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Mendelian randomization and genetic analyses reveal causal roles of immune cells and inflammatory proteins in keratoconus

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Immunity and inflammation are implicated in the progression of keratoconus (KC), but the causal relationships between inflammatory immune phenotypes and the disease remain unclear. We conducted a comprehensive Mendelian randomization (MR) analysis using GWAS data to investigate the causal effects of inflammatory and immune factors on KC. Multiple sensitivity analyses were performed to validate our findings, with significant results confirmed through meta-analyses using independent GWAS datasets. The Steiger test, LD score regression, and multivariate MR were applied to assess independent effects. Analysis of inflammatory proteins revealed that IL-12B (P_{IVW} = 8.26 × 10^-5) and IL-13 (P_{IVW} = 0.012) were associated with an increased risk of KC, whereas IL-17 A (P_{IVW} = 0.049) was inversely associated with KC risk. After FDR adjustment, the results for IL-12B (P_{FDR} = 0.007) remained significant. Twenty-two protective and eleven risk immune cells were identified. Meta-analysis supports CD20 on IgD- CD24- B cells and Central Memory CD8 + T cells %CD8 + T cells as protective factors against KC. Multivariable MR revealed independent heritability for seven inflammatory proteins and three immune cells. These findings highlight the critical role of immune and inflammatory factors in KC pathogenesis, suggesting possible targets for future investigation in KC prevention and treatment.

Keywords Keratoconus, Mendelian randomization, Immunity, Inflammation

Keratoconus (KC) is a progressive, asymmetrical corneal ectasia characterized by central corneal thinning and conical protrusion, often resulting in irreversible vision loss, irregular astigmatism, corneal scarring, and other visual impairments, severely impacting quality of life. Typically, KC manifests in adolescence, progresses over time, and frequently reaches a stable state by approximately 40 years of age[1,2]. The pathology of KC involves intricate physiological mechanisms influenced by a multitude of factors, including genetic predispositions and environmental conditions[1,3]. KC prevalence has shown an upward trend globally, indicating that it is a significant global public health concern. Currently, treatment options for KC are limited, primarily focusing on refractive correction and symptomatic management. In severe cases, corneal transplantation is a viable option, albeit with challenges such as donor shortages and the risk of postoperative immune rejection. Some patients with early or subclinical KC exhibit an insidious onset and lack typical clinical manifestations. The progressive changes in corneal morphology further complicate early diagnosis.

Initial research suggested that KC is devoid of inflammatory characteristics, suggesting that it is a noninflammatory disorder[4]. However, accumulating evidence reveals a significant correlation between KC and abnormal concentrations of different inflammatory mediators, such as immune cells, in both eye-related and systemic environments. Numerous studies have reported abnormal expression levels of different inflammatory cytokines and proteases within the tear and corneal tissues of individuals diagnosed with KC. Subsequent research revealed that the homeostasis of these components, in conjunction with extracellular matrix signaling, plays a crucial role in maintaining or restoring corneal integrity[5–7]. The contribution of genetic elements to the development of KC has received growing interest. Various immune-related genetic conditions, including allergic responses and atopic dermatitis, have been linked to KC[8,9]. Notably, disease progression in KC patients is mitigated following the correction of immune disturbances, indicating that immune and inflammatory

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mediators may play a pivotal role in KC pathogenesis. Within corneal cell populations, antigen-presenting dendritic cells, particularly Langerhans cells, play a crucial role in maintaining corneal immune homeostasis and mediating inflammation[10]. Numerous studies analyzing blood biomarkers in KC patients have revealed that systemic inflammatory markers are significantly elevated compared to those in control subjects[11,12] which further supports that alterations in the expression of inflammatory and immune mediators may be implicated in the progression of KC. Early identification of inflammatory and immune alterations and the discovery of disease biomarkers are critical for early diagnosis, effective disease management, and the development of targeted intervention strategies for KC.

Mendelian randomization (MR) represents a developing research approach that leverages genetic variability to clarify the relationships between specific phenotypes. In recent years, its application in studies related to disease etiology has grown significantly[13]. Primarily employed for causal inference within the field of epidemiology, MR represents the most compelling method for examining causal links between exposures and outcomes, particularly in situations where randomized controlled trials cannot be conducted. It effectively mitigates the limitations of sample sizes and ethical concerns, minimizes confounding and reverse causation, and reduces experimental bias[14]. This research involved an in-depth investigation into the causal influences of 91 inflammatory proteins alongside 731 immune cell phenotypes on KC, utilizing MR analyses with aggregated data from genome-wide association studies (GWAS). Additionally, we conducted linkage disequilibrium score regression (LDSC), multivariate MR, and MR-META analyses to control for the effects of genetic associations and potential confounders. The objective of this research was to clarify the inflammatory and immune-related etiology of KC, provide novel insights into its biological processes, and enhance the understanding of KC pathogenesis.

Results

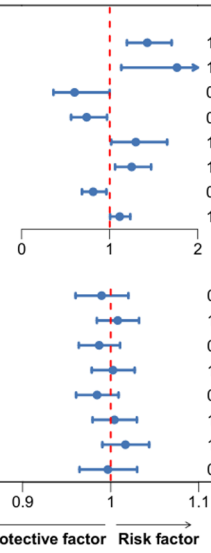
Selection and characteristics of instrumental variables

Following the instrumental variable (IV)-specific selection criteria and excluding chain-imbalanced single nucleotide polymorphisms (SNPs), 1,348 and 15,292 SNPs were included in our initial analyses of inflammatory factors and immune cells, respectively. All included SNPs demonstrated F-statistics > 10, effectively minimizing weak instrument bias, with detailed F-statistics for each SNP presented in Supplementary Tables S1 and S2, respectively. After performing a rigorous literature review and clinical epidemiologic examination, pleiotropic SNPs with potentially confounding phenotypes were excluded. For instance, rs5743604, rs1571025 and rs847888 were associated with allergic and rheumatic reactions, while rs7600322, rs139795227, rs11547311, rs9366778, and rs2524090 were associated with other ophthalmic diseases. Supplementary Table S3 lists the confounding IV information.

