Dermatology Reports

https://www.pagepress.org/journals/index.php/dr/index

eISSN 2036-7406

Please cite this article as: Zou J, Nov P, Du K. ***Mendelian randomization analysis of the causal relationship between immune cells and keloid.*** *Dermatol Rep 2024 [Epub Ahead of Print] doi: 10.4081/dr.2024.10106*

Submitted 31 July 2024 – Accepted 28 September 2024

Mendelian randomization analysis of the causal relationship between immune cells and keloid

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Key words: Mendelian randomization, keloid, immune cells, causality, myeloid cells

Ethics approval and consent to participate: not applicable.

Consent for publication: written informed consent for publication was obtained from all participants.

Availability of data and material: the datasets used and analyzed during the current study are available in the Genome-Wide Association Study (GWAS) dataset (https://gwas.mrcieu.ac.uk/datasets/ebi-a-GCST90018874/).

Competing interests: not applicable.

Funding: this work was supported by the 2024 Basic Research "nursery" Project of the Dermatology Hospital of Southern Medical University [no. BR202401].

Contributions: Conceptualization: J. Z., P. N., K. D.; Methodology: P. N.; Formal analysis and investigation: J. Z., P. N.; Writing - original draft preparation: J. Z., P. N.; Writing - review and editing: K. D.; Supervision: J. Z., K. D.

Acknowledgements: not applicable.

Abstract

Immune cells play complex roles in the formation of keloid. We aimed to investigate the causal relationship between immune cells and keloid and provide genetic evidence for the association between immune cells and keloid risk. Based on data from GWAS, we performed a comprehensive two-sample Mendelian Randomization (MR) analysis of 731 immune cell traits in 481,912 keloid cases. We used Inverse-Variance Weighted (IVW) method as the primary analysis. Then, a comprehensive sensitivity analysis was adopted to verify the results' robustness, heterogeneity, and horizontal pleiotropy. Finally, reverse MR analysis was performed. The IVW method in forward MR analysis showed that CD66b++ myeloid cell AC was negatively associated with keloid risk (OR < 1, P < 0.05). Consistently, reverse MR analysis showed keloid risk was negatively associated with CD66b++ myeloid cell AC (OR = 0.85, P = 0.012). No significant horizontal pleiotropy or heterogeneity was observed. The results of MR analysis demonstrate a bidirectional causal association between CD66b++ myeloid cell AC and keloid formation, suggesting CD66b++ myeloid cell AC is a protective factor against keloid.

Introduction

Keloids, overgrowths of scar tissue that develop at the site of a skin injury, have a variable incidence rate that is influenced by several factors, including genetic predisposition, skin type, and the location and nature of the injury.[1] Incidence rates are particularly higher among certain ethnic groups, with individuals of African, Asian, and Hispanic descent being more predisposed. Statistics show that keloid can affect between 5% to 16% of these populations.[2,3] The scar tissue is often itchy, painful, and can restrict movement if located near a joint. In addition to physical discomfort, keloid can also have a detrimental effect on an individual's psychological well-being.[4,5] The main treatment options currently available for keloid include surgical removal, laser therapy, steroid injections, cryotherapy, radiation therapy, pressure treatment and silicone gel sheeting. However, keloids tend to recur after these interventions and none of these modalities are uniformly effective.[6-8] Therefore, the research on the pathogenesis of keloid has profound clinical implications and may unlock new therapeutic directions for managing disfiguring, disabling and therapy-resistant scars.

Recent studies have shed new light on the involvement of immune cells and inflammation in the pathogenesis of keloid. It is now recognized that the post-injury immune response plays a key role in orchestrating the fibrotic process during scar formation[9,10]. Macrophages are implicated as major drivers of inflammation and fibrosis in scars through the release of profibrotic mediators like TGF-β1 and PDGF. M2 macrophages, in particular, accumulate in hypertrophic scars and stimulate collagen synthesis by fibroblasts.[11,12] Mast cells also populate scars and contribute to fibrosis by releasing pro-fibrotic cytokines when activated.[13] Additionally, lymphocytes may modulate the phenotype of other scar-resident cells via cytokine signaling. Altered inflammatory cell profiles and cytokine milieu thus help sustain the fibrotic microenvironment in hypertrophic scars.[14,15] These findings highlight

targeting aberrant immune responses and inflammation as a promising therapeutic approach for pathological scarring. More research is warranted to fully elucidate the immunological mechanisms in hypertrophic scarring and develop novel immunomodulatory therapies for improved clinical outcomes.

