting for the age of the subjects. Levels of all cytokines tested were higher in the 13 contact lens users when compared to non-contact lens users, and while the differences were moderate for some of the cytokines, they were not statistically significant except for IL-4 (Table S1). The average time since diagnosis of keratoconus was 16±14 (mean ± SD) years (Table 1).

Three of the 18 keratoconus patients had a medical history including atopy. Tear fluid samples were collected between 9 am and 4 pm, with no statistically significant differences in collection times between control and keratoconus subjects.

Table 1. Keratoconus and control samples for immuno-bead multiplex ELISA.

| Sample | Age (years) | Gender | Race | Contact lens use (years) | Diagnosis |
|--------|-------------|--------|------|-------------------------|-----------|
| 1      | KC          | Severe | 42   | M                       | Black     |
| 2      | KC          | Severe | 44   | F                       | Native American | 30.0 | 32 |
| 3      | KC          | Mild   | 52   | F                       | White     | None | 9   |
| 4      | KC          | Severe | 34   | F                       | White     | None | 9   |
| 5      | KC          | -      | 76   | F                       | Black     | 42.0 | 42  |
| 6      | KC          | Mild   | 38   | M                       | Asian     | Yes  | 10  |
| 7      | KC          | -      | 45   | M                       | White     | None | 7   |
| 8      | KC          | Severe | 30   | M                       | Black     | Yes  | ?   |
| 9      | KC          | Severe | 31   | M                       | Native American | None | 3   |
| 10     | KC          | Severe | 27   | M                       | Asian     | None | 6   |
| 11A    | KC          | Severe | 27   | M                       | Native American | None | 10  |
| 12     | KC          | Severe | 35   | M                       | White     | None | 9   |
| 13     | KC          | Mild   | 32   | F                       | Native American | 5.0  | 8   |
| 14A    | KC          | Mild   | 61   | F                       | White     | 20.0 | 25  |
| 15     | KC          | -      | 61   | M                       | White     | None | 48  |
| 16A    | KC          | Mild   | 40   | M                       | White     | 24.0 | 7   |
| 17     | KC          | Mild   | 56   | F                       | Black     | Yes  | 22  |
| 18     | KC          | Mild   | 53   | M                       | White     | 27.0 | 8   |
| Mean   |             |        | 44   |                     |          | 24.7 | 16  |
| SD     |             |        | 14   |                     |          | 12.2 | 14  |
| 1      | Control     | N/A    | 22   | F                       | White     | 10.0 | N/A |
| 2      | Control     | N/A    | 50   | F                       | White     | None | N/A |
| 3      | Control     | N/A    | 51   | M                       | White     | None | N/A |
| 4      | Control     | N/A    | 30   | M                       | Native American | None | N/A |
| 5      | Control     | N/A    | 40   | M                       | Asian     | None | N/A |
| 6      | Control     | N/A    | 31   | F                       | White     | 10.0 | N/A |
| 7      | Control     | N/A    | 24   | M                       | White     | 9.0  | N/A |
| 8      | Control     | N/A    | 30   | M                       | White     | None | N/A |
| 9      | Control     | N/A    | 31   | F                       | White     | None | N/A |
| 10     | Control     | N/A    | 30   | F                       | White     | None | N/A |
| 11     | Control     | N/A    | 24   | F                       | White     | 10.0 | N/A |
| Mean   |             |        | 33   |                     |          | 9.8  | 0.5 |
| SD     |             |        | 10   |                     |          | 0.5  | 0.5 |

A: atopy, KC: keratoconus, M: male, F: female, SD: 1 standard deviation, N/A: not applicable,
- Unable to obtain a keratometry measurement for severity due to irregular curvature of patient’s cornea.
doi:10.1371/journal.pone.0016437.t001
that the keratoconus group was ethnically more diverse than the control group. Our recent efforts are geared towards collecting tear samples from appropriately matched control subjects, and when applicable to collect tear fluids from the unaffected eye of keratoconus patients as well.

