

# Innate and Adaptive Gene Single Nucleotide Polymorphisms Associated With Susceptibility of Severe Inflammatory Complications in *Acanthamoeba* Keratitis

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**PURPOSE.** Over a third of patients with *Acanthamoeba* keratitis (AK) experience severe inflammatory complications (SICs). This study aimed to determine if some contact lens (CL) wearers with AK were predisposed to SICs due to variations in key immune genes.

**METHODS.** CL wearers with AK who attended Moorfields Eye Hospital were recruited prospectively between April 2013 and October 2014. SICs were defined as scleritis and/or stromal ring infiltrate. Genomic DNA was processed with an Illumina Low Input Custom Amplicon assay of 58 single nucleotide polymorphism (SNP) targets across 18 genes and tested for association in PLINK.

**RESULTS.** Genomic DNA was obtained and analyzed for 105 cases of AK, 40 (38%) of whom experienced SICs. SNPs in the *CXCL8* gene encoding IL-8 was significantly associated with protection from SICs (chr4: rs1126647, odds ratio [OR] = 0.3,  $P = 0.005$ , rs2227543, OR = 0.4,  $P = 0.007$ , and rs2227307, OR = 0.4,  $P = 0.02$ ) after adjusting for age, sex, steroids prediagnosis, and herpes simplex keratitis (HSK) misdiagnosis. Two *TLR-4* SNPs were associated with increased risk of SICs (chr9: rs4986791 and rs4986790, both OR = 6.9,  $P = 0.01$ ). Th-17 associated SNPs (chr1: *IL-23R* rs11209026, chr2: *IL-1β* rs16944, and chr12: *IL-22* rs1179251) were also associated with SICs.

**CONCLUSIONS.** The current study identifies biologically relevant genetic variants in patients with AK with SICs; IL-8 is associated with a strong neutrophil response in the cornea in AK, TLR-4 is important in early AK disease, and Th-17 genes are associated with adaptive immune responses to AK in animal models. Genetic screening of patients with AK to predict severity is viable and this would be expected to assist disease management.

**Keywords:** *Acanthamoeba* keratitis (AK), adaptive, complications, genetic susceptibility, inflammation, innate, keratitis, scleritis

*Acanthamoeba* keratitis (AK) results in the most prolonged and severe morbidity of any of the causes of acute microbial keratitis.<sup>1</sup> Unlike other *Acanthamoeba* infections, which usually affect immunocompromised patients, AK occurs in immunocompetent healthy individuals.<sup>2</sup> Treatment failure rates are currently in the order of 50% for a poor outcome (acuity less  $\leq 20/80 \pm$  surgery) with median overall cure times in the order of 5 months<sup>3,4</sup> and 25% of patients requiring more than 9 months of treatment.<sup>3</sup> Over 25% of patients in large series have required a keratoplasty.<sup>5,6</sup>

One of the major causes of morbidity in AK is the severe inflammatory response that develops in some patients.<sup>7</sup> This occurs in both the infected cornea, with the development of a corneal ring infiltrate, as well as in the usually noninfected adjacent sclera. Scleritis causes both severe pain, and,

in some cases, the secondary complications of scleral thinning, choroidal detachment, and hypotony.<sup>8-11</sup> Corneal ring infiltrates are reported in 13 to 24% of cases and scleritis in 9 to 18% of patients with AK.<sup>8,12,13</sup> These severe inflammatory complications are associated with poor outcomes.<sup>7</sup>

We and others have shown that single nucleotide polymorphisms (SNPs) of inflammatory genes involved in animal models of bacterial keratitis are associated with the onset and severity of corneal infection in patients who wear contact lenses (CLs).<sup>14-18</sup> CL wear is the major risk factor for AK in developed countries, accounting for around 90% of cases.<sup>19</sup> This study was designed to test our hypothesis that differences in immune gene variation in otherwise healthy CL wearers determine whether a severe inflammatory response develops in the course of AK.

**TABLE 1.** Distribution of Sample, Presenting History, and Clinical Features of Patients With and Without Severe Inflammatory Complications

| Characteristics   | SICs<br>N = 40 | Non-SICs<br>N = 65 | P Value |
|---|----------------|--------------------|---------|
| Age at diagnosis, mean y ± SD (n = 89)                    | 38.3 ± 17.3    | 37.9 ± 16.2        | 0.906   |
| Sex, n (%) (n = 105)                                      |                |                    |         |
| M   | 20 (50.0)      | 30 (46.2)          | 0.841   |
| F   | 20 (50.0)      | 35 (53.8)          |         |
| Topical corticosteroid use prior to AAT n (%) (n = 95)    |                |                    |         |
| No  | 23 (67.6)      | 52 (85.2)          | 0.065   |
| Yes   | 11 (32.4)      | 9 (14.8)           |         |
| HSV keratitis treatment prior to AAT (n = 95)             |                |                    |         |
| No  | 20 (58.8)      | 48 (78.7)          | 0.057   |
| Yes   | 14 (41.2)      | 13 (21.3)          |         |
| Bacterial keratitis treatment prior to AAT n (%) (n = 95) |                |                    |         |
| No  | 13 (38.2)      | 25 (41.0)          | 0.830   |
| Yes   | 21 (61.8)      | 36 (59.0)          |         |
| Patients referred to tertiary care (%) (n = 103)          |                |                    |         |
| No  | 19 (47.5)      | 37 (58.7)          | 0.313   |
| Yes   | 21 (52.5)      | 26 (41.3)          |         |
| Disease stage at presentation <sup>a</sup> (%) (n = 84)   |                |                    |         |
| 1   | 5 (18.5)       | 25 (43.9)          | <0.001  |
| 2   | 9 (33.3)       | 31 (54.4)          |         |
| 3   | 13 (48.1)      | 1 (1.8)            |         |

AAT = anti-amoebic therapy; HSV = herpes simplex virus.

<sup>a</sup> Stage 1 corneal epitheliopathy; stage 2, stage 1 + epithelial defect, perineural infiltrate or stromal infiltrate; and stage 3, stage 2 + corneal ring infiltrate.

## METHODS

This was a prospective study of patients with CL associated AK classified as either displaying a severe inflammatory complication (SICs) or not (non-SICs).