Mendelian randomization analysis is emerging as a useful approach to assess causal relationships between exposures and outcomes.[16,17] Applying this technique to analyze immune cells in keloid can provide pivotal insights into the pathogenic roles of various inflammatory mediators. The presence of single nucleotide polymorphisms that randomly assign levels of cytokines, immune cell subsets and other immunological factors can be leveraged to gauge their causal effects on keloid development in an unbiased manner.[18] A Mendelian randomization study design minimizes confounding and overcomes limitations of observational studies. Assessing whether genetic determinants of heightened inflammation are associated with abnormal healing risk can clarify if altered inflammatory responses play a causal role in this disorder. This knowledge can help identify and prioritize molecular immunological pathways and cell types that contribute to pathological scarring. In turn, these can serve as targets for developing novel immunomodulatory drugs and biologics to improve treatment outcomes. Therefore, harnessing Mendelian randomization analysis to probe the pathogenic immunological mechanisms of keloid can have tremendous value in guiding therapeutic strategies to address this challenging clinical problem.

This study investigated the relationship between immune cells and keloid formation using Genome-Wide Association Study (GWAS) data and two-sample Mendelian randomization analysis. Analyzing 731 immune cell traits in 481,912 keloid cases, the results showed a bidirectional causal relationship, with CD66b++ myeloid cell AC acting as a protective factor against keloid formation. This study offers new insights into the pathogenesis of keloids.

Materials and Methods

Study design

We conducted a two-sample Mendelian randomization analysis to assess the causal relationship between 731 immune cell traits (divided into 7 groups) and keloid (Figure 1). Mendelian randomization analysis uses genetic variation as instrumental variables for causal inference, and the key is to select suitable instrumental variables. The instrumental variables selected in this study need to meet the following three points: i) the genetic variation is directly related to immune cell traits; ii) the genetic variation is unrelated to potential confounding factors between immune cells and keloid; iii) the genetic variation does not affect keloid through pathways other than immune cell traits.

GWAS data sources for keloid

A Genome-Wide Association Study (GWAS) is a research method used to identify genetic variants across the genome that are associated with specific diseases or traits. Genetic summary statistics for keloid were extracted from a GWAS dataset with the

accession number ebi-a-GCST90018874 (Genome-wide association study (https://gwas.mrcieu.ac.uk/datasets/ebi-a-GCST90018874/). This study conducted a GWAS involving a large cohort of 481,912 individuals of European descent, consisting of 668 cases and 481,244 controls. After rigorous quality control measures and imputation, the analysis encompassed approximately 24,197,210 genetic variants.

Immunity-wide GWAS data sources

Summary statistics for GWAS about various immune traits are publicly accessible via the GWAS Catalog, with accession numbers spanning from GCST0001391 to GCST0002121.[18] These statistics encompass a total of 731 immunophenotypes, categorized into the following groups: Absolute Cell (AC) counts (n = 118); Median Fluorescence Intensities (MFI); representing surface antigen levels (n = 389); Morphological Parameters (MP) (n = 32); Relative Cell (RC) counts (n = 192). The AC, MFI, and RC categories encompass a range of immune cell types, including B cells, CDCs, mature T cell stages, monocytes, myeloid cells, TBNK (T cells, B cells, natural killer cells), and Treg (regulatory T cells) panels. Meanwhile, the MP category includes panels specific to CDC and TBNK. The original GWAS for immune traits involved 3,757 individuals of European descent, and it's important to note that there was no overlap in the cohorts used. Genotyping included approximately 22 million Single Nucleotide Polymorphisms (SNPs) and was conducted using high-density arrays. Imputation of genotypes was performed with a reference panel based on Sardinian sequence data.[19] Associations were evaluated after adjusting for covariates, such as sex, age, and age squared (age2).

Selection of Instrumental Variables (IVs)

Based on recent research, the significance threshold for IVs associated with each immune trait was established at $1\times10-5$.[18-20] To obtain individual IVs, we conducted clustering based on the Linkage Disequilibrium (LD) reference panel from the 1000 Genomes Project (with $R2 < 0.001$ at a distance of 1,000 kb). Given the relatively small scale of the Genome-Wide Association Study (GWAS) for immune cells, we employed a p-value cut-off of 5 × 10-8 and a more lenient clustering threshold ($R2 < 0.1$ at a distance of 500 kb).[21] Moreover, to prevent biases from weak instruments, we considered IVs with F-statistics greater than 10 as robust instruments and retained them for further analyses. We coordinated the exposure and outcome SNPs to ensure consistent estimation of effects for the same effect alleles. Alleles were excluded if they had intermediate effect allele frequencies (EAFs 0.42) or if there were allele-incompatible SNPs.