A second set of samples comprising 29 KC and 38 control samples were collected for performing individual ELISA assays for validation purposes (Table S2). In this set, the control subjects were matched more closely to the KC group for age and ethnicity. The mean ± SD ages were 38±10 and 40±12 years, for KC and control subjects, respectively. Some of the KC subjects wore contact lenses, while the control subjects for this group were not contact lens users (Table S2). Of the keratoconus and control cases (Table S2), atopy was reported for six and four of the individuals, respectively.

**Multiplex cytokine analysis of keratoconus and control subjects**

To investigate the relationship between disease status and cytokine levels, we performed a linear multivariate correlation study of natural log transformed cytokine levels and disease, after correcting for age and contact lens use, as covariates. The results were reported as significant in Table 2, only if disease was a significant independent predictor of cytokine levels. All cytokines tested by multiplex immuno-bead assay, except IL-17, were detectable in tear samples. Table 2 shows median, 25th and 75th percentile values of tear fluid cytokines of all keratoconus and control subjects. Overall, we found IL-6 levels were 3 fold elevated (based on mean values) in keratoconus samples compared to control subjects (Table 2 and Figure 1), confirming a previous study of increased IL-6 in keratoconus [8]. The increase in IL-6 was not statistically significant due to the variation in levels reported of increased IL-6 in keratoconus [8]. The increase in IL-6 was not detected in similar numbers in keratoconus and control subjects, respectively. Some of the KC subjects wore contact lenses, while the control subjects for this group were not contact lens users (Table S2). Of the keratoconus and control cases (Table S2), atopy was reported for six and four of the individuals, respectively.

**Measurements of selected cytokines in tear fluid by standard ELISA**

The multiplex data implicate reductions in Th1 cytokines (IL-12, TNF-α, IFN-γ), and possibly Th2 cytokines (IL-4 and IL-13) in keratoconus. To further explore these possibilities, we performed standard individual ELISAs for selected cytokines. In general, the conventional, standard ELISA (high sensitivity) is less sensitive than multiplex immuno-bead assays. Therefore, it was necessary to pool the tear samples from different KC and control subjects (Table S2) for these ELISA experiments. Based on the commercial availability of high sensitivity ELISA kits, we selected TNF-α and IFN-γ as Th1 cytokines (IL-12 and IL-13) in keratoconus. To further explore these possibilities, we performed standard individual ELISAs for selected cytokines. In general, the conventional, standard ELISA (high sensitivity) is less sensitive than multiplex immuno-bead assays.

**Table 2. Tear-fluid cytokine concentrations in keratoconus and control subjects.**

| Cytokine | Control (C) | Keratoconus (KC) | Fold change | p value |
|----------|-------------|-----------------|-------------|---------|
| IL-1β    | 10 [3.2, 19.4] | 6.9 [3.6, 9.6] | 0.88 | 0.87 |
| IL-4     | 111.2 [52.6, 175.1] | 38.7 [12.8, 122.7] | 0.6 | 0.43 |
| IL-6     | 66.8 [27.4, 140.4] | 72.2 [48.9, 241] | 3.2 | 0.17 |
| IL-10    | 10.0 [9.7, 19.1] | 4 [0.9, 12.1] | 0.4 | 0.4 |
| IL-12    | 48.6 [10.6, 142.8] | 8.4 [1.6, 37.1] | 0.24 | 0.03 |
| IL-13    | 14.7 [9.1, 46] | 7.8 [5.7, 12.1] | 0.35 | 0.11 |
| IFN-γ    | 1969 [643, 4859] | 76 [19.3, 401.6] | 0.2 | 0.2 |
| CCL5     | 109.4 [57, 222] | 1.2 [1.2, 131.7] | 0.53 | 0.05 |
| TNF-α    | 150 [80.5, 349.5] | 54.6 [34.4, 122.3] | 0.3 | 0.04 |

*25th and 75th percentile.

1Fold change in mean values.

