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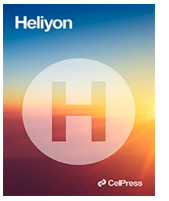

The peripheral immune cell counts and mouth ulcers: A two-sample Mendelian randomization study

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ABSTRACT

Objective: This study explored the causal association of peripheral immune cell counts with mouth ulcers (MUs) by two-sample Mendelian Randomization.
Design: The counts of 12 circulating immune cell types (leukocytes, lymphocytes, monocytes, eosinophils, neutrophils, basophils, $CD4^+$ cells, $CD8^+$ cells, unswitched memory B cells, NK cells, B cells and a derived ratio (CD4+/CD8+)) were determined as the exposure. MUs were the outcome. The analysis was conducted mostly using the inverse-variance weighted (IVW) approach. MR Egger, weighted median, weighted mode and simple mode were used to detect the horizontal pleiotropy.
Results: The IVW results for leukocytes and lymphocyte counts were OR = 0.93, 95 % CI = 0.88–0.98, $p = 0.0115$ and OR = 0.91, 95 % CI: 0.84–0.98, $p = 0.0150$, respectively. The Wald ratio result for $CD4^+$ cell and $CD8^+$ cell counts were OR = 0.70, 95 % CI: 0.65–0.75, $p = 1.05 \times 10^{-20}$ and OR = 1.25, 95 % CI: 1.19–1.31, $p = 9.99 \times 10^{-21}$, respectively.
Conclusions: This study supports a causal effect of peripheral immune cell counts on MUs. Higher leukocyte, lymphocyte and $CD4^+$ cell counts can protect against MUs, but higher $CD8^+$ cell counts enhance the risk of MUs. This finding confirms host immune factors play a crucial role in the aetiology of MUs.

1. Introduction

As a highly prevalent mouth disease in the world, mouth ulcers [MUs, also known as oral ulcers, mainly including recurrent aphthous stomatitis (RAS)] occur on any locations of oral mucosa, including the buccal, lingual mucous epithelium and lamina propria [1]. The clinical manifestation of MU is round or oval ulcers with a red inflammatory halo. Mus also can result in a burning sensation and long-lasting pain in patients [2]. In addition, MUs also strongly affected the normal physiological functions of the mouth, such as speaking, swallowing and eating. Its characteristic recurrence severely affects patients' social interaction, personal quality of life, and psychological health due to fear of cancer [3–5]. In fact, MUs with long-term nonhealing issues may develop into oral cancer because of

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local stimulation, such as sharp tooth tips. Notably, the feature of MUs, especially recurrence, pain and impact on general health of the whole body, and have motivated numerous studies on the aetiology and treatment method. However, there is no uniform, effective and mature treatment method since the aetiology of MUs is not clear. Hence, it is necessary to elucidate the pathogenesis of MU to provide important information for clinical treatment and public oral health interventions.

It is well accepted that the aetiology of MUs is probably multifactorial, including foods, trauma, stress, gastrointestinal diseases, hormonal imbalance, smoking and so on [6]. Currently, a growing number of researchers support the theory that cellular immunity disorders are an important potential risk factor for MUs. For example, Th1 cytokine production is well acknowledged to be much higher in MUs patients. and the Th1 type immunologic response play crucial roles in the development of MUs [7–11]. Innate and adaptive (humoral and cellular) immune responses both may become disordered in MUs patients, including neutrophil reactivation and hyperreactivity, increased counts of lymphocytes types (including B cells, NK cells, CD25 and T-cell receptor γδ cells), increased complement component concentrations, reduced CD4+/CD8+ ratios in peripheral blood [12,13]. In addition, compared with young MUs' patients, the reduced occurrence in elderly MUs' patients may partially result from reductions in the chemotactic and phagocytic activity of neutrophils as well as the fraction of naive T cells [14,15]. These studies together lead to an assumption that immune cell disorders, especially in T cells, may activate MUs. However, the causal relationship between peripheral immune cells and MUs remains unclear.