The causal relationships between inflammatory proteins and KC

The KC data were initially analyzed independently within the Finnish database. Initially, we confirmed the consistency of all results, using IVW as our main analysis technique and employing forest plots for result visualization. Four inflammatory proteins were initially identified as important. Further comprehensive sensitivity analyses indicated that three of these proteins were causally linked to KC (Fig. 1, Supplementary Table S4). Specifically, IL-17 A (OR 0.601, 95% CI 0.361–0.999; $P = 0.049$) was recognized as a factor providing protection against KC. Conversely, IL-12B (OR 1.427, 95% CI 1.195–1.703; $P = 8.26E\text{-}05$) and IL-13 (OR 1.764,

Exposure	Outcome	Gwas	nSNP	OR(95%CI)	Pval	Cochran's Q pval	MR–Egger Intercept pval	Steiger Test Pval	MRPRESSO GLOBAL pval
Forward MR									
IL−12B	Keratoconus	Finngen Study	23	1.427(1.195 to 1.703)	8.26E−05	0.987	0.530	<0.05	0.988
IL−13	Keratoconus	Finngen Study	10	1.764(1.132 to 2.750)	0.012	0.913	0.475	8.80E−51	0.909
IL−17A	Keratoconus	Finngen Study	10	0.601(0.361 to 0.999)	0.049	0.880	0.963	5.46E−46	0.871
4E−BP1	Keratoconus	Hardcastle et al.	6	0.738(0.562 to 0.970)	0.029	0.726	0.401	5.69E−28	0.755
IL−5	Keratoconus	Hardcastle et al.	9	1.296(1.017 to 1.653)	0.036	0.446	0.962	1.79E−24	0.462
ST1A1	Keratoconus	Hardcastle et al.	15	1.249(1.061 to 1.471)	0.008	0.664	0.489	3.76E−59	0.679
CXCL10	Keratoconus	Hardcastle et al.	19	0.812(0.685 to 0.962)	0.016	0.330	0.657	9.69E−82	0.380
FGF−5	Keratoconus	Hardcastle et al.	21	1.113(1.005 to 1.234)	0.040	0.311	0.144	1.50E−225	0.331
Reverse MR									
Exposure	**Outcome**								
Keratoconus	IL−12B	Finngen Study	12	0.990(0.960 to 1.020)	0.500	0.024	0.136	0.193	0.035
Keratoconus	IL−13	Finngen Study	12	1.008(0.984 to 1.032)	0.514	0.672	0.250	0.973	0.645
Keratoconus	IL−17A	Finngen Study	12	0.987(0.964 to 1.011)	0.275	0.830	0.362	0.788	0.814
Keratoconus	4E−BP1	Hardcastle et al.	32	1.002(0.978 to 1.027)	0.840	0.365	0.245	3.21E−100	0.340
Keratoconus	CXCL10	Hardcastle et al.	32	0.984(0.961 to 1.009)	0.210	0.330	0.784	1.56E−99	0.344
Keratoconus	FGF−5	Hardcastle et al.	32	1.004(0.979 to 1.030)	0.743	0.714	0.597	6.61E−88	0.736
Keratoconus	IL−5	Hardcastle et al.	32	1.017(0.990 to 1.044)	0.214	0.785	0.759	3.84E−88	0.763
Keratoconus	ST1A1	Hardcastle et al.	32	0.997(0.965 to 1.030)	0.845	0.027	0.268	1.41E−75	0.037



P<0.05 was considered statistically significant

Fig. 1. Relationships between inflammatory factors and KC via Mendelian randomization. *KC* keratoconus, *SNP* single-nucleotide polymorphism, *OR* odds ratio, *CI* confidence interval.

95% CI 1.132–2.750; $P = 0.012$) were found to be associated with an increased risk for KC. After comprehensive false discovery rate (FDR)-adjusted multiple testing, IL-12B (P_{FDR} = 0.007) continued to demonstrate causal significance with KC.

The causal relationships between immune cells and KC

We identified 44 significant immune cell phenotypes (31 protective and 13 risk-associated) in the initial analysis. Following sensitivity analyses, 33 immune cell phenotypes were identified as candidate outcomes, including 11 B cells, 3 conventional dendritic cells (cDCs), 3 TBNK cells, 2 monocytes, 3 myeloid cells, 8 regulatory T cells (Tregs), and 3 T cells at maturation stages (Fig. 2). After adjusting for the FDR, no immune features were identified at the significance threshold. Our analysis highlighted several protective factors against KC, including CD20 on IgD- CD24- B cells (OR 0.872, 95% CI 0.770–0.989; $P = 0.032$), CD25 on IgD + CD38- naive B cell (OR 0.902, 95% CI 0.825–0.987; $P = 0.024$), CD25 on IgD + CD38 + B cell (OR 0.792, 95% CI 0.651–0.963; $P = 0.019$), CD25 on IgD + B cell (OR 0.898, 95% CI 0.825–0.978; $P = 0.013$), CD27 on CD20- B cell (OR 0.759, 95% CI 0.635–0.908; $P = 0.003$), CD86 on CD62L + myeloid Dendritic Cell (OR 0.817, 95% CI 0.710–0.940; $P = 0.005$), CD3 on HLA DR + CD4 + T cell (OR 0.821, 95% CI 0.714–0.944; $P = 0.006$), and CD45 on HLA DR + Natural Killer (OR 0.748, 95% CI 0.616–0.908; $P = 0.003$). Other notable protective associations included CD14 on CD14 + CD16- monocyte (OR 0.771, 95% CI 0.616–0.965; $P = 0.023$), CD3 on activated & secreting CD4 regulatory T cell (OR 0.871, 95% CI 0.779–0.973; $P = 0.015$), and CD3 on effector memory CD8 + T cell (OR 0.818, 95% CI 0.705–0.950; $P = 0.009$).

Conversely, several immune cell phenotypes were identified as risk factors for KC, including BAFF-R on IgD + CD24 + B cell (OR 1.103, 95% CI 1.004–1.213; $P = 0.041$), BAFF-R on IgD + B cell (OR 1.111, 95% CI 1.008–1.226; $P = 0.035$), CD19 on IgD + CD38- unswitched memory B cell (OR 1.115, 95% CI 1.035–1.201; $P = 0.004$), CD19 on IgD + CD38dim B cell (OR 1.075, 95% CI 1.004–1.152; $P = 0.039$), and CD19 on unswitched memory B cell (OR 1.163, 95% CI 1.047–1.291; $P = 0.005$). Additional risk factors included CD86 on myeloid Dendritic Cell (OR 1.294, 95% CI 1.071–1.565; $P = 0.008$), CD62L- Dendritic Cell %Dendritic Cell (OR 1.124, 95% CI 1.034–1.222; $P = 0.006$), CD34 on Hematopoietic Stem Cell (OR 1.103, 95% CI 1.013–1.201; $P = 0.024$), and CD25 on CD39 + CD4 regulatory T cell (OR 1.281, 95% CI 1.036–1.585; $P = 0.022$). The robustness of these causal associations was supported by additional methods and sensitivity analyses. Detailed MR results are presented in Supplementary Table S5.