2p value based on a multivariate linear model after correcting for age and contact lens use covariates.

DOI:10.1371/journal.pone.0016437.t002
for TNF-α detection in the tear fluids by ELISA (data not shown). IFN-γ was detectable and showed a decrease in keratoconus compared to pooled control samples but without statistical significance (p = 0.5, data not shown). We detected 14.2 ± 8.6 pg/ml (mean ± SD) of IL-4 in the control and 7.6 ± 5 pg/ml in the KC pool (Figure 2); the IL-4 decrease in KC was statistically significant (p = 0.04), confirming the multiplex immuno-bead assay results (Figure 1, Tables 2 and 3). IL-17 was detectable in one control and 3 keratoconus pools in one experimental set. All negative values in that set, being below the level of detection, were set at the minimum detection level for this ELISA kit. Based on these limited data (Figure 3), there was a trend towards increased IL-17 (72.5 ± 59.2 pg/ml) in keratoconus tear fluids compared to control samples (18.5 ± 7.8 pg/ml).

**Discussion**

In this study, we investigated cytokines in the sera and tear fluids of keratoconus and control individuals to determine if altered inflammatory response is a factor contributing to the etiology of keratoconus. Of the cytokines measured in the serum, most were at very low levels and none showed significant differences between patients and control individuals. This is consistent with the idea that keratoconus is not associated with major systemic inflammation. However, our results indicate perturbations in immune related homeostasis in the tear film and the corneal microenvironment of keratoconus patients.

The published data on actual baseline cytokine concentrations in tear fluids is limited, and quite variable. To compare our findings to those of others, we examined the literature and found only a few instances where tear fluids were assessed for multiple cytokines. Since only a limited amount of tear fluid is obtainable without stimulation, measuring multiple cytokines is challenging. Li and coworkers used a solid phase antibody protein array-based method to estimate the relative amounts of tear fluid cytokines in Sjogren’s syndrome, without reporting actual levels of cytokines [11]. A recent study used an immuno-bead based multiplex system to obtain baseline levels of 30 different cytokines and chemokines [12]. Others have examined isolated cytokines by conventional sandwich ELISA and reported concentrations of IL-6, TNF-α and IL-10 in control and keratoconus subjects [8,13]. Our immuno-bead based assay, on average showed high levels of IL-4, IL-6, IL-
12, IFN-γ, TNF-α and CCL5 in the tear samples of control subjects. Our baseline findings for IL-6 and TNF-α by multiplex assays were 5-10 times higher than the reported single ELISA measurements. However, our assay yielded baseline ranges for IL-10, IL-13, CCL5 and IL-6 that were within the range reported by the recent immuno-bead based study [12]. On the other hand, our estimates of TNF-α and IFN-γ were higher, and that of IL-1b was lower than that reported in the earlier study [12]. Since our immuno-bead assay kit is different from that used in the other study, some of this difference may arise from different primary and secondary antibodies. In addition to the variation seen between different studies, the range for each cytokine within a given study, including ours, is very large. Some of this variability could be due to individual-to-individual variation in cytokine levels and the extent of tearing even though samples are collected without direct stimulation.

Comparing tear fluid cytokines in the control and keratoconus subjects, we noted an increase in IL-6 in keratoconus, in agreement with earlier reports [8,13]. The latter observation disagrees with an earlier report of a small increase in TNF-α of keratoconus tear fluids. The possible reasons for this difference are, 1) use of different antibody-based assays, 2) actual TNF-α levels detected in the other study is very close to the lower limit of TNF-α detection by the conventional ELISA kit used in that study, and 3) differences in patient population between the two studies. IL-12 promotes the differentiation of Th1 cells; its decrease in keratoconus is consistent with decreases in two signature Th1 cytokines, IFN-γ and TNF-α. In severe keratoconus, IL-12 and TNF-α decreases were more pronounced and this may play a role in increased IL-17 and associated tissue degenerative processes. Both IL-1 and IL-13 cytokines were also reduced in keratoconus, and the decrease in IL-4, as measured by conventional ELISA, was statistically significant. IL-4 is a key Th12 cell differentiation cytokine, and both IL-4 and IL-13 play crucial roles in amplification of the Th12 response through up regulation of STAT5, STAT6 and GATA3 transcription [14]. Thus, decreases in IL-4 and IL-13 suggest that Th12 responses may be dampened in keratoconus. IL-4 is associated with allergic response and promotes the synthesis of IgE [15]. Based on the reported link between atopy and keratoconus, it is conceivable that IL-4 levels would be higher in the keratoconus group. However, the number of patients with a history of atopy in our samples was extremely low. Thus, separate from atopy, the decrease in IL-4 we see may