Mendelian randomization (MR) was utilized to establish the causal inference between complex traits as the rapid and large-scale development of genome-wide association studies (GWAS). As we all known, the gold standard of determination causality is randomized controlled trials (RCTs). Besides RCTs, MR also is a statistical method to answering the causal relationships by the natural random genetic variants which occurs in an individual's genetic make-up. MR uses multiple single-nucleotide polymorphisms (SNPs) as instrumental variable to analyse the effect of one or multiple exposure on an or multiple outcome [16]. Because genetic variants (SNPs) are typically not associated with confounders, the analytical results of MR are more reliable compared with traditional observational studies, which are influenced by reverse causation or multiple confounding factors [17]. Based on the aforementioned benefits, this study conducted a two-sample MR to explore the possible risk of mouth ulcers by peripheral immune cell counts. These findings will become a significant source of knowledge for deciphering pathogenic pathways and locating potential novel MUs prevention and clinical treatment approaches.

2. Methods

2.1. Study design

The principle of the two-sample MR study depended on three assumptions (Fig. 1): 1) relevance assumption: genetic instrumental variables (SNPs) are strongly associated with exposure (peripheral immune cell counts); 2) independence assumption: genetic instrumental variations are not influenced by confounders; and 3) exclusion-restriction assumption: instrumental SNPs influence the outcome (mouth ulcers) only through exposure (peripheral immune cell counts) and not via other pathways [18]. According to the

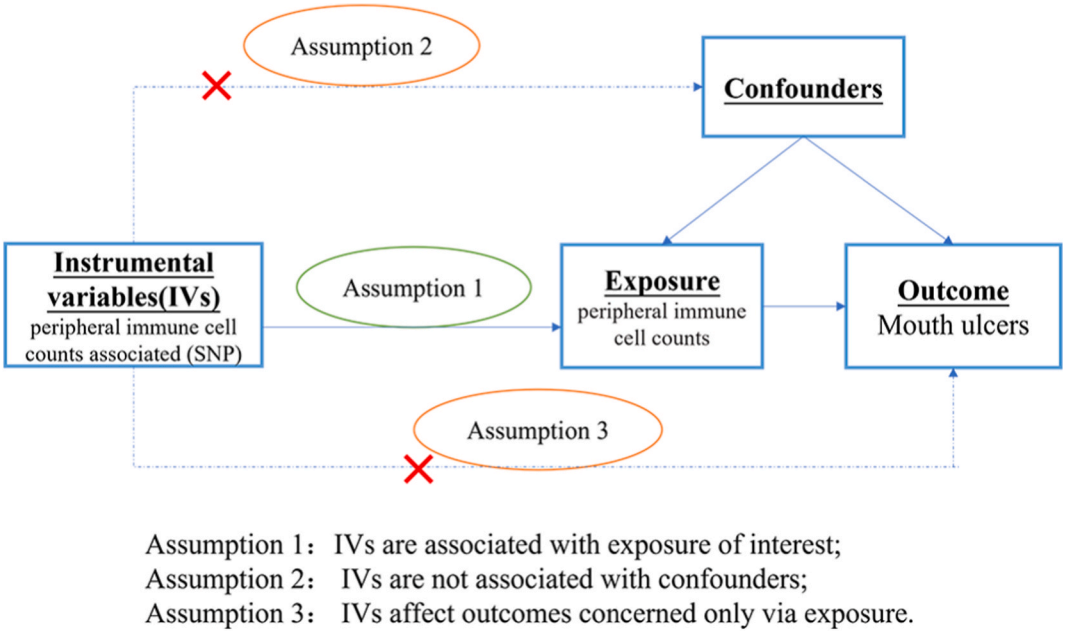

Fig. 1. Study design of the causal association of peripheral immune cell counts with mouth ulcers based on the three assumptions of Mendelian randomization (MR).

original GWAS methodology and report, all participants supplied explicit ethical review and informed consent for this study because it used publicly available summary statistics in the public study.

2.2. Data sources

In this study, for peripheral immune cell counts, white blood cell subgroup counts [including total white blood cells (WBCs), lymphocytes, monocytes, neutrophils, basophils, eosinophils] and lymphocyte subgroup cell counts [including $CD4^+$ cells, $CD8^+$ cells, B cells, unswitched memory B cells, NK cells and derived ratio (CD4+/CD8+)] were chosen to explore the causal association with MUs.