Genetic correlation analysis

We determined genetic correlations between inflammatory factors and immune phenotypes and KC using the LDSC method. Some samples were excluded from the analyses due to differences in heritability and sample size. In our preliminary examination, we acquired a sum of 87 results based on inflammatory factors and 293 results focused on immune cells. Two MR analyses revealed immune cells that exhibited genetic correlations ($rg\_p < 0.05$), but no substantial genetic correlation between inflammatory factors and KC was found. In the replication analyses, no significant genetic correlation was found between any MR analysis-determined phenotypes and KC. This finding aligns with previously reported findings in the literature and further demonstrates the robustness of our results. For detailed results, refer to Supplementary Table S6.

Examination of outliers and heterogeneity

All sensitivity analyses supported the robustness of our findings. MR-Egger intercept tests indicated no directional pleiotropy (all $P > 0.05$, Fig. 1; Table 1 and Supplementary Table S8), confirming that causal estimates were not biased by horizontal pleiotropy. MR-PRESSO detected no significant global outliers (all $P > 0.05$), further validating instrumental variable assumptions. Cochran's Q tests indicated no significant heterogeneity (all $P > 0.05$; Table 1 and Supplementary Table S7), while scatter plots (Supplementary Figs. S1 and S4) and funnel plots (Supplementary Figs. S2 and S5) visually confirmed the robustness of associations and absence of bias. Leave-one-out analysis confirmed no individual SNP bias (Supplementary Figs. S3 and S6). Steiger tests verified the assumed causal direction for all associations (all $P < 0.05$, Table 1; Fig. 1). These convergent findings demonstrate the absence of horizontal pleiotropy and support the validity of our causal inferences.

Reverse MR analysis

Reverse MR analysis identified 14 KC-associated SNPs in the discovery cohort and 37 SNPs in the replication cohort as valid instruments, with all F-statistics exceeding 10 (Supplementary Table S9). For immune cells and inflammatory proteins that demonstrated significant causal associations with KC in forward MR analyses, no significant reverse causal effects were observed (all $P > 0.05$), indicating absence of reverse causality. Comprehensive reverse MR results are presented in Supplementary Tables S10-S11.

Replication analysis and meta-analysis

We applied the same rigorous criteria in the replication analysis as in the discovery cohort analysis. After conducting comprehensive sensitivity analyses, we identified significant candidates for five inflammatory proteins and thirty-nine immune cell phenotypes (Supplementary Tables S12-S15). Referencing the published literature and considering potential differences and heterogeneity due to ethnicity and sample size, we performed a meta-analysis of notable MR outcomes from various databases to confirm the dependability of our results and to evaluate the factors that may affect the significant effects detected. For immunophenotyping, candidate results from two cohort replicates were included, encompassing a total of eight inflammatory phenotypes and three immunophenotypes. An I^2 value less than 40% indicated low heterogeneity. All the results are presented in Fig. 3.

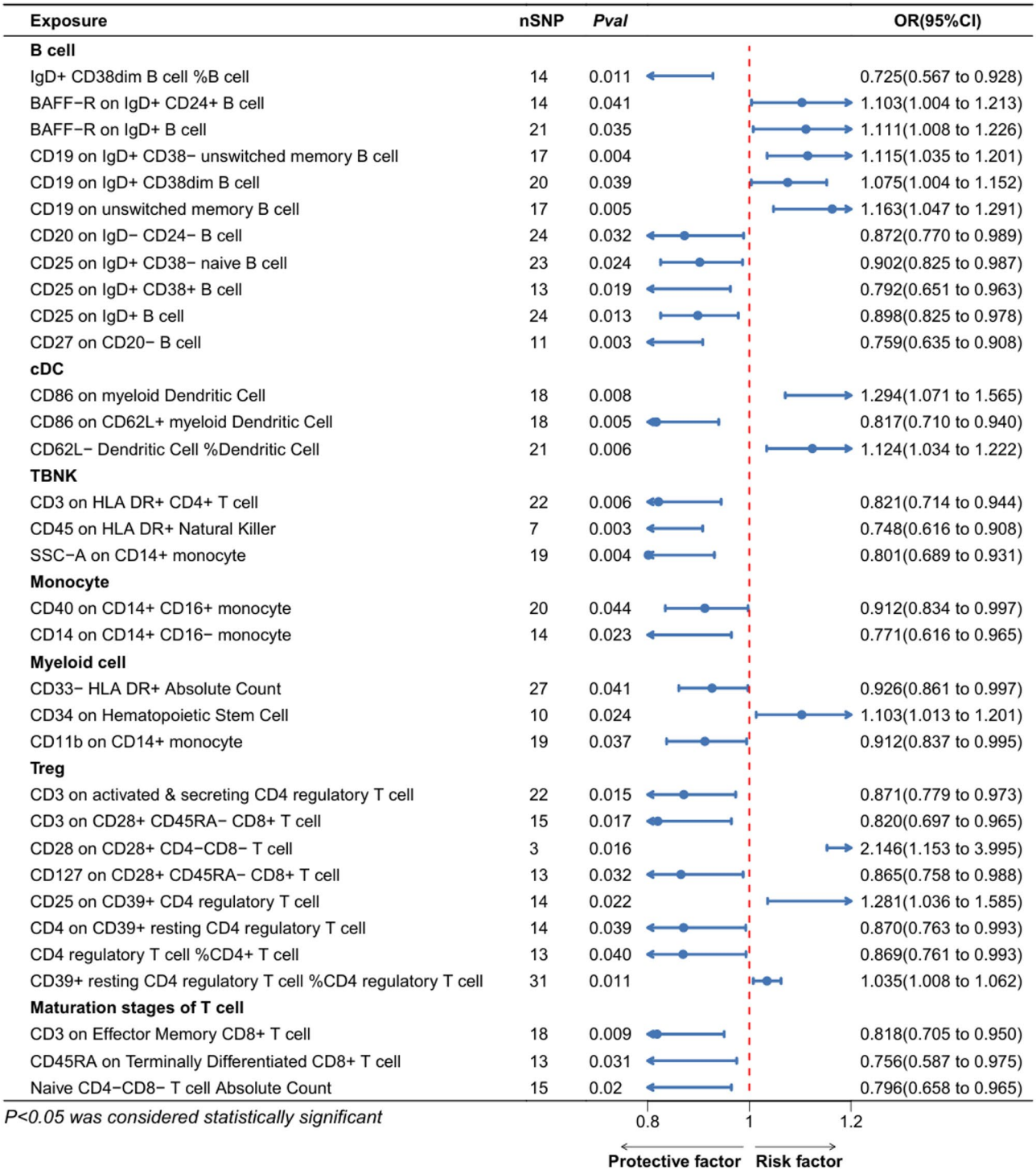


Fig. 2. Associations between immune cells and KC via Mendelian randomization. *SNP* single-nucleotide polymorphism, *OR* odds ratio, *CI* confidence interval.