Table 3. Tear-fluid cytokine concentrations in severe (K>52D) keratoconus and control subjects.

| Cytokine | Control (C) | Keratoconus (KC) | Fold change | p-value |
|----------|-------------|------------------|-------------|---------|
| IL-1b    | 12.19±11.56 | 7.46±5.58        | 6.11        | 0.83    |
| IL-4     | 116±81.8   | 33.48±49.74      | 3.09        | 0.31    |
| IL-6     | 89.64±83.36| 254±312.41       | 2.83        | 0.27    |
| IL-10    | 16.46±23.5 | 7.05±9           | 2.34        | 0.17    |
| IL-12    | 90.1±115.57| 12.57±15.25      | 7.11        | 0.007   |
| IL-13    | 32.34±35.1 | 8.79±6           | 3.74        | 0.04    |
| IFN-γ    | 2780.6±2781.3| 543.83±946.28    | 5.11        | 0.133   |
| CCL5     | 149±149.4  | 25.8±48.1        | 5.88        | 0.013   |
| TNF-α    | 253±275    | 55.06±67.72      | 4.58        | 0.04    |

*p-value based on a multivariate linear model after correcting for age and contact lens use covariates.

Table 4. Serum cytokine concentrations in keratoconus and control subjects.

| Cytokine | Control (C) | Keratoconus (KC) | Fold change | p-value |
|----------|-------------|------------------|-------------|---------|
| IL-1b    | 0.18±0.15   | 0.14±0.11        | 0.8         | 0.71    |
| IL-4     | 0.09±0.06   | 0.11±0.08        | 1.2         | 0.61    |
| IL-6     | 1.77±3.66   | 1.39±2.37        | 0.8         | 0.31    |
| IL-10    | ND          | ND               | N/A         | -       |
| IL-12    | ND          | ND               | N/A         | -       |
| IL-13    | ND          | ND               | N/A         | -       |
| IFN-γ    | 27.49±9.73  | 25.84±25.38      | 0.9         | 0.4     |
| CCL5     | 100.70±55.61| 93.19±58.0       | 0.9         | 0.73    |
| TNF-α    | 1.46±1.71   | 1.57±1.42        | 1.1         | 1.0     |

*Fold change in mean cytokine level.
*p-value based on Mann-Whitney test. ND: Not detectable. All readings were below detection levels. N/A: not applicable.

doi:10.1371/journal.pone.0016437.t003

doi:10.1371/journal.pone.0016437.t004
be pertinent to the pathophysiology of keratoconus itself. How do our tear fluid cytokine observations in keratoconus compare to other ocular surface diseases? A study reported increases in IL-1β, IL-2, IL-5, IL-6, IL-12, IL-13, in seasonal allergic conjunctivitis (SAC), vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC), while IL-4, IFN-γ and IL-10 levels were increased in SAC and VKC compared to controls [16]. These observations are certainly different from the cytokine profile we see in keratoconus. Thus, the broad decrease in inflammatory cytokines reported in our study may indeed be keratoconus disease-specific.