Statistical analysis

Analyses were conducted in R 4.3.1 to evaluate potential causal links between 731 immunophenotypes and keloid formation. Inverse variance weighting (IVW),[22] median-based weighting,[23] and mode-based weighting[24] were implemented primarily through the 'TwoSampleMR' package[25] to assess these associations. Heterogeneity

was examined via Cochran's Q tests. Random-effects IVW replaced fixed-effects IVW when heterogeneity was detected.[26] To exclude pleiotropic effects, we used MR-Egger regression, with a significant intercept indicating pleiotropy presence.[27] The MR Pleiotropy Residual Sum and Outlier (MR-PRESSO) method further excluded outliers exhibiting strong horizontal pleiotropy.[28] Visual inspection of scatter and funnel plots provided additional validation. The scatter plots showed outliers exerted minimal influence on findings. Meanwhile, funnel plots demonstrated correlation robustness and an absence of heterogeneity. Finally, we performed reverse Mendelian randomization analysis to examine causal relationships in the opposite direction, in order to validate and complement traditional methods and investigate the bi-directionality of causal associations.

Results

Exploration of the causal effect of immunophenotypes on keloid onset

We primarily used the IVW method to assess the potential causal associations between immune cells and keloid, and the results demonstrated that seven types of immune cells were related. The results of the IVW method showed that CD25 on CD39+ CD4 Treg (Treg panel) (OR = 1.17, 95% CI: 1.03-1.33, P = 0.014), CD19 on naive-mature B cell (B cell panel) (OR = 1.09, 95% CI: 1.02-1.16, P = 0.009), and CD19 on IgD+ (B cell panel) (OR = 1.08, 95% CI: 1.01-1.15, P = 0.035) were positively associated with the risk of keloid occurrence. Although several methods in the Mendelian randomization analysis did not achieve statistical significance, the OR values demonstrated consistent trends across all methods. Meanwhile, CD20 on IgD+ CD38br (B cell panel) (OR=0.89, 95%CI: 0.81-0.98, P=0.017), CD66b++ myeloid cell AC (Myeloid cell panel) (OR=0.88, 95%CI: 0.79-0.99, P=0.028), CD25 on unsw mem (B cell panel) (OR=0.93, 95%CI: 0.88-0.99, P=0.029), Activated & secreting Treg AC (Treg panel) (OR=0.95, 95%CI: 0.90-0.10, P=0.036) were negatively associated with keloid risk (Figure 2). The results from the other four methods were similar with OR values all greater than one, despite some methods not achieving statistical significance in P values. All SNPs were not weak instrumental variables. The causal effects of each genetic variation on keloid were depicted in Figure 3 and Figure S2. Additionally, the details of the sensitivity analyses demonstrated the robustness of the observed causal associations (Figure 3 and Figure S3). The scatter plots and funnel plots presented in Figure 3 and Figure S4, S5 provided additional support for the stability of these results across various analytical approaches. Furthermore, the causal effect estimates of peripheral immune cell count on keloid susceptibility are summarized in Figure S1. However, no obvious association was observed between basophil, white blood, monocyte, lymphocyte, eosinophil, neutrophil cell counts and keloid susceptibility.

Examining the reverse causal effect of keloid onset on immunophenotypes

We conducted reverse MR analysis to examine the possible reverse causal associations between the aforementioned seven immune cell phenotypes and keloid.

Using the IVW method, we only found one immune cell type that had a statistically significant association with keloid. For CD66b++ myeloid cell AC (Myeloid cell panel), a negative association was observed (OR=0.85, 95%CI: 0.74-0.96, P=0.012), which was consistent with the forward MR analysis results (Figure 4). In addition, the results calculated by other methods showed consistent trends, with OR values greater than one, although the P values of some methods did not reach statistical significance. Scatter plots and funnel plots were employed to assess the robustness and reliability of the results (Figure 5). These graphical representations confirmed the consistency and validity of the findings. The MR-Egger intercept test and Cochran's Q test did not indicate the presence of pleiotropy or heterogeneity.

Discussion

Keloids, characterized by excessive collagen deposition and genetic predisposition, offer a novel avenue for research through Mendelian analysis. By leveraging Mendelian randomization principles, researchers can investigate the causal relationship between genetic variants and keloid formation, minimizing confounding factors. This approach enhances our understanding of the genetic basis of keloids and identifies potential therapeutic targets. Utilizing Mendelian analysis for keloid research is innovative as it provides a robust framework for dissecting the genetic underpinnings of this condition, which may lead to more effective and personalized treatment strategies. Utilizing extensive genetic data publicly accessible, we investigated the causal links between 731 immune cell traits and keloid. To the best of our knowledge, this represents the inaugural MR analysis delving into the causal connection between diverse immunophenotypes and keloid. Within the scope of this study encompassing four categories of immune traits (MFI, RC, AC, and MP), it was observed that seven immunophenotypes demonstrated notably significant causal effects on keloid ($P < 0.05$), while keloid exhibited causal impacts on one immunophenotype ($P < 0.05$).