Multivariate MR and confounding analysis

We examined the independent associations between eight candidate inflammatory factors and three candidate immunophenotypes with common risk factors for KC. For instance, after adjusting for diabetes, IL-12B (adjusted OR = 1.40, P_{Robust} = 8.79E-6), FGF-5 (adjusted OR = 1.13, P_{Robust} = 0.0001) and BAFF-R on IgD + B cells (adjusted OR = 1.08, P_{Robust} = 0.024) were associated with higher KC risk. These associations remained robust even after adjusting for education, hyperlipidemia, and television viewing factors. Conversely, 4E-BP1, CXCL-10, and Central Memory CD8 + T cells %CD8 + T cells exhibited protective effects against KC in most confounding

Exposure	Heterogeneity test		Pleio test		Steiger Test Pval	MRPRESSO Global Pval
	Cochran's Q	Cochran's Q Pval	MR-Egger intercept	MR-Egger Intercept pval		
IgD + CD38dim B cell %B cell	13.2389	0.4295	0.0967	0.0713	2.84E-70	0.448
BAFF-R on IgD + CD24 + B cell	11.7592	0.5475	0.0302	0.3876	<0.05	0.619
BAFF-R on IgD + B cell	24.6129	0.2166	0.0194	0.5191	<0.05	0.28
CD19 on IgD + CD38- unswitched memory B cell	17.0665	0.3813	−0.0288	0.5070	4.19E-105	0.385
CD19 on IgD + CD38dim B cell	13.0328	0.8369	−0.0077	0.7477	5.97E-110	0.846
CD19 on unswitched memory B cell	11.4820	0.7788	0.0071	0.7755	1.53E-94	0.822
CD20 on IgD- CD24- B cell	16.2171	0.8458	0.0161	0.4931	8.56E-127	0.882
CD25 on IgD + CD38- naive B cell	13.7173	0.9112	0.0292	0.2824	8.29E-155	0.936
CD25 on IgD + CD38 + B cell	10.6090	0.5627	0.0609	0.2108	9.55E-93	0.583
CD25 on IgD + B cell	29.3371	0.1694	0.0313	0.2778	2.21E-158	0.187
CD27 on CD20- B cell	6.2815	0.7911	0.0031	0.9332	1.54E-51	0.837
CD62L- Dendritic Cell %Dendritic Cell	18.5051	0.5542	−0.0136	0.5962	1.38E-278	0.685
CD86 on myeloid Dendritic Cell	13.4756	0.7038	−0.0123	0.7592	4.37E-90	0.733
CD86 on CD62L + myeloid Dendritic Cell	14.8990	0.6028	0.0047	0.8754	1.14E-92	0.635
CD3 on HLA DR + CD4 + T cell	24.6410	0.2630	0.0434	0.1620	4.88E-182	0.214
CD45 on HLA DR + Natural Killer	4.4955	0.6099	0.0087	0.8387	5.36E-32	0.613
SSC-A on CD14 + monocyte	20.8346	0.2878	0.0252	0.4407	6.31E-286	0.35
CD40 on CD14 + CD16 + monocyte	25.4509	0.1462	0.0306	0.5141	3.86E-305	0.195
CD14 on CD14 + CD16- monocyte	16.8072	0.2083	−0.0217	0.6412	4.92E-76	0.273
CD33- HLA DR + Absolute Count	18.2604	0.8661	0.0100	0.6884	1.13E-164	0.879
CD34 on Hematopoietic Stem Cell	8.2430	0.5099	0.0439	0.2238	6.31E-83	0.651
CD11b on CD14 + monocyte	16.1567	0.5816	0.0404	0.1512	5.15E-189	0.625
CD4 regulatory T cell %CD4 + T cell	9.3426	0.6734	0.0258	0.5482	5.39E-72	0.737
CD39 + resting CD4 regulatory T cell %CD4 regulatory T cell	28.7035	0.5332	0.0198	0.2595	<0.05	0.676
CD3 on activated & secreting CD4 regulatory T cell	23.9859	0.2937	0.0536	0.0596	2.00E-289	0.247
CD3 on CD28 + CD45RA- CD8 + T cell	13.0069	0.5260	0.0579	0.2240	2.76E-120	0.424
CD28 on CD28 + CD4-CD8- T cell	0.2123	0.8993	−0.0881	0.7315	5.44E-16	NA
CD127 on CD28 + CD45RA- CD8 + T cell	9.0341	0.7000	−0.0132	0.7283	2.49E-66	0.737
CD25 on CD39 + CD4 regulatory T cell	12.6752	0.4732	0.0071	0.8802	6.66E-74	0.496
CD4 on CD39 + resting CD4 regulatory T cell	9.4074	0.7415	0.0108	0.7679	1.11E-71	0.749
CD3 on Effector Memory CD8 + T cell	13.2135	0.7218	0.0057	0.8460	4.89E-128	0.626
CD45RA on Terminally Differentiated CD8 + T cell	15.6613	0.2072	−0.0115	0.8554	8.44E-66	0.266
Naive CD4 – CD8 – T cell Absolute Count	12.2343	0.5875	0.0302	0.4304	6.17E-74	0.681

Table 1. Sensitivity analysis results of immune cells and keratoconus: heterogeneity test, horizontal Pleiotropy assessment, and the Steiger Test. *Cochran's Q* Cochran's Q test, *MR-Egger intercept* MR-Egger intercept test, *IgD+* Immunoglobulin D-positive cells, *HLA DR* Human Leukocyte Antigen-DR isotype, *BAFF-R* B-cell activating factor receptor.

adjustment scenarios, although 4E-BP1's effect was not significant after adjusting for television watching (Fig. 4, Supplementary Table S16).

Discussion

Numerous studies have shown a connection between immune and inflammatory factors and KC; however, such research often involved limited sample sizes and primarily consisted of clinical observational studies. In the present study, we analyzed the causal effects of inflammatory proteins and immune cell profiles on KC using multiple large biogenetic databases, using genetic epidemiological methods. This study constitutes the most comprehensive systematic inquiry undertaken to date regarding the influence of inflammatory and immune factors on KC. We performed bidirectional two-sample MR analyses in two independent populations, conducted meta-analyses of significant phenotypes, and assessed the robustness of the results using multiple sensitivity analyses. These results offer valuable perspectives on possible pathogenetic processes related to KC and play a crucial role in developing accurate preventive and treatment approaches.