## Ethics

The protocol was approved by the London-Hampstead National Research Ethics Service (Reference 13/LO/0032) and Moorfields Eye Hospital Research Management Committee. The trial was adopted by the UK National Institute for Health Research, Clinical Research Network Portfolio. All participants gave written informed consent and all study procedures conformed to the tenets of the Declaration of Helsinki.

## Recruitment

CL wearers who attended Moorfields Eye Hospital for diagnosis and/or ongoing treatment for AK were recruited prospectively between April 2013 and October 2014.

For the diagnosis of AK, one or more of the following criteria was satisfied:

1. A positive *Acanthamoeba* culture or histopathologic confirmation of trophozoites or cysts.
2. Culture-negative cases that were positively identified as having *Acanthamoeba* cysts on confocal microscopy, together with a typical clinical course and response to anti-amoebic treatment (AAT).
3. In the absence of criterion 1 or 2, patients with perineural corneal infiltrates or a typical clinical course with a response to AAT.

Genomic DNA was collected by a self-administered cheek swab (FLOQswabs; Copan Diagnostics, CA, USA) or a saliva kit (DNAGenotek, Ontario, Canada or DNAGard, Biomatri-

cia, CA, USA). Age, sex, and ethnicity was collected via self-report. Clinical data were collected retrospectively from the patients' with AK hospital records using the REDCap database platform (Vanderbilt University, Nashville, TN, USA).

Disease stage at presentation of AK was divided into 3 categories: stage 1, corneal epitheliopathy only; stage 2, presence of one or more of corneal epithelial defects, perineural infiltrates or stromal infiltrates, in addition to stage 1 findings; and stage 3, presence of a corneal stromal ring infiltrate in addition to one or more features of stage 2 disease.<sup>20</sup>

## Severe Inflammatory Complications

SICs were defined as documentation of scleritis and/or stromal ring infiltrate in the patients' hospital records in accordance with a previous study.<sup>7</sup>

## SNP Assay Design

Much of the understanding of the immune response to *Acanthamoeba* has come from studies in animal models. These studies indicate that innate cells and proteins, such as TLR-4, IL-8, and neutrophils<sup>21,22</sup> are crucial to mount a response in early AK disease, and that Th17 cells are involved in limiting disease severity.<sup>23</sup> We therefore designed a SNP assay targeting Th-17 pathway genes (*IL17-A*, *IL17-F*, *IL17-R*, *IL-1β*, *TNFα*, *TGFβ*, *IFNγ*, *IL-6*, *IL-23R*, *IL-22*, and *IL-27*) and *CXCL8* (encodes for IL-8). We also included Th-1 and Th-2 interleukins genes found associated with bacterial keratitis (*IL-12* and *IL-10*, respectively) and a novel cytokine (*IL-18*). Surface defense proteins defensin 1 and TLR-4 were further targeted, as expression of these proteins are upregulated in the presence of *Acanthamoeba* in both animal models and in vitro.<sup>24</sup> As a SNP of TNF-related apoptosis-inducing ligand receptor (TRAIL-R1), a negative modulator of

**TABLE 2.** Allelic Frequencies of Targeted SNPs and Association Analysis in Patients With AK With Severe Inflammatory Complications (SICs) Compared to Those Without