Decreased IL-4 in keratoconus may have broad immune and non-immune consequences [17] that should be considered in the context of corneal thinning and abnormal stromal extracellular matrix (ECM). IL-4 and IL-4 receptor engagement through the JAK-STAT and the IRS1/2 pathways regulate cell proliferation and tissue homeostasis [17]. Dermal fibroblasts stimulated with IL-4 and IL-13 up regulate production of collagens type I and III [18]. IL-4 mediated induction of STAT-6 pathway in intrahepatic cells has been shown to induce collagen synthesis [19]. While its implication in hepatic disease is over-induction of STAT-6, increased collagen, and fibrosis, reduction of normal levels of IL-4 in the corneal environment may tip the balance toward poor stromal keratocyte cell survival, oxidative stress, altered collagen and ECM stability associated with keratoconus [20,21,22,23].

In vitro and in vivo studies indicate reciprocal interactions between IFN-γ and IL-17, and between IL-4 and IL-17, and that these cytokines restrict undue amplification of the TH17 response [24]. It is possible that in the pathogenesis of keratoconus, decrease and dysregulation of several cytokines and growth factors encourage aggravated TH17 response, production of metalloproteinases and tissue damage. Furthermore, TGF-β and IL-6 levels regulate differentiation of Treg and TH17 subsets of T cell [25]. TGF-β members are present in all ocular tissues [26,27,28], and, by immunohistochemistry we detected stronger immunostaining for total TGF-β in the epithelial layer (our unpublished observations) of keratoconus corneas. Moreover, IL-6 levels were increased in the tear fluid of keratoconus patients as indicated in this study and reported earlier [8]. Indeed, whether relative TGF-β and IL-6 levels are altered sufficiently in keratoconus ocular tissues are not known at this time. On testing IL-17 by the multiplex immuno-bead assay, we could not detect it in the tear fluid. However, using an IL-17 ELISA kit, we detected higher levels of IL-17 in 3 of the five patient pools in one set of samples. An increasing number of studies link IL-17 to autoimmune and inflammatory diseases such as rheumatoid arthritis, lupus, asthma and psoriasis [29]. IL-17 is produced primarily by TH17 subset T lymphocytes, while IL-17 receptors are found in a broad array of cell types including fibroblasts and myofibroblasts [30,31,32]. Multiple studies are beginning to link IL-17 mediated induction of fibroblasts and myofibroblasts and the production of tissue degrading proteases and cytokines [31,33,34,35]. Recent studies have also linked IL-17A to ocular pathogenesis of Sjögren’s Syndrome [36]. Desiccating stress in dry eye disease appears to also involve increased MMP-9 and TH17 inflammation [37,38,39]. In dry eye, IL-17 promoting cytokines have been shown to be produced by isolated T cells in the conjunctiva and the cornea [37]. In our hands quantitative reverse transcriptase polymerase chain reaction on total RNA from scraped corneal epithelium failed to detect IL-17A. Additional immunohistology of conjunctiva impressions will be needed to further address the source of IL-17 producing cells and the validity of an IL-17 mediated inflammation and tissue damage in keratoconus. Two recent studies performed proteomic analyses on the tear fluids of unaffected individuals and keratoconus patients. In one the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lacoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lacoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40]. Using a cytokine antibody array, the authors reported down regulations in lactoferrin and immunoglobulin kappa chains [40].
focused on the cornea itself, the tear fluid changes implicate the conjunctiva and the lacrimal gland as having some paracrine effects in this disease.

Materials and Methods

Samples
All patients and normal subjects provided written informed consent according to a protocol used in the current study that was approved by the Johns Hopkins Institutional Review Board. Inclusion criterion for participation in the study was presence of keratoconus as determined by clinical examination and corneal modeling (Pentacam, Oculus, Lynnwood, WA). Participants completed a comprehensive questionnaire designed to collect information on quality of life, contact lens use, past medical history, and family history. For the multiplex cytokine analysis (Table 1) we used 18 patients (61% males, mean age ± standard deviation (SD) = 43±13 years) and 11 control (45% males, mean age ± SD = 33±10 years) tear samples. Whole blood was collected for serum extraction.