In recent decades, due to the avascular nature of the cornea, KC has been considered a noninflammatory disease that is primarily influenced by factors such as heredity, mechanical trauma, and eye habits. Further research has revealed that disruption of homeostasis in the body's inflammatory and immune systems is closely related to the pathogenesis of KC. Typically, an imbalance in proinflammatory and anti-inflammatory factors is followed by abnormal expression of endogenous matrix metalloproteinases, leading to excessive degradation

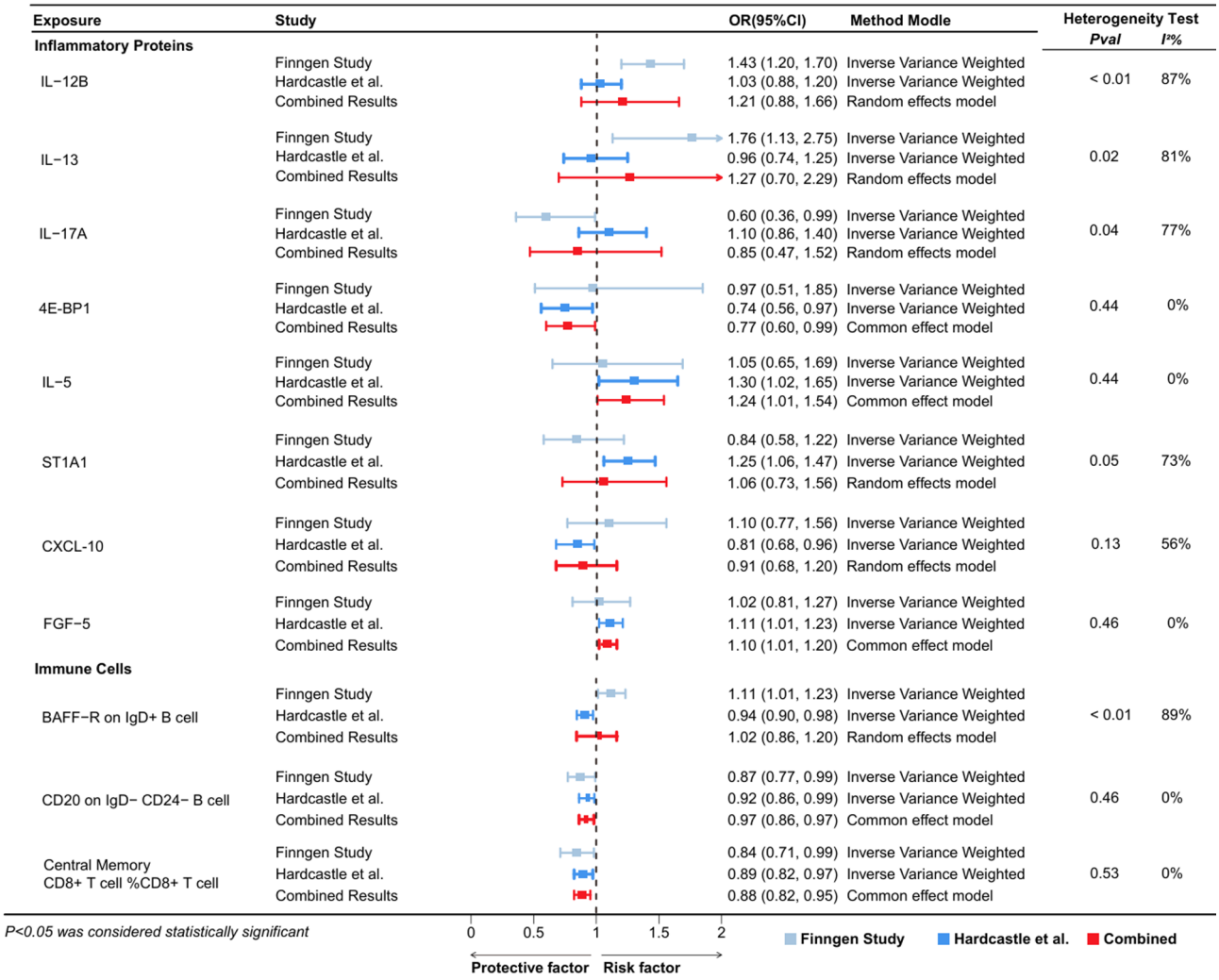


Fig. 3. Forest plots visualizing the results of the analysis. Meta-analysis was employed to assess the overall effects of inflammatory factors, immune cells, and KC and to evaluate positive or potentially positive results and their reliability.

of the extracellular matrix and collagen, which contributes to changes in corneal morphology[15,16]. Studies have shown that KC promotes inflammatory cytokines, adhesion molecules, and metalloproteinases expression, modulating the microenvironment[17].

Interleukin (IL) is a secreted protein and signaling molecule that serves as a key mediator in the regulation of immune and inflammatory responses. Research indicates that the concentration of IL receptors in corneal stromal cells of KC patients is significantly greater than that in the normal population and these cytokines can directly affect the healing of corneal wounds by regulating the proliferation, differentiation, and apoptosis of corneal stromal cells[18,19]. Eser et al.[20] recently published a prospective study analyzing tear samples from 17 KC patients and 17 healthy controls before and after CXL surgery. They found elevated levels of multiple inflammatory cytokines in KC patients, which significantly decreased following treatment. The results align with our current research, which indicated that IL-12B and IL-13 had a positive correlation with KC onset, while IL-17 A was potentially protective. This finding remained significant for IL-12B after FDR correction.