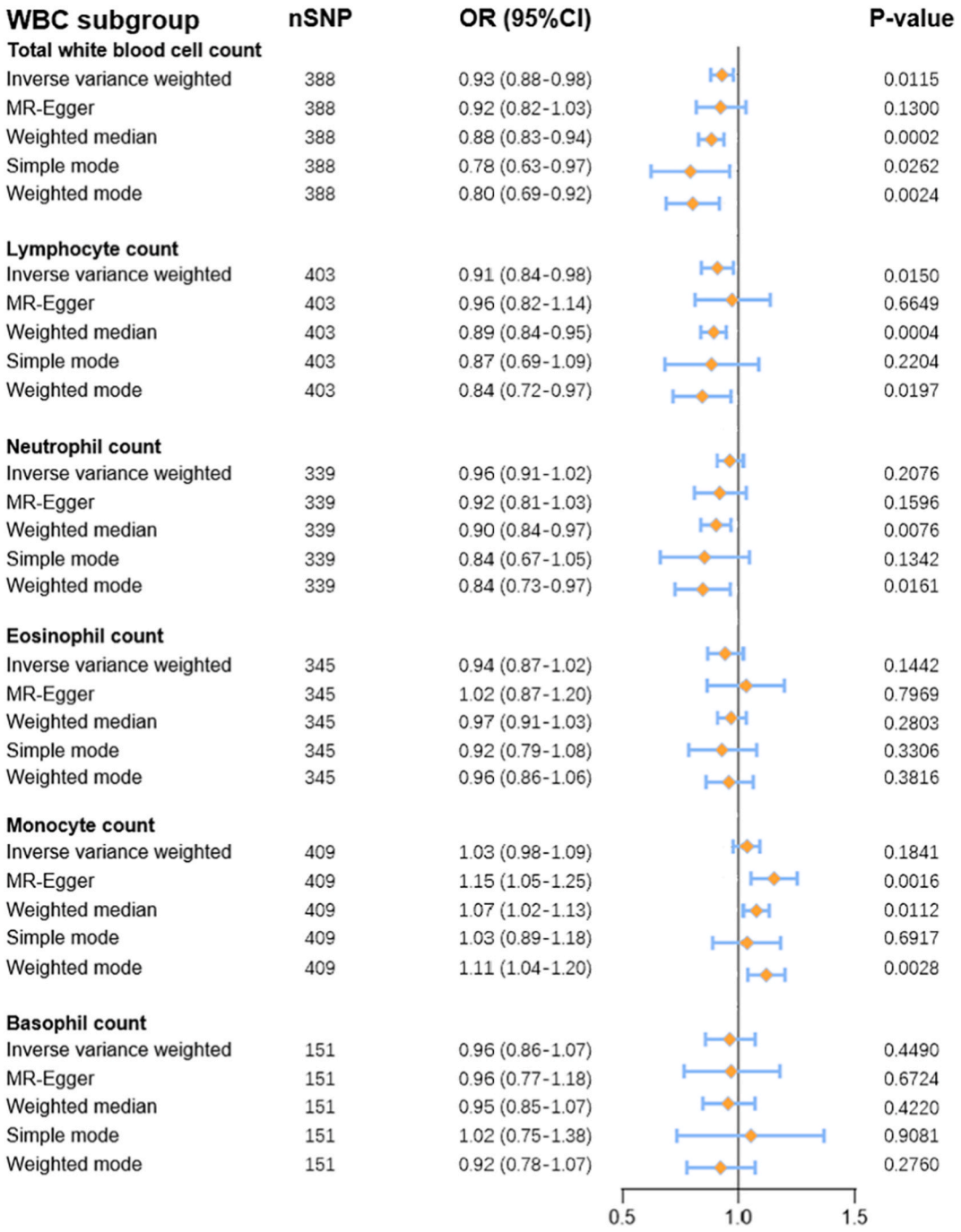

Fig. 2. The effect estimates of total white blood cells, lymphocytes, monocytes, eosinophils, neutrophils, and basophils on MUs by two-sample Mendelian randomization. nSNP, number of instrumental SNPs used to process MR analyses; OR, odds ratio; CI: confidence interval.

Among T cells, the CD4+/CD8+ ratio was also selected as a crucial immunological characteristic.