Our study revealed that the risk of keloid decreases with the increase in the proportion of CD66b++ myeloid cell AC (Antigen-Presenting Cell), and reverse MR analysis yielded consistent results. Myeloid cells include mononuclear cells (macrophages, dendritic cells and monocytes) and polymorphonuclear cells (mast cells, basophils, neutrophils and eosinophils), as well as immature myeloid progenitor cells from both lineages.[29] These cells play a crucial role in immune homeostasis and inflammation. CD66b, also known as CEACAM8 (CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 8), is a cell adhesion molecule primarily expressed on the surface of neutrophils. CD66b is commonly regarded as one of the markers on the surface of neutrophils, associated with the recognition and inflammatory processes involving neutrophils.[30] Neutrophil extracellular traps (NETs) are extracellular networks composed of DNA scaffolds decorated with granular components, histones, and cytoplasmic proteins, released by neutrophils as part of the immune response.[31,32] Some studies suggest that NETs promote fibrosis in congestive heart failures,[33] while NETs carrying IL-17 promote fibrosis in interstitial lung diseases.[34] Moreover, it has been suggested that REDD1-mediated NETs carrying bioactive TF and IL-17A can

activate and differentiate human skin fibroblasts to produce collagen.[35] In recent years, researchers have shed new light on the role of neutrophils in fibrotic diseases. Carolina Jimenez Calvente et al. have uncovered that neutrophils promote the spontaneous resolution of liver inflammation and fibrosis through microRNA-223. In their study, utilizing two models of liver inflammation resolution, it was found that mice with neutrophil depletion showed persistent liver inflammation, activated mechanisms of fibrogenesis, and early fibrosis.[36] In addition, Eiko Saijou et al. showed that neutrophils normally exacerbate inflammation in acute injury, but also show a protective effect against liver fibrosis in chronic injury, as the expression of MMP8 and MMP9 eliminates fibrosis. During the fibrosis resolution phase, exposure to neutralizing Ly6G antibodies leads to neutrophil depletion, which impinges on stromal degradation.[37] Similarly, the findings of Yi Wu et al. showed that targeting cIAP mitigated CCL4-induced liver fibrosis by increasing neutrophil-derived MMP9 expression.[38] So far, there are limited studies on the role of CD66b++ myeloid cell AC in keloid. Therefore, the function and specific mechanism of these cells in keloid remain to be further studied.

Studies have shown an increase in the number of Treg cells in keloid lesions.[39,40] Our results indicated that the risk of keloid formation decreased with an increase in the proportion of Activated & secreting Treg AC cells. However, the risk increased with the rise in the proportion of CD25 on CD39+ CD4 Treg cells. These results suggested that Treg cell subtypes may exert entirely different functions, and the dynamic balance between different subtypes plays a crucial role in the occurrence and development of keloid. In chronic inflammatory skin diseases, when IL-15 is present, Treg cells proliferate upon contact with dermal fibroblasts.[41] It is currently unclear whether the apparent excess of local Treg cells is pathogenic or simply represents a response to inflammation. do Valle Duraes *et al.* revealed the protective role of Treg cells in the process of kidney injury and fibrosis through single-cell RNA sequencing.[42] However, Glaubitz and colleagues' research has identified Treg cells as crucial regulators of the type II immune response and organ remodeling during chronic pancreatitis. The Treg/Th2 axis may serve as a therapeutic target for preventing fibrosis and protecting functional pancreatic tissue.[43] Additionally, the role of Treg cells in idiopathic pulmonary fibrosis remains contradictory and poorly defined. Some studies have found an increased quantity of peripheral blood Tregs in these patients and is positively correlated with disease severity.[44-46] In the mouse model of idiopathic pulmonary fibrosis, it has been observed that Tregs are recruited to the lung tissue.[47,48] On the contrary, some studies have observed a decrease in the quantity of Treg cells in the peripheral blood and bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis. This reduction is associated with diminished inhibitory function and correlates with the severity of the disease.[49,50] TGF-b1 and IL-10 are key cytokines secreted by Tregs, exerting autocrine functions. The former mediates the processing of matrix proteins and stimulates mast cells to produce IL-6, while the latter downregulates pro-inflammatory macrophages and promotes B cell activation and immunoglobulin secretion.[51] Interestingly, IL-10 antagonizes the effect of TGF-b1 on keloid fibroblasts.[10] IL-10 can downregulate the

synthesis of type I collagen in fibroblasts derived from human scar tissue and prevent bleomycin-induced pulmonary fibrosis.[52,53] Therefore, further research is needed to determine the role of different Treg subtypes in keloid formation.