It is worth noting that although there was an apparent protective association between IL-17 A and KC, its borderline statistical significance ($P = 0.049$) and failure to replicate in our validation cohort necessitate cautious interpretation. Meta-analysis of the combined datasets yielded non-significant results with confidence intervals crossing unity, indicating that this finding likely represents statistical variation rather than a robust biological effect. While this apparent contradiction with IL-17 A's well-established pro-inflammatory role initially appeared paradoxical, our comprehensive literature review revealed that IL-17 A can exhibit context-dependent protective functions in corneal tissue homeostasis. Specifically, IL-17 A has been demonstrated to be essential for corneal nerve regeneration and epithelial healing following acute injury[21]where it orchestrates beneficial inflammatory cascades involving VEGF-mediated repair mechanisms and promotes corneal epithelial healing through γδ T cell activation[22]. Recent comprehensive reviews have elucidated IL-17 A's dual role in tissue repair, particularly its critical function in bridging acute and chronic inflammatory responses during wound healing processes[23]. In

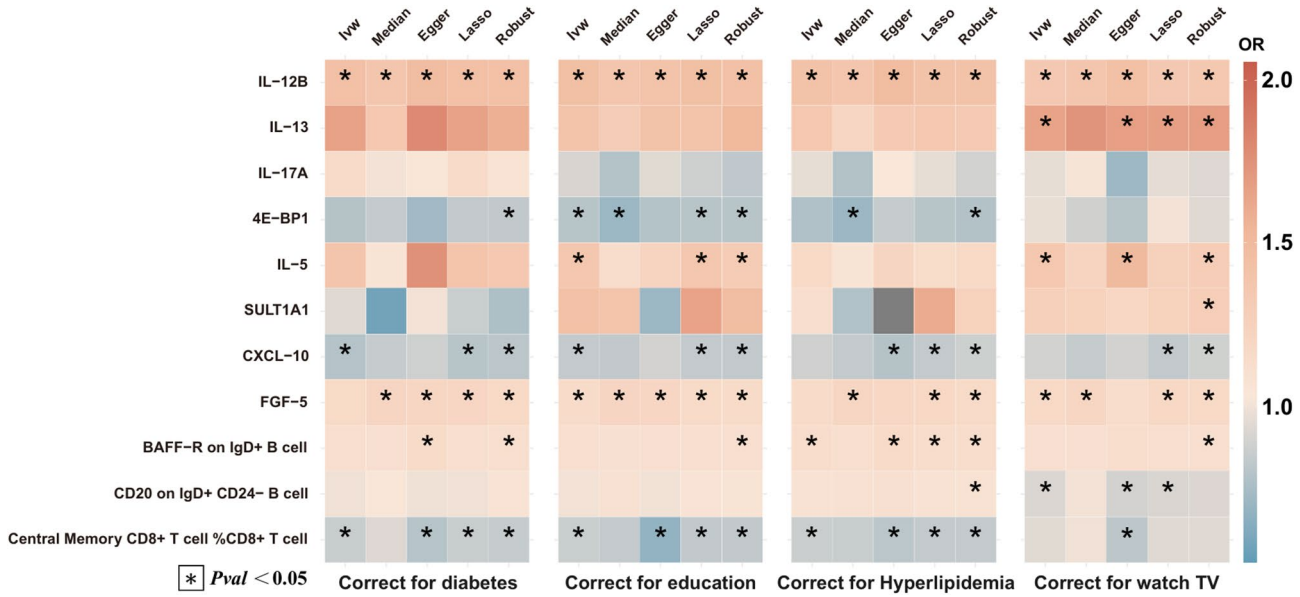


Fig. 4. Associations of the MVMR with inflammatory factors and immune cells. For each MVMR analysis, we added each genetic confounder separately. *IVW* inverse variance weighted, *LASSO* least absolute shrinkage and selection operator, *Median* Weighted Median, *OR* Odds Ratio; **Pval* < 0.05.

the specific context of KC pathogenesis, hypothetically protective mechanisms might encompass maintenance of corneal epithelial barrier integrity, modulation of matrix metalloproteinase activity, or prevention of excessive inflammatory cascades that contribute to progressive stromal degradation. Therefore, future studies using substantially larger longitudinal cohorts with pre-specified IL-17 A hypotheses are required to definitively establish whether this cytokine exerts clinically meaningful protective effects in KC pathogenesis.

Our meta-analysis showed that IL-5 retained statistical significance in the replication cohort, consistent with Eser's findings. Krok and colleagues[24] found that KC patients had lower levels of tear CXCL-10, further corroborating our findings. Notably, our study also reported for the first time the potential associations of 4E-BP1 and FGF-5 with KC. These factors may serve as potential influencers for KC intervention given their role in regulating ECM remodeling and collagen expression.

Our results indicated that the expression of IgD+, CD38, CD20, CD25, and CD27 on the surface of B cells was protective against the development of KC. In contrast, higher concentrations of BAFF-R and CD19 were linked to a greater likelihood of KC. IgD+, an immunoglobulin, is often expressed along with immunoglobulin M (IgM), both of which are involved in B-cell activation and the immune response. Serum levels of total IgD antibodies are significantly greater in allergic patients than in normal subjects, consistent with epidemiological reports that allergic symptoms may exacerbate KC[25]. However, there are no specific studies on the correlation between IgD + expression on the surface of B cells and KC. This study reports for the first time a possible protective association between IgD + and KC, which may be attributed to the pleiotropic nature of IgD antibodies[26]. It has been shown that IgD antibodies not only enhance allergen sensitization but also reduce allergic reactions, leading to allergen tolerance. Allergen-specific immunotherapy significantly increased the level of specific IgD antibodies. This effect has been demonstrated in egg-, milk-, and dust mite-specific immunotherapy[27]. The significant correlation between the improvement of clinical symptoms reported by patients and the level of dust mite-specific IgD antibodies suggests that IgD antibodies may be involved in the immune tolerance response[28].

BAFF-R has been shown to be overexpressed in desiccation syndrome, making it a current research hotspot for the development of new drugs targeting this receptor. Notably, Sjögren's syndrome and KC share overlapping genetic predispositions, and given their impact on the lacrimal glands, leading to diminished tear secretion and consequent dry eye conditions[29,30]. In our study, BAFF-R showed potential associations with different trends in the discovery and replication cohort analyses, but was nonsignificant after performing the meta-analysis. This may be related to population heterogeneity. Nevertheless, our analysis provides important new insights that may guide the search for new biomarkers for KC. Our research additionally indicated that the expression of CD20 on B cells, alongside the proportion of memory CD8 + T cells within the CD8 + T subpopulation, might contribute to a protective function in the development of KC. CD20 is mainly expressed in B lymphocytes at different developmental stages and is involved in regulating calcium influx and cell cycle progression and enhancing the B-cell antigen receptor immune microenvironment. Some drugs targeting CD20 have shown promising therapeutic effects on diseases such as lymphoma, leukemia, multiple sclerosis, and rheumatoid arthritis. However, there are no reports on the use of CD20 for KC therapy, indicating the need for more in-depth mechanistic studies.