First, for WBC counts, we obtained the GWAS data from Vuckovic D et al. [19], who reported the power of a large-scale blood cell trait GWAS that included 563,085 European ancestry participants. These helpful data were downloaded from The Lettre Lab meta-analysis website (http://www.mhi-humangenetics.org/en/resources/). Second, for T-cell subsets, the GWAS data of $CD4^+$ and $CD8^+$ cells and their ratio (CD4+/CD8+) were from Ferreira M et al. [20], who measured the five-lymphocyte subset counts in 2538 individuals from the European population. Third, the genetic instrumental variables (SNPs, p value $< 5 \times 10^{-8}$) that were strongly associated with B-cell counts (B cells and unswitched memory B cells) were obtained from He D et al. [21], who used GWAS summary statistics with 3757 Sardinian individuals [22] and determined whether genetically predicted peripheral immune cell counts may have a causal effect on Multiple Sclerosis. Fourth, the SNPs strongly associated with NK cells (p value $< 5 \times 10^{-8}$) were obtained from Gong Z et al. [23], who also used the GWAS summary statistics with 3757 Sardinian individuals [22] and determined whether genetically predicted NK cell-related immune traits may have a causal effect on amyotrophic lateral sclerosis.

For MUs, the GWAS data were obtained from Jin Y et al. [24], who analysed the genome-wide characteristics of mouth ulcers from 36,831 European ancestry participants and 323,010 controls. These data were also recorded in the UK Biobank (https://www.ukbiobank.ac.uk). In this study, mouth ulcers in this database were defined that have appeared within the past year, including RAS or other mouth ulcers.

2.3. Statistical analyses

The MR analysis was conducted by the Two-Sample MR (version 0.5.4) package in R4.2.1 (R Foundation for Statistical Computing, Vienna, Austria) [25]. First, valid instrumental SNPs of 12 markers of circulating immune cell counts were restricted to those of genome-wide significant association ($p < 5 \times 10^{-8}$). Second, SNPs were eliminated which were in high linkage disequilibrium ($r^2 > 0.001$ and clump windows <10,000 kb) in order to guarantee independent variants used in this study. To fulfill two hypotheses of Mendelian, every SNPs was checked in PhenoScanner V2 for evidence of pleiotropy, and the SNPs related with putative confounders or outcome factors at genome-wide significance ($p < 5 \times 10^{-8}$) were eliminated [26]. Supplementary Tables 1–7 showed the detailed SNPs associated with peripheral immune cell counts. Third and most importantly, among many methods of two-sample MR analysis, inverse variance weighting (IVW) was used as a mainly calculated method to estimate causal association of peripheral immune cell counts with MUs. Under a multiplicative random effects mode, IVW is a method to meta-analysed individual Wald-type ratios of IVs. If the SNPs were less than 2, the Wald ratio was used instead of IVW. In addition, in order to evaluate potential pleiotropy and directional pleiotropy, the weighted median approach and MR Egger intercept test were cited in this study, respectively. To assess the sensitivity of MR, Cochran Q was used to test heterogeneity. Finally, the result was subjected to Bonferroni correction, and the significance level was set at $p < 0.05/6 = 0.0083$ in the scenario of multiple tests. Findings with p values between 0.05 and 0.0083 were considered suggestive evidence of causality.