Conventional ELISA (Table S2) was performed for selected cytokines on pooled samples from an additional 29 keratoconus patients (76% male, mean age = 38±10) and 38 control individuals (52% male, mean age = 40±12). These samples were pooled to generate 13 control and 10 keratoconus pools. The keratoconus samples were pooled based on disease status (mild, moderate and severe).

Severity of keratoconus was graded by the steepest keratometry (K) measurement with <45 diopters (D) being mild, 45–52D being moderate, and severe >52D or not measurable. Ophthalmic modeling (Pentacam, Oculus, Lynnwood, WA) was used to detect levels of selected cytokines in pooled tear fluid samples diluted 1:2. The following ELISA kits were used for IFN-γ (Cat# DIF50, Human IFN-gamma Quantikine ELISA Kit, R&D Systems), IL-4 (Cat# HS400, Human IL-4 Quantikine HS ELISA Kit, R&D Systems) and IL-17A (Cat# D1700, human IL-17 Quantikine ELISA Kit, R&D Systems). Based on the standard curves the detection sensitivity for IFN-γ, IL-4 and IL-17 were 8 pg/ml, 0.11 and 15 pg/ml, respectively.

Serum Analysis
An aliquot of 10 ml blood was collected in a clot activating tube (Becton Dickinson, Franklin Lakes, NJ) and allowed to stand at room temperature one to two hours before spinning 15 minutes at 4°C. Serum aliquots were immediately transferred to −80°C for storage. Undiluted serum samples were assayed using the Bio-Plex Human Cytokine Assay kit, (Biorad, Hercules, CA) for IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, CCL5, TNF-α as described for tear fluid samples.

Statistical Analysis
Chi-square tests were used to determine statistical significance for categorical variables between groups. Cytokine levels in keratoconus and control subjects were first compared using a 2-tailed, Mann-Whitney nonparametric test (Prism; GraphPad Software, San Diego, CA), with a p-value <0.05 considered to be statistically significant. To determine significant (p<0.05) associations between disease status and cytokine changes, we fit a multivariate linear model using natural log transformed cytokine levels, with age in years, and contact lens use as covariates. These multivariate analyses were performed using the R statistical software (http://www.R-project.org). We found no correlation between cytokine level and time of tear fluid collection (Spearman correlation, Prism; GraphPad) and did not include collection time as a variable in the multivariate analysis.

Supporting Information
Table S1 Cytokine concentrations in contact lens users and non-users (DOC)
Table S2 Keratoconus and control samples for ELISA measurements of selected cytokines (DOC)

Author Contributions
Conceived and designed the experiments: SC ASJ AH. Performed the experiments: CS HM XF. Analyzed the data: SC LC. Wrote the paper: SC ASJ. Troubleshooting ELISAs, preparation of figures and tables: SL.