Recent studies have demonstrated anti-inflammatory effects of certain CD20 + cells, with anti-CD20 monoclonal antibodies showing therapeutic efficacy in various autoimmune conditions[31]. Recent research has indicated that memory CD8 + T cells play a role in regulating cardiac and vascular functions, as well as in

conditions such as psoriatic arthritis and multiple sclerosis[32]. Although studies on central memory CD8 + T cells in nontumor diseases in KC are scarce and have not yet been reported, we found a protective effect on KC, filling a gap in the study of memory CD8 + T cells and KC. Notably, after meta-analyzing multiple samples, the effects of CD20 on the percentages of IgD- CD24- B cells and central memory CD8 + T cells among CD8 + T cells remained significant. In our multivariable MR analysis, we further examined the robustness of these associations across different adjustment models. Interestingly, the protective effect of CD20 on IgD- CD24- B cells maintained its directional consistency across all adjustment models, though with varying levels of statistical significance. This protective association remained statistically significant after adjusting for television watching habits ($P < 0.05$), while similar effect sizes with reduced statistical significance were observed after adjustments for diabetes, education, and hyperlipidemia. This pattern suggests that while the protective relationship of CD20 on IgD- CD24- B cells appears stable in direction, its statistical significance may be influenced by complex interactions with other factors. Based on our findings, several hypotheses emerge for future investigation. The associations observed with IL-12B and IL-13 warrant experimental studies to elucidate their functional roles in KC pathogenesis. Our novel finding of protective associations for CD20 + B cells and central memory CD8 + T cells generates hypotheses regarding immune cell involvement that require functional validation. From a diagnostic perspective, these inflammatory proteins and immune cell markers represent candidates for biomarker development studies, potentially complementing current assessment methods, particularly for early diagnosis in high-risk individuals. These genetic associations provide valuable insights into KC pathogenesis, but comprehensive functional studies are essential to establish biological mechanisms before considering clinical translation.

Although candidate genes for KC have been gradually identified by scholars, these genes mostly exist in isolation, making it difficult to explain the complex pathogenesis of KC. With the continuous advancement of genetic testing technology, various genomic tests play increasingly important roles in exploring the gene expression characteristics of diseases, mining pathogenic mechanisms, and identifying therapeutic candidate molecules. While MR methodology has been increasingly applied to other ophthalmic conditions including glaucoma, age-related macular degeneration, and diabetic retinopathy[33–35]comprehensive MR studies investigating immune cells and inflammatory proteins in KC remain limited. Previous KC research primarily relied on small observational samples, such as studies by Sorkhabi et al.[36] (42 KC patients) and Santos et al.[37] (30 KC patients). While genetic studies by Bykhovskaya et al.[38].included larger cohorts (526 KC patients), they focused on candidate gene analyses rather than comprehensive immune pathway evaluation. Recent MR work by Zhang et al.[39] investigated inflammatory factors and KC, but was limited to Finnish populations, covered only 41 inflammatory proteins, and lacked LDSC analysis to exclude potential false positives. By contrast, our study integrated large biogenetic databases containing over 2,800 KC patients, systematically evaluated 91 inflammatory proteins and 731 immune cell phenotypes, and performed rigorous LDSC checks and MVMR methods. These methodological innovations enabled a more comprehensive and causally informative assessment of KC's immunopathological mechanisms. However, despite our findings, this study has several limitations. First, we relied on datasets with potential heterogeneity in sample quality control, population selection, and tissue sources, possibly introducing errors in IV selection. Second, using pooled data precluded stratification by factors such as age or sex. Third, our analysis was restricted to European populations, which may limit the generalizability of findings to other ancestries. Furthermore, despite FDR adjustments and sensitivity analyses, the sample size could still result in false-positive outcomes. Further validation through extensive experimental research is essential. Nevertheless, these discoveries enhance our understanding of KC's pathogenesis and offer fresh perspectives for identifying potential genetic biomarkers and developing prevention and treatment strategies.

Conclusion

This study emphasizes the critical role of inflammation and immune system imbalance in the development and progression of KC, deepening our understanding of its pathogenesis. By identifying key inflammatory and immune pathways, our findings provide new insights into potential intervention strategies.

Materials and methods

Study design

Figure 5 comprehensively illustrates the design of this study and the entire research process. In the initial analysis, we utilized bidirectional MR to investigate associations between immune cells, inflammatory protein factors and KC, screening for phenotypes associated with this corneal disorder. Subsequently, an independent secondary endpoint was utilized for validation, with the results tested for robustness and false positives excluded using LDSC and sensitivity analyses. Finally, positive outcome effects were thoroughly evaluated via meta-analysis. Further corrective analyses were conducted using multivariate MR (MVMR) to determine whether these outcomes independently influenced the development of KC. This research followed the three core assumptions of MR: (1) there is a notable relationship between the IV and the exposure; (2) the IV eliminates the impact of possible confounding variables and (3) the IV has no significant association with outcome[40]. Data were collected from publicly available databases, which removed the necessity for additional ethical approval.

Data sources

The immune cell data accessible to the public from Valeria et al.'s research encompassed 731 distinct immunophenotypes. This collection comprises 118 counts of cells, 192 relative cell counts, 32 parameters concerning cell morphology, and 389 median fluorescence intensities pertaining to surface antigens, with the latter indicating ratios among various cell types[41]. Data summarizing circulating inflammatory factors