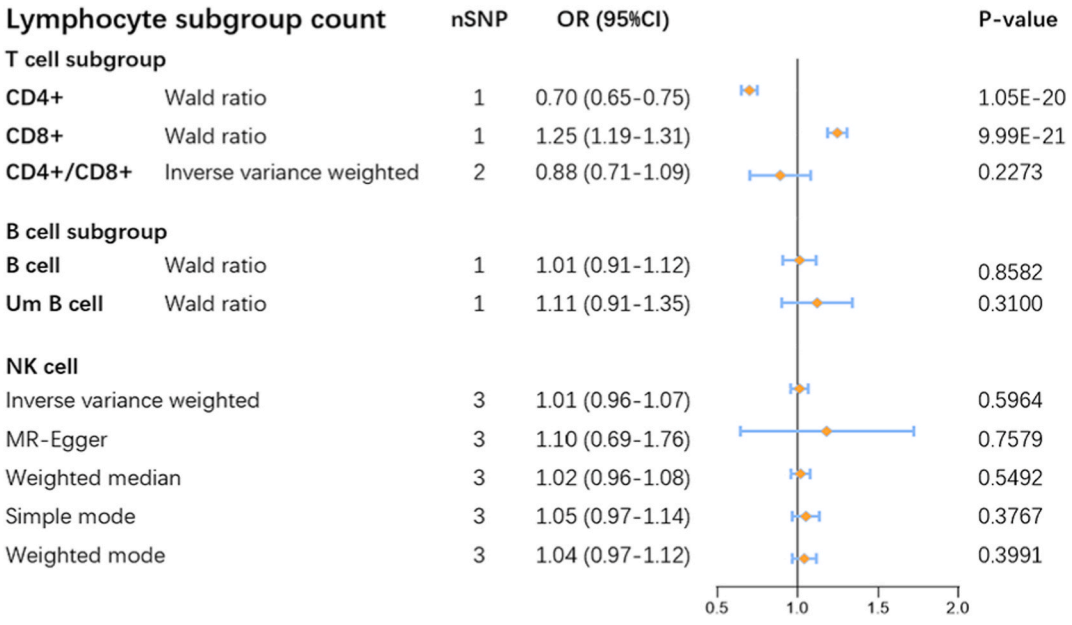

Fig. 3. The effect estimates of lymphocyte subgroups on MUs by two-sample Mendelian randomization. nSNP, number of instrumental SNPs used to process MR analyses; OR, odds ratio; CI: confidence interval. Um B cell, unswitched memory B cell.

3. Results

3.1. Mendelian randomization analyses of WBC subgroup cell counts on MUs

Fig. 2 summarizes the causal effect of WBC subgroup cell counts on MUs by MR methods. For the total WBC counts, the detailed data of IVW methods were odds ratio (OR) = 0.93, 95 % confidence interval (CI) = 0.88–0.98, $p = 0.0115$. After Bonferroni correction ($p < 0.05/6 = 0.008$), this *p value* between 0.05 and 0.008 indicated that total WBC counts were suggestive of decreased MU susceptibility. Similarly, by the IVW method, the corresponding effect size was OR = 0.91 (95 % CI: 0.84–0.98, $p = 0.0150$) for lymphocytes, which illustrated suggestive evidence for the protective effect of lymphocyte counts on MUs. In contrast, the other results showed no evidence of causal effects on MUs from the neutrophil counts (IVW: OR = 0.96, $p = 0.2076$), monocyte counts (IVW: OR = 1.03, $p = 0.1841$), basophil counts (IVW: OR = 0.96, $p = 0.4490$) or eosinophil counts (IVW: OR = 0.94, $p = 0.1442$). Using the Cochran Q test, we detected significant heterogeneity ($p < 0.05$). Hence, in order to estimate the MR effect size, the random effect model was applied [18].

3.2. Mendelian randomization analyses of lymphocyte subgroup cell counts on MUs

To determine which lymphocyte subgroup cell plays a vital role in the causal effect on MUs, this study next analysed the risk effect of the three major lymphocyte subpopulations, including B cells, T cells, and NK cells, on MUs by the same MR methods. In this study, only six lymphocyte markers ($CD4^+$ cells, $CD8^+$ cells, CD4+/CD8+ ratio, B cells, unswitched memory B cells and NK cells) were assessed because of the poor GWAS sample size. As shown in Fig. 3, after selecting the SNPs, there were only 1, 1, 2, 1, 1, and 3 SNPs strongly associated with $CD4^+$ cells, $CD8^+$ cells, the CD4+/CD8+ ratio, B cells, unswitched memory B cells and NK cells, respectively. Hence, the Wald ratio was used instead of IVW when there was only one strong IV. There was only IVW presented as a result when there were only two strong IVs. Among the T-cell subgroup, the Wald ratio result (OR = 0.70, 95 % CI: 0.65–0.75, $p = 1.05 \times 10^{-20}$) indicated that $CD4^+$ cell counts showed a negative and robust causal correlation tendency with MUs. In contrast, $CD8^+$ cell counts were strongly associated with an enhanced risk of MU susceptibility according to the Wald ratio results (OR = 1.25, 95 % CI: 1.19–1.31, $p = 9.99 \times 10^{-21}$). However, the CD4+/CD8+ ratio indicated no evidence of a causal relationship with MUs by the IVW method (OR = 0.87, 95 % CI: 0.71–1.09, $p = 0.2273$). Among the B-cell subgroups, neither B-cell counts ($p = 0.8582$) nor unswitched memory B-cell counts ($p = 0.3100$) had a causal effect on MUs. The same results were found in NK cell counts (IVW: $p = 0.5964$).