References
1. Krachmer JH, Feder RS, Belin MW (1984) Keratoconus and related noninflammatory corneal thinning disorders. Surv Ophthalmol 28: 293–322.
2. Yue BY, Sugar J, Benveniste K (1984) Heterogeneity in keratoconus: possible biochemical basis. Proc Soc Exp Biol Med 175: 336–341.
3. Rabkinowitz TS (1996) Keratoconus. Surv Ophthalmol 42: 297–319.
4. Olivares Jimenez JL, Guerrero Jurado JC, Bermudez Rodriguez FJ, Serrano Laborda D (1997) Keratoconus: age of onset and natural history. Optom Vis Sci 74: 147-151.
5. Galin MA, Berger R (1958) Atopy and keratoconus. Am J Ophthalmol 45: 904–906.
6. Kemp EG, Lewis CJ (1982) Immunoglobulin patterns in keratoconus with particular reference to total and specific IgE levels. Br J Ophthalmol 66: 717–720.
7. Rahi A, Davies P, Ruben M, Lobascher D, Menon J (1977) Keratoconus and coexisting atopic disease. Br J Ophthalmol 61: 761–764.
8. Lema I, Duran JA (2005) Inflammatory molecules in the tears of patients with keratoconus. Ophthalmology 112: 654–659.
9. Lema I, Sobrino T, Duran JA, Brea D, Díez-Feijoo E (2009) Subclinical keratoconus and inflammatory molecules from tears. Br J Ophthalmol 93: 820–824.
10. Fabre EJ, Bureau J, Pouliquen Y, Lorans G (1991) Binding sites for human interleukin 1 alpha, gamma interferon and tumor necrosis factor on cultured fibroblasts of normal cornea and keratoconus. Curr Eye Res 10: 583–592.
11. Li S, Sark R, Vijnna T, Satho S, Beaton A, et al. (2008) Antibody protein array analysis of the tear film cytokines. Optom Vis Sci 85: 653–660.
12. Carreno E, Enriquez-de-Salamanca A, Tession M, Garcia-Vazquez C, Stern ME, et al. (2010) Cytokine and chemokine levels in tears from healthy subjects. Acta Ophthalmol.
13. Lema I, Duran JA, Ruiz C, Díez-Feijoo E, Acera A, et al. (2008) Inflammatory response to contact lenses in patients with keratoconus compared with myopic subjects. Cornea 27: 738–763.
14. Paul WE, Zhu J (2010) How are T(H)2-type immune responses initiated and amplified? Nat Rev Immunol 10: 225–235.

15. Wills-Karp M, Finkelstein FD (2008) Untangling the complex web of IL-4 and IL-13-mediated signaling pathways. Sci Signal 1: p55.

16. Leonardi A, Cernow SJ, Zhan H, Calder VL (2006) Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. Clin Exp Allergy 36: 777–784.

17. Nehls K, Keegan AD, Zamorano J, Ryan JJ, Paul WE (1999) The IL-4 receptor: signaling mechanisms and biologic functions. Annu Rev Immunol 17: 701–738.

18. Bhogal RK, Bona CA (2008) Regulatory effect of extracellular signal-regulated kinases (ERK) on type I collagen synthesis in human dermal fibroblasts stimulated by IL-4 and IL-13. Int Rev Immunol 27: 472–496.

19. Aoudjehane L, Pisani A, Jr., Scatton O, Podevin P, Massault PP, et al. (2008) Interleukin-4 induces the activation and collagen production of cultured human intrahepatic fibroblasts via the STAT-6 pathway. Lab Invest 88: 973–985.

20. Steinman L (2007) A brief history of T(H)-17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nat Immunol 8: 1069–1070.

21. Yagi Y, Andoh A, Inatomi O, Tsujikawa T, Fujiyama Y (2007) Inflammatory responses induced by interleukin-17 family members in human colon subepithelial myofibroblasts. J Gastroenterol 42: 746–753.

22. Cortez DM, Feldman MD, Moutsopoulos NM, Wahl SM (2007) T lymphocytes in Sjogren’s syndrome: contributors to and regulators of pathophysiology. Clin Rev Allergy Immunol 32: 252–264.

23. De Paiva CS, Chotikavanich S, Pangelinan SB, Pitcher JD 3rd, Fang B, et al. (2009) IL-17 disrupts corneal barrier following desiccating stress. Mucosal Immunol 2: 243–253.

24. Chauhan SK, El Annan J, Ecoiffier T, Goyal S, Zhang Q, et al. (2009) Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. J Immunol 182: 1247–1252.

25. Zheng X, de Paiva CS, Li DQ, Farley WJ, Pflugfelder SC (2010) Desiccating Stress Promotes Th17 Differentiation by Ocular Surface Tissues through a Dendritic Cell-Mediated Pathway. Invest Ophthalmol Vis Sci.

26. Lema I, Brea D, Rodriguez-Gonzalez R, Diez-Frijio E, Sobrino T (2010) Proteomic analysis of the tear film in patients with keratoconus. Mol Vis 16: 2055–2061.

27. Pannebaker C, Chandler HL, Nichols JJ (2010) Tear proteomics in keratoconus. Mol Vis 16: 1949–1957.