| Gene/Protein                                | SNP Reference     | Chromosome: Base Pair<br>(hg38 Assembly) | Alleles<br>A2/A1 | MAF (A1)     |              | SICs vs. Non SICs<br>Adjusted <sup>a</sup> |              |
|---|-------------------|--|------------------|--------------|--------------|--|--------------|
|   |                   |  |                  | SIC          | Non-SIC      | OR   | P Value      |
| <i>IL10</i> /IL-10                          | rs1800872         | 1:206773062                              | G/T              | 0.175        | 0.285        | 0.595                                      | 0.192        |
|   | rs1800871         | 1:206773289                              | G/A              | 0.175        | 0.292        | 0.569                                      | 0.160        |
|   | rs1800896         | 1:206773552                              | T/C              | 0.375        | 0.385        | 0.909                                      | 0.784        |
|   | rs1800890         | 1:206776020                              | A/T              | 0.288        | 0.300        | 0.836                                      | 0.643        |
| <i>IL23R</i> /IL-23R                        | rs1884444         | 1:67168129                               | G/T              | 0.075        | 0.077        | 0.983                                      | 0.970        |
|   | rs7517847         | 1:67215986                               | T/G              | 0.450        | 0.346        | 2.035                                      | 0.073        |
|   | rs2201841         | 1:67228519                               | A/G              | 0.263        | 0.323        | 0.884                                      | 0.742        |
|   | <b>rs11209026</b> | <b>1:67240275</b>                        | <b>G/A</b>       | <b>0.113</b> | <b>0.031</b> | <b>6.294</b>                               | <b>0.015</b> |
|   | rs11465817        | 1:67255414                               | C/A              | 0.238        | 0.308        | 0.858                                      | 0.681        |
|   | rs10889677        | 1:67259437                               | C/A              | 0.288        | 0.323        | 0.884                                      | 0.742        |
| <i>IL1B</i> /IL-1 $\beta$                   | rs1143634         | 2:112832813                              | G/A              | 0.250        | 0.208        | 1.045                                      | 0.923        |
|   | <b>rs16944</b>    | <b>2:112837290</b>                       | <b>G/A</b>       | <b>0.275</b> | <b>0.362</b> | <b>0.438</b>                               | <b>0.031</b> |
| <i>CXCL8</i> /IL-8                          | rs4073            | 4:73740307                               | T/A              | 0.413        | 0.508        | 0.508                                      | 0.0703       |
|   | <b>rs2227307</b>  | <b>4:73740952</b>                        | <b>T/G</b>       | <b>0.350</b> | <b>0.500</b> | <b>0.411</b>                               | <b>0.019</b> |
|   | rs2227549         | 4:73741020                               | A/G              | 0.038        | 0.038        | 1.160                                      | 0.834        |
|   | rs2227306         | 4:73741338                               | C/T              | 0.288        | 0.439        | 0.495                                      | 0.053        |
|   | <b>rs2227543</b>  | <b>4:73742193</b>                        | <b>C/T</b>       | <b>0.263</b> | <b>0.469</b> | <b>0.365</b>                               | <b>0.007</b> |
|   | <b>rs1126647</b>  | <b>4:73743328</b>                        | <b>A/T</b>       | <b>0.250</b> | <b>0.462</b> | <b>0.336</b>                               | <b>0.005</b> |
| <i>IL12B</i> /IL-12 B                       | rs3212227         | 5:159315942                              | T/G              | 0.125        | 0.231        | 0.554                                      | 0.200        |
|   | rs10045431        | 5:159387525                              | C/A              | 0.300        | 0.339        | 0.775                                      | 0.466        |
|   | rs6887695         | 5:159395637                              | G/C              | 0.338        | 0.331        | 0.947                                      | 0.874        |
| <i>TNF<math>\alpha</math></i> /TNF $\alpha$ | rs1800629         | 6:31575254                               | G/A              | 0.188        | 0.123        | 1.497                                      | 0.334        |
| <i>IL17A</i> /IL-17A                        | rs2275913         | 6:52186235                               | G/A              | 0.300        | 0.377        | 0.842                                      | 0.588        |
|   | rs3748067         | 6:52190541                               | C/T              | 0.088        | 0.085        | 0.982                                      | 0.975        |
| <i>IL17F</i> /IL-17F                        | rs763780          | 6:52236941                               | T/C              | 0.050        | 0.031        | 2.330                                      | 0.279        |
|   | rs2397084         | 6:52237046                               | T/C              | 0.100        | 0.100        | 0.944                                      | 0.926        |
| <i>IL6</i> /IL-6                            | rs1800797         | 7:22726602                               | G/A              | 0.313        | 0.369        | 0.750                                      | 0.415        |
|   | rs1800796         | 7:22726627                               | G/C              | 0.113        | 0.108        | 1.074                                      | 0.886        |
|   | rs1800795         | 7:22727026                               | G/C              | 0.313        | 0.369        | 0.846                                      | 0.629        |
| <i>DEFB1</i> /DEF $\beta$ 1                 | rs1800972         | 8:6877901                                | G/C              | 0.175        | 0.246        | 0.701                                      | 0.395        |
|   | rs1799946         | 8:6877909                                | C/T              | 0.375        | 0.346        | 1.349                                      | 0.437        |
|   | rs2702877         | 8:6878545                                | C/G              | 0.275        | 0.269        | 1.020                                      | 0.957        |
|   | rs5743409         | 8:6879098                                | C/A              | 0.425        | 0.423        | 0.937                                      | 0.841        |
| <i>TRAIL-R1</i> /TNFRSF10A                  | rs20576           | 8:23200707                               | T/G              | 0.175        | 0.185        | 1.253                                      | 0.605        |
|   | rs20575           | 8:23201811                               | G/C              | 0.375        | 0.485        | 0.739                                      | 0.346        |
|   | rs6557634         | 8:23202743                               | C/T              | 0.363        | 0.477        | 0.711                                      | 0.284        |
| <i>TLR4</i> /TL-R4                          | rs10983755        | 9:117702392                              | A/G              | 0.038        | 0.015        | 4.065                                      | 0.282        |
|   | rs10759932        | 9:117702866                              | T/C              | 0.163        | 0.162        | 0.785                                      | 0.630        |
|   | rs11536879        | 9:117709933                              | A/G              | 0.038        | 0.054        | 0.808                                      | 0.787        |
|   | rs1927907         | 9:117710486                              | C/T              | 0.150        | 0.169        | 0.814                                      | 0.674        |
|   | <b>rs4986790</b>  | <b>9:117713024</b>                       | <b>A/G</b>       | <b>0.100</b> | <b>0.031</b> | <b>6.915</b>                               | <b>0.014</b> |
|   | <b>rs4986791</b>  | <b>9:117713324</b>                       | <b>C/T</b>       | <b>0.113</b> | <b>0.031</b> | <b>6.915</b>                               | <b>0.014</b> |
|   | rs11536889        | 9:117715853                              | G/C              | 0.163        | 0.162        | 0.780                                      | 0.593        |
| rs7873784                                   | 9:117716658       | G/C                                      | 0.175            | 0.169        | 1.412        | 0.403                                      |              |
| <i>IL18</i> /IL-18                          | rs187238          | 11:112164265                             | C/G              | 0.250        | 0.269        | 0.761                                      | 0.512        |
| <i>IFN<math>\gamma</math></i> /IFN $\gamma$ | rs2069718         | 12:68156382                              | G/A              | 0.425        | 0.369        | 1.302                                      | 0.445        |
|   | rs1861494         | 12:68157629                              | T/C              | 0.300        | 0.300        | 0.935                                      | 0.826        |
|   | rs2430561         | 12:68158742                              | T/A              | 0.463        | 0.462        | 1.109                                      | 0.755        |
| <i>IL22</i> /IL-22                          | <b>rs1179251</b>  | <b>12:68251271</b>                       | <b>C/G</b>       | <b>0.025</b> | <b>0.131</b> | <b>0.0620</b>                              | <b>0.014</b> |
|   | rs2227485         | 12:68253933                              | A/G              | 0.475        | 0.408        | 1.216                                      | 0.567        |
|   | rs2227478         | 12:68254842                              | A/G              | 0.400        | 0.292        | 1.996                                      | 0.0665       |
|   | rs2227473         | 12:68255258                              | C/T              | 0.113        | 0.131        | 1.243                                      | 0.666        |
|   | rs2227472         | 12:68255353                              | C/T              | 0.463        | 0.415        | 1.174                                      | 0.634        |
| <i>IL27</i> /IL-27                          | rs4788084         | 16:28528527                              | C/T              | 0.375        | 0.323        | 1.294                                      | 0.423        |
| <i>TGFB1</i> /TGF $\beta$                   | rs1800471         | 19:41352971                              | C/G              | 0.063        | 0.085        | 0.530                                      | 0.353        |
|   | rs1800469         | 19:41354391                              | G/A              | 0.313        | 0.277        | 1.178                                      | 0.666        |
| <i>IL17RA</i> /IL-17RA                      | rs4819554         | 22:17084145                              | A/G              | 0.238        | 0.192        | 1.428                                      | 0.387        |
|   | rs2229151         | 22:17108407                              | G/A              | 0.025        | 0.023        | 1.288                                      | 0.758        |