Numerous studies have shown that B cells play a key role in various systemic autoimmune diseases, and our results show that the risk of keloid development increases with the proportion of CD19 on naive-mature B cells and CD19 on IgD+ cells. CD19 is a central regulatory factor in B cell signal transduction and has been demonstrated to be associated with the occurrence of fibrotic diseases. B cells from patients with systemic sclerosis exhibit increased expression of CD19, leading to the induction of specific autoantibodies in transgenic mice. Furthermore, the absence of CD19 inhibits the high reactivity of chronic B cells and eliminates the production of autoantibodies, which is associated with the improvement of skin fibrosis.[54] In TSK/+ mice, chronic B cell activation induced by enhanced CD19 signaling may lead to skin sclerosis through excessive production of IL-6 and autoimmune reactions.[55] Liang Yong *et al.* found that after infection by Schistosoma japonicum, the secretion of IL-10 from liver B1 cells increased, which inhibited the infiltration of Ly6Chi monocytes into the liver, thus alleviating early liver inflammation and late fibrosis.[56] Current research indicates that eliminating B cells in systemic sclerosis patients with rituximab can reduce skin fibrosis.[57,58] However, we found that the risk of keloid development decreased as the proportion of CD20on IgD+ CD38br and CD25on unsw mem cells increased. Studies of these two subtypes of B cells are limited, and what role they play in keloid development remains to be clarified. Regulatory B cells (Breg) are a relatively newly recognized subset of B cells with immunomodulatory functions. They can inhibit the inflammatory immune responses and prevent autoimmune reactions.[59,60] Among them, Bregs that produce IL-10 are labeled as B10 cells.[61] Chen *et al.* 's study found that B10 cells play an anti-fibrotic role during heart injury by regulating extracellular matrix components, and also highlighted that B10 cells may be a promising therapeutic candidate for treating cardiac fibrosis-related diseases.[62] Bregs do not have specific surface markers. As research progresses, more and more subtypes are being identified as B10. These B cell subtypes identified as B10 include: CD19(+)CD24(high)CD38(high), CD19(+)CD24highCD27(+), CD25(high)CD71(high)CD73(-), CD19+CD1d(high)CD5(+), CD39(+)CD73(+) and CD25(high)CD27(high)CD86(high)CD1d(high)TGFβ(high).[61,63-67] Whether CD20 on IgD+ CD38br and CD25 on unsw mem belong to B10 cells needs further experimental identification.

In this research, a two-sample MR analysis was performed using data from extensive GWAS cohorts, with a sample size of approximately 480,000 individuals, ensuring a high level of statistical efficiency. The results rely on genetic instrumental variables, employing a range of MR analysis methods to draw causal inferences. The findings are robust and not susceptible to horizontal pleiotropy and other confounding factors. Nevertheless, our study still has limitations. Firstly, even with multiple sensitivity analyses, it is not possible to fully assess the level of horizontal pleiotropy. Secondly, due to a lack of individual-level information, we are unable to conduct further stratified analyses on the population. Thirdly, given that our study relies on

European databases, it is crucial to recognize that the conclusions may not be applicable to diverse ethnicities, thereby constraining the broader applicability of our results. Lastly, we employed a less stringent threshold to assess the results, which could potentially introduce some false positives. Nonetheless, this approach enables a more comprehensive evaluation of the strong correlation between immune features and keloid.

Conclusions

In summary, we have substantiated the causal relationship between various immune phenotypes and keloid by a comprehensive bidirectional MR analysis. This underscores the intricate patterns of interaction between the immune system and keloid. Additionally, our research has substantially reduced the impact of inevitable confounding factors, reverse causation and other variables. It may provide researchers with new avenues to explore the biological mechanisms of keloid formation, potentially leading to investigations into early intervention and treatment. Our findings expand the understanding of the immune microenvironment of keloid scars, offering valuable clues for the prevention of keloid formation.

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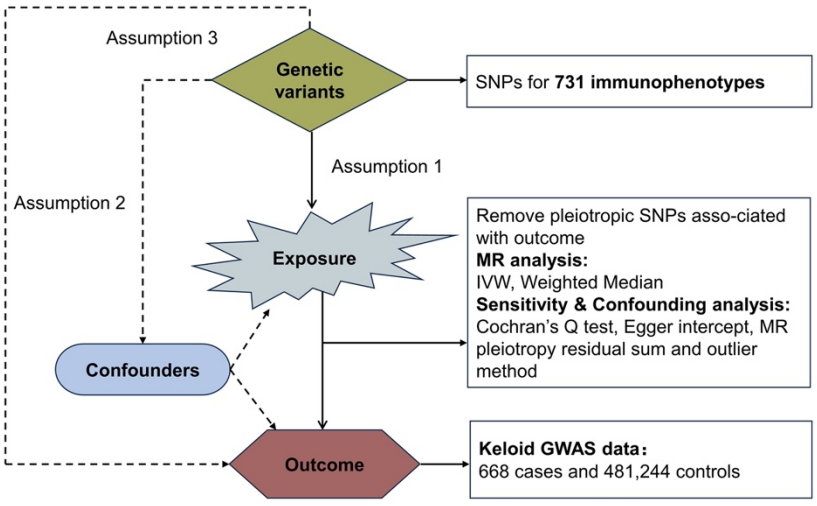

Figure 1. Overview of the overall Mendelian randomization analysis design.