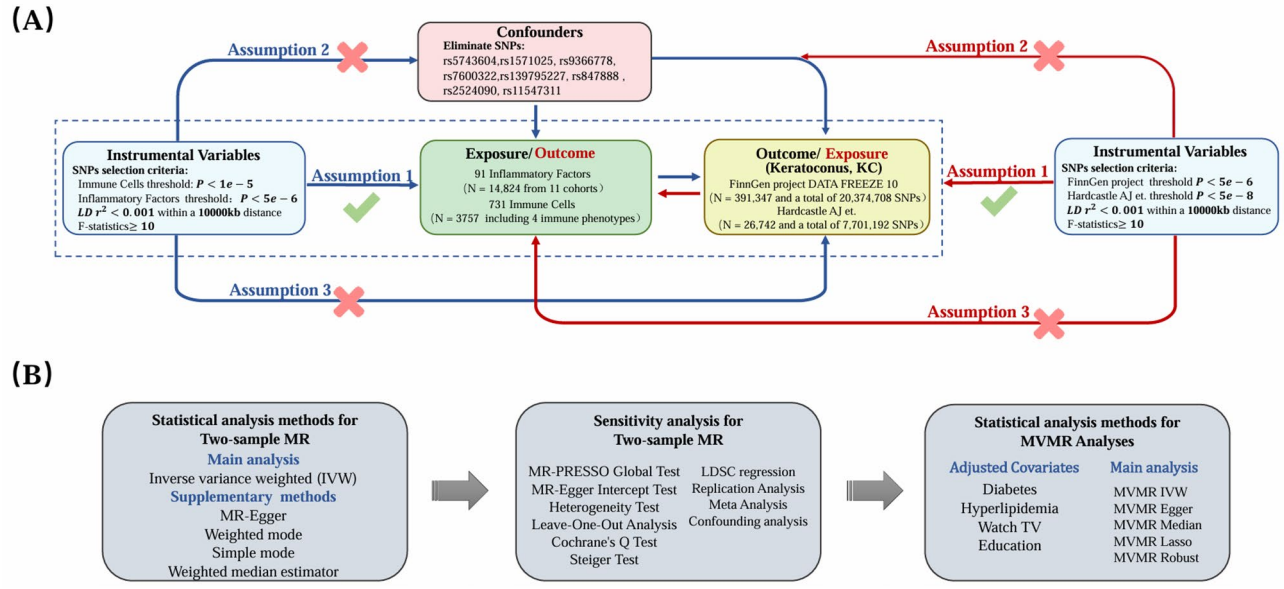


Fig. 5. Overall study design to explore associations among inflammatory factors, immune phenotypes, and genetic associations with KC. (**A**) Illustration of the foundational assumptions of Mendelian randomization. (**B**) Flowchart of the methodology used to guide this MR study.

originated from a comprehensive meta-analysis recently published, which included 14,824 participants from Europe across 11 different cohorts and investigated 91 plasma factors associated with inflammation[42].KC data were sourced from two distinct databases, with the initial analysis conducted using the FinnGen project, followed by validation in an independent cohort[43]. The FinnGen project data were derived from the FREEZE 10, which is specifically designed for a European target population, with disease classifications based on ICD-10 criteria[44]. The study encompassed 391,347 participants and 20,374,708 SNPs, while factoring in age, sex, genetic correlations, and primary components of genotyping batches. The validation dataset for KC GWAS summary statistics was obtained from a recent meta-analysis study by Hardcastle AJ, which included 2,116 European KC patients and 24,626 healthy controls[45]. Pooled GWAS data for all adjusted covariates were sourced from the UK Biobank. Detailed information on the phenotypes is provided in Supplementary Table S17.

Instrumental variable selection

According to previous studies, genome-wide significance levels ($P<1\times10^{-5}$ for immune cells and $P<5\times10^{-6}$ for inflammatory proteins) were employed as screening criteria for global IVs to ensure an adequate number of IVs for subsequent analysis[46–48]. LD was eliminated using European 1000 Genomes ($R^2<0.001$, 10,000 kb window)[48,49]. Echo sequences and allele-incongruent SNPs were removed, and exposure and outcome SNPs were harmonized. To meet the third Mendelian assumption, SNPs exhibiting a strong correlation with the outcome were excluded[50]. IV strength was evaluated using F statistics with a threshold of 10 to minimize potential bias[51]. The LDlink platform was utilized to validate SNPs and eliminate potentially confounding variants that might have independent causal associations with the outcome[52].

For reverse MR analysis, KC-associated SNPs were selected as instrumental variables using genome-wide significance thresholds of $P<5\times10^{-6}$ for the discovery cohort and $P<5\times10^{-8}$ for the replication cohort to ensure adequate statistical power. The same LD clumping parameters ($R^2<0.001$, 10,000 kb window) and harmonization procedures were applied as described above. All selected KC SNPs demonstrated F-statistics > 10, confirming sufficient instrument strength for reverse causality assessment.

Statistical analysis

Univariable MR analyses

We performed two-sample MR using IVW as primary method, with four additional approaches for validation[53]. Results were considered reliable if consistent trends were observed across all methods. Multiple comparisons were adjusted using FDR[54]. FDR-adjusted *P values* (P_{FDR}) below 0.05 were considered to indicate statistical significance. Conversely, *P values* less than 0.05 that corresponded to P_{FDR} exceeding 0.05 were interpreted as suggestive associations.

Sensitivity analysis

Various sensitivity testing approaches were implemented to evaluate heterogeneity and pinpoint possible outliers in the initial results. We utilized IVW analysis as our primary method due to its statistical efficiency when all genetic variants are valid instruments. MR–Egger regression was employed both to evaluate heterogeneity and to assess potential directional pleiotropy, while the Cochrane Q statistic provided a quantitative assessment of heterogeneity across individual SNP effects. The MR-PRESSO technique was chosen specifically to detect and

correct for horizontal pleiotropy by identifying and removing outliers. Individual SNP effects were evaluated using LOO analysis. MR Steiger directional test was implemented to confirm the causal direction, ensuring that IVs were primarily associated with exposure rather than outcome, which is critical for valid causal inference[55–57].

Genetic correlation analysis

LDSC is a validated method for estimating heritability and genetic correlation components and is widely employed in the analysis of complex diseases[58]. This method offers a sophisticated means of examining genetic correlations in complex diseases or phenotypic traits by utilizing summary-level data from GWAS. Genome-wide genetic correlations (RGs) measure the average genetic effect shared between two traits, independent of environmental confounders[59].

Reverse MR and meta-analysis

Reverse MR analysis was conducted using KC-associated SNPs as instrumental variables, following the same rigorous criteria as forward analysis. Meta-analysis facilitated the assessment of the combined effects of results from both the initial and replication analyses. Through an examination of existing literature and taking into account possible variations stemming from ethnicity and the size of the samples, we performed a meta-analysis of MR findings related to potential outcomes across both groups to assess whether distinguishing population factors impacted significant effect trends[60].

Multivariable MR analyses

Epidemiologic analyses have demonstrated that KC is influenced by genetic factors, allergic diseases, and other variables. In this study, despite eliminating potentially confounding SNPs during the IV screening stage and conducting rigorous sensitivity analyses, the expression of inflammatory proteins and immune cells remained subject to multiple influences. We employed the MVMR method to examine characteristics such as diabetes status, hyperlipidemia status, television viewing status, and education level, all of which have been reported as KC risk factors. In brief, MVMR estimates the independent effects of individual risk factors by incorporating the genetic variants of each risk factor into a unified model[61]. In our study, we linked candidate inflammatory factors and immune phenotypes with each KC risk factor, constructed MVMR analysis models, and evaluated whether inflammatory factors and immune phenotypes remained independently associated with KC when these risk factors were accounted for. Five MVMR methods were applied to evaluate independent causal effects[62]. The findings were considered strong when at least one of these techniques demonstrated significant evidence of causality.

Data availability

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

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Author contributions
J.L.C. and X.L.Q. conceptualized the study. J.L.C. performed data collection and analysis. Y.N.J. assisted with figure preparation. J.L.C. drafted the manuscript. Y.N.J. and X.L.Q. reviewed and revised the manuscript. X.L.Q. supervised the research.

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Declarations

Competing interests
The authors declare no competing interests.

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