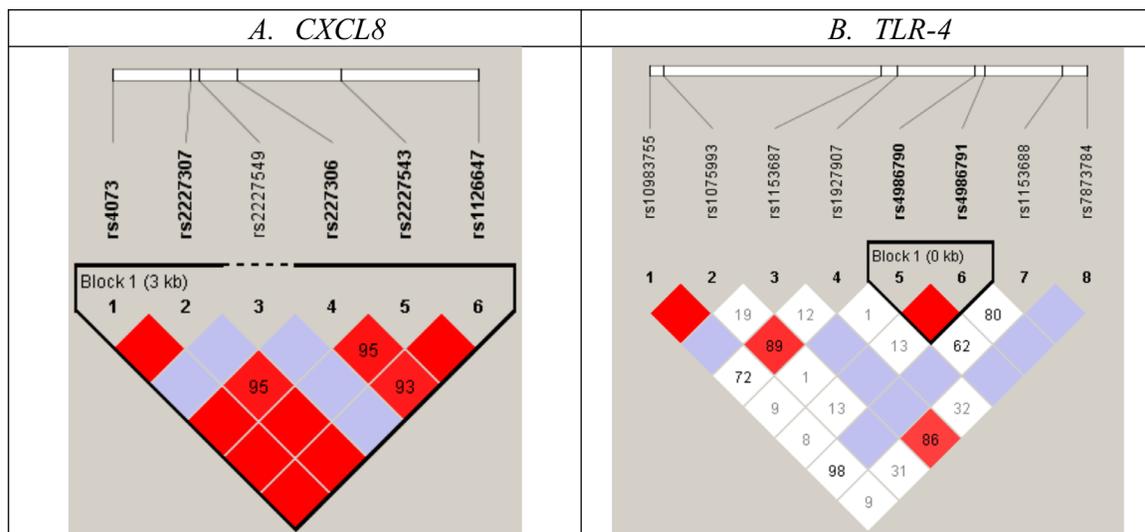
Statistically significant differences in bold typeface.

MAF = minor allele frequency.

<sup>a</sup> SICs versus age at diagnosis, sex, topical corticosteroid use prior to anti-amoebic therapy and HSV keratitis treatment prior to anti-amoebic therapy.

**TABLE 3.** Genotype Frequencies of *CXCL8* (Encodes IL-8) and *TLR-4* in Patients With Severe Inflammatory Complications (SICs) and Controls

| Gene/Protein     | SNPs                     | Genotypes                | SICs (%)<br>(n = 40) | Non-SICs (%)<br>(n = 65) | Adjusted P Value<br>(Dominant Model) |         |        |
|------------------|--------------------------|--------------------------|----------------------|--------------------------|--------------------------------------|---------|--------|
| <i>CXCL8/IL8</i> | rs2227543 C/T4:73742193  | TT                       | 3 (7.5)              | 14 (21.5)                | 0.0145                               |         |        |
|                  |                          | TC                       | 15 (37.5)            | 33 (50.8)                |                                      |         |        |
|                  |                          | CC                       | 22 (55.0)            | 18 (27.7)                |                                      |         |        |
| <i>CXCL8/IL8</i> | rs1126647 A/T4:73743328  | TT                       | 2 (5.0)              | 14 (21.5)                | 0.0111                               |         |        |
|                  |                          | TA                       | 16 (40.0)            | 32 (49.2)                |                                      |         |        |
|                  |                          | AA                       | 22 (55.0)            | 19 (29.2)                |                                      |         |        |
| <i>TLR4/TLR4</i> | rs4986790 A/G9:117713024 | GG                       | 0                    | 0                        | 0.0541                               |         |        |
|                  |                          | GA                       | 8 (20.0)             | 4 (6.2)                  |                                      |         |        |
|                  |                          | AA                       | 32 (80.0)            | 61 (93.8)                |                                      |         |        |
|                  |                          | rs4986791 C/T9:117713324 | TT                   | 0                        |                                      | 0       | 0.0290 |
|                  |                          |                          | TC                   | 9 (22.5)                 |                                      | 4 (6.2) |        |
|                  |                          | CC                       | 31 (77.5)            | 61 (93.8)                |                                      |         |        |



**FIGURE.** Linkage disequilibrium structure of selected SNPs in regions of interest in *CXCL8* (encodes IL-8) (A) and *TLR-4* (B). LD blocks were created with the default algorithm in HaploView program (version 4.1) that creates 95% confidence bounds on the normalized coefficient of linkage disequilibrium ( $D'$ ) considered being in strong LD where 95% of the comparisons made are informative. The coloring of the boxes depends on two scores, the logarithm of the odds (LOD) and  $D'$ , high association (LOD  $\geq 2$  and  $D' = 1$ , red), low association (LOD  $< 2$  and  $D' = 1$ , blue), and no association (LOD  $< 2$  and  $D' < 1$ , white). LOD score are shown within the box if  $D' < 1$ .

inflammation, has recently been associated with trachoma infection in humans<sup>25</sup> and it was also included in the SNP panel.

Following collection via a cheek swab or a saliva kit, genomic DNA was extracted and processed using the Illumina MiSeq platform. Detailed gDNA sample processing and bioinformatics methods are included in Supplementary File S1.