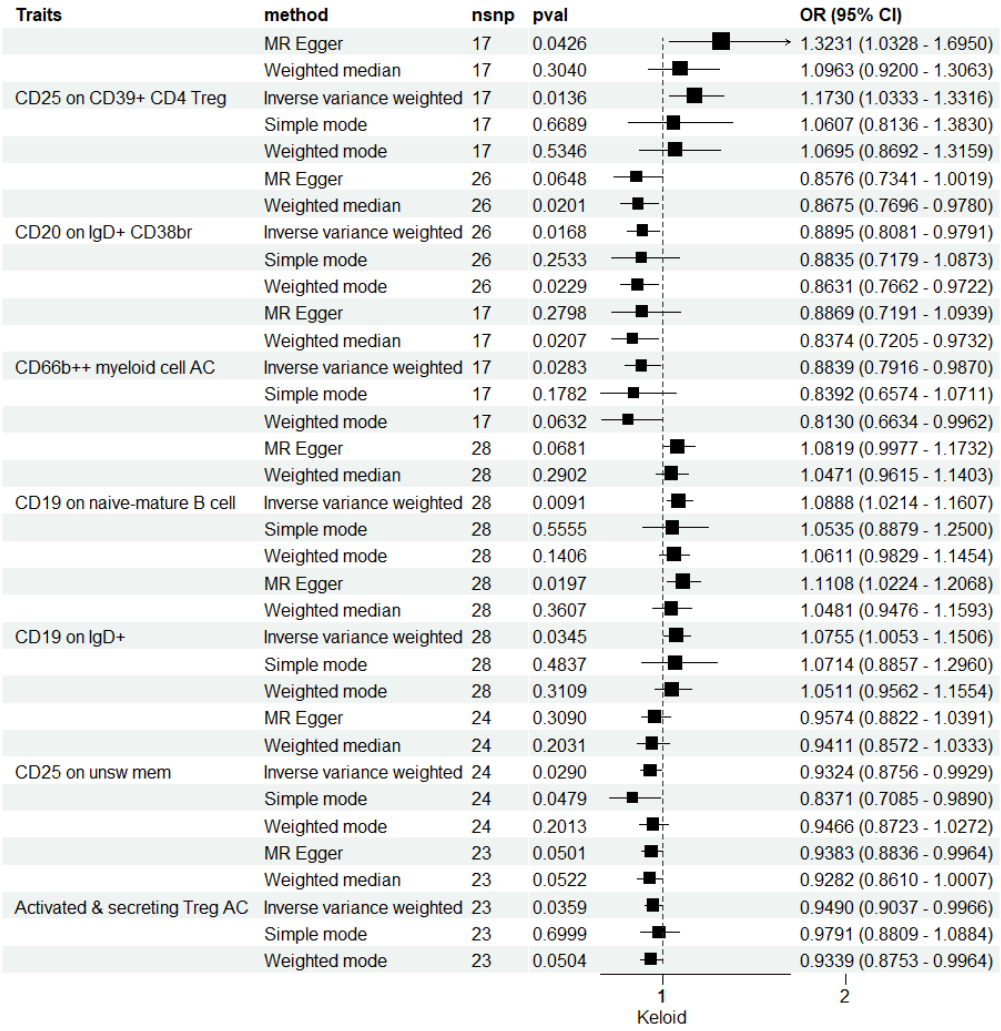

Figure 2. Forest plot for the causal effect of immune cell traits on the risk of keloid. IVW: Inverse Variance Weighting; CI: Confidence Interval.