4. Discussion

Accumulating epidemiologic and observational evidence implicates peripheral immune cell counts in the risk of mouth ulcers. However, whether there is a causal effect of peripheral immune cell counts on MUs remains controversial because of the existence of reverse causation and confounders. To illustrate the above doubt, we assessed the causal effect of the six peripheral WBC counts and six lymphocyte subpopulations (including one derived ratio) on MUs by two-sample MR analysis. Our results showed that peripheral total WBC counts were suggestive of decreased MU susceptibility, which indicated that WBC counts could protect against MUs. This finding is consistent with other MR studies, such as a causal relationship between peripheral leukocyte and lymphocyte counts and multiple sclerosis [21], and a decreased risk of amyotrophic lateral sclerosis is linked to NK cells with greater levels of CD16CD56+ and HLA-DR + expression [23]. However, this result regarding total WBCs was in contrast with recent observational research that reported that the median peripheral WBC count of 39 Turkish patients with MUs was considerably higher in the active lesion group in comparison to 60 healthy control subjects [27]. The likely explanations are the difference in ancestry and fairly small sample size. Compared with 39 samples in the above observational study, our research used 563,085 European ancestry participants, which was more convincing and trustworthy.

Because we speculated that the regulatory influence of variants is often dependent on the cell subgroup, five main blood cell subpopulations were further analysed. Among those five blood cells, only lymphocyte counts had a suggestive causal relationship with MUs. With the increase in lymphocyte counts, the risk of MUs susceptibility decreased, following the same trend as the effect of total WBC counts on MUs. However, the counts of the other four WBC subpopulations (neutrophils, monocytes, basophils and eosinophils) had no evidence of a causal effect on MUs, which indicated that lymphocyte counts made the largest contribution to the causal effect of total WBC counts on MUs. A single-centre, case–control study in Turkey reported that there was no differences in peripheral neutrophils, lymphocytes, and monocyte cells in MU patients (n = 97) compared with controls (n = 90) [28]. Another retrospective study demonstrated that white blood counts and neutrophil counts were significantly higher in RAS patients (n = 80), with no significant difference in terms of lymphocyte counts or platelet counts [29]. The results from the observational study were paradoxical. This difference in outcomes with our MR analysis may also be greatly interfered with by different ancestries and poor sample sizes. For inferring causality, MR has more credibility than case–control studies. In addition, many studies have reported that the platelet/lymphocyte ratio and neutrophil/lymphocyte ratio were significantly higher in the RAS group than in the controls [30–32]. This also explained the different results and implied that the ratio between two cell types was more critical than the cell type itself. Hence, more GWAS data on the two blood cell ratios are greatly needed in the future.

We also determined which subgroup cell counts play a vital role in the causal effect on MU from lymphocytes. There has been mounting evidence in recent years that immunological diseases, particularly those involving T lymphocytes, contribute to the emergence and growth of MUs. For example, when compared to normal control participants, patients in the exacerbation stage of recurrent aphthous ulcers had significantly higher percentages of $CD4^+$ cells and a higher CD4+/CD8+ ratio [33]. Another study's

results showed that $CD4^+$ cells were lower in recurrent aphthous ulcers than in controls [34,35]. Moreover, the CD4+/CD8+ ratio normalized with significant improvement in the clinical symptoms of patients with recurrent aphthous ulcers after levamisole treatment [36]. Whether peripheral T-cell counts have a causal effect on MUs or whether they are only reflective of MU progression remains unknown. We discovered a causal association between higher $CD4^+$ counts and a lower MU risk, and an increase in circulating $CD8^+$ cells as measured in the blood would increase MU risk, although only one SNP could be used as an IV. However, there was no evidence of other lymphocyte subgroup (CD4+/CD8+ ratio, B cell, unswitched memory B cell and NK cell) counts having a causal effect on MUs by MR methods. However, there is inconsistency between these observational studies. The relatively small size ($n < 100$) and different MU types of observational studies may explain the null finding. Regarding B cells and NK cells, there have been few observational studies, and only one older study reported increased NK cell activity in the exacerbation of major aphthous ulcers [37]. However, our proposition was that there were no causal effects of peripheral B cell and NK cell counts on MUs using MR with limited present GWAS data on those two cell types. With the supplementation and expansion of GWAS information, more studies and new perspectives will emerge in the future.