### SNPs and Phenotype Association Analysis

The PLINK (version 1.07) software was used for association analysis.<sup>26</sup> SNPs with a minor allele frequency  $< 0.01$  in the study cohort or not conforming to Hardy-Weinberg Equilibrium ( $P < 0.001$ ) were removed. Remaining SNPs were assessed for association with SICs using logistic regression. Topical corticosteroid use prior to the initiation of AAT, bacterial keratitis treatment prior to AAT, herpes simplex virus (HSV) keratitis treatment prior to AAT, disease stage at presentation, and tertiary referral (i.e. referred from another hospital for tertiary management at Moorfields Eye Hospi-

tal) were tested for univariate associations and included in the multivariate model if significant ( $P < 0.2$ ) along with sex and age as covariates. Analysis of linkage disequilibrium between multiple SNPs in the same gene was performed using HaploView (version 4).<sup>27</sup>

### RESULTS

Genomic DNA was obtained and analyzed for 105 cases of AK, 40 (38%) of whom experienced SICs (Table 1). The average age at the time of presentation for all AK cases was  $36.9 \pm 15.5$  years, and there was no difference in age between those with SICs compared to those without ( $38.3 \pm 17.3$  compared to  $37.9 \pm 16.2$ ,  $P = 0.906$ ). Likewise, there was no difference in sex between the two groups (women: 20/40, 50% vs. 35/65, 54%,  $P = 0.841$ ). “Topical corticosteroid use” and “herpes simplex virus (HSV) keratitis treatment prior to the initiation AAT” was significant  $P < 0.2$  and was included in the genotype model. “Disease stage at presentation” was more advanced in the SICs group ( $P < 0.001$ ) so this variable was not included

in the final model due to collinearity. There was no difference between “bacterial keratitis treatment prior to AAT” and “patients referred to tertiary care” for SICs versus non-SICs and so these characteristics were also not included in the multivariate model.

Of the 72 targeted SNPs, 6 were not able to be sequenced with the assay (*IL1B* rs1143627; *IL10* rs6703630; *TLR4* rs12377632; *IL18* rs5744292; *IL22* rs2227483; and *IFN $\gamma$*  rs2069707), 5 did not conform to Hardy-Weinberg Equilibrium ( $P > 0.001$ , *IL12B* rs17860508; *TNF $\alpha$*  rs1799964; *IL22* rs1012356; *IFN $\gamma$*  rs2069705; and *TGFB1* rs1800470), and three had minor allele frequencies of  $< 0.01$  (*CXCL8/IL-8* rs2227532; *TRAIL-R1* rs2230229; and *IFN $\gamma$*  rs2069709) in this cohort. The remaining 58 SNPs were included in the analysis. Table 2 details the allele frequencies of the analyzed SNPs and adjusted multivariable analysis by allele. The adjusted analysis accounted for the a priori factors of age at diagnosis, sex, topical corticosteroid use prior to AAT, and HSV keratitis treatment prior to AAT. Significant associations (adjusted  $P < 0.05$ ) after adjustment for covariates were found in SNPs in genes *IL-23R*, *IL-1 $\beta$* , *CXCL8*, *TLR-4*, and *IL-22*.

The highest significance was found for *CXCL8* (encodes IL-8) and *TLR-4* SNPs and because an association was found with more than one SNP for each of these genes, they were investigated further for inheritance and linkage disequilibrium (LD).

Associated SNPs in *CXCL8* (encodes IL-8) and *TLR-4* demonstrated dominant inheritance models (Table 3). LD was performed in HaploView (version 4) for the targeted SNPs in *CXCL8* (encodes IL-8) and *TLR-4* (Fig.). All SNPs in *CXCL8* (encodes IL-8) were in LD apart from rs2227549 (4:73741020). For *TLR-4* only the 2 associated SNPs were in an LD block with low LD across the rest of the gene.

## DISCUSSION

This study investigated SNPs in genes encoding innate and adaptive immune responses thought to play key roles in the severity of AK from in vitro and in vivo animal and human studies. SNPs in five genes encoding biologically relevant molecules, *CXCL8* (encodes IL-8, rs2227543 C/T, rs1126647 A/T, and rs2227307 T/G, all in 3'UTR gene region) and *TLR-4* (rs4986790 A/G, rs4986791 C/T, missense coding gene region), *IL-1 $\beta$*  (rs16944, G/A, upstream regulatory region), *IL-23R* (rs11209026, G/A, missense coding gene region), and *IL-22* (rs1179251, C/G, intron gene region) were identified that were associated with SICs in AK after adjusting for covariates. SNPs of *CXCL8* (encoding IL-8) and *TLR-4* had two significantly associated SNPs, each in LD and exhibited the strongest association with SICs.

The *CXCL8* (encodes IL-8) SNPs were protective for SICs in this population. Carriers of the minor allele (T) were around 70% less likely to experience SICs than noncarriers in this study. In this same AK patient cohort, increased levels of IL-8 protein in tears were associated with more severe AK disease.<sup>28</sup> However, tear IL-8 levels were not correlated with specific genotypes in patients in this study ( $P = 0.324$ ). Similarly, a study of sepsis susceptibility in Chinese men<sup>29</sup> found that the T allele for SNP rs1126647 was protective and increased levels of serum IL-8 were associated with sepsis in their population, but found no correlation between specific genotypes and the serum IL-8 protein levels. These findings indicate that this SNP may not be the causal SNP

that increases the transcriptional capacity for IL-8. Other studies have suggested that rs4073 and rs2227306 may be causal SNPs.<sup>30</sup> Although significance associations of these SNPs were not found in this study, they are in LD with those identified as significant.

IL-8 is produced in the cornea by epithelial cells, keratocytes,<sup>31</sup> and macrophages<sup>22</sup> and has been shown to be upregulated in other ocular surface diseases, such as allergy<sup>32</sup> and dry eye.<sup>33</sup> IL-8 mobilizes and activates neutrophils, which is crucial in the early stage of corneal infection. However, activated neutrophils can also release metalloproteinases, which can degrade the extracellular matrix.<sup>34</sup> This is evidenced by an immunodeficient mouse model, which when infected with adenovirus vector encoding human IL-8, results in neutrophil infiltration and corneal ulceration.<sup>35</sup> Thinning of the cornea and persistent epithelial defects, whereas not by definition part of SICs, are often present in severe cases of AK.<sup>19</sup> IL-8 is produced early in the inflammatory cycle, but unlike most other inflammatory cytokines, it can remain active up to weeks at the site of inflammation.<sup>36,37</sup> In contrast to the transitory effect of other proinflammatory cytokines that may last only a few hours, IL-8 may have a sustained influence<sup>36,37</sup> and therefore could be an important contributor to severe inflammatory outcomes in AK. In addition, IL-8 may be repeatedly produced by epithelial and inflammatory cells, leading to prolonged inflammation and collateral tissue damage.