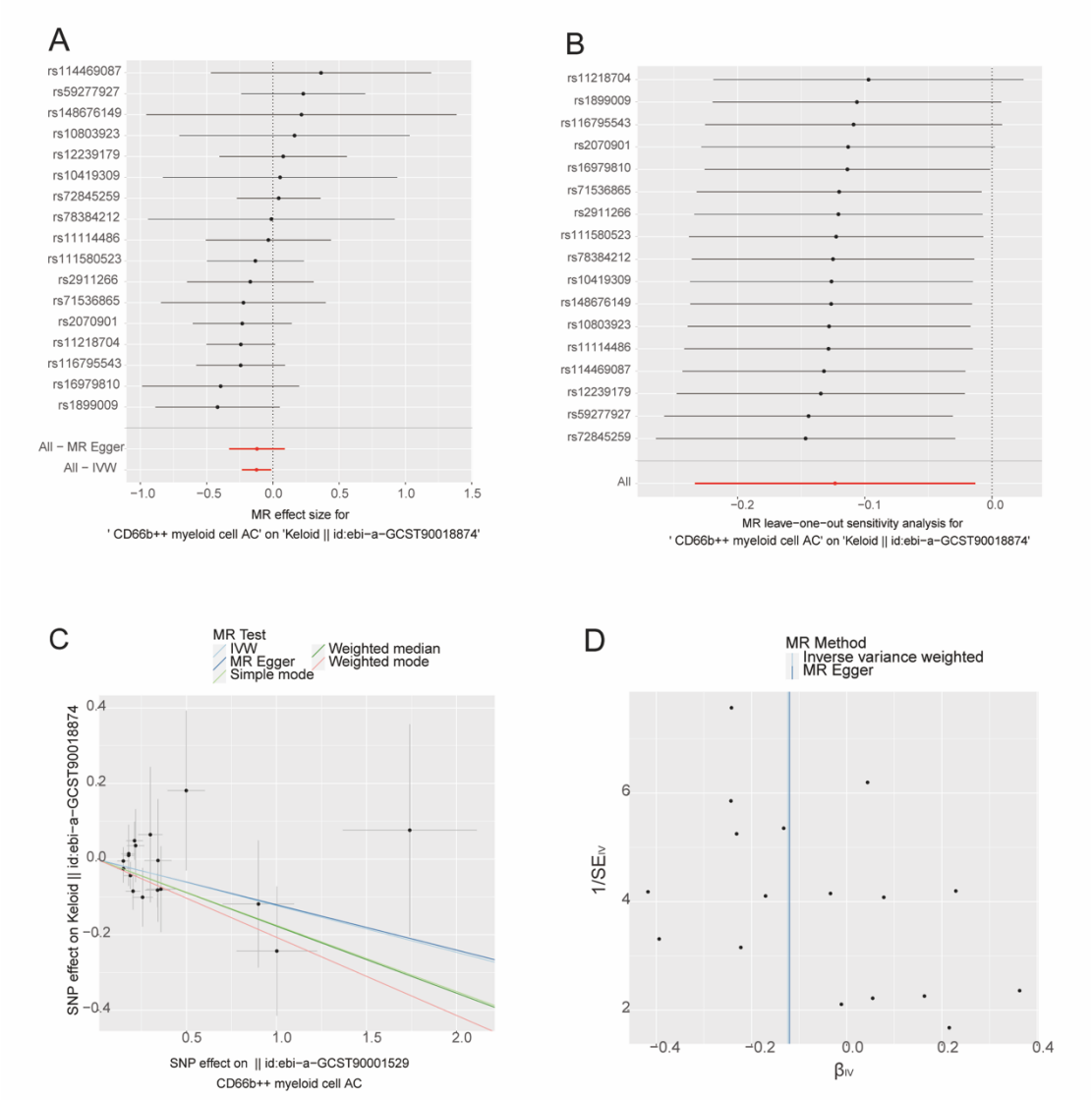

Figure 3. Robustness verification of the results. (A) Forest plot showing the causal effect of each SNP on the risk of keloid. (B) Leave-one-out plot to visualize causal effect of CD66b++ myeloid cell AC on keloid risk when leaving one SNP out. (C) Scatter plot showing the causal effect of CD66b++ myeloid cell AC on the risk of keloid. (D) Funnel plots to visualize the overall heterogeneity of MR estimates for the effect of CD66b++ myeloid cell AC on keloid.

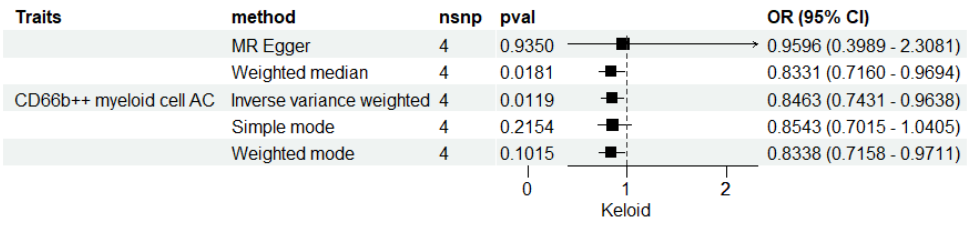

Figure 4. Forest plot for the causal effect of keloid on immune cell traits. IVW: Inverse Variance Weighting; CI: Confidence Interval.