Above all, this study found a causal association between lower peripheral lymphocyte T-$CD4^+$ counts and higher MUs risk and between higher lymphocyte $CD8^+$ counts and increased MUs risk. The possible pathogenetic mechanisms of this results maybe that decreased $CD4^+$ cells maybe led to lower IL-10 and TGF-beta anti-inflammatory cytokine production [11]. T lymphocytes also can produce pro-inflammatory cytokines (such as IL-2, IL-12 and IFN-γ) [7]. This imbalance in pro- and anti-inflammatory cytokines' production may contribute to the development of autoimmunisation and MUs [38]. But the mechanisms for those effects still need more studies in the future. Meanwhile, immunopotentiator (e. g. thymosin) or immunosuppressant (e. g. thalidomide), as the Th1 type immunologic response inhibitors, may be useful in the treatment of MUs.

This study had several strengths. This study enrolled a recent, high-quality GWAS data on the peripheral white blood subgroup cell spectrum. In addition, MR study methods can reduce the potential confounding effects because of the lack of reverse causation. However, there were some limitations in this MR study. First, GWAS datasets of $CD4^+$ cells, $CD8^+$ cells and CD4+/CD8+ ratios were obtained from an older study published in 2010. Although relatively old, these data were also used due to the absence of a recent, relevant GWAS for any of the three T-cell subgroups mentioned above. Second, there were only 1–3 SNPs strongly associated with the six lymphocyte subgroups after setting the association threshold with a p value $< 10^{-8}$. To achieve the first MR assumption, we did not relax the association threshold and sacrifice the number of SNPs. Hence, the Wald ratio was used instead of IVW. Considering the low evaluation effectiveness of the Wald ratio, future research also needs more SNPs to verify from a large GWAS in the future. Third, the Cochran Q test detected significant heterogeneity in some cell types (including the WBC subgroups). The MR effect magnitude was calculated using the random effect model. In addition, to reduce the potential bias from pleiotropic effects, additional MR techniques were employed, which should be evaluated cautiously. Fourth, peripheral immune cell counts recorded at a particular time period might not accurately represent a person's innate immunological characteristics. Fifth, there are many types of mouth ulcers according to different clinical manifestations. However, the GWAS data in the UKB do not consider and separate the different types of MUs. Considering that RAS is the major type of MU and other MUs are less likely to be genetic (such as traumatic mouth ulcers), our results tend to illustrate the causal effect of peripheral immune cell counts on RAS.

5. Conclusions

In conclusion, our study utilized two-sample MR analyses and demonstrated the peripheral immune cell counts disorder would increase the risk of mouth ulcers. Among these peripheral immune cell counts, total WBC and lymphocyte counts were suggestive of decreased MU susceptibility. Among lymphocyte subsets, $CD4^+$ cell counts showed a negative and robust causal correlation tendency with MUs. In contrast, $CD8^+$ cell counts were strongly associated with an increased risk of MUs susceptibility. This result will provide strong new evidence for dentists and clinical physicians to discover and use new promising potential immunotherapeutics to treat MUs patients.

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Additional information

We should submit any Supplementary Tables 1–7 together with the manuscript.

Data availability statement

The data associated with this study has been deposited into a publicly available repository. The GWAS summary data of WBC subgroup cell counts are available at Resources – The Lettre Lab (mhi-humangenetics.org). T cell counts GWAS data are available at http://genepi.qimr.edu.au/staff/manuelF/gwas_results/main.html. MU GWAS data also been recorded in UK Biobank (https://www.ukbiobank.ac.uk).

CRediT authorship contribution statement

Yajing Wang: Writing - original draft, Validation, Software, Methodology, Funding acquisition, Data curation, Conceptualization. **Yuanyuan Hu:** Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Mengxuan Shen:** Visualization, Validation, Software, Methodology, Investigation, Formal analysis. **Yang Cai:** Writing - review & editing, Supervision. **Zhiyuan Li:** Software, Methodology. **Changyue Xue:** Software, Methodology. **Xu Tan:** Writing - review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Jukun Song:** Writing - review & editing, Visualization, Validation, Software, Resources, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23430.

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