Although IL-8 may have detrimental proinflammatory effects mediated through neutrophils, several animal studies have shown the powerful anti-acanthamoeba action of neutrophils in the early stages of disease. Neutrophils are chemotactic to *Acanthamoeba* trophozoites and cyst lysates, and are capable of killing the organism in vitro through myeloperoxidase (MPO).<sup>38</sup> Upon injection of one million *Acanthamoeba castellanii* trophozoites into the anterior chamber of a mini pig model, AK was eliminated by a robust neutrophil response.<sup>39</sup> Intraocular infection has only been reported in a handful of human cases<sup>8</sup> indicating this neutrophil response in the anterior segment may be a factor in limiting the organism from infecting the posterior eye in humans.

In contrast to *CXCL8*, minor alleles of *TLR-4* SNPs were associated with more severe AK in this study. Carriers of the minor allele were around 6.9 times more likely to experience SICs than noncarriers. The two significant *TLR-4* SNPs are in almost complete LD. These SNPs are not common, however, the non-SIC minor allele frequencies are similar to population controls (minor allele frequency 0.06 from phase III 1000 Genomes) with increased frequency in SICs, reflecting a representative sample.

*TLR-4* is a surface recognition receptor present on corneal and conjunctival cells. In the presence of *Acanthamoeba*, in vivo and in vitro models both show activation of *TLR-4* receptor.<sup>40</sup> In mouse models of AK, *TLR-4* is also involved in initiating the cytokine complex.<sup>40</sup> It is likely that in patients with AK, decreased recognition of *Acanthamoeba* allows a greater inoculum load into the cornea with dysregulation of the cytokine response, increasing disease severity. *TLR-4* is primarily associated with a pro-inflammatory response, however, chronic activation of *TLR-4* can lead to an anti-inflammatory response in some circumstances.<sup>41</sup> If this is the case in AK, patients with milder disease may be able to mount this anti-inflammatory response, whereas those with the SNP and more severe disease may not, leading to chronic hyperinflammation.

A study of patients with bacterial keratitis in India found higher carriage of *TLR-4* rs4986791 SNP compared with healthy controls.<sup>18</sup> However, another study did not find an association in rs4986790 (in LD with rs4986791) between CL patients with bacterial keratitis compared with healthy CL wearing controls in a Caucasian population.<sup>14</sup> The differences in results in these two studies are intriguing, but may be attributable to the rarity of the minor alleles. Interestingly, carriers of *TLR-4* SNPs have been shown to have a decreased response to endotoxins in several studies.<sup>42,43</sup> Of note, patients with *TLR-4* SNPs show a blunted airway response to inhalation of lipopolysaccharide (LPS).<sup>42</sup> The minor (T) allele of rs4986791 is associated with lower *TLR-4* expression in blood plasma of a large German cohort, which is replicated in a Arab and Asian cohort of healthy individuals.<sup>44</sup>

Three Th17-related genes were associated with SICs in this study, *IL-1β*, *IL-23R*, and *IL-22*. *IL-1β* and *IL-22* are pro-inflammatory cytokines and associated with the differentiation of CD4<sup>+</sup> T cells into effector Th17 cells. For *IL-22*, the SNP (rs1179251), as for *CXCL8* (encodes IL-8) was protective. *IL-22* tear fluid protein was also expressed more often in this cohort of patients with AK who experienced severe disease, as for IL-8.<sup>28</sup> However, we did not find an association between *IL-22* tear protein levels and rs1179251 genotypes ( $P = 0.07$ ). To our knowledge, there are no studies that correlate *IL-22* protein to rs1179251. Of note is that serum *IL-22* protein is increased in idiopathic scleritis.<sup>45</sup> A study of *IL-22* gene variations in autoimmune patients found the CC wild type genotype was associated with Graves' ophthalmopathy, conferring protection by the minor allele, similar to our findings.<sup>46</sup> Serum levels of *IL-22* are associated with the minor allele of rs2227484,<sup>47</sup> and stimulated peripheral blood mononuclear cells (PBMC) from patients carrying the minor allele of rs2227473 produce more *IL-22*.<sup>48</sup> However, both these SNPs are in the promoter region of the gene, whereas rs1179251 is in intron 4, rendering it difficult to draw comparisons.

In alignment with other cytokines in this study, the significant detection of SNP in *IL-1β* (rs16944) is associated with a lower risk of SICs. This SNP minor allele is associated with increased gene expression in PBMCs.<sup>49</sup> However, *IL-1β* tear protein was not detectable in any of the tear samples from AK cases or nonaffected healthy controls in this current study population.<sup>28</sup> This SNP and others in *IL-1β* have been associated with susceptibility to early onset periodontitis<sup>50</sup> and osteoarthritis.<sup>51</sup> In a previous study in a Caucasian population, an association was not found with *IL-1β* SNPs between patients with CL associated bacterial keratitis and healthy CL wearing controls.<sup>14</sup> Further work is required to understand the role of *IL-1β* in AK.

For *IL-23R* rs11209026, carriage of the A allele was associated with a 6 times increased risk of SICs. The presence of the A allele increases expression of the soluble form of *IL-23R* mRNA (which then functions as a decoy receptor) and thus impairs the function of *IL-23* in its ability to promote and maintain Th-17 cells.<sup>52</sup> This may mean that the Th-17 responses observed in an animal model of AK<sup>23</sup> are hampered in patients with this SNP.

There are several limitations of this study, in particular the sample size. Although this is a rare disease and we have a relatively large sample size per se, this limits power. In addition, the small sample size did not enable a more comprehensive analysis, such as genomewide association study (GWAS) or exome screening. Similarly, we have not

made a correction for multiple testing of the 58 SNPs. All of the SNPs analyzed in this study had a priori hypothesis of association based on previous literature, but none reach significance under strict multiple testing criteria, despite the large effect sizes in many instances. Replication of these findings in similar, but independent, cohorts is critical and meta-analyses will likely be required to reach true statistical significance. Another limitation in this rare cohort was the self-report of ethnicity, which was completed poorly by participants (76/105, 72% identified ethnicity), however, 70/76, 92% identified as Caucasian. In a previous genetic study of bacterial keratitis in CL wearers at Moorfields Eye Hospital in 2012, 82% were Caucasian,<sup>14</sup> adding certainty that the cohort in this current study is predominantly Caucasian.