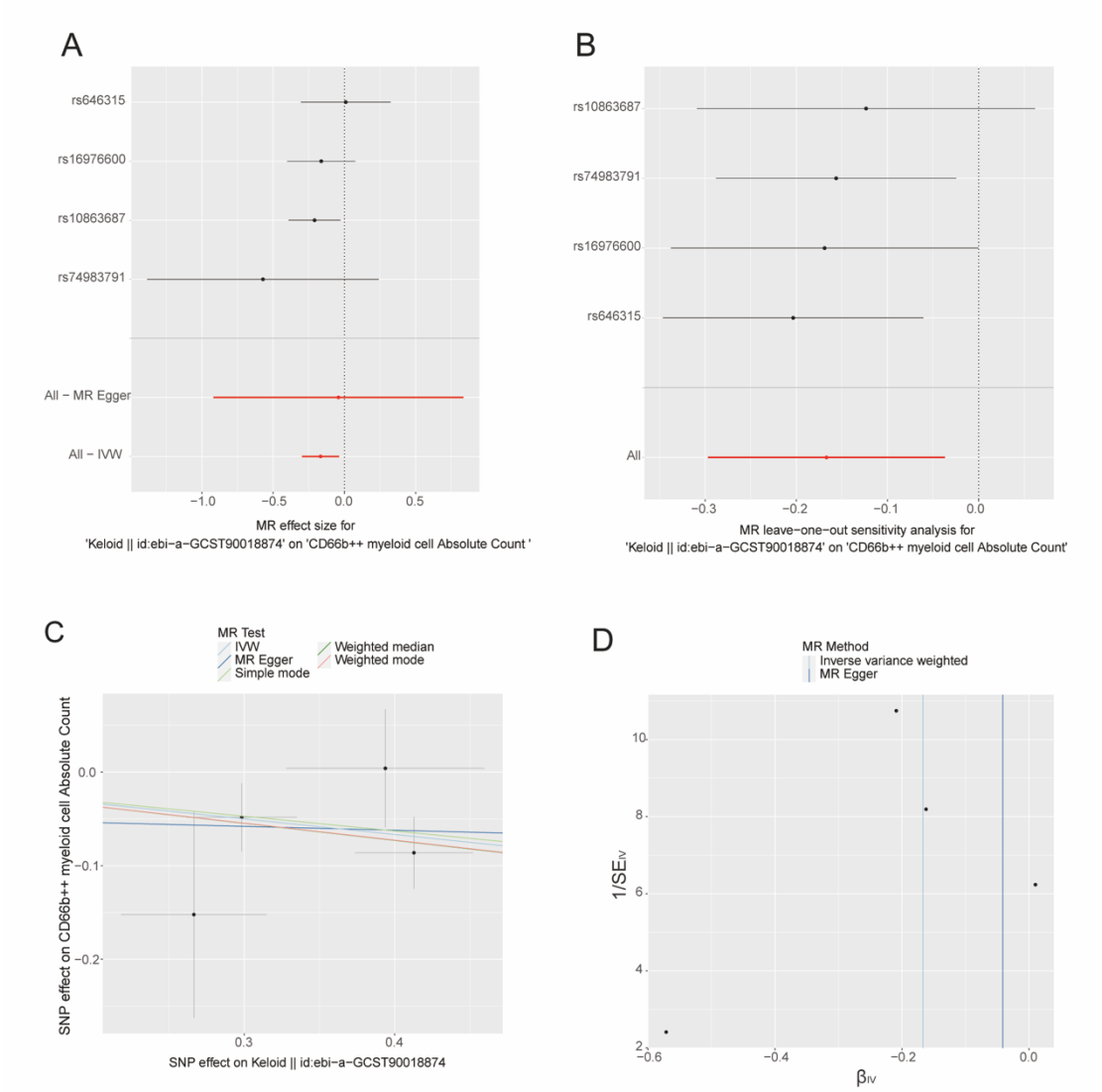

Figure 5. Robustness verification of the results. (A) Forest plot showing the causal effect of each SNP on CD66b++ myeloid cell AC. (B) Leave-one-out plot to visualize causal effect of keloid on CD66b++ myeloid cell AC when leaving one SNP out. (C) Scatter plot showing the causal effect of keloid on CD66b++ myeloid cell AC. (D) Funnel plots to visualize overall heterogeneity of MR estimates for the effect of keloid on CD66b++ myeloid cell AC.

Online Supplementary Materials

Figure S1. Forest plot for the causal effect of peripheral immune cells on the risk of keloid. IVW: Inverse Variance Weighting; CI: Confidence Interval.

Figure S2. Forest plot showing shows the causal relationship of each SNP in different immune cells traits to keloid risk.

Figure S3. Leave-one-out plot to visualize causal effect of the six immune cell traits on keloid risk when leaving one SNP out.

Figure S4. Scatter plot showing the causal effect of the six immune cell traits on the risk of keloid.

Figure S5. Funnel plots to visualize overall heterogeneity of MR estimates for the effect of the six immune cell traits on keloid.