Nevertheless, the study author's clinical experience in this area meant a very well phenotyped cohort. In addition, a well-matched cohort in terms of sex and age between the SICs and non-SIC groups were recruited. Our results are further strengthened by an earlier published tear fluid protein study carried out on the same patient cohort.<sup>28</sup>

In summary, our study has highlighted the importance of innate and Th17 immunity in patients with AK. A study of gene expression in late stage bacterial and fungal keratitis also found persistence of innate immune pathways.<sup>53</sup> Further work will be important to define cause and effect in tissue and animal models.

Furthermore, this study demonstrates that genetic screening of patients with AK for the presence of these SNPs is viable and could predict the risk of patients developing the severe inflammatory complications of the disease. The presence of these SNPs in an individual patient could assist clinicians managing AK by identifying patients susceptible to developing SICs in whom the prompt treatment of inflammation can be expected to improve outcomes.

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### References

1. Carnt N, Hoffman JM, Verma S, et al. *Acanthamoeba* keratitis: confirmation of the UK outbreak and a prospective case-control study identifying contributing risk factors. *Br J Ophthalmol*. 2018;102:1621–1628.

2. van Klink F, Taylor WM, Alizadeh H, Jager MJ, van Rooijen N, Niederkorn JY. The role of macrophages in *Acanthamoeba* keratitis. *Invest Ophthalmol Vis Sci.* 1996;37:1271–1281.
3. Papa V, Rama P, Radford C, Minassian DC, Dart JKG. *Acanthamoeba* keratitis therapy: time to cure and visual outcome analysis for different antiamebic therapies in 227 cases. *Br J Ophthalmol.* 2020;104:575–581.
4. Randag AC, van Rooij J, van Goor AT, et al. The rising incidence of *Acanthamoeba* keratitis: a 7-year nationwide survey and clinical assessment of risk factors and functional outcomes. *PLoS One.* 2019;14:e0222092.
5. Robaei D, Carnt N, Minassian DC, Dart JK. Therapeutic and optical keratoplasty in the management of *Acanthamoeba* keratitis: risk factors, outcomes, and summary of the literature. *Ophthalmology.* 2015;122:17–24.
6. Roozbahani M, Hammersmith KM, Rapuano CJ, Nagra PK, Zhang QE, Siu SY. *Acanthamoeba* keratitis: are recent cases more severe? *Cornea.* 2018;37:1381–1387.
7. Carnt N, Robaei D, Minassian DC, Dart JKG. *Acanthamoeba* keratitis in 194 patients: risk factors for bad outcomes and severe inflammatory complications. *Br J Ophthalmol.* 2018;102(10):1431–1435.
8. Iovieno A, Gore DM, Carnt N, Dart JK. *Acanthamoeba* sclerokeratitis: epidemiology, clinical features, and treatment outcomes. *Ophthalmology.* 2014;121:2340–2347.
9. Ebrahimi KB, Green WR, Grebe R, Jun AS. *Acanthamoeba* sclerokeratitis. *Graefes Arch Clin Exp Ophthalmol.* 2009;247:283–286.
10. Herz NL, Matoba AY, Wilhelmus KR. Rapidly progressive cataract and iris atrophy during treatment of *Acanthamoeba* keratitis. *Ophthalmology.* 2008;115:866–869.
11. Mannis MJ, Tamaru R, Roth AM, Burns M, Thirkill C. *Acanthamoeba* sclerokeratitis. *Arch Ophthalmol.* 1986;104:1313–1317.
12. Hollhumer R, Keay L, Watson SL. *Acanthamoeba* keratitis in Australia: demographics, associated factors, presentation and outcomes: a 15-year case review. *Eye (Lond).* 2020;34:725–732.
13. Szentmary N, Daas L, Shi L, et al. *Acanthamoeba* keratitis - clinical signs, differential diagnosis and treatment. *J Curr Ophthalmol.* 2019;31:16–23.
14. Carnt NA, Willcox MD, Hau S, et al. Association of single nucleotide polymorphisms of interleukins-1beta, -6, and -12B with contact lens keratitis susceptibility and severity. *Ophthalmology.* 2012;119:1320–1327.
15. Carnt NA, Willcox MD, Hau S, et al. Immune defense single nucleotide polymorphisms and recruitment strategies associated with contact lens keratitis. *Ophthalmology.* 2012;119:1997–2002.
16. Carnt N, Cipriani V, Stapleton F, Calder V, Willcox M. Association study of single nucleotide polymorphisms in IL-10 and IL-17 genes with the severity of microbial keratitis. *Contact Lens Anterior Eye.* 2019;42(6):658–661.
17. Keijser S, Kurreeman FA, de Keizer RJ, et al. IL-10 promotor haplotypes associated with susceptibility to and severity of bacterial corneal ulcers. *Exp Eye Res.* 2009;88:1124–1128.
18. Konda N, Kaur I, Garg P, Chakrabarti S, Willcox MDP. Toll-like receptor gene polymorphisms in patients with keratitis. *Cont Lens Anterior Eye*, <https://doi.org/10.1016/j.clae.2020.07.003>.
19. Dart JK, Saw VP, Kilvington S. *Acanthamoeba* keratitis: diagnosis and treatment update 2009. *Am J Ophthalmol.* 2009;148:487–499.e482.
20. Tu EY, Joslin CE, Sugar J, Shoff ME, Booton GC. Prognostic factors affecting visual outcome in *Acanthamoeba* keratitis. *Ophthalmology.* 2008;115:1998–2003.
21. Hurt M, Apte S, Leher H, Howard K, Niederkorn J, Alizadeh H. Exacerbation of *Acanthamoeba* keratitis in animals treated with anti-macrophage inflammatory protein 2 or antineutrophil antibodies. *Infect Immun.* 2001;69:2988–2995.
22. Ghasemi H, Ghazanfari T, Yaraee R, Faghihzadeh S, Hassan ZM. Roles of IL-8 in ocular inflammations: a review. *Ocul Immunol Inflamm.* 2011;19:401–412.
23. Suryawanshi A, Cao Z, Sampson JF, Panjwani N. IL-17A-mediated protection against *Acanthamoeba* keratitis. *J Immunol.* 2015;194:650–663.
24. Hoti SL, Tandon V. Ocular parasitoses and their immunology. *Ocul Immunol Inflamm.* 2011;19:385–396.
25. Al-Kuhlani M, Rothschild J, Pal S, et al. TRAIL-R1 is a negative regulator of pro-inflammatory responses and modulates long-term sequelae resulting from Chlamydia trachomatis infections in humans. *PLoS One.* 2014;9:e93939.
26. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a toolset for whole-genome association and population-based linkage analysis. *Am J Hum Genet.* 2007;81(3):559–575.
27. Barrett JC, Fry B, Maller J, MJ D. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005;21:263–265.
28. Carnt N, Montanez VM, Galatowicz G, Veli N, Calder V. Tear cytokine levels in contact lens wearers with *Acanthamoeba* keratitis. *Cornea.* 2017;36:791–798.
29. Hu D, Wang H, Huang X, et al. Investigation of association between IL-8 serum levels and IL8 polymorphisms in Chinese patients with sepsis. *Gene.* 2016;594:165–170.
30. Hacking D, Knight JC, Rockett K, et al. Increased in vivo transcription of an IL-8 haplotype associated with respiratory syncytial virus disease-susceptibility. *Genes Immun.* 2004;5:274–282.
31. Cubitt CL, Tang Q, Monteiro CA, Lausch RN, Oakes JE. IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes. *Invest Ophthalmol Vis Sci.* 1993;34:3199–3206.
32. Leonardi A, Curnow SJ, Zhan H, Calder VL. Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. *Clin Exp Allergy.* 2006;36:777–784.
33. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of inflammatory cytokines in the tears of dry eye patients. *Cornea.* 2009;28:1023–1027.
34. Li Q, Fukuda K, Lu Y, et al. Enhancement by neutrophils of collagen degradation by corneal fibroblasts. *J Leukoc Biol.* 2003;74:412–419.
35. Oka M, Norose K, Matsushima K, Nishigori C, Herlyn M. Overexpression of IL-8 in the cornea induces ulcer formation in the SCID mouse. *Br J Ophthalmol.* 2006;90:612–615.
36. DeForge LE, Fantone JC, Kenney JS, Remick DG. Oxygen radical scavengers selectively inhibit interleukin 8 production in human whole blood. *J Clin Invest.* 1992;90:2123–2129.
37. Apostolakis S, Vogiatzi K, Amanatidou V, Spandidos DA. Interleukin 8 and cardiovascular disease. *Cardiovasc Res.* 2009;84:353–360.
38. Hurt M, Proy V, Niederkorn JY, Alizadeh H. The interaction of *Acanthamoeba castellanii* cysts with macrophages and neutrophils. *J Parasitol.* 2003;89:565–572.
39. Clarke DW, Alizadeh H, Niederkorn JY. Failure of *Acanthamoeba castellanii* to produce intraocular infections. *Invest Ophthalmol Vis Sci.* 2005;46:2472–2478.
40. Hoti SL, Tandon V. Ocular parasitoses and their immunology. *Ocul Immunol Inflamm.* 2011;19:385–396.
41. Gurung P, Li B, Subbarao Malireddi RK, Lamkanfi M, Geiger TL, Kanneganti TD. Chronic TLR stimulation controls NLRP3 inflammasome activation through IL-10 mediated regulation of NLRP3 expression and caspase-8 activation. *Sci Rep.* 2015;5:14488.

42. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet.* 2000;25:187–191.
43. Michelsen KS, Arditi M. Toll-like receptor signaling and atherosclerosis. *Curr Opin Hematol.* 2006;13:163–168.
44. Suhre K, Arnold M, Bhagwat AM, et al. Connecting genetic risk to disease end points through the human blood plasma proteome. *Nat Commun.* 2017;8:14357.
45. Sainz-de-la-Maza M, Molins B, Mesquida M, et al. Interleukin-22 serum levels are elevated in active scleritis. *Acta Ophthalmol.* 2016;94:e395–399.
46. Song RH, Li Q, Wang W, Yao QM, Shao XQ, Zhang JA. Variants of interleukin-22 gene confer predisposition to autoimmune thyroid disease. *Int J Endocrinol.* 2017;2017:3428236.
47. Liu F, Pan X, Zhou L, et al. Genetic polymorphisms and plasma levels of interleukin-22 contribute to the development of nonsmall cell lung cancer. *DNA Cell Biol.* 2014;33:705–714.
48. Zhang G, Chen X, Chan L, et al. An SNP selection strategy identified IL-22 associating with susceptibility to tuberculosis in Chinese. *Sci Rep.* 2011;1:20.
49. Hameed I, Masoodi SR, Malik PA, Mir SA, Ghazanfar K, Ganai BA. Genetic variations in key inflammatory cytokines exacerbates the risk of diabetic nephropathy by influencing the gene expression. *Gene.* 2018;661:51–59.
50. Parkhill JM, Hennig BJ, Chapple IL, Heasman PA, Taylor JJ. Association of interleukin-1 gene polymorphisms with early-onset periodontitis. *J Clin Periodontol.* 2000;27:682–689.
51. Solovieva S, Kamarainen O-P, Hirvonen A, et al. Association between interleukin 1 gene cluster polymorphisms and bilateral distal interphalangeal osteoarthritis. *J Rheumatol.* 2009;36:1977–1986.
52. Yu RY, Brazaitis J, Gallagher G. The human IL-23 receptor rs11209026 A allele promotes the expression of a soluble IL-23R-encoding mRNA species. *J Immunol.* 2015;194:1062–1068.
53. Chidambaram JD, Kannambath S, Srikanthi P, et al. Persistence of innate immune pathways in late stage human bacterial and fungal keratitis: results from a comparative transcriptome analysis. *Front Cell Infect Microbiol.* 2017;7:193.